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Arcachon, France, September 1–5, 1998

From *Torpedo* electric organ to human brain: fundamental and applied aspects

De l'organe électrique de torpille au cerveau humain : aspects fondamentaux et appliqués





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Foreword

The International Symposia on Cholinergic Mechanisms (ISCM) were established in Stockholm, Sweden, in 1970 and have been held every three years to discuss progress in the understanding of molecular, pharmacological, behavioural and clinical aspects of cholinergic mechanisms. The volumes published after each symposium have been landmarks in this field (the proceedings of the last two meetings were special issues of Progress in Brain Research). Following Sweden, Switzerland, USA, Italy, Great Britain, Canada and Germany, France hosts the Tenth ISCM in September 1998, in Arcachon. The marine laboratory in Arcachon has played an important role in cholinergic research since David Nachmansohn discovered there, in 1938, that the electric organs of *Torpedo* are a rich source of cholinergic synapses.

The meeting addresses cholinergic mechanisms in central and peripheral nervous systems, sensory organs and muscles. The development and the structure of the vertebrate neuromuscular junction are discussed, as well as its pathologies (myasthenia gravis and myasthenic syndromes). In the brain, cholinergic transmission or neuromodulation is involved in memory, sleep and arousal. Its dysfunction underlies nicotine addiction, as well as various neurological and psychiatric disorders such as dementia and some epilepsies. The molecular genetics of cholinergic mechanisms (nicotinic and muscarinic receptors, cholinesterases, acetylcholine synthesis and release) are progressing rapidly. Cholinergic drugs are being used or evaluated for treatment of myasthenia gravis and Alzheimer's disease. Cholinergic toxicology includes poisoning by organophosphate pesticides and nerve gases, with the intriguing Persian Gulf Syndrome. The Tenth ISCM in Arcachon is an opportunity to discuss exciting new insights into fundamental aspects of cholinergic mechanisms and their consequences for toxicology, pharmacology, medicine and the environment. This volume offers an up-to-date account of current research in the cholinergic field, from the molecular level to the most integrated levels, and its applications in toxicology and medicine.

Dr. Jean Massoulié
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Arcachon, 1938

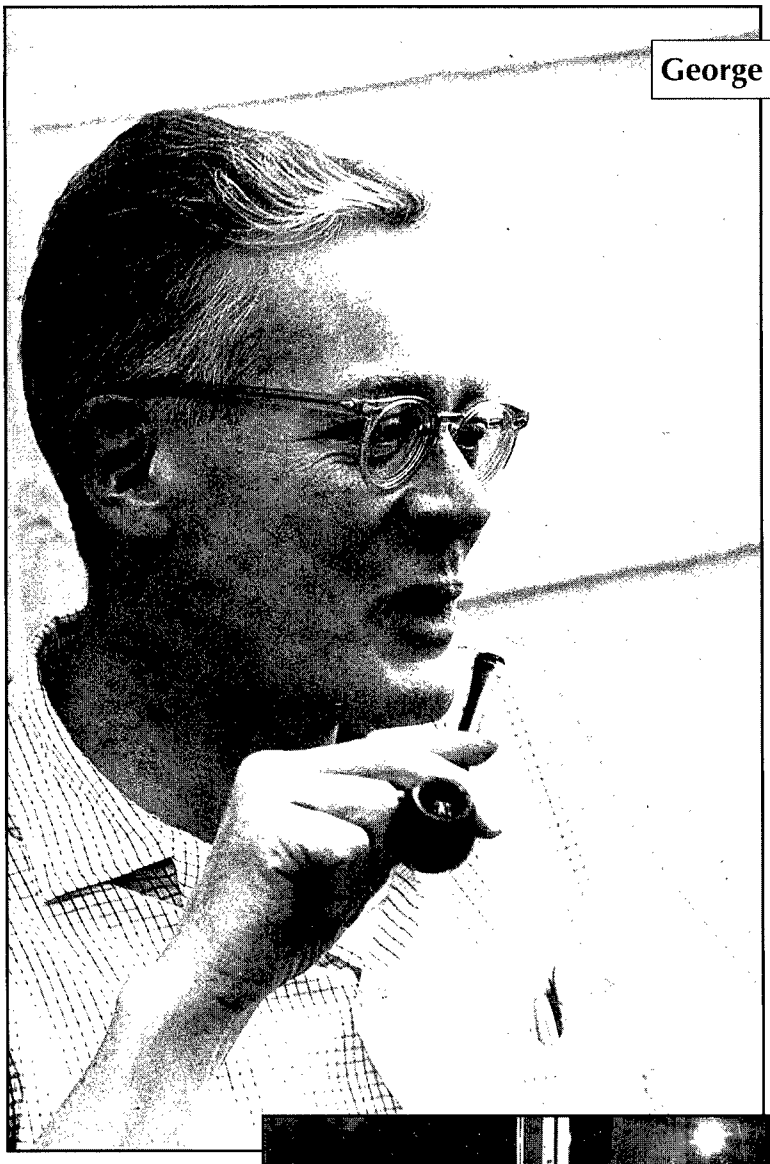
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La Grande-Motte, 1990.
From left to right: Miro Brzin, Winifred Koelle and George Koelle.



George Koelle



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Arcachon and cholinergic transmission

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Abstract — The cholinergic nature of transmission at the electromotor synapse of *Torpedo marmorata* was established at Arcachon in 1939 by Feldberg, Fessard and Nachmansohn (J. Physiol. (Lond.) 97 (1939/1940) 3P–4P) soon after transmission at the neuromuscular junction had been shown to be cholinergic. In 1964, after a quarter of a century of neglect, workers in Cambridge, then in Paris, Göttingen and elsewhere, began to use this system, 500–1000 times richer in cholinergic synapses than muscle, for intensive studies of cholinergic transmission at the cellular and molecular level. (©Elsevier, Paris)

Resumé — Arcachon et la transmission cholinergique. La nature cholinergique de la transmission à la synapse électromotrice de la torpille a été établie à Arcachon en 1939 par Feldberg, Fessard et Nachmansohn (J. Physiol. (Lond.) 97 (1939/1940) 3P–4P), peu après la découverte du caractère cholinergique de la transmission à la jonction neuromusculaire. Après une période assez longue de désuétude, les chercheurs de Cambridge, Paris, Göttingen et d'ailleurs ont commencé, vers 1964, à utiliser ce système, 500–1000 fois plus riche en terminaisons cholinergiques que le muscle, pour étudier intensivement la transmission cholinergique au niveau cellulaire et moléculaire. (©Elsevier, Paris)

***Torpedo marmorata* / electric organ / electromotor synapse / cholinergic transmission**

1. Introduction

The electric ray, *Torpedo marmorata*, is fairly common in the Bay of Biscay (Baie de Gascoigne): although a slow breeder (6–12 young every 2 years), it is not fished commercially. Its chief interest for the neurobiologist is that it possesses paired electric organs on each side of the midline which are capable of giving shocks (up to 40 V in air), strong enough to stun prey and deter predators. These organs are under the control of the electromotor nerves which originate in the electric lobes, paired nuclei on the brain stem placed caudally to the cerebellum. The electromotor neurons are in turn under the control of the oval nucleus in the brain-stem. This integrates sensations of hunger, detection of prey and nociceptive stimuli and decides whether to release a train of electric shocks (short trains at a frequency of 100–300 Hz). The electric organ consists of stacks of flattened cells known as electrocytes, electroplaques or electroplax; these are densely innervated on their ventral surfaces by fine branches of the electromotor axons.

Torpedo spp. are not the only fish to have electric organs; members of at least seven other diverse families have acquired them by a process of convergent evolution (for a recent review see [21]). However, in terms of availability, amount of tissue per specimen and density of synaptic material, the Torpedinidae, especially *T. marmorata* (Eastern Atlantic), *T. ocellata* (Mediterranian) and *T. californica* (Pacific), are the family of choice.

Electric organs have excited the interest of biologists since earliest times: the puzzling nature of their discharge was only resolved with the discovery of electricity and indeed led to the invention of the battery or Voltaic pile which A. Volta (for attributions in this form, see Whittaker [20] for original references) himself described, in a memoire to the Royal Society of London published in 1800, as an artifical electric organ. J. Bernstein correctly postulated in 1912 that the discharge was caused by a transient increase in the permeability of the electroplaque membrane to positively charged ions.

It has been known since the 1870s, mainly from the work of A. Babuchin in 1876, that the electrocytes are derived embryonically from myoblasts; it is therefore not surprising that the electromotor synapse is cholinergic. Grundfest [5] in a definitive review, reinterpreted Bernstein's theory of the discharge in the light of contemporary knowledge of the excitation of muscle; he described it as the summation in series and in parallel of normal excitatory postsynaptic potentials (EPSPs) evoked by the release of transmitter from the presynaptic nerve terminals. Unlike some other electric organs, the electrocytes of Torpedinidae are electrically inexcitable; thus the discharge in this type of electric organ is

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pure summed EPSP unmodified by a conducted response corresponding to a muscle action potential.

Apart from a few autonomic vascular endings, the innervation of the *Torpedo* electric organ is purely cholinergic with a synaptic content 500–1000 times that of muscle; indeed the organ resembles a huge mass of hypertrophied motor endplates. With 400–500 g of tissue per fish to work with, the electric organ is an ideal resource for studies of the cellular and molecular biology of all aspects of cholinergic transmission: the synthesis and storage of transmitter, its release and postsynaptic action, its hydrolysis to inactive products (choline and acetate) and their eventual reutilization. The electric lobes, though available in smaller quantities (ca. 400 mg/specimen), contain large (~40 μm diameter) cholinergic cell bodies studded with putative glutamergic nerve terminals; they contain the complete genome for cholinergic function and the mechanisms for the formation and transport of synaptic vesicles. In only one important respect does the electromotor terminal differ from terminals in muscle (including *Torpedo* muscle): its synaptic vesicles are larger (~90 nm in diameter versus ~50 nm in muscle). This facilitates a study of their function which appears to be similar in all respects to that of their smaller congeners.

2. Discovery of the cholinergic nature of transmission at the electromotor synapse

The discovery that transmission at the electromotor synapse is cholinergic was made during 3 weeks of research in June 1939 as the coming World War cast its shadow over Europe. Three neurobiologists of very different backgrounds came together at the Station Biologique d'Arcachon where *Torpedo* from the Bassin was readily available. They were A. Fessard, an electrophysiologist from the Collège de France, Paris, D. Nachmansohn, a biochemist and German Jewish emigrant working at the Sorbonne, Paris, and W. Feldberg, a pharmacologist and also a German Jewish emigrant working with H.H. Dale in London.

Fessard had worked extensively with electric tissue (for literature citations see Feldberg and Fessard [4]). He showed that excitation could only be brought about via the nerve – denervated tissue was unexcitable mechanically and electrically – and that nerve action could be blocked by drugs (curare, eserine) that block the cholinergic transmission in muscle discovered by H.H. Dale, W. Feldberg and M. Vogt in 1936. He surmised that the discharge must be triggered by the release of a depolarizing substance from the electromotor nerve terminals, probably acetylcholine, as in muscle.

Nachmansohn, a medical graduate of Berlin University, had received his early research training with O. Meyerhof, who had done classical work on the energy-yielding metabolic reactions sustaining muscular contractions. Nachmansohn thought that biochemical methods should be applied to nerve activity and argued that if acetylcholine were indeed a transmitter at the neuromuscular junction, the released transmitter must be destroyed by cholinesterase within the refractory period of muscle. With his student-assistant Annette Marnay he showed that the cholinesterase activity of the innervated portion of the frog sartorius muscle was several thousand times greater than that of the non-innervated portion and great enough to destroy released acetylcholine within the muscle's refractory period. Being aware that the electrocytes of electric tissue resembled motor endplates, he asked Marnay to test for the presence of cholinesterase in electric tissue. She found very high levels of activity in this tissue, exceeding that of muscle several hundred-fold.

Fessard heard about these results and invited Nachmansohn to work with him at Arcachon on chemical transmission in the electric organ. However, if the techniques the Dale group had used so successfully with muscle were to be applied to the electric organ, a third collaborator who could assay acetylcholine in tissue and perfusates and apply it to the organ by close arterial injection would be needed.

Accounts differ [20] on how Feldberg came to be invited to join Fessard and Nachmansohn but no-one better qualified than he could have been chosen, in view of his participation in the work on muscle.

The results showed unequivocally that transmission at the electromotor synapse is cholinergic. Publication was delayed by the outbreak of the Second World War in September 1939 and Nachmansohn's hasty departure for America, but the results were eventually written up by Feldberg and were published, together with some additional work Feldberg did in London on extracts of electric organ he brought back with him, in a now classical paper in the Journal of Physiology [4].

Three lines of evidence justify the conclusion that transmission at the electromotor synapse is cholinergic. Firstly, extracts of electric organ under condtions which prevent the breakdown of acetylcholine contain a substance which precisely matches the action of acetylcholine in a variety of assay systems: the frog rectus abdominis muscle; the dorsal muscle of the leech; slowing of the frog's heart; a fall in the cat's blood pressure; adrenaline release from the adrenal medulla. No other known substance would

match acetylcholine in all of them. The substance behaved like acetylcholine in its lability in alkaline solutions and its sensitivity to cholinesterase. The acetylcholine equivalence of the substance (400 nmol g of tissue^{-1}) is within the range found by later authors. In the second line of evidence it is shown that what we can now assume to be acetylcholine is released into perfusates of the organ on stimulation and in the third, close arterial injection of authentic acetylcholine excited discharges similar to those evoked by nerve stimulation.

Some years later Woodin (cited by Whittaker [19]) confirmed the identity of the acetylcholine-like substance in electric organ as acetylcholine by paper chromatography and more recently, it has been unequivocally identified by gas chromatography-mass spectrometry [18].

Nachmansohn continued to work on electric organs in the US, but switched to the electric eel, *Gymnotus electricus*, a denizen of the Amazon. His views on the role of acetylcholine in the nervous system began to deviate sharply from those of Dale and most other neurobiologists (for a critique and references, see Whittaker [20]); nevertheless, his group made many important contributions to cholinergic neurobiology. The comparative electrophysiology of electric organs attracted sporadic attention during the 1950s and with the coming of electron microscopy, the fine structure of the electromotor synapse was studied in several Torpedinidae: *Narcine* [17]; *T. marmorata* [9, 13]. Nevertheless, the potentialities of the electromotor system for the investigation of the cellular and molecular biology of cholinergic transmission remained largely neglected for 25 years.

3. The electromotor innervation of *Torpedo* as a model cholinergic system

3.1. The electromotor synapse: cytoplasmic and vesicular pools of acetylcholine

My own involvement with the *Torpedo* electric organ started in 1963 soon after my discovery of the synaptosome and the successful isolation from synaptosomes of pure preparations of mammalian cortical synaptic vesicles, a proportion of which stored acetylcholine in amounts consistent with their being the morphological basis of quantal transmitter release [24, 26]. In 1963 a colleague, R.D. Keynes, suggested that *Torpedo* electric organ might be a better source of cholinergic vesicles than mammalian brain. The tissue proved difficult to homogenize, but with M.N. Sheridan and M. Israël [14, 27] synaptosomes in low yield and reasonably homogeneous preparations of synaptic vesicles were obtained. Purer preparations of the latter were subsequently obtained by M. Israël on his return to France. Meanwhile, the large-scale isolation of pure synaptic vesicles was made possible [25] by three technical innovations: comminution of the tissue by crushing after rendering it brittle by freezing it at low temperatures; extraction of the crushed tissue with solutions iso-osmotic to *Torpedo* fluids; and high-resolution separation of the resultant cytoplasmic extracts on continuous density gradients in a large-capacity zonal rotor. This enabled the composition, biophysics and recycling of synaptic vesicles to be intensively studied [20]. Briefly, it was established by the Göttingen group that acetylcholine exists in two pools: the cytoplasmic, where it is synthesized, and the vesicular, in which it is stored and from which it is released. Small differences in density and composition enabled three pools of vesicles to be separated: largely empty vesicles newly arrived from the cell body by fast axonal transport [6], a reserve pool and a stimulus-induced recycling pool [29] which preferentially takes up acetylcholine newly synthesized in the cytoplasm. By using choline analogues it was possible to label the cytoplasmic and the two transmitter-containing vesicular pools differentially and to show that transmitter released by stimulation arises exclusively from the recycling pool [7].

The vesicular uptake of acetylcholine is driven by a proton gradient generated by a vacuolar-type ATPase [28] and is facilitated by a vesicular acetylcholine transporter (vAChT), the sequence of which is encoded within the first intron of the gene locus for choline acetyltransferase [3].

3.2. Synaptosomes and presynaptic plasma membranes

Improved methods of preparing synaptosomes were developed both by our group and that of Israël; when well sealed these were able to develop membrane potentials comparable to those in intact neurons [10, 11]. The specificity and properties of the choline uptake system were extensively studied [2, 23]. The plasma membranes are rich in a family of minor gangliosides containing a sialylated *N*-acetylgalactosamine residue; these are specific for mammalian cholinergic neurons and have permitted the immunoisolation of cholinergic synaptosomes from a mixed population (for review see [22]).

3.3. Electromotor cell bodies and axons

The cell bodies of electromotor neurons are ~40 μm in diameter and thus among the largest ver-

tebrate motor neurons known. They are readily isolated and though shorn of their dendrites are well sealed. Their choline acetyltransferase activity is twice that of cholinergic cell bodies in *Aplysia* and about 40 times greater than mammalian ganglion cells, but only one quarter that of electromotor nerve terminals.

The perikarya of these cells are rich in Golgi membranes and synaptic vesicles and contain readily translatable mRNA which code inter alia for several synaptic vesicle proteins [12]. Choline acetyltransferase is transported down the axon at the slow rate [1]; synaptic vesicles are transported at the fast rate and take up acetylcholine only when they reach the terminal [6].

The nerve terminals in the lobe are those of afferents from the command or oval nucleus. They are probably glutamergic and have been isolated in low yield by conventional methods.

3.4. Cotransmission in the electromotor synapse

Many cholinergic neurons are now known to utilize various neuropeptides as co-transmitters. The electromotor neurons are no exception; as in mammalian brain and autonomic ganglia, VIP is the co-transmitter. It is transported to the terminal in dense-cored vesicles (for references see Whittaker [20]). Another vesicular constituent which may act as a cotransmitter or regulator is ATP, present in vesicles in a 1:5 ratio to acetylcholine.

3.5. The nicotinic acetylcholine receptor (nAChR)

In one of the major advances of modern neurobiology the acetylcholine receptor in the muscle and electrocyte postsynaptic membrane is now known to be a ligand-gated pentameric ion channel composed of four polypeptide subunits, α, β, γ, δ of known sequences in which the α unit is represented twice. Early work was done on *Electrophorus* (*Gymnotus*) electric organ but when it was realised (from about 1975) that *Torpedo* membranes are much richer in receptor than *Electrophorus*, there was a shift to *Torpedo* spp. (*T. californica* and *T. marmorata*) by the many groups working in this field. The complete receptor is a rivet-like structure which undergoes a tilting conformational change as it opens and closes [16].

3.6. Acetylcholinesterase

The type of cholinesterase present in electrocytes hydrolyses acetylcholine faster than other choline esters and is thus known as an acetylcholinesterase. It has been fully sequenced and its tertiary structure and mode of catalysis are known. The choline moiety of acetylcholine is firmly held in a trough lined with 14 aromatic residues whose π electrons interact with the positive charge on the quaternary nitrogen of acetylcholine. Hydrolysis is effected by a charge relay system; the catalytic triad has been identified as Glu-327, His-440 and Ser-200. These widely separated residues are brought together by folding [15].

Massoulié and coworkers (for review see Massoulié and Toutant [8]) have shown that acetylcholinesterase can exist in several forms in electric organ and other tissues. The catalytic subunit (G_1) is a highly glycosylated soluble globular protein of molecular mass 70–85 kDa. In tissues a tetramer (G_4) of this unit is normally conjugated through S-S bonds to a collagen tail giving an asymmetric form (A_4). Further aggregation via interaction of the tails gives larger forms culminating in A_{12}, an aggregate of three A_4 units. Such forms are extractable by solutions of high ionic strength. In another conjugate, dimeric catalytic units are linked through ethanolamine, glycan and glucosamine to phosphatidylinositol (PI); this form is inserted via the lipid end of its tail into lipoprotein membranes. Such tails are found in several unrelated membrane-bound molecules with the common property of being solubilized by PI-specific phospholipase C (PIPLC).

4. The development of the electromotor system

This has been extensively studied by J. Melllinger in Rheims and by the Göttingen group (for references see Whittaker [20]). Nineteenth-century observations have been confirmed and extended by electronmicroscopic, electrophysiological and biochemical techniques. Briefly, four stages in development have been recognized: I, myogenic; II, electrocytogenic; III, synaptogenic divided into IIIa, penetration of stacks of electrocytes by ingrowing neurites, and IIIb, functional synaptogenesis. During phases II and IIIa, extensive apoptosis of electromotor neurons takes place as neurites are matched with their targets. Two trophic factors have been identified: a heat-stable neuronotrophic factor essential for neuronal survival and a heat-sensitive cholinotrophic factor that stimulates the expression of the cholinergic phenotype. The molecular structure of these factors has not been identified.

5. Conclusion

Although a vast amount of important work has been done with the electromotor system of *Torpedo*

since its cholinergic character was first established by Feldberg, Fessard and Nachmansohn, its potentiality is far from exhausted. While fish may not be so convenient to work with as small rodents, any difficulties in transporting and keeping them can be overcome by careful organization.

'Cholinologists' have reason to be grateful to the Station Biologique d'Arcachon for their exceptional willingness to provide *Torpedo* alive or as frozen tissues to so many laboratories and for the local organization and facilities which have made this possible.

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Early days in the research to localize skeletal muscle acetylcholinesterases

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Abstract — Very early in the study of the mechanism of neuromuscular transmission in skeletal muscles, it was clear that the hydrolysis of acetylcholine by muscle cholinesterases within the time of the refractory period required a very high concentration of the enzyme near the motor terminals. David Nachmansohn and George B. Koelle and their collaborators obtained the first biochemical and histochemical data consistent with this prediction. Now that the various molecular forms of AChE have been satisfactorily described, it is possible to analyse the mechanisms by which they are anchored to the structures of the neuromuscular junction, and in particular, to the synaptic basal lamina. (©Elsevier, Paris)

Résumé — Les premières étapes des recherches dans la localisation des acétylcholinestérases du muscle squelettique. En étudiant le mécanisme de la transmission neuromusculaire des muscles squelettiques, il est très tôt apparu que l'hydrolyse de l'acétylcholine par les cholinestérases musculaires dans les délais de la période réfractaire impliquait l'existence d'une très forte concentration enzymatique au voisinage immédiat des terminaisons motrices. C'est à David Nachmansohn et à George B. Koelle, ainsi qu'à leurs collaborateurs, que nous devons les premières données biochimiques et histochimiques qui s'accordent pleinement avec cette prévision. Maintenant que les diverses formes moléculaires de l'AChE ont été définies de manière satisfaisante, il est devenu possible d'entreprendre l'étude des modalités de leur ancrage dans les structures de la jonction neuromusculaire et, en particulier, dans la lame basale synaptique. (©Elsevier, Paris)

acetylcholinesterases / neuromuscular junction / synaptic transmission

Cholinesterases were among the first proteins to be studied when investigations of the mechanism of synaptic transmission began. Their localization and their function were lively debated between partisans and opponents of a 'neurohumoral' model of transmission at the neuromuscular junction of skeletal muscle.

As Dale first indicated in 1914, if acetylcholine is a major agent of synaptic transmission, it must be rapidly inactivated, presumably by an enzyme. Brown, Dale and Feldberg [3] believed that the elimination of ester liberated by stimulation associated with such neuromuscular transmission must occur with a 'flashlike' suddenness, within the limits of the refractory period, i.e., within one or a few ms. To explain such a rapid hydrolysis, they suggested that the esterase must be concentrated at the motor nerve endings. In 1936, this was only a hypothesis, which many physiologists and biochemists still considered highly unlikely. David Nachmansohn and George B. Koelle, who will be honoured at the next Arcachon Symposium, made decisive contributions to the validation of this hypothesis.

For the occasion of this Symposium on cholinergic mechanisms, where there will be frequent reference to the work of Nachmansohn and of Koelle, it seems to me to be appropriate to return, briefly, to these early days in the search to identify the sites of muscular cholinesterases, in which both were outstanding figures. My own research on skeletal muscle repeatedly benefited from my direct and personal contact with these two pioneers in neurobiology, and particularly from my collaboration with Nachmansohn during his stay in France, at the Sorbonne, which was interrupted by the war.

This retrospective of what may seem to be prehistoric research can also be justified in view of the world events which often disrupted the publication and discussion of their results, as was also the case for work on the cholinesterases of the *Torpedo* electric organ, one of the central themes of the Arcachon Symposium of 1939.

David Nachmansohn and his student, Annette Marnay, first used the frog sartorius muscle to study the distribution of muscle cholinesterases. This muscle contains regions rich in motor nerve terminals, usually called 'neural' parts of the muscle, and regions poor in such terminals, called 'aneural' parts (Keith Lucas, 1907; Pezard and May, 1937). They hoped to find significantly different rates of hydrolysis of acetylcholine in the neural and aneural regions. The first results obtained by Marnay and Nachmansohn [17], presented to the meeting of the Physiological Society in London in February 1937, were disappointing. A difference of only 20% to 40% was found between neural and aneural regions. These values appeared too low to be considered evidence of a particular concentration of the enzyme

at motor nerve endings. In addition, when the rates of acetylcholine hydrolysis in the tissues of various guinea-pig organs were determined, the cholinesterase activities were not very different from those in skeletal muscle tissue. New experiments with the frog sartorius, but using homogenized samples rather than small pieces of muscle, were more conclusive [18, 19, 21]. Acetylcholine hydrolysis was three to four times higher in neural than aneural samples prepared in this way. In view of the extremely small volume of neuromuscular junctions, these observations were consistent with a very high concentration of cholinesterase in each junction. At the same time, Marnay and Nachmansohn performed analogous experiments with the dog gastrocnemius. This was a muscle whose motor innervation I had previously studied and so I could supply Marnay and Nachmansohn with neural and aneural part of this muscle. Despite only few assays, the difference between these two preparations of dog muscle was concordant with that found between analogous preparations of the frog sartorius.

A further work with the internal section of the guinea-pig gastrocnemius confirmed clearly these first results and allowed the conclusions drawn from the studies of frog end-bushes to be extended to mammalian motor end-plates [4, 9, 10, 11]. By measuring cholinesterase activity in a series of slices obtained with a freezing microtome with a suitable orientation, the distribution of the enzyme activity could be plotted and compared to that of the motor end-plates. The correlation between these two distributions was also established by treating alternate slices from the same muscle for biochemical assay of enzyme activity and staining to reveal nerve endings.

The high cholinesterase activity at the neuromuscular junctions was initially attributed to the nerve terminals themselves. The best way to confirm this appeared to be to study the changes in this activity following section of the motor nerve, during the subsequent loss of nerve endings. The first studies of denervated muscle cholinesterases were conducted by Martini and Torda [22] in an attempt to explain the hypersensitivity of denervated muscle to acetylcholine. Two weeks after sectioning the motor nerve of the rat gastrocnemius, they observed a loss of enzyme activity, which fell to half its normal value. At first view, these findings suggested that the cholinesterase at the junctions was mainly located in the nerve endings themselves.

To elucidate the role of acetylcholine in neuromuscular transmission, it was obviously important to determine whether the enzyme was indeed in the nerve endings of the junctions, or elsewhere. If acetylcholine were the neurotransmitter, it would have to act and be inactivated outside the nerve endings. Repeating this denervation experiment with the guinea-pig gastrocnemius, Marnay and Nachmansohn [20] unexpectedly found an increase in the concentration of cholinesterase in the weeks following section of the motor nerve and degeneration of the axons.

To explain this increased concentration in guinea-pig muscles, Marnay and Nachmansohn [20] at first suggested that it might be linked to the increase in the number of nuclei observed following denervation (T. Cahn, 1927; S.Tower, 1935). Work on the development of the muscles of the hind leg of the chick embryo led Nachmansohn [25, 26] however to abandon his first hypothesis. In the muscles of the chick embryo, the cholinesterase concentration increases progressively during incubation, and then declines after hatching so that after 3 weeks the concentration is only 10% of that on the day of hatching. Recent publications concerning the formation of mammalian motor end-plates suggested to Nachmansohn an interpretation of these changes in cholinesterase concentration. To quote: 'the observations of Couteaux indicate that the end-plates reach nearly their final size at birth, so that while the muscle fibres of an embryo are small, the end-plates in a given weight of muscle are relatively both large and numerous' [26]. Variations in enzyme concentration could thus be explained to a large extent by changes in the ratio of the volume of the motor end-plates to that of the muscle fibres and consequently by differences in the number of motor end-plates per unit weight. The decrease in concentration following hatching would thus be due mostly to the increase in size of the muscle fibres.

By analogy, for muscles which become smaller following denervation (as in the case for the guinea-pig gastrocnemius) the variations in the concentration of enzyme following denervation were suggested to be mainly due to changes in the muscle fibre volume (*figure 1*). Two weeks after sectioning the sciatic nerve, the change in the concentration of the enzymatic activity was indeed found to be inversely proportional to the change in volume of the denervated muscle fibre: the former increasing while the latter decreased. Thus, the activity at each motor end-plate was almost unchanged, and the high concentration of cholinesterase at the normal neuromuscular junction was thus not in the nerve ending itself but outside and around it [4, 9, 10, 11].

This interpretation was satisfactorily consistent with findings for muscles in which the fibres decrease in volume only slowly after denervation. For example, in the toad sartorius (Feng and Ting, 1938) and the rat gastrocnemius (Stoerk and Morpeth, 1944) denervation does not lead to a rapid change

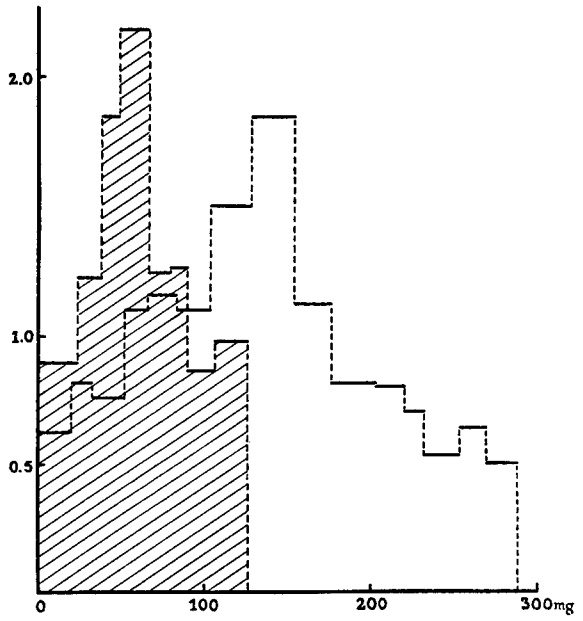


Figure 1. Concentration of cholinesterase activity in the middle portion ('neural' part) of the internal sections of two gastrocnemius muscles of the same guinea-pig. Each muscle was cut in consecutive slices and each horizontal line corresponds to the weight of a slice. Ordinates: cholinesterase activity (QChE). One of the muscles (hatched zone) was denervated by section of the sciatic nerve 16 days before. The comparison of the curves shows the persistence of a high enzymatic activity in the region of denervated endplates.

in the volume of the muscle fibres and thus not to a correlative increase in the number of denervated end-plates per unit weight. In both these muscles, the degeneration of motor axons is not followed by an increase in the concentration of the cholinesterase, but indeed by a decrease in its activity, most of which is expressed at the denervated end-plates.

The accumulation of biochemical data and the progress of morphological studies of the neuromuscular junction led to the suggestion some years later that the high cholinesterase activity of the neuromuscular junction was mainly located in the sub-neural or sub-synaptic apparatus [5]. Such an assumption could only be verified by histochemistry. Two years after it was formulated, Koelle and Friedenwald [16], using acetylthiocholine as the substrate, developed an appropriate histochemical technique. The images of sites of high concentration obtained by this approach, as viewed with the light microscope, were largely superimposable on those of the subneural apparatus obtained with certain aniline stains. This technique also revealed another, sometimes very major, site of cholinesterase concentration at muscle-tendon junctions [7, 8]. The muscle fibre at this junction, where the sarcolemma presents folds comparable in certain respects to the subsynaptic folds, is more sensitive than in non-synaptic regions to acetylcholine [14].

Using selective inhibitors in association, this acetylthiocholine technique was highly specific, and could distinguish between acetylcholinesterases and other cholinesterases. However, the significance of the locations identified was more debatable. The technique could lead to major artefacts, particularly resulting from diffusion, which could lead to incorrect conclusions as to the site of the enzymatic activity. In the case of skeletal muscles, the reaction products (and not the enzymes) could diffuse to neighbouring sites. Various modifications of the technique were used and substantially reduced the extent of this problem [6, 12, 15]. Nevertheless, these artefacts limit the accuracy of the localization particularly as concerns sites which are close together, as in the case for the neuromuscular junction, the presynaptic plasma membrane, the synaptic basal lamina (BL), the postsynaptic plasma membrane and the Schwann cells.

Despite the use of numerous new histochemical techniques derived from the acetylthiocholine method, or using other substrates, doubts remained about the interpretation of the locations observed, in particular for the postsynaptic plasma membrane and the synaptic BL in the synaptic cleft. Correlations were established between the effects of the action of proteolytic enzymes on the synaptic BL and variations in the intensity of cholinesterase staining of end-plates. They suggested a link between the enzyme and synaptic BL [1, 2, 13]. This association between the junctional cholinesterase and the synaptic BL was demonstrated histochemically by McMahan, Sanes and Marshall [24] on the cutaneous pectoris muscle of the frog in which the neuromuscular junctions were deprived *in vivo* of their cellular components (nerve terminal, Schwann cell and muscle cell): only subsisted in the preparation the sheaths of BL which surrounded the muscle fibres. Using this preparation, histochemical staining revealed the persistence of the enzymatic activity in the synaptic BL. The reduction in staining intensity following inhibition by a specific inhibitor of 'true AChE' demonstrated that 'some if not all' of the cholinesterase in the BL was AChE.

To characterise the nature of the association of AChE with the synaptic BL and other muscle sites, it was necessary to investigate its molecular forms. Such studies showed that there were multiple forms of AChE with diverse quaternary structures and solubility characteristics [23]. The means of anchorage

of various asymmetric and globular AChE have been investigated in detail, and the results will be discussed during the Arcachon Symposium.

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Allosteric nicotinic receptors, human pathologies

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Abstract — Nicotinic acetylcholine receptors are ligand-gated ion channels present in muscle and brain. These allosteric oligomers may exist in several conformational states which include a resting state, an open-channel state, and a desensitized refractory state. Recent work has shown that point mutations in the nicotinic receptor may, altogether, abolish desensitization, increase apparent affinity for agonists and convert the effect of a competitive antagonist into an agonist response. These pleiotropic effects are interpreted in terms of the allosteric model. This paper reviews recent evidence that such mutations occur spontaneously in humans and may cause diseases such as congenital myasthenia or familial frontal lobe epilepsy. In addition, nicotinic receptors are involved in tobacco smoking. Accumulating evidence, including experiments with knock-out animals, indicates that addiction to nicotine is linked to the activation of β2-subunit containing nicotinic receptors in the dopaminergic mesolimbic neurons which are part of the reward systems in the brain. Current research also indicates that nicotinic agonists might serve as therapeutic agents for Alzheimer's disease and Tourette's syndrome, as well as for schizophrenia. This paper extends and updates a recently published review. (©Elsevier, Paris)

Résumé — Récepteurs nicotiniques allosteriques et pathologies humaines. Les récepteurs nicotiniques sont des canaux activés par un neurotransmetteur présents dans le muscle et dans le cerveau. Ces oligomères allostériques peuvent exister dans différentes conformations telles qu'un état de repos, un état avec le canal ouvert et un état désensibilisé, réfractaire à l'activation. Des travaux récents ont montré que des mutations ponctuelles dans les récepteurs nicotiniques peuvent, d'un même coup, supprimer la désensibilisation, augmenter l'affinité apparente des agonistes et convertir l'effet des antagonistes compétitifs en réponse à un agoniste. Ces effets pléiotropiques sont interprétés à l'aide du modèle allostérique. Le présent article résume la découverte récente de l'occurence spontanée de telles mutations chez l'humain et des pathologies qu'elles causent telles que des myasthénies congénitales et des épilepsies du lobe frontal. De plus, les récepteurs nicotiniques sont impliqués dans la tabagie. Un nombre croissant de travaux, notamment faisant usage d'animaux recombinants, démontre que la dépendance à la nicotine est liée à l'activation de récepteurs nicotiniques contenant la sous-unité β2, dans les neurones dopaminergiques mésolimbiques qui participent aux systèmes de récompense dans le cerveau. Les travaux contemporains indiquent également que les agonistes nicotiniques pourraient remplir le rôle de médicament pour le traitement de la maladie d'Alzheimer, du syndrome de Gilles de la Tourette, et de la schizophrénie. Cet article est une mise à jour étendue d'une revue publiée antérieurement. (©Elsevier, Paris)

nicotinic acetylcholine receptors / allosteric proteins / congenital myasthenia / frontal lobe epilepsy / Alzheimer's disease / schizophrenia / nicotine addiction

1. Introduction

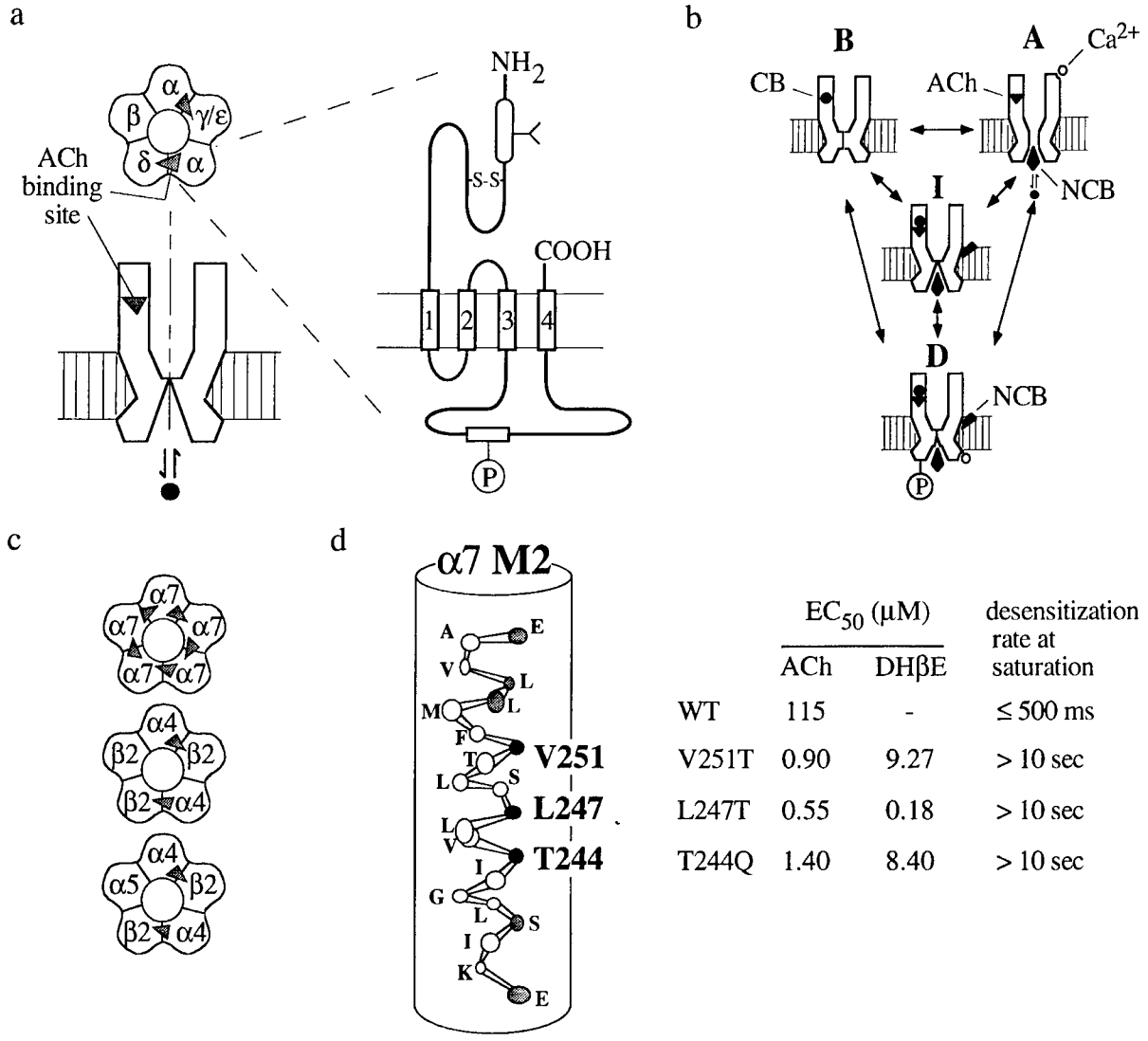
The nicotinic acetylcholine receptor (nAChR) was the first neurotransmitter receptor biochemically and functionally identified [2, 3], in part because high amounts of the receptor protein were available in the fish electric organ [4] and also because snake venom α-toxins had been identified as highly selective markers of nAChRs [5]. Recombinant DNA technologies further led to the demonstration that the structural and functional properties of this membrane allosteric protein from fish are, to a large extent, paralleled by those of brain nicotinic receptors thus opening the field to human brain pathologies [6, 7].

In this review, two aspects of nAChRs relevant to medicine are presented. First, point mutations in muscle and brain nAChRs may produce congenital myasthenia and familial epilepsies. The phenotypes of the mutated nAChRs are interpreted in terms of changes in the properties of the allosteric transitions. Second, nicotinic drugs, despite their addictive properties, could potentially alleviate neurologic and psychiatric disorders.

2. Nicotinic receptors as allosteric membrane proteins

The nAChRs compose a family of ligand-gated ion channels differentially expressed in skeletal muscle and nerve cells (reviewed in [8–10]). They form 300 kDa transmembrane hetero- (or homo-) pentamers from a repertoire of 16 known different types of subunits referred to as α1–α9, β1-β4, γ, δ, and ε. The subunits are regularly distributed around an axis of quasi-symmetry delineating the ion channel (*figure 1a, d*). Each subunit contains a large N-terminal hydrophilic domain exposed to the synaptic cleft, followed by three transmembrane segments

* Correspondence and reprints.



	EC_{50} (μM)		desensitization rate at saturation
	ACh	DHβE	
WT	115	-	≤ 500 ms
V251T	0.90	9.27	> 10 sec
L247T	0.55	0.18	> 10 sec
T244Q	1.40	8.40	> 10 sec

Figure 1. Nicotinic acetylcholine receptors (nAChRs) are ligand-gated ion channels with allosteric properties [30]. **a.** Muscle and *Torpedo* nAChRs are pentameric oligomers. The five homologous subunits are organized around an axis of quasi-symmetry perpendicular to plane of the plasma membrane, that delineates the ion channel pore. Each subunit exhibits a similar transmembrane organization sketched on the right. The binding sites are located at the interface of the extracellular N-terminal domains of the subunits, and the ion channel is lined by the M2 transmembrane segment. **b.** The nAChRs undergo allosteric transitions between a small number of states: resting (B), active (A) and desensitized (I, D) states [30, 31]. Various ligands preferentially bind to different states as indicated. CB, competitive blockers; NCB, non-competitive blockers; ACh, acetylcholine. **c.** Putative organization of three different types of neuronal nAChRs: homopentamers of α7 subunit, heteropentamers of α4 and β2, heteropentamers of α4, β2 and α5. **d.** The M2 transmembrane segment is putatively organized in an α-helix. Mutations of residues in M2 facing the channel pore increase the apparent affinity for nicotine, convert the antagonist dihydro-β-erythroidine into an agonist and drastically slow the desensitization rate of mutated α7-nAChRs (adapted from [41]). Adapted from [1].

(M1–M3), a large intracellular loop and a C-terminal transmembrane segment (M4). Acetylcholine binding sites are located at the interface between α and non-α subunits in the N-terminal regions [8, 11–13]. They include a principal component of three loops A, B, and C and a complementary component of at least three loops, D, E and F, on the non-α subunit. In homo-oligomeric receptors, the two components are carried by identical subunits [11, 14]. A wide diversity of binding properties in hetero-oligomers

results from the combinatorial diversity of the active site structure (e.g., [14]). The ion channel is lined by the M2 segment from each of the five subunits [15–21]. Neuronal nAChRs are more permeable to calcium ions than muscle nAChRs (neuronal nAChRs: pCa/pNa from 15 to 0.5 depending on the subunit composition; muscle nAChRs: pCa/pNa of about 0.2) [8, 9, 22].

Muscle nAChRs have a fixed composition $[\alpha 1]_2[\beta 1][\delta][\gamma$ or $\varepsilon]$ in vertebrates. Neuronal nicotinic receptors are composed of neuron-specific subunits homologous to the muscle subunits. To date, ten neuronal subunits have been identified in mammals ($\alpha 2$–$\alpha 7$, $\alpha 9$, $\beta 2$–4) (e.g., [10, 23]). Of the more than 20 000 possible combinations of subunits, only a few yield functional receptors. The $\alpha 7$ and $\alpha 9$ subunits form functional homo-oligomers when expressed in *Xenopus* oocytes, while the $\alpha 2$–4, $\alpha 6$ subunits produce hetero-oligomers with the $\beta 2$ or the $\beta 4$ subunit (*figure 1c*, reviewed in [24, 25]). The $\alpha 5$ subunit can associate with $\alpha 3\beta 2/4$ and $\alpha 4\beta 2$ subunits and thus form hetero-oligomers with three different subunits (*figure 1c*; [26, 27]). The sequence homology of $\beta 3$ with the $\alpha 5$ subunit suggests that $\beta 3$ possesses a similar function [28], and is also integrated into functional nAChRs [29].

Upon application of nicotinic agonists, both muscle and neuronal nAChRs undergo fast activation leading to an open-channel state, and a slow desensitization reaction leading to a closed-channel state refractory to activation. Activation and desensitization of muscle and brain nAChRs correspond to transitions between a small number of discrete structural states with distinct binding properties and ion channel conductance [30]. Consistent with the allosteric MWC model and its extension to membrane receptors [30, 31], the different conformational states may spontaneously exist in the absence of ligands, and nicotinic effectors cooperatively modify the equilibrium and kinetic constants for the transitions between the states (*figure 1b*). The pharmacological and kinetic characteristics of these states depend upon the subunit composition. Indeed, the two main subtypes of brain nAChRs strikingly differ: the human $\alpha 4\beta 2$ and $\alpha 7$ have respectively a low and a high EC_{50} for nicotine (0.3–5 μM versus 40–110 μM); at saturation, they desensitize respectively in the 10-s and in the 10–100 ms range (or less) [32–36]. The kinetic constants governing the ligand binding and the transitions between the different states (14 independent rate constants for a four state model) have been estimated for muscle nAChR [31] and the analysis extended to neuronal nAChR mutants [31, 37, 38].

Site-directed mutagenesis of affinity-labeled residues in the channel and active site domains [39–43] revealed that mutations of single amino acids can modify multiple functions of the nAChR. For instance, mutations in the channel lining region M2 (e.g., $\alpha 7$T244Q, $\alpha 7$L247T, $\alpha 7$V251T) produce a 100-fold increase in apparent affinity for agonists, a loss of desensitization and a conversion of competitive antagonists to agonists (*figure 1d*) [39, 41–43] (reviewed in [8, 37]). The allosteric model accounts for these pleiotropic phenotypes. Different classes of phenotypes may be associated with selective changes either in the binding properties (K phenotype), in the biological activity of the ion channel (γ-phenotype) or in the isomerization constants between receptor conformations (L-phenotype) and possibly in both [37, 44].

The neuronal nicotinic receptor subunits are expressed differentially in the mammalian brain. In situ hybridization in the rat brain shows that $\alpha 4$, $\beta 2$ and $\alpha 7$ are widely expressed, that $\alpha 3$ and $\alpha 5$ are less ubiquitous and that $\alpha 6$, $\beta 3$, $\beta 4$ and $\alpha 2$ are only expressed in a few brain structures (*table I*). In contrast, $\alpha 3$ and $\beta 4$ are the most abundant nAChR subunits in the autonomic peripheral nervous system [45]. The distribution of nAChRs has also been studied with radiolabeled nicotinic ligands. At least four different types of nicotinic-ligand binding activity with different distributions have been distinguished: the high affinity binding sites for nicotine that correspond to $\beta 2$-containing nAChRs [46, 47] are found throughout the brain (e.g., [48]) and are also labeled by three other nicotinic ligands: acetylcholine (in the presence of atropine to block muscarinic ligands), cytisine and epibatidine. The high-affinity binding sites for epibatidine (but not for nicotine) have a much restricted localization limited mostly to the habenulo-interpeduncular system and the dorsal medulla oblongata; these sites may be separated into two families, since only a subset of them binds cytisine with a high affinity [47] (see also [49]). They correspond presumably to $\beta 4$-containing nAChRs [4]. The α-bungarotoxin binding sites that correspond to $\alpha 7$-containing nAChRs [50] are distributed throughout the brain with a prevalent localization in the limbic system. As a consequence of such diversity in function and distribution, nAChRs may contribute to a wide array of brain functions [51]. Conversely, dysfunction of a single nAChR subunit may produce diverse deficits.

3. Congenital myasthenia and familial epilepsies result from nAChR point mutation

Genetic analysis of several human (and animal) pathologies has revealed nAChR mutations yielding pleiotropic phenotypes (*figure 2*). The mutations are

Table I. Differential distribution of nAChR subunit mRNA in the rat brain ([28] and references therein). Recent work suggests a broader expression pattern of the subunits α3 and β4 observed by in situ hybridization [112]. However such observations do not seem to be consistent with recent binding experiments in β2-knockout mice [47]. The expression of α7 in the dopaminergic nuclei (DA nuclei) was recently demonstrated [91]. NTS, nucleus of the tractus solitarius. Adapted from [1].

	α2	α3	α4	α5	α6	α7	β2	β3	β4
Telencephalon									
olfactory bulb	+	++	+	++	-	++	++	-	+
isocortex									
layer II-III	-	-	+	+	-	+	++	-	-
layer IV	-	+	+	+	-	+	++	-	-
layer V	-	-	++	+	-	++	++	-	-
layer VI	-	-	++	++	-	++	++	-	-
hippocampal formation	(+)	(+)	+	+	-	+++	++	-	-
striatum	-	-	-	-	-	-	+	-	-
septum	-	-	+	-	-	+	+	-	-
hypothalamus	-	-	+	-	-	-	+	-	-
supraoptic n.	-	-	+	-	-	+++	+	-	-
Diencephalon									
pineal gland	-	+++	-	+	-	-	+	-	+++
habenula	-	+++	++	+	(+)	(+)	++	++	+++
thalamus	-	+	+++	-	+	-	+++	+	-
Mesencephalon									
DA nuclei	-	(+)	++	++	+++	(+)	++	+++	-
mesenc V nucleus	-	-	+	-	+	-	++	+++	-
interpeduncular nucleus	++	+	+	++	+	(+)	++	+	+
Rhombencephalon									
vestibular nuclei	-	-	+	+	-	++	+	-	-
cerebellum	-	+	-	+	-	-	+	+	+
locus coeruleus	-	(+)	-	-	+++	-	++	+++	(+)
motor nuclei	-	+	+	+	-	-	++	-	-
NTS	-	++	(+)	+	-	-	++	-	++
area postrema	-	++	++	+	-	-	++	-	+

homologous or even identical to those initially studied in reconstituted α7 homo-oligomers and their phenotype may be also interpreted in terms of the allosteric model.

A mutation in the *deg-3* gene coding for a putative nAChR subunit of the nematode *C. elegans* results in neurodegeneration [52]. This *deg3*-I293N mutation is likely to cause an 'increase-of-function' similar to that initially found with the vertebrate α7V251T mutation [41]. The neurotoxicity could plausibly arise from a large toxic influx of calcium associated with a non-desensitizing and/or spontaneously open nAChR channel [39, 41].

In humans, myasthenia gravis is a sporadic disease caused by an auto-immune reaction directed against muscle nAChRs. However, some congenital myasthenic syndromes are associated with point mutations in the muscle α1, β1 or ε subunits. Mutations reducing channel opening transitions (e.g., εP121L, ε1254ins18), or affecting nAChR assembly (εR147L) cause myasthenic symptoms only when combined with a null mutation of the other allele [53–55]. Null mutations [56] result in symptoms only when expressed on both alleles. In accordance, animal models with a knock-out of the subunit ε express obvious myasthenic symptoms only in a homozygous genotype [57]. In these myasthenic patients and animal models, neurotransmission is partially rescued at the neuromuscular junction by the persistence of expression of the fetal nAChR γ subunit.

Mutations increasing the time spent by nAChRs in the open state also produce myasthenic syndromes, even as heterozygous mutations [58–63] (but see [54]). Mutations causing such 'increase-of-function' phenotypes occur near the ligand binding region (α1G153S, α1V156M), in the transmembrane M2 segment delineating the ion channel (α1T254I,

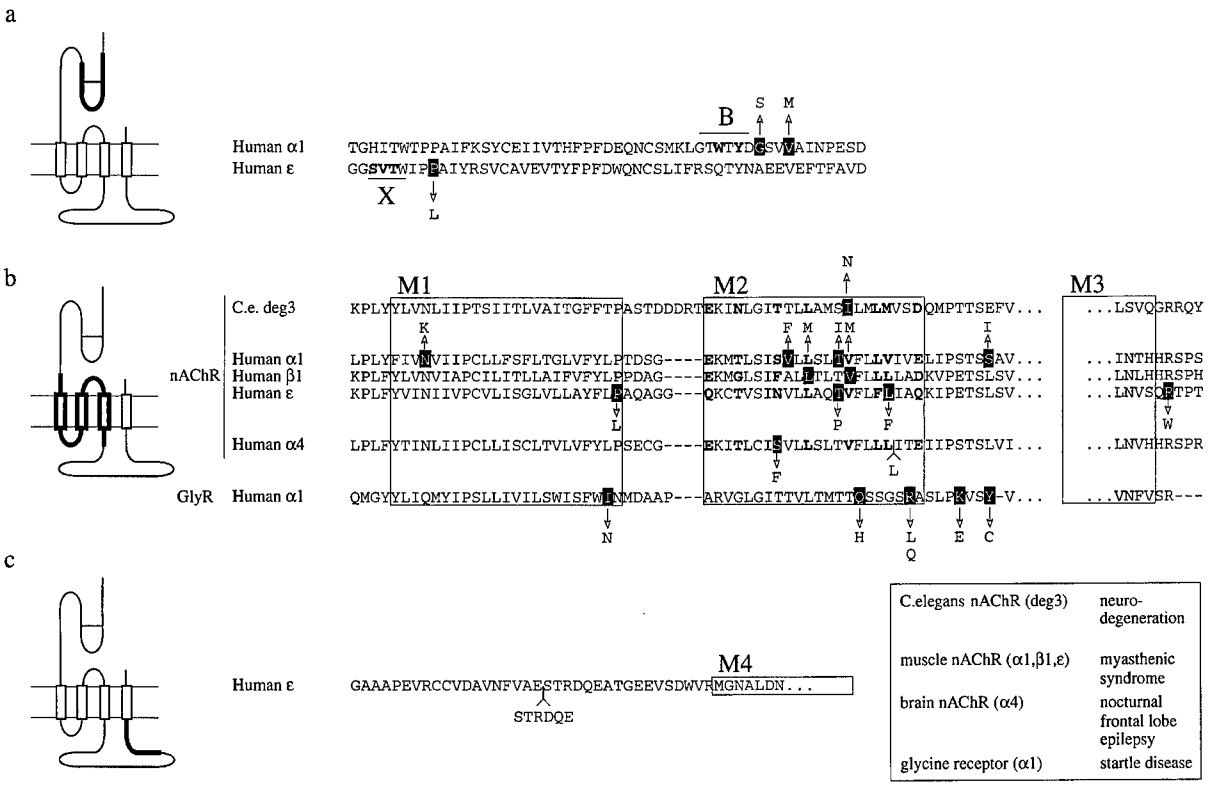


Figure 2. Pathogenic mutations affecting allosteric transitions in the muscle and neuronal nicotinic receptors and in the homologous glycine receptor. Each disease is caused by a single mutation among the mutations indicated. The drawings on the left indicate the protein domain concerned. The mutations are indicated by the amino acid symbol above/under the sequence with an arrow pointing from the wild-type highlighted residue. Null mutations are not presented here. **a.** Mutations near the ligand binding regions. B, B-loop in the principal component of the binding site. X, unnamed region in the complementary component of the binding site [12]. The mutations drawn are: εP121L [53], α1G153S [59, 62] and α1V156M [62]. **b.** Mutations in/near the transmembrane domains. Note the large number of mutations in the M2 region. The amino acids facing the channel pore are indicated in bold. The mutations in nAChR subunit gene are *deg3*I293N [52], α1N217K [60], α1V249F [63], α1T254I [62], α1S269I [62], β1L262M[61], β1V266M [60], εP245L [54], εT264P[58], εL269F [60, 111], εR311W [54], α4S248F [66], α4-776 (ins3) [69]. The mutations in the α1 glycine receptor gene are: GlyRα1-I244N, GlyRα1-Q266H, GlyRα1-R271Q, GlyRα1-R271L, GlyRα1-K276E, GlyRα1-Y279C (references in [70]). Alignment was performed with the program Clustalw of DG Higgins and PM Sharp. **c.** The mutation in the intracellular loop between the M3 and M4 segments is ε1254ins18 [55]. Adapted from [1].

α1V249F, β1V266M, β1L262M, εL269F) or in adjacent regions (α1N217K, α1S269I, εP245L) (*figure 2*). They may affect both intrinsic ligand binding (K phenotype) and opening transition/desensitization (L phenotype) processes or both [37, 38, 44]. Neighboring mutations may produce different phenotypes; for instance, α1G153S slows agonist dissociation while α1V156M decreases the rate of channel closing [59, 62]. Some mutations (α1V249F, β1V266M, εT264P, εL269F) produce a high rate of spontaneous openings in the absence of ligand [58, 60, 63], a phenotype consistent with the allosteric model on the basis of a shift of the allosteric equilibrium in favor of the open state [37, 38, 64]. The confirmation that such 'increase-of-function' mutations in muscle nAChRs are pathogenic was recently obtained in an animal model of transgenic mice carrying the εL269F mutation [65].

Recently, some familial epilepsies have been linked to mutations in the α4 nAChR subunit. In an Australian family, an α4S248F mutation was found to produce autosomal dominant frontal lobe epilepsy [66]. The mutated serine faces the channel pore, as initially demonstrated by chlorpromazine labeling in

Torpedo nAChRs (references in [8]). The mutation of the homologous residue in brain α7 nAChRs (α7T244, *figure 1d*) causes drastic changes in the affinity for ACh and the desensitization properties of the nAChRs [41]. In the human α4 gene, α4S248F produces a limited change in the apparent affinity for acetylcholine and a five-fold increase in the desensitization rate of α4β2 nAChRs [67], a decrease in the mean single channel conductance and a loss of calcium permeability [68]. Furthermore, this mutant exhibited an 'use-dependent potentiation' of the electrophysiological response to nicotinic agonists [68]. In a Norwegian family, the same epileptic syndrome was linked to the insertion of a GCT triplet at nucleotide 776, resulting in the insertion of a leucine at codon 260 [69]. In oocyte experiments, this insertion causes a 12-fold increase in the apparent affinity for ACh of human α4β2. As the insertion is adjacent to a pair of leucines previously identified as a critical element for calcium permeability [22], it might also reduce calcium permeability, though definitive evidence is lacking for this mutant. It is unclear for both mutations α4S248F, α4 (776ins3) whether the phenotype is due to a 'loss of function' (increased desensitization rate and loss of calcium permeability) or to an 'increase of function' (increase in apparent affinity, 'use-dependent' potentiation).

Point mutations that change allosteric properties occur in other ligand-gated ion channels, such as the glycine receptor α1 subunit. Human hereditary hyperekplexia is caused by mutations in the M1–M2 and M2–M3 loops that lead to a dramatic reduction of efficacy of the agonist ([70] and references therein). Overall, these results show that point mutations can cause either a loss of function or an apparent 'increase of function' by altering the allosteric transitions of the nAChRs. Increase-of-function mutations occur frequently and may be as pathogenic as null mutations. Since each allosteric state of the nAChR possesses a distinct pharmacological profile, one may anticipate the development of novel pharmacological agents targeted not only to a particular combination of subunits but to each of the diverse conformations spontaneously accessible by the various receptor oligomers.

4. Null mutation of neuronal nAChRs, Alzheimer's disease and memory

The role of defined nAChR subunits in brain function has been examined using knock-out mice. Mice lacking the most widely expressed β2 subunit survive, feed and mate normally [46]. Their brains are of a normal size and morphology. The high affinity nicotine binding sites (classically attributed to α4β2 nAChRs) completely disappear from the brain of homozygous mutant mice, whereas the α-bungarotoxin sites (corresponding to the α7-containing nAChRs) persist. Electrophysiological responses to nicotine are no longer recorded in the thalamus but persist in a few structures expressing the β4 nAChR subunit (such as the medial habenula) [46, 47]. Further analysis of the β2 mutant mice has shown that the β2-containing nAChRs are expressed both in the somato-dendritic compartment, and in the axonal compartment of neurons as presynaptic nAChRs [71]. The absence of the β2 subunit affects the performance in associative memory (passive avoidance) tests of mutant animals and suppresses the improvement of the performance by nicotine [46]. Activation of β2-containing nAChRs by endogenous ACh is thus likely to take place in the course of these memory tasks.

Preliminary results indicate that the knock-out of the α7 subunit yields animals that survive normally but display an anomalous synchronisation during EEG recordings [72].

Nicotine enhancement of memory processes has motivated clinical trials of nicotinic treatment in the Alzheimer's disease (AD). The severity of symptoms in AD is well correlated with a reduction in cortical acetycholine ([73] and references therein) and AD patients exhibit a marked reduction in the number of high affinity nicotine binding sites [74]. Nicotine treatment partially relieves the cognitive deficits of AD [75, 76]. The site of this beneficial action of nicotine is not yet clearly established. For instance, nicotine may increase the levels of ACh in the cortex by recruiting presynaptic nAChRs on ACh terminals in the cortex (e.g., [77]). As the use of nicotine presents a number of side effects linked to the activation of peripheral nAChRs, attempts are made to find nicotinic drugs specific for brain subtypes such as ABT418 [78], SIB-1508Y [79] and RJR 2403 [80].

5. Nicotinic receptors in the reward system and tobacco abuse

The nAChRs subunits are abundantly expressed in the mesencephalic dopaminergic nuclei ([28] and references therein). These nuclei are part of the mesostriatal reward system. Theoretical work has underlined the critical function of reward systems in learning of behavioral rules by selection [81, 82]. Dysfunction or anomalous chemical stimulation of these systems strongly affects brain function. Indeed, the mesostriatal dopaminergic system is a common target of many addictive drugs (reviewed in [51, 83]).

Accumulating data suggest that both tobacco smoking in humans and nicotine self-administration in animals are associated with an increase in dopamine release following nicotinic actions on the mesencephalic dopaminergic neurons (reviewed in [84]). Self-administration of nicotine shares common mechanisms with that of other addictive drugs. Minimal doses of nicotine, comparable to those causing self-administration behavior, trigger a specific increase of metabolism and the release of dopamine in the nucleus accumbens, as observed with strongly addictive drugs such as cocaine or amphetamines [85]. Nicotine and cocaine self-administration activates a number of common brain structures, as visualized with c-Fos immunoreactivity, notably the terminal fields of the mesencephalic dopaminergic neurons [86]. It should be noted that tobacco smoking is not only associated with nicotine intake but also with respiratory sensations of smoke intake (review in [84]); nicotine action could also be amplified by changes in dopamine metabolism, since smokers display a 40% reduction of monoamine oxidase B compared to former smokers or non-smokers [87].

What is the composition of the nAChRs involved in the self-administration of nicotine? The concentration of nicotine in the plasma of smokers is in the 100–500 nM range [88]. Oocyte experiments with human nAChRs reveal which combinations of subunits may respond to such low concentration of agonists in vivo. EC_{50} values below 10 μM have been observed for α4β2, α4β4 α3β2 ([33] but see [36]) and α3β2α5 [27]. In situ hybridization experiments (references in [28]) indicate that the nAChR subunits forming these combinations (except β4) are expressed in the mesencephalic nuclei. These nuclei also contain high amounts of the α6 and β3 subunit mRNAs, suggesting the contribution of an α6β3β2 subtype [28]. Recent work on the β2-knock-out mice show that the β2-mutant animals no longer self-administer nicotine after priming by cocaine, indicating that β2 is part of the nAChRs involved in nicotine reinforcement [89]. Furthermore, in the mutant animals low concentrations of nicotine fail to stimulate the mesencephalic dopaminergic neurons recorded on slices in vitro and systemic nicotine injection no longer enhances the striatal release of dopamine [89].

The chronic intake of nicotine results in a so-called 'neuronal adaptation'. This includes desensitization of nAChRs (reviewed in [90]) [91]. Desensitization of neuronal nicotinic receptors may recover only in tens of minutes (e.g., [91–94]) and thus likely contributes to short term adaptation to nicotine. Furthermore, nAChRs may directly contribute to endogenous reward processes. Indeed, they contribute to cholinergic synaptic transmission between the posterior cholinergic nuclei (latero-dorsal tegmental nucleus, pedonculopontine nucleus) and the mesencephalic dopaminergic neurons (e.g., [95, 96]). Chronic nicotine treatment thus likely affects the brain reward mechanisms as shown with self-stimulation protocols [97] or nicotine withdrawal experiments [98].

6. The nicotinic receptors in psychiatric and neurological disorders

The high prevalence of tobacco-smoking in schizophrenic patients suggests that nicotine intake by cigarette consumption may be a form of self-medication. By stimulating the mesencephalic dopaminergic system (see above), and more specifically by increasing the burst firing of dopaminergic neurons to burst activity, nicotine might compensate for the hypofrontality observed in schizophrenia [9]. Nicotine has been found to reverse the cognitive deficits produced by haloperidol in schizophrenics [100]. A synergy between nicotine and dopaminergic neuroleptics also exists in the treatment of Tourette's syndrome [101]. Nicotine has been proposed as an alternative to drugs increasing the brain levels of dopamine in the treatment of attention deficit/hyperactivity disorder [102]. Finally, nicotine and various nicotinic agonists might help to compensate the deficit in striatal dopamine in Parkinson's disease patients and might, in some instances, relieve the symptom of the disease ([103] but see [104]). The interaction of nicotine with the mesencephalic dopaminergic system might thus explain its action on psychiatric and neurological disorders.

Schizophrenic patients often exhibit, among diverse symptoms, a diminished habituation to auditory stimulation (reviewed in [105]). Experiments in the rodent have shown that auditory gating is impaired by antagonists of α7 nAChRs. Furthermore, the number of α-bungarotoxin sites is reduced in post-mortem brains of schizophrenics. The deficit of sensory gating in schizophrenics might thus be due to a reduction, or a loss, of α7 nAChR function. Consistent with this hypothesis, genetic analysis in nuclear families with at least two cases of schizophrenia has shown that the deficit in auditory gating is significantly linked to a genetic marker neighboring the locus of the α7 gene [106]. A study of the relatives of schizophrenics sharing the deficit in auditory gating revealed that nicotine could reverse the deficit, presumably by activating α7 nAChRs (references in [105]). This result is quite unexpected, since human α7 nAChRs exhibit a low sensitivity to nicotine (see *Introduction*). However, recent experiments in the chick [107] and rat [108] have

shown that low doses of nicotine can activate α-bungarotoxin sensitive nAChRs in glutamatergic nerve terminals. The α7 nAChR subunit, possibly together with still unidentified subunit(s) (see however, [109]), may thus form another relevant target for nicotinic therapies of psychiatric disorders.

7. Conclusion

Over 25 years after the identification and purification of the *Torpedo* nAChR (see [30]), the accumulating knowledge on the nAChRs in vertebrates has led to the demonstration that alterations of these receptors are responsible for a variety of familial disorders of the central and peripheral nervous system. Conversely, these receptors are now considered as relevant targets for nicotinic therapies of brain disorders.

Previous experiments combining photoaffinity labeling and site-directed mutagenesis had shown that changes of critical amino acids, in the nAChR channel or ligand binding site, may markedly affect its function in a pleiotropic manner. They may for instance, either reduce or increase channel opening in the presence and sometimes in the absence of acetylcholine by altering the allosteric properties of the protein. Interestingly, analogous, if not identical, point mutations in human nAChR genes (and glycine receptor genes) have been shown to cause pathologies either by a loss or by an 'increase-of-function'. Mutations causing pathologies via changes in allosteric properties have also been described for G-protein linked receptors [110]. Development of novel nicotinic therapies with pharmacological agents targeted to these diverse 'allosteric' phenotypes may thus be anticipated.

While the strategic location of nAChRs in the dopaminergic reward system renders nicotine an addictive drug, it also underlies potential beneficial effects of nicotine in the treatment of psychiatric disorders. Furthermore, nAChRs may relieve symptoms of AD or schizophrenia via pathways different from the dopaminergic system; nicotinic agents which specifically activate nAChR subtypes absent from the dopaminergic system, and thus with no (or diminished) addictive properties should, therefore, be looked for.

Fundamental research on properties of nAChRs in normal and pathological situations opens many new strategies to design drug therapies targeted not only to specific nAChRs in defined brain circuits but also to specific allosteric transitions impaired by nAChR gene mutations in humans.

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Dissection of active zones at the neuromuscular junction by EM tomography

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Abstract — We used EM tomography to examine the fine structure of the apparently amorphous electron dense material that is seen at active zones of axon terminals when viewed by conventional 2D electron microscopy. Serial 1-nm optical slices from 3D reconstructions of individual thin tissue sections reveal that the material is composed of an interconnecting network of elongate components directly linked to synaptic vesicles and the presynaptic membrane. Each vesicle at the active zone that lies adjacent to the presynaptic plasma membrane has several such connections. Information provided by reconstruction data may be useful in generating experiments aimed at understanding the mechanisms involved in the docking of synaptic vesicles and their exocytosis during synaptic transmission. (©Elsevier, Paris)

Résumé — Dissection des zones actives à la jonction neuromuculaire par tomographie ME. Nous avons utilisé la tomographie ME pour examiner aux électrons la structure fine du matériel dense apparemment amorphe qui est observé au niveau des zones actives des terminaisons axonales en microscopie électronique conventionnelle 2D. Des reconstructions 3D obtenues à partir de coupes sériées de 1 nm font apparaître un matériel constitué d'un réseau connectif de composés directement liés aux vésicules synaptiques et à la membrane présynaptique. (©Elsevier, Paris)

neuromuscular junctions / active zones (3D fine structure of) / EM tomography

1. Introduction

Conventional 2D electron microscope images of neuromuscular junctions made from thin sections of fixed, metal stained and plastic embedded tissue reveal that the synaptic vesicles in the axon terminal are focused on patches of electron dense material lining the cytoplasmic surface of the presynaptic membrane [1]. It is along side these patches that the vesicles undergo exocytosis to release their acetylcholine into the synaptic cleft during synaptic transmission [1, 3]. Thus, the patches of dense material and their associated vesicles mark the 'active zones' [1] of the presynaptic membrane. The close and constant positioning of synaptic vesicles adjacent to the dense material has led to the concept that vesicles are directly associated with the material prior to exocytosis and that such an association is necessary for normal synaptic transmission to occur. Indeed, one would expect the recently identified proteins [11] thought to be involved in vesicle docking and exocytosis to be at or near the dense material. The dense material in 2D images of thin sections does often appear to have substructure, but because the dimensions of the components are considerably less than the thickness of a section, their outlines are usually indistinct and they are not obviously organized. Moreover, although the dense material seems to contact the vesicles and plasma membrane, the likelihood of such connections is made uncertain by the absence of z axis information; e.g., vesicles and portions of the material that seem to contact each other in the x-y axis of even the thinnest sections that can be cut (30–50 nm) might actually lie in different z axis planes.

We have begun to examine the patches of active zone material in 3D reconstructions of sections from muscles prepared by the same techniques used for conventional 2D electron microscopy. The reconstructions are generated by electron microscope tomography. This is part of a long range EM tomography study of the entire synaptic apparatus, including the basal lamina of the synaptic cleft and the band of electron dense material that lines the cytoplasmic surface of the postsynaptic membrane. Like the active zone material, these structures appear nearly amorphous in 2D images and they are known to contain aggregates of proteins that are involved in synaptic function. If this material has relationships and organization that are obscured in 2D images by the absence of z axis information, they might be ap-

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parent in 3D reconstructions. The exposure of such architecture might then make it possible to accurately map at the nanometer level the relative position of synapse specific proteins within it through the use of, for example, antibodies, toxins, or gene deletions. Such information is crucial for a thorough understanding of the mechanisms involved in the assembly of synaptic apparatus and how each of its components functions.

EM tomography involves the integration of multiple 2D images of a section made at different degrees of tilt relative to the electron beam [2]. It has been extensively used by others over the last decade to determine the structure of isolated macromolecules, organelles and viruses [7, 12–14]. It has also been used on tissue sections for examining the contractile apparatus of muscle fibers [9] and the membrane arrangement in mitochondria [8]. Here we present our initial findings on the structure of the active zone material and its relationship to synaptic vesicles at the frog's neuromuscular junction, which has long been a model for studies on synaptic structure and function [1, 3–5].

2. Materials and methods

2.1. Fixation, staining, embedding and microscopy for the reconstruction

Immediately after killing a *Rana pipiens*, the skin overlying the thin cutaneous pectoris muscles was slit open and tissue paper soaked in 1% phosphate buffered glutaraldehyde (240 mOsM) was placed directly on the muscles. 30 min later the muscles were removed and bathed in the same solution for 30 min more. After rinsing them with phosphate buffer, the muscles were immersed in phosphate buffered osmium tetroxide, rinsed in distilled water, treated with saturated aqueous uranyl acetate, dehydrated in ethanol and propylene oxide and embedded in Epon. Thin sections having silver interference colors were mounted on single slot Formvar coated grids and stained with uranyl acetate followed by lead citrate. They were then coated with dilute 10 nm colloidal gold particles to provide fiducials for the alignment of tilt images. The data were collected with a Philips CM200 electron microscope, which was equipped with a FEG, cryoholder and 1024 × 1024 CCD. The cryoholder was filled with liquid nitrogen which maintained the specimen at ~ –186 °C to inhibit beam-induced specimen shrinkage during data collection. Tilt images were collected at 2° intervals to plus and minus 66° from horizontal. The images were made at a microscope magnification of × 34 000; a single pixel on the CCD corresponded to 0.26 nm. The total beam dosage during data collection was ~ 350 000 e^-/nm^2.

2.2. Filtering, alignment and reconstruction

Each 2D image in a tilt series was first filtered to reduce noise using a steerable pyramid-scheme [10]. Next, the images were coaligned using an automatic computer algorithm that detects the location of the fiducial gold on each image. The accuracy of alignment is a major determining factor in the spatial resolution in the volume; the alignment accuracy on the data set presented here is 1 nm RMS. Finally, weighted back projection was used to convert the coaligned set of 2D images into a 3D volume.

3. Results and discussion

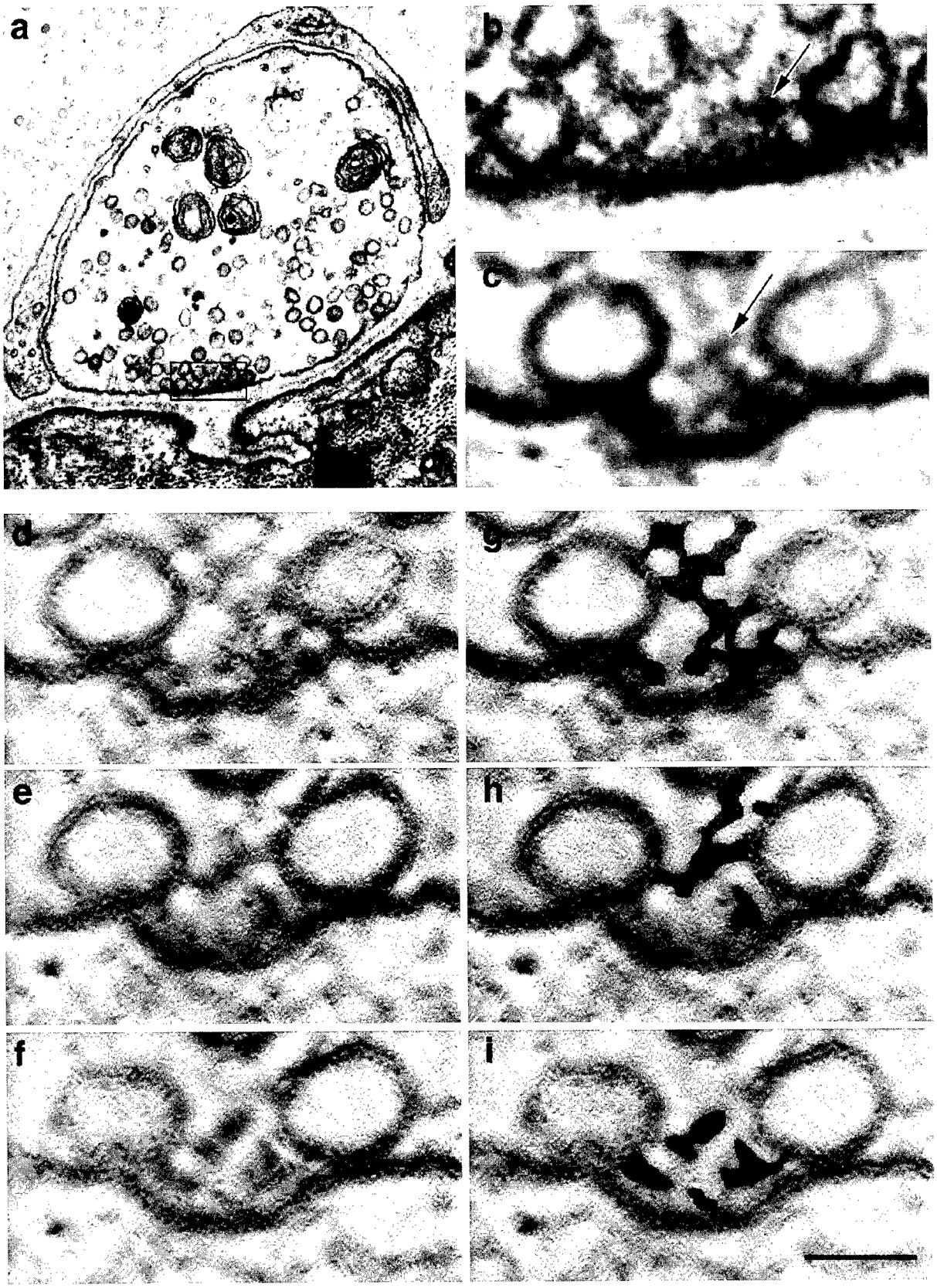
Figure 1a is a 2D image of a section from a neuromuscular junction made by conventional electron microscopy. It shows the synaptic vesicle-containing axon terminal and the surface of the muscle fiber characterized by its infolding of the postsynaptic plasma membrane. The plasma membranes of the terminal and muscle fiber are separated by the basal lamina-filled synaptic cleft. This axon terminal profile has a single active zone (box). Typically, the active zone is situated just opposite the infolding in the muscle fiber's plasma membrane.

Figure 1b is a higher magnification view of the active zone in *figure 1a*; the field corresponds to that outlined by the box in *figure 1a*. The patch of electron dense active zone material (arrow) is amorphous and appears to bear a close relationship to the vesicles associated with it, some of which are also closely associated with the plasma membrane.

Figure 1c is a 2D image of a 3D reconstruction of a section of a neuromuscular junction made by EM tomography. It was formed by summing the central 40 nm of the volume along the z axis. The field is similar to that in *figure 1b*; a synaptic vesicle closely apposed to the presynaptic membrane is situated on each side of the active zone material (arrow). By counting voxels in the z axis of the full reconstruction, it was determined that the total thickness of

Figure 1. Electron dense material at active zones of the frog's neuromuscular junction in images made by conventional 2D EM and by EM tomography. **a.** Low power view of an axon terminal and muscle fiber surface made by 2D EM. Box outlines active zone. **b.** Higher power view of active zone in box in **a** showing electron dense active zone material (arrow). **c.** 40 nm thick reconstruction of an active zone made by EM tomography showing electron dense active zone material (arrow). **d, e, f.** 1 nm thick optical sections from different z axis planes of the reconstruction shown in **c**. **g, h, i.** Same optical sections as in **d, e, f** with certain components of the active zone material are emphasized. Scale bar, **a,** 400 nm; **b,** 88 nm; **c–i,** 50 nm.




a
b
c
d
g
e
h
f
i

the section was 50 nm; the top and bottom 5 nm were discarded because they contained obvious artifact. The contrast and delineation of the plasma and vesicle membranes in the reconstruction compares favorably to that of the same structures in conventional 2D images (*figure 1b*) as does the active zone material.

Figure 1d, e and *f* show three optical slices, 1 nm (4 voxels) thick, from the reconstruction in *figure 1c*. All are in the x-y plane but each is from a different level in the z-axis. They illustrate what we have now observed at all of several active zones so far examined in this way: 1) the active zone material is composed of elongate interconnecting structures; 2) the structures abut synaptic vesicles and the presynaptic plasma membrane; and 3) each vesicle is contacted by several such structures. *Figure 1g, h* and *i* are duplicates of *figure 1d, e* and *f*; the gray-scale density of some components of the active zone material was selectively increased to facilitate viewing.

Although images from the reconstruction leave little doubt that the patch of active zone material consists of a complex arrangement of measureable subcomponents and that certain of these components are linked to synaptic vesicles and presynaptic plasma membrane, we have not yet identified a pattern of component shapes and organization common to all patches of active zone material. This might be expected if, for example, the active zone material associated with each vesicle were a functional unit. In order to be able to recognize any such patterns within the complexity of structures at an active zone, we are currently developing volume segmentation methods to extract and quantify shapes and relationships. It may well be that aldehyde fixation, as used for the reconstruction demonstrated here, distorts the material at different active zones in a random way, hindering recognition of a common organizational scheme. Accordingly we have also begun to reconstruct active zones in muscles prepared by 'ultra-rapid' freezing and acetone freeze-substitution, which provide better preservation of certain cellular structures than aldehyde fixation [6].

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Toxins selective for subunit interfaces as probes of nicotinic acetylcholine receptor structure

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Abstract — The pentameric structure of the nicotinic acetylcholine receptor with two of the five subunit interfaces serving as ligand binding sites offers an opportunity to distinguish features on the surfaces of the subunits and their ligand specificity characteristics. This problem has been approached through the study of assembly of subunits and binding characteristics of selective peptide toxins. The receptor, with its circular order of homologous subunits ($\alpha\gamma\alpha\delta\beta$), assembles in only one arrangement, and through mutagenesis, the residues governing assembly can be ascertained. Selectivity of certain toxins is sufficient to readily distinguish between sites at the $\alpha\gamma$ and $\alpha\delta$ interfaces. By interchanging residues on the γ and δ subunits, and ascertaining how they interact with the α-subunit, determinants forming the binding sites can be delineated. The α-conotoxins, which contain two disulfide loops and 12–14 amino acids, show a 10 000-fold preference for the $\alpha\delta$ over the $\alpha\gamma$ subunit interface with $\alpha\varepsilon$ falling between the two. The waglerins, as 22–24 amino acid peptides with a single core disulfide loop, show a 2000-fold preference for $\alpha\varepsilon$ over the $\alpha\gamma$ and $\alpha\delta$ interfaces. Finally, the 6700 Da short α-neurotoxin from *N. mossambica mossambica* shows a 10 000-fold preference for the $\alpha\gamma$ and $\alpha\delta$ interfaces over $\alpha\varepsilon$. Selective mutagenesis enables one to also distinguish α-neurotoxin binding at the $\alpha\gamma$ and $\alpha\delta$ subunits. This information, when coupled with homology modeling of domains and site-directed residue modification, reveals important elements of receptor structure and conformation. (©Elsevier, Paris)

Résumé — Liaison sélective de toxines aux interfaces entre sous unités : structure du récepteur cholinergique. La structure pentamérique du récepteur nicotinique, dans laquelle deux des cinq interfaces entre sous-unités servent de sites de liaison pour l'acétylcholine, offre la possibilité de distinguer les caractéristiques des deux sites et leur spécificité pour les ligands. Nous avons abordé l'étude de l'assemblage des sous-unités par la liaison de toxines peptidiques sélectives. Le récepteur s'assemble d'une seule manière avec un ordre circulaire unique de sous-unités ($\alpha\gamma\delta\alpha\beta$), et il est possible d'identifier les résidus qui contrôlent ces interactions par mutagenèse dirigée. La sélectivité de certaines toxines est suffisante pour distinguer clairement les sites des interfaces $\alpha\gamma$ et $\alpha\delta$. En échangeant des résidus entre les sous-unités α et δ, et en analysant leurs associations avec la sous-unité α, on peut définir les déterminants des sites de liaison. Les conotoxines α formées de 12–14 acides aminés et contenant deux ponts disulfure se lient 10 000 fois mieux au site $\alpha\gamma$, et de façon intermédiaire à $\alpha\varepsilon$. Les waglérines, formées de 20–24 acides aminés et possédant un seul pont disulfure se lient 2 000 fois mieux à $\alpha\varepsilon$ qu'à $\alpha\gamma$ et $\alpha\delta$. Finalement, la neurotoxine α courte de 6 700 Da de *Naja mossambica mossambica* présente une préférence de 10 000 fois pour les interfaces $\alpha\gamma$ et $\alpha\delta$, par rapport à $\alpha\varepsilon$. La mutagenèse permet aussi de distinguer la liaison de l'α neurotoxine aux sites $\alpha\gamma$ et $\alpha\delta$. Ces informations, associées à la modélisation des domaines par homologie et la modification de résidus par mutagenèse dirigée, donnent d'importants éléments pour comprendre la structure et la conformation du récepteur. (©Elsevier, Paris)

homologous subunits / nicotinic acetylcholine receptor / ligand binding sites

The nicotinic acetylcholine receptor (nAChR) is a circular pentamer composed of four homologous subunits with the stoichiometry of $\alpha_2\beta\gamma\delta$ arranged around a central channel [4, 8]. In the mature neuromuscular junction of mammals, the ε-subunit replaces γ with minimal changes in specificity for acetylcholine and the alkaloid antagonists. The two binding sites exist at the $\alpha\gamma$ (or $\alpha\varepsilon$) and $\alpha\delta$ interfaces; simultaneous occupation of the two sites gives rise to channel opening. Occupation of one of the two sites by an antagonist is sufficient to result in very low probabilities of channel opening, even in the presence of agonist.

The close sequence identity of the γ, δ and ε subunits and the fact that homologous residues should exist in the same three-dimensional space in a oligomer composed of related subunits offer the possibility of residue substitution to examine the relation between structure and specificity of binding events occurring at the subunit interface.

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Our previous studies have examined several determinants of subunit assembly, both in terms of a preferential subunit order [9] and kinetics of assembly [9, 16]. These studies have identified and confirmed the likely presence of particular residues at the subunit interface. The requirements of a specific subunit order revealed that Lys 145 and Lys 150, found in the δ subunit but not in γ and ε, form the structural basis for the δ subunit's resistance to be located between the two α-subunits [9]. Moreover, residues 145 and 150 reside on the non-ligand binding surface of the δ subunit.

When studying whether subunit glycosylation would affect α-conotoxin association as it does for the larger α-neurotoxins (*figure 1*), we noted that α-conotoxin M1 only associated with high affinity at one of the two subunit interfaces [10]. More detailed studies showed that it bound to the αδ subunit with 30 000 times the affinity of αγ. $K_{D,\alpha\delta} = 6.2 \times 10^{-10}$ M and $K_{D,\alpha\gamma} = 2.0 \times 10^{-5}$ M (*figure 2A*). Preferential binding to the αδ site was also demonstrated by transfection of αδ or αγ subunit pairs into null cells and examining ^{125}I-α-conotoxin M1 (Tyr 12) binding to the isolated subunit pairs in permeabilized cells [17]. An extensive set of studies initially with chimeras of γ and δ subunit sequences followed by site-specific mutants revealed that three regions in the γ and δ subunits, corresponding to residues 34, 111 and 172 in γ, form the basis for subunit specificity; the residues are likely found at the γ and δ interfaces of the two binding sites [15]. Studies on the α-subunit, whose opposing face should constitute the corresponding portion of the binding site showed that positions in the vicinity of residues 93, 152–154 and 180–200 were contributory to ligand binding [17]. These general regions had been identified previously through photolytic labeling with agonists [5, 6] and site-specific mutagenesis [12, 14, 18]. The influence of combined α + δ and α + γ subunit mutations appeared additive in terms of free energy changes [17].

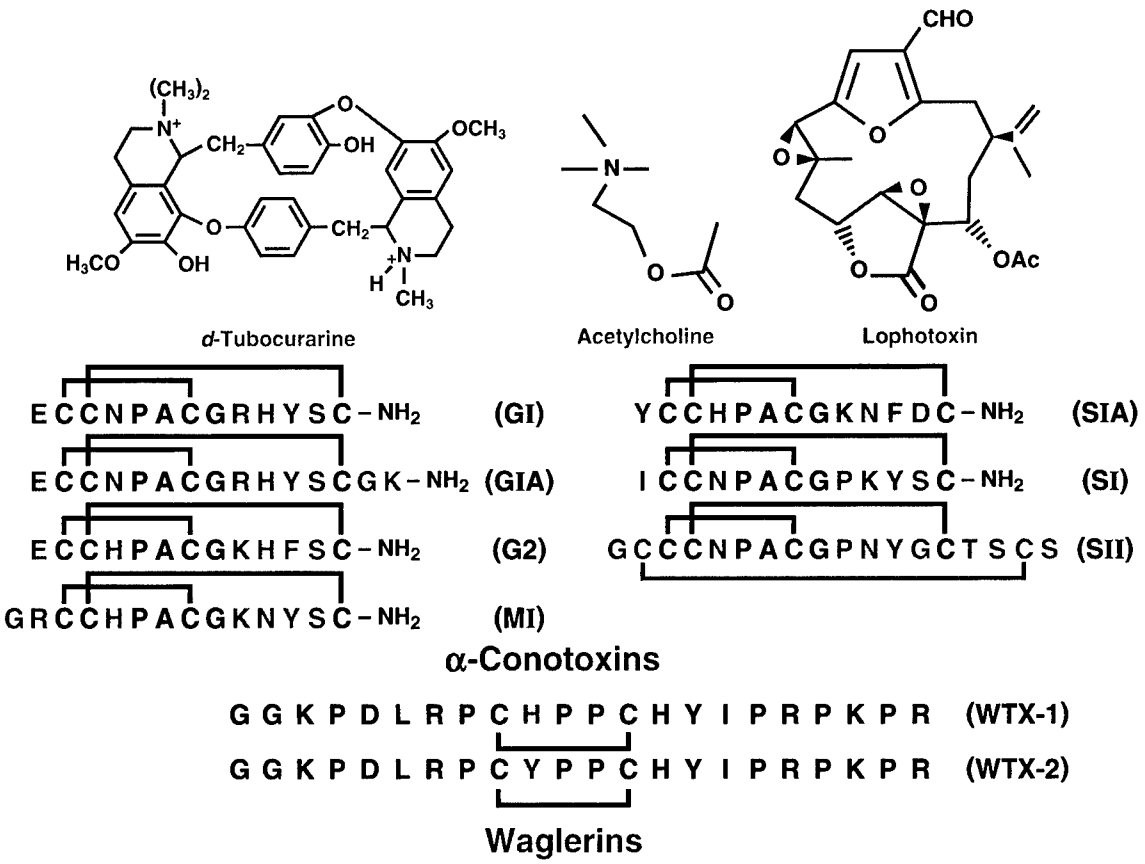


Figure 1. Structures of acetylcholine and various antagonists of the nicotinic receptor. *d*-Tubocurarine is an alkaloid from a plant source, lophotoxin is found in coral of the genus *Lophogorgia*, α-conotoxins are from a Pacific snail, *Conus* sp., and waglerins are from the pit viper, *Trimeresurus wagleri*. Not shown are the 65–75 amino acid, three fingered α-neurotoxin peptides such as α-bungarotoxin and *Naja mossambica mossambica* I α-toxin.

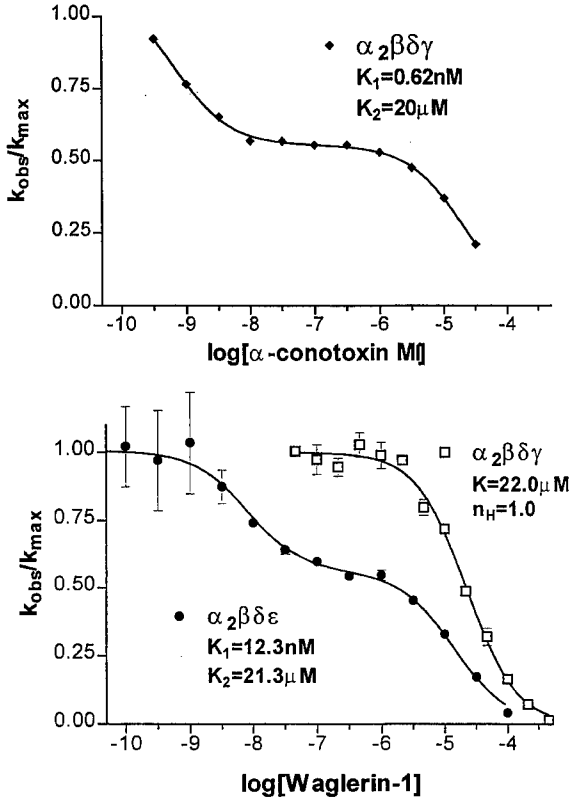


Figure 2. Measurement of small peptide antagonist binding to nicotinic receptors by competition with the initial rate of α-bungarotoxin binding. **A.** Binding of α-conotoxin M1 to the nicotinic receptor expressed as $\alpha_2\beta\gamma\delta$ after prior transfection of the cDNAs encoding the α, β, γ and δ subunits into HEK-293 cells. **B.** Binding of waglerin 1 to the nicotinic receptor expressed as $\alpha_2\beta\varepsilon\delta$. Dissociation constants are calculated from the competition curves assuming an equal distribution of binding sites.

While most mutations of the α subunit affected α-conotoxin M1 binding equally at the two sites, certain mutations in the common α-subunit only or primarily affected α-conotoxin binding at one of the two interfaces. For example, the mutations αY93F and αV188K enhanced and reduced α-conotoxin M1 affinity, respectively, but only at the low affinity, αγ site. In contrast, the mutation αD152N only affected α-conotoxin M-1 affinity at the high affinity, αδ site. This would indicate that the actual orientation of the α-conotoxins, when bound at the αγ and αδ interfaces, are not identical, a point to which we will return later.

A low molecular mass family of toxins, the waglerins, appeared to be a second candidate for these specificity considerations since the waglerins were shown to be toxic to adult mice [20] (containing ε and δ subunits), but not to embryonic and newborn animals (containing γ and δ subunits) (McArdle J.J., personal communication). Since binding at one subunit interface is sufficient for functional antagonism, toxicity should arise from a preferential interaction at the αε interface. That this is the case is revealed by expression of receptors of compositions αεαδβ and αγαδβ (*figure 2b*). Studies with chimeras of ε sequence with either γ or δ sequences reveal that two areas corresponding to residues 57–61 and again 172–174 in ε are responsible for the specificity of the waglerins (Molles et al., abstract, this conference).

Waglerins also show greater toxicity in mice than rats. Rat and mouse ε-subunits only differ by 10 residues in their 210 residue extracellular domain. The analysis, through site specific mutagenesis, shows residues that reside at positions 115 and 59 in the ε and γ subunits contribute to the differences between mouse and rat in binding waglerin 1.

A third toxin, *Naja mossambica mossambica* (N.m.m.I toxin), an α-neurotoxin with a molecular mass of 6700, reveals a selectivity for the αγ, αδ and αε binding interfaces that is precisely the opposite of waglerin. The α-neurotoxins of this family were the primary ligands which enabled the identification and initial purification of the nicotinic receptor [4, 11]. Early chemical cross-linking studies involving the α-toxins with the *Torpedo* receptor showed likely contact surfaces with both the α and the γ/δ subunits [13]. The extensive mutagenesis studies of Menez and colleagues [19] have shown many of the α-toxin residue positions involved in the multipoint attachment. Typically the α-toxins, such as α-bungarotoxin and the cobra α-toxins, show near equivalent dissociation constants for their two binding sites in the intact receptor; this is also the case for N.m.m.I toxin with the mouse $\alpha_2\beta\gamma\delta$ receptor where we observe equivalent binding constants whether measured directly or by competition [1]. Surprisingly, the $\alpha_2\beta\varepsilon\delta$ combination shows high and low affinity binding sites with N.m.m. α-toxin; hence the ε subunit interface is resistant to forming a high affinity complex (Osaka et al., this volume). Studies with chimeras and site-specific mutants pinpoint residues 175 and 176 as influencing N.m.m.I toxin binding, a region just beyond the 172 and 174 residues responsible for specificity of the shorter toxins, α-conotoxin and waglerin 1. Mutations of P175T and E176A substituted into a γ-subunit template yield a low affinity for N.m.m.I α-toxin, equivalent to that of the ε subunit. The inverse substitutions T175P and A176E in an ε-template give rise to a return of the high affinity. This points to a common region in the γ, δ and ε subunits with

which the α-toxin associates, and it should be possible to identify the complementary α-toxin residues presumably in apposition with this region on the receptor.

We have also embarked on studies where we have examined pair-wise interactions between residues on the α subunit of the receptor and the α-toxin [2]. Since Coulombic forces are long range, electrostatic forces lacking specific orientational constraints, we have begun with charged substitutions on both the receptor and α-toxin in order to examine residue proximity. To date, we have examined residues on loop II (K27, R33, R36) and loop III (K47) and residues on the receptor α-subunit between 180 and 200. *Figure 3* shows the influence of three toxin mutations on N.m.m.I binding to the receptor. In the case of two of the mutations, shallow curves are observed showing that the binding constants are no longer the same. K_D's shown in the legend assume equal population of two sites. If the αδ site is protected with α-conotoxin M1, only 50% of the sites remain and the binding curve steepens. The calculated dissociation constant for the unprotected site corresponds to $K_{D\alpha\gamma}$ and should reflect one of the two dissociation constants calculated from *figure 3* [1]. Hence, toxin modification (*figure 3*) and certain modifications of the receptor in the α-subunit (not shown) reveal a non-equivalence in the binding sites. This is also indicates that α-toxin binding orientations may not be equivalent at the two sites.

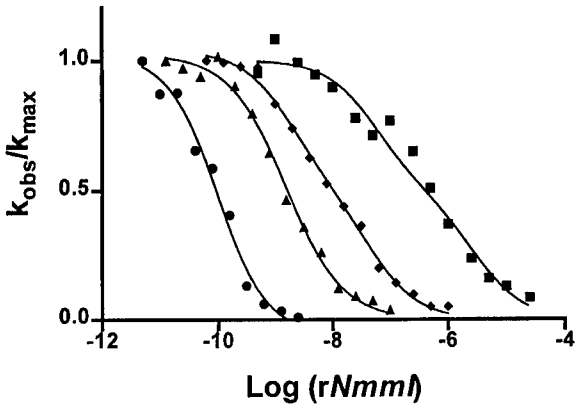


Figure 3. Equilibrium binding of wild-type rNmmI and rNmmI mutants to wild-type nAChR. Binding of either wild-type rNmmI (●), K47A (▲), K27E (◆), or R33E (■) was measured as the fractional reduction in the initial rates of [^{125}]-α-bungarotoxin binding in the absence of rNmmI (k_{max}). The curves for wild-type K_D = 0.14 nM and for K47A K_D = 1.5 nM are least-squares fits to the Hill equation with n_H = 1.0. The two sites behave equivalently for the toxins. The curves for K27E and R33E are least-squares fits to two sites present in equal populations. The dissociation constants were determined as: K27E, $K_{D\alpha,\delta}$ = 1.8 nM; $K_{D\alpha,\gamma}$ = 54 nM: R33E, $K_{D\alpha,\delta}$ = 95 nM; $K_{D\alpha,\gamma}$ = 2.2 μM. The assignment of γ or δ to the site comes from α-conotoxin protection experiments as described in the text (modified from Ackermann and Taylor [1]).

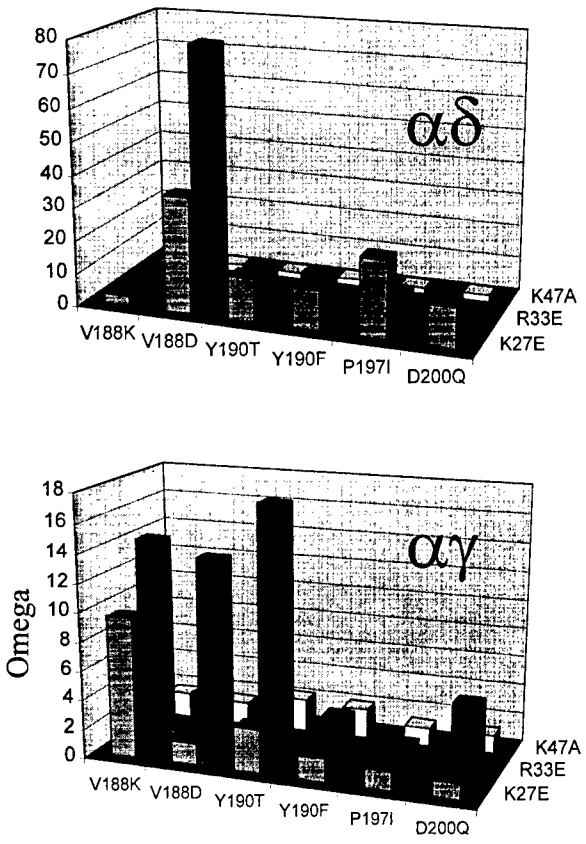


Figure 4. Plot of the Σ values calculated according to equations below for each of the mutant pairs at the αδ binding site (upper panel) or αγ binding site (lower panel). Note that the scale for Σ values differs between the two plots. The value for the R33E/V188D pair at the αγ site is 12, with the exact number not determined due to the very large loss in the overall binding affinity.

$$\Omega = \frac{K_{D(WT-T,WT-R)} \cdot K_{D(MT-T,MT-R)}}{K_{D(MT-T,WT-R)} \cdot K_{D(WT-T,MT-R)}}$$

where the subscripts WT and MT designate wild-type and mutant and T and R designate toxin and nAChR. The pairwise interaction free energy may be calculated from: $\Delta\Delta G_{int} = RT\ln\Omega$ (adapted from Ackermann et al. [2]).

A compilation of interaction energies from the respective K_D values should delineate pair-wise toxin-receptor interactions enabling one to identify interactive (*figure 4*) and proximal residues on the two molecules in the complex [2]. Minimally, the

position of the α-toxin loops with respect to the subunit faces should emerge from this approach. Second, we have built a model of the extracellular portion of the receptor based on sequence homology of a limited segment of 100 residues with a family of copper binding proteins and residue positions inferred from mutagenesis and site directed labeling [21]. A future challenge is presented in the fitting of the three peptide toxins within the framework of the model structure. Although such an approach will not achieve structural detail comparable to direct physical measurements, the X-ray crystallographic structure of the homologous peptide, fasciculin, with its target acetylcholinesterase provides an interesting template for understanding how the three loops of these toxins interact with a macromolecular target surface [3, 7].

Acknowledgment

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The role of subunit interfaces for the nicotinic acetylcholine receptor's allosterism

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Abstract — Cross-linking of nicotinic acetylcholine receptors, combined with binding studies and patch-clamp electrophysiology, has proven the existence of a 'pre-existing equilibrium' of functional states and the functional role of subunit interfaces, two key postulates of the allosteric model. (©Elsevier, Paris)

Résumé — Le rôle des interfaces entre les sous-unités du récepteur cholinergique nicotinique. Des expériences de cross-linking des sous-unités du récepteur cholinergique nicotinique associées à des études de liaison et électrophysiologiques (patch-clamp) ont demontré l'existence d'un équilibre pré-existant entre plusieurs états fonctionnels et un rôle fonctionnel des interfaces entre les sous-unités, deux postulats clés du modèle allostérique. (©Elsevier, Paris)

acetylcholine receptor / allosterism / quaternary structure / cross-linking

1. Introduction

Neurotransmitter receptors, especially the ligand-gated ion channels are allosteric proteins. Allosterism, a concept developed to explain regulatory properties of proteins [1], includes several fundamental features which must be proven to make a protein an 'allosteric protein'. The most important of these are the following: i) the existence of a ligand-binding site sterically separated from the active site, which influences through conformational changes properties of the active site; ii) the existence of at least two functional states, active and inactive, in a dynamic equilibrium ('preformed', i.e., the equilibrium is present already in the absence of ligands), which is affected by ligand binding; iii) allosteric proteins are symmetrical complexes of at least two subunits, undergoing all-or-none conformational changes preserving the symmetry of the quaternary structure; and iv) ligand binding is cooperative (homotropic or heterotropic). The degree of cooperativity is expressed in terms of the Hill coefficient.

The nicotinic acetylcholine receptor (AChR) according to these criteria is an allosteric protein [2], although an 'unconventional' one [3]. Sterically separate ligand binding sites affecting each other, a dynamic equilibrium between states affected by ligand binding, a (not quite symmetrical) quaternary structure, cooperativity of ligand binding and activation (channel opening), are all well documented. The structural basis of the receptor's allosterism is beginning to emerge. A key role presumably is played by the subunit interfaces: the ion channel to be opened by cooperative agonist binding is formed by the helices M2 contributed by the five subunits [4, 5]; and agonist binding itself is cooperative. With the agonist binding sites located on the two α-subunits which have been shown to be separated in the circular quaternary structure by the γ-subunit, communication between sites (and with the channel-forming domains) has to be 'long-range' across subunit boundaries.

Evidence has been accumulated that the agonist and competitive antagonist-binding sites are located at the interfaces between the α-subunits and its neighboring γ- and δ-subunits, respectively (reviewed in [6]). Here we show that mobility at subunit interfaces indeed is essential for allosteric action of the AChR.

2. Materials and methods

AChR was prepared from the electric organ of *Torpedo californica*. Cross-linking of receptor-rich membranes with dimethylsuberimidate (DMS) and all other chemical reactions were performed as described in [7]. Binding assays with tritiated acetylcholine were performed by ultracentrifugation after the acetylcholinesterase had been blocked by incubation with eserine [7]. Patch clamp electrophysiology with the xhole-cell configuration was performed with rat muscle AChR expressed in *Xenopus* oocytes [7].

3. Results

Membrane-bound AChR was cross-linked with DMS. Binding studies showed (*figure 1*) that this re-

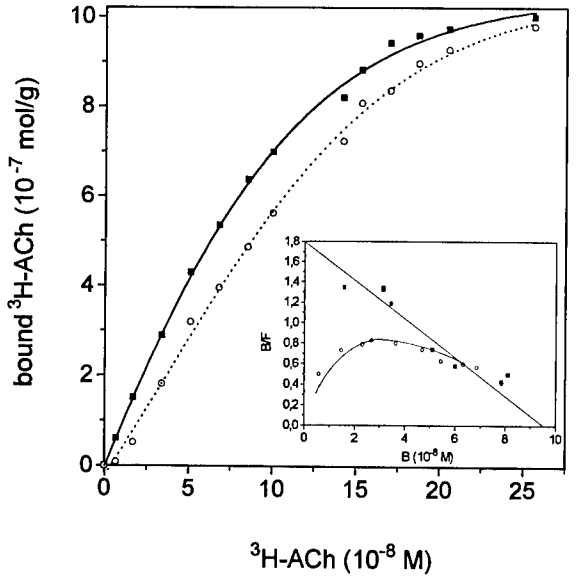


Figure 1. Properties of the nAChR from *Torpedo californica* after cross-linking with DMS. [^{3}H] ACh binding of native receptor (○) and after cross-linking with DMS (■). Inset. Scatchard plot (B, bound [^{3}H] ACh; F, free [^{3}H]ACh). The linear Scatchard plot after cross-linking indicates the loss of cooperativity (K_d = 50 nM; Hill coefficient n_H = 1.0).

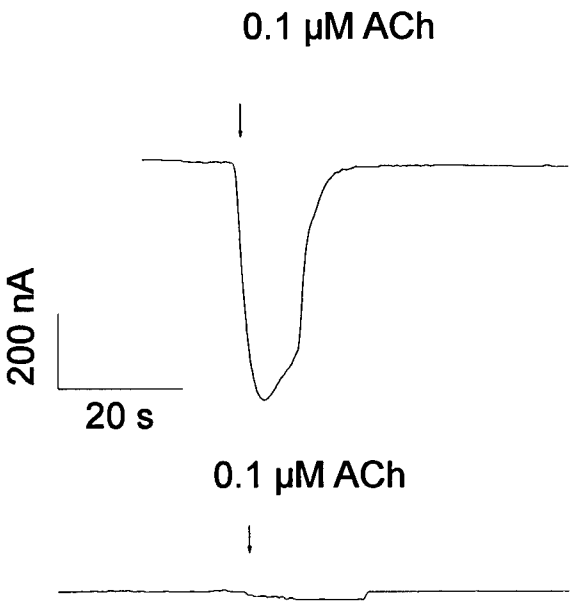


Figure 2. Effect of DMS cross-linking on ACh-induced current at nAChRs from rat muscle expressed in *Xenopus* oocytes. Whole-cell current induced by ACh application before (upper trace) and after (lower trace) 30 min of DMS cross-linking (< 10% remaining current). The oocytes were washed for at least 10 min after cross-linking, to remove excess of free cross-linker.

sulted in high affinity binding (K_D = 50 nM). Binding was non-cooperative (n = 1, as compared to n = 1.6 for the native control). High affinity in combination with a closed channel are the characteristics of the desensitized receptor [3]. Whole-cell current measurements with cross-linked rat muscle AChR expressed in *Xenopus* oocytes showed indeed that cross-linking had fixed the receptor in a channel-closed state (*figure 2*). This poses the question: why was the AChR in the absence of an agonist shifted to an apparently desen-

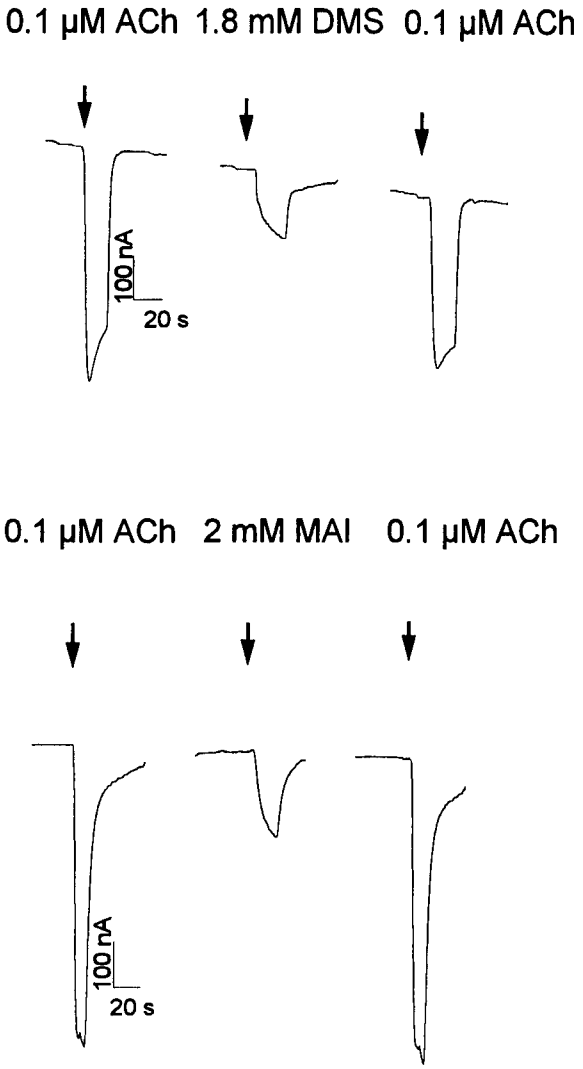


Figure 3. Agonistic effects of the imidates DMS and MAI with rat muscle nAChR expressed in *Xenopus* oocytes. Whole-cell current (rat muscle nAChR) induced by DMS application (upper trace) and by MAI application (lower trace). Whole-cell current induced by ACh application is shown before and after DMS/MAI application.

sitized state? The answer became obvious by appropriate electrophysiological measurements. DMS itself, perhaps through its positively charged imidate groups, acts as a weak agonist (*figure 3*).

The fixation in a desensitized state is due to bifunctional covalent cross-linking and not to monofunctional blockage of a functional group essential for desensitization. This was shown by using methylacetoimidate (MAI), the monofunctional homologue of DMS. This reagent is also an agonist, with about the same potency as DMS, but it was not able to irreversibly fix the receptor in the high affinity non-cooperative, channel closed state (*figure 3*). The imidate's hydrolysis products had no effect on the AChR.

If the hypothesis were right that a positively charged bifunctional cross-linking agent acts as an agonist and fixes the receptor in a desensitized state, other bifunctional reagents without this charge should fix other states. Indeed, we observed that glutardialdehyde fixed a low-affinity, channel-closed, non-cooperative state (*figure 4*). But incubation with the agonist carbamoylcholine at desensitizing concentrations prior to cross-linking with glutardialdehyde resulted in irreversible fixation of the desensitized state (data not shown).

More detailed analysis of the ^{3}H-acetylcholine binding curve of the receptor cross-linked with glutardialdehyde in the absence of any agonist showed an interesting phenomenon (*figure 4*). The binding curve was biphasic; besides the low affinity part (K_D = 5 μM) Scatchard analysis indicated that about 12% of the binding sites possess a high affinity (K_D = 35 nM). Obviously, the receptor in the absence of cholinergic ligands contains AChR in dif-

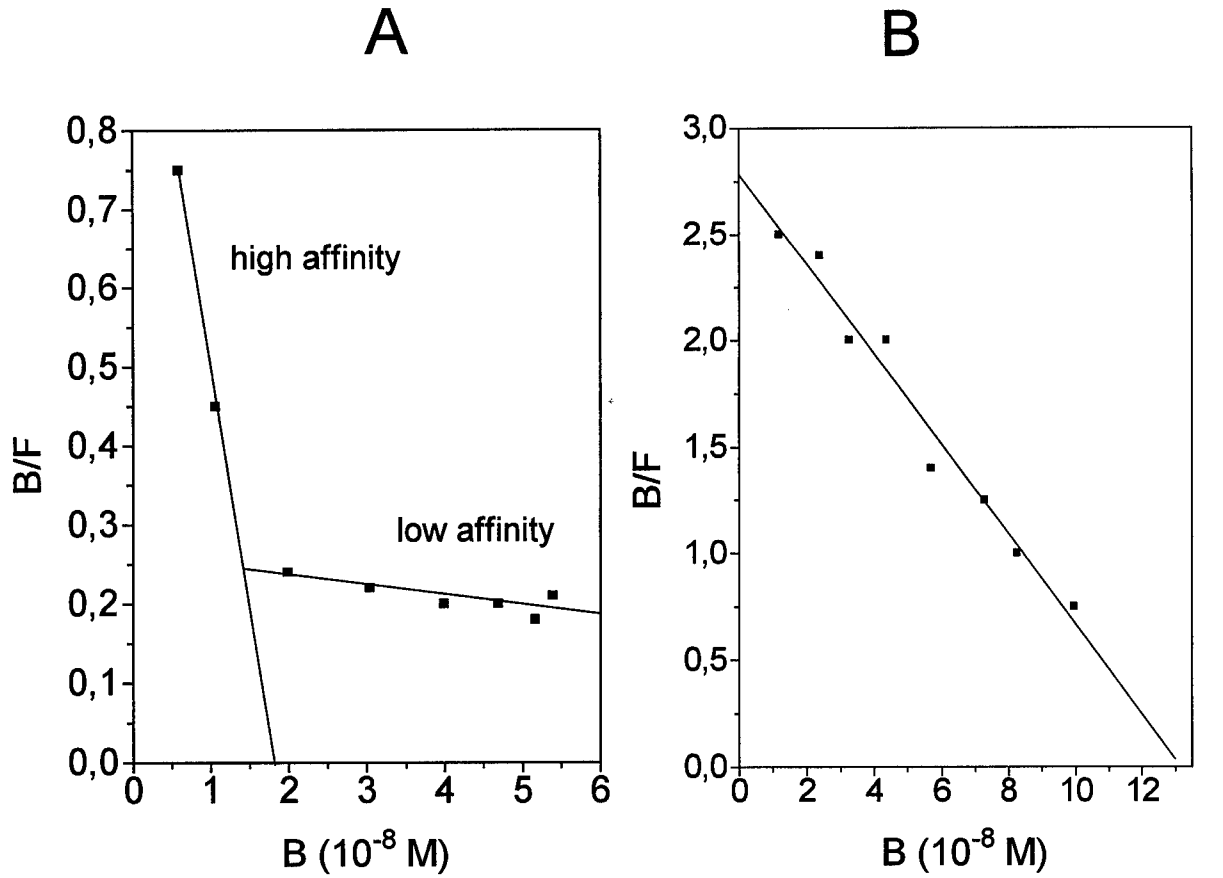


Figure 4. Properties of the nAChR from *Torpedo californica* after cross-linking with the non-agonistic cross-linker glutardialdehyde. **A.** Scatchard plot after cross-linking with glutardialdehyde in the absence of carbamoylcholine. A low-affinity binding site ($K_d = -1/m$ = 2–5 μM; n_H = 1.0) and a high-affinity binding site can be seen. Inset. Scatchard plot of the high-affinity binding site alone after glutardialdehyde cross-linking, as measured by using a much higher amount of protein to achieve higher concentrations of bound [^{3}H]ACh (K_d = 35 nM; n_H = 1.0). The low-affinity binding site can not be seen under these conditions. **B.** Scatchard plot after cross-linking with glutardialdehyde in the presence of carbamoylcholine (K_d = nM; n_H = 1.0)

ferent functional states, which we tentatively interpret as resting and desensitized. If this interpretation is correct, we have here a direct proof for the preformed equilibrium, postulated by the allosteric theory. Because of the slowness of the cross-linking reaction the intermediate active (channel-open) state can not be fixed by this method.

4. Conclusion

Immobilization of the AChR's subunits by covalent cross-linking with bifunctional reagents abolishes allosteric properties. From our experiments we conclude that the quaternary structure and especially the mobility at the subunit interfaces is essential for the regulatory properties of the receptor. Covalent fixation of receptor states may be a useful tool for quantifying the components of an equilibrium. Shifting the equilibrium to and fixing it in a single state might also reduce the heterogeneity of a receptor preparation and facilitate certain experiments as for example attempts at receptor crystallization.

Acknowledgments

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Allosteric modulation of neuronal nicotinic acetylcholine receptors

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Abstract — The structure-function relationship of the neuronal nicotinic acetylcholine receptor is examined in the light of the allosteric concepts. Effects of site-directed mutagenesis as well as those caused by allosteric effector of the physiological and pharmacological receptor properties are discussed. (©Elsevier, Paris)

Résumé — Modulation allostérique des récepteurs nicotiniques neuronaux. La relation entre la structure et la fonction du récepteur nicotinique neuronal à l'acétylcholine est examinée à la lumière des concepts allostériques. Les effets de mutations dirigées ainsi que ceux causés par des effecteurs allostériques sur les propriétés physiologiques et pharmacologiques du récepteur sont examinées en fonction de ces concepts. (©Elsevier, Paris)

nicotinic acetylcholine receptors / allosteric concepts / site-directed mutagenesis

1. Introduction

The discovery of the nervous electrical activity and physiological description of the action potential [50] led to rapid progress in studies of the nervous system. It was soon realized, however, that neuronal firing represents only one of the steps in the elucidation of the nervous system activity but that comprehension of signal transmission across neurons was another major breakthrough required to approach the understanding of brain function. Transmission of electrical activity in the mammalian brain almost exclusively relies on the chemical synapses. Propagation of the action potential at the synaptic bouton causes the release of neurotransmitter, which passively diffuses across the synaptic cleft and binds to integral proteins inserted in the postsynaptic membrane. Regeneration of an electrical signal by the neurotransmitter molecules depends upon the opening of ions permeable channels. Thus, along the evolution, some highly specialized proteins, expressed in the postsynaptic membrane, have developed a dual function with the capacity of ligand binding and the formation of an aqueous ionic pore. These molecules are identified as ligand-gated channels (LGCs). Neuronal nicotinic acetylcholine receptors (nAChRs) belong to a broad family of neuroreceptors that includes $GABA_A$, glycine and serotoninergic 5-HT_3 receptors [38]. They have been demonstrated to be activated within microseconds [20, 33] by the presence of acetylcholine (ACh) at micromolar concentrations. Cloning and sequencing of the neuromuscular junction nAChR subunits [81] represented an important step in the understanding of their structure. Derived from these studies it was proposed that a single receptor complex is made by the assembly of five subunits, each of which spans four times the membrane. These proteins, which are inserted into the cytoplasmic membrane, form at the same time the ligand binding site, in the extracellular domain, and the ionic pore, aligned with the pseudosymmetrical axis of the pentamer [14]. Library screening using low stringency hybridization with partial sequences of the DNA coding for the neuromuscular nAChR led to the identification of several related genes coding for the neuronal nAChRs. Up to now a total of 11 closely related cDNAs coding for neuronal nAChRs proteins have been identified in vertebrates [62, 67].

Neuronal nAChRs can be divided in two main groups depending upon their sensitivity to the snake toxin α-bungarotoxin (α-Bgt) [16]. Sensitivity to α-Bgt was employed for a long time as criteria to identify neuronal nAChRs from muscular receptors, which are blocked by this competitive inhibitor in the nanomolar range. In contrast, neuronal nAChRs, such as ganglionic receptors, were shown to be insensitive to this toxin even for concentration a 1000-fold higher [1]. With the identification of a group of a subunits (α7-α9) that can reconstitute functional nAChRs when expressed alone in *Xenopus* oocytes or in cell lines it was also possible to demonstrate that this class of receptors is inhibited by α-Bgt [24, 32, 43, 84, 87, 100]. Several studies have further illustrated that these initial findings could take into account the important α-Bgt labeling observed in the central nervous system [17, 117]. Given their capacity to reconstitute homomeric receptor, α7 subunits were employed as a model in many structure function relationship studies. The main advantage provi-

ded by this receptor is that engineering of a single subunit is sufficient to alter the entire receptor complex (reviewed in [6]).

Rapid progresses in molecular biology and the introduction of reconstitution techniques (reviewed in [7, 11]) allowed to investigate physiological and pharmacological properties of neuronal nAChRs in isolation. Combination of site-directed point mutagenesis and electrophysiological recordings further increased the possibilities of studies of these LGCs at the molecular level. The purpose of this work is to review, in the light of the allosteric model, the properties of the neuronal nAChRs and to place them in perspective of both our current knowledge of the wider family of neuroreceptors as well as related neurological disorders.

2. The nAChR: a model of allosteric protein

High resolution electron micrograph studies, at 9 Å resolution, of the *Torpedo* electric organ realized by Unwin [110] confirmed the hypothesized protein structure of the neuromuscular junction receptor [27] and is used as a tridimensional model for other receptors comprising the same protein characteristics (reviewed in [5, 38]). Schematic organization of a single receptor complex, based on these studies, is presented in *figure 1A*.

Since nAChRs result from the assembly site of five subunits, they constitute an excellent prototype of allosteric proteins in which the ligand binding is distinct from the 'reaction site', that is, the ionic pore. Allosteric proteins, as firstly proposed by Monod, Wyman and Changeux (MWC), have the particularity to undergo spontaneous transitions between different conformations, some of which being preferentially stabilized by the presence of ligand(s). Such transitions are possible when assuming a 'concerted' rearrangement of the protein subunits (i.e., the different subunits change their conformation in cooperativity). Although initially formulated for cytosolic proteins, such as hemoglobin [28], the allosteric theory can be extended to several types of molecules, ranging from DNA to LGCs [38, 63, 96]. In the case of the nAChRs, it is assumed that binding of the natural ligand acetylcholine (ACh) stabilizes the receptor in the active open state. To take into account the channel closure observed upon prolonged exposure to high agonist concentrations, a three-state model, presented in *figure 1B*, represents the minimal number of states required for the description of the channel properties [48]. This phenomenon, typically observed for LGCs, is referred as 'receptor desensitization'. According to this three-state model the receptor can take any of the three conformations, basal (closed), active (open) or desensitized (closed). Thus, and deriving from the allosteric concept, it can be assumed that some of the nAChR 'binding sites' will display different properties according to the state which is considered.

Studies of the muscle nAChR using photoaffinity labeling [41] or other techniques [25, 55, 103] have revealed that the ligand binding site lies at the interface between an alpha and the adjacent subunit (γ or δ (reviewed in [6])). Sequence alignment of the N-terminal amino acid sequences of muscle and neuronal nAChRs unveiled the high conservation of residues identified as determinant of the ACh binding site. Mutation of these aromatic residues in the $\alpha 7$ neuronal nAChR caused an important reduction of the receptor sensitivity to ACh [36], as shown by the decrease in the EC_{50} (the concentration of agonist required to produce half activation of the receptor). These studies lead to the conclusion that in the alpha subunit the agonist binding pocket comprises at least three main loops referenced to as A, B and C (*figure 1C*). Further site-directed mutagenesis studies have confirmed that, as predicted from the muscle experiments, ACh binds at the interface between alphas and the adjacent subunits. Namely, it was observed that mutations in the so-called complementary domain, in the adjacent subunit, cause important changes in the receptor EC_{50} as well as its pharmacological profile [21]. Concomitant with these studies, other experiments conducted on the muscle nAChRs have illustrated the high degree of homology observed between nAChRs [14]. Attempts of modelisation of the N-terminal structure using a bacterial copper binding protein template [107] further support the initial schematic representation of the ACh binding site. Thus, although the exact tridimensional structure of the ACh binding pocket remains to be determined at the atomic level, all evidence suggests that the ligand is binding in the extracellular domain of the proteins at the interface between two adjacent subunits.

3. The Monod-Wyman-Changeux model

Modeling of the allosteric proteins properties in steady state conditions, as proposed by MWC, can readily be done using the following equation:

$$\overline{A} = \frac{1}{1 + L \cdot \left\{ \frac{1 + [ACh]/K_b}{1 + [ACh]/K_a} \right\}^n}$$

where $\overline{A}$ is the fraction of receptors in the open state, L is the isomerization constant, K_a and K_b are the respective microscopic dissociation constants for

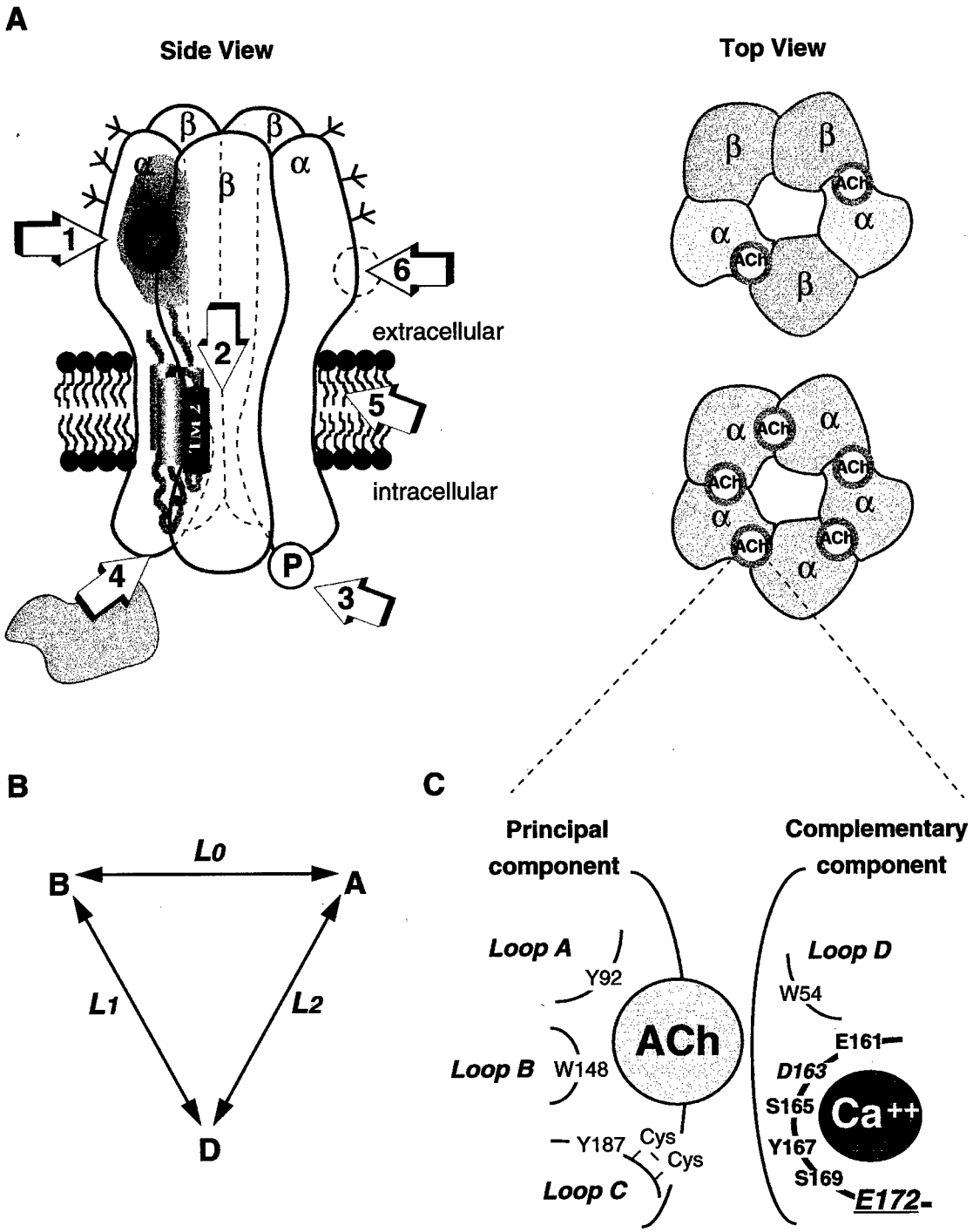


Figure 1. The neuronal nAChRs: a prototype of an allosteric protein. **A.** Schematic organization of the receptor. Transmembrane organization and possible interaction sites are represented on the side view (left panel). Arrows correspond to: 1) ligand-binding site(s); 2) ionic pore; 3) putative phosphorylation domain(s); 4) possible site(s) of interaction with cytosolic or protein(s) of the cytoskeleton; 5) phospholipid interaction site(s); and 6) extracellular allosteric binding site(s). Heteromeric and homomeric receptors with possible ACh binding sites are represented in the top view (right panel). **B.** Three-state allosteric model where: B, basal (closed) state; A, active (open) state; D, desensitized (closed) state; L_0, L_1, L_2, isomerization constants. **C.** Schematic representation of the ligand binding site at the interface between an alpha and the adjacent subunit. Residues participating to the formation of the principal and complementary components are represented.

the agonist while [ACh] is the agonist concentration. *n* corresponds to the number of binding sites for the agonist (ACh).

When considering the transition from the close resting state to the open conducting state, a first prediction derived from this equation is that, depending upon the value of L, the EC_{50} as well as the fraction of receptors that can be stabilized in the open conformation will vary. The lowest is the L value, the easiest is the transition. Two alternative conditions can be considered in which the L value may be modified. First, mutations of amino acid residues may cause a modification of the protein stability and thereby may affect the concerted transitions between different conformational states and, as a correlate, may change the value of the intrinsic isomerization constant (L). Second, the presence of a molecule that could bind to the protein at a site near or distant from the ACh binding pocket may affect the L coefficient, as initially described by Rubin and Changeux [95]. It is of value to recall that the Greek prefix 'allo' means 'elsewhere' because the allosteric model postulates that different regions of one molecule are cross-linked in their behavior. As we shall see, the two conditions listed above have been experimentally observed with the neuronal nAChRs.

Among the different methods that allow to investigate the properties of LGCs, the electrophysiological approach offers the main advantage of a real time monitoring, with high time resolution, of the almost synchronous behavior of up to 1000 of proteins expressed in a cell membrane. Using the so-called 'voltage clamp' methods, it is possible to follow the net and macroscopic ion flux evoked by the opening of many LGCs while clamping the membrane potential at the same time. Ultimate resolution is reached, with the patch clamp technique, by recording small patches of membrane where the opening of a single channel can be observed. In optimal conditions, the transitions of a single LGC molecule can be followed with a pico-Amper and microsecond resolution [7, 11, 20]. In the case of the neuronal nAChRs, investigations at the single channel level are limited by an important run down, that is, the 'loss' of channel activity (in outside out patches) even in the absence of agonist [9, 79, 85].

4. Electrophysiological recordings: a probe for the allosteric model

Site-directed mutagenesis of a single amino acid comprised within the second transmembrane segment (TM2) of α7, with the replacement of a leucine in threonine (L247T), was found to produce pleiotropic alterations of the receptor properties [4, 91]. Most important modifications being a 200-fold increase in the agonist sensitivity, loss of desensitization, transformation of the receptor pharmacological profile and presence of a new conductance at the single channel level (reviewed in [6]). Interestingly, all these modifications could be taken into account assuming that the L247T mutation renders conductors a desensitized state of the nAChR.

When analyzed on the basis of the allosteric model, effects of the L247T mutation can be described assuming a new conductive state and a low isomerization constant [40]. A further prediction of the allosteric model is that for a low L value a fraction of the receptor corresponding to 1/L should be open even in absence of agonist. Although this fraction is close to zero for L values as large as 10^6-10^8 needed for the modeling of the wild type (WT) α7 receptor this fraction becomes non-negligible when a L coefficient as low as 20 is employed for the description of the L247T mutant. Detailed analysis of the properties of oocytes expressing the L247T mutant revealed that a constant current must be injected to hold at −100 mV cells expressing this nAChR. This leakage current being several fold larger than that needed for non-injected oocytes [8]. Furthermore, it was found that this current was reduced by incubation of the cell in presence of the competitive inhibitor methyllycaconitine (MLA; [82]). From these and other observations it was concluded that, as predicted by the allosteric model, a fraction of receptors are spontaneously open in cells expressing the L247T mutant [8].

Another important finding obtained from the analysis of the L247T mutant concerns the modification of the pharmacological profile of the receptor. While the WT α7 nAChR is inhibited in a competitive manner by dihydro-β-erytrhoidine (DHβE), this compound is a full agonist at the L247T mutant. Although at first surprising, these data are readily explained assuming that in the WT receptor DHβE stabilizes a desensitized state which becomes conductor in the L247T mutant. In contrast to DHβE the other competitive antagonist MLA does not evoke large amplitude currents when applied to the L247T but, as described above, closes the channels. The differential effect of these two competitive antagonists can be explained by assuming that they may stabilize different conformational states, namely the 'closed resting state' for MLA and one of the 'desensitized state' for DHβE. Further support for this hypothesis is provided by the observation that α-Bgt behaves like MLA, either on the α7 WT or on the L247T mutant receptor. At WT α7 nAChRs, MLA and DHβE are identified as 'competitive antagonists'. At the L247T mutant, MLA and α-Bgt may be classified as 'inverse agonists' whereas

DHβE is a full agonist. Thus, the allosteric interpretation of the α7 nAChR mutants points, once again, to the semantic limits of the current pharmacological nomenclature.

Simultaneous introduction of two mutations in TM2 (L247T and V251T) causes a further alteration of the receptor properties. Oocytes expressing this type of receptor display very large leak currents, in the μA range, that are reduced by applications of low ACh concentrations [8]. As confirmed by measurements of the cell-input resistance, this weak current is attributable to spontaneous opening of an important fraction of the receptors. Furthermore, current-voltage relationships (I-V) recorded in the absence or presence of agonist revealed that spontaneously open channels display a reversal potential similar to that of the α7 WT nAChR. Increasing the agonist concentration results first in smaller reduction of the channel closure and then in an inward current. This complex channel behavior can be explained by the allosteric model assuming that spontaneous opening reflects the fraction of receptor which is in the desensitized, high affinity, D state while current activation by high agonist concentrations results from the stabilization of the receptor in the active and low affinity A state.

As described above, another important prediction of the allosteric model is that substances that can bind to the protein at a site distinct from that of the native ligand binding pocket may also affect the overall state equilibrium [95]. Compounds that increase the apparent isomerization L coefficient are called negative allosteric effectors whereas substances that reduce the apparent L coefficient are called positive allosteric effectors. Application of a negative allosteric effector should result in a reduction of the protein activation while a positive effector would reinforce the agonist effect.

Electrophysiological studies of native nAChRs had shown that these receptors are potentiated, at physiological concentration, by extracellular calcium [80, 114]. A high calcium concentration (i.e., > 2 mM) induces both a decrease of the single channel conductance and an increase of the mean open time. At the macroscopic level, an increase of the ACh-evoked current is observed. In the view of these results it was suggested that calcium acts as a positive allosteric effector on the neuronal nAChRs where it could bind with an affinity in the millimolar range [65]. Based on putative sequence homology with natural calcium binding proteins, such as calmodulin, site-directed mutagenesis experiments conjugated with functional studies were designed. Results obtained with this approach confirmed the existence in the extracellular domain of the α7 nAChR of a consensus calcium binding motive, the mutation of which suppresses calcium potentiation [37]. Moreover, introduction of this consensus sequence in the extracellular N-terminal part of the 5-HT_3 receptor, that is natively insensitive to the extracellular calcium concentration, was sufficient to cause calcium potentiation [37].

Other studies had previously pointed out that steroids, and more specifically progesterone, are negative allosteric effectors at the major brain α4β2 nAChR [3, 112]. Although these studies were conducted on the chick nAChRs it was recently shown that progesterone also behaves as a negative allosteric effector on human nAChRs reconstituted either with the α4β2, α3β2 or α7 subunits (Buisson et al., submitted). In agreement with these observations, dose-responses to ACh recorded in control conditions or in presence of progesterone are readily fitted with an allosteric equation assuming that progesterone reduces the apparent isomerization coefficient L_0 of the B ↔ A equilibrium. Nevertheless, since progesterone increases the nAChRs desensitization without affecting the receptor apparent affinity for ACh, it is likely that this steroid may stabilize one the nAChRs desensitized states with a concomitant reduction of the isomerization constant L_2 (*figure 1B*). Of more interest is the observation that another 'female' steroid, 17β-oestradiol, potentiates human α4β2 nAChRs while it inhibits α3β2 and homomeric α7 nAChRs (Buisson et al., submitted). The 17β-oestradiol potentiation of α4β2 nAChRs occurs with no change in the receptors apparent affinity for ACh and of the desensitization profile. Thus, 17β-oestradiol may preferentially stabilize the A state. In favor of this hypothesis is the observation that the fit of the 17β-oestradiol dose-response profile with the allosteric equation [95] yields an isomerization constant of 60 for the B ↔ A equilibrium, whereas it yields L_o = 220 in control conditions. With an identical β2 subunit, 17β-oestradiol mediates two opposite allosteric modulations that are related to the presence of one α subunit: α4 for the potentiation and α3 for the inhibition. At human α4β2 nAChRs 17β-oestradiol is a positive allosteric modulator whereas Ca^{2+} (at concentrations > 2 mM) is a negative allosteric modulator [9] (Buisson et al., submitted). However, the same compounds behave in opposite directions at human α3β2 nAChRs where 17β-oestradiol is a negative allosteric modulator and Ca^{2+} a positive one [59]. Although performed with reconstituted receptors, these experiments raise the notion that according to their subunit composition as well as to their cellular location (post- versus presynaptic), modulation of neuronal nAChRs could have opposite effects on the synaptic transmission mechanisms in a given physiological background. This illustrates that, as previously suspected, the

composition and the distribution of the nAChRs subunits around the axis of pseudo-symmetry is determinant for the interaction of allosteric modulators [6].

More recently it was shown that the anthelmint ivermectin (IVM) is a powerful allosteric potentiator at both chick and human homomeric α7 nAChR [58]. The direct mode of action of IVM on the α7 receptor was supported by the observation that application of this drug does not modify the reversal potential of the current-voltage relationship indicating that increase in the ACh-evoked responses cannot be attributed to the activation of another ionic channel. In addition, an important potentiation was also observed with the calcium impermeant mutant E237A [4], indicating that the IVM effects are independent of the calcium influx.

Taken together these data confirm the existence of negative as well as positive allosteric effectors on the neuronal nAChRs and support the hypothesis that these integral membrane proteins can take several conformations, some of which being preferentially stabilized by the ligand or compounds that can bind in the ligand-binding pocket. In this respect it is of value to note that ligands are typically classified in three categories, depending on their physiological effects. The first class being the natural agonist, which is thought to fully activate the receptor. The second class corresponds to the competitive inhibitors, which are thought to stabilize the receptor in an inactive configuration. The third class are the partial agonists, which can only evoke a fraction of the activation caused by the natural agonist. Several studies, as in the case of the glutamate receptor [71], have revealed that, when applied concomitantly to the receptor natural ligand, partial agonists provoke an inhibition. Although at first surprising, this observation can be taken into account assuming that, given their chemical nature, partial agonists can stabilize only a fraction of the receptor in the active state. It can therefore be concluded that, in term of allosteric model, partial versus full agonist effects should depend upon the isomerization coefficient L. As a consequence it can be proposed that exposure to a positive allosteric effector that reduces the apparent L coefficient should also cause a modification of the receptor pharmacological profile. This hypothesis that was indeed confirmed with IVM as shown by the transformation of the pharmacological profile of chick α7 to dimethyl-phenyl-pyperazinium (DMPP). In control conditions DMPP behaves as a very weak agonist at the chick α7 nAChR. However, short pre-applications of IVM can 'transform' this compound as a full agonist [58].

5. Discussion

Neurotransmission in the vertebrate central nervous system (CNS) is almost exclusively mediated by synapses with their cohort of neuroreceptor molecules. LGCs with their rapidly activating ionic pore have been shown to play an important role in fast signal transmission. Neuronal nAChRs, which belong to the so-called four transmembrane LGC superfamily, are homologous of $GABA_A$, glycine and $5\text{-}HT_3$ receptors [38]. Within this superfamily, conservation of the tridimensional protein organization as well as its functional properties are such that a switch of the ionic selectivity of α7 nAChR, from cation to anion, can be engineered by substituting a few amino acids that face the ionic pore for their $GABA_A$ counterparts [39]. The conservation of the functional domains is illustrated by the production of a chimeric receptor between the homomeric α7 nAChR and the $5\text{-}HT_3$ receptor. The chimera displays the pharmacological specificity of a nAChR but the ionic pore properties of the native $5\text{-}HT_3$ [31]. Moreover, repetition of the chick α7 L247T mutation in the muscle nAChR [60] as well as in the $GABA_A$ [106] and $5\text{-}HT_3$ [119] receptors produces equivalent phenotypes.

An important paradigm that has to be kept in mind in modeling receptor properties is their ability to be in multiple functional states. Models that can be considered for the description of LGCs are subdivided in two basic classes with: a) induced fit reactions: and b) allosteric processes. While induced-fit models [57] assume that binding of the ligand causes a modification in the protein conformation, the allosteric scheme postulates that, in absence of ligand, the protein undergoes spontaneous conformational changes and that addition of the ligand preferentially stabilizes one or more conformational states [76]. For an allosteric analysis of its properties, a LGC must fulfill the following criteria: i) it results from the assembly of at least two protomers; ii) it possesses one or more axis of rotational symmetry; iii) it exists in, at least, two freely interconvertible conformational states; and iv) transition from one state to the other involves a 'concerted' conformational change of the protomers.

Isolation of genes coding for nAChRs and their reconstitution in host systems unveiled that indeed these receptors result from the assembly of five subunits (reviewed in [6, 38]). Moreover, an axis of pseudo-symmetry can be observed at the ionic pore level, which lies in the center of the subunit assembly [14, 110]. A clear distinction can be made between the induce-fit and allosteric models regarding the possibility that the protein spontaneously undergoes conformational changes. In the case of the al-

losteric model, spontaneous channel opening should be present, even if these occurrence may be very low, whereas induce-fit models can hardly predict such openings. In agreement with this observation spontaneous single channel activity was observed for the muscle receptor [54]. For the neuronal nAChR, site directed mutagenesis done in TM2 have further illustrated that cells expressing the α7 L247T mutant display a significant fraction of current even in the absence of agonist [8]. Similar mutation performed in the $GABA_A$ receptor yielded proteins that also showed a significant fraction of opening in absence of ligand [83, 106]. Although the observation of 'open' *Torpedo* nAChRs using electronic microscopy suggests a conformational rearrangement of the transmembrane domains [111], the functional characterization of concerted transitions remains to be established. Strategies employing concatemers of subunits allowed to demonstrate the cooperative interaction of the potassium channel subunits in the gating mechanism [108]. Nevertheless, it is very likely that equivalent concerted mechanisms occur during the allosteric transitions of LGCs. Altogether, these data strongly support the hypothesis that LGCs represent typical allosteric proteins since they can undergo spontaneous transitions.

Recent studies performed with cGMP gated channels indicate that such channels also display a spontaneous activity in the absence of cGMP (the intracellular ligand) and this was also invoked as a clear demonstration of the allosteric nature of the transitions that occur during the receptor activation [96]. Spontaneous receptor activity has also been reported for G-protein coupled receptors (GPCRs) [15, 29, 42, 86, 98]. Thus, the ability of undergoing allosteric transitions seems a common property of many membrane receptor proteins.

One important feature, that has been poorly explored up to now, is the allosteric modulation of neuronal nAChRs by intracellular factors. Investigations performed with the glutamate NMDA receptor have demonstrated that intracellular proteins (such as actin filaments of the cytoskeleton) are able to modulate the receptor properties [94]. The analysis of the amino acid in the long intracellular loop comprised between transmembrane segments 3 and 4 (TM3 and TM4) reveals the presence of a number of consensus sequences for kinase phosphorylations. Preliminary works have shown that activation of the cyclic AMP (cAMP) pathway by peptidic hormones such as calcitonin gene-related peptide (CGRP) or vasoactive intestinal peptide (VIP) can modify the properties (agonist affinity and/or desensitization profile) of muscle and neuronal nAChRs, respectively [46, 69, 78]. These effects may be related to phosphorylation/dephosphorylation events following the cAMP activation of protein-kinase A (PKA). The rat nAChR α4 subunit is phosphorylated by PKA [53] and PKC [30]. Moreover, the up-regulation of human α4β2 nAChRs expressed in K177 cells involve PKA- and PKC-mediated mechanisms [44]. In vitro experiments indicate that the α7 intracellular loop (TM3-TM4) is a substrate for PKA [77]. Since careful investigations of phosphorylation/dephosphorylation effects on neuronal nAChRs are needed, it is reasonable to assume that, as observed for other membrane receptors, phosphorylations may modify the intrinsic properties of nAChRs (changing the L value) and thus the pharmacological profile and/or gating properties. Moreover, phosphorylations may modify the interaction of nAChRs with other intracellular factors such as Grb2 [19] or agrin [35]. In myoblasts, cytoplasmic proteins that are closely associated with the nAChRs such as agrin and rapsyn have been characterized for targeting and/or stabilizing nAChR molecules in postsynaptic regions [13, 90, 121]. Gephyrin has recently been implicated in the clustering of glycine receptors in brain neurons [56] while proteins of the PDZ family play an identical role at ionotropic glutamate receptors [26]. The possible influence of these docking molecules on the receptor functions awaits a further characterization, since in many studies, recordings of the nAChRs are performed with the presence of endogenous factors that are hardly controlled. Studies performed with GPCRs indicate that their pharmacology is influenced by the association-dissociation with trimeric G-proteins. Most of the time, GPCRs have a lower affinity for their natural agonist when they are associated to 'inactive' G-proteins [92, 116]. Moreover, the overexpression of G-proteins is able to stabilize GPCRs in their active state [12]. Thus, similar interactions may be postulated between neuronal nAChRs and intracellular proteins that remain, however, to be identified.

Investigations performed either with animals or with freshly isolated slices/neurons pointed out that many physiological and/or synthetic compounds could modify the LGCs properties and therefore, the synaptic transmission efficacy. Among these molecules, the benzodiazepines (BZDs) represent the prototype of allosteric effectors. Identified for their anti-convulsant and anxiolytic properties, the binding site of these compounds has been located on the $GABA_A$ receptor but at a site that is distinct from the agonist one [101], suggesting that, as observed a few years ago for the enzymes (reviewed in [28]), complex receptor proteins can present alternative binding sites for allosteric effectors. Moreover, the sensitivity toward BZDs depends on the receptor subunits: as an example, $GABA_A$ receptors containing the α4 subunit are less sensitive to BZDs [102, 115].

The discovery that steroid binds and modulates $GABA_A$ receptors further confirmed the idea that LGCs could be negatively and positively regulated by different allosteric molecules belonging to a same family [47, 68]. As observed for the BZDs, a steroid- and subunit-specific modulation was observed in experiments performed with reconstituted receptors [61, 89]. The allosteric modulation by steroids is a feature that is shared by many LGCs since these cholesterol-derived compounds are still active at glutamate NMDA receptors [72] and also at neuronal nAChRs (see above). Thus, steroids originating either from the gonads and/or de novo synthesized in the brain [2] can target and modulate different types of LGCs. The potential interest of using such compounds for the treatment of neuropathologies [97] is emphasized by the empirical, but very promising results obtained with the estrogen replacement therapy (ERT) for people suffering from Alzheimer's disease [49].

Immunocytochemistry as well as in situ hybridization studies have revealed that neuronal nAChR subunits are broadly expressed in the fetal and adult brain [74, 93] and that, most of the time, a given region expressed more than three different subunits [22, 23, 51, 52, 88, 93, 113]. From a theoretical point of view, the nAChRs composition and subunit rearrangement could be relatively complex [6] as well as their cellular location (pre- versus post-synaptic in neurons). Moreover, all nAChRs are, to some degree, permeable to Ca^{2+} with the highest permeability observed for the homomeric α7 nAChRs [4, 100]. Thus, and according to the pivotal role of calcium in synaptic transmission/plasticity, neuronal nAChRs may constitute determinant actors/modulators of the synaptic transmission [93, 118]. Electrophysiological investigations performed with rodent brain tissues have indicated that presynaptic nAChRs containing the α7 subunits control the release of glutamate [45, 73]. However, α7-containing nAChRs have been identified both in post and presynaptic membranes at chick ciliary ganglion synapses [18, 52] where they mediate up to 90% of the fast cholinergic transmission [109, 122]. Thus, α-Bgt sensitive nAChRs can be present on both sides of synaptic cleft. Non-α7 nAChRs subunits have been implicated in the release of other neurotransmitters such as GABA [64, 66, 75, 120], dopamine [70], serotonin [99] and also ACh [18, 34]. Thus, the distribution and the roles of nAChRs follow a complex scheme. With the exception of a few compounds (which can be counted on one hand), pharmacological investigations performed on reconstituted or on native receptors did not allow the identification of agonists/competitive antagonists that are specific to one nAChR subtype. Moreover, non-competitive antagonists, such as open channel blockers, can display a much larger spectrum of action than initially expected [10]. Therefore, the characterization of allosteric effectors such as 17β-oestradiol, that are able to specifically potentiate one type of nAChR, opens alternative pharmacological pathways for the identification/design of molecules that can be target of one type of nAChR. This observation is of particular relevance when knowing that patients affected by autosomal dominant nocturnal frontal lobe epilepsy (ADNFLE) present mutations in the nAChR α4 subunit [104, 105]. Thus, and as recently illustrated for the $GABA_A$ receptor [102], alteration of a single subunit can lead to a neuropathological profile. Since presynaptic boutons and postsynaptic membranes express nAChRs that could be of different compositions (see above), physiological as well as pharmacological controls of these synapses must implicate compounds highly specific for a given receptor subtype, and that allow a precise adjustment of the synaptic transmission efficacy. The identification and comparison of the 17β-oestradiol binding sites on human α4 and α3 nAChRs subunits shall constitute a determinant step in the design of targeted allosteric modulators for the neuronal nAChRs.

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Molecular dissection of subunit interfaces in the nicotinic acetylcholine receptor

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Abstract — Ligand binding sites in the muscle nicotinic acetylcholine receptor are generated by pairs of α and non-α subunits. The non-α subunits, γ, δ and ε, contribute significantly to overall affinity of agonists and antagonists, and confer selectivity of these ligands for the two binding sites. By constructing chimeras composed of segments of the various non-α subunits and determining ligand selectivity, we have identified four loops, well separated in the linear sequence, that contribute to the non-α portion of the binding site. Studies of point mutations in these loops and labeling of engineered cysteines show that the peptide backbones of each non-α subunit fold into similar basic scaffolds. Studies of mutations of the peptide antagonists α-conotoxin MI and ImI reveal pairs of residues in the binding site and the toxin that stabilize the complex. (©Elsevier, Paris)

Résumé — Dissection moléculaire des interfaces entre sous-unités dans le récepteur nicotinique à l'ACh. Les sites de liaison des ligands cholinergiques au récepteur nicotinique musculaire résultent d'interactions entre des paires de sous-unités α et non-α. Les sous-unités β, γ et ε contribuent ainsi à former deux sites de liaison d'affinité différente pour les agonistes et les antagonistes. En étudiant la sélectivité de liaison de chimères composées de segments des différentes sous-unités non-α, nous avons mis en évidence quatre boucles espacées dans la séquence primaire, qui participent à la partie non-α des sites de liaison. Des mutations ponctuelles dans ces boucles et le marquage de cystéines introduites par mutagenèse montrent que les peptides non-α se replient tous suivant le même motif. De plus, l'étude de mutations d'antagonistes peptidiques, les αconotoxines MI et ImI, montre que des interactions entre paires de résidus de la toxine et du site de liaison stabilisent le complexe. (©Elsevier, Paris)

molecular dissection / subunit interfaces / α-conotoxins / curare

1. Introduction

Acetylcholine receptors (AChR) from vertebrate skeletal muscle are pentamers of homologous subunits with compositions $\alpha_2\beta\gamma\delta$ in fetal or $\alpha_2\beta\varepsilon\delta$ in adult muscle. The ligand binding sites are generated by apposition of pairs of non-equivalent subunits, αδ and either αγ or αε. Antagonists of the curare or α-conotoxin families select between the two sites of each receptor type, with the degree of selectivity ranging from 100- to 10 000-fold. Because the α subunit is common to each binding site, differences in affinity are attributed to different contributions of the non-α subunits. Over the past several years, we have used subunit chimeras and point mutations to determine the origin of the site-selectivities of these antagonists [2, 4, 5].

The present work describes identification of residues in the non-α subunits that confer site selectivity of curare for the adult muscle AChR. Cysteine mutagenesis is then used to identify residues in close proximity to the site of α-bungarotoxin binding. Finally, mutant α-conotoxins are used in combination with receptor mutations to probe binding sites in both the muscle and neuronal α_7 receptors. The results reveal pairs of residues in both the toxins and the receptors that interact to stabilize the α-conotoxin-receptor complex.

2. Materials and methods

2.1. Materials

Dimethyl-*d*-tubocurarine (DMT) was generously provided by the Eli Lilly Co. ^{125}I-labeled α-bungarotoxin (btx) was purchased from Dupont NEN, and the 293 human embryonic kidney cell line (293 HEK) from the American Type Culture Collection.

2.2. Plasmids and mutagenesis

Sources of the mouse AChR cDNAs and subcloning into the cytomegalovirus-based expression vector, pRBG4, were as described [4]. Human α_7 and rat 5HT-3 subunit cDNAs were generously provided by Drs. John Lindstrom and William Green. Chimeric subunit cDNAs were constructed by bridging naturally occurring or mutagenically installed restriction sites with synthetic double stranded oligonucleotides [4]. The chimeras are designated as follows: the first letter gives the subunit from which N-terminal sequence is taken, the following number gives the position of the chimeric junction, and the final letter gives the subunit from which C-terminal sequence is taken.

2.3. Expression of mutant receptors and ligand binding measurements

293 HEK cells were transfected with mutant or wild type AChR subunit cDNAs using calcium phosphate precipitation.

Three days after transfection, ligand binding to intact cells was measured by competition against the initial rate of ^{125}I-α-btx binding [4]. After harvesting, the cells were briefly centrifuged, resuspended in high potassium Ringer's solution, and divided into aliquots for ligand binding measurements. Specified concentrations of ligand were added 30 min prior to addition of ^{125}I-α-btx, which was allowed to bind for 30 min to occupy approximately half of the surface receptors. Binding was terminated by addition of 2 mL of potassium Ringer's solution containing 300 μM *d*-tubocurarine chloride. Cells were then harvested by filtration through Whatman GF-B filters using a Brandel Cell Harvester and washed four times with 3 mL of potassium Ringer's solution. Non-specific binding was determined in the presence of 10 mM carbamylcholine. The initial rate of α-btx binding was calculated to yield fractional occupancy by competing ligand. Binding measurements were analyzed by fitting to either the monophasic Hill equation or the sum of two distinct binding sites using the program UltraFit (BIOSOFT).

2.4. Synthesis and purification of α-conotoxin ImI and derivatives

α-Conotoxins (CTx) were synthesized by standard FMOC (9-fluoreny-methoxycarbonyl) chemistry on an Applied Biosystems 431A peptide synthesizer. During synthesis, cysteine (S-triphenylmethyl) protecting groups were incorporated at cysteines 3 and 12, and acetamidomethyl (ACM) protecting groups were incorporated at cysteines 2 and 8. The linear peptide was purified by reversed phase HPLC using a Vydac C18 preparative column with TFA/acetonitrile buffers. The two intramolecular disulfide bridges were formed as follows: the cysteine S-triphenylmethyl protecting groups of cysteines 3 and 12 were removed during TFA cleavage of the linear peptide from the support resin, and the peptide was oxidized by molecular oxygen to form the 3–12 disulfide by stirring in 50 mM ammonium bicarbonate buffer, pH 8.5 at 25 °C for 24 h. The peptide was lyophilized prior to the formation of the second bridge. The ACM protecting groups on cysteine 2 and 8 were removed oxidatively by reaction with iodine for 16 h prior to carbon tetrachloride extraction. Residual iodine was separated from the pure product by HPLC. Formation of disulfide bonds was confirmed by lack of reaction with Ellman's reagent, and molecular mass was confirmed by mass spectrometry.

3. Results

3.1. Identification of residues in the adult muscle AChR that confer selectivity for DMT-

Previous work showed that the pair of equivalent residues γY117/δT119 are major determinants of DMT selectivity in the fetal AChR [4]. γY117 contributes to high affinity of the αγ site, whereas δT119 contributes to low affinity of the αδ site. Because serine occupies the equivalent position in the ε subunit of the adult AChR, and high affinity requires an aromatic side chain at this position, the αε site is not expected to bind DMT with high affinity. To determine the origin of high affinity conferred by the ε subunit, we constructed a series of ε-δ chimeras (*figure 1*), coexpressed them with complementary subunits and measured DMT binding by competition against the initial rate of ^{125}I-α-btx binding [1].

Selectivity of the adult AChR for DMT is illustrated in *figure 1*; distinct affinities of the αε and αδ sites are clearly resolved, with the two-site fit disclosing affinities different by 70-fold. Substituting ε sequence into the N-terminal 63 positions of the δ subunit increases DMT affinity to approach that conferred by the pure ε subunit (*figure 1*, ε63δ). Conversely, substituting δ sequence between positions 43 and 63 of the ε subunit decreases affinity to approach that of the pure δ subunit (*figure 1*, ε43δ63ε). Thus, determinants of DMT selectivity in the adult AChR are located in the major extracellular domain between residues 43 and 63 of the ε subunit and equivalent residues of the δ subunit.

To identify the key residues within the 43 to 63 segment, we constructed additional ε-δ chimeras and point mutants in the ε and δ subunits. The overall results show that two pairs of equivalent residues, εI58/δH60 and εD59/A61, fully account for site-selectivity of DMT for the adult receptor. To confirm that these pairs of residues are solely responsible for DMT selectivity of the adult receptor, we expressed the double mutant subunits, ε (I58H + D59A) and δ (H60I + A61D), alone or together, and measured DMT binding. Incorporating both the ε and δ double mutants into a single receptor mimics the selective binding of DMT to the wild type adult receptor [1]. Thus, the pair of equivalent residues εI58/δH60 and εD59/A61 account entirely for DMT selectivity of the adult receptor.

3.2. Probing the non-α subunits for residues close to the site of α-btx binding

To identify residues in the non-α subunits close to the site of α-btx binding, we carried out cysteine mutagenesis of the predisulfide region of the γ subunit (residues 116–121). As this region contains endogenous cysteines at positions 106 and 115, these were first mutated to serine, and the remaining residues individually mutated to cysteine. We applied one of three methanethiosulfonate reagents to HEK cells expressing each mutant subunit plus complementary α, β and δ subunits, and measured the total number of ^{125}I-α-btx sites. Of the 18 combinations of mutant and reagent, only receptors harboring γL119C treated with the quaternary ammonium reagent MTSET show a significant decrease of the total number of α-btx sites (*table I*). Neither the positively charged aminoethyl (MTSEA) nor the nega-

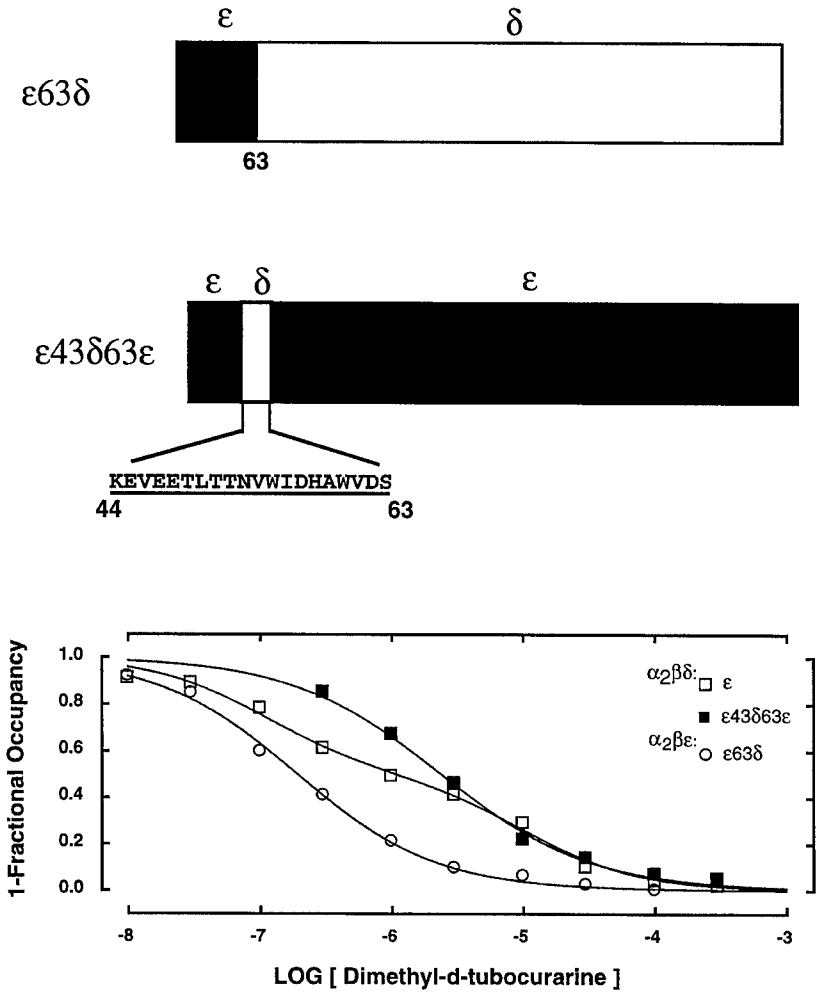


Figure 1. DMT binding to receptors containing ε-δ chimeras. Upper panels are schematic drawings of the ε-δ subunit chimeras: ε63δ contains ε sequence from the N-terminus to position 63 followed by δ sequence to the C-terminus, whereas ε43-δ63ε contains δ sequence between residues 43 and 63 of the ε subunit. Shaded portions represent ε sequence, and unshaded portions δ sequence. Bold underlined text indicates δ sequence. Lower panel: binding of DMT to surface receptors with the indicated compositions: wild type ε (open squares), ε43-δ63ε (filled squares), and ε63δ (open circles). For wild type, the curve through the data is a least squares fit to a two-site equation with the fitted parameters K_A = 0.11 μM and K_B = 10.5 μM, and the fraction of each site constrained to 0.5. For receptors containing chimeric subunits, the curves through the data are fits to the Hill equation with the following fitted parameters: ε43δ63ε, K_{App} = 2.4 μM and n = 0.76; ε63δ, K_{App} = 0.28 μM and n = 0.90.

tively charged sulfonatoethyl (MTSES) reagents affect the number of α-btx sites. The decrease of approximately 50% of α-btx sites suggests that MTSET selectively blocks one of the two ligand binding sites in γL119C receptors.

3.3. MTSET selectively modifies cysteines at equivalent positions of the ε and δ subunits

The high degree of homology among AChR subunits suggests that the polypeptide chains of each subunit fold into similar basic scaffolds. Support for this basic scaffold hypothesis comes from the observation that site-selectivity of DMT, CTx and carbamylcholine can be exchanged between the γ and δ subunits by exchanging a small number of residues at equivalent positions of the primary sequence. To determine whether residues in equivalent positions of the δ and ε subunits are functionally equivalent to γL119C, receptors containing either εL119C or δL121C were treated with MTSET and the total number of α-btx sites was measured. As observed for receptors containing γL119C, MTSET reduces by 50% the total number of α-btx sites in receptors containing either δL121C or εL119C, but has no effect on receptors containing βQ119C or αT119C (*table I*).

3.4. Probing the neuronal α_7 binding site with CTx ImI and synthetic mutants

α-Conotoxins offer the opportunity to modify both the competing ligand and the receptor binding site to identify pairs of residues that stabilize the toxin-receptor complex. To begin study of the α_7 neuronal receptor binding site, we introduced conservative substitutions for each non-cysteine residue in CTx ImI and measured binding of each mutant toxin to α_7/5HT-3 receptors expresed in 293 HEK

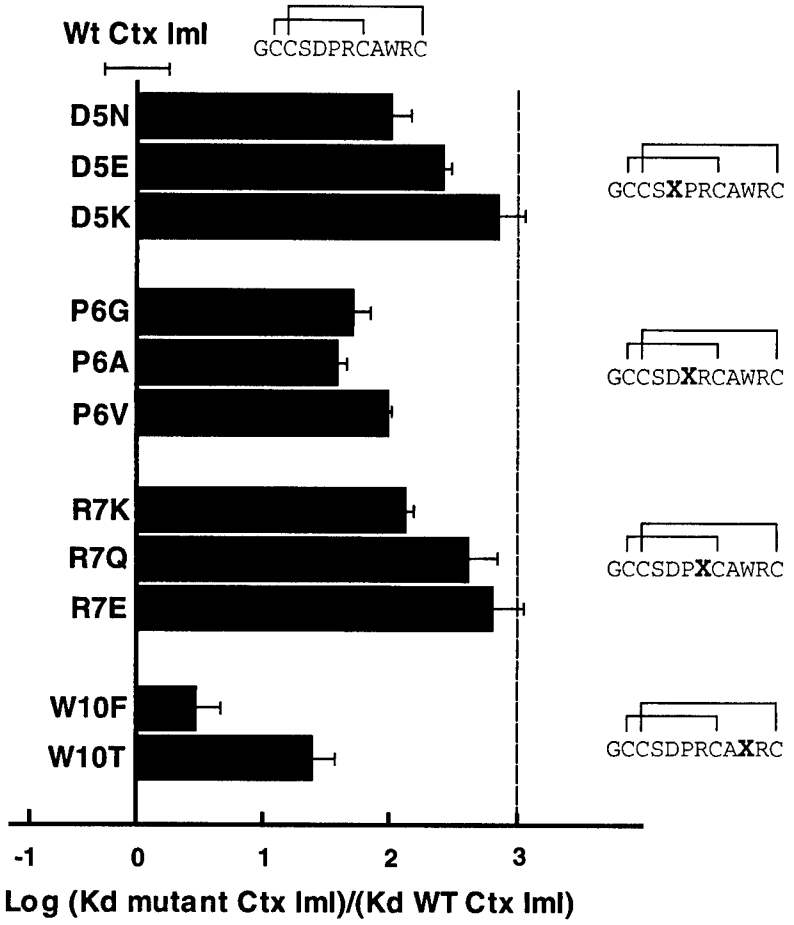


Figure 2. Side chain specificity of the determinants of CTx ImI affinity. For each CTx ImI mutant, dissociation constants are expressed as the log ratio relative to wild type CTx ImI. The affinity of wild type CTx ImI for α_7/5HT-3 receptors is shown by the vertical bold line, and the error bars indicate ± SD. In the schematic representations to the right, **X** indicates the mutant residue.

cells [3]. The results reveal two key regions in CTx ImI essential for high affinity binding (*figure 2*). The first region is the triad Asp-Pro-Arg in the first loop of CTx ImI, where individual mutations decrease affinity by 70- to 500-fold. The second region is the single tryptophan in the second loop, which when mutated to threonine decreases affinity by 30-fold. On the other hand, mutating the four remaining non-cysteines in CTx ImI does not significantly alter affinity for α_7/5HT-3 receptors. These null mutations include acetylation of the amino-terminal glycine and neutralization of arginine in the second loop (R11Q). Thus mutation of four of the eight non-cysteines in CTx ImI alters affinity for α_7/5HT-3 receptors.

We carried out systematic mutations of each of the four bioactive residues in CTx ImI. The overall results reveal that within the first loop, the triad Asp-Pro-Arg is conformationally restricted by the proline, and that the flanking charged residues require maintenance of both charge sign and side chain length (*figure 2*). Within the second loop, mutation of the lone tryptophan shows that it contributes its aromatic ring to stabilize the α_7-CTx ImI complex.

4. Discussion

The experiments described herein probe the structure of the ligand binding site of the nicotinic AChR using: 1) chimeric subunits combined with the site-selective competitive antagonist DMT; 2) cysteine mutagenesis followed by reaction with methylthiosulfonate reagents and measurements of the number of α-btx binding sites; and 3) mutagenesis of α-CTx ImI followed by measurements of binding to α_7/5HT-3 chimeric receptors.

Studies of chimeric ε-δ subunits reveal residues in the ε and δ subunits that give the two binding sites of the adult mouse AChR different affinities for the curariform antagonist DMT. Previous work identified a different set of residues in the γ and δ subunits that confer site-selectivity in the fetal mouse AChR [4]. Two sets of determinants were

Table I. Total α-bungarotoxin sites following methanethiosulfonate treatment.

	α-btx binding sites (%)		
Mutant	*MTSET*	*MTSEA*	*MTSES*
Fetal AChR:			
αβγδ	102 ± 3	107 ± 4	103 ± 4
γC106S + C115S	95 ± 7	105 ± 3	103 ± 7
γI116C	98 ± 3	105 ± 1	103 ± 1
γY117C	86 ± 11	94 ± 1	105 ± 6
γW118C	85 ± 1	105 ± 2	112 ± 7
γL119C	43 ± 6	94 ± 10	103 ± 2
γP120C	100 ± 5	99 ± 2	105 ± 1
γP121C	97 ± 5	101 ± 6	96 ± 1
Adult AChR:			
αβεδ	99 ± 3	117 ± 6	–
αT119C	97 ± 6	98 ± 3	–
βQ119C	98 ± 8	100 ± 13	–
εL119C	50 ± 4	94 ± 5	–
δL119C	52 ± 4	96 ± 11	–

The indicated mutant subunit cDNAs were coexpressed with complementary wild type subunit cDNAs. The total number of α-btx sites is expressed as a percentage of that determined for the corresponding untreated receptor. Values are means ± standard deviations for 3–12 determinations.

identified, with each set flanking the disulfide loop common to all members of the AChR superfamily; the predisulfide set is γI116/δV118 and γY117/δT119 and the postdisulfide set is γS161/δK163. By contrast, selectivity determinants in the adult receptor are far from these in the linear sequence, and comprise εI58/δH60 and εD59/δA61, showing that alternative residues confer DMT selectivity in fetal and adult receptors. The results support a basic scaffold hypothesis because selectivity can be exchanged between the ε and δ subunits by exchanging a small number of residues at equivalent positions of the primary sequence.

The cysteine mutagenesis experiments identify a localized region of the γ, ε and δ subunits that contributes to the site of α-btx binding. This region is located within one of four loops of the γ, ε and δ subunits that contribute to binding of agonists and competitive antagonists. Cysteines placed at the equivalent positions γL119, δL121 and εL119 are chemically modified in a site-specific manner by MTSET, and the modifications block binding of α-btx to the binding site harboring the mutation. Prior to modification, cysteine mutant subunits assemble normally with complementary wild type subunits, and form binding sites with high affinity for α-btx and normal selectivities for the competitive antagonists DMT and CTx. Following modification by MTSET, α-btx no longer binds with high affinity to sites harboring γL119C. The results support the hypothesis that residues at equivalent positions in the primary sequences occupy equivalent positions in three-dimensional space. In addition to supporting the basic scaffold hypothesis, the findings demonstrate that the γ, δ and ε subunits contribute to one face to the binding site, whereas equivalent portions of the α and β subunits do not. The overall findings indicate that γL119 is close to or within the site of α-btx binding, and that MTSET modification of γL119C creates a localized point of interaction with α-btx that prevents its binding.

Studies of mutant α-conotoxins reveal two regions of CTx ImI that confer specificity for α_7/5HT-3 chimeric receptors. The first region is the conformationally sensitive triad Asp-Pro-Arg within the first loop of CTx ImI. Subtle changes in side chain length of the aspartic acid and arginine reduce affinity, and their side chains appear to be held in place by the intervening proline. Thus, the triad must maintain a specific conformation to fit properly into a specific and focal counterpart in the α_7 binding site. The second region is the tryptophan at position 10 within the second loop of CTx ImI. Studies of side chain specificity at position 10 indicate the requirement of an aromatic ring. Current mutant cycles analysis with mutant toxins and receptors aim to identify pairs of residues that stabilize the toxin-receptor complex.

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Functional determinants by which snake and cone snail toxins block the α7 neuronal nicotinic acetylcholine receptors

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Abstract — Snakes and cone snails produce toxins which block muscular and/or neuronal nicotinic acetylcholine receptors (AChRs). This paper mostly focuses on the determinants by which a snake long chain curaremimetic toxin and the cone snail toxin ImI bind specifically to the α7 neuronal receptor. In both cases, the site involves a small turn-like structure constrained by two half-cystines. (©Elsevier, Paris)

Résumé — Déterminants fonctionnels de toxines de serpents et de cônes qui bloquent le récepteur nicotinique de l'acétylcholine de type α7. Les serpents et les cônes marins produisent des toxines qui bloquent les récepteurs cholinergiques nicotiniques (AChRs) musculaires et/ou neuronaux. Nous avons identifié les déterminants majeurs par lesquels une toxine longue curarisante de serpents et la toxine de cônes ImI, reconnaissent le récepteur neuronal α7. Dans les deux cas, le site comporte une petite structure organisée en coude, encadrée par deux demi-cystines. (©Elsevier, Paris)

nicotinic acetylcholine receptors / snake curaremimetic toxins / α-conotoxins

1. Introduction

Although they are phylogenetically distant, chordates (snakes) and molluscs (cone snails) include venomous species which elaborate various toxins that block muscular and/or neuronal nicotinic acetylcholine receptors (AChRs) [6, 15]. Understanding of the mode of action of these toxins, at the molecular level, requires elucidation of both their architecture and residues that are directly implicated in the specific recognition of AChRs. By introducing appropriate substitutions in amino acid sequences, we have elucidated: i) the specific functional elements which provide long chain curaremimetic toxins from snakes with high affinity for α7 neuronal AChR; and ii) functional residues by which the conotoxin α-ImI from *Conus imperialis* recognizes α7 neuronal receptor. Evidence suggests that this specific recognition involves in both cases a small turn-like structure that may differ from the determinants by which snake and cone snail toxins bind to *Torpedo* AChRs.

2. Materials and methods

2.1. Solid phase peptide synthesis

Conotoxin α-ImI and its analogues were synthesized by solid phase using the Fmoc-chemistry. The SPPS was carried out on an ABI 430A automated synthesizer (Perkin-Elmer/Applied Biosystems Division) with the HBTU/HOBt *Fast-moc* coupling program [7]. Pairing of the two disulphide bridges was achieved stepwise by specific chemistry as described previously [12]. The production yield of the α-ImI conotoxin and analogues was 10% as calculated from the starting Fmoc-Rink-AM- amide resin obtained from Novabiochem. Synthetic α-ImI and analogues were characterized by amino acid sequence analysis, analytical HPLC and electro spray ionization mass spectrometry (not shown).

2.2. α7 expression in HEK cells

The chimeric α7-5HT3 receptor was expressed transiently in HEK cells after calcium precipitation as described previously [2].

2.3. α-Cobratoxin expression, purification and mutagenesis

The cDNA encoding α-Cbtx was subcloned into pCP vector (fusion ZZ-Cbtx) as previously described [3, 4], expressed into the BL21 *E. coli* strain and the recombinant fused toxin was purified onto a IgG-Sepharose column and treated in 0.1 N HCl with CNBr. The toxin was refolded in phosphate buffer (0.1 M, pH 7.8) containing GSH/GSSG (4 mM/2 mM) and purified to homogeneity on a reverse phase (C4) column. The α-Cbtx mutant described here was prepared using the Stratagene kit (Quick Change) and the sequence of the entire gene was checked by automatic sequencing (ABI PRISM™ 310 Genetic Analyses, Applied Biosystem).

2.4. Binding assays

We determined the effect of increasing concentrations of: i) the wild-type and derivatized α-Cbtx; and ii) α-ImI and its analogues, on the initial rate of [^{125}I]-α-Bgtx binding to the

α7-5HT3 chimeric receptor [18]. The competitors were preincubated at different concentrations for at least 45 min with the cells suspension and filtrated 6 min after addition of 5 nM of ^{125}I-α-Bgtx. The protection constant calculated by fitting the competition data by the Hill equation reflects the K_d value [21].

3. Results and discussion

3.1. On the determinants by which long chain curaremimetic toxins bind to α7 receptor with high affinity

Snake curaremimetic toxins include both long chain toxins (66-74 residues and five disulphide bonds) and short chain toxins (60-62 residues and four disulphide bonds). They are all folded into three adjacent loops which emerge from a small globular core. The loops are rich in β-pleated sheet which provides the toxins with a leaf-like shape and two faces. While both short chain and long chain curaremimetic toxins from snakes bind to *Torpedo* AChR with high affinity [6], only long chain toxins display high affinities for neuronal α7 receptor [18].

Two lines of evidence indicate that this property is associated with the presence of the extra disulfide bond (Cys26-Cys30), called the fifth disuphide, that is uniquely found in long chain toxins. First, selective reduction of the fifth bond of α-cobratoxin (α-Cbtx), a prototype of long chain toxins, followed by modification of its two free cysteines by a chemical reagent, caused a substantial affinity decrease for the α7 receptor without modifying the binding property of the toxin for the *Torpedo* AChR [18]. Second, mutation of each of the two extra half-cystines into serine of α-Cbtx caused a 25-fold affinity decrease uniquely for the α7 receptor (*figure 1*, *table I*). Therefore, we suggest that the fifth disulphide controls an appropriate conformation of some key residues, most probably belonging to the small loop 26-30, and which may interact with the α7 receptor. This loop adopts a β-turn structure [1], whose residues (Asp^{27}-Ala^{28}-Phe^{29}) protrude at the tip of the toxin central loop. Whether additional determinants contribute to the high affinity binding of long chain toxins to AChR remains to be determined.

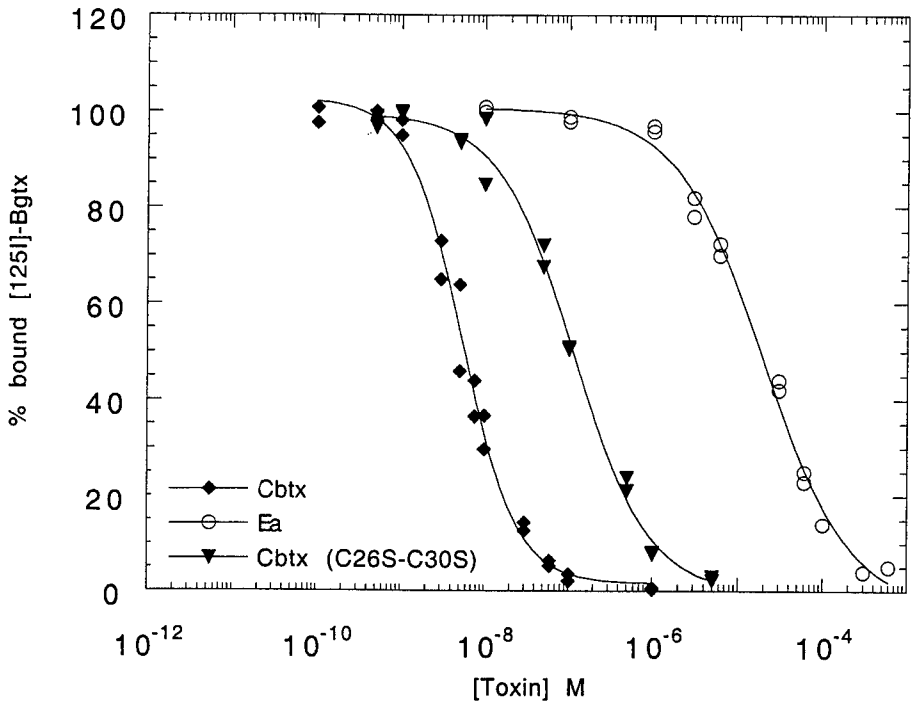
The determinants by which long chain curaremimetic toxins bind to muscular-type *Torpedo* AChR have not yet been elucidated. In contrast, the functional architecture of a erabutoxin a from Laticanda semifasciata (Ea), short chain toxin is becoming well documented [5, 14, 20]. It involves at least 10 functionally important residues which constitute a homogeneous surface located only on the concave face of the toxin but spread on its three loops. Most of the functionally important residues of Ea that are located on the second and third loops of the toxin are highly conserved in most long chain toxins, suggesting that they are also part of the site by which long chain curaremimetic toxins bind to muscular-type *Torpedo* AChR. In agreement with this proposal are the numerous chemical modifications that were previously performed on various long chain curaremimetic toxins [6]. Therefore, the small β-turn region which contributes to the high affinity binding of long-chain toxin to α7 receptor is adjacently located to the conserved functional determinants of curaremimetic toxins.

3.2. On the determinants by which α-conotoxin ImI binds to α7 receptor

Cone snails are recognized for their remarkable capacity to elaborate a diversity of toxins with a large spectrum of biological activities [15]. Like snakes, they produce toxins that act on muscular and/or neuronal receptors AChRs. However, in sharp contrast to reptile toxins, cone snail toxins are usually small peptides which often contain less than 20 residues and two disulphide bonds which define two small loops [13]. The functional sites of conotoxins acting on muscular AChRs have been previously subjected to extensive studies [8, 19] whereas the functional anatomies of neuronal conotoxins remain unknown.

α-ImI is a 12 residue-containing peptide with two disulphide bonds, isolated from venom of *Conus imperialis* [13]. This peptide has been described as a specific ligand of some neuronal AChRs, including the α7 subtype [11]. To identify the site by which this peptide binds to this neuronal AChR, we substituted all its positions, but the four half-cystines, by an alanine residue and determined the affinity constants of the analogues for the α7 neuronal AChR, using as receptor the chimeric construction previously described [2]. The two disulphide bonds of α-ImI analogues were paired as in the native toxin. In addition, however, we prepared two peptides, called I and II which respectively possessed none and one disulphide bond (Cys^{2}SSCys^8).

The K_d value for the synthetic α-ImI was 4 μM (*figure 2*, *table I*), in agreement with previous result obtained for chick muscle α7 receptors expressed during development [17]. This value, however, was approximately 20-40-fold higher as compared to that determined from electrophysiological recordings for mammalian (rat or mouse) α7 receptors [11, 16]. The competition curves performed with the alanine-containing analogues are shown in *figure 2* and the derived inhibition constants are reported in *table I*. The substitutions G1A, S4A and R11A virtually unaffected the competition potency of α-ImI. In contrast, the mutation W10A caused a six-fold affinity decrease and the substitutions D5A, P6A and R7A, caused respectively 38-fold, 70-fold and 165-fold affinity decreases.



Ea : RICFNHQSSQPQTTKTCSPGESSCYNKQWSD-F---RGTIIERGC--GCPTVKPGIKLSCCESEVCNN

Cbtx: IRCFITPDITSKD---CPNGH-VCYTKTWCDAFCSIRGKRVDLGCAATCPTVKTGVDIQCCSTDNCNPFFPTRKRP

Figure 1. Inhibition of binding of ^{125}I-α-bungarotoxin to the chimeric α7 receptor (α7-V201-5HT$_3$) [2], by: i) wild type α-cobratoxin (Cbtx); ii) Cbtx whose both half cystines C26 and C30 were replaced by a serine; and iii) erabutoxin a (Ea). The recombinant receptor was produced at the surface of HEK 293 cells. Varying concentrations of the competitors were incubated for 45 min with cell suspension and the mixtures were filtered through GF/C filters, 6 min after addition of ^{125}I-α-Bgtx at a final concentration of 5 nM. The continuous lines correspond to theoretical dose-response curves fitted through the data points using the non-linear Hill equation. The amino acid sequences of a short chain toxin (Ea) and a long chain toxin (Cbtx) are presented under the figure. For sake of clarity, the four disulphide bonds that are conserved in all curaremimetic toxins have been presented in Ea sequence only. The fifth disulphide that is only found on long chain toxins is also shown in the Cbtx sequence. Bold letters indicate the functional residues of Ea that are conserved in at least 90% of 84 amino acid sequences of short chain and long chain curaremimetic toxins [6]. These functionally highly conserved residues are Lys-27, Trp-29, Asp-31, Arg-33, Glu (or Asp)-38 and Lys-47 (using the numbering in the amino acid sequence of Ea).

The sequence D5-P6-R7 therefore, is a major determinant which, assisted by W10, allows α-ImI to bind to the α7 receptor.

Two lines of evidence further support the view that the sequence D5-P6-R7 is functionally important. First, introduction of the additional individual substitutions R7K, R7Nle, D5N and P6S also caused affinity decreases (see *table I*). Second, suppression of the bond Cys3-Cys12 had no effect on the toxin affinity whereas the additional deletion of the bond Cys2-Cys8, which is suspected to control the local organization of the sequence D5-P6-R7 (see below), substantially affected toxin affinity (*table I*).

What is the structural organization associated with the functionally critical sequence D5-P6-R7? At present, the three-dimensional structure of α-ImI is unknown. However, as inferred from an unpublished modeling study, based on both the known architectures of conotoxins sharing the same disulphide framework, such as α-conotoxin G1 [9] and secondary structure prediction

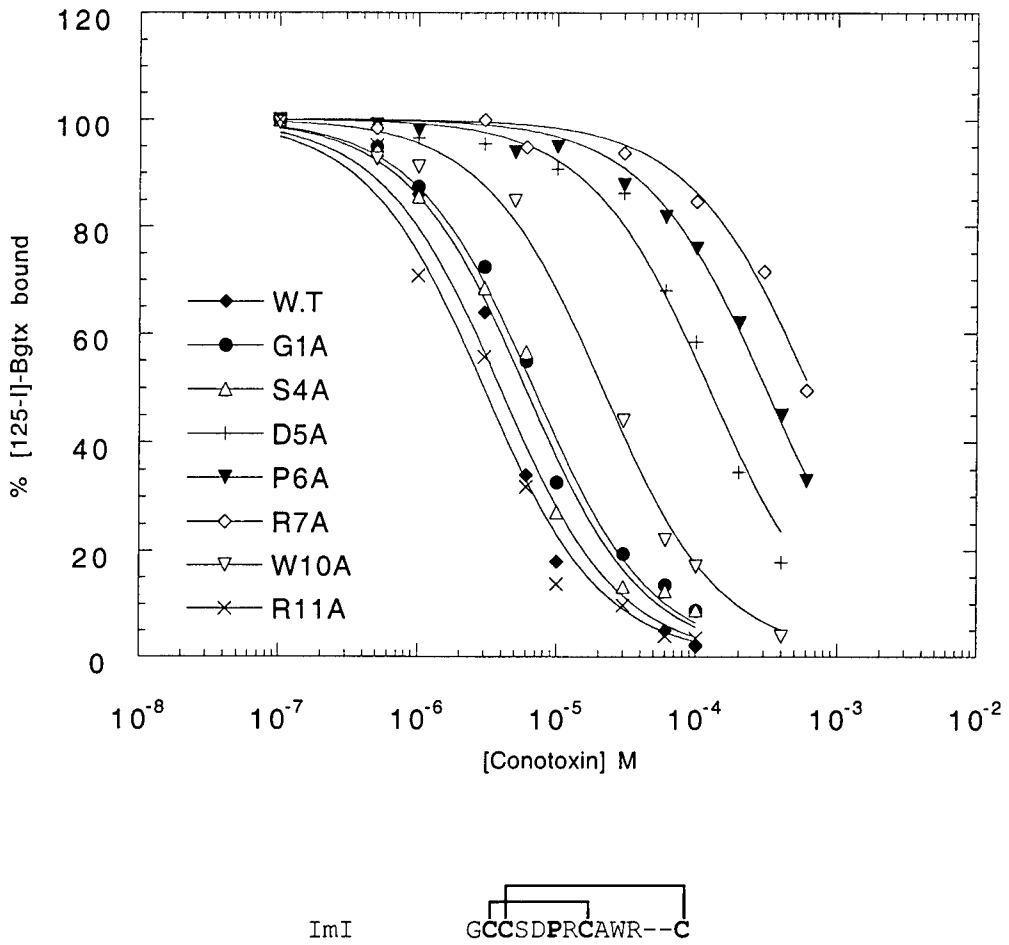


Figure 2. Inhibition of binding of ^{125}I-α-bungarotoxin to the chimeric α7 receptor by the wild type α-ImI conotoxin and its alanine-containing analogues. Binding experiments were performed as described in the legend to *figure 1*. The amino acids sequence and disulfide bond pairing of α-conotoxins ImI are presented.

methods [10], the region 5-8 is anticipated to adopt a β turn-like structure.

Previous studies have shown that the functional site by which α-G1 recognizes the muscular-type AChR involves primarily residues that are located between the half-cystines 8 and 13 [8, 19]. In contrast, we have shown that the α7 neuronal AChR-specific binding site of ImI is predominantly located in the small loop cyclized by the two half-cystines 2 and 8. Therefore, the major determinants by which α-conotoxins preferentially bind to α7 neuronal and muscular-type AChRs seem to be differently located on the conotoxin scaffold.

4. Concluding remarks

At least two types of toxins have some selectivity toward the α7 receptor. These are the snake long chain toxins, including curaremimetic toxins and K-neurotoxins [18] and some cone snail toxins, like ImI [11]. Though the two types of toxins share no three-dimensional analogy, their capacity to recognize the neuronal α7 receptor seems to be primarily associated with presentation of few specific critical residues on a small β-turn structure, stabilized by two adjacent half-cystines. Whether or not these structurally similar functional determinants recognize the same region of the α7 receptor remains to be determined.

Acknowledgments

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Table I. Affinity constants of α-Cbtx, α-ImI and their substituted analogues for the α7 receptor. Cbtx and Cbtx (C26S-C30S), stand respectively for α-cobratoxin and its mutant in which each of its two disulphide bonds 26 and 30 were mutated into serine. The peptides I and II correspond respectively to the α-conotoxin ImI in which: i) the two disulphide bonds were reduced and acetamidomethylated; and ii) only the disulphide 3-12 was reduced and subsequently modified.

	Kp (μM)	*Kp mutant/Kp W.T*
Cbtx		
Wild-type	0.0055 ± 0.001	1
Cbtx (C26S-C30S)	0.125 ± 0.015	23
α-ImI		
Wild-type	4 ± 0.26	1
G1A	5.6 ± 0.6	1.4
S4A	5.3 ± 0.8	1.3
D5A	150 ± 28	37.5
P6A	280 ± 151	70
R7A	660 ± 235	165
W10A	24 ± 3.1	6
R11A	2.9 ± 0.6	0.75
D5N	31 ± 7.2	8
P6S	170 ± 44	42.5
R7Nle	380 ± 81	95
R7K	295 ± 43	74
Peptide I	70 ± 9.8	17.5
Peptide II	3.7 ± 0.2	0.92

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Congenital myasthenic syndromes: Experiments of nature

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Abstract — Congenital myasthenic syndromes (CMS) can arise from presynaptic, synaptic, or postsynaptic defects. Recent studies indicate that mutations in the acetylcholine receptor (AChR) subunit genes are a common cause of the postsynaptic CMS. The mutations, which increase or decrease the response to acetylcholine, are experiments of nature that highlight functionally significant domains of the AChR. (©Elsevier, Paris)

Résumé — Les syndromes myasténiques congénitaux : Expériences de la nature. Les syndromes myasténiques congénitaux (SMC) peuvent être liés à des défauts présynaptiques, synaptiques ou postsynaptiques. Des études récentes indiquent que des mutations dans les gènes codant les sous-unités du récepteur à l'acétylcholine (RACh) sont une cause commune des SMC postsynaptiques. Les mutations, qui augmentent ou diminuent la réponse à l'acétylcholine, sont des expériences de la Nature qui rendent signifiantes les domaines fonctionnels de l'AChR. (©Elsevier, Paris)

congenital myasthenic syndromes / acetylcholine receptor / mutation analysis / expression studies

1. Introduction

Congenital myasthenic syndromes (CMS) are heterogeneous disorders arising from presynaptic, synaptic, or postsynaptic defects. In each CMS, the specific defect compromises the safety margin of neuromuscular transmission by one or more mechanisms. The clinical phenotypes of CMS are often similar; therefore, precise diagnosis requires correlation of clinical, in vitro electrophysiological, morphological, and, whenever possible, molecular genetic studies [3].

Prior to 1990, the investigation of CMS patients was based on clinical, morphologic, and conventional microelectrode studies. Since then, three developments paved the way for molecular analysis of postsynaptic CMS. First, by 1993, the cDNA sequences of the α, β, δ and ε subunits of adult and of the γ subunit of fetal human AChR were known, allowing molecular genetic analysis. Second, in the early 1990s, Milone et al. [10] succeeded in patch-clamping endplates (EPs) in human intercostal muscles, permitting analysis of the activity of single AChR channels. Third, the use of mammalian expression systems facilitated detailed analysis of how human AChR mutants alter the kinetics of the AChR channel. Coincident with this, we hypothesized that a kinetic abnormality of AChR at the single channel level predicts, and that severe EP AChR deficiency may predict, one or more mutations in the subunits of AChR. This hypothesis was subsequently confirmed by the discovery of mutations in different subunits of AChR that either increase [5, 13, 18, 21, 23] or decrease [4, 12, 14-17, 21, 22] the synaptic response to ACh.

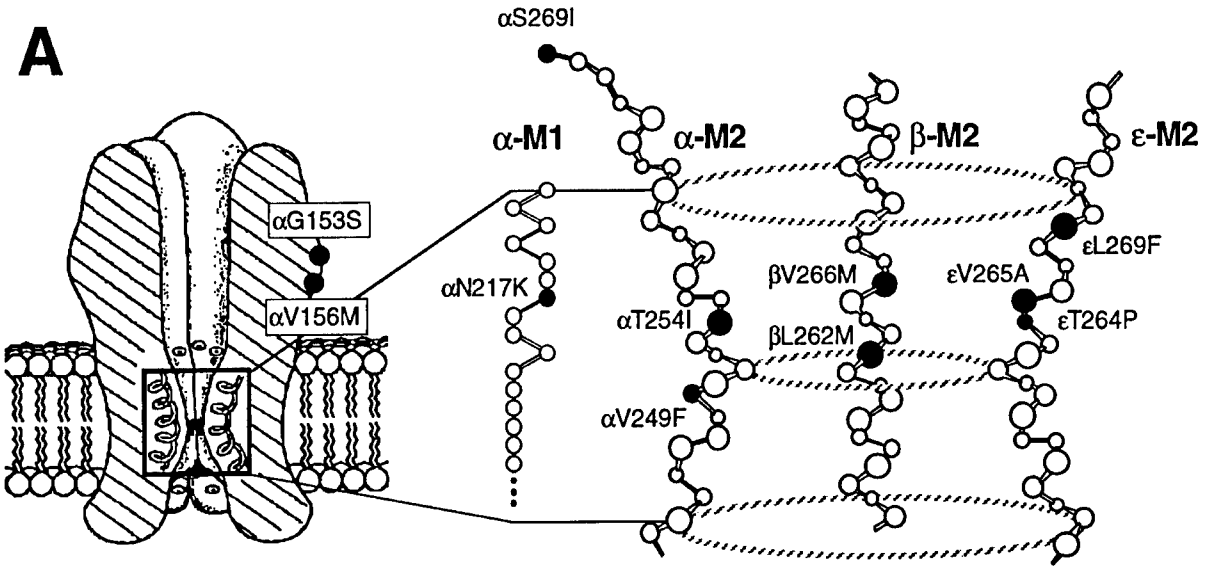
2. Mutations in AChR subunits cause postsynaptic CMS

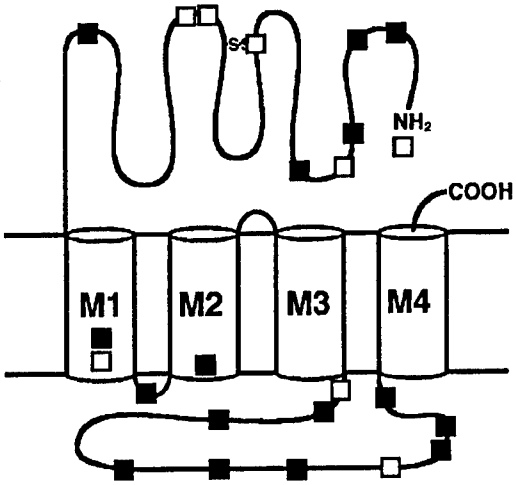
Since 1994, we and other investigators identified 56 AChR subunit gene mutations in 69 CMS kinships. *Table I* indicates the identified mutations according to their functional consequences and subunit locations. Interestingly, 38 of the 56 mutations and all 27 null mutations occur in the ε subunit of AChR, highlighting the susceptibility of the gene to mutation.

3. Increased response to ACh: slow-channel mutations

The clues for the diagnosis of an SCCMS consist of selectively severe weakness of the forearm extensor muscles, a repetitive compound muscle action potential response to single nerve stimuli that is accentuated by edrophonium, and a prolonged and biexponentially decaying miniature EP current. Eleven SCCMS mutations have been reported to date [2, 5, 7, 13, 18-20, 23, 25] . The different mutations occur in different AChR subunits and in different functional domains of the subunits (*figure 1A*). Each is dominant, causing a pathologic gain of function.

The phenotypic consequences of the SCCMS mutations stem from prolonged opening episodes of the





Mutations	Domains	Reference
❑ εG-8R	Signal peptide	22
■ ε59ins5	Extracellular domain	15
■ ε70insG	Extracellular domain	15
■ ε127ins5	Extracellular domain	21
❑ εT51P	Extracellular domain	9
■ εR64X	Extracellular domain	21
❑ εC128S	Disulfide loop	14
❑ εS143L	N-glycosylation site	22
❑ εR147L	Extracellular domain	21
■ ε553del7	Extracellular domain	21,16,12
■ ε723delC	M1 domain	9
❑* εP245L	M1 domain	21
■ εIVS7+2T→C	Link between M1 and M2	15
■ ε760ins8	M2 domain	9
❑* εR311W	Long cytoplasmic loop	21
■ εIVS9-1G→C	Long cytoplasmic loop	16
■ ε1012del20	Long cytoplasmic loop	17
■ ε1101insT	Long cytoplasmic loop	4
■ εIVS10+2T→G	Long cytoplasmic loop	9
■ ε1206ins19	Long cytoplasmic loop	15
❑* ε1254ins18	Long cytoplasmic loop	14
■ ε1267delG	Long cytoplasmic loop	9, 1
■ ε1276delG	Long cytoplasmic loop	15
■ ε1293insG	Long cytoplasmic loop	4

❑ Missense mutations, reduced expression
■ Premature chain termination, null mutations
* Mutation also has significant kinetic effects

Figure 1. A. Schematic diagrams of slow-channel CMS mutations reported to date. Cartoon on the left shows a section through AChR lodged in a lipid bilayer. The extracellular part of AChR forms a funnel-shaped channel that narrows to a gate formed by the alpha-helical M2 domain of each subunit. Two α subunit mutations in the extracellular domain near the ACh binding site are indicated. Cartoon on the right shows slow-channel mutations detected between the M2 and M3 domains of the α subunit, in the M2 domains of the α, β and ε subunits, and in the M1 domain of the α subunit. **B.** Schematic diagram and list of 24 low-expressor or null mutations in the ε subunit.

Table I. Fifty-six AChR subunit gene mutations in 69 kinships.

Mutation	α	β	δ	ε	*Total*
Point mutations					
Slow channel mutation	8	2		3	13
Fast channel mutation	**1**			1	2
Null mutation				2*	2
Reduced expression	**4**		**2**	5	11
Inframe rearrangement		**1**		**2**	3
Premature chain termination (null mutations)					
Frameshifting rearrangement				**16***	16
Splice-site mutation				**6***	6
Nonsense mutation				**3***	3
Total	13	3	2	38	56

Includes 34 published and 17 unpublished CMS mutations observed in our laboratories, three slow-channel mutations in the α subunit [2] and a frameshifting rearrangement in the ε subunit [1] described by Croxen et al., and a slow-channel mutation in the β subunit detected by Gomez et al. [7]. Numbers in bold face indicate recessive mutations that reduce AChR expression.
* Null mutations.

AChR channel. These cause: 1) cationic overloading of the junctional sarcoplasm and an EP myopathy with loss of AChR from degenerating junctional folds; and 2) a depolarization block due to staircase summation of prolonged end-plate potentials [5, 18, 23]. Patch clamp studies at the EP, mutation analysis, and expression studies in human embryonic kidney fibroblast (HEK) cells dissected three types of SCCMS. Those residing in the M2 domain, which lines the channel pore, act predominantly by slowing channel closure [5, 18]. A mutation near the ACh binding site on the α subunit increases affinity of AChR for ACh causing repeated reopenings of the channel during the prolonged ACh occupancy [23]. Another type of CMS has features of the two preceding types and the mutations reside in the M1 or M2 domain [5, 13, 25].

Recent studies indicate that quinidine, a long-lived open-channel blocker of AChR, is beneficial in the SCCMS. Fukudome et al. [6] demonstrated that drug levels attainable in clinical practice shorten and even normalize the prolonged opening episodes of mutant SCCMS AChRs expressed in HEK cells, and Harper and Engel [8] found that doses of the drug producing serum levels of 0.7-2.5 μg/mL (2.1-7.7 μM/L) benefit SCCMS patients by clinical and EMG criteria.

4. Decreased response to ACh: the low-affinity fast-channel mutations

Mutation analysis in two patients revealed two mutations in different alleles of the ε subunit gene: a common εP121L mutation plus a null mutation in the second ε allele, so that εP121L defined the clinical phenotype. In these patients the postsynaptic response to ACh is markedly diminished although the number of AChR per EP is normal [22, 24]. Patch-clamp studies show infrequent AChR channel events, abnormally brief activation episodes due to diminished channel reopenings during ACh occupancy, and increased resistance to desensitization by ACh [22]. Genetically engineered εP121L-AChR expressed in HEK cells have markedly decreased rate of channel opening and show greatly reduced affinity for ACh in the open-channel and desensitized states [22].

It is interesting to note that the εP121L mutation and the SCCMS mutations have opposite effects: SCCMS mutations increase the duration of activation episodes, enhance ACh binding affinity, increase desensitization by ACh, and cause an EP myopathy; εP121L shortens the duration of activation episodes, reduces ACh binding affinity, decreases desensitization by ACh, and leaves no anatomic footprint.

Recently we encountered a second low-affinity fast-channel mutation, αV285I, combined with a low-expressor mutation, αF233V, in the other α allele [11]. Detailed kinetic studies of αV285I are in progress.

5. Decreased response to ACh: a mutation causing mode-switching kinetics

In this disorder, an inframe duplication in the long cytoplasmic loop of ε, ε1254ins18, appears in com-

bination with a cysteine-loop null mutation, εC128S [14]. The ε1254ins18 mutation, which determines the phenotype, causes mode switching in the kinetics of receptor activation in which the normal high efficiency of gating is accompanied by two new modes that gait inefficiently. In the two abnormal modes the channel opens more slowly and closes more rapidly than normal. The ε1245ins18 AChR at the endplate shows abnormally brief activation episodes during steady state agonist application, and appears electrically silent during the synaptic response to acetylcholine. The phenotypic consequences are endplate AChR deficiency, simplification of the postsynaptic region, and compensatory expression of fetal AChR that restores electrical activity at the endplate and rescues the phenotype.

6. AChR deficiency caused by recessive mutations in AChR subunits

Severe EP AChR deficiency can result from different types of recessive mutations in AChR subunit genes. The mutations are either homozygous or, more frequently, heterozygous. Morphologic studies show an increased number of EP regions distributed over an increased span of the muscle fiber. The integrity of the junctional folds is preserved but some EP regions are simplified and smaller than normal. The distribution of AChR on the junctional folds is patchy and the density of the reaction for AChR is attenuated. Conventional microelectrode studies reveal a decreased amplitude of the miniature EP potentials and currents, and frequently high or higher than normal quantal release by nerve impulse. Single channel recordings at the EP [12, 14, 21] or immunocytochemical studies [4] often reveal the presence of fetal AChR that harbors the γ instead of the adult ε subunit (γ-AChR) at the EP.

Different types of recessive mutations causing severe EP AChR deficiency have now been identified (*figure1B*): 1) mutations causing premature termination of the translational chain. These mutations are frameshifting [4, 9, 15, 17, 21], occur at a splice site [9, 15, 16], or produce a stop codon directly [21]; 2) missense mutation in a signal peptide region (εG-8R) [22]; 3) missense mutations in residues essential for assembly of the pentameric receptor. Mutations of this type were observed in the ε subunit at an N-glycosylation site (εS143L) [22]; in cysteine 128 (εC128S), a residue that is an essential part of the C128-C142 disulfide loop in the extracellular domain [14]; and in arginine 147 (εR147L) in the extracellular domain, which lies between isoleucine 145 and threonine 150, residues that contribute to subunit assembly [21]; and 4) missense mutations affecting both AChR expression and kinetics. For example, εR311W [21] in the long cytoplasmic loop between M3 and M4 decreases, whereas εP245L in the M1 domain [21] increases the open duration of channel events. In the case of εR311W and εP245L, the kinetic consequences are modest and are likely overshadowed by the reduced expression of the mutant gene.

There are two possible reasons for why recessive mutations causing AChR deficiency are concentrated in the ε subunit. First, expression of the fetal type γ subunit, although at a low level, may compensate for absence of the ε subunit [4, 14, 21], whereas patients harboring null mutations in subunits other than ε might not survive for lack of a substituting subunit. Second, the gene encoding the ε subunit, and especially the exons coding for the long cytoplasmic loop, have a high GC content that likely predisposes to DNA rearrangements.

7. Future prospects

Since 1994, molecular analysis of the postsynaptic CMS provided clear insights into disease mechanisms and highlighted functionally significant domains of AChR. In the coming years molecular studies will undoubtedly be applied to presynaptic and synaptic forms of CMS. It is also likely that the molecular studies will provide clues for conventional and gene therapy and lead to the recognition of novel types of CMS.

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Regulated exocytosis in neurons and neurosecretory cells: Structural events and expression competence

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Abstract — This paper summarizes the contribution of the laboratory first in the development of the 'kiss-and-run model' of exocytosis, with its fascinating aspects of specificity and rapidity, then in proposing the existence of 'competence factors' that appear to govern the appearance of the secretory vesicles and exocytic process in neurosecretory cells. (©Elsevier, Paris)

Résumé — Exocytose contrôlée dans les neurones et les cellules neurosécrétrices : événements structuraux et compétence cellulaire. Cet article résume les contributions de notre laboratoire, d'une part dans le développement du modèle d'exocytose *'kiss-and-run'*, avec ses aspects fascinants de spécificité et de rapidité, et d'autre part dans l'hypothèse de l'existence de « facteurs de compétence » qui semblent contrôler la formation de vésicules de sécrétion et les processus d'exocytose dans les cellules neurosécrétrices. (©Elsevier, Paris)

regulated exocytosis / vesicle fusion / vesicle recycling / neurosecretion competence

1. Introduction

Exocytosis in neurons and neurosecretory cells, i.e., the process by which quanta of neurotransmitter are released in bulk and made available to the specific receptors in the surrounding membranes, has been a major interest of our group. Studies were initiated by Bruno Ceccarelli in the early seventies and later developed in multiple directions by his and my laboratory. Soon after the exocytic process was identified, it became clear that the cellular aspects are more complex and elaborate than initially thought inasmuch as the step of vesicle fusion is rapidly followed by the recycling of the fused membrane, necessary to maintain the plasmalemma specificity in terms of both surface extension and molecular composition. The nomenclature of exo-endocytosis (or exo-endocytic cycle) was thus proposed, and attention was focused more and more on the molecular mechanisms and on their regulation, with progressive identification of the proteins involved, both in the membranes and in the cytosol. Surprisingly, however, for quite sometime the structural features and the time-course of the cycle did attract only few specific studies. Even at synapses the recycling of fused vesicles was largely believed to occur by a process of conventional endocytosis: formation of a coated cage around the plasmalemmal invagination resulting from an exocytic fusion, detachment of the resulting coated vesicle from the plasmalemma, shedding of the coat followed by fusion of the naked vesicle with a cisterna of the endocytic compartment from which new synaptic vesicles were believed to originate by budding (for a classical discussion of the problem see Ceccarelli and Hurlbut [2]). Such a sequence of events, although necessarily slow in its development, was so successful that it is still the only one reported in most textbooks, even in some of the most advanced.

Among the few scientists that remained not convinced by the 'coated vesicle model' were those of our group. For quite sometime the success of alternative explanations remained limited. Yet, intriguing suggestions were offered even by plain electron microscopy, when applied however not to conventionally fixed samples but to preparations that had been quick frozen and thus blocked within 1 ms in the course of their stimulated exocytic process. With the latter approach many of the vesicles more intimately continuous with the plasmalemma appear in fact not as invaginations, open to the extracellular space, but still as vesicles, sealed however by thin, clearly discernible diaphragms in direct continuity with the cell surface. The interpretation that Ceccarelli and his colleagues provided to the images was that of transient and incomplete fusions, adequate for the release of the neurotransmitter quanta. Such a mechanism can assure the rapid recycling of the vesicles, with

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no risk of molecular membrane intermixing with the plasmalemma [2, 7]. In our more recent review, written a few years after Bruno Ceccarelli's death, this exo-endocytic process was first referred to as 'kiss-and-run' [4], a definition that is now encountering favor in the scientific community. The favor is not only due to the suggestive power of the definition. Rather, new and independent evidence has accumulated that appears at least compatible with the 'kiss-and-run' model of exocytosis. In fact, very transient openings of secretory organelles do indeed take place in at least some secretory systems, as revealed by capacitance measurements in patch clamping [1, 5]; exocytosis can be dissociated from recycling of membrane invaginations [6]; interactions of recycled vesicles with endosomes have now been excluded [8].

To sum up, the exo-endocytic field appears now more open than a few years ago (see also Schweitzer et al. [9]). Kiss-and-run can be envisaged as one (the most convenient for the cell!) of the processes by which exo-endocytosis can take place. When in contrast the opening of a fused vesicle proceeds from the transient to a more persistent stage, possibly because of the disassembly of an initial, channel-like structure around the vesicle opening [5], recycling requires more complex events involving the contractile protein, dynamin together with clathrin and its coats [10]. Whatever the frequency of the two processes (and possibly of others that remain to be identified), the lesson for future work is to pay attention to properties, such as timing and number of vesicles involved, that in most studies carried out until recently had been let aside, with ensuing missing of important aspects of the exo-endocytic process.

2. Regulated exocytosis: what about its cell expression?

Regulated exocytosis with release of specific products is widespread, but not ubiquitous among eukaryotic cell types. The question then is: how do cells acquire the ability to assemble specialized organelles (dense and clear vesicles in the case of neurons and neurosecretory cells) together with the molecular apparatus (not only in vesicles but also in the plasmalemma and in the cytosol) that, in response to appropriate stimulation, governs exocytosis followed by recycling? Since in the appropriate cells the appearance of vesicles of either type takes place during embryonic development, exocytosis is often thought to represent a step in phenotype acquisition. However, results that we have obtained during the last several years with a clone of PC12, a cell line derived from a rat pheochromocytoma, suggest the story to be more complex. Our clone, in fact, was found to lack completely the secretory organelles together with their membrane and cargo components, and to be also free of the specific cytosolic and plasmalemma proteins participating in regulated exocytosis. Yet, the clone is not de-differentiated with respect to the other PC12 cells. In contrast, it does express a variety of specific markers, thus its cells can only be defined as neurosecretory but defective of regulated neurosecretion competence [3].

The latter conclusion suggests that regulated exocytosis is a property expressed independently from the others of the neurosecretory cell phenotype, governed by specific factor(s) that operate 'in block', by switching on (or off) the expression of multiple and variously targeted components of the cell. The nature of the 'secretion competence factor(s)', although intensively investigated during the last few years, is at the moment still mysterious. Evidence of its existence has however been obtained by cell fusion experiments. Reappearance of neurosecretion, specific in molecular terms, has been observed in chimerae obtained by the fusion of defective PC12 not only with secretory but also with non-secretory cells (unpublished results). A new, so far unexplored line of exocytosis research, concerning the cell competence and expression of the process, has thus been initiated. Although at the moment its success is hard to predict, the study appears nevertheless fascinating, also because it necessarily requires the combination of multiple advanced experimental approaches, focused on a single, well defined and original issue. Let's wait for the developments.

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Acetylcholine release. Reconstitution of the elementary quantal mechanism

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Abstract — Choline acetyltransferase and vesicular acetylcholine transporter genes are the products of two adjacent genes defining a cholinergic locus. The release mechanism is expressed independently of this locus in some cell lines. A cholinergic neuron will therefore have to coordinate the expression of release with that of the cholinergic locus. Transfection of a plasmid encoding *Torpedo* mediatophore in cells that are unable to release this transmitter endows them with a Ca^{2+}-dependent and quantal release mechanism. The synchronization of mediatophore activation results from a control of calcium microdomains by the synaptic vesicles. It is therefore dependent on the proteins that dock vesicles close to calcium channels. (©Elsevier, Paris)

Résumé — Libération d'acétylcholine. Reconstitution du mécanisme quantique élémentaire. La choline acétylase et le transporteur vésiculaire d'acétylcholine sont codés par deux gènes adjacents qui définissent un locus cholinergique. Certaines lignées cellulaires expriment le mécanisme de libération de l'acétylcholine indépendamment de ce locus cholinergique. Un neurone cholinergique devra donc coordonner l'expression du locus cholinergique et celle du mécanisme de libération. La transfection de cellules incapables de libérer l'acétylcholine avec un plasmide codant pour le médiatophore de Torpille leur confère un mécanisme de libération Ca^{2+}-dépendant et quantique. La synchronisation des médiatophores est liée au contrôle de microdomaines calciques par les vésicules synaptiques. Elle dépend donc des protéines qui assurent l'ancrage des vésicules à proximité des canaux calciques. (©Elsevier, Paris)

acetylcholine release / cholinergic locus / mediatophore / transfection

1. Introduction

It has long been known that proteins supporting cholinergic transmission are expressed in a variety of cells that are not directly involved in synaptic transmission. For example, acetylcholinesterase is present in erythrocytes [1], choline-acetyltransferase in the placenta [34] and nicotinic receptors at the tendon [25]. Unexpected localization of the release machinery itself has also been suspected from recent experiments showing that glial cells, fibroblasts and other cell types, loaded in culture with acetylcholine (ACh), are subsequently able to release the neurotransmitter, either in response to a calcium influx or after electrical stimulation [11–13, 20]. Also fibroblasts transfected with choline-acetyltranferase cDNA become able to release the synthesized transmitter [14] and myocytes injected with ACh can generate miniature end-plate potentials [9]. More recently, both spontaneous and evoked ACh release were demonstrated in fibroblast cell lines [16, 27]. Moreover, proteins that are typical for the active zone of nerve terminals belong to protein families whose other members are found even in yeast, suggesting a very broad role in cellular communication [31].

2. Results and discussion

2.1. Unexpected expression of the release mechanism in different cell lines

The localization of choline acetyltransferase (ChAT) and vesicular acetylcholine transporter (VAChT) genes within a single genomic locus and the co-expression of both proteins throughout the cholinergic system strongly suggests that synthesis and storage of ACh are tightly co-regulated [2, 4, 10, 26]. But what about regulation of the release mechanism? A variety of cell lines that do not express the ChAT and VAChT genes nonetheless display a Ca^{2+}-dependent quantal release mechanism for ACh, as discovered after loading the cells in culture with ACh [12, 20] or after transfection with ChAT cDNA [14, 21]. Taken together, these data demonstrate that the proteins involved in release can be expressed independently of ChAT or VAChT.

Ca^{2+}-dependent ACh release was monitored from four different cell lines by using the choline-oxidase procedure. Cells were loaded with ACh during culture [20] and release was elicited with a calcium ionophore (A23187), followed by calcium. In these experiments, the amount of ACh was the same in

the different cell samples. It was measured before the release challenge and the sample size adjusted accordingly. As seen in *figure 1* (top traces), cells from the glioma C6BU-1, the fibroblast L-M (TK$^-$) and the hybrid NG108-15 lines were found to possess a release mechanism, whereas neuroblastoma N18TG-2 cells were unable to release the transmitter. The neuroblastoma-glioma NG108-15 hybrid line (which is issued from C6BU-1 and N18TG-2 lines) might therefore have 'inherited' the release machinery from its glial parent. The pattern of release-competent and release-incompetent cells presented here was a constant finding with these cell lines.

An electrophysiological approach enabled us to characterize the release mechanism with a more adequate time resolution. The results were in full agreement with those of the biochemical assay (*figure 1*, bottom traces). ACh-filled cells were individually put into contact with a *Xenopus* myoball that served as a real-time ACh detector. On electrical stimulation of C6BU-1, L-M (TK$^-$) and NG108-15 cells, rapid inward currents were recorded from the patched myoball. These 'postjunctional' currents were reversibly suppressed by addition of tubocurarine or when calcium was replaced by 10 mM magnesium in the solution. In series of responses generated by a given cell, the evoked currents showed a tendency to peak at preferential steps, suggesting a quantal composition. In contrast to the other lines, N18TG-2 cells did not express stimulation-evoked ACh release. No response, or only responses of a negligible amplitude, were observed after testing more than 130 ACh-filled N18TG-2 cells.

2.2. Acetylcholine release from mediatophore-transfected cells

Mediatophore is a protein we had characterized in previous works. Purified from the plasma mem-

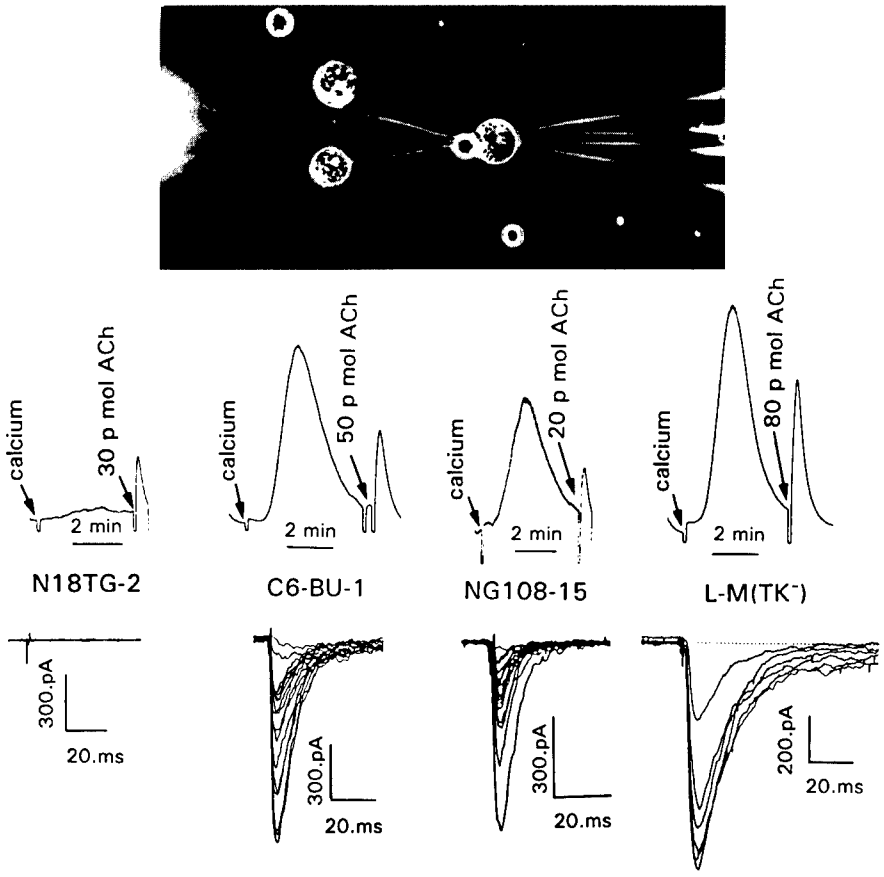


Figure 1. ACh release elicited from various cell lines loaded with the transmitter. Top traces: release was triggered by calcium after treatment with ionophore A23187 and measured continuously with the luminescence assay. Bottom traces: release from the same cell lines, but in this case release was evoked by electrical stimulation and measured in real-time by using a patched *Xenopus* myocyte. Top picture: a releasing cell (on the left) in contact with the *Xenopus* myocyte. The neuroblastoma N18TG-2 cells were not release-competent, while the rat glioma C6-Bu-1, the glioma-neuroblastoma hybrid NG108-15 and the fibroblast L-M (TX) lines expressed Ca^{2+}-dependent and quantal transmitter release.

brane of *Torpedo* electric organ synaptosomes, mediatophore is able to translocate ACh upon calcium action [19, 20]. Mediatophore is localized at the active zone of nerve terminals [6]. It is a homo-oligomer of a 16-kDa subunit that is also found in association with other components in the membrane sector of the V-ATPase [35]. Antisense probes hybridizing the mediatophore messenger inhibit ACh release [7, 8, 20].

We have recently shown that cells with a low 16-kDa proteolipid content in their membrane have a low capacity for ACh release. This was the case for N18GT-2 cells. They consequently represented an ideal material for a reconstitution of ACh release. N18GT-2 cells were transfected with a plasmid encoding the *Torpedo* mediatophore. The Western blot in *figure 2* demonstrates that the transfected cells express the *Torpedo* protein. Several clones were selected and tested for release. The N18TG-2 cells that expressed *Torpedo* mediatophore gained the capability to release ACh in response to a calcium influx (*figure 2*, top traces). Control clones, transfected with the plasmid alone, did not release the transmitter although they contained the same amount of ACh.

Control and transfected cells were also tested for release by using patched *Xenopus* myoballs. Stimulation elicited a pulse-like release of transmitter from mediatophore-transfected cells, but not from controls (*figure 2*, bottom traces). In series of successive responses, as those illustrated in *figure 2*, the currents

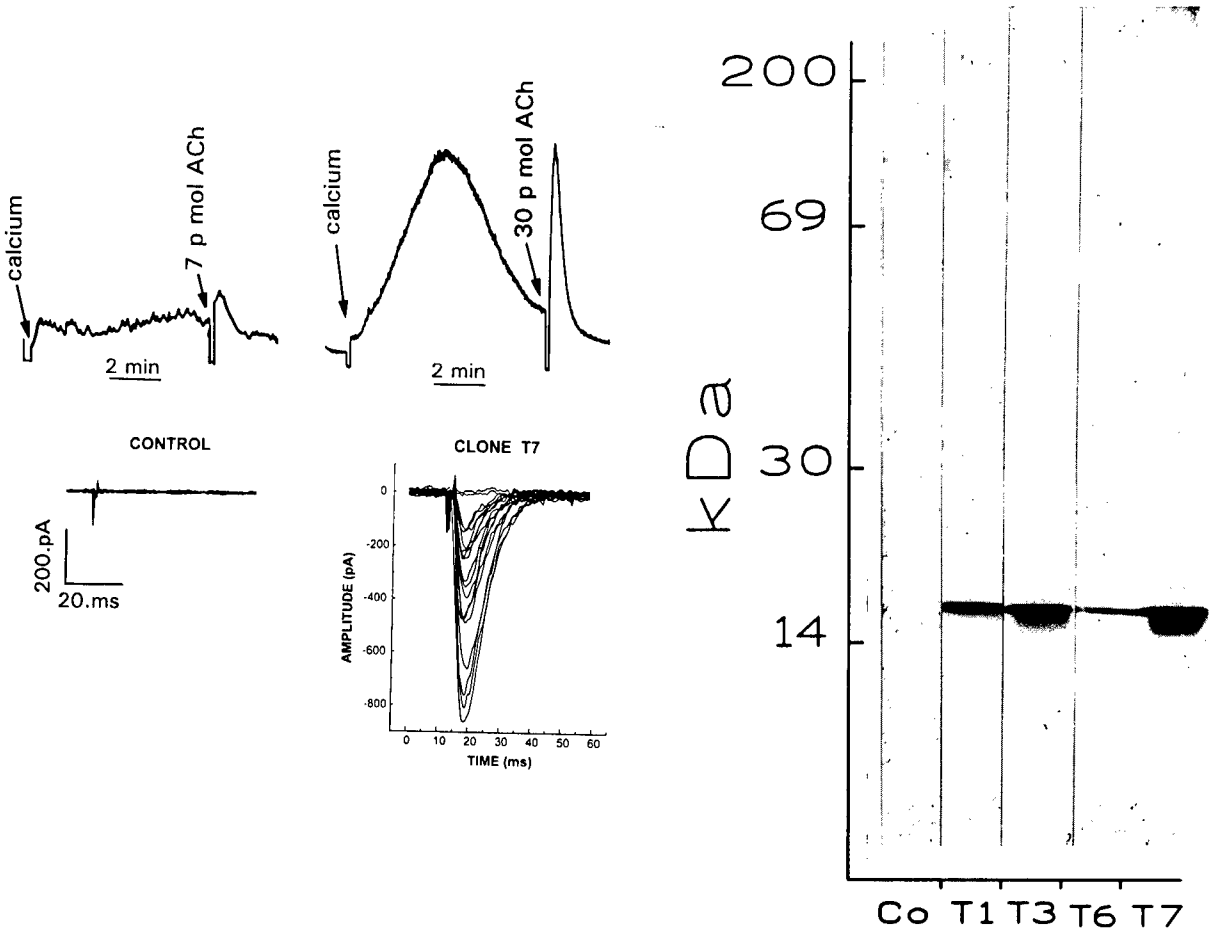


Figure 2. Right part: Western blot showing that *Torpedo* mediatophore was expressed in several clones (T1 to T7) of N18TG-2 cells after transfection. 'Co' is a control clone containing the transfection vector without the mediatophore insert. The control cells did not release ACh, while the mediatophore-transfected clones acquired a new release capacity. Release was measured by the choline oxidase chemiluminescent procedure (top traces) and recorded electrophysiologically by using a patched myoball (bottom traces). Transfection with the 16-kDa subunit of the mediatophore re-established a ACh release which was typically Ca^{2+}-dependent and quantal, like in natural synapses.

peaked at preferential amplitude values, suggesting a quantal composition of transmitter release. This was further demonstrated by analyzing the successive responses using the Poisson's distribution [11]. As with the cell lines described above, it was verified that evoked ACh release induced by transfection in N18TG-2 cells was curare-sensitive and Ca^{2+}-dependent. Hence, release-deficient cells which had acquired by transfection the *Torpedo* mediatophore in their plasma membrane became able to release ACh in a pulse-like and quantal form, which is the most characteristic property of transmitter release in naturally-occurring synapses.

2.3. A common regulation is required to turn on both the cholinergic locus and the release mechanism

In spite of the fact that the cholinergic release mechanism can be expressed independently of synthesis and storage, cholinergic neurons will have to turn on both the release apparatus and the cholinergic locus by some coordinated regulation. For the release, the problem is complex since many proteins might be involved. Nevertheless, the approach can be simplified by separately considering those proteins that are more specifically related to the release of ACh and those that are common to many cells. The latter include the components of the T-and-V-SNAREs [28] that govern the traffic of intracellular membranes at the active zone as they do in the Golgi apparatus. These proteins (the T-SNAREs syntaxin and SNAP-25 at the plasma membrane, and the V-SNARE VAMP/synaptobrevin on synaptic vesicules) are expressed in many cell types. The same is true for V-ATPase, which functions to accumulate protons in intracellular organelles. In neurons, it is this process which is responsible for the driving force concentrating the transmitters into granules or vesicles.

The protein supporting more specifically ACh release has been identified and named mediatophore (see above). However, mediatophore is a homo-oligomer of a 16 kDa proteolipid that is also part of the membrane sector of the V-ATPase. The 16-kDa element in itself cannot therefore be considered as a cholinergic-specific protein. What may be actually more specific for cholinergic neurons is the mechanism that directs the 16-kDa to the plasma membrane. This question was recently addressed by Leroy and Meunier [22] who expressed the 16-kDa in *Xenopus* oocytes. They showed that injecting the mRNA of the 16-kDa alone was less efficient in inducing ACh release than injecting the whole set of poly (A*) mRNAs extracted from cholinergic neurons. In the former, the 16-kDa protein accumulated chiefly in intracellular membranes. With the total poly (A*) mRNAs, substantial amounts reached the plasmalemma. Therefore, another protein is probably required for directing the 16-kDa subunit at the plasma membrane where it is assembled to form the mediatophore homo-oligomer. This process may coordinate the events associated with expression of the cholinergic locus and that of a specific step for ACh release. In this respect, cAMP-dependent systems may represent an important regulation pathway since they can upregulate both ACh synthesis and ACh release, as well as Ca^{2+} channels in some cells [3, 12, 20].

2.4. Vesicular docking mechanisms

The three proteins, syntaxin, SNAP-25 and VAMP/synaptobrevin constitute a clostridrial toxin-sensitive site, since they are cleaved by botulinum and tetanus toxins. This complex has been purified by fractionation and immunoprecipitation experiments [29, 31]. There are, in addition, two possible systems that may anchor the vesicles to the plasma membrane: the 'C2' attachment site and the synaptophysin-mediatophore link.

Several proteins share a homologous stretch of sequence, the C2 domain. It has been found in the vesicular protein synaptotagmin, in protein kinase C and in the cytoplasmic protein rabphilin. This last protein undergoes reversible binding to Rab3-GTP and thereby, to vesicle membranes. The C2 anchor binds directly or indirectly to the calcium channel as shown by immunoprecipitation experiments [17, 23]. Decoy peptides homologous to the C2 domain or protein kinase C itself perturb this C2 docking and alter transmission [5]. Similarly, interference with Rab-rabphilin attachment affects release in pituitary cells [24].

The third type of docking may involve two channel-forming proteins, synaptophysin and mediatophore. This possibility was suggested by the fact that synaptophysin (and also synaptobrevin) can be immunoprecipitated with 16-kDa protein [15, 29] which is common to mediatophore and to the c-subunit of the V-ATPase membrane sector (V0). In addition, another subunit of V-ATPase (39 kDa) was previously identified as physophilin, a synaptophysin-binding protein [30, 33]. This docking system might link in series, through mediatophore and synaptophysin-V0, the vesicle lumen to the extracellular space. Such a link is expected to be resistant to clostridial toxins. It may still function, like the C2 anchor, when the clostridial anchor (T- and V-SNAREs) is absent or cut. In fact, vesicles still dock to the membrane when the clostridial link is damaged [32]. However, a physiologically intact

clostridial link is likely to be essential for controlling the shape and kinetics of the Ca^{2+} microdomains, needed for ensuring synchronization of release and protecting mediatophores against desensitization. When this link is disrupted, quantal ACh release can still be elicited, but it becomes desynchronized and poorly Ca^{2+}-sensitive; hence, nerve-evoked transmission fails.

3. Conclusion

In order to develop its 'communication apparatus', a cholinergic neuron has to turn on several genes in parallel. The cholinergic locus provides ACh synthesis and storage. Mediatophore molecules represent the elementary pores that translocate ACh either from the cytosol, or from vesicles if they are connected to the pore. The mediatophores seem to be clustered at membrane spots [6], probable close to a calcium channel. This organization is not sufficient for an efficient and fully regulated transmission since the activity of individual mediatophores has to be synchronized. The vesicles docked at the membrane by specific proteins in close contact to the calcium channel are important for this task. First, they efficiently take calcium, 'shaping' in space and time the local calcium microdomains which are determinant for phasic transmitter release. In addition, the vesicles perform a variety of controls according to the phase of their endo-exocytic cycle. They provide a reserve pool of transmitter. After a period of activity, they deliver, or take up, proteins and other compounds to, and from, the plasma membrane and the extracellular matrix.

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Protein interactions implicated in neurotransmitter release

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Abstract — Biochemical evidence indicates that the exocytotic release of neurotransmitters involves both evolutionary conserved membrane proteins, the SNAREs, as well as ubiquitous cytosolic fusion proteins, NSF and SNAPs. We have analyzed the biochemical properties and the physiological effects of these proteins. Our data suggest models how NSF, SNAPs and SNAREs may function in neurotransmitter exocytosis. (©Elsevier, Paris)

Résumé — **Interactions protéiques impliquées dans la libération des neurotransmetteurs.** Des données biochimiques ont montré que des protéines membranaires (les SNAREs) et ubiquitaires cytosoliques (NSF et SNAPs) conservées au cours de l'évolution sont impliquées dans la libération des neurotransmetteurs. L'analyse des propriétés biochimiques et des effets physiologiques de ces protéines nous permettent de proposer des modèles sur la façon dont les SNAREs, les SNAPs et NSF pourraient être impliquées dans l'exocytose. (©Elsevier, Paris)

protein interactions / neurotransmitter release / SNAREs

1. Introduction

The regulated release of neurotransmitters at presynaptic nerve terminals provides the elementary process, upon which fast synaptic transmission is based. Its high speed and efficient regulation are crucial for intercellular communication on a millisecond time-scale. Work by Katz, Whittaker and others has established that this release process involves the Ca^{2+}-regulated exocytosis of neurotransmitter from specialized storage organelles, the small synaptic vesicles. Rapid freezing of stimulated nerve terminals has allowed visualization of synaptic vesicles captured at various stages of fusion with the plasma membrane and identified a pool of synaptic vesicles that are clustered around specialized regions of the presynaptic plasmalemma [8]. These 'active zones' are thought to contain a sub-pool of readily releasable vesicles, which are 'docked' close to the channels that mediate the Ca^{2+} influx that triggers release [10, 11].

Recent biochemical and genetic studies indicate that an evolutionary conserved protein network utilized in all eukaryotic vesicle-based membrane traffic also mediates synaptic vesicle-plasma membrane fusion [3]. Experiments conducted to define the membrane receptors for ubiquitous cytosolic fusion factors, the ATPase *N*-ethylmaleimide sensitive fusion protein (NSF) and the α-, β- and γ-soluble NSF attachment proteins (SNAPs), identified a synaptosomal complex composed of the plasma membrane proteins syntaxin and synaptosomal associated protein of 25 kDa (SNAP-25) and the synaptic vesicle protein synaptobrevin as the major SNAP binding component of rat brain extracts [23]. These proteins were therefore collectively named as SNAP receptors (SNAREs) and proposed to act as both synapse-specific membrane recognition molecules and acceptors for fusion catalyzing proteins.

In detergent extracts, the synaptosomal SNAREs form a complex that migrates at 7S upon density gradient centrifugation and is thought to represent the biochemical correlate of synaptic vesicles docked at the plasma membrane [23, 24]. Under conditions that prevent ATP hydrolysis, the SNARE complex binds SNAP and NSF to form a multimeric complex that migrates at 20S. ATP hydrolysis by NSF disrupts this 20S complex in a process that has been postulated to drive membrane fusion [23, 24]. Further evidence for an important role of the SNAREs in synaptic vesicle exocytosis comes from their identification as the targets of clostridial neurotoxins, the most potent inhibitors of neurotransmitter release known. The light chains of these toxins act as Zn^{2+} dependent endoproteases, with each light chain specifically cleaving one of the three synaptosomal SNAREs (*figure 1*). Synaptobrevin is cleaved by tetanus toxin and botulinum toxins B, D, F and G;

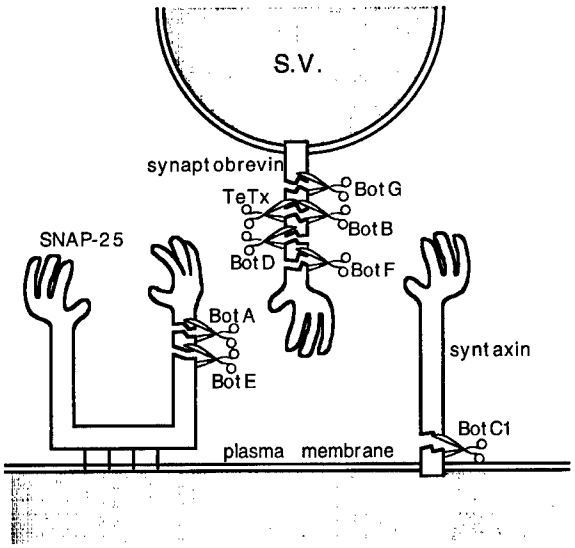


Figure 1. Clostridial toxin cleavage sites. The proteins cleaved by the clostridial neurotoxins are shown to highlight their juxtaposed membrane orientations in the synaptic vesicle (synaptobrevin) and plasma (syntaxin and SNAP-25) membranes. This oversimplifies the in vivo situation as fractions of cellular syntaxin and SNAP-25 are found on synaptic vesicles [25]. Transmembrane domains insert syntaxin and synaptobrevin into the membrane, whereas palmitoylation of cysteine residues is responsible for the membrane localization of SNAP-25. Note that Bot C1 may cleave both syntaxin and SNAP-25.

syntaxin by botulinum toxin C1; and SNAP-25 by botulinum toxins A and E [13, 15].

In this report we summarize biochemical and microinjection experiments aimed at demonstrating a physiological role of the SNAREs and the soluble fusion proteins in a living synapse and clarifying the importance of their various interactions.

2. SNAREs have a post-docking role in synaptic vesicle fusion

To elucidate whether the SNAREs synaptobrevin and syntaxin are involved in synaptic vesicle exocytosis, we used the giant synapse of the squid stellate ganglion. The enormous size of this synapse allows simultaneous recording of pre- and postsynaptic potentials in a preparation amenable to microinjection of recombinant proteins, peptides and other molecular probes. First, we tested the function of the synaptic vesicle SNARE synaptobrevin. cDNA cloning of squid synaptobrevin revealed that the cleavage site for the clostridial Zn^{2+} proteases tetanus toxin and botulinum toxin B light chain is conserved in this invertebrate [9]. Upon microinjection into the giant terminal, both toxins produced a slow irreversible inhibition of neurotransmitter release that was prevented by coinjection of an inhibitor peptide that corresponds to the sequence of synaptobrevin that spans the cleavage site. Electron microscopy revealed that the toxin-poisoned terminals contained increased numbers of synaptic vesicles around their active zone profiles. Importantly, the number of vesicles apposed to the presynaptic membrane, i.e., the docked vesicle population, was significantly higher than in control injected terminals [9]. Thus, toxin cleavage severed synaptobrevin function at a late step of vesicle exocytosis, which lies downstream of the vesicle-plasma membrane recognition and/or docking process.

The same approach was also used to unravel a role for the plasma membrane SNARE syntaxin [17]. Presynaptic injection of the light chain of botulinum toxin C1 irreversibly blocked neurotransmission. This is consistent with the conservation of the botulinum toxin C1 cleavage site in the amino acid sequence of the squid protein deduced from cDNA. Moreover, injection of the highly conserved C-terminal H3 domain of syntaxin also produced a potent reversible inhibition of neurotransmitter release. Biochemical experiments revealed that the recombinant H3 domain competed syntaxin binding to SNAP-25 and thus prevented the formation of 20S fusion complexes. In contrast to observations made upon tetanus toxin light chain injection, however, ultrastructural analysis of nerve terminals microinjected with either botulinum toxin C1 or the H3 domain failed to reveal changes in the number or distribution of synaptic vesicles at presynaptic active zones. These data are consistent with syntaxin-SNAP-25 interactions functioning in an early reversible post-docking step of the exocytotic process [17].

In vitro data also support the conclusion that the SNAREs function at post-docking steps of exocytotic membrane fusion. SNAREs are accessible to proteolysis by clostridial neurotoxins only in their free non-complexed state [19]. Surprisingly, however, SNARE complex formation was not abolished in brain extracts, in which synaptobrevin was completely cleaved by tetanus toxin treatment [20]. Rather both cleavage fragments of synaptobrevin were recovered in the resultant 7S SNARE complex. Also, assembly and disassembly of the 20S complex, i.e., binding of SNAP and NSF by the complexed SNAREs and their dissociation upon NSF-catalyzed ATP hydrolysis, was indistinguishable in tetanus toxin cleaved and control preparations. Apparently, synaptobrevin cleavage does not disrupt the SNAREs' principal conformational transitions. However, a highly characteristic property of the complexed

SNAREs, their ability to form a SDS-resistant trimer, was severely impaired as revealed by a different temperature sensitivity to SDS dissociation [7, 20]. This shows that the stability, i.e., the low intrinsic energy, of the SNARE complex is altered upon incorporation of toxin-cleaved synaptobrevin. We have therefore proposed that clostridial neurotoxins impair SNARE function by compromising the ability of the SNARE complex to form an 'activated' high-energy intermediate upon NSF-catalyzed ATP hydrolysis state [20]. Accordingly, the conformational energy stored in this complex is postulated to drive membrane fusion upon Ca^{2+} triggering [16] of a release event. This idea is consistent with recent ultrastructural [6] and reconstitution [26] experiments, which assign NSF-driven ATP hydrolysis a central role in the generation of free SNAREs capable of forming parallel hairpin-like SNARE complexes postulated to be essential for close apposition of the membranes to be fused.

3. The soluble fusion proteins snap and nsf: determinants of the size and kinetics of neurotransmitter release?

We also have employed the squid giant synapse to test the functions of the ubiquitous soluble fusion proteins NSF and SNAP. The cloning of a full-length squid SNAP cDNA allowed to produce a recombinant squid SNAP protein [5]. Microinjection of either this squid SNAP or recombinant mammalian α-SNAP into the giant presynaptic terminal produced an enhancement of transmitter release as assayed by postsynaptic recording. This indicates that SNAP is a rate-limiting component of the release apparatus. In contrast, peptides corresponding to potentially surface-exposed regions of squid SNAP caused a rapid and reversible inhibition of synaptic vesicle exocytosis. Examination by imaging and electron microscopy of the effects of one of these peptides revealed that its blocking action was not related to alterations in Ca^{2+} influx, but correlated with an about 3-fold increase in the number of synaptic vesicles docked at the active zones of the presynaptic plasma membrane. Assuming that this accumulation of docked vesicles in the peptide-inhibited terminals was the consequence of a competitive inhibition of SNAP interaction with other components of the vesicle release machinery [1, 21], our data are consistent with a post-docking role of SNAP in synaptic vesicle exocytosis.

Cloning of sequences encoding the squid homologue of mammalian NSF [22] revealed that, like SNAREs and SNAPs, this trimeric ATPase is highly conserved in invertebrates, a conclusion that also emerged from studies in *Drosophila melanogaster*. There, the temperature-sensitive comatose mutant phenotype has been shown to result from point mutations in one of two NSF genes [18]. The protein sequence deduced from a partial squid NSF cDNA allowed to synthesize peptides corresponding to subdomains of the highly conserved D1 region which are predicted to be surface-exposed. Microinjection of two of these peptides into the presynaptic terminal of squid giant synapse produced not only a reduction in neurotransmitter release, but also slowed its kinetics [22]. Specifically, both peptides increased the synaptic delay and slowed both the onset and the decay of excitatory post-synaptic currents. These effects were highly specific, since substitution of a single glycine residue by glutamate in one of the peptides caused a loss of peptide inhibition and slowing. Interestingly, the same substitution is found in one of the aforementioned Drosophila comatose mutants [18]. These data indicate that NSF has a key role in the regulation of the release process. Biochemical experiments revealed that the active NSF peptides inhibited SNAP stimulation of NSF's ATPase activity [22]. Electron microscopy showed a modest increase in the number of synaptic vesicles directly docked at the presynaptic plasma membrane, whereas the more distant vesicle pool was severely depleted. This is consistent with NSF functioning not only in the release process, but also being required for replenishing the pool of vesicles associated with synaptic zones. In conclusion, our experiments suggest that NSF-catalyzed ATP hydrolysis regulates both the kinetics of the exocytotic process and other steps of intraterminal vesicle traffick.

4. Discussion

The work reported here shows that both the SNAREs and the soluble fusion proteins NSF and SNAP have essential roles in neurotransmitter release. From our electrophysiological and ultrastructural data, all these proteins may be concluded to act at post-docking steps of synaptic vesicle exocytosis (*figure 2A*). This is consistent with the original postulates of the SNARE hypothesis [23, 24], although Ca^{2+} triggering is likely to occur after SNAP/NSF action [4, 20]. Recent studies on constitutive membrane fusion in yeast, however, have challenged this view by showing that NSF action can precede SNARE complex formation [12, 14]. It therefore has been proposed that NSF acts as a chaperone [2] to unfold SNARE complexes after fusion and thus to generate reactivated SNAREs to drive another fusion cycle (*figure 2B*). This model is consistent with ultrastructural data on recombinant SNARE complexes [6]

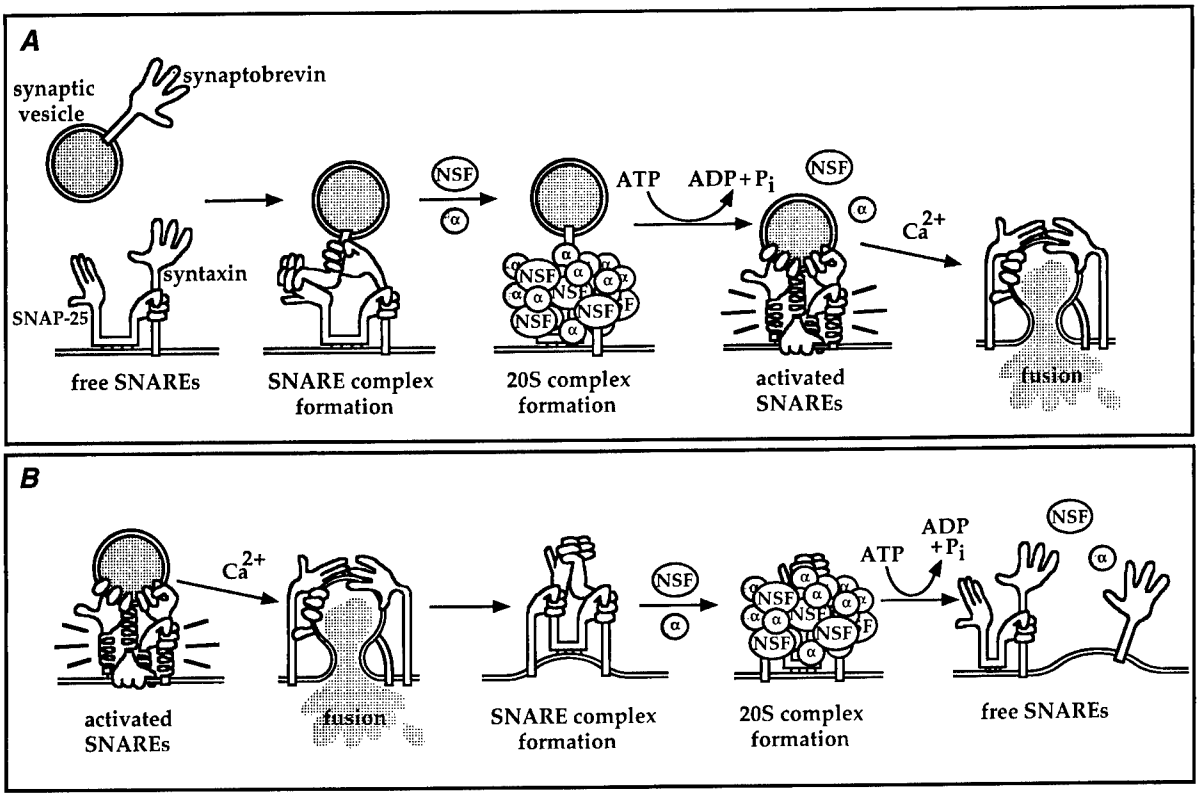


Figure 2. Pre- or post-fusion activation of SNARE complexes by NSF? **A.** Activation of SNARE complexes by NSF is assumed to occur after docking but prior to fusion. **B.** A post-fusion mechanism that reconciles data obtained from analysing SNARE complex function in yeast is shown. In both models, the activated complex is triggered by Ca^{2+} to initiate fusion. For B, this is postulated to cause a re-orientation of the SNAREs to result in a post-fusion complex that subsequently requires NSF action to liberate individual SNAREs. According to B, NSF generates a fusion-competent complex by re-activating SNAREs. The two models envisage NSF to act at distinct steps of the synaptic vesicle cycle. Model B is consistent with ultrastructural data obtained by negative staining [6].

which revealed a parallel orientation of synaptobrevin and syntaxin cytoplasmic domains. This parallel configuration might be crucial to overcome the energy barrier imposed by the repulsion of phospholipid head groups in the apposed lipid bilayers. Accordingly, the extraordinarily stable association of complexed SNAREs is a prerequisite for the fusion process [6, 26]. The effects of SNAP and NSF inhibition seen upon peptide injection into the squid giant synapse then might reflect a reduced number and/or interaction competence of partially reactivated SNAREs. A prediction of model B is that the 7S SNARE complex alone should be sufficient to drive membrane fusion. Recent reconstitution experiments with isolated SNAREs [26] are consistent with this interpretation.

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On the action of botulinum neurotoxins A and E at cholinergic terminals

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Abstract — Botulinum neurotoxins type A and E (BoNT/A and /E) are metalloproteases with a unique specificity for SNAP-25 (synaptosomal-associated protein of 25 kDa), an essential protein component of the neuroexocytotic machinery. It was proposed that this specificity is based on the recognition of a nine-residue sequence, termed SNARE motif, which is common to the other two SNARE proteins: VAMP (vesicle-associated membrane protein) and syntaxin, the only known substrates of the other six clostridial neurotoxins. Here we report on recent studies which provide evidence for the involvement of the SNARE motif present in SNAP-25 in its interaction with BoNT/A and /E by following the kinetics of proteolysis of SNAP-25 mutants deleted of SNARE motifs. We show that a single copy of the motif is sufficient for BoNT/A and /E to recognise SNAP-25. While the copy of the motif proximal to the cleavage site is clearly involved in recognition, in its absence, other more distant copies of the motif are able to support proteolysis. We also report on studies of poisoning human neuromuscular junctions with either BoNT/A or BoNT/E and describe the unexpected finding that the time of recovery of function after poisoning is much shorter in the case of type E with respect to type A intoxication. These data are discussed in terms of the different sites of action of the two toxins within SNAP-25. (©Elsevier, Paris)

Résumé — Action des neurotoxines botuliniques A et E sur les terminaisons cholinergiques. Les neurotoxines botuliniques de type A et E (BoNT/A et BoNT/E) sont des métalloprotéases qui clivent de façon extrêmement spécifique la SNAP-25 (*'synaptosomal associated protein of 25 kDa'*), qui joue un rôle essentiel dans les processus de neuro-exocytose. Il a été proposé que cette spécificité repose sur la reconnaissance d'un motif de neuf acides aminés, appelé motif SNARE, qui se retrouve dans les deux autres protéines 'SNARE', la VAMP ('vesicle-associated membrane protein') et la syntaxine, car ces protéines constituent les seuls substrats connus des six neurotaxines clostidiales. Nous avons étudié la protéolyse de mutants de SNAP-25 dans lesquels le motif SNARE est délété, et nous montrons que ce motif est impliqué dans l'interaction de la protéine avec BoNT/A et BoNT/E. Une seule copie de ce motif est suffisante pour que ces neurotoxines reconnaissent la SNAP-25 : le motif le plus proche du site de clivage joue un rôle évident dans cette reconnaissance, mais en son absence, d'autres copies plus éloignées de ce motif peuvent assurer la protéolyse. Nous décrivons aussi l'effet neurotoxique de BoNT/A et BoNT/E sur des jonctions neuromusculaires humaines : de façon inattendue, la récupération fonctionnelle est beaucoup plus rapide après l'action de la BoNT/E qu'après intoxication par la BoNT/A. Nous discutons ces résultats en fonction des sites de clivage de la SNAP-25 par ces deux toxines. (©Elsevier, Paris)

SNAP-25 / SNARE motif / botulism / neurotoxin / zinc-endopeptidases / dystonia

1. Introduction

Botulinum neurotoxins cause the flaccid paralysis typical of botulism by blocking acetylcholine release at the neuromuscular junction. They are di-chain zinc endopeptidases secreted by different bacteria of the genus *Clostridium*. The neurotoxins reach their intracellular targets by translocating the light chain (50 kDa) into the cytosol after having been endocytosed via interaction of the heavy chain (100 kDa) with a high affinity receptor complex [7, 8]. Serotypes B, D, F and G proteolyse VAMP/synaptobrevin [18, 20], serotypes A and E specifically cleave SNAP-25 [1, 2, 19] and type C acts on syntaxin and SNAP-25 [5, 11, 22]. Syntaxin and SNAP-25, associated with the cytoplasmic face of the plasma membrane of presynaptic nerve terminals, and VAMP, inserted into the synaptic vesicle membrane, have been postulated to act as synaptic vesicle docking receptors (t- and v-SNAREs). They have been implicated in the steps leading to membrane fusion for release of neurotransmitter into the synaptic cleft [16, 17]. SNAP-25, however, seems not only involved in neuroexocytosis, but is also required for axonal growth and synaptogenesis [10].

The three SNAREs, SNAP-25, syntaxin and VAMP are the only eukaryotic proteins to possess a distinct and conserved motif [14]. This short motif which is present at four positions in SNAP-25 (S1 to S4) (*figure 1*) is predicted to form an amphipathic

* Correspondence and reprints
Abbreviations: BoNT, botulinum neurotoxin; DTT, dithiothreitol; GST, glutathione S-methyl transferase; SNAP-25, synaptosome associated protein of 25 kDa; SNAREs, soluble NSF accessory protein receptors; TeNT, tetanus toxin; VAMP, vesicle associated membrane protein.

α-helix, providing a surface of negative charge on one-third of the surface and a group of hydrophobic residues on the adjacent third of the cylinder.

BoNT/A is the best available therapeutic agent of a variety of human diseases that benefit from a functional inhibition of cholinergic terminals [6, 9]. Hence, there is a particular importance in understanding the molecular basis of the action of BoNT/A. Here, we report on mutagenesis studies of SNAP-25 which demonstrate that recognition of the SNARE motif is essential for SNAP-25 proteolysis by BoNT/A and /E.

2. Materials and methods

BoNT/A and /E were obtained from Wako (Germany) and further purified via immobilized-metal-ion affinity chromatography to remove traces of contaminant proteases [15]. Anti C-terminal SNAP-25 antibodies were prepared as detailed previously [13]. Primary antibodies were detected by immunostaining with an anti-rabbit conjugated with alkaline phosphatase (Sigma) using nitrotetrazolium blue and bromo-chloro-indolylphosphate (Sigma). Bacterial strains, plasmid construction and SNAP-25 deletions and mutagenesis were as described before [21].

2.1 Proteolytic assays

Mouse SNAP-25 and its mutants were expressed as GST fusion proteins and were purified by affinity chromatography on GSH-sepharose matrix (Pharmacia) as before [12]. After treatment with 10 mM DTT for 30 min at 37 °C, BoNT/A or BoNT/E (50 nM final concentration) were added to each of the GST-fusion SNAP-25s (0.5 μg/μL final concentration) in phosphate buffer: 10 mM NaH_2PO_4, 150 mM NaCl, pH 7.4 and incubated at 37 °C for 60 min. The concentrations of the two proteases were selected after preliminary experiments to provide rates of cleavage capable of revealing differences among SNAP-25 mutants. Samples were analysed by Western blotting onto nitrocellulose after electrophoresis in a 13% polyacrylamide SDS gel and were probed with an anti SNAP-25 C-terminal antibody.

2.2 Neuromuscular block with BoNT/A and /E

The EDB muscles of human volunteers were injected with BoNT/A in one side and with BoNT/E in the other side as described before [3]. At 7, 14, 30, 45, 60 and 90 days the recovery of neuromuscular function was assayed by measuring muscular contraction elicited by a supramaximal stimulation of the peroneal muscle.

3. Results and discussion

Figure 1 illustrates the location and sequence of the four copies of the SNARE motif present in the SNAP-25 molecule, together with the cleavage sites of BoNT/A and /E. Beginning from the amino-terminus, the four copies of the motif are termed S1, S2, S3 and S4. To determine which of the four copies of the SNARE motif present in neuronal SNAP-25 is important for the recognition by the SNAP-25 specific clostridial neurotoxins, we constructed a series of N-terminal deletions of mouse SNAP-25b expressed as recombinant GST-fusion proteins and measured their rate of proteolytic cleavage. *Figure 1* also shows the various chimeric GST-SNAP-25 constructs that have been prepared (top panel), expressed in *E. coli* and purified by affinity chromatography on Sepharose-GSH columns, together with the extent of their proteolytic cleavage by BoNT/A (left panel) and BoNT/E (right panel) after 1 h of incubation. Progressive deletions beginning from the distal amino-terminal copy of the motif do not alter the rate of cleavage of the substrate both by BoNT/A and by BoNT/E. Moreover, the deleted SNAP-25 substrates are cleaved as long as a single copy of the SNARE motif (S4) is present. However, removal of a further 14 residues containing the S4 motif from Δ1–140 to produce Δ1–154 totally abolishes proteolytic activity. Hence, the minimal segment of the SNAP-25 molecule required for the proteolytic cleavage by both BoNT/A and /E has to include S4.

To determine whether the other SNARE motif copies present in the SNAP-25 molecule could substitute for S4, N-terminal deletions were generated using a ΔS4 mutant (*figure 2*). As shown, the rate of cleavage by BoNT/A is reduced progressively for each SNARE motif removed, but cleavage is completely abolished when all remaining SNARE motifs were deleted. BoNT/E shows a smaller dependence of the rate of proteolysis on sequential removal of the N-terminal SNARE motifs, but it is entirely inactive on a SNAP-25 mutant not containing any copy of the motif. These results indicate that the N-terminal SNARE motifs, S1, S2 and S3, can substitute to different extents for the absence of S4.

BoNT/A is the best available treatment for a variety of human dystonias and other cholinergic syndromes [9]. However, a sizeable proportion of patients are resistant to BoNT/A from the first injection, while others become resistant following the production of antibodies specific for BoNT/A. For this reason, we have introduced the use of BoNT/C and have proven that this toxin is as effective as BoNT/A with therapeutic benefits lasting for just as long [4]. To extend this approach, we have tested the effect of BoNT/E on humans. This toxin induces a very effective paralysis in humans. However, neuromuscular junctions of EDB muscles recovered their full function after 30–45 days after the injection of BoNT/E, compared to the more than 90 days needed to recover from BoNT/A treatment. This result

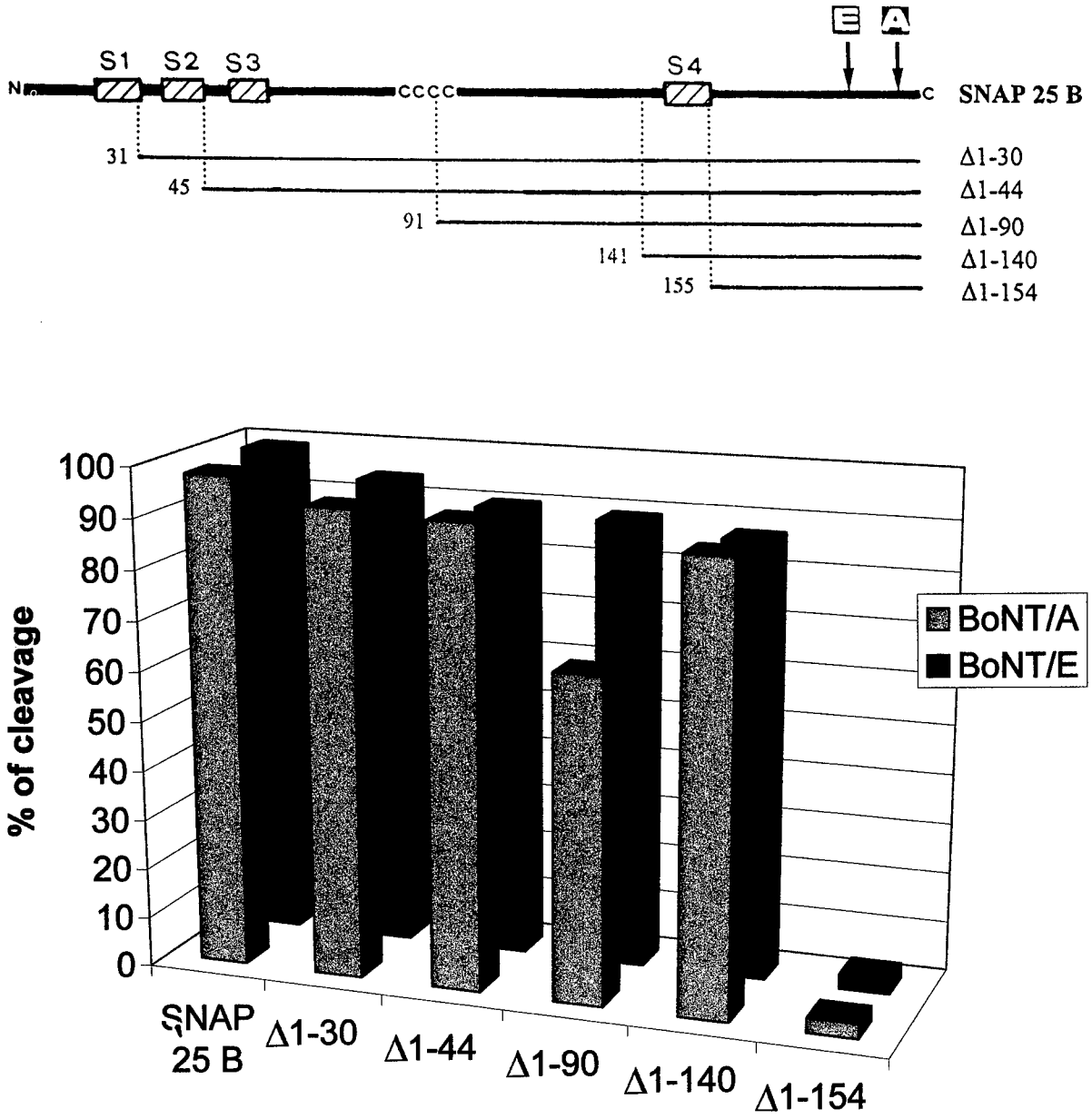


Figure 1. Proteolysis by BoNT/A and /E of N-terminal deletion mutants of SNAP-25. The upper diagram shows the deletion mutants that have been fused to GST and assayed for their susceptibility to BoNT/A and /E. All constructs were incubated with 50 nM BoNT/A and 50 nM BoNT/E, samples were taken after 60 min, submitted to electrophoresis and Western blot. The alkaline phosphatase stained blots were quantified by densitometric scanning. Data are average of three independent experiments.

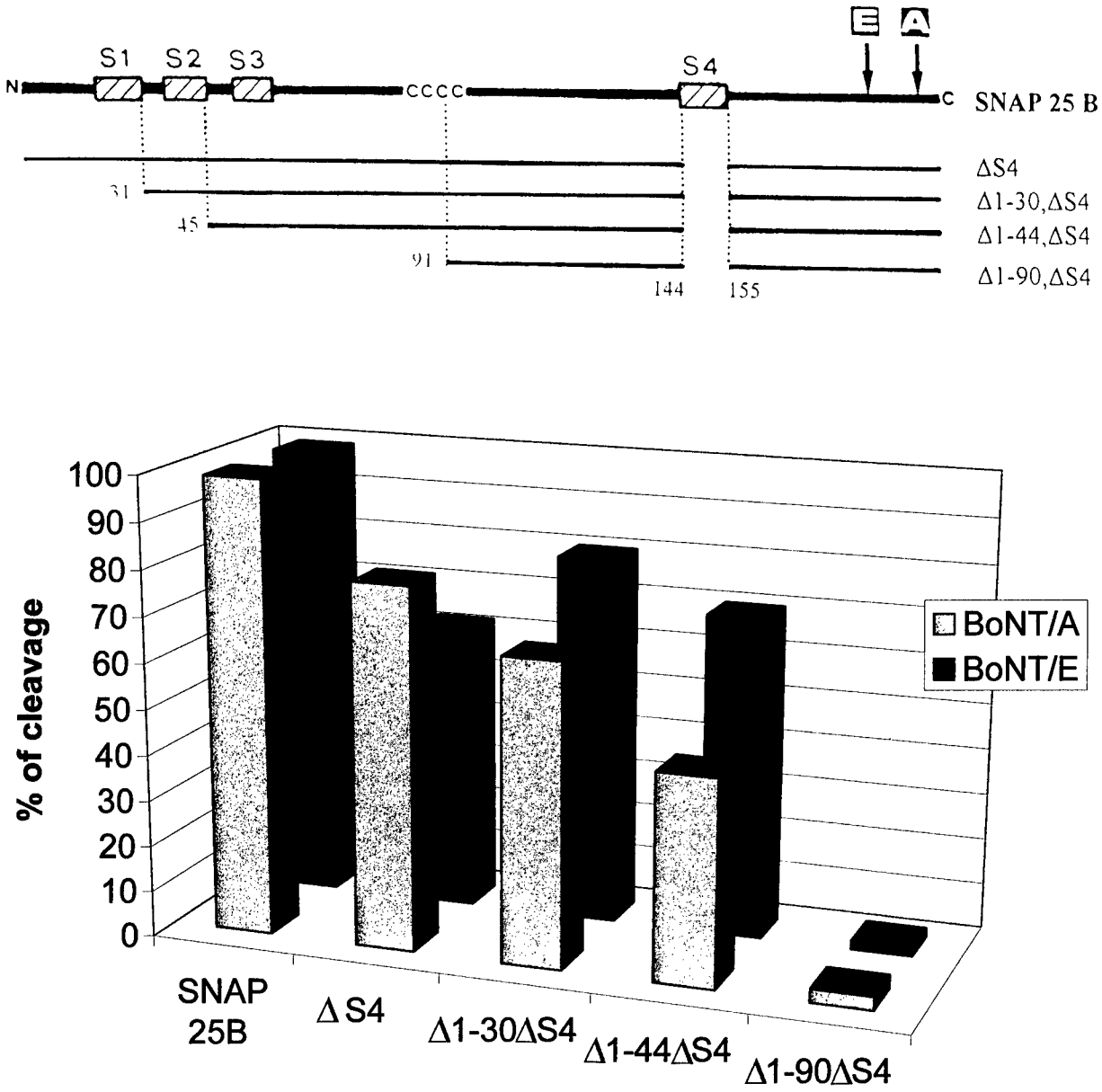


Figure 2. Neurotoxin proteolysis of deletion mutants that progressively remove the three N-terminal SNARE motifs in the absence of S4. The upper diagram shows deletion mutants of SNAP-25, but which include a loop out deletion of amino acids 141 to 154 (ΔS4). All constructs were incubated with 50 nM BoNT/A and 50 nM BoNT/E, samples were taken after 60 min, submitted to electrophoresis and Western blot. The alkaline phosphatase stained blots were quantified by densitometric scanning. Data are average of three independent experiments.

is not due to a different life-time of the two toxins inside the motoneuron terminals. Rather, it appears that removal of 26 residues, caused by BoNT/E, from the SNAP-25 C-terminus induces a much more rapid replacement of the damaged SNAP-25 molecule with respect to the removal of nine residues, caused by BoNT/A.

Acknowledgments

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Dissociation of the vesicular acetylcholine transporter domains important for high-affinity transport recognition, binding of vesamicol and targeting to synaptic vesicles

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Abstract — Chimeras between the human vesicular acetylcholine transporter (hVAChT) and the neuronal isoform of the human vesicular monoamine transporter (hVMAT2) have been constructed and stably expressed in a rat pheochromocytoma cell line (PC12) in an effort to identify cholinergic-specific domains of VAChT. Examination of the transport properties of a chimera in which the N-terminal portion (up to putative transmembrane domain II and including the lumenal glycosylated loop) of hVAChT was replaced with hVMAT2 sequences (2/V@*Nhe*I) revealed that its apparent affinity for acetylcholine (ACh) was reduced approximately seven-fold compared to wild-type. However, the affinity of this chimera for vesamicol did not significantly differ from hVAChT. Similarly, the 2/V@*Nhe*I chimera retained its preferential targeting to the small synaptic-like vesicles found in PC12 cells in agreement with our recently reported observations that the synaptic vesicle targeting domain resides in the cytoplasmic tail of VAChT. (©Elsevier, Paris)

Résumé — Dissociation des domaines du transporteur vésiculaire d'acétylcholine impliqués dans la reconnaissance du substrat, la liaison du vesamicol, et l'adressage vers les vesicules synaptiques. Nous avons cherché à identifier les régions spécifiquement cholinergiques du transporteur vésiculaire d'acétylcholine (VAChT) à l'aide de proteines chimériques humaines composées de fragments de hVAChT et de l'isoforme neuronal du tranporteur vésiculaire de monoamines (hVMAT2), exprimées de façon stable dans des cellules PC12 (pheochromocytomes de rat). L'affinité apparente pour l'acétylcholine de la chimère 2/V@*Nhe*I (hVMAT2 jusqu'au début du domaine transmembranaire II, puis hVAChT) est réduite 7 fois par rapport à hVAChT. L'affinité de cette chimère pour le vésamicol est quant à elle inchangée. De plus, cette chimère 2/V@*Nhe*I, comme hVAChT, est adressée préférentiellement vers les petites vésicules de type synaptiques dans les cellules PC12. Ce resultat est en accord avec notre récente observation que le signal d'adressage vers les vésicules synaptiques se situe à l'extrémité C-terminale cytoplasmique de hVAChT. (©Elsevier, Paris)

vesicular acetylcholine transporter (VAChT) / neuronal isoform of the vesicular monoamine transporter (VMAT2) / acetylcholine / vesamicol / synaptic vesicles / targeting

1. Introduction

The vesicular acetylcholine transporter (VAChT) is responsible for packaging cytoplasmic acetylcholine (ACh) into the small synaptic vesicles of cholinergic neurons where it is available for regulated neurosecretion. Vesamicol, a neuromuscular blocking agent, disrupts this process by specifically inhibiting vesicular accumulation of ACh. Recently, the molecular cloning and functional identification of VAChT has revealed that vesicular ACh transport and vesamicol binding properties are encoded by a single polypeptide [11].

VAChT exhibits approximately 40% identity with the two other members of this gene family, the endocrine-specific (VMAT1) and neuronal (VMAT2) isoforms of the vesicular monoamine transporter [8]. Since these proteins all utilize a common H+/antiport mechanism for vesicular substrate accumulation, chimeric transporters offer an opportunity to identify regions of these proteins which are important in determining substrate and inhibitor specificity as well as specific targeting to cholinergic versus monoaminergic secretory organelles.

Rat pheochromocytoma cells (PC12) synthesize both dopamine and ACh with expression of rVMAT1 on large dense core vesicles (LDCV) and rVAChT on the small synaptic vesicle clusters which can be observed following treatment with nerve growth factor [13]. Recently, we have shown that structural information resides within the terminal cytoplasmic domain of hVAChT, which specifically targets it to the PC12 synaptic vesicles [12]. Overexpression of hVAChT in PC12 cells results in approximately a 20-fold increase in vesamicol-sensitive vesicular accumulation of ACh [10]. This expression system should prove useful for the structure/function analysis of VAChT and help to further establish a relationship between the activity of vesicular neurotransmitter transporters and vesicular storage and levels of neurotransmitter available for regulated neurosecretion.

2. Materials and methods

Polyclonal antipeptide antibodies against the C-terminal amino acids of hVAChT [6] were affinity purified and previously characterized [10, 12]. Polyclonal antibodies against chromogranin B (CgB) were a generous gift of Reiner Fischer-Colbrie (Innsbrück, Austria). Monoclonal antibodies directed against synaptophysin (p38) were purchased from Sigma.

2.1. 2/V@NheI chimeric construction

An *Nhe*I restriction site was introduced into both hVAChT and hVMAT2 cDNAs at an equilavent site within the putative 2nd transmembrane domain of these proteins (conserved amino acids Ala^{149} and Ser^{150}) by site-directed mutagenesis [4]. Mutagenesis was performed using the full-length hVMAT2 cDNA in pCDM7amp. Since hVAChT has two internal *Nhe*I sites, the 5′ *Hin*dIII/*Sse8387*I fragment of hVAChT was first subcloned into pUC18 where mutagenesis was performed. Transformants were screened by restriction analysis and verified by cDNA double-stranded sequencing using Sequenase II (U.S. Biochemical Corp.). The 5′ region of hVMAT2 was then subcloned into the hVAChT fragment (at *Hin*dIII/*Nhe*I in pUC18) and the 2/V chimeric fragment (*Hin*dIII/*Sse8387*I) was subcloned back into hVAChT in the mammalian expression vector Rc/CMV (Invitrogen).

2.2. Transfection and selection of stable PC12 cell lines

Rat PC12 cells were transfected with the 2/V@*Nhe*I construct using Lipofectin (10 mg/mL: Life Technologies, Inc.), and stable transformants were selected with 0.5 mg/mL Geneticin (G418; Life Technologies, Inc.) and screened by immunocytochemistry as previously described [10, 12].

2.3. Vesicular acetylcholine transport and vesamicol binding assays

ATP-dependent vesicular transport of [^{3}H]-ACh (55.2 mCi/mmol, DuPont NEN) and the binding of [^{3}H]-vesamicol (AH5183, L-[piperidinyl-3,4-^{3}H]vesamicol; 31 Ci/mmol, DuPont NEN) were measured using postnuclear supernatants (800 *g* for 10 min) prepared from control, hVAChT-expressing or 2/V@*Nhe*I-expressing PC12 cells as previously described [9, 10]. Uptake of ^{3}H-acetylcholine by control PC12 cells was subtracted. Non-specific binding of ^{3}H-vesamicol was determined in the presence of 30 μM L-vescamicol and was subtracted from the total binding.

2.4. Subcellular localization

Postnuclear supernatants were centrifuged at 10 000 *g* for 10 min, and the resulting supernatant (about 2 mg of protein) was loaded onto a 4.6 mL 5–25% glycerol gradient prepared in homogenization buffer for a 45-min-long centrifugation at 55 000 rpm (SW55 rotor), which enables separation of endosomal membranes from synaptic vesicles [2]. Successive 350-μL fractions were collected and processed for Western blot analysis as previously described [12].

3. Results

The hVAChT and 2/V@*Nhe*I chimera could be distinguished from the endogenously expressed rVAChT in stably transfected PC12 cells lines by immunocytochemistry and Western blotting using a human-specific antibody. Furthermore, the binding of [^{3}H]vesamicol and transport of [^{3}H]ACh in postnuclear supernatants of PC12 cells expressing the human proteins was significantly higher than levels detected in PC12 cells containing only the endogenous rVAChT. Uptake mediated by the endogenous rVAChT protein was less than two-fold greater than that observed in the presence of vesamicol or at 4 °C.

3.1. ACh transport and vesamicol binding properties of 2/V@NheI chimera

Specific vesicular uptake of [^{3}H]ACh was observed in homogenates from the 2/V@*Nhe*I chimera. Uptake of [^{3}H]ACh was completely inhibited by 2 mM L-vesamicol, bafilomycin A_1 (1 μM), a specific inhibitor of the vesicular H^+ ATPase, and the proton ionophore FCCP (2.5 μM). Furthermore, [^{3}H]ACh transport was reduced approximately 90% in the absence of exogenous ATP.

The kinetic analysis of the uptake of [^{3}H]ACh by the 2/V@*Nhe*I chimera is shown in *figure 1A*. The initial rate of [^{3}H]ACh uptake was measured during the linear portion of the time course (6 min) and was saturable and displayed an apparent K_m of 7.4 mM. The apparent affinity of hVAChT for ACh (K_m = 0.9 mM) is shown for comparison. Thus, the presence of hVMAT2 sequences in the N-terminal portion of hVAChT significantly reduces the affinity of this chimeric transporter for ACh. The 2/V@*Nhe*I chimera does not however transport monoamines when expressed in digotonin-permeabilized CV-1 fibroblasts (data not shown).

The affinity of [^{3}H]vesamicol for the 2/V@*Nhe*I chimera was not significantly different from that observed with hVAChT and exhibited a K_d of approximately 5 nM (*figure 1B*).

3.2. Subcellular localization of 2/V@NheI chimera

Western blot analysis of fractions obtained following velocity sedimentation of high speed supernatants of stably transfected PC12 cells through glycerol is shown in *figure 2*. The 2/V@*Nhe*I immunoreactivity sedimented in synaptic vesicles fractions containing synaptophysin (p38), similarly to that observed with hVAChT-expressing PC12 homogenates. Thus, the presence of hVMAT2 sequences in the N-terminal portion of hVAChT does not in-

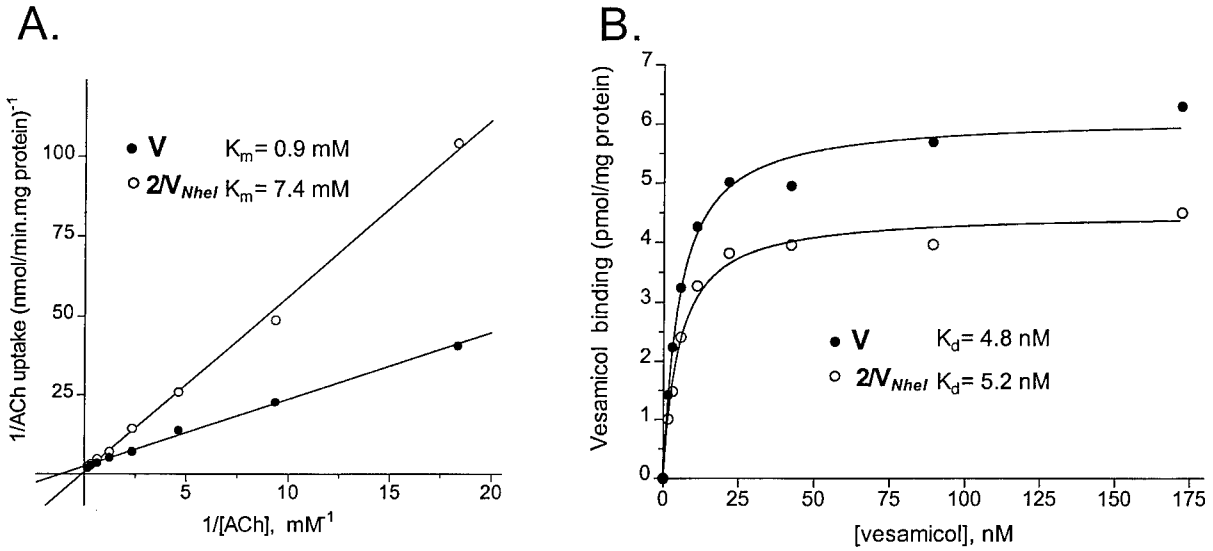


Figure 1. 2/V@*Nhe*I chimera dissociates high-affinity ACh recognition from vesamicol binding. **A.** Lineweaver-Burk analysis of initial [^{3}H]ACh (0.03–10 mM) uptake velocity. **B.** Saturation isotherm of binding of [^{3}H]vesamicol (0.08–116 nM). Data are from a representative experiment performed in duplicate.



Figure 2. 2/V@*Nhe*I chimera retains targeting to PC12 synaptic vesicles. Membrane protein from synaptic vesicle enriched supernatants fractionated through glycerol were immunoblotted with affinity-purified anti-hVAChT. Each gradient was controlled for p38 and CgB localization.

terfer with its targeting to the small synaptic vesicles in PC12 cells.

4. Discussion

The results presented here indicate that high-affinity vesicular transport of ACh requires cholinergic-specific amino acids within the N-terminal portion of hVAChT. It is likely that this specificity lies within the first putative transmembrane domain (TMD) as the amino terminus and large lumenal glycosylated loop between TMDs I and II are poorly conserved. The conserved aspartic acid residue (Asp^{33}) in TMD I of VMATs has been implicated in high-affinity monoamine recognition [5]. Our data suggest that other amino acids within TMD I of VAChT, in addition to the aspartic acid residue (Asp^{46}), are also important for ACh transport function.

The binding of vesamicol does not require these cholinergic-specific amino acids in the N-terminal portion of hVAChT. Vesamicol is a non-competitive inhibitor of active ACh transport in synaptic vesicles isolated from *Torpedo* [1, 3]. Our results are consistent with earlier work suggesting that ACh and vesamicol do not bind to the same site in hVAChT. However, preliminary evidence suggests the presence of a second, lower affinity ACh binding site in hVAChT which may interact with vesamicol.

It has recently been observed that the VAChT-specific aspartic acid residue (Asp^{193}) in TMD IV and the conserved aspartic acid residue (Asp^{398}) in TMD X are required for ACh transport function [7]. We are currently examining the role these residues play in high- and low-affinity ACh recognition and in the binding of vesamicol. The intramembrane aspartic acid residues as well as other polar amino acids (e.g., serine, glycine, cysteine) of VAChT are likely to be part of a 'pore' and be important for the binding and exchange of H^+ and ACh.

When expressed in PC12 cells, hVAChT is preferentially targeted to the small synaptic vesicles while hVMAT2 is confined to the LDCVs [12]. Here we show that the presence of the lumenal glycosylated loop of hVMAT2 does not interfere with the targeting of the 2/V@*Nhe*I chimera to the small synaptic vesicles. The synaptic vesicle targeting information instead resides within the terminal cytoplasmic domain of VAChT [12]. Future work will determine what consequence a reduced affinity of hVAChT for ACh has on regulated ACh secretion from the small synaptic vesicles of PC12 cells.

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The cholinergic locus: ChAT and VAChT genes

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Abstract — The gene encoding the vesicular acetylcholine transporter has been localized within the first intron of the gene encoding choline acetyltransferase and is in the same transcriptional orientation. These two genes, whose products are required for the expression of the cholinergic phenotype, could therefore be coregulated. The promoters of both genes have been identified. The mechanisms that account for the regulation of the expression of both genes are now being investigated. (©Elsevier, Paris)

Résumé — Le locus cholinergique: les gènes ChAT et VAChT. Le gène codant le transporteur vésiculaire de l'acétylcholine a été localisé dans le premier intron du gène codant la choline acétyltransférase, dans la même direction transcriptionnelle que celui-ci. Ces deux gènes, dont les produits sont indispensables à l'expression du phénotype cholinergique, pourraient donc être co-régulés. Les promoteurs de ces deux gènes ont été identifiés. Les mécanismes de régulation de l'expression de ces gènes sont actuellement étudiés. (©Elsevier, Paris)

choline acetyltransferase / vesicular acetylcholine transporter / cholinergic locus / regulatory mechanisms

1. Introduction

The expression of the cholinergic phenotype depends on the activity of a high affinity choline transporter as well as that of proteins involved in the synthesis, storage and release of the neurotransmitter acetylcholine (ACh). ACh is synthesized in the cytoplasm of presynaptic neurons by choline acetyltransferase (ChAT), then translocated into synaptic vesicles by the vesicular ACh transporter (VAChT). cDNAs encoding ChAT have been cloned in different species which has led to the subsequent isolation of the ChAT gene (for review see [17]). The expression of this gene is complex. In rodent, the ChAT gene contains three 5′ non-coding exons designated as R, N and M. The use of different promoters and alternative mRNA splicing within the 5′ region gives rise to the synthesis of several mRNAs with different 5′ untranslated sequences (*figure 1*) [10, 14]. Two promoters of the murine ChAT gene have been identified upstream from the exons R and M ([2, 9, 14]; Bejanin et al., unpublished results).

Analysis of the sequence of the first intron of the rat ChAT gene has revealed the presence of an open reading frame of 1590 bp encoding the VAChT protein and directed in the same transcriptional orientation as the ChAT gene (*figure1*) [3]. This unique gene organization has been demonstrated in parallel in *Caenorhabditis elegans* and in man [1, 8] and more recently in mouse and *Drosophila* [11, 16]. The conservation of these gene arrangements between evolutionary separated species suggests its functional significance which may be the coordinated expression of the ChAT and VAChT genes.

2. Transcription of the rat ChAT and VAChT genes

The study of the regulatory mechanisms of these two embedded genes first requires the understanding of their mode of transcription. Are ChAT and VAChT genes transcribed from independent promoters or from one common promoter region?

Northern blot analysis of rat VAChT mRNAs has revealed their molecular diversity [3, 7]. Three mRNAs of 2.6, 3 and 3.9 kb have been detected in spinal cord and brainstem, and detailed analysis of VAChT transcripts indicated that this diversity relies in their 5′ non coding region (see below).

To test whether ChAT and VAChT originate from the same transcription unit, RT-PCR experiments were performed with rat spinal cord mRNA using oligonucleotides complementary to the sequences of VAChT and of the exon R. Two forms of VAChT mRNAs were identified that contain the sequence of

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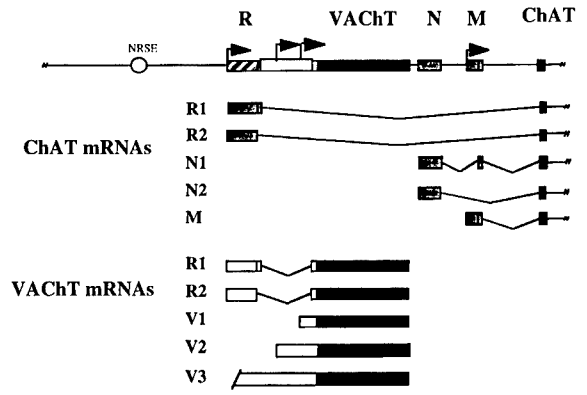


Figure 1. Schematic representation of the 5′ region of the rat ChAT gene (top line) and the different ChAT and VAChT mRNA species. Black boxes indicate coding sequences, gray and open boxes represent non-coding sequences of ChAT and VAChT genes, respectively. Exon R, which is shared by both genes, is gray and white. Additional vertical bars represent splice sites. Arrows indicate the transcriptional start sites identified for the ChAT and VAChT genes. The approximate position of the neuron restrictive silencer (NRSE) element is represented by a circle.

the exon R immediately followed by the sequence of an exon beginning at 308 bp upstream from the ATG translation initiation codon of VAChT (*figure 1*) [3]. The existence of R-type VAChT mRNAs suggests that ChAT and VAChT genes can be transcribed from the same promoter region.

RT-PCR have also been carried out with primers complementary to VAChT and to a sequence at 410 bp upstream from the ATG codon. The obtained amplification product revealed another type of VAChT mRNA designated as V [3]. RNase protection experiments were then performed to localize the transcription initiation sites of the V-type VAChT mRNAs [7]. Two clusters of start sites separated by about 450 bp have been identified for the VAChT gene transcription and that give rise to two VAChT mRNA designated as V1 and V2 (*figure 1*). Surprisingly, an additional VAChT mRNA species, V3, has been identified whose sequence covers that of the first ChAT intron until at least 16 nucleotides from the 3′ end of the exon R (*figure 1*). This mRNA may be produced from the same promoter as the R-type mRNAs.

The existence of V-type mRNAs has suggested the presence of VAChT promoters within the first intron of the ChAT gene. Transient transfection experiments have revealed the presence of two different promoter regions within this intron that generate the V1 and V2 VAChT transcripts [7].

Together, these data led us to propose the following model for the transcription of the rat ChAT/VAChT gene locus. Both ChAT and VAChT genes may be transcribed from a promoter region located upstream from the exon R. VAChT mRNAs are generated when transcription is stopped at the polyadenylation site of the VAChT gene and these mRNAs either are spliced to generate R-type mRNAs or remain unspliced (V3 mRNA). Alternatively, transcription may continue until the polyadenylation site of the ChAT gene giving rise to R-type ChAT mRNAs by removing a large fragment containing the VAChT open reading frame. In addition, the VAChT gene may be transcribed from the two promoters localized in the first intron of the ChAT gene to produce the VAChT mRNA V1 and V2. Finally, the ChAT gene may be transcribed from two promoters located downstream from the VAChT gene, thus producing the ChAT N- and M-type mRNAs.

3. Regulation of ChAT and VAChT gene expression

The VAChT promoter regions described above are highly active in non cholinergic cell lines [7]. Therefore, they lack the regulatory motifs responsible for the cell-specificity of the VAChT gene expression. These motifs may be located upstream from the R-type promoter. Indeed, a rat genomic region upstream from the R exon has been shown to confer cholinergic specificity to the R-type promoter in vitro [9]. This same region directs the in vivo expression of a heterologous downstream promoter in cholinergic neurons [12]. Moreover, it contains a neuron restrictive silencer element (NRSE) that, when fused to a heterologous promoter, acts as a silencer in non-neuronal cells [13]. This raises the question of whether this region also restricts the expression of the VAChT gene to cholinergic cells.

This question is being addressed by transient transfection experiments of different cell lines with constructs of the VAChT promoters fused to the luciferase gene. These constructs contain the VAChT promoters upstream and/or downstream from the exon R, that include or not the NRSE-containing region. Our results reveal that this region down regulates, but does not completely inhibit, the activity of the VAChT promoters in non-neuronal cells. This suggests that other regulatory domains of the VAChT gene are required to strictly restrict the expression of this gene to cholinergic cells. Mutation of the NRSE element will allow us to test its involvement in the regulation of the VAChT gene transcription.

The proximity of the ChAT and VAChT genes, which encode different proteins required for the same neuronal phenotype, suggests the existence of regulatory mechanisms that coordinate their concomitant expression. To investigate this possibility, we ascertained that signals that induce the expression of the ChAT gene also induce that of the VAChT gene. Using cultured sympathetic neurons as an in vitro model, several classes of factors, such as the cholinergic differentiation factor/leukemia inhibitory factor (CDF/LIF) or retinoic acid (RA), have been shown to induce ChAT activity and to increase the level of the multiple ChAT mRNA species in these neurons ([6] and references cited therein). Both CDF/LIF and RA increased the amount of VAChT protein, parallely and with comparable induction ranges to that of ChAT activity. Moreover, these two factors increased concomitantly VAChT and ChAT mRNA levels [4]. Similar findings have been reported by others [5, 15]. These data are consistent with the notion that transcription of both the ChAT and VAChT genes is subject to common regulatory mechanisms.

In order to identify the *cis*-active elements involved in the regulation of these genes by CDF/LIF and RA in cultured sympathetic neurons, it was necessary to transfect these neurons. The fact that classical transfection methods were ineffective led us to develop a protocol of microinjection of plasmids with reporter genes in these neurons, followed by the quantification and the normalization of the reporter gene activity. Reproducible reporter gene activities as well as induction of a CDF/LIF responsive promoter could be measured in microinjected neurons (Pajak et al., in preparation). This strategy is now used to identify the CDF/LIF and RA responsive elements of the ChAT and VAChT gene locus.

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Regulation of the cholinergic gene locus

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Astract — DNase I hypersensitive site mapping of the human cholinergic gene locus has been used to detect cholinergic specific potential regulatory sites. Analysis of mutant PC12 cell lines provides evidence that protein kinase A II is required and coordinately regulates basal expression of both the ChAT and VAChT genes. (©Elsevier, Paris)

Résumé — Régulation du locus génétique cholinergique Nous avons utilisé la cartographie des sites hypersensibles à la DNAse I dans le locus génétique cholinergique humain pour identifier des sites régulateurs potentiels. L'analyse de lignées mutantes de cellules PC12 indique que la protéine kinase A II est impliquée dans la régulation coordonnée de l'expression des gènes ChAT et VAChT. (©Elsevier, Paris)

choline acetyltransferase / vesicular acetylcholine transporter / transcriptional regulation / coordinate regulation / protein kinase A

1. Introduction

The cholinergic gene locus is comprised of the genes for the biosynthetic enzyme choline acetyltransferase (ChAT) and the gene for the vesicular acetylcholine transporter (VAChT). The VAChT gene, whose open reading frame is within a single exon, lies within the intron between the first and second exons of the ChAT gene. This unusual gene arrangement is conserved across such diverse species as the nematode, *Drosophila*, and mammals, and suggests potential coordinate regulation of the gene.

The mechanisms responsible for the transcriptional regulation of the cholinergic gene locus have been studied by both transient cell transfection analysis as well as through transgenic mice models. Based on studies from a number of laboratories a complex regulatory pattern at this locus is emerging. Recent transgenic mouse models [10, 13], each utilizing different non-overlapping regions of the locus, were reported to provide cell specific expression of the locus. This suggests the existence of multiple cell specific regulatory elements in the gene.

We report here two approaches to study regulation of the cholinergic gene locus. In one we analyzed the chromatin structure of the human cholinergic gene locus by DNase I hypersensitive mapping to identify potential regulatory sites in the gene. In the other we have used mutant PC12 cell lines to demonstrate coordinate regulation of the ChAT and VAChT genes by a protein kinase A dependent mechanism.

2. Materials and methods

2.1. Cell lines

The cell lines used in this study include CHP134, a cholinergic neuroblastoma cell line, HeLa, MCF-7, a human breast adenocarcinoma cell line, HeLa, PC12, and two PC12 mutant cell lines. PC12/ A123.7 expresses a mutant regulatory PKA subunit [2, 5] and contains reduced levels of PKA I and PKA II. PC12/A126-1B2 was generated by nitrosoguanidine mutagenesis and lacks PKA II activity, but contains normal levels of PKA I [15].

2.2. DNase I hypersensitivity mapping

Cell nuclei were isolated from detergent lysed cells by centrifugation and resuspended in digestion buffer (10 mM Tris-HCl, pH 7.6, 5 mM $MgCl_2$, 0.5 mM $CaCl_2$, 25 mM KCl, 0.5 mM DTT and 0.35 M sucrose). Nuclei were digested with 1, 5, or 10 units of DNase I for 10 min at 25 °C. Following termination the sample was digested with proteinase K followed by phenol, phenol/chloroform, and chloroform extraction, and precipitation with ethanol. The DNA pellet was treated with RNase, followed by digestion with proteinase K, and extraction and precipitation as noted above. This DNA was digested with a suitable restriction enzyme and electrophoresed on a 0.8% agarose gel. The DNA was transferred to a Hybond N (+) membrane and hybridized with a probe radiolabeled by the random priming method [14]. The washed membrane was exposed to Kodak XAR-5 film.

2.3. Determination of choline acetyltransferase activity

Choline acetyltransferase was assayed by a modification of the method of Fonnum [4] as described by Hersh [6].

2.4. Competitive PCR

Total cellular RNA was isolated from cultured cells by the method of Chomczynski and Sacchi [1]. Single-stranded cDNAs were synthesized using Molony murine leukemia virus (M-MLV) reverse transcriptase using specific primers for VAChT or ChAT. Single-stranded cDNA (0.1 μg) was amplified by PCR in a programmable thermal cycler that included variable amounts of an appropriate internal standard and a labeled nucleotide. Following amplification the PCR product was electrophoresed on a 5% polyacrylamide gel and autoradiographed. Signal intensities were quantified directly from the bands with a Storm™ PhosphorImager and Image Quant.

2.5. Other materials

RNase inhibitor, dibutyryl cAMP, and deoxynucleotides were purchased from Boehringer Mannheim (Indianapolis, IN, USA). DNase I was from Promega (Madison, WI, USA). H-89 and H-9 dihydrochloride were obtained from Calbiochem (La Jolla, CA, USA). M-MLV reverse transcriptase and Taq DNA polymerase were purchased from Promega (Madison, WI). 8- (4-chlorophenylthio) adenosine-3′,5′-cyclic monophosphate (8-CPT-cAMP), 8-piperidinoadenosine -3′,5′-cyclic monophosphate (8-PIP-cAMP), 8- (4-aminohexyl) aminoadenosine-3′,5′-cyclic monophosphate (8-AHA-cAMP), and N^6-benzoyladenosine-3′,5′-cyclic monophosphate (N^6-BNZ-cAMP) were purchased from BioLog Life Science Institute (Bremen, Germany). All other reagents were from Sigma Chem. Co. (St. Louis, MO, USA).

3. Results

DNase I hypersensitive site mapping was employed as a method to locate specific elements involved in the regulation of the human cholinergic gene locus. CHP134 cells were used as a cholinergic cell line and HeLa cells and MCF-7 cells as non-cholinergic cell lines. A variety of probes were employed to detect the DNase I dependent fragmentation pattern of nuclear DNA digested with restriction enzymes. This analysis led to the identification of eight DNase I hypersensitive sites in the cholinergic cell line (*figure 1*). In contrast the two non-cholinergic cell lines showed two DNase I hypersensitive sites (*figure 1*).

Analysis of the two DNase I hypersensitive sites seen in the inactive cholinergic gene from the non-cholinergic cell lines indicated that site 1′ corresponds to the neuronal restrictive silencer element, NRSE, previously identified in the gene [11, 3]. The other site, site 6, corresponded to an enhancer element identified in this laboratory [9]. Of the eight DNase I hypersensitive sites found in active chromatin from CHP 134 cells six of these appear to correspond to core promoter regions, site 2, R promoter, site 3, VAChT promoter, site 4, V1 VAChT promoter, site 7, N promoter, site 8, M promoter. In addition site 1 is a cholinergic specific site which resides in the vicinity of the NRSE, site 5 appears to be an enhancer element, while site 6 is the previously identified enhancer element noted above. These latter sites may be key regulators in the cell specific expression of this locus.

In a different approach to study regulation of the cholinergic gene locus we utilized two protein kinase A (PKA) mutant cell lines, A126-1B2 which is deficient in PKA II and A123.7 which has decreased levels of PKA I and PKA II. A PCR based assay was used to demonstrate that both ChAT and VAChT mRNA levels in the mutant cell lines were significantly decreased (*figure 2*). Treatment of the parental PC12 cell line, but not the PKA mutants, with dbcAMP increased ChAT mRNA levels 3.6-fold and VAChT mRNA levels 4.2-fold. Treatment of the parental PC12 cell line with two selective PKA inhibitors H-9 or H-89 reduced both ChAT and VAChT mRNA levels to ∽1/3 of that in the untreated cells (*figure 2*). The reduction of VAChT mRNA again paralleled that of ChAT mRNA. The dbcAMP induced increase in ChAT mRNA was paralleled by a 5-fold increase in ChAT activity, and H89 blocked this increase. H89 and H9 alone decreased ChAT activity to 1/4 and 1/5 the control level, respectively.

The above results suggest that PKA II, but not PKA I, may regulate the cholinergic gene locus. We confirmed this by demonstrating 2.5 fold increase increases in ChAT activity produced by a combination of 8-CPT-cAMP and N^6-BNZ-cAMP, which selectively activates PKA II, with no effect of a combination of 8-AHA-cAMP and 8-PIP-cAMP which selectively activates PKA I.

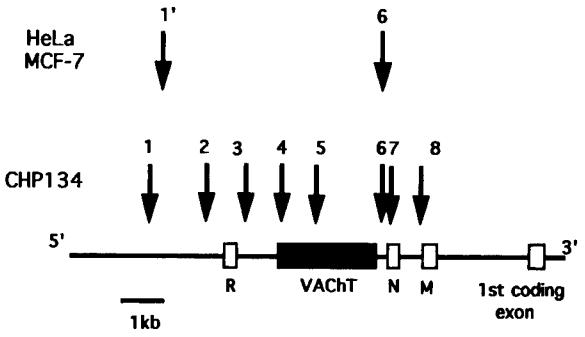


Figure 1. DNase I hypersensitive sites of the cholinergic gene locus in CHP134, HeLa and MCF-7 cells. The R, N, M, and first coding exons are illustrated as well as the VAChT gene open reading frame. Arrows indicate the DNase I hypersensitive sites.

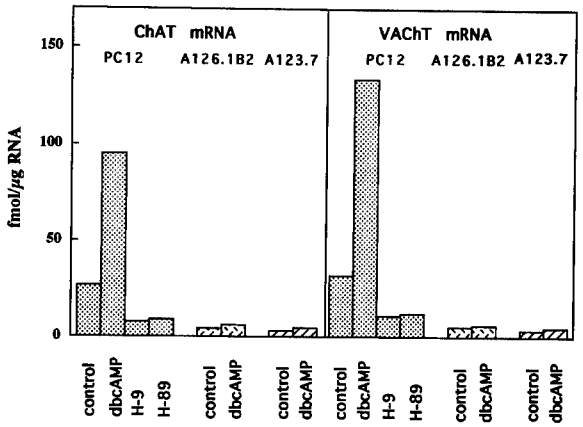


Figure 2. ChAT and VAChT mRNA levels in PC12 cells and PKA mutant cell lines.

4. Discussion

For many genes, it has been reported that the elements which correspond to DNase I hypersensitive sites play an important role for transcriptional regulation. We observed a number of cholinergic specific DNase I hypersensitive sites in regions of the gene which do not exhibit cell specific reporter gene activity [7]. It is possible that these sites are cholinergic cell specific as a result of the chromatin structure of the cholinergic gene locus.

An interesting finding is the presence of a DNase I hypersensitivity site, site 1, which is located more than 1 kb upstream of the R exon. This is the region of the cholinergic gene locus shown to contain a cholinergic specific repressor in the rat gene [8]. The presence of site-1′ in non-cholinergic cells, which corresponds to the NRSE, is understandable since this element can repress expression of the cholinergic gene in non-neuronal cells [11]. However, the presence of site 6, which corresponds to an enhancer element, is unexpected. We suggest that this site might act in conjunction with the NRSE to silence this locus in non-cholinergic cells.

Studies with protein kinase A deficient PC12 cells establish the requirement for this kinase for basal expression of both the ChAT and VAChT genes. Furthermore this regulation appears to be coordinate with these two genes. In the parental PC12 cell line, dbcAMP induced both ChAT and VAChT mRNAs in a parallel fashion. Similarly a parallel reduction in ChAT and VAChT mRNA levels was produced by the PKA inhibitors H-89 and H-9. In the protein kinase A deficient cell lines considerably lower ChAT and VAChT mRNA levels were observed compared to the PC12 parental line. These findings demonstrate that both ChAT and VAChT gene transcription are regulated by protein kinase A, and that this regulation is coordinate. The finding that ChAT and VAChT mRNA levels are reduced in a cell line which contains wild type levels of PKA I, but is devoid of PKA II activity, and the ability of PKA II agonists, but not PKA I agonists, to increase ChAT activity shows that regulation of the cholinergic gene is through a PKA II signaling pathway. Transient transfection analysis indicates that protein kinase A can act through a site on the human ChAT gene upstream of the VAChT promoter. This is clearly a different site than the CRE-like element shown by Misawa et al. [12] to be localized between the M exon and the first coding exon. The mechanism by which PKA II regulates the cholinergic gene locus is currently under investigation.

Acknowledgments

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The Schwann cell at the neuromuscular junction

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Abstract — Synapses obtained in vitro in a system of co-culture of muscle cells and neurons are of embryonic type. We prepared a monoclonal antibody (6.17) which recognizes a molecule synthesized by Schwann cells and used it to show that the main characteristics of maturity (decrease in number of synapses, appearance of junctional folds, and suppression of butyrylcholinesterase expression) are under the control of Schwann cells. In addition, Schwann cells have the capacity to aggregate the acetylcholine receptors in myotube cultures. (©Elsevier, Paris)

Résumé — La cellule de Schwann à la jonction neuromusculaire. Les synapses obtenues dans un système de co-cultures cellules musculaires-neurones sont de type embryonnaire. En utilisant un anticorps monoclonal obtenu au laboratoire (le 6.17), dirigé contre un antigène schwannien, nous avons montré que certains caractères de maturité (diminution du nombres de synapses, apparition de plis sous-neuraux et répression de l'expression de la butyrylcholinestérase) sont contrôlés par la cellule de Schwann. Enfin, la cellule de Schwann possède la capacité d'agréger les récepteurs à acétylcholine dans des myotubes en culture. (©Elsevier, Paris)

acetylcholinesterase / butyrylcholinesterase / acetylcholine receptor / neuromuscular junction / Schwann cells

Schwann cells wrap around nerve axons to provide electrical insulation and are essential for the formation, maintenance and repair of the neuromuscular junction (NMJ) [1]. At the NMJ, they are juxtaposed with nerve terminal and muscle cell, with which they interact during synaptogenesis. We focused on their role in maturation of the NMJ.

Establishment of an NMJ involves a sequence of complex mechanisms. The progression of events, from the first neuromuscular contact (14–15 embryonic days) to the mature synapse (15 days post-natal), occurs over 3 weeks. As soon as the first nerve-muscle contact is established, acetylcholine receptors (AChR) and cholinesterases (acetylcholinesterase (AChE) and butyrylcholinesterase (BChE)) accumulate at synaptic sites. The accumulation of AChR in the muscular membrane is a prerequisite for normal accumulation of AChE in the basal lamina of the synaptic cleft: in different systems of cultures, the conditions which prevented clustering of AChR (anti-AChR antibodies, deficiency in AChR of the variant C2 cell line) also suppressed AChE clustering [5]. AChR mature up to 2 weeks after birth under the control of molecules synthesized by the motoneuron (reviewed in [8, 14]). AChR consists of α, β, γ, and δ subunits at the embryonic stage, and the γ subunit is replaced by the ε subunit 2–3 days after innervation. This substitution affects the channel properties of the AChR (conductance and open time). Two molecules synthesized by the motoneuron are known to induce expression of the ε gene: acetylcholine receptor-inducing activity (ARIA)/heregulin and agrin. Another neuronal molecule, calcitonin-gene related peptide (CGRP), also increases transcription of the AChR α-subunit gene, but not of other subunits. Agrin also induces the clustering of AChR, AChE, BChE and heparan sulphate proteoglycan (HSPG), a component of the basal lamina.

Cholinesterases exist in several oligomeric molecular forms, distinguished by their sedimentation coefficient: the globular and the asymmetric forms (for review see [12]). The globular forms correspond to the monomer G1, dimer G2 and tetramer G4, and the asymmetric (A) forms to one, two or three tetramers linked to a collagen-like tail (A4, A8 and A12, respectively). The catalytic subunits corresponding to AChE and BChE are encoded by distinct genes. The role of AChE in cholinergic transmission is unambiguous, but the function of BChE is not clear, except as an adhesion molecule [11]. BChE, which is little expressed in the adult NMJ, also hydrolyses the neurotransmitter acetylcholine, and may thus participate in neurotransmission in vitro, and in vivo during development, and to a lesser degree in the adult NMJ. During development of chicken synapses, Tsim et al. [15] have demonstrated the transition of heterogeneous collagen-tailed forms, containing both AChE and BChE subunits, to homogeneous AChE collagen-tailed molecules. Berman et al. [2] showed that, in whole rat skeletal muscle (i.e., without distinguishing synaptic and extrasynaptic zones), there is a gradual increase in

muscle AChE activity, and a concomitant decrease in BChE activity, between 7 and 15 days after birth. We demonstrated that AChE and BChE are initially present in equal amounts at the synapses, but BChE levels diminish sharply between 7 and 15 days after birth [4], the stage at which the synaptic Schwann cell membrane becomes juxtaposed with the muscle membrane [6].

Synapses obtained in vitro, by conventional techniques of contact between myoblasts and spinal cord neurons, have the morphological, biochemical and electrophysiological characteristics of embryonic synapses. The events of postnatal maturation, such as disappearance of polyinnervation, changes in levels of AChE and BChE, and appearance of junctional folds, have never been observed, whatever the duration of culture or the age (embryonic or adult) of the cells used. In co-cultures of adult human muscles with explants from whole cord and dorsal root ganglia of 13- to 14-day-old rat embryos, Kobayashi et al. [9]) observed the formation of mature muscle fibers, as characterized by contractions and striations, well-organized AChE clusters, and the trend from multifocal to unifocal innervation. This result suggested that Schwann cells of dorsal root ganglia participate in synaptic maturation.

The factors involved in the morphological maturation (disappearance of polyinnervation, appearance of junctional folds) and changes of synaptic cholinesterases were not identified and we postulated that Schwann cells were implicated in these events. We prepared mixed cultures, combining muscle cells (from the hindleg of 18-day-old rat embryos), spinal cord cells (from 14-day-old embryos) and Schwann cells (from the sciatic nerve of new-born animals or from the Schwann cell line TSC2, a gift from Prof. Tennekoon, Philadelphia, USA). Schwann cells and spinal cord cells were added to the muscle cells at day 5 of culture, the stage at which the myotubes were formed, contracting and morphologically differentiated. We prepared a monoclonal antibody (6.17) which recognizes a molecule synthesized by Schwann cells and is co-localized with the AChR at the NMJ. 6.17 antigen appears to be localized in the synaptic space, but not in the basal lamina: on cryostat sections of adult rat lens, 6.17 antibody labels the membranes of the lens cells but not the basal lamina. In vivo in the rat, 6.17 antigen is initially distributed diffusely and concentrates at the synapse 15 days after birth, and its maintenance at the NMJ depends on innervation: antigen 6.17 is present in the endomysium in the extrasynaptic zones of muscle fibers during maturation of the muscle and after denervation [10].

We have shown that during in vitro synaptogenesis, the addition of Schwann cells to muscle-neuron co-cultures: 1) allows visualization of antigen 6.17 at the synapse and induces synaptic maturation (decrease in number of synapses (50%) and appearance of junctional folds) [3]; and 2) induces the disappearance of BChE at synaptic clusters, leaving only AChE activity, as in the adult neuromuscular junction [4]. This maturation is inhibited by the presence of antibody 6.17. Antigen 6.17 is secreted by Schwann cells and TSC2 cells, since medium conditioned by these cells reproduced the effect of the cells themselves: only AChE patches could be observed in the co-cultures in the presence of conditioned medium. 6.17 antibody inhibited the effect of Schwann cells and the effect of medium conditioned by these cells and TSC2 cells. It may be wondered whether antigen 6.17 corresponds to a previously characterized molecule [4], and its molecular characterization is in progress (in collaboration with H. Chneiweiss, INSERM U114, Paris). These results prompted us to postulate that Schwann cells could mediate changes in synaptic maturation during development. Heregulins and their specific receptors, members of the erbB family of tyrosine kinases,

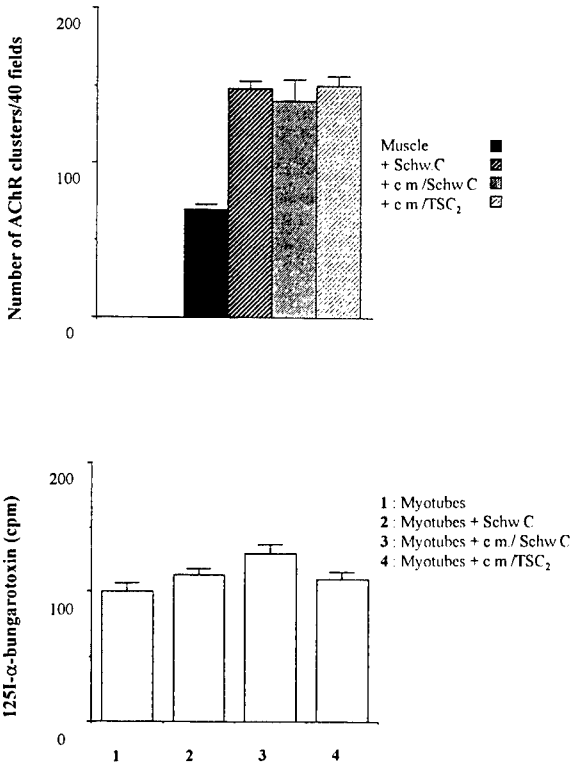


Figure 1. Number of AChR clusters (top) and ^{125}I-α-bungarotoxin binding sites (bottom) in 7-day-old myotubes cultured for 2 days with Schwann cells or with conditioned medium (c.m.) by Schwann or TSC2 cells.

have been implicated in the control of growth and development of Schwann cells. Homozygous erbB3 mutant embryos lack Schwann-cell precursors and Schwann cells that accompany peripheral axons of motor (and sensory) neurons [13]. It will be of interest to look at the possible presence of high levels of BChE in such mutants.

AChR clustering may be induced by neuronal agrin, electric fields, latex beads, and basal lamina components (collagen, laminin) (for review see [7]. As the Schwann cell is closely associated with the nerve terminal, we investigated whether it plays a role in the formation of AChR clusters, and found that it does. When primary Schwann cells or medium conditioned by Schwann cells or by TSC2 cells were added to muscle cells in culture, the number of AChR sites increased by only 10%, but the number of AChR clusters increased by 100% (*figure 1*). Antibody 6.17 does not inhibit the Schwann cell-mediated increase in the number of AChR clusters. This strengthens the idea that antigen 617 is specifically implicated in synaptic maturation. We have tested the possibility that agrin synthesized by the Schwann cell is the molecule responsible for this increase. But the agrin isoform expressed by the Schwann cell is the inactive one (B0) (in collaboration with G. Escher, Institut d'Embryologie, Lausanne, Switzerland). We are currently testing different factors synthesized and released by Schwann cells.

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Lipid peroxidation and changes in cytochrome c oxidase and xanthine oxidase activity in organophosphorus anticholinesterase induced myopathy

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Abstract — A possible role of radical oxygen species (ROS) initiated lipid peroxidation in diisopropylphosphorofluoridate (DFP)-induced muscle necrosis was investigated by quantifying muscle changes in F_2-isoprostanes, novel and extremely accurate markers of lipid peroxidation in vivo. A significant increase in F_2-isoprostanes of 56% was found in the diaphragm of rats 60 min after DFP-induced fasciculations. As possible source of ROS initiating lipid peroxidation, the cytocrome-c oxidase (Cyt-ox) and xanthine dehydrogenase-xanthine oxidase (XD-XO) systems were investigated. Within 30 min of onset of fasciculations Cyt-ox activity was reduced by 50% from 0.526 to 0.263 µmol/mg prot/min and XO activity increased from 0.242 to 0.541 µmol/mg prot/min. Total XD-XO activity was unchanged, indicating a conversion from XD into XO. In rats pretreatment with the neuromuscular blocking agent d-tubocurarine, prevented DFP-induced fasciculations, increases in F_2-isoprostanes and changes in Cyt-ox or XD-XO. The decrease in Cyt-ox and increase in XO suggest that ROS are produced during DFP induced muscle fasciculations initiating lipid peroxidation and subsequent myopathy. (©Elsevier, Paris)

Résumé — Peroxydation des lipides et modification de la cytochrome C oxydase et de la xanthine oxydase dans la myopathie induite par les inhibiteurs anticholinestérasiques organophosphorés. Nous avons examiné le rôle possible de la peroxydation de lipides par des résidus libres de l'oxygène (ROS) dans la nécrose musculaire induite par le diisopropylphosphorofluoridate (DFP), en quantifiant le niveau d'isoprostane F2 musculaire, qui constitue un nouveau marqueur, extrêmement précis, de la peroxydation lipidique in vivo. Nous avons trouvé des augmentations significatives d'isoprostane F2 (56%) dans le diaphragme de rat, 1 heure après l'induction de fasciculations par le DFP. La cytochrome C oxydase (Cyt-ox) et la xanthine déshydrogénase-xanthine oxydase (XD-XO) peuvent produire des radicaux libres (ROS) entraînant la peroxydation des lipides. En 30 minutes après le début des fasciculations, l'activité de la Cyt-ox était réduite de 50% (de 0,526 à 0,263 µmole/mg de protéine/min) et l'activité de la XO était augmentée de 0,242 à 0,541 µmole/mg de protéine/min. L'activité XD-XO totale n'était pas modifiée, ce qui indique une conversion de XD en XO. Chez les rats, un pré-traitement par la d-tubocurarine bloque l'activité musculaire et les fasciculations induites par le DFP, et empêche l'augmentation d'isoprostane F2 et les changements de l'activité Cyt-Ox et du système XD-XO. Nous suggérons que l'hyperactivité cholinergique induite par le DFP provoque l'apparition de radicaux libres et la peroxydation des lipides. La diminution de Cyt-Ox et l'augmentation de XO suggèrent que les ROS sont produits pendant les fasciculations et que la peroxydation des lipides qui en résulte est responsable de la myopathie induite par le DFP. (©Elsevier, Paris)

lipid peroxidation / free radicals / myopathy / muscle hyperactivity

1. Introduction

Systemic injection of non-lethal concentrations of organophosphorus anticholinesterases (OP-antiChE) causes neuromuscular injury consequent to induced sustained muscle fasciculations [10]. The chain of events linking prolonged acetylcholine (ACh) receptor activation to neuromuscular pathology is complex and not fully understood. Recent evidence supports the assumption that over-production of reactive oxygen species (ROS) and lipid peroxidation may have an important role in initiation of the neuromuscular lesions [20, 21].

In skeletal muscle, several sources of ROS have been identified, such as mitochondrial electron transport, oxidases in sarcoplasmic reticulum and sarcolemma and cytosolic xanthine oxidase.

During normal conditions ROS, such as superoxide anion, hydrogen peroxide and hydroxyl radicals, are generated at a low rate by cytochrome c oxidase (Cyt-ox) and can be taken care of by the scavenger and antioxidant systems. However, a reduced capacity of this enzyme can lead to incomplete reduction of oxygen and thus increased ROS production [18], which may exceed the capacity of the cellular defence system.

Another source of free radicals is xanthine oxidase (XO). This enzyme exist as NAD^+ dependent dehydrogenase (XD, EC1.1.1.2.0.4) and is converted into xanthine oxidase (EC1.1.3.2.2). Xanthine oxidase directly transfers electrons from the oxidation of hypoxanthine to molecular oxygen, producing ROS such as superoxide and hydrogen peroxide; both of these are very toxic and can interact with

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free fatty acids such as arachidonic acid which via prostaglandin synthesis generates more ROS [4, 6]. Conversion from XD to XO has been shown to occur during muscle ischemia [6], seizure activity [2] and muscle activity [17]. Previous studies have shown that a decrease in the endogenous antioxidant glutathione by buthionine sulfoxime (BSO) increased the DFP-induced lipid peroxidation and the severity of the myopathy [20].

The present investigation was undertaken to determine through appropriate measurements whether Cyt-ox, XD and XO activities are changed in muscle taken from DFP-treated rats. To rule out direct effects of DFP on these enzymes, the neuromuscular blocking agent d-tubocurarine was used in another series of experiments to prevent DFP-induced muscle hyperactivity.

2. Materials and methods

2.1. Animals

Male Sprague-Dawley rats weighing 200-250 g were maintained on a 12 h light/dark cycle with food and tap water available ad libitum. For biochemical studies rats were decapitated 30–120 min following the DFP administration (0.5–1.7 mg/kg, s.c.). d-Tubocurarine (0.07 mg/kg) was given i.p. 30 min prior to the DFP injection and immediately after the DFP injection. Buthionine sulfoxime (BSO) 900 mg/kg, i.p. was given 3.5 and 1.5 h before DFP. For histochemistry rats were decapitated 24 h after the start of the experiment.

2.2. Enzyme determination

Cyt-ox activity was determined by measuring spectrophotometrically the oxidation rate of ferrocytochrome c at the absorbance of it's α-band, 550 nm, as described by Wharton and Tzagoloff [19]. 0.1 M 2-(N-morpholine) ethanesulfonic acid (MES, pH 6.0) with 10 μM EDTA was used as buffer. XO or XD activity was determined spectrophotometrically based on the production of uric acid at 295 nm [15]. XO activity was assayed in the absence of NAD^+, and the XD-XO was measured in the presence of NAD^+.

2.3. Lipid peroxidation

A series of prostaglandin F_2-like compounds termed F_2-isoprostanes are formed in vivo from the free radical-catalyzed peroxidation of arachidonic acid and are useful markers of oxidant stress [13]. We have used these F_2-isoprostanes as an index of lipid peroxidation.

2.4. Statistical evaluation

Student's *t*-test was used to establish significance at $P < 0.05$.

3. Results

3.1. DFP induced lipid peroxidation and muscle necrosis

Rats treated with DFP (1.5-1.7 mg/kg, s.c.) developed fasciculations within 10 min lasting for 4–8 h. Histochemical studies revealed necrotic lesions involving endplates. The number of necrotic lesions was reduced significantly when rats were pretreated with d-tubocurarine in order to prevent fasciculations (*table I*). Glutathione (GSH), an endogenous antioxidant, protects against oxidative stress in vivo, its depletion should increase lipid peroxidation and the number of necrotic fibers in our experiments. In rats when pretreated with BSO 3.5 and 1 h before DFP, the number of necrotic fibers was significantly higher than seen with DFP only (*table I*). The combined treatment of BSO and DFP reduced GSH from 1.36 ± 0.20 (100%) to 0.39 ± 0.11 (28%) μmol/g muscle.

Significant increases of F_2-isoprostanes were detected following exposure of rats to 1.7 mg/kg over 30, 60 and 120 min. The largest increase of 56% from control 1.66 ± 0.20 ng/g tissue in F_2-isoprostanes was observed in rats treated with 1.7 mg/kg DFP following 60 min of exposure ($P < 0.01$). Fasciculations also were detected under these conditions. Lower doses of DFP, such as 1 mg/kg, which did not induce fasciculations, produced no increases in

Table I. Modification of DFP myopathy by pretreatment with d-tubocurarine or BSO.

	Treatment[a]			
	Control	*DFP*	*d-Tubocurarine + DFP*	*BSO + DFP*
Fasciculations	0	++++	0	++++
Number of necrotic fibers/1000 muscle fibers	2 ± 2 (0.2%)	479 ± 45[b] (47.9%)	73 ± 19[c, d]	655 ± 67[b, e] (65.5%)

[a] Treatment: DFP (1.7 mg/kg), d-tubocurarine (0.07 mg/kg), BSO (900 mg/kg). All drugs were given i.p. with the exception of DFP which was given s.c. Rats were killed 24 h after DFP treatment for histochemistry. Values are the mean ± S.E.M. of seven muscles. [b] $P < 0.001$ and [c] $P < 0.01$, between control and treated rats; [d] $P < 0.01$ and [e] $P < 0.05$ between DFP-only-treated rats and the rats with drug pretreatments.

formation of F_2-isoprostanes when compared to control animals.

Pretreatment with the neuromuscular blocking agent d-tubocurarine prevented the muscle hyperactivity and increases in F_2-isoprostanes formation otherwise elevated by DFP treatment.

3.2. Sources for ROS production-cytocrome c oxidase c reduction

In diaphragm, following DFP, Cyt-ox was transiently reduced to 51% of control activity after 15 min ($P < 0.001$) and to 77% within 30 min ($P < 0.01$). By 60 min, the Cyt-ox activity had completely recovered (*figure 1*). The 0.5 mg/kg DFP treatment did neither cause muscle hyperactivity such as fasciculations, nor any significant Cyt-ox reduction (*figure 1*), myopathic lesions or increases in lipid peroxidation.

As shown in *figure 2*, following pretreatment with d-tubocurarine (0.07 mg/kg), only a 7.6% decrease in Cyt-ox activity was seen within 15 min following DFP (1.7 mg/kg). This is a significant protection of Cyt-ox activity when compared to the 51% reduction seen after DFP alone, Under these conditions, muscle fasciculations, increases in F_2-isoprostanes as well as the muscle necrosis were prevented.

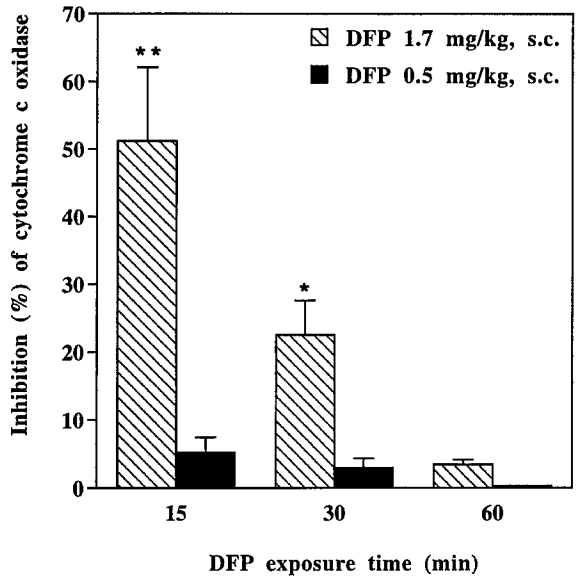


Figure 1. Reduction (%) of cytochrome c oxidase in rat diaphragm following various in vivo DFP treatments. Control activity of cytochrome c oxidase: 0.526 ± 0.093 µmol/min/mg protein. *$P < 0.01$; ** $P < 0.001$.

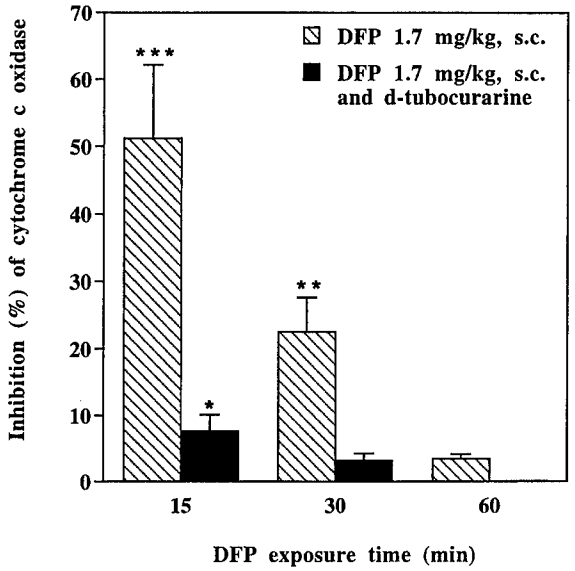


Figure 2. Effect of d-tubocurarine pretreatment on the cytocrome c oxidase reduction in rat diaphragm following various *in vivo* DFP treatments. d-Tubocurarine (0.07 mg/kg, i.p.) was given twice: 30 min before DFP injection and immediately after DFP injection. Control activity of cytochrome c oxidase was: 0.526 ± 0.093 µmol/min/mg protein. *$P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

3.3. Xanthine dexydrogenase/xanthine oxidase conversion

Within 30 min of DFP-induced fasciculations, an increase of XO activity was seen. During DFP-induced fasciculations, however, XO activity rose to 70% of the total XD + XO activity (*table II*). This increase in XO activity appears to have resulted from conversion of XD to XO, since the total activity of XD + XO remained constant throughout the 60 min observation period. Prevention of muscle hyperactivity through pretreatment with d-tubocurarine inhibited the conversion of XD to XO otherwise seen with DFP-induced muscle fasciculations (*table II*). These findings rule out a direct effect of DFP on Cyt-ox or XD-XO.

4. Discussion

Radical oxygen species are considered to be active mediators in the hyperactivity-induced skeletal muscle damage and inflammation, and in various muscle diseases [17]. While the potential sources of

Table II. Conversion of xanthine dehydrogenase (XD) to xanthine oxidase (XO) in rat diaphragm following in vivo DFP treatments, with or without the d-tubocurarine pretreatment[a].

	Control	*DFP 30 min*	*DFP 60 min*	*DFP 30 min + d-tubocurarine*	*DFP 60 min + d-tubocurarine*
XO[b]	0.242 ± 0.05	0.541 ± 0.13[d]	0.553 ± 0.09[d]	0.264 ± 0.07	0.236 ± 0.07
XD+XO[c]	0.794 ± 0.18	0.768 ± 0.20	0.803 ± 0.14	0.771 ± 0.22	0.825 ± 0.21
XO/XD	0.44	2.39[d]	2.21[d]	0.52	0.40
Increase of XO/XD from control (%)	–	443	402	18	

[a]DFP was given 1.7 mg/kg, s.c. Rats were killed after 30 or 60 min of exposure to DFP. The drug pretreatment was same as in *table I*. [b]XO, μmol/min/mg protein. [c]XO + XD: μmol/min/mg protein. Values were mean ± S.D. of five rats.
[d]$P < 0.001$ between control and treated animals.

ROS in muscle are numerous, such as mitochondrial electron transport, oxidases in sarcoplasmatic reticulum and sarcolemma and cytosolic xanthine oxidase, the mechanisms that control the reactive oxidant production and the sites at which oxidative stress causes dysfunction remain little understood.

The present data demonstrate that DFP-induced muscle hyperactivity results in a rapid, significant reduction in the activity of Cyt-ox (*figure 1*) and an increased conversion of XD to XO (*table II*), suggesting the generation of ROS. This agrees with previous observations of increased levels of ROS following exercise-induced muscle damage [1, 5, 9, 14, 16].

Cytochrome c oxidase is the terminal complex in the mitochondria respiratory chain, which generates ATP by oxidative phosphorylation. During intensive muscle activity, the activity of Cyt-ox is reduced [7, 18], leading to an increase in the electron pressure within the electron transport chain and to the ROS production.

The significant increase in the ratio XO/XD following the onset of DFP-induced muscle fasciculations appear to be due to a conversion from XD to XO as no significant changes in the total activity (XO + XD) were observed. The shift of XD to XO begins with the onset of fasciculations and is still present after 60 min. As previously observed, DFP-induced fasciculations caused a rapid fall in ATP [8], leading to increased accumulation of Ca^{2+} [11]. The increased calcium may initiate the conversion of XD to XO through a calcium-dependent protease [3]. The loss of ATP is followed by an increase in ADP, which is further metabolized to adenosine, inosine and hypoxanthine, a substrate for XO [12]. Xanthine oxidase through the oxidation of hypoxanthine produces ROS as superoxide and hydrogen peroxide leading to lipid peroxidation and membrane damage.

A greatly increased rate of free radical production may exceed the capacity of the cellular defence system such as antioxidants and permit a substantial attack of free radicals on lipids in the cell membrane causing lipid peroxidation leading to cell injury. This suggestion is supported by findings that BSO induced loss of GHS increased lipid peroxidation and the severity of the myopathy due to DFP [20].

The prevention of the DFP-induced muscle hyperactivity through the neuromuscular blocking agent d-tubocurarine attenuated the increase of F_2-isoprostanes [20, 21], prevented the change in Cyt-ox (*figures 1, 2*) and the conversion of XD into XO (*table II*). These data suggest that free radical generation during DFP-induced fasciculation is associated with AChE inhibition by DFP, and is not due to direct effects of DFP on Cyt-ox and XD/XO.

No studies to date have reported a relationship between OP-antiChE-induced muscle hyperactivity, lipid peroxidation, and muscle lesions. From the present studies, however, it is concluded that the accumulation of oxygen free radicals and the related increases in lipid peroxidation are caused by the DFP-induced muscle hyperactivity and that lipid peroxidation is a contributing initial factor in the mechanism of OP-antiChE-induced cell injury.

In summary, the results of this study demonstrated: 1) that the OP-antiChE, DFP, caused fasciculations leading to lipid peroxidation and muscle necrosis; 2) prevention of DFP-induced muscle hyperactivity by d-tubocurarine inhibits lipid peroxidation and necrosis and rules out a direct effect of DFP on Cyt-ox and XD-XO and thus support the hypothesis that OP-antiChE-induced muscle hyperactivity stimulates ROS production, through changes in cytochrome c oxidase and xanthine oxidase activity.

Acknowledgment

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Regulation of gene expression by trans-synaptic activity: A role for the transcription factor NF-κB

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Abstract — Earlier studies in the sympathetic ganglion have led to the proposal that adaptation of transcription to trans-synaptic activity is controlled by a signal transduction pathway featuring a transcription factor which translocates to the nucleus upon its release from the post-synaptic membrane by after-hyperpolarization. In light of recent progress, it is proposed here that NF-κB constitutes the postulated transcription factor. (©Elsevier, Paris)

Résumé — Régulation de la transcription par l'activité trans-synaptique: un rôle pour le facteur de transcription NF-κB. Des études antérieures ont amené à formuler l'hypothèse que, dans les neurones sympathiques, la transcription s'adapte à l'activité trans-synaptique grâce à un mécanisme de transduction par lequel un facteur de transcription, attaché à la membrane postsynaptique, est libéré par l'hyperpolarisation post-stimulatoire et migre dans le noyau. A la lumière de données nouvelles, il est proposé ici que NF-κB constitue ce facteur de transcription. (©Elsevier, Paris)

NF-κB / trans-synaptic regulation / gene expression / superior cervical ganglion

1. Introduction

It is now well established that activation of synaptic receptors by neurotransmitter can modify gene transcription and protein synthesis. However, the mechanisms whereby receptor activation sends transcriptional instructions from the post-synaptic membrane to the nucleus remain to be elucidated. One difficulty arises from the existence of several types of neurotransmitter-driven adaptations, each with its own mode of signal transduction. A textbook example is long-term potentiation (LTP), in which synaptic stimuli induce transcriptional changes through a second messenger signalling system, to eventually alter synaptic responsiveness [20, 22]. However, there is also a more basic and common type of neurotransmitter-driven adaptation whereby, as already hinted by Nissl more than a century ago [23], neurons adjust their protein content to their activity. Increasing data support the notion that all mature innervated excitable cells continuously adapt their protein composition and content to the amount of activity imposed upon them trans-synaptically [27, 31] (see references in [22, 29]). A dose-response relationship has been reported, for instance, between RNA synthesis and the amount (frequency and duration) of trans-synaptic stimulation in sympathetic ganglion [8, 12]. Altogether, the available data suggest that, in excitable cells, transcription of a number of genes is constitutively locked on synaptic activity and continuously adjusts to its fluctuations.

This basic mode of adaptation to elicited activity has been extensively studied in the rat superior cervical ganglion (SCG), starting in the late sixties with the findings that trans-synaptic activation of sympathetic neurons increases the synthesis of RNA [13] and that of tyrosine hydroxylase (TH) [32]. These two independent lines of investigations yielded strikingly parallel results [3, 7, 12, 30, 33], suggesting that both RNA and TH adaptations are induced through the same regulatory mechanisms. The RNA studies allowed for the identification of the specific ionic event triggering the activity-mediated induction of RNA synthesis [9] and led to the proposal that sympathetic neurons adapt to activity through a direct synapse-to-nucleus signal transduction system involving the translocation of a protein able to modify gene transcription [8, 10–12].

More recently, reports about the transcription factor NF-κB in the central nervous system (for review see [25]) have renewed interest in this proposal. Indeed, the properties displayed by NF-κB in nervous tissue closely match those predicted for the regulatory protein in the postulated signal transduction system. Revisiting the earlier data in light of these recent developments improves the current understanding of the signal transduction pathway by which SCG adapts to trans-synaptic activation.

2. The regulation of RNA synthesis by neuronal activity

The regulation of RNA synthesis by trans-synaptic activation was investigated in incubated rat SCG by monitoring the changes in RNA radiolabelling brought about by preganglionic stimulation, or application of ACh or carbachol [8] ([12] and references cited therein). The results of these studies may be summarized as follows.

Prolonged stimulation of SCG induces a sequence of three changes in RNA synthesis: an initial decrease, followed by an increase developing after about 90 min of activation, and another and larger increase taking place after the end of the stimulation (see *figure 2* in [12]).

The three changes are specifically initiated by activation of the nicotinic receptors and the consecutive passive influx of Na^+ generating the fast EPSP. In particular, the effects of stimulation are abolished by replacing Na^+ of the medium by Tris or sucrose, or by clamping by KCl depolarization the membrane potential of the neurons near the reversal potential. Conversely, the RNA adaptations are independent of the generation of action potentials, membrane depolarization per se, activation of muscarinic receptors, as well as second messengers such as Ca^{2+} and cAMP.

Each of the three changes has been linked to a specific ionic event. The initial fall in RNA synthesis is caused by the Na^+ influx mediated by opening of the nicotinic receptors and, indeed, both elevation of internal Na^+ concentration ($[Na^+]_i$) [4] and decrease in RNA synthesis develop along parallel time-courses. Moreover, the initial ACh-mediated reduction in RNA synthesis can be reproduced without synaptic activation by application of aconitine, an alkaloid which, like batrachotoxin, prolongs the opening of fast Na^+ channels [2, 16]. Interestingly, the initial decrease in RNA synthesis affects all species of RNA and is accompanied by a broad, non-specific reduction in protein synthesis [14], suggesting that elevation of $[Na^+]_i$ depresses both transcription and translation machineries.

The increase in RNA synthesis occuring during the late stages of synaptic activation requires high external concentrations of Cl^-. Substitution of Cl^- by impermeant anions (sulfate or isethionate) abolishes this particular increase in RNA synthesis without affecting the two other ACh-mediated changes. As for the post-stimulation increase in RNA synthesis, it is related to the long-lasting after-hyperpolarization generated by the electrogenic extrusion of Na^+ by the Na^+-K^+ pump [5, 28]. There is a close parallelism between the development of both phenomena as well as the conditions which favour or prevent their occurrence ([12] and references therein). Especially important, the post-stimulation increase in RNA synthesis is abolished by replacing NaCl in the incubating medium by LiCl, a condition which also prevents the after-hyperpolarization [5]. In contrast, the LiCl substitution does not affect the two changes, reduction and increase in RNA synthesis, occurring during the simulation. In addition, an enhancement of RNA synthesis can be obtained without stimulation of the nicotinic receptors, by lowering the external KCl concentration or by applying tetrodotoxin to ganglia previously treated with aconitine, i.e., two conditions which generate an hyperpolarization in the sympathetic neurons [5] (references in [12]).

Both increases in RNA synthesis during and after activation, as monitored by 1-h radiolabelling, concern exclusively pre-messenger nuclear RNA [8, 10]. It was also demonstrated by autoradiography that these increases affect essentially sympathetic neurons, and not glial cells. Nevertheless, among the two increases in RNA synthesis, the one triggered by after-hyperpolarization is obviously that bearing the main functional significance. After-hyperpolarization appears to be a most appropriate signal for informing a regulation system of the level of activity to which to adapt. Indeed, the integral of amplitude and duration of the after-hyperpolarization is directly proportional to the amount of Na^+ extruded, which mirrors the influx of Na^+ [5]. Therefore, after-hyperpolarization provides the trans-synaptic regulation system with constantly up-dated information about the level of activity to which to adapt. This is especially relevant in vivo where the activation of sympathetic neurons occurs in a succession of irregularly spaced short trains of pulses [28].

Several facts, including the need for an adequate external Cl^- concentration to increase RNA synthesis during stimulation, make it unlikely that activation of the Na^+-K^+ pump or the reduction in $[Na^+]_i$ by themselves play a direct role in RNA regulation. The specific signal triggering the induction of RNA synthesis appears to be the hyperpolarization itself, i.e., the accumulation of negative charges in the subsynaptic region. The regulation system controlling the adaptation to trans-synaptic activity should therefore be able to convert hyper-electronegativity into a signal bearing metabolic significance, and to transfer it to the nucleus within the lag of 15–30 min separating the generation of hyperpolarization and the actual onset of the RNA induction. For these reasons, a signal transduction pathway was postulaled that comprises a transcription factor attached to the internal side of the post-synaptic membrane [8, 10, 12]. In this hypothesis, the accumulation of negative charges underneath the membrane generated by the

after-hyperpolarization would liberate this factor through an ion-exchange or electrostatic effect. The freed factor would then be channelled through the retrograde dendritic transport to the nucleus where it would associate with the promoter of the various genes subject to trans-synaptic regulation. The identity of this factor remained unknown. However, recent reports have revealed that the transcription factor NF-κB displays properties in CNS neurons that match well those required by the model put forward [25]. Furthermore, rat SCG contains an inducible form of this factor (Gisiger and Bendayan, in preparation). Therefore, NF-κB appears a likely candidate for the postulated factor transducing hyperpolarization into induction of RNA synthesis in rat SCG.

3. Role of the transcription factor NF-κB in adaptation to activity

NF-κB is an ubiquitous transcription factor specialized in generating rapid coordinated activation of genes in response to situations critical for survival (for reviews see [1, 25, 35]). Briefly, NF-κB is stored in the cytoplasm, from where it rapidly translocates to the nucleus upon extracellular signaling, to enhance the transcription of numerous target genes. To accomplish this, NF-κB carries two important sites: a nuclear localization signal (NLS), which directs the translocation to the nucleus, and a high affinity site toward a specific decameric sequence of DNA, the κB site. Prior to the stimulus, NF-κB remains sequestred in the cytoplasm in a latent form, because it is complexed to a specific inhibitory protein, IκB, which covers these two sites. IκB binds to the NLS of NF-κB through an ankyrin-like region containing an acidic domain [6, 35]. Activation of NF-κB occurs when, following an extracellular signal, IκB is modified and dissociates from NF-κB, allowing NF-κB to translocate to the nucleus and to bind to the κB DNA sequence.

Three recent observations are noteworthy in the present context. First, NF-κB has been found associated with synaptic structures in adult cerebral cortex and hippocampus [15, 18]. Second, a 1–5 min stimulation of glutamate receptors in cultured cerebellar granule cells induces translocation of NF-κB to the nucleus of these neurons within a 30–45 min period, but not in glial cells [15, 19]. Third, it has been shown in *Aplysia* that soluble cytoplasmic proteins containing the NLS are taken up by the retrograde axonal transport, probably through a receptor, and rapidly carried into the nucleus [26].

These facts fit well into the proposed model of synapse-to-nucleus signal transduction. Nevertheless, further implications need to be considered. In order for NF-κB to remain close to the synapses, as already described [15, 18, 19], there is a need for a synaptic variant of IκB anchored to synaptic structures such as postsynaptic densities, for example [29]. In this instance, hyperpolarization, by counteracting the negative charges of the acidic domain of the IκB ankyrin regions, would release NF-κB at least as long as hyper-electronegativity persists. Having its NLS exposed, NF-κB would then associate with the NLS receptors to undergo retrograde dendritic transport and translocation into the nucleus. Because of its ability to carry NF-κB away from synaptic IκB before hyperpolarization disappears, the proposed mechanism provides an efficient way to transform an ephemeral event, lasting no longer than a few seconds, into middle or long-term alterations of transcription.

Interestingly, a kB-like motif is present within the promoter of TH [17, 21]. This suggests that the transcription of TH is directly enhanced by NF-κB without the intermediary of other early genes. This is consistent with the observation that the AP-1 transcription factor does not mediate trans-synaptic induction of TH transcription in adult SCG [34]. Indirect evidence suggests that other genes, including choline-acetyl-transferase [24] and dopamine-β-hydroxylase ([33] and references therein), have their transcription controlled by the same synapse-to-nucleus signalling system. The promoter of these genes should contain the specific κB DNA sequence, which will be helpful for their identification. Taking into account the recently reported localization of NF-κB at central synapses, it is possible that a similar synapse-to-nucleus signal transduction pathway accounts for adaptations to trans-synaptic activation in the CNS.

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Development of the neuromuscular junction: Genetic analysis in mice

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Abstract — Formation of the skeletal neuromuscular junction is a multi-step process that requires communication between the nerve and muscle. Studies in many laboratories have led to identification of factors that seem likely to mediate these interactions. 'Knock-out' mice have now been generated with mutations in several genes that encode candidate transsynaptic messengers and components of their effector mechanisms. Using these mice, it is possible to test hypotheses about the control of synaptogenesis. Here, we review our studies on neuromuscular development in mutant mice lacking agrin αCGRP, rapsyn, MuSK, dystrophin, dystrobrevin, utrophin, laminin α5, laminin β2, collagen α3 (IV), the acetylcholine receptor ε subunit, the collagenous tail of acetylcholinesterase, fibroblast growth factor-5, the neural cell adhesion molecule, and tenascin-C. (©Elsevier, Paris)

Résumé — Développement de la jonction neuromusculaire : analyse génétique chez la souris. La formation de la jonction neuromusculaire des muscles squelettiques est un processus à étapes multiples qui requiert une communication entre nerf et muscle. Les études de nombreux laboratoires ont conduit à l'identification de facteurs qui semblent capables d'assurer ces interactions. Chez la souris, nous avons inactivé ('knock out') les gènes de plusieurs messagers trans-synaptiques potentiels et de composants de leurs mécanismes effecteurs. Grâce à ces souris, il est possible d'analyser le contrôle de la synaptogénèse. Nous décrivons ici les résultats obtenus chez les souris mutantes dépourvues des protéines suivantes : agrine, αCGRP, rapsyne, MuSK, dystrophine, dystrobrévine, utrophine, laminine α5, laminine β2, collagène α3 (IV), sous-unité ε du récepteur nicotinique, queue collagénique de l'acétylcholinestérase, FGF-5, N-CAM et ténascine-C. (©Elsevier, Paris)

acetylcholine receptor / agrin / basal lamina / laminin / synapse formation

1. Introduction

The vertebrate skeletal neuromuscular junction (NMJ) is the best understood of all synapses. Its accessibility and simplicity led to its being studied intensively over most of this century, so we now know an enormous amount about its structure, function and molecular architecture. For example, numerous proteins have been identified as components of the pre- and postsynaptic membranes, the synaptic vesicles that fill the nerve terminal, the Schwann cell that caps the terminal, the cytoskeleton that coats the infolded postsynaptic membrane, and the basal lamina that runs through the synaptic cleft (*figure 1*).

This rich background, in turn, has facilitated developmental analysis of the NMJ. Studies carried out over the past 30 years have shown that the NMJ develops in a series of steps that involve and require complex interactions between nerve and muscle. That is, the growth cone of a motor axon initiates formation of the postsynaptic apparatus, the muscle cell in turn provides retrograde signals that organize the transformation of a growth cone into a nerve terminal, the nerve terminal promotes postsynaptic maturation, and so on. Attempts to elucidate the mechanisms underlying these interactions have led to identification of numerous molecules that may serve as intercellular signals or components of their intracellular signal transduction pathways. Many of these molecules have bioactivities in vitro and localizations in vivo consistent with their involvement in synaptic development. It has been difficult, however, to determine what roles, if any, these candidates play in vivo.

Recently, the 'knockout' technique has provided a way to circumvent this limitation in mice. Mutations can be engineered in genes by homologous recombination in embryonic stem (ES) cells. The ES

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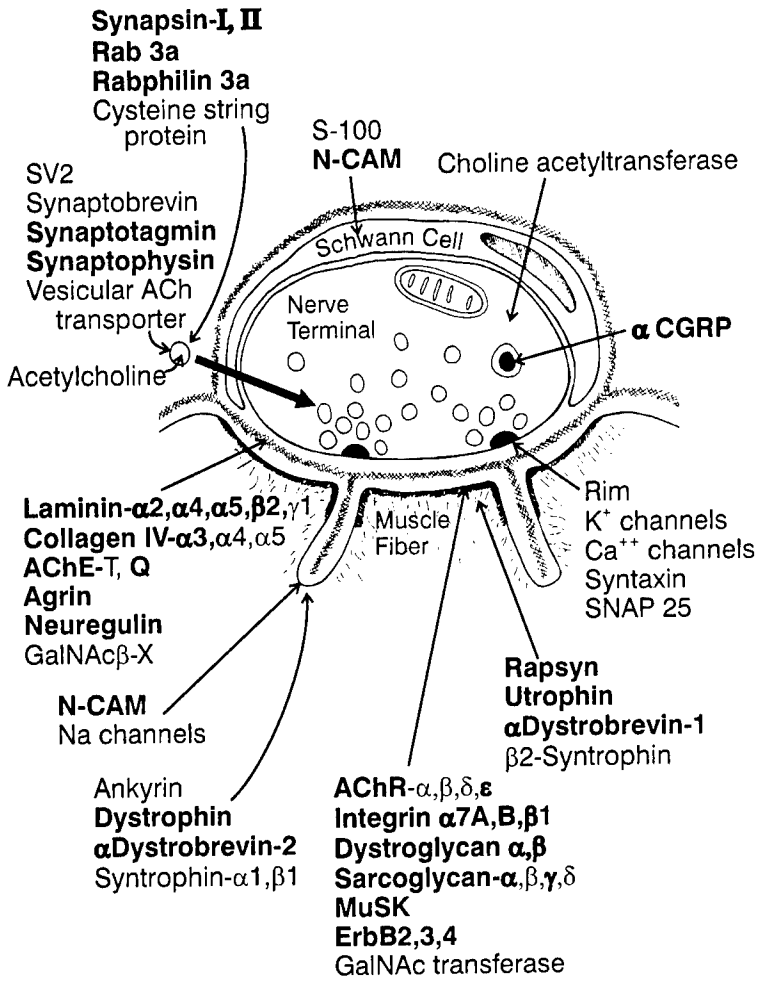


Figure 1. Structure and molecular architecture of the NMJ. The nerve terminal occupies a shallow gutter in the muscle fiber and is capped by processes of Schwann cells. Active zones in the nerve terminal directly appose junctional folds in the postsynaptic membrane. Some of the proteins concentrated at the synapse are shown, with their subcellular localizations indicated by arrows. Those for which 'knock-out' mice have been generated are indicated in bold-face. See Hall and Sanes [15] and Sanes and Lichtman [25] for primary references and names of additional components.

cells are then injected into the blastocyst to generate, in sequence, germ-line chimeras, mutant heterozygotes (which usually exhibit no detectable phenotype) and homozygotes (which sometimes do). In work begun in collaboration with the late John Merlie, our group has generated and/or analyzed several of these mutants. This chapter summarizes some of these studies. Limitations of space preclude citing the studies that led to our work; we refer the reader to reviews by Hall and Sanes [15], Duclert and Changeux [6], Bowe and Fallon [2], Fischbach and Rosen [8], Daniels [4], and Sanes and Lichtman [25].

2. Agrin: a hypomorph, a null, and an isoform-specific mutant

Nerves and components of synaptic basal lamina can induce clustering of acetylcholine receptors (AChRs) in the postsynaptic membrane of the NMJ. Several groups therefore sought factors that could stimulate the clustering of initially diffuse AChRs on aneural cultured myotubes. Using this assay, McMahan and colleagues isolated a protein that they called agrin. They then generated antibodies, and used them to show that agrin is synthesized by motoneurons, transported down motor axons, and stably incorporated into the basal lamina of the synaptic cleft. Based on these studies, McMahan proposed that agrin is a critical nerve-derived organizer of postsynaptic differentiation [2].

We generated agrin-deficient mutant mice to test this hypothesis. The original mutants we analyzed were severe hypomorphs, that expressed no 'active' agrin (see below) at all, and only ~10% of wild-type levels of other agrin isoforms [9]. More recently, we have generated a protein null and obtained similar results [3]. In both the hypomorph and the null, post-

synaptic differentiation is drastically impaired: neither clustered AChR clusters nor other synaptic specializations are detectable at most motor axon-myotube contacts. Those AChR clusters that are present are smaller and less densely packed than normal, and most often occur on uninnervated portions of the myotube surface. These results support McMahan's 'agrin hypothesis'. In addition, mutant motor nerve terminals fail to arborize or differentiate normally.

Although agrin is confined to synaptic sites in adult muscle fibers, it is synthesized by muscle as well as nerve and is present throughout the basal lamina of developing myotubes. Initially, this distribution seemed inconsistent with the hypothesis that agrin serves as a nerve-derived signal. A likely resolution to this paradox came with the discoveries that an alternatively spliced product of the agrin gene (which includes exons called 'B' in birds and 'z' in mammals) greatly potentiates the AChR clustering activity of agrin, and is expressed only by neurons. Our initial analysis did not distinguish between roles of neural and muscle agrin. We addressed this issue in two ways [3]. First, agrin$^{-/-}$ muscles were transplanted to wild-type hosts, where they were reinnervated by agrin-expressing axons. Synapses in these surgical chimeras displayed neither pre- nor postsynaptic defects. Second, a novel allele was generated in which the 'z' exons were deleted with only minor effects on the levels of 'z-minus' muscle agrin. Both pre- and postsynaptic defects in these 'second generation' mutants were indistinguishable from those in the original allele. Thus, it is nerve-derived z agrin that is essential for synaptic development. Moreover, the effects of agrin on motor axons are not mediated by muscle agrin, but are more likely to be an indirect consequence of impaired postsynaptic differentiation.

3. Agrin signalling: rapsyn and MuSK

Rapsyn is a 43 kDa cytoplasmic protein originally isolated by virtue of co-purification with AChRs. It is precisely co-localized with AChRs at the NMJ, and causes redistribution of diffusely distributed AChRs into high-density patches when co-expressed with them in heterologous cells. These results suggested an involvement of rapsyn in AChR clustering at the synapse. Genetic analysis supported this idea: AChRs were synthesized but failed to cluster in muscles of rapsyn$^{-/-}$ mice or in myotubes cultured from the mutants, even in the presence of high levels of agrin [10]. Likewise, numerous components of the postsynaptic membrane and cytoskeleton remained diffusely distributed in mutants [19]. Thus, rapsyn is necessary for agrin to exert its effects.

How does agrin influence rapsyn? Agrin interacts with numerous components of the myotube surface, several of which seemed reasonable candidates to mediate agrin's effects on postsynaptic differentiation [24]. However, mutations that disrupt some of them fail to show severe neuromuscular defects ([18] and see below). In contrast, the phenotype of 'knockout' mice lacking a muscle-specific tyrosine kinase (MuSK) exhibited a phenotype similar to, and if anything, more severe than that of the agrin mutants described above [5] (Gautam et al., submitted). Based on this phenotype, MuSK, which had been isolated as part of an unrelated study, became the leading candidate agrin receptor. In support of this idea, MuSK$^{-/-}$ myotubes are unresponsive to agrin, responsiveness is restored by reintroduction of MuSK, and a dominant negative MuSK protein inhibits agrin signaling in wild-type muscle cells [11, 12] (Zhou and Sanes, in preparation).

Although genetic studies place agrin, MuSK and rapsyn in a common pathway, they leave open questions of what steps intervene between agrin and MuSK and between MuSK and rapsyn. Isolated MuSK cannot bind agrin, so it is likely to be only one component of a multi-subunit receptor. Other subunits (called MASCs for muscle-associated specificity components; [12]) have not yet been identified. Likewise, the interaction of MuSK with rapsyn is complex: although MuSK and rapsyn co-cluster in heterologous cells, it is the extracellular rather than the cytoplasmic domain of MuSK that is essential for clustering. Because rapsyn is entirely cytoplasmic, this result implies the existence of a rapsyn-associated transmembrane linker (RATL; [1]). Rapsyn does not appear to localize MuSK to synapses, however, in that MuSK is clustered at synaptic sites in rapsyn$^{-/-}$ mice [1]. Thus, MuSK is a critical component of a primary synaptic scaffold and rapsyn recruits other synaptic components to that scaffold. A final complexity is that rapsyn and AChRs cluster spontaneously in heterologous cells, but fail to cluster in myotubes unless agrin (or another exogenous agent is added). The implication is that muscles normally exert some inhibitory influence on clustering, which agrin relieves via MuSK.

4. The dystrophin-glycoprotein complex: utrophin, dystrophin, and α-dystrobrevin

Dystroglycan was isolated as part of a multimolecular complex (the dystrophin glycoprotein complex or DGC) that co-purifies with dystrophin, the cyoskeletal protein mutated in humans with Duchenne or Becker muscular dystrophy and in mdx mice. Dystroglycan binds to laminin extracellularly

and dystrophin intracellularly, forming part of a system that links the extracellular matrix to the cytoskeleton. The DGC is also present at the NMJ, but with alterations, the most striking of which is that dystrophin is replaced by an autosomal homologue, utrophin at the synapse.

In 1994, several groups reported that agrin binds tightly and specifically to dystroglycan, leading to the speculation that dystroglycan might be the functional agrin receptor, and to the specific hypothesis that utrophin transforms a widely distributed laminin receptor into an agrin receptor. Dystroglycan$^{-/-}$ mutants die before muscles form, and are therefore unsuitable for studies of the NMJ. However, it was possible to test the specific hypothesis that utrophin is essential for synaptic development. In fact, utrophin$^{-/-}$ mice are viable, fertile, and apparently healthy. Overall, their NMJs are qualitatively normal, but the density of AChRs in the postsynaptic membrane is reduced by ~30% in mutants compared to controls, and the number of junctional folds is reduced by half [13]. One possible explanation for the subtlety of the defects in utrophin$^{-/-}$ mice was that the presence of dystrophin provided compensation for loss of its homologue. We tested this possibility by generating and analyzing double mutants lacking both utrophin and dystrophin. The double mutants were severely dystrophic, but their NMJs were not significantly more affected than those of utrophin$^{-/-}$ or dystrophin$^{-/-}$ mice [14]. Thus, utrophin and dystrophin are dispensable for formation of the NMJ.

More recently, we generated mice lacking another cytoplasmic component of the DGC, α-dystrobrevin. These mice exhibit a mild dystrophy similar to that seen in dystrophin$^{-/-}$ mice. Interestingly, however, AChR-rich regions at α-dystrobrevin$^{-/-}$ NMJs have a feathered appearance not seen in utrophin$^{-/-}$, dystrophin$^{-/-}$ or double mutant synapses. Moreover, agrin induces significantly smaller AChR clusters in αdystrobrevin$^{-/-}$ than in wild-type myotubes [26]. These results raise the possibility that the DGC may be involved in the maturation or maintenance of the NMJ, perhaps by stabilizing postsynaptic specializations once they have been generated through a MuSK-dependent pathway.

5. AChR synthesis: neuregulins and αCGRP

Motor nerves not only cluster diffusely-distributed AChRs but also induce selective transcription of AChR genes by subsynaptic nuclei. Three molecules have been proposed as nerve-derived inducers of AChR gene expression: neuregulin, αCGRP, and agrin itself. All three are reasonable candidates based on their localizations and bioactivities. Evidence in favor of neuregulin and agrin is reviewed elsewhere [8, 25]. αCGRP is a peptide generated from an alternatively spliced product of the gene that also encodes calcitonin. αCGRP is synthesized by motoneurons, released by motor nerve terminals, and capable of stimulating AChR expression by cultured myotubes. αCGRP has also been proposed to regulate activity-dependent sprouting of motor axons. However, we detected no defects in synaptic development or regeneration in mutant mice lacking αCGRP (Lu et al., in preparation). In particular, synaptic nuclei became transcriptionally specialized in the mutants. One potential caveat to this finding is that the closely related peptide βCGRP might have compensated for lack of its homologue. However, antisera that recognize both α and βCGRP stained motor nerve terminals in wild-type but not in αCGRP$^{-/-}$ mice, implying that neither CGRP is required for synaptogenesis.

6. Synaptic basal lamina: laminins α5 and β2 and collagen α3 (IV)

Studies on regeneration on nerve and muscle revealed that synaptic basal lamina contains components capable of organizing postsynaptic differentiation in the absence of the nerve and presynaptic differentiation in the absence of muscle. In addition, components of basal lamina account, at least in part, for the preferential reinnervation of original synaptic sites following axotomy [15]. Apparently, nerve terminals and muscle fibers insert components into synaptic basal lamina to influence each other's differentiation or regeneration. Agrin, which is inserted by the nerve, is clearly a major organizer of postsynaptic differentiation. Likely candidates for muscle-derived retrograde signals are synapse-specific isoforms of widely-distributed basal lamina components. The entire muscle fiber basal lamina is rich in laminin and collagen IV, but isoforms differ between synaptic and extrasynaptic regions. Specifically, extrasynaptic laminin is rich in laminin 2 (composed of the β2, β1 and γ1 subunits) whereas laminin 11 (α5/β2/γ1) is one of a few major synaptic laminins. Similarly, extrasynaptic and synaptic basal lamina are rich in the α1/β2 and α3-α5 chains of collagen IV, respectively. Moreover, native laminin 11 and the isolated β2 chain serve as stop signals for growing motor neurites, and β2 fragments trigger differentiation of growth cones into motor nerve terminals [23].

To test roles of laminins and collagens in synaptic development, we generated mutants lacking laminin α5, laminin β2, and collagen α3 (IV). Laminin α5 mutants die of placental defects just as NMJs are

beginning to form, and collagen α3 (IV) show no obvious neuromuscular defects, but laminin β2 mutants have been informative. Formation of nerve terminals is severely impaired in laminin $\beta2^{-/-}$ mice: vesicles fail to aggregate near the presynaptic membrane, few active zones form, and levels of spontaneous and evoked transmitter release are decreased several-fold [20]. The consequent weakness is a major contributor, along with renal defects [21] to failure of the mutants to thrive and to their death during the third postnatal week.

Synaptic laminins also provide an inhibitory signal to the terminal Schwann cell. In normal muscle, Schwann cells cap nerve terminals, but in laminin $\beta2^{-/-}$ mutants, Schwann cell processes invade the synaptic cleft. Although we initially speculated that this defect was a secondary consequence of decreased adhesion of nerve to basal lamina, experiments in vivo and in vitro showed that synaptic laminins actively inhibit extension of Schwann cell processes [22]. Thus, maintenance of normal nerve-muscle apposition requires not only tight adhesion of nerve to muscle via the intervening cleft, but active repulsion of the Schwann cell from the cleft. This interaction, in turn, may be necessary because Schwann cells need to be in close apposition to nerve terminals to provide them with with trophic sustenance. Repulsion from the cleft allows the nerve terminal-Schwann cell relationship to be intimate without becoming suffocating.

7. Retrograde messengers: N-CAM, FGF-5, and tenascin-C

Several proteins originally shown to be neuroactive in other systems were later implicated in the control of neuromuscular development. For example, the neural cell adhesion molecule (N-CAM) is coregulated with AChRs during development and following denervation, binds agrin tightly and specifically, and promotes neurite outgrowth. Moreover, infusion of antibodies to N-CAM into denervated muscle interferes with reinnervation. Fibroblast growth factor 5 (FGF-5) is a potent promoter of motoneuron survival and differentiation in vitro, and accounts for a large fraction of the survival-promoting activity extractable from neonatal skeletal muscle. Tenascin C is synthesized by a population of perisynaptic fibroblasts that is stimulated to proliferate following denervation; this intriguing distribution along with antibody-blocking experiments suggested that tenascin-C might help guide regenerating axons back to original synaptic sites (see [18] for reference).

We tested these hypotheses by analyzing mutant mice lacking N-CAM, FGF-5, tenascin-C, or both N-CAM and tenascin-C. In brief, synaptic development, reinnervation, and sprouting following partial denervation proceeded normally in all three mutants. Two aspects of postnatal synaptic maturation were slowed in the $\text{N-CAM}^{-/-}$ mutants: the generation of junction folds and the elimination of polyneuronal innervation. Nonetheless, all three molecules are dispensable for most aspects of synaptic development.

8. Machinery of neurotransmission: AChRs and acetylcholinesterase

The point of neuromuscular development is to form the specializations required for neurotransmission. Prominent among these are AChRs and acetylcholinesterase (AChE). In addition to their roles in synaptic function, however, both of these proteins have been hypothesized to play roles in synaptic development. We used genetic methods to test this idea.

The subunit composition of AChRs changes postnatally: AChRs containing a gamma subunit ($\alpha_2\beta\gamma\delta$) are replaced by AChRs containing a homologous epsilon subunit ($\alpha_2\beta\varepsilon\delta$) during the first postnatal week. The significance of the switch may be in biophysical differences between the subunts: ε-containing AChRs open more briefly but with higher conductance than γ-AChRs. Another possibility, though, is that the transition is important for structural maturation of the synapse. For example, the γ and ε subunits might interact with different proteins. We tested this idea in ε-deficient mutants. Levels of AChRs are initially normal in these mutants, and γ-AChRs are retained long beyond the time they normally disappear. Even while the γ subunit is providing compensation, the formation of the raised end-plate and of junctional folds are impaired. Later, as AChR levels decline, other synaptic components are lost in parallel, and the remaining components are collected into micro-islands [17]. Together, these results reveal novel structural roles of the AChR.

Much of the AChE of the NMJ is anchored to the synaptic basal lamina via a collagenous subunit called 'Q.' We generated Q-deficient mutants, and showed that they lacked all detectable synaptic AChE. Homozygous mutants were weak and survival was variable, but some mutants lived for several months. In view of several reports suggesting that AChE modulates neurite outgrowth, we were particularly interested in the possibility that motor axons might fail to stop growing or to arborize properly at AChE-deficient end-plates. In fact, arborization

was grossly normal in the mutants, but the precise apposition of pre- to postsynaptic specializations was degraded. Ongoing work is aimed at determining whether this phenotype reflects a signaling function of AChE or whether it is secondary to altered activity patterns.

9. Conclusion

Analysis of the mutant phenotypes is by no means straightforward, in that signficant roles can be masked by redundancy or compensation, and apparently significant defects can result from indirect effects. Nonetheless, mutant mice have allowed some tests of hypothesis about mechanisms that regulate neuromuscular development. For example, they have provided evidence for a pathway of postsynaptic differentiation in which agrin is a nerve-derived signal, MuSK is a component of the receptor, rapsyn is an effector, and the DGC is involved in stabilization. Genetic studies have also allowed tests of candidate retrograde signals, and implicated the synaptic laminins in this process. An intriguing question for the future, which the mutants may help us answer, is whether similar mechanisms regulate formation of neuron-neuron synapses in ganglia or the brain [7].

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Transcriptional pathways for synapse-specific, neuregulin-induced and electrical activity-dependent transcription

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Abstract — Innervation-dependent expression of acetylcholine receptor (AChR) genes in skeletal muscle is mediated by multiple transcriptional pathways. One pathway leads to activation of AChR genes selectively in synaptic nuclei and requires an Ets binding site that binds GABP. A second pathway leads to repression of AChR transcription in nuclei throughout the myofiber and requires inactivation of E-box-binding proteins, including myogenic bHLH proteins. Taken together, these studies indicate that separate pathways regulate innervation-dependent transcription. (©Elsevier, Paris)

Résumé — Les voies de la transcription synapse-spécifique, induit par la neuréguline et dépendant de l'activité électrique. L'expression des gènes codant les récepteurs à l'acétylcholine (RACh) dépendante de l'innervation dans le muscle squelettique est engendrée par de multiples voies transcriptionnelles. Une de ces voies aboutit à l'activation des gènes RACh sélectivement dans les noyaux synaptiques et nécessite la présence de protéines liant les sites Ets, y compris GABP. Une deuxième voie aboutit à la répression de la transcription des RACh dans les noyaux dans toute la myofibre et nécessite l'inactivation des protéines liant la boite E. (©Elsevier, Paris)

acetylcholine receptors / neuromuscular synapses / Ets proteins / GABP

1. Introduction

Localization of acetylcholine receptors (AChRs) to neuromuscular synapses is mediated by multiple transcriptional pathways. One pathway, which is stimulated by a signal that is concentrated at synaptic sites, induces expression of AChR genes in myofiber nuclei that are positioned near the synaptic site (for reviews see [4, 5, 8, 10]). Neuregulin (NRG) is the best candidate for the extracellular signal that induces synapse-specific gene expression, since NRG activates AChR gene expression in cultured muscle cells and NRG and its receptors are concentrated at synaptic sites [2, 7, 9, 11, 14, 17, 25]. A second pathway, which depends upon propagated electrical activity in the myofiber, represses AChR gene expression in nuclei throughout the myofiber (for review see [10]). These two pathways act together to restrict AChR expression to synaptic sites.

181 bp of 5′ flanking DNA from the AChR δ-subunit gene are sufficient to confer both synapse-specific and electrical activity-dependent transcription in transgenic mice [19, 21]. These two aspects of innervation-dependent expression, however, are controlled by distinct regulatory elements since an E-box is required for electrical activity-dependent but not synapse-specific transcription and an Ets-binding site is required for synapse-specific but not electrical activity-dependent transcription [21] (Fromm and Burden, submitted). Taken together, these results indicate that separate transcriptional pathways mediate synapse-specific and electrical activity-dependent gene expression.

2. Materials and methods

Myoblasts were transfected, induced to differentiate into myotubes, and the stably transfected cells were treated with NRG for 2 days [11, 18] (Fromm and Burden, submitted). The amount of hGH secreted from treated and untreated cells was measured by a radioimmunoassay [11].

Nuclear extracts from Sol8 myotubes were incubated with a radiolabeled oligonucleotide probe (nucleotides –62 to –47 in the AChR δ subunit gene) [11, 18] (Fromm and Burden, submitted), and complexes were resolved by electrophoresis. The specificity of the binding reaction was determined by the addition of unlabelled competitor DNA to the reaction mixture.

Antibodies to Ets proteins, which were either kindly supplied by colleagues or purchased from Santa Cruz Biotechnology, were incubated with nuclear extracts prior to the addition of the labeled probe [11, 18] (Fromm and Burden, submitted).

We stained muscles from transgenic mice carrying AChR d subunit-hGH transgenes with antibodies to hGH and Texas Red α-bungarotoxin [19, 21] (Fromm and Burden, submitted), and we measured the level of hGH, AChR δ subunit and actin mRNA expression by RNase protection [19, 21] (Fromm and Burden, submitted).

3. Results

181 bp of 5′ flanking DNA from the AChR δ subunit gene are sufficient to confer synapse-specific transcription in transgenic mice and NRG-induced

transcription in cultured muscle cells [11, 19, 21]. Changeux and colleagues showed that a sequence (CGGAA; nucleotides –54 to –58) that conforms to a consensus binding site (C/AGGAA/T) for Ets proteins is important for synapse-specific expression [12]. We showed that this potential binding site for Ets proteins is required for NRG-induced transcription, since NRG induces a ~10-fold increase in gene expression from myotubes transfected with a wild-type AChR δ regulatory region (–1823 ± 25) but only a modest (1.8-fold) increase in gene expression from myotubes transfected with an AChR δ regulatory region containing mutations (CAAAA) in nucleotides that are critical for binding Ets proteins (Fromm and Burden, submitted).

To determine whether the sequence requirements for NRG-induced transcription conform to the sequence specificity for binding Ets proteins, we transfected myotubes with gene fusions containing a C, A or T at the 5′ end of the core Ets-binding site. We found that mutation of the C to an A results in a reduced but substantial (5-fold) response to NRG, whereas mutation of the C to a T results in a weak NRG response (1.7-fold) (Fromm and Burden, submitted). Since Ets proteins bind sequences containing a C or an A, but not a T (C/AGGAA/T) at the 5′ end of a core Ets-binding site [25], these results are consistent with the idea that the NRG-response element (NRE) binds Ets proteins and that this binding is important for NRG-induced expression of the AChR δ subunit gene.

We found that mutation of the NRE reduces expression from untreated cells by ~7-fold, and these results suggest that this NRE is also required for maximal expression from myotubes not treated with NRG (Fromm and Burden, submitted). Taken together with studies showing that muscle cells synthesize NRG [14, 16], these results raise the possibility that AChR gene expression from untreated cells is nevertheless NRG-dependent and regulated by autocrine signaling.

We analyzed synapse-specific transcription by producing transgenic mice carrying gene fusions between the mouse AChR δ subunit gene and the hGH gene [19, 21]. Because hGH is processed in the endoplasmic reticulum and Golgi apparatus and because these organelles are closely associated with nuclei, it is possible to infer the nuclear source of hGH transcription by studying the spatial pattern of intracellular hGH using immunohistochemistry. We produced transgenic mice carrying wild-type or mutated AChR δ subunit-hGH gene fusions and showed that transgenes containing a mutation in the NRE, unlike wild-type transgenes, are not expressed selectively from synaptic nuclei, demonstrating that the NRE is required for synaptic expression (Fromm and Burden, submitted).

An absence of synaptic expression could be owing to a specific role for this NRE in synapse-specific transcription or to a requirement for this NRE in transcription per se. We denervated muscle from mice carrying transgenes with a mutated NRE and showed that the mutant transgenes, like wild-type transgenes are induced following denervation (Fromm and Burden, submitted). These results demonstrate that the transgenes containing a mutation in this NRE are transcriptionally competent and not simply inactive, indicating that the NRE is required specifically for synapse-specific transcription. Further, these results show that the NRE is not required for electrical activity-dependent gene expression.

E-boxes are important regulatory elements for activating muscle genes, including AChR subunit genes [15], during myogenesis. We asked whether E-boxes in the AChR δ subunit gene may therefore be required for synapse-specific expression. We produced transgenic mice carrying AChR δ subunit-hGH gene fusions with a mutation in the E-box near the transcription start site and showed that these transgenes, like wild-type transgenes are expressed selectively from synaptic nuclei, demonstrating that this E-box is not required for synaptic expression [21]. This E-box, unlike the NRE, however, is required for electrical activity-dependent transcription, since transgenes with a mutated E-box, unlike wild-type transgenes or transgenes with a mutated NRE, are not induced following denervation [3, 21]. These results demonstrate that this E-box is required specifically for electrical activity-dependent transcription and are consistent with the idea that electrical activity represses myogenic bHLH proteins either by decreasing their expression and/or activity.

We found that protein(s) in myotube nuclear extracts bind an oligonucleotide probe containing the NRE. Binding is competed by an excess of unlabelled, competitor DNA containing a wild-type NRE but not by competitor DNA containing mutations in nucleotides that are important for NRG responsiveness (Fromm and Burden, submitted). Thus, there is a good correlation between nucleotides required for NRG-responsiveness and protein-binding.

NRG stimulation does not appear to recruit the protein(s) to the NRE, since a similar protein/DNA complex is detected with nuclear extracts from untreated myotubes (Fromm and Burden, submitted). These data favor the idea that NRG increases the transcriptional activity rather than the DNA-binding activity of the protein(s) that bind to this NRE.

We incubated nuclear extracts from NRG-stimulated myotubes with antibodies to Ets proteins to determine whether known Ets proteins are present in

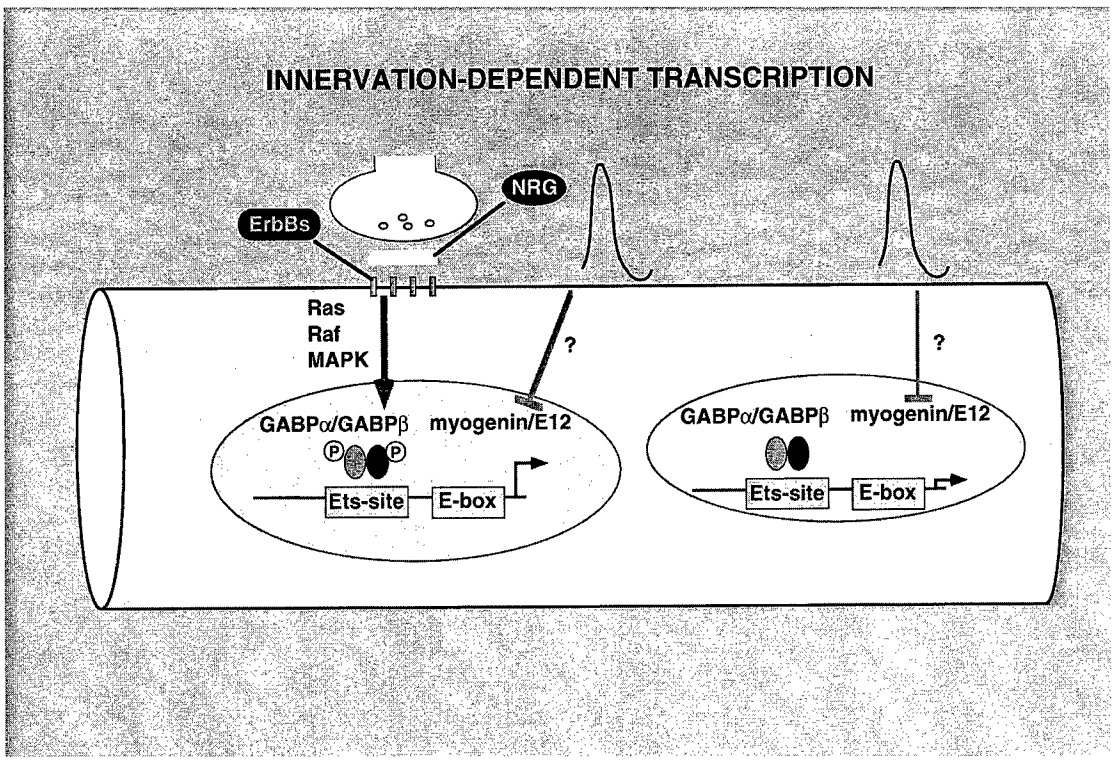


Figure 1. Separate pathways regulate synapse-specific and electrical activity-dependent transcription. NRG, which is present in the synaptic basal lamina, activates ErbB receptors in the postsynaptic membrane and leads to activation of a Ras signaling pathway. Our experiments suggest that this pathway leads to activation of GABPα/GABPβ and an increase in AChR gene expression selectively in synaptic nuclei. The pathway activated by electrical activity is thought to result in a decrease in the activity and/or abundance of transcriptional activators that bind E-boxes, such as myogenin and E12, leading to a decrease in AChR gene expression in nuclei throughout the muscle fiber.

the protein/DNA complex detected in the EMSA. We found that antibodies against either GABPα, an Ets protein, or GABPβ, a protein which lacks an Ets domain but dimerizes with GABPα [23], both inhibit the formation of the protein/DNA complex and super-shift the complex (Fromm and Burden, submitted). In contrast, antibodies against eight other Ets proteins, four of which are known to be expressed in skeletal muscle cells, fail to inhibit formation of the protein/DNA complex (Fromm and Burden, submitted). These results indicate that GABPα is the major, if not the only Ets protein in this complex and that a complex of GABPα/GABPβ binds to the NRE.

4. Discussion

An Ets site in the AChR δ subunit gene binds GABP and is required for transcriptional induction of the AChR gene by NRG and for synapse-specific gene expression in transgenic mice. These results provide further evidence for the idea that NRG is a signal for synapse-specific transcription and suggest that GABPα/GABPβ respond to NRG signaling by stimulating transcription of the AChR δ subunit gene.

NRG stimulation results in activation of a Ras/Raf/MAP kinase signaling cascade, and both Ras and MAP kinase kinase are required for induction of AChR genes by NRG [1, 20, 22]. Ets proteins, including GABPα, are known targets of Ras/MAP kinase signaling, and in vitro studies have shown that Ets proteins, including GABPα, can be phosphorylated directly by MAP kinase. Taken together, these studies raise the possibility that GABPα/GABPβ binds the NRE in the AChR δ subunit gene and that Ras signaling leads to transcriptional activation of GABP and induction of AChR genes.

GABP is present in myotube nuclear extracts and binds to the NRE in the AChR δ subunit promoter. Although Elf-1, Elf-2, ERP, NERF, ER81, Ets-2 and GABP are known to be expressed in skeletal muscle, GABPα/GABPβ appears to be the major, if not only

Ets protein that binds the NRE. The apparent preference for GABP could be owing to a greater abundance of GABP than other Ets proteins in skeletal muscle or to a higher affinity of GABP for the NRE.

An E-box is important for electrical activity-dependent but not synapse-specific transcription of AChR genes [3, 21]. In contrast, the NRE is required for synapse-specific but not electrical activity-dependent transcription (Fromm and Burden, submitted). Taken together, these studies indicate that separate pathways regulate innervation-dependent transcription: an electrical activity-dependent pathway that leads to a decrease in the activity and/or expression of myogenic bHLH proteins and a synapse-specific pathway that leads to the activation of GABPα/GABPβ.

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Targeting of acetylcholine receptor and 43 kDa rapsyn to the postsynaptic membrane in *Torpedo marmorata* electrocyte

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Abstract — In this study we have investigated the intracellular routing of two major components of the postsynaptic membrane in *Torpedo* electrocytes, the nicotinic acetylcholine receptor and the extrinsic 43 kDa protein rapsyn, and of a protein from the non-innervated membrane, the Na^+,K^+ ATPase. We isolated subpopulations of post-Golgi vesicles (PGVs) enriched either in AChR or in Na^+,K^+ ATPase. Rapsyn was associated to AChR-containing PGVs suggesting that both AChR and rapsyn are targeted to intracellular organelles in the secretory pathway before delivery to the postsynaptic membrane. In vitro assays further show that rapsyn-containing PVGs do bind more efficiently to microtubules compared to Na^+,K^+ ATPase-enriched PVGs. These data provide evidence in favor of the contribution of the secretory pathway to the delivery of synaptic components. (©Elsevier, Paris)

Résumé — Adressage du récepteur de l'acétylcholine et de la rapsyne à la membrane postsynaptique de l'électrocyte de torpille. Nous avons étudié le trafic intracellulaire de deux protéines synaptiques : le récepteur de l'acétylcholine et la rapsyne-43kDa dans l'électrocyte de torpille ainsi que l'ATPase Na^+,K^+, la protéine majeure de la membrane non-innervée. Nous avons isolé différentes sous-populations de vésicules post-golgiennes, enrichies respectivement en AChR et en ATPase Na^+,K^+. La rapsyne est associée spécifiquement à des vésicules riches en AChR, ce qui suggère son adressage à la voie d'exocytose avant son adressage à la membrane plasmique. La liaison *in vitro* des vésicules contenant la rapsyne à des microtubules suggère la contribution des voies d'exocytose dans l'adressage direct des protéines synaptiques. (©Elsevier, Paris)

acetylcholine receptor / rapsyn / intracellular trafficking / microtubules / synaptogenesis

1. Introduction

Accumulation of acetylcholine receptors (AChRs) and other synaptic proteins at the neuromuscular junction (NMJ) results from several regulatory mechanisms. In situ hybridization and promoter studies in transgenic mice have demonstrated that the restricted distribution of several synaptic proteins at the NMJ results in part from differential transcription in synaptic vs. extrasynaptic regions of the muscle fiber (reviewed in [4]). This mechanism suggests a local biosynthesis of synaptic components, as well as a polarized sorting in the exocytic pathway, the latter event being a key feature of polarized cells which achieve the differentiation of plasma membrane domains by way of protein sorting in the Golgi apparatus or in the endosomes (reviewed in [9]).

In addition to the AChR, the mature post-synaptic membrane accommodates a host of extrinsic proteins. Among them, the 43 kDa rapsyn is particularly important since it plays a major role in the regulation of the clustering and in the maintenance of high densities of AChRs at the synapse. Rapsyn lacks a signal sequence for membrane insertion but possesses an amino-terminal myristoylation site [2] responsible in part for its targeting to the plasma membrane [13]. As such, rapsyn could possibly be translated on free polysomes, acylated and inserted into the plasma membrane. Recent evidence shows, however, that processing and targeting of several acylated proteins depends on a functional secretory pathway [5, 11]. In this line, rapsyn may associate with intracellular organelles followed by vesicular transport to the plasma membrane.

The electrocyte of the electric ray *Torpedo marmorata* has long been used as a model system for the study of cholinergic synapses. Here, we have taken advantage of its intrinsic polarity to study the intracellular trafficking and the delivery of AChR and rapsyn to the innervated membrane, compared to that of the Na^+,K^+ ATPase, a marker of the non-innervated membrane.

2. Materials and methods

2.1. Animals

Torpedoes (*Torpedo marmorata*) were obtained from the Stations de Biologie Marine, Roscoff (Université Paris 6) and Arcachon (Université Bordeaux I), France.

2.2. Antibodies

Polyclonal antibodies directed against *Torpedo* Na^+,K^+ ATPase and AChR were obtained in our laboratory [1]. A monoclonal antibody directed against rapsyn (mAb 1234) was kindly provided by Dr. S. Froehner [12]. A monoclonal antibody directed against glutaminated α-tubulin (mAb GT335;) was a generous gift of Dr. P. Denoulet [18]. Monoclonal antibodies directed against cytoplasmic epitopes of AChR α-subunit (mAbs 111 and 155) were kindly provided by Dr. S. Tzartos [17].

2.3. Immunoisolation of post-Golgi vesicles enriched in AChR, rapsyn or Na^+,K^+ ATPase

Crude post-Golgi vesicles were isolated following the protocol described in Camus et al. [1]. The AChR, rapsyn and Na^+,K^+ ATPase-enriched post-Golgi vesicles (V_R, V_{43} and V_A) were immunopurified on magnetic beads (Dynabeads M-280, Dynal AS Oslo, Norway) coated by mAbs 111/155, mAb 1234 and polyclonal anti- Na^+,K^+ ATPase, respectively (see Camus et al. [1] for details). For biochemical analysis by SDS-PAGE and Western blotting, beads were directly resuspended in Laemmli buffer [10].

2.4. Mono-dimensional SDS-PAGE and Western blotting

Proteins from various membranes preparations were separated on 10% monodimensional sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) in a slab cell (Mini Protean II, BioRad Richmond CA, USA). After separation, proteins were electrotransferred onto nitrocellulose paper (Schleicher and Schuell, Dassel, Germany) according to Towbin et al. [16]. Immunoblot experiments were performed as described elsewhere [3] and the immunodetections were achieved using the chemiluminescent reaction (ECL, Amersham, UK) using X-ray films.

2.5. Microtubule binding assays

Phosphocellulose (PC)-purified pig brain tubulin was polymerized with 20 μM taxol for 30 min at 37°C in PEMT (80 mM K-Pipes, 1 mM EGTA, 1 mM $MgCl_2$, 20 μM taxol, pH 6.8). Fragments of microtubules obtained by sonication were incubated at room temperature with AChR-enriched post-Golgi vesicles immuno-isolated on magnetic beads (see above), in PEMT in a final volume of 0.5 mL. Cytosol from *Torpedo* electric organ was added at a final concentration of 3 mg/mL and parallel experiments without cytosol were performed as control. Magnetic beads were collected, washed once in PEMT, and treated for electron microscopy analysis as described below. Quantitative microtubule-binding assay was performed as follows: microtubules (see above) were immuno-adsorbed on magnetic beads using mAb GT335. Incubations with crude PGVs in the presence of cytosol (3 mg/mL in PEMT) or with 0.3% BSA in the control were performed at room temperature. After two gentle washes, immunoblot analysis of the bound vesicles was carried out as described.

2.6. Electron microscopy and immunogold labeling

Vesicles immuno-isolated on magnetic beads were labeled in batch using secondary antibodies conjugated to 10 nm gold particles [15] (Slot and Geuze, 1985) and then fixed in 2.5% gluteraldehyde, 0.1% tannic acid in 0.1 M cacodylate buffer, pH 7.4, and post-fixed with 1% osmium tetroxide. Embedding in epoxy resin was performed after dehydration in a series of ethanol solutions. Thin sections were stained with 5% uranyl acetate and observed with a Philips CM12 electron microscope operating at 80 kV.

3. Results and discussion

3.1. Biochemical and immunocytochemical characterization of post-Golgi vesicles (PGVs) immunopurified with anti-AChR, anti-rapsyn and anti-ATPase antibodies

To unravel the exocytic pathway of synaptic proteins, we first isolated crude post-Golgi vesicles (PGVs) using sucrose equilibrium gradient centrifugation. As in other tissues, these vesicles have a buoyant density of 1.09–1.10 g/mL and a mean diameter of 80–100 nm [1]. Distinct populations of PGVs were purified, by using immunomagnetic beads, on the basis of their content in AChR, rapsyn and Na^+,K^+ ATPase, the major components of the innervated and non-innervated membrane domains of the electrocyte, respectively. The protein content of each population was determined using Western blotting experiments. *Figure 1* shows that PGVs isolated either with anti-AChR or anti-rapsyn antibodies were almost devoid of Na^+,K^+ ATPase. Conversely, PGVs isolated with anti-Na^+,K^+ ATPase were devoid of AChR or rapsyn. These data suggest that these classes of PGVs result from a sorting in the Golgi apparatus. Surprisingly, AChR-purified PGVs contained low and variable amounts of rapsyn, while rapsyn-purified PGVs always contained equal amounts of AChR and rapsyn (*figure 1*). This indicates that several subpopulations of AChR-containing PGVs contain variable amounts of rapsyn. These populations of PGVs most likely result from a gradient of maturation of AChR-containing PGVs to which rapsyn molecules bind, after budding from the *trans* Golgi network. This could be accounted for by the presence of coat proteins necessary for the budding of PGVs from the *trans* Golgi compartment, assuming that the association of rapsyn occurs only after uncoating. In agreement with this hypothesis, Golgi fractions purified from *Torpedo* electrocytes usually have a low rapsyn content (not shown).

Electron microscope analysis was used to show that the immunopurified membranes corresponded to uncoated vesicles and not to Golgi stacks (*figure*

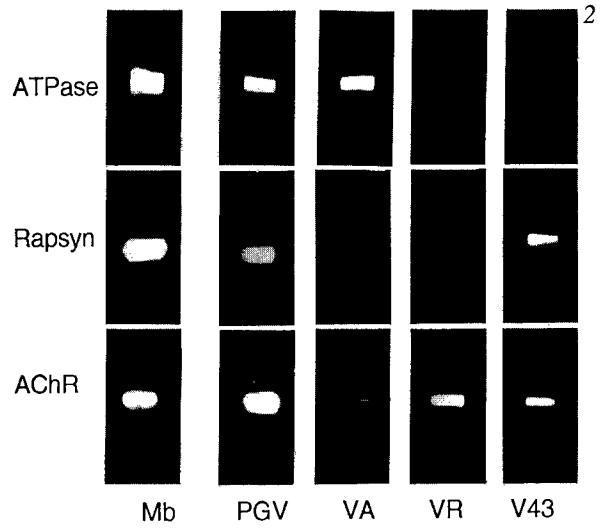


Figure 1. Biochemical characterization of PGV fractions. The content of immunopurified PGV fractions (V_A, V_R and V_{43}) was analyzed by immunoblotting after SDS-PAGE using polyclonal anti-Na^+, K^+ ATPase and anti-AChR antibodies and mAb 1234 anti-rapsyn, and compared to crude *Torpedo* plasma membranes (Mb) and crude PGVs. To allow quantitative comparison between different fractions, the immunodetection of the proteins in a given fraction was carried out on the same nitrocellulose strip. All blots were exposed for ECL detection during the same period of time. V_A contained very low amounts of AChR and rapsyn; V_R contained traces of Na^+, K^+ ATPase and variable but always low amounts of rapsyn, whereas V_{43} contained low amounts of Na^+, K^+ ATPase but equal amounts of AChR and rapsyn.

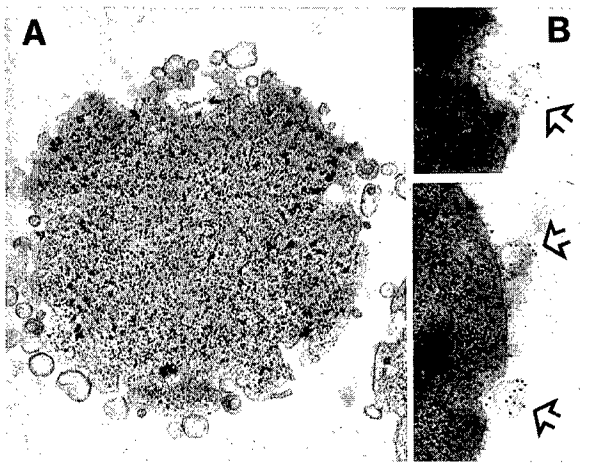


Figure 2. EM analysis of AChR and rapsyn-enriched PGV fractions. Uncoated vesicles were visualized around magnetic beads coated with anti-AChR mAbs (**A**, magnification = 34 000). Vesicles immunopurified with anti-rapsyn mAb displayed a similar aspect (not shown). Immunogold labeling with anti AChR antibodies, revealed that most of these vesicles contained AChR protein (arrows in **B**; magnification = 50 000).

A). Immunogold labeling confirmed that most of the rapsyn-purified vesicles contained AChR (*figure 2B*).

3.2. Role of microtubules in the intracellular routing of postsynaptic proteins

In polarized cells, microtubules are involved in the direct delivery of newly synthesized surface proteins, by facilitating their intracellular routing to the apical (and also basolateral) domains and by limiting missorting (see [6] and references therein). Previous data from our laboratory and others have reported the presence of a specialized meshwork of stable microtubules in the subneural sarcoplasm in skeletal muscle fibers [7, 14]. Similarly, a polarized microtubule network is localized in the subneural region of the electrocyte [8]. To address the question of the role of microtubules in the intracellular trafficking of synaptic proteins, we have carried out in vitro binding experiments with microtubules polymerized from pig brain tubulin and PGVs. In a first approach, AChR-containing PGVs immunopurified on magnetic beads were tested for their competence to bind microtubules. As monitored by thin-section electron microscopy, this assay reveals the presence of microtubules at the surface of AChR-containing PGVs (*figure 3A*). In a second series of experiments, microtubules attached to magnetic beads were used in an in vitro binding assay including crude PGVs and electrocyte cytosol. The protein content of the bound vesicles in this assay was analyzed using Western blotting (*figure 3B*). By comparison with the crude PGV fraction (or the binding in the absence of cytosol), the rapsyn/Na^+,K^+ ATPase ratio in the fraction bound to microtubules was increased in the presence of cytosol. These data show that the subpopulation of PGVs enriched in rapsyn binds to microtubules in a cytosol-dependent manner. Given the vectorial organization of the microtubular network in electrocytes, this selective binding is likely to favor the proper delivery of synaptic proteins.

4. Concluding remarks

In this study, we have investigated the intracellular routing of two significant proteins of the postsynaptic membrane, the nicotinic acetylcholine receptor and rapsyn. The AChR being an intrinsic membrane component, it was reasonable to assume that it follows the secretory pathway. In this line, we isolated subpopulations of post-Golgi vesicles that were enriched either in AChR or in Na^+,K^+ ATPase, an intrinsic protein from the non-innervated membrane. These vesicles are likely to result from a sorting in the Golgi apparatus. The targeting of

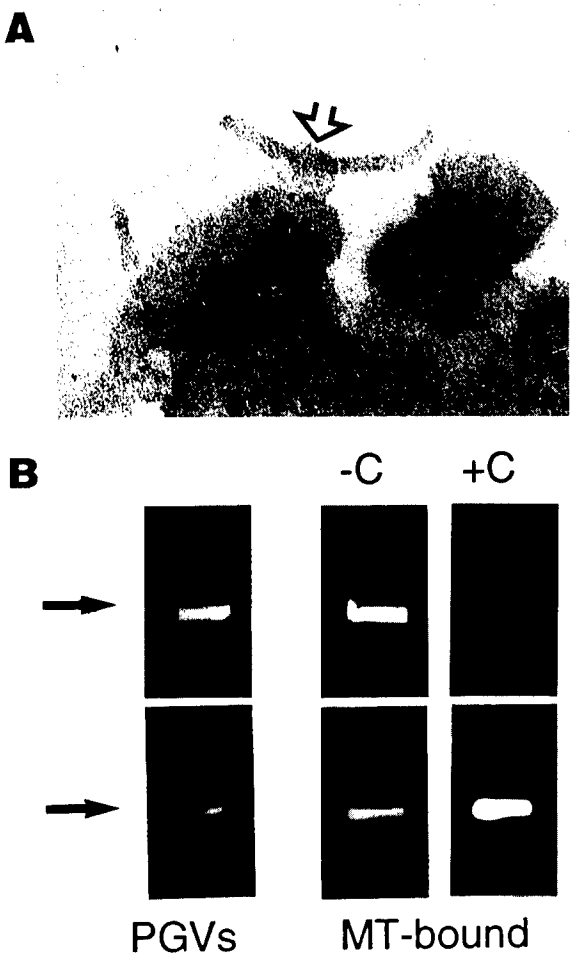


Figure 3. AChR and rapsyn-containing PVGs bind to microtubules. **A.** Vesicles immunopurified with anti-AChR coated beads are able to bind microtubules (arrow, magnification = 65 000). **B.** In vitro binding assay was carried out to identify PGV subpopulations able to bind to microtubules in the presence of cytosol. Taxol-polymerized microtubules were attached to magnetic beads. The protein content of the bound vesicles was analyzed using Western blotting. Immunoblotting analysis of vesicles recovered with microtubules revealed that, in the presence of cytosol (+C), rapsyn-containing vesicles are predominant. In the absence of cytosol (–C), non-specific binding of all classes of PGVs probably accounted for the high Na^+, K^+ ATPase/rapsyn ratio, a pattern similar to that observed for crude PGVs.

rapsyn, an extrinsic component of the postsynaptic membrane was also studied. As a myristoylated protein, it is synthesized on free polysomes and could either be targeted to the postsynaptic membrane, independently of the AChR, or to intracellular compartments en route to the plasma membrane. We showed that rapsyn associated to AChR-containing PGVs, suggesting that AChR and rapsyn are targeted to intracellular organelles in the secretory pathway before delivery to the postsynaptic membrane. Since we isolated PGVs containing AChR and a low amount of rapsyn as well as PGVs containing both proteins, it is possible that rapsyn associates with AChR-containing vesicles only after their budding from the TGN and uncoating. As in other polarized cells, a vectorial organization of the microtubular network, which is thought to facilitate vesicle trafficking between the Golgi apparatus and the cell surface, was observed in *Torpedo* electrocytes [1, 8]. The present in vitro assays further show that rapsyn-containing PVGs do bind more efficiently to microtubules, compared to Na^+,K^+ ATPase-enriched PVGs. Considered together, these data bring strong evidence that the secretory pathway contributes to the localization of synaptic proteins.

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Acetylcholinesterase: C-terminal domains, molecular forms and functional localization

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Abstract — Acetylcholinesterase (AChE) possesses short C-terminal peptides that are not necessary for catalytic activity. These peptides belong to different classes (R, H, T, S) and define the post-translational processing and targeting of the enzyme. In vertebrates, subunits of type H ($AChE_H$) and of type T ($AChE_T$) are the most important: $AChE_H$ subunits produce glycolipid (GPI)-anchored dimers and $AChE_T$ subunits produce hetero-oligomeric forms such as membrane-bound tetramers in the mammalian brain (containing a 20 kDa hydrophobic protein) and asymmetric collagen-tailed forms in neuromuscular junctions (containing a specific collagen, ColQ). The T peptide allows the formation of tetrameric assemblies with a proline-rich attachment domain (PRAD) of collagen ColQ. These complex molecular structures condition the functional localization of the enzyme in the supramolecular architecture of cholinergic synapses. (©Elsevier, Paris)

Résumé — Acétylcholinestérase : domaines C-terminaux, formes moléculaires et localisation fonctionnelle. L'acétylcholinestérase (AChE) possède de courts peptides C-terminaux qui ne sont pas indispensables pour l'activité enzymatique. Ces peptides, classés en différents types (R, H, T, S), déterminent la maturation et le devenir de l'enzyme. Chez les vertébrés, les sous-unités de type H ($AChE_H$) et de type T ($AChE_T$) sont les plus importantes, et produisent respectivement des dimères ancrés par un glycolipide (GPI) et des formes hétéro-oligomériques telles que les tétramères membranaires du cerveau des mammifères (comprenant une protéine hydrophobe de 20 kDa) et les formes asymétriques des jonctions neuromusculaires (comprenant un collagène spécifique, ColQ). Le peptide T permet la formation d'un assemblage tétramérique avec un domaine riche en prolines ('proline-rich attachment domain', PRAD) du collagène ColQ. Ces structures moléculaires complexes conditionnent la localisation fonctionnelle de l'enzyme dans l'architecture supramoléculaire des synapses cholinergiques. (©Elsevier, Paris)

acetylcholinesterase / anchoring / basal lamina / collagen

1. Introduction

An efficient hydrolysis of acetylcholine by cholinesterases depends on the precise localization of these enzymes, particularly in synaptic structures. The catalytic domain of cholinesterases is organized as an α/β hydrolase fold [1, 2], possessing the conformation of a globular protein without any transmembrane or other anchoring feature. However, cholinesterase subunits present a variety of quaternary associations and can be attached to cell membranes or with extracellular structures in various ways, because of the presence of small C-terminal domains [3], as shown in *figure 1*.

2. Multiplicity of C-terminal domains: species and tissue distribution

The cholinesterase genes that have been analyzed so far contain three types of exons encoding distinct C-terminal domains, called H ('hydrophobic'), T ('tailed') and S ('soluble', or 'snake'). In addition, R ('readthrough') transcripts, which have been found in *Torpedo* and mammals, are not spliced after the last exon encoding the catalytic domain.

Invertebrates may possess one cholinesterase gene (*Drosophila*) [11], two genes like *Culex* [36] and amphioxus [37] or four cholinesterase genes like *Caenorhabditis* [38], but each gene seems to produce a single C-terminal domain. While insects and the prochordate amphioxus only possess cholinesterases of type H, the various genes of the nematode *Caenorhabditis* produce either subunits of type T, for the major *ace-1* gene [16], or of type H, for the less expressed *ace-2* gene, and probably also the minor *ace-3* and *ace-4* genes [38].

Vertebrates possess two cholinesterase genes, producing the enzymes acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) that differ in their substrate specificity: BChE hydrolyzes butyrylcholine, in addition to acetylcholine, while AChE is essentially inactive on the larger ester. This difference is now perfectly explained by the size of an acyl pocket in the active site of these enzymes, that accommodates a butyryl moiety in the case of BChE, but not in AChE because it is limited by two bulky phenylalanine side chains [39]. Both AChE and BChE genes produce subunits of type T [3]. In addition, the AChE genes of some species may

Type of subunit	Peptide sequence of C-terminal region		Molecular forms	Tissue distribution
R	*Torpedo* AChE	GNVFAFHMQKVRTPAKTYHFGVIVAHLLLLSLPTASDVPRLA SSKWWAHSDPL@S-RR@WESWGRIL [4]	- Soluble monomer (G_1^{na}) [8]	- R transcripts in *Torpedo* electric organ [4], in mouse embryonic muscle [9]; expression level increased by stress in mouse brain [10]
	Rat AChE	GRRGVGKQGMHKAARVGRTGERKGGKHRM [5]		
	Mouse AChE	GRRMEWGEQGMHKAARVGRRGERWGAKHRV [6]		
	Human AChE	GMQGPAGSGWEEGSGSPPGVTPLFSP [7]		
H	*Drosophila*	GT@DGDSG<u>SASISPRLQLLGIAALIYICAALRTKRVE</u> [11]	- Amphiphilic, intracellular precursors (G_2^a) - GPI-anchored dimers, exposed at the cell surface (G_2^a, type I) - Soluble, secreted C-terminal cleaved dimers (G_2^{na})	- nervous tissue in insects [14] - muscles, nervous tissue and electric organs in *Torpedo* [4,15] - hematopoietic cells in mammals [8]
	Torpedo AChE	A@DGELS@<u>SSTSSSKGIIFYVLFSILYLIFY</u> [4,12]		
	Rat AChE	ATEVP@T@PSPAH@<u>EAAPRPGPALSLSLLFFLFLLHSGLRWL</u> [5,13]		
T	*C. elegans* ACE-1	ADVGDPYLVWKQQMDKWQNEYITDWQYHFEQYKRYQTYRQSDSET@GG [16]	- Amphiphilic monomers, dimers and tetramers (G_1^a, G_2^a, G_4^a, type II), cellular and secreted [25,26] - Truncated nonamphiphilic forms (G_1^{na}) [27] - Nonamphiphilic tetramers (G_4^{na}) [26] - Heteromeric associations with anchoring subunits: □ collagen ColQ (collagen-tailed, asymmetric forms, A_4, A_8, A_{12}) [28,29] □ 20 kDa hydrophobic anchor (G_4^a) [13,30-33]	- muscles and nervous tissues in all vertebrates [3]
	Torpedo AChE [17,18]	ETIDEAERQWKTEFHRW-SSYMMHWKNQFDQY--------SRHEN@AEL		
	Electrophorus AChE	ENIDDAERQWKAEFHRW-SSYMMHWKNQFDHY--------SKQER@TNL [19]		
	Bungarus AChE	DNIEEAERQWKLEFHLW-SAYMMHWKSQFDHY--------NKQDR@SEL [20]		
	Quail AChE	GPTEDAER-WRLEFHRW-SSYMGRWRTQFEHY--------SRQQP@ATL [21]		
	Rat AChE	DTLDEAERQWRAEFHRW-SSYMVHWKNQFDHY--------SKQDR@SDL [22]		
	Human BChE [23,24]	GNIDEAEWFWKAGFHRW-NNYMMDWKNQFNDYT-------SKKES@VGL		
S	*Bungarus* AChE	VDPPRADRRRSARA [20,34]	- Soluble monomer (G_1^{na}) [34]	- venom glands and other tissues in Elapid snakes (*Bungarus*) [35]

Figure 1. Different types of C-terminal peptides in cholinesterases. The underlined regions are removed from the mature proteins; the last residue of the mature protein (ω) is shown in bold type, and the C-terminal region is shown in italics. A C-terminal part of the T peptide may be removed upon secretion, yielding amphiphilic or non-amphiphilic forms, but the sites of cleavage have not been determined. Conserved aromatic residues in T peptides are shown in bold letters; in H and T peptides, cysteines that may establish intercatenary disulfide bonds are shaded. For additional sequences, see the ESTHER server [7].

produce other types of subunits, by alternative splicing of the 3′ coding exon. *Torpedo* and mammals possess AChEs of types H, T and possibly R, although R subunits have not been characterized in vivo. Elapid snakes, that secrete AChE in their venom, possess AChEs of types T and S [20]. Only AChE of type T seems to exist in bony fishes (*Danio*, *Electrophorus*) [19, 40] and birds (chicken, quail) (Anselmet, unpublished result). In *Torpedo* and mammals, exons H are located between the last catalytic exon and exon T [4, 6]. Examination of the corresponding sequences in the teleost fish *Electrophorus* [19], in the snake *Bungarus* [20] and in the chicken did not reveal the presence of any potential region that might constitute an exon H encoding a C-terminal peptide with the required features.

In fact, the sequences of H exons in *Torpedo* and mammals do not appear homologous, and only resemble each other by the presence of a cysteine and a GPI addition signal, which is extremely flexible in its amino acid sequence (*figure 1*). Since mammals are descended from reptiles and teleost fishes, it seems that during evolution of the vertebrate lineages, the exon H as it exists in *Torpedo* was lost, and that a new exon H appeared de novo in mammals.

The C-terminal domains determine the post-translational processing and the fate of the enzyme. Accordingly, when different types of subunits exist in an organism, they are expressed in distinct territories, in a tissue- and cell-specific manner. In *Torpedo*, $AChE_H$ and $AChE_T$ subunits coexist in the electric organ: $AChE_H$ is produced in the electric motoneurons, and also post-synaptically together with $AChE_T$. In contrast, the striated muscle of *Torpedo* only seems to contain $AChE_H$ subunits (Bon, unpublished result). In the elapid snake *Bungarus*, $AChE_S$ is expressed in the venom gland, but also in the liver and in muscles, together with $AChE_T$ [20]. In adult mammals, $AChE_H$ is mostly expressed in blood cells (lymphocytes and erythrocytes), while $AChE_T$ is mainly expressed in muscles and in the central and peripheral nervous tissues [5, 22, 41]. In mammalian muscle, however, an exclusive splicing of AChE transcripts towards T exons is only established progressively: in the mouse embryonic diaphragm, R and H transcripts coexist with T transcripts and disappear completely at birth [9].

Thus, the tissue expression of the different types of AChE subunits differs considerably, according to the species. *Drosophila* uses $AChE_H$ in its nervous system, *Torpedo* uses $AChE_H$ in its muscles, while the corresponding tissues in adult mammals only contain $AChE_T$.

3. Post-translational processing

In this section, we will discuss the manner in which the different C-terminal domains of cholinesterases affect their post-translational fate. It is im-

portant to note that these C-terminal domains are not required for catalytic activity. Truncated subunits ($AChE_D$), which are essentially reduced to their catalytic domain, may be constructed by introducing a stop codon either at the end of the catalytic domain, or after the first few residues of one of the C-terminal peptides. Such subunits produce active AChE when expressed in transfected cells, in the form of soluble monomers that are readily secreted into the medium [42]. If a cysteine is present after the catalytic domain, such subunits are able to produce disulfide-linked dimers. This is the case if a stop codon is introduced after the cysteine(s) of an H peptide, or in abnormally spliced subunits obtained in the case of *Electrophorus* AChE [19]: when the T exon is deleted or when its acceptor site is mutated, the last catalytic exon is spliced to upstream sites, located 6 bp apart from each other, which introduce very short coding sequences containing two adjoining cysteines, VLECC and ECC.

We will examine the various types of natural subunits, in the order of increasing complexity, which is also the order of increasing physiological importance in higher vertebrates.

3.1. R subunits

R transcripts have been found in *Torpedo*, mouse and rat [4, 5, 8], whose AChE genes contain two alternative C-terminal exons. It is therefore possible that they represent a default product and do not produce a physiologically significant version of AChE. The actual protein has not been identified in tissues, but this is not conclusive. It is intriguing that the R transcripts are specifically up-regulated in the mouse brain after exposure to anticholinesterases or to stress [10]. When synthesized in transfected cells, the mouse R subunits form soluble monomers [8], in agreement with the fact that their C-terminal domain (R peptide) contains neither a cysteine that might form intercatenary disulfide bonds, nor an hydrophobic sequence that might constitute a membrane anchor.

3.2. S subunits

The S exon has recently been discovered in the *Bungarus* AChE gene, and is alternatively spliced with a T exon [20]. The S exon is expressed in the venom glands, and produces a soluble monomeric form of AChE. It encodes a short C-terminal peptide of 15 residues, which is very hydrophilic and does not contain any cysteine. Curiously, this peptide is cleaved upon secretion so that the mature venom protein retains only its first seven amino acids. The secreted enzyme produced by transfected COS cells is also cleaved, as indicated by the loss of a flag epitope that was introduced at the C-terminus by genetic construction in expression vectors. This cleavage does not seem necessary for an efficient expression and secretion of the enzyme, since we observed a similar yield in the case of a truncated form, in which a stop codon was introduced at the end of the catalytic domain.

3.3. H subunits

Subunits of type H are characterized by the fact that their C-terminal peptide contains one or two cysteines near the catalytic domain, and a signal for addition of a glycophosphatidylinositol anchor (GPI). The $AChE_H$ subunits thus generate disulfide-linked, GPI-anchored dimers. Since these dimers are globular and amphiphilic, we note them G_2^a; they correspond to amphiphilic forms of type I, as opposed to those produced from subunits of type T.

The GPI addition signal consists of a cleavage/addition site, located about 10–12 residues upstream of a C-terminal hydrophobic region. At the cleavage/addition site, a peptide bond is replaced by an amide bond to the ethanolamine moiety of a preassembled GPI anchor. This anchor thus becomes attached to the last remaining residue of the mature protein, which is called ω. Analyses of GPI addition signals and site-directed mutagenesis studies have shown that ω must possess a small side chain, and that a similar restriction may also apply to the adjoining residues, ω + 1 and ω + 2 [43, 44].

We have analyzed the environment of ω sites in *Torpedo* and rat H peptides by mutagenesis and this led us to reconsider some of the previously proposed consensus rules (Bon et al., in preparation). For example, we found that the ω-1 residue has a strong influence on processing. It is particularly interesting that the production of GPI-anchored AChE, which becomes exposed at the cell surface, is accompanied by release of a soluble form in the culture medium. The efficiency of processing, i.e., the ratio of GPI-anchored molecules to uncleaved precursors, which remain trapped in vesicles within the cells, appeared generally correlated with the release of soluble enzyme, and with the total yield of AChE activity. This yield varied more than 10-fold from unprocessed or poorly processed mutants to the better processed ones.

In these analyses, we used an operational distinction between amphiphilic (G^a) and non-amphiphilic (G^{na}) molecules: amphiphilic molecules are defined by their capacity to bind micelles of non-denaturing detergents, such as Triton X-100 and Brij-96, resulting in clear changes in their hydrodynamic parameters. The sedimentation of amphiphilic molecules is retarded in the presence of Triton X-100, and even more so in the presence of Brij-96; the Stokes radius

is increased and the rate of migration of amphiphilic molecules in non-denaturing polyacrylamide gels is decreased in the presence of Triton X-100. According to these criteria, the GPI-anchored forms and the precursors which had retained their C-terminal hydrophobic region are amphiphilic, while the released enzyme is nonamphiphilic. This released form may constitute a large fraction of the enzyme produced from $AChE_H$, and thus it may be physiologically significant, e.g., by contributing to the soluble AChE activity that exists in the sera of some mammalian species.

GPI-anchored AChE is clearly essential in invertebrates where it is the only or predominant cholinesterase in the nervous system, and also appears to serve an essential role in *Torpedo*, since this is the only form of enzyme in the striated muscles. In mammals, the expression of $AChE_H$ in hematopoietic cells may be related to their differentiation [45, 46]. However, the physiological significance of GPI-anchored AChE on the surface of blood cells is not clear, especially since its activity varies widely with the species (and does not exist in reptiles and birds). In fact, the presence of AChE on red blood cells may be redundant with that of soluble AChE and BChE in the plasma. It would be interesting to specifically inactivate the exon H in mouse, in order to observe whether the absence of GPI-anchored molecules would induce any functional defect.

3.4. T subunits

As already indicated, subunits of type T seem to exist in all vertebrates AChEs. Vertebrate BChEs and the product of the major *ace-1* gene in *C. elegans* also belong to this category. The characteristic C-terminal regions of these subunits, the T peptides, are 40 residues long in vertebrate AChEs, and remarkably well conserved throughout evolution, with a series of aromatic residues and a cysteine located near the C-terminus (at position –4 in vertebrate AChEs and BChEs) (*figure 1*).

The first part of the T peptide probably organizes as an amphiphilic α-helix, with all aromatic side chains forming an hydrophobic patch [3], as shown by spectroscopic analyses of a synthetic peptide, in the presence of detergent and lipid micelles (Bon et al., in preparation). In a proposed theoretical model, the α-helix bends upon itself, creating an hydrophobic zone [47]. This type of conformation probably explains the multiple interactions of cholinesterase subunits of type T. These subunits generate various oligomeric forms, ranging from monomers to the complex heteromeric collagen-tailed and hydrophobic-tailed molecules, in which they are associated with structural proteins.

Monomers and dimers occur in tissues and in transfected cells that express $AChE_T$ subunits, in variable proportions depending on the species. For example, dimers are predominant in the case of *Torpedo* $AChE_T$ [42],whereas monomers are predominant in the case of rat $AChE_T$ [22, 26]. These molecules are amphiphilic, but differ from GPI-anchored forms because they are more easily solubilized in an aqueous buffer without detergent, and do not form aggregates. They were thus called amphiphilic forms of type II, as opposed to GPI-anchored amphiphilic forms of type I [48–50]. Transfected COS cells expressing $AChE_T$ secrete amphiphilic monomers (G_1^a) and dimers (G_2^a), which resemble their cellular counterparts but differ in their electrophoretic migration in non-denaturing polyacrylamide gels, and probably undergo a proteolytic cleavage at their C-terminus, as shown by the loss of a flag peptide epitope, which was added by mutagenesis at the end of some constructs [26].

In cultures of murine neural cells, it was shown that monomers and dimers are rapidly renewed, presenting several metabolic components with short half-lives (less than 8 h), while the half life of tetramers exceeds 20 h [51].

In addition to monomers and dimers, transfected COS cells, expressing $AChE_T$ subunits, also produce tetramers and heavier oligomers [26]. The tetramers exist as both amphiphilic and nonamphiphilic molecules, which possibly differ by the organization of their T peptides: in amphiphilic molecules, at least part of the hydrophobic region of the T peptide would remain exposed, while they would be occluded within an internal hydrophobic core in the nonamphiphilic molecules. These interpretations assume that such tetramers only consist of $AChE_T$ subunits; there is, however, another possibility, as discussed below.

The transfected cells also produce a '13 S' component, which might correspond to an hexamer of $AChE_T$ subunits. This type of molecule has been observed in COS cells, in *Xenopus* oocytes (Simon et al., in preparation), and also in primary cultures of murine nerve cells (Lazar, unpublished result). It is not amphiphilic and may be secreted, e.g., from transfected oocytes. It is quite unstable, being readily dissociated into tetramers, dimers and monomers, even around room temperature. The proportion of resulting amphiphilic tetramers is increased when dissociation occurs in the presence of detergent, suggesting that interaction with micelles favors a conformation in which the amphiphilic α-helix is exposed.

The presence of the T peptide also endows $AChE_T$ subunits to form heteromeric associations. In the collagen-tailed or asymmetric (A) forms, each strand

of a triple helical collagen may be attached to a tetramer of $AChE_T$ subunits: two of these subunits are disulfide-linked to each other, while the other two are disulfide-linked to the tail subunit. In the A_{12} form, each of the three strands of the collagen is attached to a catalytic tetramer. When one or two of these strands remain unoccupied, the corresponding molecular forms are called A_8 and A_4, indicating their respective number of catalytic subunits. The hydrophobic-tailed, membrane-bound tetramers, which represent the major AChE species in mammalian brain, contain an hydrophobic protein of 20 kDa [30, 31] and present the same type of disulfide-bond organization [13].

4. Heteromeric assembly between T subunits and the collagen tail, ColQ

The AChE-associated collagen of asymmetric forms has been cloned in *Torpedo* [28] and in mammals [29]. The collagen subunit, ColQ, consists of a signal peptide, an N-terminal region, Q_N, a collagen central domain that is bracketed by cysteines, and a C-terminal region, Q_C, containing proline-rich and cysteine-rich conserved motifs.

In mammals, we found a single ColQ gene, and showed that ColQ subunits form homomeric triple helices that constitute the collagen tail in asymmetric forms of both AChE and BChE. The ColQ gene generates a number of splicing variants, some of which do not encode the Q_N or the Q_C regions. Their significance has not yet been elucidated. The Q_N region is responsible for the binding of $AChE_T$ tetramers. We could, for example, generate soluble tetramers by co-expressing $AChE_T$ with a Q_N construct, without the collagen and Q_C regions, or GPI-anchored tetramers with a Q_N/H_C construct, in which Q_N was combined with the C-terminal signal for addition of a GPI anchor, as encoded by an H exon. The Q_N domains of *Torpedo* and rat are able to associate with T subunits from various origins, indicating a strong conservation of their complementarity through evolution.

Comparison of the Q_N sequences showed a conserved motif of 17 residues, that includes two adjoining cysteines and strings of five and three consecutive prolines. Deletion studies showed that this domain is sufficient for interaction with cholinesterase subunits of type T, and we called it a proline-rich attachment domain, PRAD [52]. Mutation studies further showed that the cysteines were dispensable, so that disulfide bonding between the catalytic subunits and the tail is not necessary, and that the only critical element of the PRAD is the presence of a sufficient number of successive prolines. We could in fact replace the PRAD by synthetic polyproline, inducing the recruitment of monomers and dimers of $AChE_T$ into tetramers, both in cultures of living cells, and in cell extracts.

Having established that the complementation between catalytic subunits and tail subunits relies on the small PRAD motif of the tail, we wondered whether, similarly, the T peptide might be sufficient for this interaction (Simon et al., in preparation). This appeared possible, because: i) the T peptide is required for this interaction and contains the cysteine that may form disulfide bonds between the two partners; ii) it is very well conserved throughout vertebrates, markedly more than the catalytic domains, since the catalytic domains of *Torpedo* and rat AChE present 59% identity, whereas their T peptides present 75% identity; and iii) the small size of the PRAD suggests that it interacts with an appropriately small domain.

We demonstrated that, indeed, an isolated T peptide, which was labeled with a flag epitope, could associate with the Q_N/H_C protein and was thus GPI-anchored at the cell membrane of transfected COS cells. We further showed that addition of a T peptide at the C-terminus of foreign proteins such as alkaline phosphatase and the green fluorescent protein, GFP, endowed them with the capacity to associate with the PRAD, when the two partners were synthesized in the secretory pathway.

Sedimentation analyses showed that mixed oligomers were formed in *Xenopus* oocytes, when the PRAD was expressed together with $AChE_T$ subunits and isolated T peptides or the GFP-T protein. The mixed oligomers contained three, two, or one $AChE_T$ subunits, showing that a PRAD combines with four T peptides. We observed, however, a bias in favor of the 2/2 combination, suggesting that the PRAD associates sequentially with dimers of T peptides, probably forming unstable intermediates before the stabilization of the tetrameric assembly. The replacement of the C-terminal cysteine by an alanine did not affect these interactions, showing that disulfide bonds between T peptides dimers are not required, any more than disulfide bonds between the PRAD and the T peptides. The formation of mixed 2/2 oligomers certainly explains the existence of AChE/BChE asymmetric forms, which were characterized in chick embryos [53].

The interaction between the PRAD and T peptides superficially resembles the binding of proline-rich ligands to SH3 and WW domains [54]. It is, however, quite original in several respects. Firstly, it does not occur in the cytoplasm, but in the secretory pathway, between proteins that are destined to be externalized. Secondly, it presents a one to four stoichiometry. In the case of the trimeric ColQ collagen, this leads to the A_{12} collagen-tailed molecule, combining three

structural subunits and 12 catalytic subunits. This type of association clearly offers the potential to generate complex supramolecular structures.

5. Generality of the PRAD/T peptide interaction

As mentioned above, the $AChE_T$ subunits associate with a 20 kDa hydrophobic membrane anchor [13, 30–33], called P [55], with the same type of disulfide bond organization as with the PRAD [13]. This suggested that the hydrophobic P subunit might be encoded by a splice variant generated from the ColQ gene, and contained the PRAD. However, this possibility was not confirmed by an examination of the splice variants produced in mammalian brain, and the fact that membrane-bound AChE tetramers do not contain a peptide epitope which is encoded by the same exon as the PRAD [29]. It is therefore likely that the P subunit is produced by a distinct gene, and it will be interesting to see whether it also contains a proline-rich domain. In any case, this indicates the existence of several peptide motifs that may associate with T subunits and induce the formation of $AChE_T$ tetramers.

An intriguing hypothesis is that $AChE_T$ subunits might never be able to form tetramers by themselves, in the absence of such an 'organizer' molecule. This would explain the fact that tetramers are clearly not in equilibrium with dimers and monomers and that their proportion varies widely with differentiation, in vivo, or according to the cell type in transfected cell cultures. For example, the ratio of tetramers to monomers and dimers increases considerably during maturation of the brain, in birds [21] and mammals [41].

Transfected COS cells produce a variable, but significant level of G_4^a and G_4^{na} forms, while *Xenopus* oocytes produce essentially no tetramers. These differences would be explained perfectly if the formation of tetramers depends on the expression of organizer proteins. The presence of such a protein has not been detected in cholinesterase tetramers, apart from the membrane-bound tetramers of mammalian brain. This may be due to its small size, to the fact that it is not necessarily disulfide-linked to catalytic subunits and to its stoichiometry of only one to four catalytic subunits.

In view of the possible existence of multiple, perhaps ubiquitous, PRAD-like motifs, we may also imagine the existence of T peptides in association with proteins that are unrelated to cholinesterases. A possible example is the 100 kDa protein that was found to be a component of the AChE collagen-tailed forms in *Torpedo* electric organs. Thus, the PRAD/T peptide association may be the prototype of a family of similar protein-protein associations, organizing supramolecular structures in the extracellular space.

6. Physiological importance of AChE anchoring

In any case, associations of AChE catalytic subunits with structural proteins, the collagen tail ColQ and the hydrophobic membrane anchor P, play a major role in the positioning and function of the enzyme in cholinergic transmission. This is perfectly illustrated by a congenital myasthenic syndrome (CMS of type 1c), which is characterized by a defect in the accumulation of AChE at neuromuscular junctions. This syndrome is usually correlated with a complete or partial lack of collagen-tailed forms [56]. An analysis of the AChE gene of a CMS-1c patient showed that it presented no anomaly and produced $AChE_T$ subunits that normally associated with a PRAD in transfected cells [57]. We therefore proposed that the genetic defect might reside in the ColQ gene. This was confirmed in a recent genetic study of a large kindred in which several siblings are affected by a mild form of CMS-1c (Donger et al., in preparation). It is particularly interesting that muscle biopsies from these patients contain a normal complement of collagen-tailed AChE, although this enzyme is not properly focalized at the neuromuscular junctions. (Note that human and primate muscles contain an important proportion of collagen-tailed AChE in extrajunctional regions of muscles, in contrast with the rat.) In these patients, we identified a point mutation which changes a conserved tyrosine residue in the C-terminal Q_C region of ColQ, and we suggest that this mutation compromises the interaction of Q_C with specific elements of the junctional basal lamina. This would be consistent with the 'parking lot' hypothesis, according to which collagen-tailed molecules attach to a limiting number of 'receptor' sites in the architecture of the neuromuscular junction [58], possibly in connection with the receptor sites.

The collagen-tailed forms, however, do not constitute the only type of physiologically active AChE species in muscles: the studies of Gisiger et al. [59] have shown that exercise induces specific changes in the membrane-bound G_4 form of rat muscles, but not in the other molecular forms of AChE, strongly suggesting that the level of G_4 has an adaptive role, related to muscle activity.

These examples show the importance of the stoichiometric and spatial organization of AChE catalytic sites and acetylcholine receptor sites for cholinergic function. Although AChE is produced from a single gene, its various molecular forms, generated by alternative splicing and by combinations with structural proteins, allow multiple modes of anchoring. This multiplicity is probably the functional counterpart of the

various types of nicotinic and muscarinic receptors, which differ widely in their affinity for acetylcholine and time course of response.

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Three-dimensional structure of a complex of E2020 with acetylcholinesterase from *Torpedo californica*

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Abstract — The 3D structure of a complex of the anti-Alzheimer drug, E2020, also known as Aricept®, with *Torpedo californica* acetylcholinesterase is reported. The X-ray structure, at 2.5 Å resolution, shows that the elongated E2020 molecule spans the entire length of the active-site gorge of the enzyme. It thus interacts with both the 'anionic' subsite, at the bottom of the gorge, and with the peripheral anionic site, near its entrance, via aromatic stacking interactions with conserved aromatic residues. It does not interact directly with either the catalytic triad or with the 'oxyanion hole'. Although E2020 is a chiral molecule, and both the S and R enantiomers have similar affinity for the enzyme, only the R enantiomer is bound within the active-site gorge when the racemate is soaked into the crystal. The selectivity of E2020 for acetylcholinesterase, relative to butyrylcholinesterase, can be ascribed primarily to its interactions with Trp279 and Phe330, which are absent in the latter. (©Elsevier, Paris)

Résumé — Structure d'un complexe de E2020 avec l'acétylcholinestérase de *Torpedo californica.* La structure 3D d'un complexe formé par une drogue anti-Alzheimer E2020 aussi connue sous le nom de Aricept® avec l'acétylcholinestérase de *Torpedo californica* est décrite. La structure aux rayons X, à la résolution de 2.5 Å, montre que la molécule allongée E2020 s'étire sur l'entière longueur de la gorge catalytique de l'enzyme. Elle interagit donc à la fois avec le sous-site anionique, au fond de la gorge et avec le site périphérique anionique, près de l'entrée par les interactions avec les résidus conservés aromatiques empilés. (©Elsevier, Paris)

Alzheimer's disease / drug design / peripheral site

1. Introduction

Acetylcholinesterase (AChE) terminates synaptic transmission at cholinergic synapses by rapid hydrolysis of acetylcholine (ACh) [15]. Anticholinesterase agents are used in the treatment of various disorders [21], and have been proposed as therapeutic agents for the management of Alzheimer's disease [7]. Two such anticholinesterase agents, both of which act as reversible inhibitors of AChE, have been licensed by the FDA: tacrine (1,2,3,4-tetrahydroacridine) [6], under the trade name Cognex®, and, more recently, E2020 ((R,S-1-benzyl-4-[5,6-dimethoxy-1-indanon-2-yl]methylpiperidine) [18], under the trade name Aricept®. Tacrine and E2020 share the same target, but, whereas tacrine must be administered up to four times a day, and displays hepatotoxic side effects, E2020 may be administered once daily, and has fewer side effects. Furthermore, E2020 displays very high selectivity, ~1000-fold, for AChE relative to butyrylcholinesterase (BChE), whereas THA has similar affinity for the two enzymes. This may be important, since it has been suggested that inhibition of human plasma BChE may cause potentiating side effects [23].

The active site of AChE contains a catalytic subsite, and a so-called 'anionic' subsite, which binds the quaternary group of ACh [15]. A second, 'peripheral', anionic site is so named since it is distant from the active site [22]. Bisquaternary inhibitors of AChE derive their enhanced potency, relative to homologous monoquaternary ligands [13], from their ability to span these two 'anionic' sites, which are ca. 14 Å apart.

The 3D structure of *Torpedo californica* (*Tc*) AChE [20] reveals that the active site is located at the bottom of a deep and narrow cavity; named the 'aromatic gorge', since > 50% of its lining is composed of the rings of 14 conserved amino acids [1, 20]. The peripheral site is located at the entrance to the gorge [8].

X-ray crystallographic studies of complexes of AChE with drugs of pharmacological interest can reveal which amino acid residues are important for binding the drug, and where space might exist for modifying the drug itself, information crucial for structure-based drug design. In the following, we describe the crystallographic structure, at 2.5 Å resolution, of a complex of E2020 with *Tc*AChE.

2. Materials and methods

2.1. Protein preparation and crystallization

*Tc*AChE was purified and crystallized as described previously [16]. E2020, as the hydrochloride salt of the pure racemate, was a generous gift from Dr. B.P. Doctor (Division of

Biochemistry, Walter Reed Army Institute of Research, Washington, DC, USA). *Tc*AChE crystals were soaked in ~10 mM (R,S)E2020 for 5 days at 4 °C, and flash cooled to 100°K.

2.2. X-ray data collection and processing

Data were collected 'inhouse', at the Weizmann Institute of Science, on a Rigaku FR300 generator set at 50 mA, 50 kV and 1.54184 Å wavelength (Cu K radiation), equipped with a R-AXI-SII detector.

2.3. Model refinement and analysis

The structure was refined on the basis of the starting model of native *Tc*AChE (PDB ID 2ACE), using only the polypeptide and none of the solvent atoms, employing XPLOR [2]. Voids were calculated between the inhibitor molecule as one entity, and the protein and solvent molecules as a second entity [12].

3. Results and discussion

The 3D structure of the complex shows more detail of the AChE structure than the starting native model (2ACE). Primarily, residues 2 and 3, at the N-terminus, and the 484–490 loop, which were not seen in the original model, can be discerned. In addition, it was possible to model the proximal Nacetyl glucosamine (NAG) moiety at four out of five putative glycosylation sites, viz. at residues Asn59, Asn416 (where two NAG moieties could be fitted), Asn457 and Asn533.

3.1. All three segments of E2020 interact with AChE

E2020 binds along the active site gorge. All three major segments of the elongated molecule make specific interactions with the enzyme, and each of these interactions involves discrete watermediated contacts which appear crucial for specificity (*figure 1*). E2020 makes principal interactions with the enzyme through: 1) the benzyl moiety; 2) the piperidine nitrogen; and 3) the dimethoxyindanone moiety. It utilizes the conserved aromatic residues which line the gorge for hydrophobic and stacking interactions, and does not make direct contact with the protein through H-bonds or salt-bridges other than via water molecules.

3.2. Interactions at the bottom of the gorge

Near the bottom of the gorge, one face of the benzyl ring stacks against the six-membered ring of the indole moiety of Trp84, similarly to THA [8]. On the opposite face, it makes a classic aromatic hydrogen bond with a water molecule (WAT1160). This water is held firmly by a hydrogen bond to another water molecule (WAT1161), in the 'oxyanion hole', and to WAT1159. WAT1161 is another example of a tightly bound water molecule; it hydrogen bonds to residues in the 'oxyanion hole' and to S200*O*. E2020 does not interact directly either with the catalytic triad or with the 'oxyanion hole'.

3.3. Interactions in the middle of the gorge

In the constricted region, halfway up the gorge, the charged nitrogen of the piperidine ring makes a cation-π interaction [5] with the phenyl ring of Phe330. The ring nitrogen also makes an in-line H-bond with WAT1159. The principal binding site for the quaternary nitrogen of ACh within the active site, and for homologous ligands, is the indole ring of Trp84 [9]. These data suggest that Phe330 may serve as an additional quaternary binding site, of possible functional significance, midway down the gorge, between the peripheral binding site and the anionic subsite of the active site.

3.4. Interactions at the entrance to the gorge

At the top of the gorge, the indanone ring stacks against the 6-membered ring of the indole moiety of Trp279, in the peripheral binding site, in a classic parallel π-π interaction. The fact that the binding of E2020 is strongly dependent on interaction with Trp279 and Phe330, which are absent in BChE, may explain its high relative specificity for AChE versus BChE. The carbonyl function on the indanone is not in direct contact with the protein, but appears to make a water-bridged contact with F288N.

3.5. Only one enantiomer of E2020 is bound to AChE

The reported pharmacological studies on E2020 emphasize that both enantiomers are active, and exhibit similar, but not identical, binding affinities for AChE [11, 19]. Since we used the racemate in our crystallographic study, we expected either to find evidence, in the form of electron density, for the presence of both enantiomers in the crystal structure, or partial disorder which would support the presence of both. Yet, when we attempted to fit a number of plausible inhibitor conformations within the active-site gorge, a unique fit to the experimental electron density was found for one conformation of the R form, which is very similar to our energetically minimized E2020 conformation, with the indanone carbonyl group pointing towards F288N as mentioned above. Thus, *Tc*AChE appears to have bound the R form selectively, despite the similarity in binding constants. Selective binding cannot be due to a limitation in the amount of the S form, since the racemate was soaked into the crystal at 10 mM, i.e.,

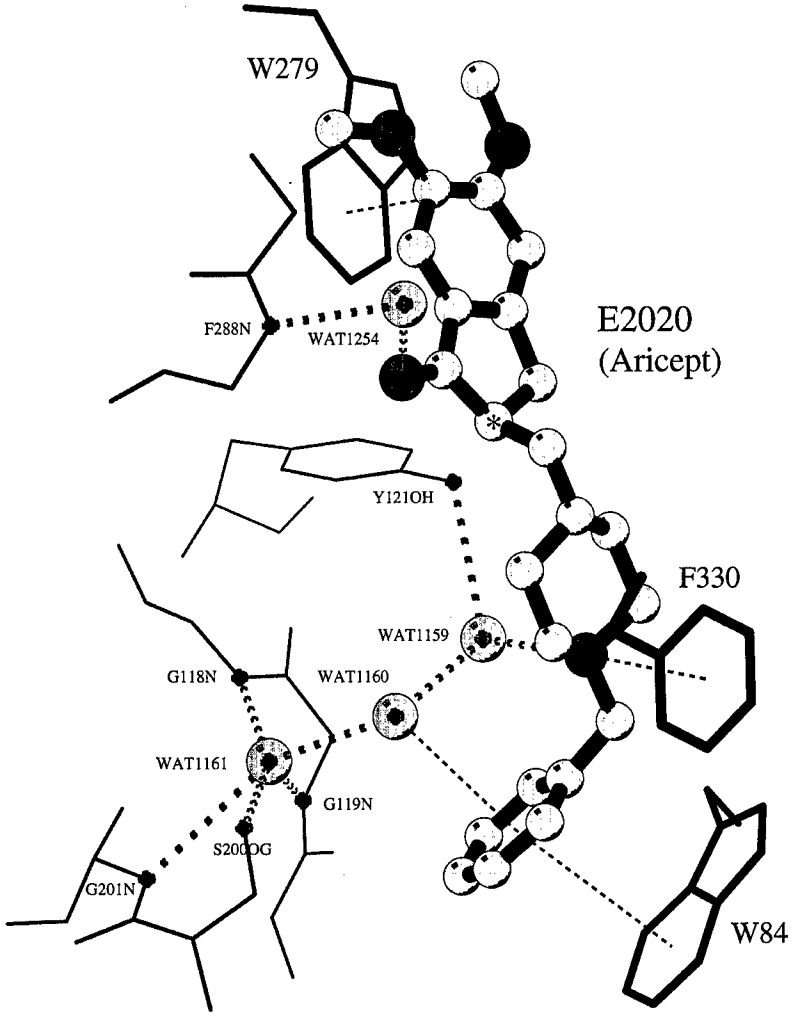


Figure 1. Binding modes of E2020 to *Tc*AChE. E2020 are displayed as a ball-and-stick model (chiral center marked with black star); direct binding residues are represented as dark grey sticks; water-mediated binding residues as light grey sticks; water molecules as light grey balls; 'standard' H-bonds as heavy dashed lines; aromatic H-bonds, -cation and -stacking as light dashed lines.

at a great molar excess. Although the two of E2020 interconvert readily in aqueous solution, via a ketoenol intermediate [14], it is not immediately obvious what could cause such a preferential binding of one form, taking into account their similar affinities. It should, however, be borne in mind that not only are the ligand and active site chiral, but also the entire lattice of the crystalline enzyme. Such a chiral environment might cause the stereoisomers to tautomerize at different rates. Based on steric considerations, it appears that both the R and S enantiomers could bind in very similar conformations, with only the position of the carbonyl function on the 5-membered ring of the indanone moiety distinguishing between them. A model which we built displaying a carbonyl on the other side of the indanone, representing the S form, does not make contact with any protein atom, and thus might be bound less tightly than the observed R form, even though this is inconsistent with the published inhibition data [11]. Furthermore, the 4.0 Å link between the indanone carbonyl and F288N, whether bridged by a water molecule or not, combined with the fact that the carbonyl 'nests' amongst three aromatic systems of residues Phe288, Phe290 and Phe331, might be sufficient to introduce a bias in favor of preferential binding of the R form. As already mentioned, R-S interconversion, via tautomerization, is known to occur, and might indeed take place under the experimental conditions employed. Thus, one explanation for the inhibitory potency of the S form would invoke AChE-induced S-to-R tautomerization.

3.6. *Possible effect on amyloid deposition*

Recently, evidence was presented that AChE may contribute to the generation of amyloid proteins and/or physically affect the process of fibril assembly which results in the formation of the senile plaques characteristic of AD [10]. The acceleration in fibril assembly produced by AChE could be retarded by the peripheral site inhibitor, propidium, and it was suggested that a hydrophobic environment close to the peripheral binding site might be involved in this process [17]. This raises the possibility that E2020, which our data clearly show as stacking against Trp279, might also moderate the rate of fibril formation. Many of the compounds synthesized and tested by the Eisai company involved modification of this segment of the molecule [3, 4]. It should be noted, however, that the screening which they performed involved assessment of affinity for AChE, together with selectivity for AChE relative to BChE, not a possible effect on amyloid fibril assembly or deposition.

Acknowledgments

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Targeting acetylcholinesterase molecules to the neuromuscular synapse

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Abstract — The functional integrity of the neuromuscular synapse requires that sufficient numbers of acetylcholinesterase (AChE) molecules be localized on the specialized extracellular matrix between the nerve terminal and the post-synaptic membrane. Multiple interrelated levels of regulation are necessary to accomplish this complex task including the spatial and temporal restriction of AChE mRNA expression within the muscle fiber, local translation and assembly of AChE polypeptides, and focused accumulation of AChE molecules on the extracellular matrix. This is accomplished in part through the organization of other extracellular matrix molecules into a complex which further associates with acetylcholine receptors and their accompanying molecules. Finally, the mature neuromuscular junction contains molecules which can act as receptors for the attachment of AChE which in turn may allow for the turnover of this enzyme at the synapse. This brief review will focus mainly on contributions from our laboratory towards understanding the mechanisms involved in organizing AChE molecules at the neuromuscular synapse. (©Elsevier, Paris)

Résumé — L'adressage des molécules d'acétylcholinestérase à la jonction musculaire. L'intégrité fonctionnelle de la synapse neuromusculaire nécessite que suffisamment de molécules d'acétylcholinestérase (AChE) soient localisées sur la matrice extracellulaire spécialisée entre la terminaison nerveuse et la membrane post-synaptique. Plusieurs niveaux de régulation sont nécessaires pour réaliser cette tâche et incluent la restriction spatiale et temporelle de l'expression de l'ARNm codant l'AChE dans la fibre musculaire, la traduction et l'assemblage des polypeptides d'AChE et la focalisation des molécules d'AChE dans la matrice extracellulaire. Ceci est réalisé en partie par l'organisation avec d'autres molécules de la matrice extracellulaire dans un complexe qui est ensuite associé aux récepteurs d'acétylcholine. Finalement, la jonction neuromusculaire mature comporte d'autres molécules qui peuvent agir comme un récepteur pour l'ancrage de l'AChE et pour le renouvellement de l'enzyme à la synapse. Ce bref compte-rendu est focalisé principalement sur les contributions de notre laboratoire à la compréhension des mécanismes engagés dans l'organisation des molécules d'acétylcholinestérase à la jonction neuromusculaire. (©Elsevier, Paris)

extracellular matrix / perlecan / basal lamina / localized gene expression

1. Introduction

The physiological function of acetylcholinesterase (AChE) at the neuromuscular junction (NMJ) is to terminate neurotransmission. To accomplish this, AChE molecules must be properly localized at the synapse where they are organized on the specialized extracellular matrix interposed between the nerve terminal and the post-synaptic membrane. The expression of AChE in nerves and muscle, the appearance of this enzyme at synapses or other specialized locations on the cell surface, is thus is the endpoint in a series of events, each of which can be viewed as potentially or actually regulatory. However, it should be remembered that unless these molecules are correctly localized within their appropriate domains on the cell surface, their physiological function is abrogated. Targeting AChE to the neuromuscular synapse therefore includes all the events, from the initial transcription of the AChE gene to the final attachment of the assembled AChE molecules on the synaptic basal lamina. Here we shall review the major steps in targeting AChE to the synaptic basal lamina and our understanding of the molecular events that ensure that AChE molecules are correctly organized at nerve-muscle synapses.

2. Differentiation of muscle and the transcriptional and post-transcriptional control of AChE

Skeletal muscle fibers are unique in many respects because of their large size and complex organization. For example, skeletal muscle fibers can be upwards of several millimeters long, and normally contain hundreds or thousands of nuclei. The neuromuscular synapse, on the other hand, covers but a small portion of the fiber surface, usually less than 0.1%, and therefore molecules destined for the NMJ must arise from a correspondingly smaller region of the cell.

Multinucleated skeletal muscle fibers arise from the fusion of many monucleated myoblasts which themselves do not express AChE [4]. Upon fusion to form multinucleated myotubes the cells increase their expression of AChE mRNA by processes which can involve either stabilization of the transcripts [4] or increases in the rate of transcription ([19]; in pre-

paration) depending upon the species or cell type used. At this point the AChE protein is expressed as active enzyme. AChE transcript levels are initially high throughout the length of the myotube. With further differentiation of the myotubes to form mature contracting skeletal muscle fibers, the levels of AChE transcripts decrease once again to very low levels along the length of the fiber with the exception of the innervated region where elevated levels persist throughout the lifespan of the organism [6, 8, 11]. Thus activity of the muscle fibers can suppress expression of the AChE transcripts by at least two different mechanisms, transcriptional controls and transcript destabilization. However, the mechanisms responsible for maintaining elevated levels of AChE mRNA expression, as well as other synapse-specific proteins, at sites of nerve-muscle contact are as yet unknown.

3. Compartmentalization of AChE expression in muscle

Compartmentalization in skeletal muscle refers to the fact that many cellular and biochemical functions within these complex multinucleated cells appear to be carried out in a spatially-restricted manner, as if each nucleus were still a mononucleated cell acting within the context of an organized tissue. That is to say that, although many nuclei are receiving the same signals at any one time and thus behaving in a coordinated manner, each nucleus is capable of acting somewhat independently. Likewise, gene expression in multinucleated skeletal muscle fibers also appears to be somewhat restricted in that transcripts from an individual nucleus tend to remain in the vicinity of that nucleus and be translated locally on either the rough endoplasmic reticulum associated with that nucleus or cytoplasmically [12, 14, 20] (for review see [5]).

Evidence that the transcription and translation of AChE is compartmentalized comes from experiments in which mosaic myotubes were prepared from two populations of myoblasts, each expressing a different allelic variant of the AChE catalytic subunit [20]. These allelic variants, α and β, differ by approximately 10 kDa and can easily be distinguished by their mobilities on SDS polyacrylamide gels. Moreover, these subunits assemble to form disulfide-bonded dimers which can also be resolved by SDS gel electrophoresis to quantitate the relative proportions of the $\alpha\alpha$, $\alpha\beta$, and $\beta\beta$ dimers. Myotubes resulting from the fusion of all heterozygous myoblasts express the several dimeric AChE forms in the ratio of 25:50:25, as would be predicted assuming random assembly in the rough endoplasmic reticulum. In contrast, dimeric AChE forms assembled in multinucleated myotubes arising from the fusion of equal numbers of $\alpha\alpha$ and $\beta\beta$ myoblasts resulted in more than 90% of the disulfide-bonded dimers being in the $\alpha\alpha$ and $\beta\beta$ forms. Labeling of the nuclei with tritiated thymidine, followed by autoradiography, showed that the nuclei were randomly mixed within the myotubes and therefore the results were not due to segregation of the nuclei expressing each allelic variant. These experiments show that once expressed, AChE transcripts are highly localized and tend not to diffuse very far from the nucleus of origin. Moreover, the transcripts are locally translated, and the subunits locally assembled in the rough endoplasmic reticulum prior to export to the cell surface. These observations are important for understanding the localization of AChE mRNA to the region of the neuromuscular junction.

Much more is known about the biogenesis and localization of AChE in tissue-cultured myotubes because of the ease with which the culture system can be experimentally manipulated. Once the AChE catalytic subunits are assembled into complex oligomeric forms (see Massoulié et al., this volume) they are transported and either secreted or localized on the surface of the myotubes [21]. At least some of the cell surface AChE is concentrated in clusters on the cell surface, a subset of which also contain ACh receptors. These clusters tend to localize within one nuclear diameter (about 20 μm) of a nucleus and thus form part of what can be called a nuclear domain [15]. Studies using mosaic mouse-quail myotubes showed that the AChE expressed by a specific nucleus is clustered on the region of membrane/extracellular matrix overlying that nucleus. Thus each nucleus is responsible for assembling the ECM on its overlying plasma membrane and targeting the AChE molecules to that domain.

4. Organization of cell surface AChE molecules in skeletal muscle

Cell surface AChE molecules can be classified into several types depending upon their oligomeric organization, post-translational modifications, and association with non-catalytic subunits (for review see [10]). The specific forms expressed depend in part upon the specific transcripts expressed and the cell type that expresses them. In tissue cultured skeletal muscle, most, if not all of the AChE forms that reach the cell surface appear to be either collagen-tailed forms that are rapidly attached to the surrounding extracellular matrix, or dimeric/ tetrameric forms that are secreted into the surrounding milieu. In vivo, tetramers appear to be secreted under some physiological conditions. The collagen-tailed AChE form that associates with the synaptic basal lamina

has been more difficult to study once it is externalized by the cell because of its tight association with the cell surface [16]. For this reason it is still not clear exactly how it becomes attached to the ECM, and even less is known about the mechanisms that specifically organize the AChE molecules on the extracellular matrix.

In the absence of nerves, spontaneous clusters of AChE molecules will form on the surface of tissue-cultured myotubes [15, 23]. These clusters consist predominantly, if not uniquely, of the collagen-tailed A_{12} AChE form [15]. It appears that following externalization the collagen-tailed AChE can undergo transient electrostatic interactions with the glycan portion of a heparan sulfate proteoglycan before immobilization on the extracellular matrix [2, 18]. Although normally the collagen-tailed AChE is not found secreted into the medium, inclusion of heparin in the culture medium results in secretion of this complex enzyme form. The interpretation of these experiments is that the soluble heparin, interacting with the heparin-binding domains of the collagen tail, prevents the transient interactions between the tail and the extracellular matrix from occurring.

Recently, progress has been made in identifying the molecule(s) to which AChE might attach on the synaptic basal lamina ([17]; Peng et al., submitted). AChE co-localizes on the surface of tissue-cultured myotubes as well as the adult neuromuscular junction with a specific heparan sulfate proteoglycan. This HSP has now been identified as perlecan [13] and is the same HSP recognized by mAb 33 described by Bayne et al. [1] (Peng et al., submitted). The collagen-tailed A_{12} AChE form can bind directly to perlecan, whereas the G_4/G_2 globular forms cannot. Although these studies do not prove that perlecan is the sole binding site for AChE, in fact other NMJ-localized proteoglycans such as agrin may also act as an AChE acceptor, they do suggest that it may be a major binding site for localizing this enzyme to sites of nerve-muscle contact.

Together, these studies suggest a mechanism for clustering AChE molecules at developing neuromuscular junctions. The collagen-tailed AChE molecules can bind to the sulfated glycan side chains of perlecan, which in turn binds to α-dystroglycan [13]. The α-dystroglycan itself is part of a large transmembrane complex which includes dystrophin on the cytoplasmic side of the membrane and can interact with several components of the extracellular matrix on the extracellular side. This complex is mobile in the plane of the lipid bilayer and can thus translocate together with other associated macromolecules. Studies in which endogenous cell surface AChE has been labeled with fluorescent fasciculin, or exogenous collagen tailed AChE added followed by detection with appropriate antibodies, show that spinal chord neurons or growth factor-coated beads can induce the accumulation of AChE at newly formed synapses in culture. This induced clustering of AChE could provide not only a mechanism for localizing this enzyme to domains of ACh receptor accumulation, it also provides a mechanism for initiating formation of the specialized extracellular matrix that forms the synaptic basal lamina.

5. Targeting AChE to the mature neuromuscular junction

AChE has been known to be concentrated at the neuromuscular junction for many decades, since the early studies by Marnay and Nachmansohn [9]. That proper innervation is also required to maintain normal levels of this enzyme at the synapse has also been known for a long time [3]. However, remarkably, very little is known about the dynamics of this enzyme at the adult neuromuscular synapse, or at any other cholinergic synapse for that matter. Although the density and distribution of AChE has been carefully measured at the NMJ, only one study has accurately determined its half-life in vivo using metabolic labeling techniques [7]. In fact, it was not until fairly recently that the almost irreversible attachment of the enzyme to the synaptic basal lamina was appreciated [16, 18].

Once the neuromuscular junction with its specialized extracellular matrix has formed, it is unlikely that many molecules will be capable of freely diffusing in the plasma membrane. Likewise for the extracellular matrix itself, where constituent molecules are often covalently cross-linked and the entire assembly is remarkably resistant to extraction even with chaotropic agents such as 8 M urea or 5 M guanidine HCl and strong ionic detergents. The mechanisms that allow for the orderly removal and replacement of these extracellular matrix molecules must therefore be complex.

For the collagen-tailed A_{12} AChE, it would appear that the assembled molecules must be secreted intact and somehow diffuse to their sites of attachment on the synaptic basal lamina. This is based on the observations that collagen-tailed AChE purified from avian muscle can be 'transplanted' onto the frog neuromuscular junction by simply incubating sections of frog muscle, or even isolated basal lamina, with the enzyme [22]. Unlike higher vertebrates, the frog does not have an accumulation of 'junctional' nuclei beneath the neuromuscular junction which can extend out along the muscle fiber for over 300 μm. Thus in some cases AChE molecules must diffuse over distances greater than 100 μm from their sites of secretion to sites of attachment on the synaptic

basal lamina. In higher vertebrates, where there is an accumulation of nuclei beneath the neuromuscular junction, it would appear that secretion followed by some diffusion would still be necessary for the AChE forms to reach their attachment sites on the basal lamina.

6. Conclusions

Ensuring that the appropriate concentration of AChE is maintained at the neuromuscular synapse requires multiple coordinated steps including spatial regulation of gene expression in the multinucleated skeletal muscle fibers. During development the expression of AChE transcripts becomes suppressed in non-innervated regions of the fibers and maintained at high levels in the vicinity of the neuromuscular junctions. This ensures that the translated proteins are assembled in the region of the neuromuscular synapse, and that the newly-synthesized and assembled AChE molecules are transported to the plasma membrane and released into the junctional space. Probably only the A_{12} forms are retained and attached to the synaptic basal lamina where they associate with heparan sulfate proteoglycans such as perlecan and possibly agrin. During initial stages of synapse formation the AChE molecules bound to HSPs may be mobile in the plasma membrane and be capable of diffusing into clusters containing other extracellular matrix molecules as well as acetylcholine receptors and associated molecules. This accumulation of extracellular, transmembrane, and cytoplasmic synaptic components may be the earliest stages of formation of the complex specialized synaptic basal lamina. Subsequently, at the mature NMJ, A_{12} AChE molecules may be released where they bind to pre-formed sites established by the HSPs, a form of molecular parking lot where the enzyme can attach and turn over as necessary.

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Imprinting of hippocampal metabolism of choline by its availability during gestation: Implications for cholinergic neurotransmission

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Abstract — Choline supplementation during the second half of the gestational period in rats permanently improves visuospatial memory. Choline availability during this period also alters the turnover of choline and acetylcholine in the hippocampus in 3–4-week-old animals in a complex pattern consistent with the notion that cholinergic neurotransmission is enhanced by prenatal choline supplementation. (©Elsevier, Paris)

Résumé — Effet à long terme du métabolisme de la choline dans l'hippocampe par sa disponibilité pendant la gestation : implications dans la neurotransmission cholinergique. L'apport de choline pendant la seconde moitié de la gestation chez le rat améliore de façon permanente la mémoire visuospatiale. La disponibilité en choline pendant cette période altère également le turn-over de la choline et de l'acétylcholine dans l'hippocampe chez les animaux de 3-4 semaines. Ceci est en accord avec l'idée que la neurotransmission cholinergique est augmentée par l'apport prénatal de choline. (©Elsevier, Paris)

acetylcholine / phosphatidyl choline / memory / nutrition

Choline is an essential nutrient for animals and humans [27] and the 1998 report on B vitamins of the Food and Nutrition Board of the Institute of Medicine of the US National Academy of Sciences, for the first time, issued recommendations for the Adequate Intake of choline [10]. Recent studies have shown that the availability of choline during a specific prenatal period in rats (embryonic (E) days 11 through 17 of the 22-day gestation period) has profound effects on cognitive performance throughout the lifespan. In brief, prenatally choline-supplemented adult rats are characterized by improved performance relative to prenatally-deficient and control animals in tasks measuring spatial memory, temporal processing, and attention [15–18, 21, 26]. In contrast, prenatally deficient animals are impaired in attentional and certain memory tasks [16]. The behavioral effects of choline availability in utero persist beyond the age of 2 years [16], which is regarded as old in the rat. Thus, prenatal supplementation with choline prevents the characteristic memory decline of old age. These data indicated that prenatal availability of choline permanently influences brain organization and function. Data consistent with this hypothesis have been obtained using neuroanatomical [25], neurophysiological [20], and neurochemical [6, 12] approaches. For example, studies of hippocampal long-term potentiation (LTP), a measure of synaptic plasticity and a model for learning and memory [2, 8], showed that in young adult rats prenatally supplemented with choline, induction of LTP was facilitated due to a reduction in the stimulus threshold for LTP generation, while the stimulus threshold for LTP generation was increased in prenatally choline-deficient animals [20].

The neurochemical mechanisms by which choline supplementation in utero leads to an improvement in memory are not known. Choline serves several biological functions [27]: 1) it is the precursor of the neurotransmitter, acetylcholine (ACh), in cholinergic neurons [3]; 2) it is the precursor of phosphatidylcholine (PC) and sphingomyelin, which are structural phospholipids in biological membranes, and which act as precursors for intracellular messengers such as diacylglycerol or ceramide; 3) it is the precursor of two signaling lipids, sphingosylphosphocholine and platelet-activating factor (PAF). The latter appears to be important in brain development because mutations in PAF acetylhydrolase (the enzyme that inactivates PAF) cause Miller-Dieker lissencephaly [11], a genetic disorder characterized by smooth cerebral hemispheres; and 4) it can be enzymatically oxidized to betaine and the methyl groups of betaine can then be used to resynthesize methionine from homocysteine, thereby providing methionine for protein synthesis and transmethylation reactions. The latter pathway also serves as an alternative to one which uses methyltetrahydrofolate, and thus spares methyltetrahydrofolate for its role in the synthesis of nucleic acids. It is possible that choline availability in utero affects one or more of these functions during brain development, resulting in changes in the brain's organization. Since the period of E11-17 coincides with the peak of cell divi-

sion and apoptosis in the developing brain, this explanation is likely. Indeed, choline supplementation during E11-17 stimulates cell division in the embryonic brain (assessed immunohistochemically on E18 following the injection of pregnant dams on E16 with the DNA precursor bromodeoxyuridine) [22], while choline deficiency during this period increases the rate of apoptosis (observed on E18) in hippocampus and septum [13, 22], two brain regions involved in memory processing.

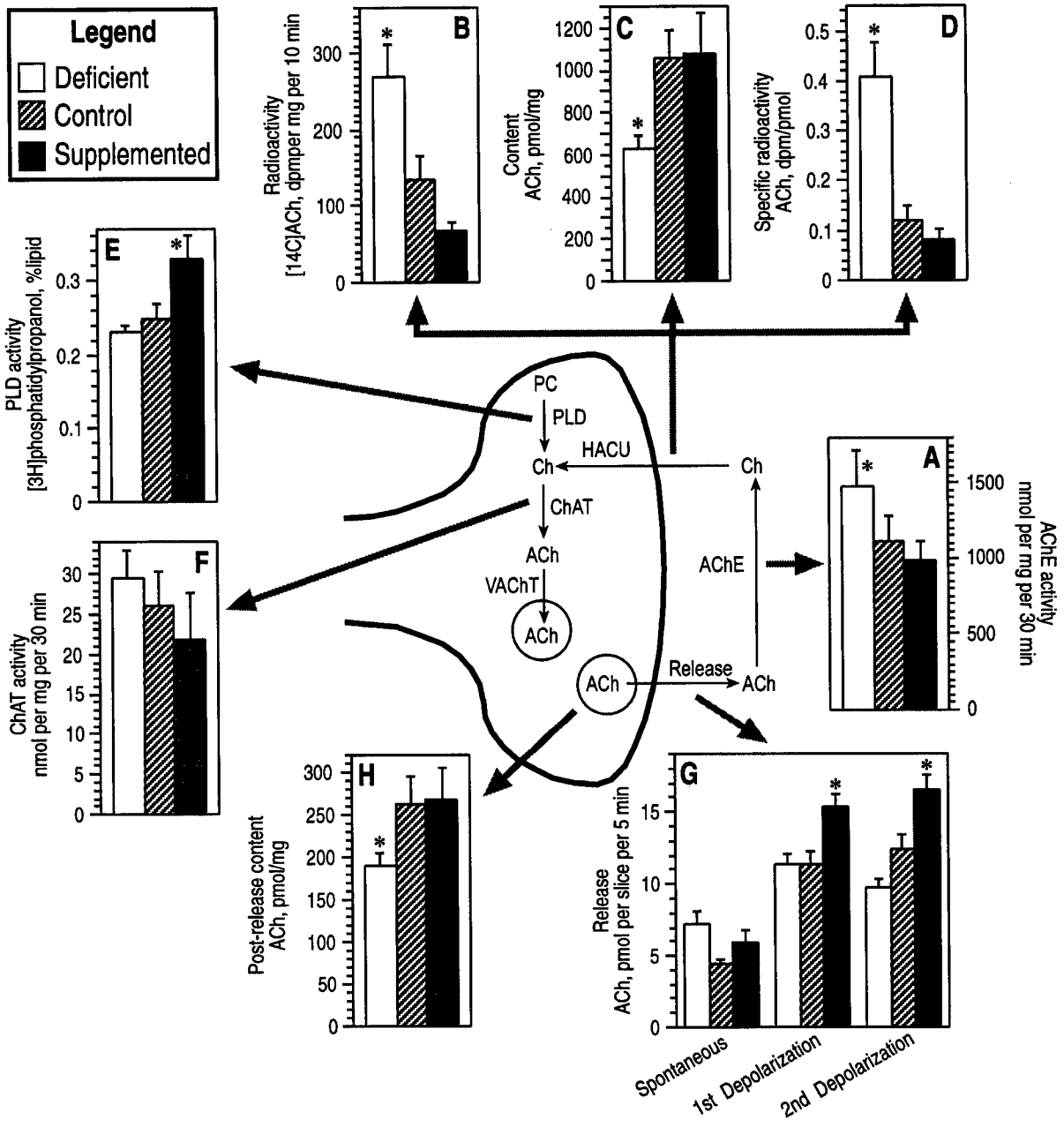
Another possible explanation for the long-term actions of prenatal choline availability on brain function that will be explored here, is that in addition to the permanent organizational changes in brain, prenatal choline status causes long-term metabolic adaptations in choline metabolism, resulting in changes in cholinergic neurotransmission. Long-term metabolic adaptations of fat and carbohydrate metabolism to the availability of carbohydrate and fat during the pre-weaning period have been observed previously [19]. This type of adaptation has been termed 'metabolic imprinting'.

In order to determine the effects of maternal choline intake on choline and ACh turnover in the hippocampus of the offspring we used pregnant rats (Sprague-Dawley *Crl*:CD (SD)BR-CD) fed varying amounts of choline during the period E11-17. We measured several indices of ACh synthesis, degradation and release, as well as the activity of a PC-hydrolyzing enzyme phospholipase D (PLD) in the offspring approximately 1 month after termination of treatment. PLD was chosen because choline liberated from PC by PLD can be used for ACh synthesis [4, 14].

The results, summarized in *figure 1*, show that prenatal choline availability altered choline and ACh turnover in the hippocampus. Prenatally choline-deficient animals displayed elevations in AChE (*figure 1A*) and ChAT (*figure 1F*) activities, and increased synthesis of ACh from choline transported by high-affinity choline uptake (HACU) (*figure 1B, D*), concomitant with reductions in hippocampal ACh content (*figure 1C, G*) and a relative inability to sustain depolarization-evoked ACh release (*figure 1H*).

Figure 1. Prenatal choline availability alters the indices of choline turnover in the hippocampus. In the center of the figure is a schematic representation of a cholinergic nerve terminal. Choline is taken up from the extracellular space by a sodium-dependent high-affinity uptake system (HACU) and then acetylated to ACh by choline acetyltransferase (ChAT). The ACh is taken up into the secretory vesicles by a specific carrier, the vesicular ACh transporter (VAChT), and then released into the synapse. Extracellular ACh is hydrolyzed by acetylcholinesterase (AChE) to free acetate and choline. The hydrolysis of phosphatidylcholine (PC) by phospholipase D (PLD) provides additional choline precursor for ACh synthesis. **A-H.** The individual assays were performed as described previously [6, 12] on animals (at 21 days of age (**E**) or 27 days of age (all other panels)) whose mothers consumed no choline (deficient), 1.3 mmol per kg per day of choline (control), or 4.6 mmol per kg per day of choline (supplemented) during days 11-17 of pregnancy. **A.** Hippocampal AChE activity. AChE activity was measured in homogenates by the method of Fonnum [9] as described [6]. AChE activity was highest in the prenatally choline-deficient group as compared to control and supplemented groups ($*P < 0.05$; $P < 0.001$, respectively). **B-D.** Acetylcholine synthesis in hippocampal slices from choline transported by HACU. **B.** Incorporation of [^{14}C]choline into ACh. Hippocampal slices were incubated for 10 min at 37 °C with 1 μM [^{14}C]choline in the presence or absence of 10 μM hemicholinium-3, a specific inhibitor of HACU. ACh was extracted, purified by HPLC and its radioactivity was determined as described [6]. The data are reported as dpm of [^{14}C]ACh accumulated in the absence of hemicholinium-3 minus that accumulated in its presence, as an index of ACh synthesis from choline taken up by HACU. The amount of [^{14}C]choline incorporated into [^{14}C]ACh was increased in the prenatally choline-deficient groups compared to prenatally choline-supplemented and control groups ($*P < 0.001$). **C.** Acetylcholine content in slices incubated as in **B** in the presence of hemicholinium-3 was determined by HPLC. The amount of ACh in hippocampal slices was decreased in the prenatally choline-deficient groups compared to the prenatally choline-supplemented and control groups ($*P < 0.01$). **D.** Specific radioactivity of [^{14}C]ACh was obtained by dividing the values in **B** by those in **C**. The specific radioactivity of [^{14}C]ACh was increased in the prenatally choline-deficient groups compared to the prenatally choline-supplemented and control groups ($*P < 0.001$). **E.** Phospholipase D activity in hippocampal slices [12]. The slices were preincubated with [^{3}H]glycerol to label phospholipids, and subsequently incubated in the presence of 1-propanol for 30 min. [^{3}H]Phosphatidylpropanol, the product of PLD, was purified by TLC and its radioactivity determined and expressed as a percentage of that in the total lipids. PLD activity was higher in prenatally choline-supplemented group as compared to the control and choline-deficient groups ($*P < 0.05$). **F.** Hippocampal ChAT activity. ChAT activity was measured in homogenates by the method of Fonnum as described [6]. ChAT activity was higher in choline-deficient group compared to the prenatally choline-supplemented group ($P < 0.05$). **G.** Spontaneous and depolarization-evoked ACh release was measured as described [6]. Hippocampal slices from the prenatally choline-supplemented group had an increased 1st and 2nd depolarization-evoked ACh release compared to the prenatally choline-deficient and control groups ($*P < 0.05$). **H.** Acetylcholine content of hippocampal slices. ACh was measured in hippocampal slices by HPLC as described [6], following the 2nd depolarization period described in **G**. The prenatally choline-deficient group had reduced ACh content compared to the control and choline-supplemented groups ($*P < 0.05$).





These results indicate that in the hippocampus of prenatally choline-deficient animals ACh turnover is accelerated (i.e., there is more rapid synthesis, degradation, and choline reutilization by HACU as indicated by the high specific radioactivity of newly-synthesized ACh (*figure 1D*)). In contrast, prenatally choline-supplemented animals showed less pronounced changes in their hippocampal cholinergic system, however the direction of those changes was consistent with the above model. AChE (*figure 1A*) and ChAT activities (*figure 1F*), and ACh synthesized from choline transported by HACU (*figure 1B,D*), were lowest in prenatally choline supplemented rats. However, depolarization-evoked ACh release was highest in these animals (*figure 1H*). The latter result, together with the reduced AChE activity,

suggest that intrasynaptic ACh concentrations and dwell times are increased, possibly resulting in enhanced cholinergic neurotransmission. The observations that ACh turnover in prenatally choline-supplemented animals is relatively slow (as indicated by low specific radioactivity of ACh newly-synthesized from exogenous choline (*figure 1D*)), but that cholinergic neurotransmission is well maintained (as evidenced by robust ACh release (*figure 1H*)), suggest that the pool of choline used for the synthesis of ACh in these animals may include that stored in membrane PC [23, 24], and may be generated by the hydrolysis of PC catalyzed by PLD [4, 14]. Consistent with the latter possibility, hippocampal PLD activity was two-fold higher in prenatally choline-supplemented rats relative to control and prenatally choline deficient animals (*figure 1E*) [12]. However, it is not yet clear if this PLD is in fact involved in supplying choline for ACh synthesis in hippocampal cholinergic nerve terminals.

Together the data show that the availability of an essential nutrient, choline, in utero causes multiple changes in its own metabolism (i.e., exerts a form of metabolic imprinting) in the hippocampus later in life. In prenatally choline-deficient animals this imprinting results in efficient recycling of choline, while in prenatally choline-supplemented rats choline recycling is reduced and there appears to be greater reliance on PC-stored choline for ACh synthesis. These adaptations seem appropriate for the periods when choline availability is altered (i.e., prenatally), however they are long-lasting, i.e., they are observed at a time when all animals consume the control diet, 1 month after the termination of treatment. The molecular mechanisms governing metabolic imprinting by choline are not known. During the period of E11-17 the brain grows rapidly due to cell division and it is possible that choline can become a rate-limiting factor in this process as it does in cell culture models [1, 5, 7, 13]. It is conceivable that when this happens, the transcriptional control of enzymes involved in choline and ACh metabolism is adjusted by unknown feedback mechanisms that operate in dividing cells, setting up the expression level of those enzymes. However, as neuronal precursor cells differentiate into neurons and become postmitotic, they no longer respond to the availability of choline and the expression of the enzymes involved in choline turnover remains at the level set during development. In the cholinergic septohippocampal pathway studied here, these changes in gene expression would have to occur in the septal neurons. Indeed, changes in the size and shape of these neurons (identified by the expression of the p75 nerve growth factor receptor) have been observed in animals prenatally supplemented with choline [25]. This model of the mechanism of metabolic imprinting by choline remains hypothetical at present, however the model is testable with available molecular tools.

Since the activities of the macromolecules involved in hippocampal ACh turnover are imprinted by choline, cholinergic neurotransmission in the adult may be differentially sensitive to the availability of choline in adulthood, based on its supply in utero. The results presented in figure 1 suggest that elevated dietary choline in adult animals that were prenatally choline-deficient would result in large increases of ACh synthesis, whereas it might have a lesser effect in prenatally choline-supplemented animals. Conversely, choline deficiency in adults that were prenatally choline-supplemented might result in a large reduction of ACh synthesis concomitant with a depletion of their PC pool. Thus prenatal choline status would impart differential vulnerability to the choline supply in adulthood.

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TrkA antagonists decrease NGF-induced ChAT activity in vitro and modulate cholinergic synaptic number in vivo

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Abstract — Cholinergic neurons are known to respond in vivo to the administration of nerve growth factor (NGF) by a prominent and selective increase of choline acetyl transferase activity and by cholinergic synaptogenesis in the rat brain. By using a synthetic TrkA antagonist we demonstrated that endogenously produced NGF is involved in the continual re-modeling of cholinergic neuronal connections during adulthood, acting through TrkA receptors. (©Elsevier, Paris)

Résumé — Inhibition, par des antagonistes du recepteur TrkA, des propriétés trophiques du NGF in vitro et in vivo. Dans le cerveau de rat, les neurones cholinergiques répondent in vivo au NGF exogène, par une augmentation importante et sélective de l'activité de l'acétylecholine transférase et par la formation de nouvelles synapses cholinergiques. En utilisant un antagoniste synthétique du récepteur TrkA, nous avons démontré qu'au cours de l'âge adulte, le NGF endogène participe, via le récepteur TrkA, au processus permanent de plasticité synaptique des neurones cholinergiques. (©Elsevier, Paris)

NGF/ TrkA / antagonist / cortex / cholinergic neurons / VAChT / synaptophysin

1. Introduction

Several lines of evidence indicate that nerve growth factor (NGF) plays an important physiological role in regulating the phenotypic development and growth of the septohippocampal projection neurons [4]. The administration of NGF to neonate rats in vivo or to septal neurons in culture induces a several-fold increase in the activity of the acetylcholine biosynthetic enzyme, choline acetyltransferase (ChAT) [1, 8–10]. In addition, we have also provided evidence that exogenous NGF induces cholinergic synaptogenesis and hypertrophy of presynaptic boutons in the CNS of adult animals [6, 7].

The purpose of this study was to evaluate the role of endogenous NGF in the maintenance of cholinergic terminals in the cerebral cortex of adult animals by the blockade of TrkA receptors in situ. For this, we have applied a recently developed low molecular mass TrkA receptor antagonist which has been shown to counteract NGF effects in PC12 cells and to target TrkA receptors in vivo [11, 12]. The TrkA antagonist is the cyclic peptide C(92–96) which mimics the conformation of the third beta-turn (variable region) of NGF [11]. In order to validate the effectiveness of this compound in regulating NGF-sensitive cholinergic phenotype we investigated its actions in vivo on dissociated embryonic septal cells. After validating its efficacy, we applied it by infusion into the cerebral cortex and analyzed its effects on the number of presynaptic elements (cholinergic and non-cholinergic).

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2. Materials and methods

2.1. Materials

In these experiments, we used a cyclic conformationally constrained peptide C(92–96) (YCTDEKQCY; [11]).

2.2. In vitro

2.2.1. Septal neuronal cultures

Cell cultures were established from the septal area of 17-day-old rat embryos (Sprague-Dawley) using procedures described by Debeir et al. [3]. Cultures of septal neurons were treated 1 day after plating. The incubation was continued until 6 days in vitro (DIV) at which time ChAT activity was evaluated.

2.2.2. Measurement of ChAT activity

At 6 DIV, ChAT activity assays were performed directly in the wells using Fonnum's method [5], where ChAT activity is defined as the radioactivity obtained in the presence of eserine minus that seen in the presence of acetylcholine esterase.

2.3. In vivo

2.3.1. Drug treatment

Adult male Wistar rats (340–360 g) were anaesthetized and then implanted with cannula according to the following coordinates from Bregma [15]: anterior/posterior –1.3 mm, lateral 3, vertical 2.2 (hindlimb area). The cannula was connected to

an Alzet 2004 osmotic pump (0.25 µL /h). The C(92–96) peptide was diluted to have a delivery rate of 28 µg in 24 h. Two weeks after implantation, the pumps and tubing were removed from the anaesthetized rats. Rats were killed 2 weeks after the treatment ended.

2.3.2. Perfusion and fixation

The rats were perfused as described by Wong et al. [17].

2.3.3. Synaptophysin and VAChT immunostaining

The sections were incubated with a mouse monoclonal antibody against synaptophysin (1:40, overnight, Boehringer) or with a rabbit polyclonal antibody against VAChT (1:10000, 4 °C, 72 h, gift of Dr. R. Edwards, UCSF). Immunoreactivity was detected by using a PAP reaction for synaptophysin and a Vectastain ABC kit (Vector) for VAChT.

2.3.4. Quantification of the VAChT and synaptophysin stained presynaptic boutons

The immunopositive punctae (presynaptic boutons and varicosities) are referred to here as VAChT-immunoreactive (IR) and synaptophysin-IR sites. They were detected by the image analysis system using software devised for silver grain counting (Imaging Research Inc., St. Catharine, Ontario, Canada) as described by Wong et al. [17].To analyze the effects of the peptide on the number of boutons, a two-way ANOVA test was employed.

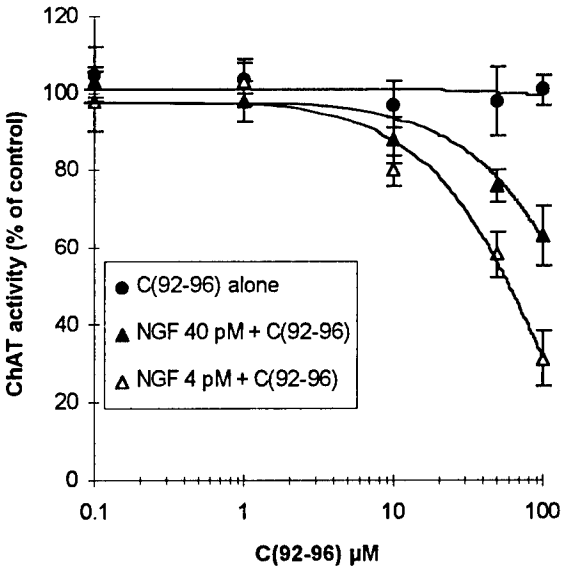


Figure 1. Effect of C(92–96) on NGF-induced cholinergic activity of septal neurons. The drugs were added to the culture medium 1 day after plating. ChAT activity was measured after 6 DIV. Values represent mean ± S.E.M. $n = 4$.

3. Results

The TrkA antagonist C(92–96) was shown to be effective in blocking NGF-induced cholinergic differentiation on primary cultures of dissociated embryonic septal cells. In the conditions applied, basal ChAT activity in septal neurons was increased by approximately two-fold in the presence of 40 pM NGF and by 50% with 4 pM NGF. The TrkA antagonist C(92–96), per se, did not provoke any effect on either ChAT activity or on cell density (data not shown). However, in the presence of 40 pM or 4 pM of NGF, the cyclic peptide C(92–96) prevented the neurotrophin induced effects on ChAT activity in a concentration dependent manner (*figure 1*). Concentrations 10^6-fold higher than those of NGF were required for the optimal blockade of neurotrophin activity by C(92–96), demonstrating that C(92–96) can also antagonize the biological effect of NGF cholinergic phenotype in vitro.

To investigate NGF actions in vivo, C(92–96) was delivered (28 µg/day) for 2 weeks into the rat cortex (hindlimb area) via a chronically implanted cannula connected to an osmotic mini-pump. Two weeks after the cessation of treatment with C(92–96), we observed a significant depletion in the number VAChT-IR sites (–23%) in the ipsilateral cortex. No significant changes in the number of synaptophysin-immunoreactivity sites were observed (*table I, figure 2*). The treatments did not provoke any contralateral effects.

Table I. Effect of cortical injection of C(92–96) on the number of synaptophysin-IR and VAChT-IR sites in the hindlimb cortex.

	Contralateral		*Ipsilateral*	
	Synapto-physin	*VAChT*	*Synapto-physin*	*VAChT*
C(92-96) 28 µg/day	106%	103%	107%	77%*

The number of sites is expressed as a percent of control. For synaptophysin-IR the contralateral and ipsilateral control values are, respectively, 720 ± 25 and 744 ± 14 sites in 1000 µm^2. For VAChT-IR, the control values are 26.4 ± 1.8 and 27.3 ± 1.8 sites in 1000 µm^2. *$P < 0.05$ versus control.

4. Discussion

Neurotrophins are responsible for inducing synaptogenesis during development (for review see [13]) and are responsible for maintaining the phenotype of some neuronal sets (for review see [14, 16]). Exogenously applied neurotrophins in the mature and fully differentiated CNS are capable of rescuing de-

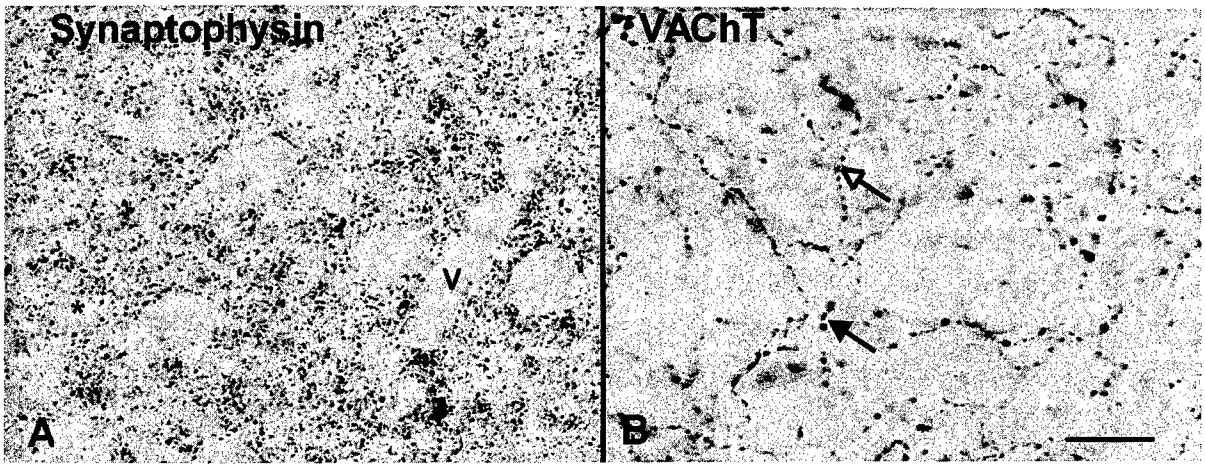


Figure 2. Synaptophysin (**A**) and VAChT (**B**) immunoreactivity in the ipsilateral cortex of control animals. Empty areas in **A** indicate vessels (V) or cells bodies (*) and small abundant punctae label non-cholinergic boutons. The solid arrow in **B** indicates three separate cholinergic boutons and the open arrow indicates an axon with multiple cholinergic varicosities. Scale bar, 20 μm.

generating or dying neurons in the lesioned CNS (for review see [2]) and are also capable of generating new synapses in the cerebral cortex of adult, fully differentiated animals [6, 7].

The analysis of the role played by endogenous neurotrophins in the maintenance of neuronal phenotype and synaptic patterns in the adult brain has been hampered by a lack of adequate antagonists. These reagents are now becoming available. In the present study, we have shown that the blockade of the TrkA receptors of embryonic cholinergic neurons counteracts the NGF-induced differentiation and up-regulation of the acetyl choline biosynthetic enzyme. More importantly, when this TrkA blockade takes place in the cerebral cortex of otherwise naïve animals in vivo, it causes an effect of its own, provoking a noticeable loss of VAChT-IR but not of synaptophysin-IR sites. These results indicate that a TrkA blockade does not affect the overall, non-cholinergic, presynaptic boutons in the adult CNS. These effects appear to be rather selective in removing the NGF-sensitive, TrkA-expressing, presynaptic cholinergic boutons which account for some 3 to 5% of the total synaptic population in the cerebral cortex. These results are consistent with the expected potency of this first generation of TrkA antagonist, based on published in vitro data [11]. They also indicate that endogenous NGF could define the steady state number and pattern of cholinergic synapses. Furthermore, it is conceivable that neurotrophic factors in general have a role in the maintenance of synaptic contacts in the adult and fully differentiated CNS.

Acknowledgments

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Nicotine modifies the activity of ventral tegmental area dopaminergic neurons and hippocampal GABAergic neurons

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Abstract — While trying to mimic the dose and time course of nicotine as it is obtained by a smoker, we found the following results. The initial arrival of even a low concentration of nicotine increased the firing rate of dopaminergic neurons from the ventral tegmental area (VTA) and increased the spontaneous vesicular release of GABA from hippocampal neurons. Longer exposure to nicotine caused variable, but dramatic, desensitization of nicotinic receptors and diminished the effects of nicotine. The addictive properties of nicotine as well as its diverse effects on cognitive function could be mediated through differences in activation and desensitization of nicotinic receptors in various areas of the brain. (©Elsevier, Paris)

Résumé — La nicotine modifie l'activité des neurones dopaminergiques de l'aire tegmentale ventrale et des neurones gabaergiques de l'hippocampe. L'arrivée initiale de même une faible concentration de nicotine augmente la décharge des neurones dopaminergiques de l'aire tegmentale ventrale (VTA) et augmente la libération vésiculaire spontanée de GABA par les neurones de l'hippocampe. Une exposition prolongée à la nicotine cause une désensibilisation des récepteurs nicotiniques variable mais prononcée et diminue les effets de la nicotine. (©Elsevier, Paris)

nicotine / dopamine / GABA / VTA / hippocampus

1. Introduction

Neuronal nicotinic acetylcholine receptors (nAChRs) were originally classified into two broad groups, the α-bungarotoxin (α-BTX)-binding sites and the more standard high-affinity nicotine binding sites [7, 18, 27, 28]. The nAChR subunits expressed in neurons include eight α (α2–α9) and three β (β2–β4) subunits. In heterologous expression systems, most subunits associate in combinations of α and β subunits to form functional nAChRs. The α7, α8, and α9 subunits are capable of forming homomeric receptors. In mammalian brain most of the α-BTX-binding sites are probably synonymous with α7-containing nAChRs.

Although the endogenous transmitter for nAChRs is acetylcholine, these receptors are also a site of action for nicotine obtained exogenously through tobacco. Smoking tobacco can deliver a small pulse of nicotine associated with each cigarette that is superimposed on a lower steady-state nicotine level maintained during the day. Nicotine is generally acknowledged as the addictive component of cigarettes [11, 29]. In addition, nicotine has numerous effects on cognitive function that may be mediated through nAChRs [17].

Ventral tegmental area (VTA) neurons of the mesolimbic dopamine system are thought to play an integral role in reinforcing rewarding behaviors. Drugs of addiction (such as cocaine, amphetamine, and nicotine) can exert influence over the mesolimbic system. Many addictive drugs increase dopamine release in the nucleus accumbens, which receives dopaminergic input from the VTA [24]. In vivo studies have shown that nicotine self-administration is reduced by lesions of the mesolimbic pathway or by nicotinic antagonists microinfused into the VTA [8, 9]. VTA neurons express nAChRs and can be stimulated to fire action potentials by nicotine [4–6, 23].

Using addictive drugs accrues learned behaviors, and contextual cues can elicit cravings long after drug use has ceased [17]. The hippocampus is an important center for contextual and spatial learning that receives cholinergic innervation and richly expresses nAChRs [13, 31]. GABAergic inhibitory interneurons in the hippocampus express nAChRs and nicotinic currents [2, 12, 14]. Nicotine has been shown to increase GABA release from several types of neurons from mouse, rat, and chick [3, 15, 16, 19] (for review see [30]). In CA1 hippocampal neurons, high concentrations of nicotine were found to increase spontaneous GABA release [2].

In both the VTA and the hippocampus, there is little information regarding the effects of sustained low concentrations of nicotine, as would be typically achieved by smokers. Therefore, we examined the

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ability of low concentrations of nicotine to modify the activity of the dopaminergic VTA neurons and GABAergic hippocampal neurons.

2. Materials and methods

2.1. Ventral tegmental slice preparation

Slices were prepared from Sprague-Dawley rats (12–25 days) using standard techniques as previously described [23]. The external bath solution contained (in mM): 135 NaCl, 2.5 KCl, 2.5 $CaCl_2$, 1 $MgCl_2$, 21 $NaHCO_3$, and 10 dextrose. The bath solution was continuously flowing and was bubbled with 95% O_2 and 5% CO_2. In most cases atropine (0.25 or 1 μM) was present to inhibit muscarinic acetylcholine receptors. The patch pipettes were filled with an internal solution containing (in mM): 120 $CsCH_3SO_3$, 10 CsCl, 10 EGTA, 5 Mg-ATP, 0.3 Na_3GTP, 10 HEPES, with pH 7.3. For current-clamp recordings half of the $CsCH_3SO_3$ was replaced by KCH_3SO_3.

2.2. Hippocampal primary culture

Hippocampal cell cultures were prepared from 1–3-day-old Sprague-Dawley rats as previously described [32]. Dissociated neurons were plated onto coverslips coated to have microislands of poly-D-lysine and collagen. The neurons were used for experiments after 15–30 days in culture. The external solution was composed of (in mM): 150 NaCl, 2.5 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 glucose, 10 HEPES, 0.5–1 μM TTX, 10 μM CNQX, with pH 7.3. The internal solution contained (in mM): 150 $CsCH_3SO_3$, 5 NaCl, 0.2 EGTA, 2–5 MgATP, 0–2 Na_2ATP, 0–0.3 Na_3GTP, 10 HEPES, with pH 7.3. Standard patch-clamp techniques were used.

3. Results

3.1. Effect of nicotine on the activity of dopaminergic VTA neurons

Dopaminergic neurons in the VTA were identified by two characteristics. In slice preparations, these neurons exhibit an inward current (I_h) in response to hyperpolarizing steps (*figure 1A*) [20]. In addition, some neurons were back-filled with biocytin, then positively labeled with antibodies for tyrosine hydroxylase, an enzyme required for dopamine synthesis. During current clamp recording under control conditions, spontaneous action potentials were observed. Bath application of 0.1 or 0.5 μM nicotine caused a depolarization of the neuron and an increase in the frequency of spontaneous action potentials (*figure 1B*). On average, 0.1 μM nicotine produced a depolarization of 4.3 ± 0.2 mV (n = 6) while 0.5 μM nicotine caused a larger depolarization of 8 ± 1 mV (n = 12). The depolarization was due to an inward current in response to nicotine that was observed in voltage-clamp recordings [23]. The majority of the nicotinic currents were inhibited by mecamylamine (5 μM), which at low concentrations is selective for the standard nAChRs. A smaller component of the current in a minority of neurons was inhibited by methyllcaconitine (10 nM), which selectively inhibits α-BTX-sensitive, α7-containing nAChRs.

Because nicotine obtained from tobacco has a long half-life, we examined the effect of a longer exposure to low concentrations of nicotine. The membrane voltage of a VTA neuron was monitored in whole-cell mode, while brief injections of 1 mM ACh were applied locally to a small portion of the soma (*figure 1C*). The local injection of ACh caused a depolarization that induced a burst of 3–5 action potentials. Then, a low concentration of nicotine (0.5 μM) was applied to the bath. In the continued presence of nicotine, the response to ACh decreased. The neuron began to fire action potentials continuously and, then, the neuron became silent even during the local ACh injection. After washing for > 10 min, the ACh injection again could elicit action potentials. It is likely that the nAChRs became desensitized by the low concentration of nicotine and, consequently, were unable to respond to the ACh injections. These results suggest that the exposure to nicotine observed after smoking a cigarette could serve to boost activity initially. Subsequently, however, long exposure to nicotine could reduce the responsiveness of these VTA neurons to nicotine and to endogenous cholinergic afferent input.

3.2. Effect of nicotine on spontaneous vesicular release of GABA from hippocampal neurons

Spontaneous GABA miniature inhibitory postsynaptic currents (mIPSCs) were observed in cultured hippocampal neurons. Microisland cultures were used so that synaptic connections could be made only among neurons on that same island [25]. This allowed applications of nicotine to a single island to reach all the neurons that might form connections with the voltage-clamped neuron. Microislands used for these experiments typically contained 2–5 neurons. Inhibitors of ionotropic glutamate receptors (10 μM CNQX) and voltage-gated Na^+ channels (0.5 μM TTX) were included to isolate the spontaneous GABA-mediated currents. At a holding potential of –20 mV, outward currents were commonly observed (*figure 2A*). Net Cl^- movement was inward at this potential (giving and outward positive current) because the internal solution contained a relatively low concentration of Cl^-. The mIPSCs had a relatively long duration (25–100 ms), typical of $GABA_A$ receptor mediated synaptic currents. We have

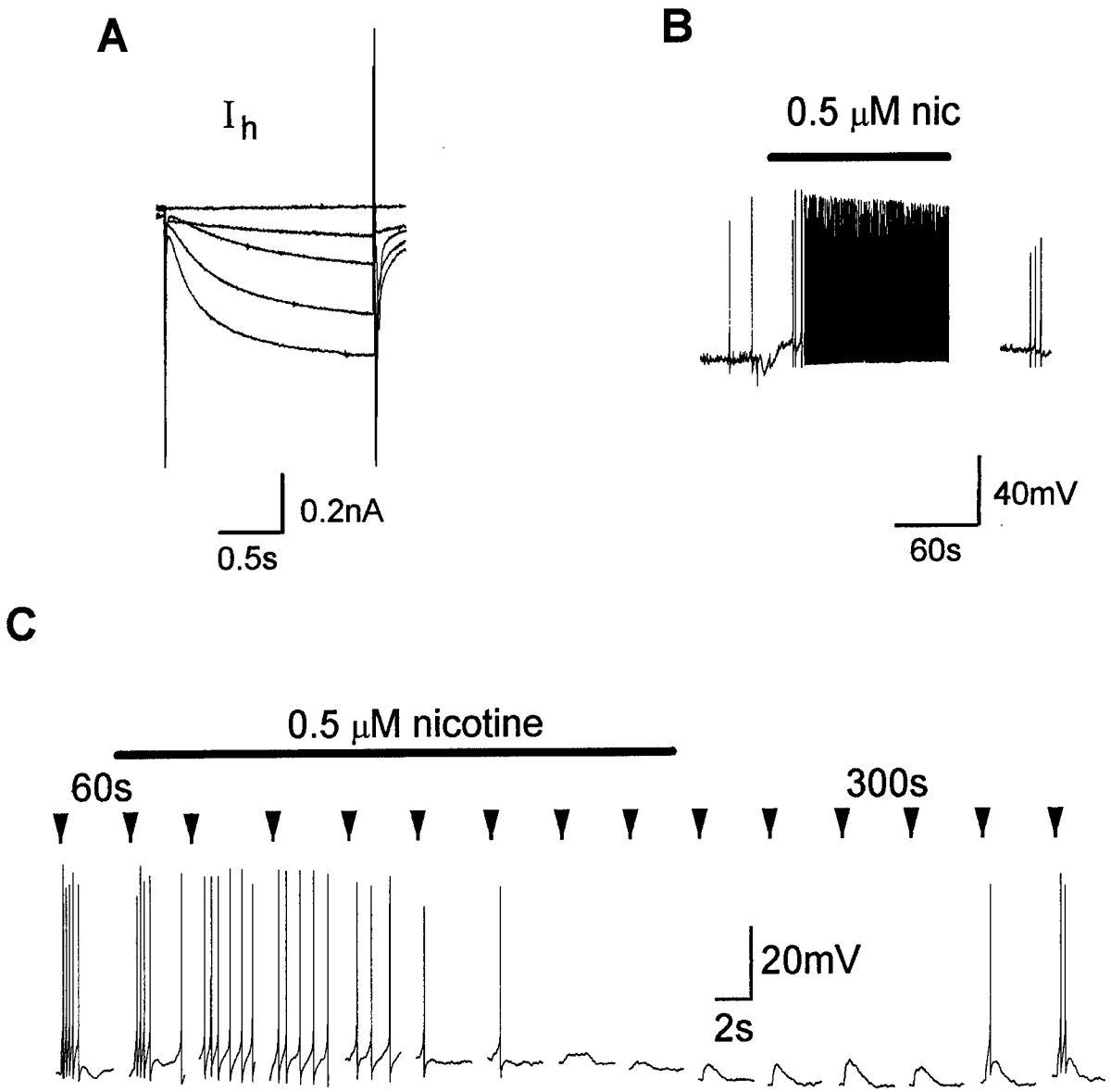


Figure 1. Nicotine initially increases the activity of dopaminergic VTA neurons, but desensitization reduces the effect of nicotine over time. **A.** Whole-cell voltage-clamped VTA neurons respond to hyperpolarizing voltage steps with an inward I_h current, characteristic of dopaminergic neurons. The neuron was held at –60 mV and negative 10 mV steps were applied (from –60 to –100 mV) to induce I_h. **B.** Bath application of 0.5 μM nicotine, as indicated by the solid bar, caused a depolarization and stimulated a burst of action potentials from a current-clamped VTA neuron. Following a 10-min washout period, activity returned to baseline levels. **C.** During a longer bath application of 0.5 μM nicotine, multiple effects were observed. Local injection of 1 mM ACh for 40 ms (arrow heads) onto a small area of the soma of a current-clamped VTA neuron caused an initial depolarization that stimulated a small number of action potentials. Bath application of 0.5 μM nicotine (solid bar), gradually reduced the responsiveness to ACh and caused the neuron initially to fire a train of action potentials. Subsequently, the neuron stopped responding to the local ACh injections and the bath applied nicotine. The injections of ACh were given every 20 s, but the time interval between displayed traces is altered for clarity from 60 to 300 s. Figures adapted from Pidoplichko et al. [23]. Reprinted by permission from Nature, copyright 1997 Macmillan Magazines Ltd.

previously shown that these events are blocked by picrotoxin, confirming that they are mediated by $GABA_A$ receptors [26].

The baseline frequency of mIPSCs was obtained by recording for several minutes before the application of nicotine. Only cells in which the frequency of events remained stable during this period were analyzed further. Nicotine (0.5 μM) was applied to hippocampal neurons for 3 – 4 min., and the mIPSCs were recorded continuously during this period (*figure 2*). The frequency of events increased an average of 1.8 ± 0.2-fold during the nicotine application ($P \leq 0.05$, paired *t*-test, $n = 3$). The frequency of events returned to the baseline level during or immediately following the nicotine application period. In some cases, after washing for a few minutes, another application of nicotine could again increase the frequency of spontaneous events. Although the frequency of the events increased, their amplitudes were unchanged [26].

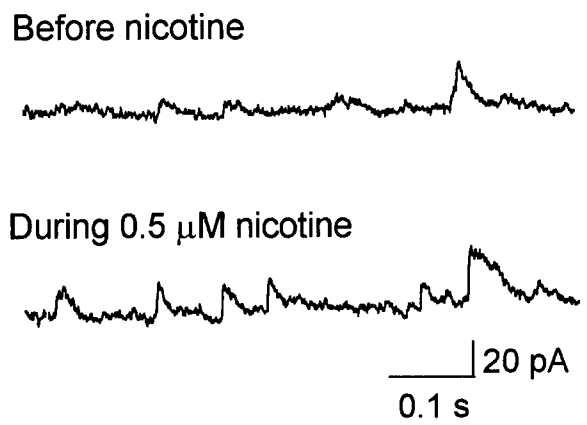


Figure 2. Nicotine increased the frequency of mIPSCs, but did not affect the amplitude. Representative traces of mIPSCs before and during bath application of 0.5 μM nicotine. Hippocampal neurons in primary culture were voltage-clamped and held at –20 mV. Due to the low concentration of intracellular Cl^-, opening of $GABA_A$ receptor channels results in a net outward current.

4. Discussion

Nicotine has a wide range of psychopharmacological effects that may be mediated by nAChRs throughout the CNS [11]. The low concentrations of nicotine achieved by smokers could alter the activity of neurons in many of the diverse regions of the brain that express nAChRs. Our results show that low concentrations of nicotine can influence synaptic activity in both the hippocampus and the VTA. Nicotine increased the firing frequency of dopaminergic neurons from the VTA and increased spontaneous synaptic activity of GABAergic hippocampal neurons.

Current evidence suggests that the dopaminergic release from the VTA is increased by addictive drugs (including nicotine), and that the increased activity of these neurons is associated with reinforcement of the addictive behavior [9, 10, 24]. Our results show that nicotine initially increases the activity of VTA neurons even at the low nicotine concentrations commonly achieved by smokers. The pharmacological profile of the response to nicotine suggested that α4β2 receptors represent most of the nAChRs in the VTA, with a smaller component of α7-containing receptors [23]. It has recently been shown that mice that do not express the β2 subunit are less susceptible to addictive properties of nicotine [22]. Unlike the short-lived exposure to high concentrations of agonist that would be expected from activity of a cholinergic neuron, the nicotine levels of smokers remain at detectable levels for over 2 h. Our results indicate that longer exposure to nicotine causes desensitization and diminishes the stimulatory effect on the VTA neurons.

Nicotine also produces a variety of effects on cognitive function, including changes in learning and memory that may be mediated through nAChRs in the hippocampus. We have shown previously that nicotine acts on α7-containing nAChR and directly increases the presynaptic concentration of Ca^{2+}, which in turn enhances the release of glutamate [13, 25]. The nicotine-induced Ca^{2+} influx was monitored with Fura-2 from single mossy-fiber presynaptic terminals in hippocampal slice while inhibiting glutamate, Na^+, and Ca^{2+} channels [13].

Our results with GABAergic neurons are similar to those reported for glutamatergic hippocampal neurons. Nicotine increased mIPSC frequency without affecting the amplitude of the events. The response to nicotine occurred in the presence of TTX, suggesting a presynaptic site of action for nicotine in these neurons. Based on related work, the effects of nicotine are predominantly mediated by α7-containing receptors [13, 25, 26]. This finding is consistent with evidence that nAChRs in the hippocampus are predominantly of the α7-containing type [1, 21, 33]. Regulation of the activity of both glutamatergic and GABAergic neurons in the hippocampus by nicotine suggests that cholinergic inputs have the potential to tightly regulate synaptic transmission in the hippocampus, providing mechanisms for modulation of both excitatory and inhibitory systems.

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The hypothesis of an ambient level of acetylcholine in the central nervous system

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Abstract — Recent ultrastructural data demonstrate the largely asynaptic character of the cholinergic innervation in many regions of adult rat brain. These data favour the hypothesis of a diffuse transmission/modulation by acetylcholine in the CNS and, by way of consequence, that of a persistent, low level of acetylcholine in the extracellular space. (©Elsevier, Paris)

Résumé — L'hypothèse d'un niveau ambiant d'acetylcholine dans le système nerveux central. Des données ultrastructurales récentes démontrent le caractère largement asynaptique de l'innervation cholinergique de plusieurs régions du cerveau chez le rat adulte. Ces données favorisent l'hypothèse d'une transmission/modulation diffuse par l'acétylcholine et, par voie de conséquence, celle de la persistance d'une faible concentration d'acétylcholine dans l'espace extracellulaire. (©Elsevier, Paris)

axon terminals / innervation / ultrastructure / diffuse transmission / acetylcholinesterase

1. Introduction

Much of the current knowledge about the properties and function of acetylcholine (ACh) in mammalian brain has been acquired before it was even possible to identify the neurons which synthetize and release this molecule as transmitter/modulator. Already in the sixties, biochemical and electrophysiological studies had revealed that ACh was implicated in cortical activation [25, 33] and sleep and wakefulness [5, 23, 24]. Subsequently, ACh was shown to play a role in learning [48], memory [4, 10] and cortical plasticity [3]. In the early eighties, specific antibodies were produced against the biosynthetic enzyme of ACh, choline acetyltransferase (ChAT), allowing for the immunocytochemical identification of these neurons [9, 17, 28]. Until the nineties, however, the light and electron microscopic demonstration of central ACh axon terminals (varicosities) remained difficult (reviewed in [22]), presumably because of a relatively low affinity of available antibodies. It is only in 1989 that Costantino Cozzari, working with Boyd K. Hartman in Minneapolis, raised and characterized a highly sensitive monoclonal antibody directed against purified rat ChAT [8], which we were privileged to use for examining the ultrastructural features of these terminals in rat brain.

Some of these results are briefly reviewed here, as a basis for re-expounding a recently proposed hypothesis according to which the spontaneous and evoked release of ACh from predominantly asynaptic axon terminals might permanently maintain a basal level of ACh in the extracellular space, at least in brain regions densely innervated by ACh neurons [7, 11, 13, 14].

2. Cerebral cortex

The ACh innervation of rat cerebral cortex (*figure 1A*) arises mainly from the nucleus basalis magnocellularis (also designated as substantia innominata, nucleus basalis of Meynert, or group Ch4) [27, 32]. The density of this innervation appears to be somewhat higher than that of its serotonin counterpart, estimated to be in the order of 5.8×10^6 varicosities per mm^3 [1]. In rodents, intracortical neurons [18, 26, 29] would account for approximately 20% of these terminals [19].

In an extensive electron microscopic investigation [44], we examined the intrinsic and relational features of ChAT-immunostained axon varicosities in the primary somatosensory area (S1 or Par 1) of adult rat cortex. 812 terminals from the different cortical layers were scrutinized in long, uninterrupted series of thin sections across their whole volume. Several were actually reconstructed in three dimensions (*figure 1B*); about 200 were visualized as doublets or triplets of varicosities along the same axon.

A totally unexpected finding was that, in every layer of the cortex, only a low proportion of these ACh varicosities displayed a synaptic membrane differentiation (junctional complex). As indicated in *table I*, their mean synaptic incidence across layers was 14%. Only layer V showed a slightly higher proportion of synaptic varicosities (21%). As a whole, cortical ACh varicosities were relatively small,

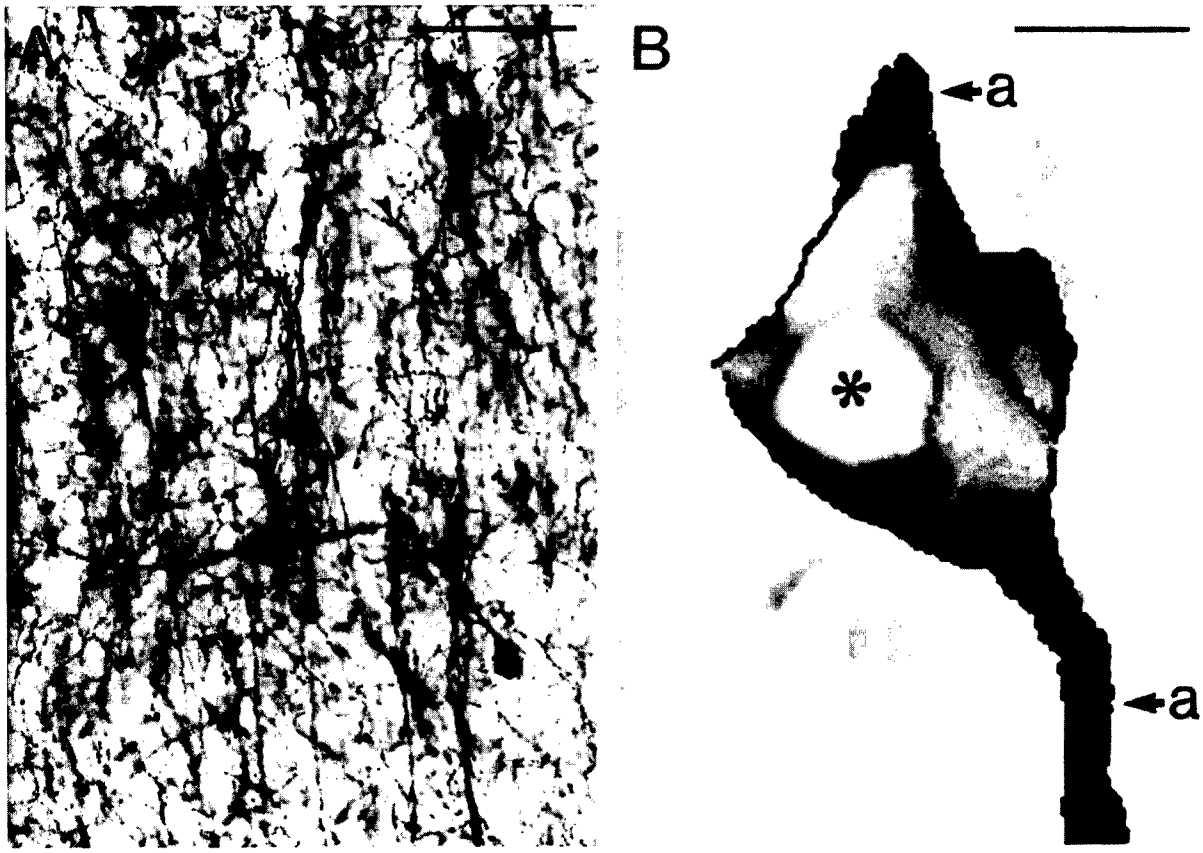


Figure 1. A. Light microscopic overview of the ChAT-immunostained innervation in layers II-III of adult rat parietal cortex (Par 1). Four immunoreactive cell bodies are visible in this field. The center one exhibits the typical features of smooth dendrite, fusiform ACh interneurons. The intricate network of thin varicose fibers pervading the neuropil arises in large part from the nucleus basalis magnocellularis. Note the irregular course of these axonal branches, which seemingly run in every direction, forming a relatively tight criss-crossing pattern with no predilection for the immediate vicinity of neuronal somata or the microvessels. Numerous bifurcations are visible (e.g., arrowheads). Smaller and larger varicosities may be observed on the same axons. Magnification × 450. Scale bar, 50 μm. **B.**Three dimensional electron microscopic reconstruction of a ChAT-immunostained axonal varicosity from layer I of the parietal cortex. This varicosity, visualized along its parent axon (a), was examined in its entirety in a series of 11 ultrathin sections (see figure 8 in [44]). It was reconstructed with the Volume Analysis Station ICAR 80.8 (ISG Technologies) from Nissei Sangyo Canada. A reference section from the middle of the series was included as background for purposes of orientation. The whole surface of the varicosity was observed and there was no hint of a synaptic specialization. The large, smooth protrusion directed toward the observer (*) marked the presence of an underlying mitochondrion. Magnification × 46 000. Scale bar, 0.5 μm (reproduced with permission from [44]).

Table I. Comparative data on the synaptic incidence of ACh axon terminals in adult rat CNS.

	ACh (%)	*NA* (%)	*5-HT* (%)	*GABA* (%)
Cerebral cortex (Par 1)	14	17	46	–
Hippocampus (CA1)	7	16	23	108
Neostriatum	9	–	13	–

From Umbriaco et al. [44] and Séguéla et al. [40, 41] for the cerebral cortex, Umbriaco et al. [45] for the hippocampus, and Contant et al. [7] and Soghomonian et al. [43] for the neostriatum.

averaging 0.6 μm in diameter. In general, those bearing a synaptic junction were slightly larger than their non-synaptic counterparts, but both junctional and non-junctional varicosities could be observed on the same axons. The junctional complexes formed by cortical ACh terminals were single, almost always symmetrical, and occupied a small fraction of the total surface of varicosities (< 3%). These synaptic varicosities usually contacted dendritic branches (76%), less often spines (24%), and none were seen on cell bodies.

The low synaptic incidence of cortical ACh varicosities in rat was confirmed in two subsequent investigations focused on the neurovascular relationships of these terminals [6, 46]. Both reports emphasized the fact that ChAT-immunostained boutons in the immediate vicinity of capillaries or microarterioles seldom exhibited synaptic specializations. The synaptic incidence of these perivascular ACh varicosities was then shown to be low not only in the frontoparietal (14%), but also in the perirhinal cortex (9%).

None of the previous ChAT immunocytochemical studies had actually been devised to determine the synaptic incidence of ACh terminals. In 1995, however, Mrzljak et al. [35] reported on 100 serially sectioned ChAT immunoreactive boutons at the border of layers II and III in macaque monkey prefrontal cortex. Only 44% of these varicosities were found to make synapse, 56% being without any visible junctional specialization, even if frequently juxtaposed to dendrites or spines receiving asymmetrical synapses. Very recently, Smiley et al. [42] performed a similar study on two samples of human anterior temporal lobe removed at surgery. They found 67% of 42 varicosities from layers I and II endowed with small but identifiable synaptic specializations. Whether such differences from the rat reflect sampling biases, regional differences or species differences remains to be determined. In all events, the results allow the inference that, in primates as well as rat cortex, ACh may subserve at least two types of modulatory effects: some dependent on the existence of symmetrical synaptic junctions, mostly made with dendritic shafts and spines; the others exerted on neuronal targets merely apposed to asynaptic ACh terminals, or even more remote from these presumed release sites.

3. Hippocampus

The ACh innervation of hippocampus originates mainly from cell bodies located in groups Ch1 and Ch2, ie, in the medial septal nucleus and nucleus of the vertical limb of the diagonal band of Broca [31, 39]. The density of this ACh innervation is again similar to that of its serotonin counterpart, estimated to average 2.7×10^6 varicosities per mm^3 [38]. In this region, the main purpose of our investigation was to compare the ACh terminals with three other types of endings also defined by their content in transmitter: noradrenaline (NA), serotonin (5-HT) and gamma-aminobutyric acid (GABA), as respectively immunostained with antibodies against NA, 5-HT, and glutamic acid decarboxylase [45]. The four types of varicosities were examined in the same portion of the stratum radiatum of CA1. The synaptic incidence was estimated from single thin sections with the stereological formula of Beaudet and Sotelo [2], the reliability of which had been experimentally verified in the serial section study of cortical terminals [44].

As shown in *table I*, the hippocampal ACh varicosities were even more asynaptic than their NA and 5-HT counterparts, in contrast to the GABA terminals which were all synaptic. The extrapolated synaptic incidence for the GABA endings exceeded 100% because of the occasional occurrence of more than one junction per varicosity.

4. Neostriatum

The ACh innervation of neostriatum is entirely issued from a fraction of the large interneurons which represent less than 2% of the total neuron population of this region [30, 49]. Yet, the ChAT immunoreactivity in neostriatum is the strongest in brain. Prior to our 1996 study [7], one could only imagine how profuse an axonal network might account for such labeling. This became immediately apparent when observing the multitude of small varicosities which pervaded the neostriatal neuropil. These were particularly small, as their mean diameter (0.43 μm) was significantly less than that of their cortical congeneers and of striatal dopamine or serotonin varicosities (all averaging about 0.6 μm in diameter).

Once again, a vast majority of these ACh varicosities were asynaptic. In single thin section, only 2.7% exhibited a junctional complex compared with 57% of unlabeled varicosity profiles selected at random from the same micrographs. The stereological extrapolation to the whole volume of ACh varicosities indicated a real synaptic incidence of less than 10% (*table I*), whereas that for the unlabeled varicosities was much higher than 100% because of the frequent occurrence of multiple junctions. Direct appositions between immunostained varicosities were not uncommon, reflecting the extreme density of this ACh innervation. Striatal ACh varicosities were often juxtaposed to unlabeled varicosities, many of which were synaptic.

5. An ambient level of ACh in brain

As initially postulated in the case of monoamine terminals in the cerebral cortex (for review, see [12]), the lack of synaptic junction on a large proportion of ACh axon varicosities in adult rat cerebral cortex, hippocampus and neostriatum is suggestive of a diffuse mode of transmission, complementary

to classical synaptic transmission. Such transmission by ACh will account for direct axo-axonic effects in the absence of axo-axonic synapses, for the presence of specific receptors located on somatodendritic membrane away from synaptic junctions, and for prolonged modulatory effects onto neurons, glial cells and microvessels. In this regard, it is noteworthy that a recent immunoelectronmicroscopic study by Mrzljak et al. [34], using subtype-specific antibodies to m1 and m2 muscarinic receptors in the prefrontal cortex of macaque monkey cortex, has indeed demonstrated the frequent localization of these receptors on dendritic spines, dendritic branches and cell bodies, as well as non-homologous (i.e., non-ACh) axon varicosities, remote from ACh terminals.

In view of the observed density of the ACh innervation, particularly in neostriatum, a complementary aspect of the diffuse transmission paradigm must also be considered. According to this hypothesis, a low level of ambient ACh would be present throughout the extracellular space, at least in brain regions densely innervated by predominantly asynaptic ACh terminals. This ambient level would be permanently maintained by the spontaneous or evoked release from the majority of non junctional ACh varicosities and spillover from the minority that are junctional. The release events would result in local and transient fluctuations of this ambient level, representing superimposed signals over the preexisting information conveyed by ACh.

In this context, as recently discussed in detail [14], brain acetylcholinesterase (AChE), which predominantly consists of the tetrameric G4 molecular form, would primarily serve to maintain the ambient level of ACh within physiological limits, rather than totally eliminate ACh from synaptic clefts and the extracellular space. This would be in keeping with the notion that this same G4 form plays a similar role in skeletal muscle, where, in contrast to the A12 form, it is concentrated outside and around endplates [20], and therefore does not significantly contribute to the rapid removal of ACh from synaptic clefts. Actually, there is increasing evidence to suggest that the perijunctional G4 compartment deals with ACh molecules spilling over from (or diffusing out of) the synaptic cleft, thus preserving the excitability of endplates, notably during high frequency activity [15, 21].

In many parts of CNS, microdialysis data seems consistent with the existence of a steady-state, resting or ambient level of ACh in the extracellular space. Spontaneous dialysis outputs of ACh in the nanomolar range have been repeatedly measured in the cerebral cortex, hippocampus or neostriatum of freely moving rat, in the absence of AChE inhibitor (references in [14]). Preliminary results obtained with the so-called zero net flux method also estimate this basal level to be in the nanomolar range [36]. Experimental and theoretical models have been proposed to evaluate the diffusion of transmitter molecules and answer the often-asked question of the distance that can be reached by these molecules in living brain [37]. In the case of dopamine, it has been inferred that a 10 μm distance might be reached within 50 ms [47]. Based on currently available estimates of the density of dopamine innervation in striatum [16], and considering that the density of striatal ACh innervation is at least equivalent, it may be calculated that a sphere of striatal neuropil of 10 μm radius contains about 400 dopamine and 400 ACh axon terminals, five to ten times more unidentified terminals, and at least several thousand dendritic spines. If ACh diffuses that far, this may explain why there would be such a variety of receptors for this single transmitter, and presumably different subtypes on any given neuron, perhaps for detecting different concentrations as informative signals.

A low ambient level of ACh could serve to regulate the expression and/or functional state of high-affinity receptor subtypes located on ACh (autoreceptors) or other neurons (heteroreceptors), glial cells and microvessels. Widely distributed homologous or heterologous receptors, activated by fluctuations of the basal level of ACh, could then be the ones to mediate complex behavioral effects produced by drugs acting on AChE or on the choline transporter. The existence of the ambient level could also explain the fact that certain pathological disorders attributed to loss of ACh neurons seem to become manifest only when a major proportion of these release sites have disappeared. Similarly, it might explain the beneficial effects of substitution, pharmacological or graft therapies, otherwise difficult to account for in the absence of restored synaptic connectivity.

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The role of neuronal nicotinic acetylcholine receptors in antinociception: Effects of ABT-594

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Abstract — ABT-594, a nicotinic acetylcholine receptor agonist, has antinociceptive effects in rat models of acute thermal, persistent chemical, and neuropathic pain. Direct injection of ABT-594 into the nucleus raphe magnus (NRM) is antinociceptive in a thermal threshold test and destruction of serotonergic neurons in the NRM attenuates the effect of systemic ABT-594. However, lidocaine-inactivation of the NRM prevents the antinociceptive effect of systemic (–)-nicotine but not that of systemic ABT-594. (©Elsevier, Paris)

Résumé — Le rôle des récepteurs nicotiniques neuronaux à l'acétylcholine dans l'antinociception. Effets de l'ABT-594. ABT-594, un agoniste du récepteur nicotinique à l'acétylcholine a des effets antinociceptifs dans des modèles de douleur induite par une température aiguë, un traitement chimique persistant et une neuropathie chez le rat. L'injection d'ABT-594 dans le grand noyau du Raphe (N.R.M.) est antinociceptif dans un test de sensibilité à la température et la destruction des noyaux sérotoninergiques atténue l'effet d'ABT-594. Cependant, l'inactivation du N.R.M. par la lidocaïne empêche l'effet antinociceptif de la nicotine mais pas de l'ABT-594. (©Elsevier, Paris)

ABT-594 / nucleus raphe magnus / nicotinic acetylcholine receptor / serotonin / antinociception

Most commonly used analgesics come from two classes: opioids and non-steroidal anti-inflammatory drugs (NSAIDs). Opioids are fully efficacious across a spectrum of pain states, but tolerance, side effects, and dependence issues are viewed as important limitations. NSAIDs, on the other hand, have more limited efficacy, being most useful in treating mild to moderate pain associated with inflammation, and have dose-limiting gastrointestinal effects.

Compounds acting at nicotinic acetylcholine receptors (nAChRs) also have antinociceptive activity, but have not been developed clinically. (–)-Nicotine, for example, is antinociceptive in rodents, but has low potency and efficacy and a short duration of action [1, 17]. More recently, however, interest in the analgesic potential of nAChR ligands has increased with the discovery that epibatidine, a potent nAChR agonist isolated from frog skin by John Daly, is fully efficacious and about 100-fold more potent than morphine in rodent models of acute thermal pain (e.g., hot-plate, tail-flick) [2, 16].

Unfortunately, epibatidine is a toxic compound. The separation between the doses producing antinociception and those producing seizures and death is relatively small in mice, and toxicity is potentiated with repeated dosing [15]. The key to the toxicity of epibatidine may be its high potency across a broad range of nAChR subtypes, including those present in autonomic ganglia and voluntary musculature [2, 16].

Given the evidence for nAChR diversity that has emerged over the last decade, however, it may be possible to achieve greater separation between desirable and undesirable effects of nAChR activation with improved subtype selectivity [9]. An example of a step in this direction is ABT-594 [10]. This compound has affinity comparable to that of (±)-epibatidine for the high affinity nicotine binding site present in rat brain, but has 3000-fold lower affinity than (±)-epibatidine for the neuromuscular-type nAChR (torpedo electroplax) [7]. Perhaps as a result of this improved selectivity, ABT-594 has better separation between antinociceptive and lethal doses in mice than (±)-epibatidine [6].

As shown in *figure 1*, ABT-594 also produces dose-dependent ameliorative effects in a variety of pain models in rats [3, 4]. ABT-594 increases latencies to respond to acute thermal stimuli in a Hargreaves paw withdrawal apparatus and markedly decreases nocifensive responses to an injection of formalin in the hindpaw. In both cases, these antinociceptive effects are prevented by pretreatment with the nAChR antagonist, mecamylamine, but not by the opioid receptor antagonist, naltrexone. Simi-

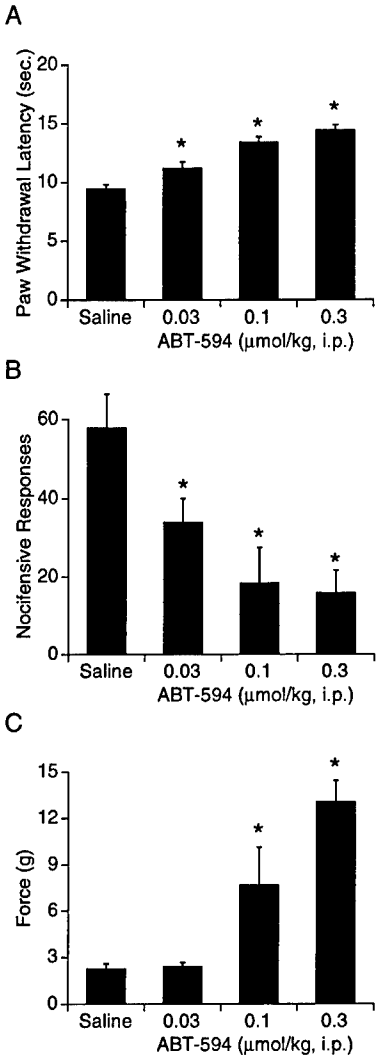


Figure 1. Shown are antinociceptive effects of ABT-594 administered i.p. on measures of acute thermal pain (**A**, paw withdrawal from a focused light source), persistent chemical pain (**B**, formalin injection in hindpaw), and neuropathic pain (**C**, Chung nerve ligation). Data are redrawn from data previously presented in [4]. Shown are means (± S.E.M.). *Difference from control, $P < 0.05$.

larly, in the Chung nerve ligation model of neuropathic pain, ABT-594 reverses tactile allodynia in the affected paw. Again, the effect can be prevented by mecamylamine, demonstrating that it is mediated by activation of nAChRs.

The antinociceptive efficacy of ABT-594 is maintained with repeated dosing [3]. In both the paw withdrawal and the neuropathic pain models, the effects of ABT-594 are maintained after a period of twice daily dosing for 5 days. In contrast, the effects of morphine are reduced under this same dosing schedule, particularly in the neuropathic pain model. Interestingly, effects of ABT-594 on motor performance and body temperature are signficantly attenuated with repeated dosing, suggesting that the mechanisms underlying these effects of ABT-594 are distinct from those underlying its antinociceptive actions.

The antinociceptive effects of ABT-594 in the paw withdrawal and formalin models are significantly attenuated by i.c.v. pretreatment with chlorisondamine [3], a compound that produces a long-lasting blockade of central nAChRs. Thus, at least some of the antinociceptive effects of ABT-594 appear to be mediated by interactions with nAChRs in the central nervous system (CNS). One potential central site of action for ABT-594 is the brainstem, a site of origin for multiple descending inhibitory influences on pain transmission [8]. Pharmacological studies suggest that the nucleus raphe magnus (NRM) may be of particular importance in nAChR-mediated antinociception [11, 12]. Consistent with this proposal, systemically-administered ABT-594 increases expression of c-fos in the NRM, and microinjection of ABT-594 into the NRM produces antinociception in the paw withdrawal model [4, 5].

The antinociceptive effects of intra-NRM ABT-594 in the paw withdrawal model are prevented by mecamylamine coadministered into the NRM. Thus, effects of intra-NRM ABT-594 appear to be mediated by nAChRs in the NRM. However, when this dose of mecamylamine is injected into the NRM prior to systemic ABT-594, the antinociceptive effect of ABT-594 is unaltered, suggesting that activation of nAChRs outside of the NRM also plays a role in the antinociceptive effects of systemically-administered ABT-594.

Activation of AChRs can increase release of serotonin in rat brain [13], and intrathecal administration of serotonin antagonists attenuates the antinociceptive effects of systemic (–)-nicotine [14]. These observations, coupled with recent evidence that serotonergic neurons in the NRM express the α4-containing nAChRs [5], suggest that nAChR-mediated antinociception involves stimulation of serotonergic neurons in the NRM. This hypothesis is supported by the finding that intra-NRM administration of the serotonergic neurotoxin, 5,7-DHT, significantly attenuates the antinociceptive effect of systemically-administered ABT-594 in the paw withdrawal model, albeit incompletely (*figure 2*). This lesion results in a loss of serotonin-containing neurons in the NRM, as measured by tryptophan hydroxylase immunostaining, but not in the dorsal raphe, so the effect appears to involve the serotonergic

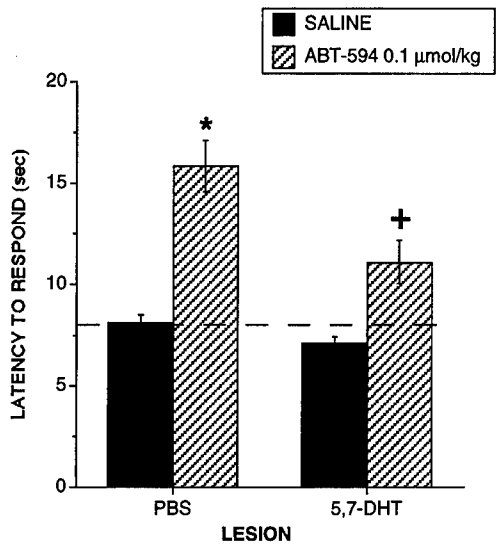


Figure 2. The antinociceptive effects of systemic ABT-594 (0.1 µmol/kg, i.p.) on paw withdrawal latencies were significantly attenuated but not abolished by an NRM lesion 15-22 days prior. For lesion surgery, phosphate buffered saline (PBS; 0.3 µL) or 5,7-DHT (3.75 nmol) was injected into the NRM (coordinates from interaural zero: –2.5 mm A-P, 0.0 mm lateral, and –0.5 mm D-V). Shown are mean latencies to respond (± S.E.M.) to an acute thermal stimulus assessed using a commercially available paw thermal stimulator (Dept. of Anesthesiology, Univ. California, San Diego, La Jolla, CA, USA) as previously described [4]. Latencies were measured 15, 30, and 45 min after systemic injections and averaged for presentation. n = 7–8/group. *Difference from PBS/saline ($P < 0.05$); +Difference ($P < 0.05$) from both PBS/ABT-594 and PBS/saline rats.

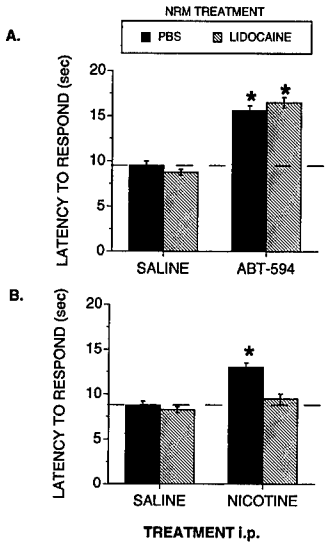


Figure 3. The antinociceptive effects of systemic ABT-594 (0.1 µmol/kg, i.p.) on paw withdrawal latencies were not blocked by an injection of lidocaine (74 nmol in 0.3 µL) into the NRM 5 min prior to ABT-594 (A). However, the antinociceptive effects of systemic (–)-nicotine (1.9 µmol/kg, i.p.) were blocked by intra-NRM lidocaine. Shown are mean latencies to respond (± S.E.M.), as described in legend to figure 2. n = 7–8/group. *Difference from PBS control, $P < 0.05$.

neurons in the NRM specifically. Interestingly, the lesion does not alter all effects of ABT-594, since no lesion effects on locomotor activity effects of systemic ABT-594 are found.

Whereas a serotonin-specific lesion of the NRM attenuates the antinociceptive effect of systemic ABT-594, reversible inactivation of the NRM with lidocaine does not. The antinociceptive effects of systemic ABT-594 are not prevented by inactivation of the NRM with lidocaine (*figure 3A*). In contrast, lidocaine-inactivation of the NRM prevents the antinociceptive effects of systemic (–)-nicotine (*figure 3B*). These results may suggest that the antinociceptive effects of ABT-594 are less dependent on the NRM than are the antinociceptive effects of (–)-nicotine. The distinct nature of the disruption of NRM function by injections of lidocaine and 5,7-DHT may account for the difference in the effects of these two manipulations on ABT-594-induced antinociception. Reversible inactivation of the NRM with lidocaine may not prevent the release of serotonin in the spinal cord since terminals in the spinal cord are still intact and serotonin could be released by activation of presynaptic nAChRs. In contrast, this potential mechanism would not be available in 5,7-DHT-treated rats with permanent destruction of the NRM-spinal serotonergic pathway.

Overall, these findings suggest that ABT-594 can produce antinociception in an acute thermal pain model by activating nAChRs in the NRM and that serotonergic neurons in the NRM play a role in ABT-594-induced antinociception. However, activation of the NRM is not necessary for the effects of systemically-administered ABT-594, implicating the involvement of other mechanisms. Differences in the effects of NRM lidocaine on antinociception produced by systemic ABT-594 and nicotine may be a reflection of more broadly distributed mechanisms of antinociception for ABT-594 than for (–)-nicotine.

Thus, ABT-594 may be acting at several sites. Effects at other brainstem nuclei, such as the locus coeruleus, are under exploration. In addition, there is the possibility that the compound may act directly on primary afferents. In vitro studies have demonstrated that ABT-594 can attenuate capsaicin-induced release of CGRP from spinal cord slices [7] or from

buccal mucosa (C. Flores, personal communication). Reduction of the release of this 'nociceptive' neuropeptide in both the periphery and the spinal cord suggests that antinociceptive effects of ABT-594 could be mediated by direct actions on either end of nociceptive afferents. This view is supported by the demonstration that the responses of wide dynamic range neurons in the spinal dorsal horn to noxious stimulation can be attenuated by direct administration of ABT-594 into either the spinal cord or the peripheral site of noxious stimulation [4]. Moreover, the effects of ABT-594 appear to be selective for nociceptive afferents since the responses of these same neurons to innocuous stimuli are not affected by ABT-594.

ABT-594 has a broad spectrum of antinociceptive activity in rodent models of pain. Moreover, there are multiple potential mechanisms implicated in the preclinical studies conducted thusfar. One interesting possibility is that distinct mechanisms underlie effects in different pain models and that actions at different nAChR subtypes underlie these diverse effects. This added level of complexity makes the ellucidation of mechanisms a more challenging, but also a potentially more rewarding, enterprise.

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Neural substrate of nicotine addiction as defined by functional brain maps of gene expression

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Abstract — The distributed neural networks involved in the intravenous self-administration of nicotine and cocaine, and in a model of relapse of nicotine-taking after abstinence, were compared in Wistar rats. Post-mortem brain maps of c-fos-related antigens expression showed specific activation in prefrontal cortex, anterior cingulate and nucleus accumbens for both drugs, but of the anterior cingulate cortex only during relapse, suggesting that a subset of the neural network involved in drug self-administration is activated during relapse. (©Elsevier, Paris)

Résumé — Activation différentielle des régions cérébrales pendant l'auto-injection de la nicotine chez le rat. La mobilisation de différentes structures cérébrales cibles du système dopaminergique mésocorticolimbique chez le rat qui s'injecte nicotine ou cocaïne, ainsi qu'un modèle de rechute de la prise de la nicotine, ont été étudiés avec l'analyse du changement de distribution de l'expression de la famille des facteurs de transcription c-fos. La cortex cingulate antérieure, et non l'accumbens, sera la seule région activée dans les trois modelés. (©Elsevier, Paris)

nicotine / cocaine / drug self-administration / c-fos / FRAs / dopamine / rat

1. Introduction

Nicotine is critical in the maintenance of tobacco smoking. Nicotine, like cocaine, activates the mesocorticolimbic dopamine (DA) system [1, 4]. Experiments with animals that voluntary press a lever to receive cocaine infusions strongly indicate that the mesocorticolimbic DA system is also a key neuroanatomical substrate for drug-seeking behavior itself. Recent data indicate that also nicotine is self-administered in rats and that the mesocorticolimbic DA system is likely to be involved in nicotine self-administration [2, 10]. However, it is not clear what the involvement of the mesocorticolimbic DA system in the relapse of nicotine self-administration after a period of abstinence is.

A powerful approach to the identification of the neural network implicated in the control of a given behavior is the extensive mapping of brain structures for immediate early gene expression, e.g., c-fos [9] and other members of the c-fos family, the Fos-related antigens [11]. In particular, acute injections of cocaine and nicotine are known to produce transient expression of the c-fos proteins in the nucleus accumbens and striatum, two of the regions of the terminal fields of the mesocorticolimbic DA system.

The goals of our investigations, briefly reviewed in this article, were to produce post-mortem brain maps measuring the expression of c-fos related transcription factors in the terminal fields of the mesocorticolimbic DA system during nicotine self-administration, and to compare them to the maps produced by: 1) cocaine self-administration; and 2) reinstatement of nicotine self-administration after a period of 'abstinence'.

2. Materials and methods

Wistar rats were individually housed in a temperature-controlled environment on a 12-h light-12-h dark cycle with the light switched on at 6:00 a.m. Food and water was freely available until the beginning of the experiments.

2.1. Nicotine and cocaine self-administration

Behavioral testing was conducted in 16 operant chambers. Each chamber was equipped with two levers. Right lever presses ('active lever presses') produced reinforcement delivery (food pellet or i.v. infusion). Data acquisition and schedule parameters were controlled by a Med-PC software. Self-administration techniques were performed as described by Corrigall et al. [2] with minor modifications. Rats ($n = 4$–13) were first trained to lever press for food as a reinforcer, and then, after surgery for catheter implantation, they were re-trained for nicotine (0.03 µg/kg/infusion), cocaine (0.25 mg/kg/infusion), or saline intravenous self-administration with an FR3 schedule, 1 h/day. One group of rats was not trained for lever press, but only placed in the cage for 1 h. About 2 weeks later, rats were killed 60–90 min after the last self-administration session. Their performances, measured in lever press/h, were: nicotine, 53 ± 10; cocaine 49 ± 9, saline 12 ± 5; untrained 6 ± 3. For more details, see Merle Pich et al. [5].

2.2. Model of relapse of nicotine self-administration after abstinence

To assess the relevance of external cue in relapse of self-administration for nicotine, a model based on classical conditioning was established in rats. A group of rats was trained using an auditory stimulus paired with nicotine infusion using the same self-administration paradigm described above, and operated by the same lever controlling the nicotine injector for self-administration. After stabilization of responding (average: 87.3 ± 20.1 lever presses/h), both nicotine and auditory stimuli were removed for 7 days. During this period of 'abstinence' the frequency of lever press in most rats was significantly reduced (18.9 ± 4.3, $P < 0.01$), indicating extinction. When a group of rats was re-exposed to the auditory stimulus previously paired with nicotine infusions, the frequency of lever press in absence of nicotine recovered towards pre-'abstinence' values (38.2 ± 8.4, $P < 0.01$). Rats were killed 90 min after the last session.

2.3. Immunohistochemistry for Fos-like immunoreactivity and image analysis

Ninety min after the end of the last self-administration session, rats were deeply anaesthetized and transcardially perfused with 4% paraformaldehyde. The time chosen for perfusion approximately coincides with the peak levels of Fos antigen after stimuli [8]. C-fos was recognized using a polyclonal sheep antibody (#OA-11-823 Cambridge Research Biochemicals), whereas FRAs were recognized using a polyclonal antibody (gift from Dr. Iadarola). The terminal fields of the mesocorticolimbic DA system were identified using antibodies against tyroxine Hydroxilase (TH, Chemicon), and double-labeling immunocytochemistry was performed using TH and Fos or FRAs antibodies. Computer-assisted morphometrical techniques were used to count the number of Fos-like immunoreactive profiles (IL) in specific anatomical regions in at least three adjacent sections/rat [5, 7] (see *figure 1A–C*). The anti-FRAs antibody was also used for Western blot analysis performed on tissue extract from dissected brain regions, as previously described [5].

3. Results

In summary, exposure to nicotine self-administration produced an increase of Fos-LI in 43 brain regions when compared to 'sham' untrained control, and in 33 brain regions when compared with rats trained previously for food but then exposed to saline. Among these regions, the anterior cingulate, the infralimbic cortex (see *figure 1F*), the shell of nucleus accumbens, all regions of the terminal field of the mesocorticolimbic DA system were found significantly activated (*figure 2*, top) (for more details, see Pagliusi et al. [7]). Interestingly, rats trained for food self-administration and then exposed to saline instead to nicotine showed significant activation when compared with the 'sham' untrained control (*figure 2*).

Exposure to cocaine self administration did not increase the Fos-LI expression when compared to the saline group (*figure 2*), a result consistent with previous reports of downregulation of Fos response to repeated injection of cocaine [3]. However, both nicotine and cocaine increased the levels of FRAs-IL in anterior cingulate cortex, infralimbic cortex, shell and core of the

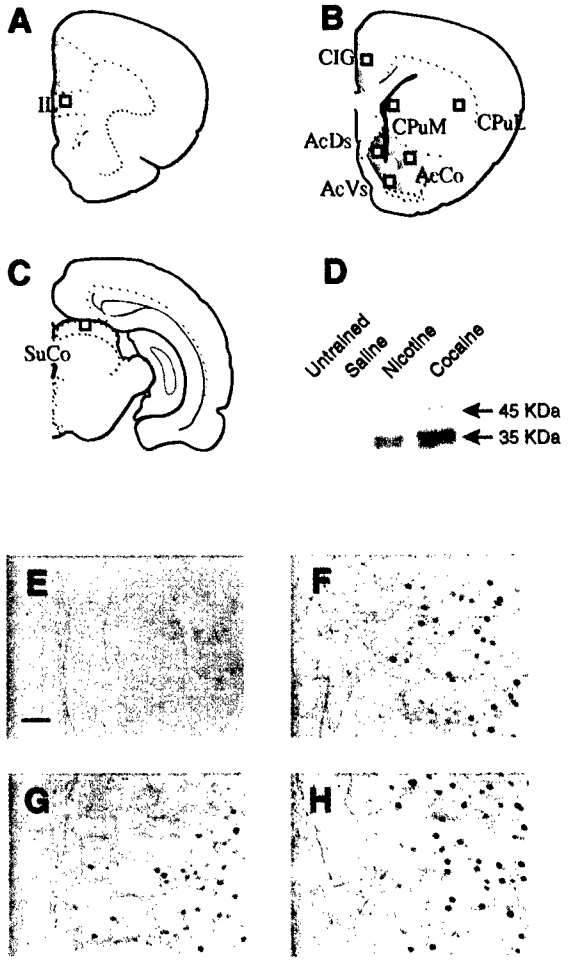


Figure 1. A–C. Cartography of rat brain structures sampled for computer-assisted image analysis. Colored areas indicate the terminal fields of dopamine mesocorticolimbic system, i.e., the prefrontal cortex (blue: IL, infralimbic cortex; CIG, cingulate cortex), the nucleus accumbens (green: AcDs, dorsal shell; AcVs, ventral shell; AcCo, core), the caudate-putament (yellow: CPuM, medial; CPuD, dorsal), and a region devoid of dopamine terminals but rich in nicotinic receptors, the superior colliculus (orange: external layer, SuCo). **D.** Western blot using anti-FRAs antibody on extracts from prefrontal cortex (blue). Data from Merle Pich et al. [5]. **E, F.** Examples of immunocytochemistry for Fos-LI in the infralimbic cortex of 'sham' control rat (**E**); nicotine self-administration rat (**F**). **G.** Saline rat. **H.** Cocaine self-administration rat. Calibration bar 50 μm.

nucleus accumbens, medial caudate, but not amygdala, all terminal fields of the mesocorticolimbic DA system, while only cocaine activated the dorsolateral striatum, the terminal field of the nigrostriatal DA system (*figure 2*, bottom). As shown with Fos-LI, some increase of FRAs-LI was observed in rats trained for food but exposed to saline instead of nicotine or cocaine, suggesting that motivational learning of an operant task can activate the same structures strongly activated by nicotine or cocaine. Gel-shift assays and Western blots on dissected brain structures confirmed these results (*figure 1D*) (for more details, see Merle Pich et al. [5]).

When mapping of Fos-IL expression was performed in brains of rats exposed to the acoustic cue previously paired with nicotine infusion after a period of 'abstinence', an increase of Fos-IL profiles was found neither in the accumbens nor in the infralimbic cortex, but only in anterior cingulate cortex (Chiamulera, in preparation).

4. Discussion

Functional mappings by immediate early genes, in particular Fos and FRAs, provide the identification of neurons transcriptionally active during nicotine self-administration. Newly translated Fos and FRAs heterodimerize with members of the Jun family to form the activating protein-1 (AP-1) complexes, important transcriptional regulators in neurons [6]. Interestingly some FRAs, i.e., the 35 kDa component, does not behave as immediate early

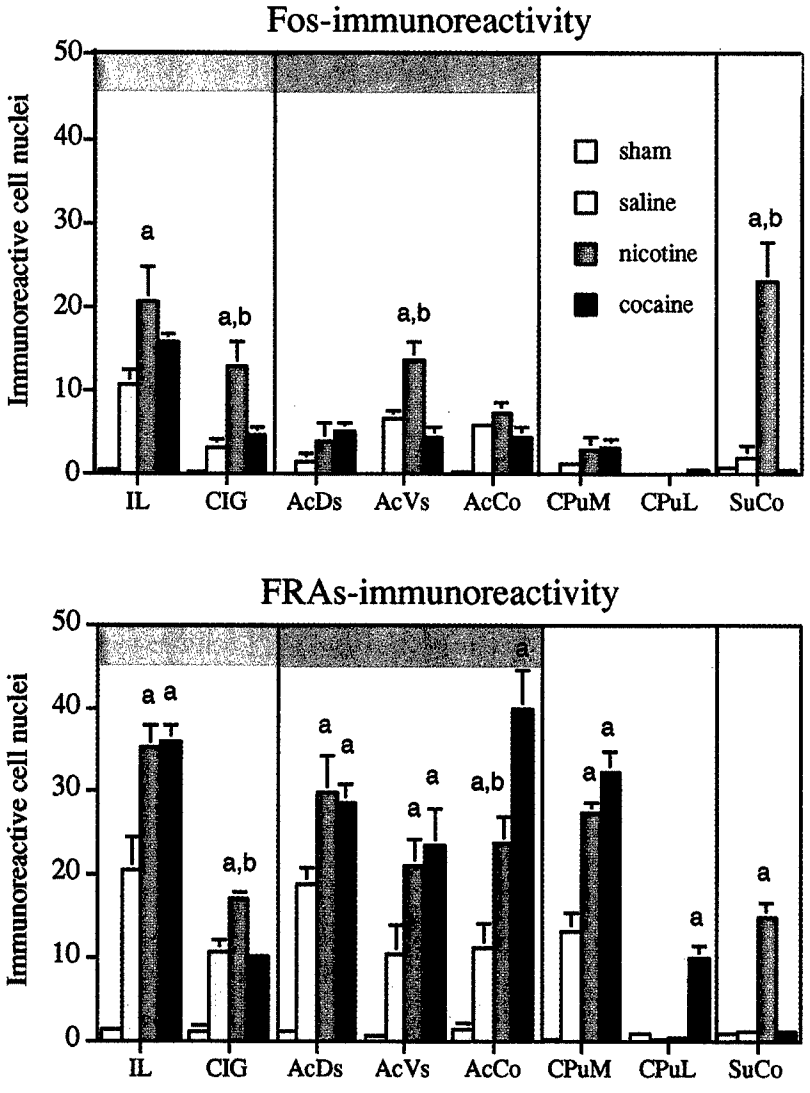


Figure 2. Counts of nuclei of neurons positively stained for Fos-LI (upper panel) and FRAS-LI (lower panel) in the brain regions described in *figure 1*. The colored strip on the top of the histograms indicates the brain areas colored in *figure 1*. Analysis of variance showed significant difference versus saline ('a', $P < 0.01$), or versus cocaine self-administration ('b', $P < 0.01$). Saline, nicotine self-administration and cocaine self-administration groups were significantly different in prefrontal cortex and nucleus accumbens from 'sham' control group ($P < 0.01$, not shown). Part of these data are from Merle Pich et al. [5].

genes, but its product, once induced in neurons, may last for several days [3]. In our studies two antibodies, one that was mainly recognizing Fos (55 kDa) [7], the other recognizing FRAs, and in particular the 35 kDa FRA band [3, 11], were used to identify to substrates of the reinforcing effects of nicotine and cocaine. The two drugs produced largely overlapping maps of 35 kDa FRAs on the terminal fields of the mesocorticolimbic DA system (see also *figure 1D*), indicating a prolonged stimulation of these neurons, and confirming the central role of such system in the motivational aspect of drug-taking. Since repeated cocaine exposure produces a downregulation of Fos response (*figure 2*, top), no comparison was possible between short-term maps of Fos produced by nicotine self-administration versus cocaine self-administration.

However, Fos mapping was used to compare the effects of an external cue paired with nicotine infusion after a period of 'abstinence' in a model of nicotine-taking relapse. Comparisons between the brains of rats exposed or not exposed to the conditioned stimulus alone after 1 week of 'abstinence' revealed that, among the various terminal fields of the mesocorticolimbic DA system, only the anterior cingulate cortex was activated, whereas the nucleus accumbens was not.

In conclusion, the Fos maps of self-administration behavior produced by the presentation of a conditioned cue previously associated with the reinforcing effect of nicotine were more restrictive than the Fos maps of nicotine self-administration, supporting the anterior cingulate cortex involvement in association between the external cue and the reinforcing effect of nicotine.

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Is dopamine important in nicotine dependence?

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Abstract — Nicotine, like other drugs when abused, can produce a wide array of behaviours, some of which collectively propel 'drug-seeking behaviour'. This review focuses on three stimulus properties of nicotine and examines the role of dopamine in mediating each effect with respect to D1 and D2 receptor subtypes. Dopamine appears to be critical in mediating the reinforcing effects of nicotine, which is in line with other commonly abused psychomotor stimulants. However, evidence derived from studies with local microinjections of nicotine suggests that the origin of nicotine action to produce its other stimulus properties may be via multiple neuroanatomical substrates. The aversive stimulus effects are resistant to dopamine receptor antagonists. The discriminative stimulus effects of nicotine, despite showing some modification with dopaminergic compounds, appear not to be solely mediated via the mesolimbic dopamine system. Taken together, the neurobiology of nicotine dependence remains complex. Nonetheless, such dissociation between stimulus properties may permit the development of more effective therapies in combating tobacco dependence. (©Elsevier, Paris)

Résumé — La dopamine intervient-elle dans la dépendance à la nicotine? Comme d'autres drogues induisant une dépendance, la nicotine peut induire des comportements variés, dont l'ensemble produit un 'comportement de recherche de drogue'. Cette revue concerne principalement trois activités stimulatrices de la nicotine et le rôle éventuel de la dopamine dans ces effets, en relation avec les récepteurs D1 et D2. La dopamine est essentielle pour les effets de renforcement, ce qui est aussi observé avec d'autres drogues produisant une stimulation psychomotrice. Cependant, les effets de microinjections locales de nicotine suggèrent que les actions stimulantes de la nicotine passent par de multiples voies neuroanatomiques. Les stimuli répulsifs résistent aux antagonistes des récepteurs dopaminergiques. Les effets discriminatifs de la nicotine sont peu affectés par les agents dopaminergiques et ne semblent pas faire exclusivement intervenir le système mésolimbique dopaminergique. La neurobiologie de la dépendance à la nicotine est donc complexe. Le fait qu'il soit possible de dissocier les différents effets de la nicotine pourrait permettre de mettre au point de nouvelles thérapies pour lutter contre la dépendance au tabac. (©Elsevier, Paris)

reinforcing stimulus / discriminative stimulus / aversive stimulus / neurobiology / mesolimbic dopamine / rodent

1. Introduction

The neurotransmitter dopamine, by far, has occupied a centre stage as the key neural substrate mediating the dependence-producing effects of most commonly abused substances. With cocaine and amphetamines, dopamine is regarded as the critical substrate; manipulations of dopaminergic systems produce dramatic changes in behaviour. However, the evidence to support the involvement of this catecholamine in other drug classes has generated some concern. In line with the proposal that all abused substances share the ability to elevate dopamine levels in the nucleus accumbens [5], the alkaloid nicotine found ubiquitously in tobacco products, was also observed to elevate dopamine levels [6]. This primary observation has led authors to categorise nicotine alongside other illicit substances [11]. Not surprisingly, nicotine has dependence-producing properties; it produces a diverse spectrum of behavioural effects, some of which contribute significantly towards the development of dependence. However, it has since become apparent that dopamine may not mediate all of these behaviours. This paper reviews and presents evidence that supports the notion that dopaminergic processes may not be the sole neural substrate in mediating the behavioural effects of nicotine. In addition to the positive reinforcing stimulus effects of nicotine, the other stimulus properties namely the discriminative and aversive properties, which also play an important role in maintaining and regulating drug intake [21], are considered.

2. Positive reinforcing effects

The ability of nicotine to serve as a positive reinforcer is indicative of all drugs with abuse liability. In humans and laboratory animals, intravenous nicotine, under certain conditions can maintain self-administration behaviour (further details reviewed by [18]). Two key neuroanatomical studies conducted in rodents suggest that a strong dopaminergic basis underlie the reinforcing properties of nicotine. Bilateral lesions of the dopamine terminal regions, the nucleus accumbens (NAC) markedly attenuated the selfadministration of nicotine in rats. The neurotoxin 6-hydroxy-dopamine depleted dopamine by approximately 90% in the nucleus accumbens, while only 25% dopamine was lost in the caudate nucleus. Mo-

reover, the same lesions produced modest changes upon responding for food [2]. These results supported earlier findings from the same authors who had examined dopamine receptor antagonists on nicotine self-administration. The dopamine selective D1 SCH23390 and D2 receptor antagonist spiperone both attenuated responding maintained by intravenous nicotine. However, the administration of these antagonists also reduced both operant responding for food and locomotor activity [1]. Despite these non-specific effects of dopamine antagonists, it was argued that the motor impairment was not the cause of decreases in nicotine self-administration since responding was attenuated in the latter half of the sessions. A study conducted in cigarette smokers confirmed the involvement of dopamine receptors in the rewarding effects of nicotine. Compared to placebo, the administration of the dopamine antagonist haloperidol increased blood levels of nicotine, an effect thought to reflect a compensatory increase in smoking in order to obtain the rewarding effect of nicotine [4].

3. Aversive stimulus properties

As discussed above, the positive reinforcing effects of nicotine represent the key element that drives drug-taking behaviour, however, paradoxically nicotine can also serve as an aversive stimulus. These aversive effects have been implicated in the regulation of nicotine intake, perhaps by setting an upper limit to the amounts consumed [9, 15]. Conditioned taste aversions have been used to demonstrate the aversive stimulus properties of a drug, in which rats will readily learn to avoid a distinctively flavoured solution when its consumption was previously paired with administration of nicotine [7, 10].

In the context of dopamine, studies have been limited to those with dopamine receptor antagonists. Indirect evidence has also come from studies inducing taste aversions by microinjecting nicotine locally into dopaminergic nerve terminal regions. *Figure 1* illustrates results from such a taste aversion conditioning experiment in which nicotine was injected into a variety of brain regions [17]. Following two conditioning trials, CTAs were observed in two groups that had received injections into the interpeduncular nucleus or the nucleus accumbens. However, only intraaccumbens administered nicotine produced CTA consistently; an effect that was blocked by mecamylamine, indicating a nicotinic receptor mediated phenomenon. This result was initially perceived as intriguing given the role of the nucleus accumbens in mediating the positive reinforcing effects. Nonetheless, several investigators have shown that the nucleus accumbens can also mediate aversive affects of drugs [16] or of drug withdrawal states [8, 20]. However, the CTAs induced by intra-accumbens administration of nicotine do not provide direct evidence that it is mediated by dopamine.

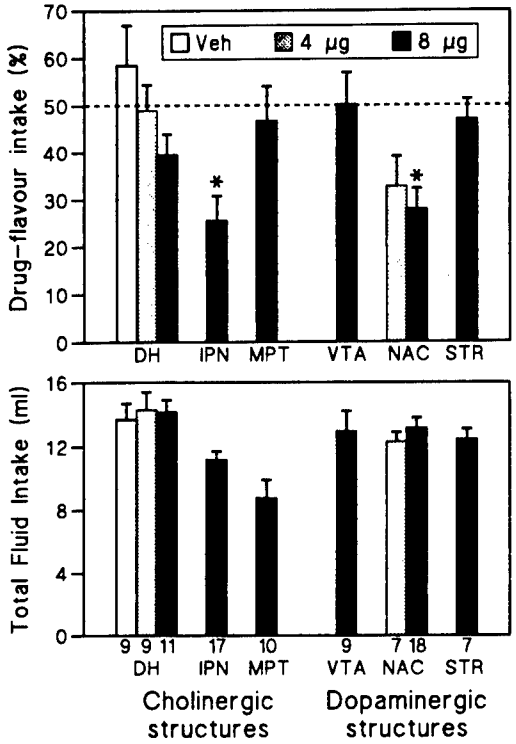


Figure 1. Taste aversion conditioning with nicotine injected into dorsal hippocampus (DH), interpeduncular nucleus (IPN), mesopontine tegmentum (MPT), ventral tegmental area (VTA), nucleus accumbens (NAC) or striatum (STR). The top section shows the percentage of total fluid intake that was in form of drug-paired flavoured solution in two-stimulus tests where both nicotine- and saline-paired flavoured solutions were presented simultaneously; asterisks denote significant CTA ($P < 0.001$) consumption less than 50%. The lower section shows the total fluid intake; the number of rats for each region is shown on the horizontal axis. Reproduced with permission from Shoaib and Stolerman [17].

In an early study, doses of the dopamine D2 receptor antagonist pimozide that blocked apomorphine-induced CTAs were ineffective against nicotine-induced CTA [12]. Furthermore, pretreatment with the D1 receptor antagonist SCH23390 failed to block nicotine-induced CTAs. *Figure 2* shows the effects of pretreatment with various doses of SCH23390 (0.01–0.09 mg/kg SC) administered 30 min prior to nicotine injection (0.4 mg/kg SC). In all four groups, rats showed significant avoidance of the flavoured solutions previously paired with ni-

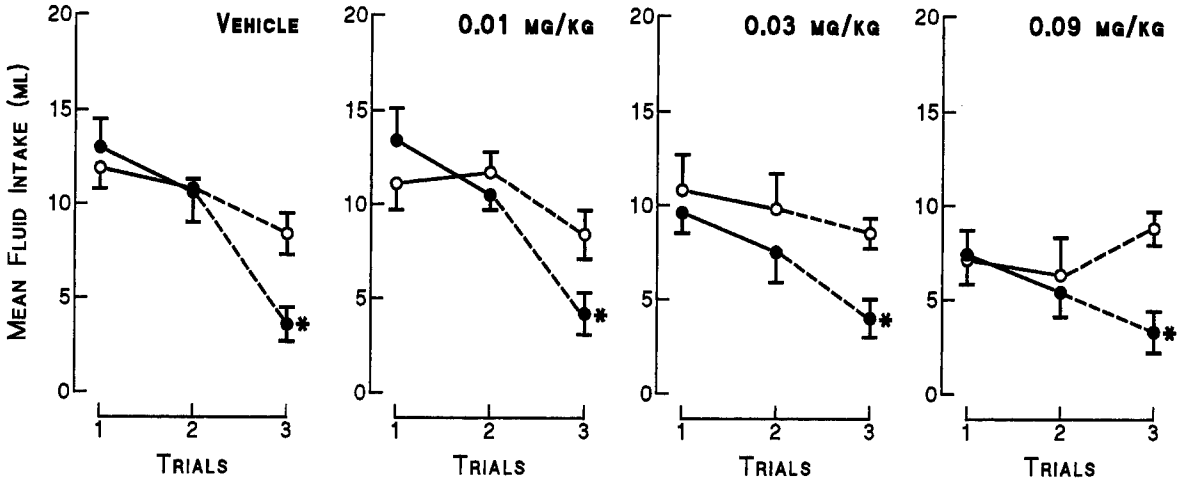


Figure 2. Effect of SCH23390 pretreatment on nicotine-induced conditioned taste aversions (n = 8). Trials 1 and 2 were conditioning sessions and trial 3 was two-stimulus test with simultaneous presentation of both flavoured solutions. Vertical bars represent the mean ± S.E.M. The lines show intakes of flavoured solutions associated with nicotine (●) or saline (○) for each group of rats. The far left figure shows a conditioned taste aversion induced by nicotine (0.4 mg/kg SC). The other figures show taste aversions induced by the same dose of nicotine that was not blocked by various doses of SCH23390 (0.01–0.09 mg/kg SC) administered 30 min prior to presentation of flavoured solutions on conditioning trials 1 and 2.

cotine administration (*figure 2*). A one-way ANOVA confirmed the apparent absence of effect by SCH23390 pretreatment [F(3,28) = 0.19, *n.s.*]. The doses of SCH23390 were behaviourally active since SCH23390 significantly reduced the amounts of fluid consumed during the conditioning trials [F(3,28) = 21.2, $P < 0.0001$].

4. Discriminative stimulus effects

The observation that humans and laboratory animals are capable of discriminating nicotine from saline has been thought to be an important attribute of a drug that supports drug-seeking behaviour. In studies exploring the neural basis of nicotine discrimination, Rosecrans and Meltzer [14] demonstrated dosedependent generalisation to intracerebral microinjections of nicotine in rats trained to discriminate systematically administered nicotine. Both the dorsal hippocampus and the reticular formation were implicated as neural substrates of the discriminative properties of nicotine. More recently, Shoaib and Stolerman [19] confirmed the involvement of the dorsal hippocampus as a mediator of the discriminative stimulus function. Infusion of similar doses of nicotine into the mesolimbic dopamine terminal failed to produce nicotine-like effects. *Figure 3* illustrates the degree of substitution observed with intrahippocampal and intra-nucleus accumbens injections.

The lack of effect associated with local injection of nicotine into the mesolimbic dopamine system appears inconsistent with findings using dopamine receptor agonists and antagonists. Both D1 and D2 receptor antagonists have been shown to attenuate the discriminative stimulus effect of nicotine [13], however Corrigall et al. [3] have suggested that the effects of dopamine receptor antagonist effects are nonspecific. Moreover, the same study showed an absence of substitution of nicotine to GBR 12909, a dopamine reuptake inhibitor [3]. With dopamine receptor agonists, partial substitution was observed with SKF 38393, a D1 agonist, while no significant effect was observed with quinpirole, a D2/D3 agonist [13]. To date, no studies have examined the influence of neurotoxin lesions to resolve the involvement of dopamine in the cueing effects of nicotine.

5. Discussion

From the evidence presented in this short review it is clear that like other abused drugs, nicotine also relies on dopamine to mediate its reinforcing effects via the mesolimbic pathway originating from the ventral tegmental area and projecting to the nucleus accumbens. However, this projection may not be critical in the transduction of the other stimulus properties of nicotine. The aversive stimulus despite showing some involvement with the nucleus accumbens may rely upon non-dopaminergic processes.

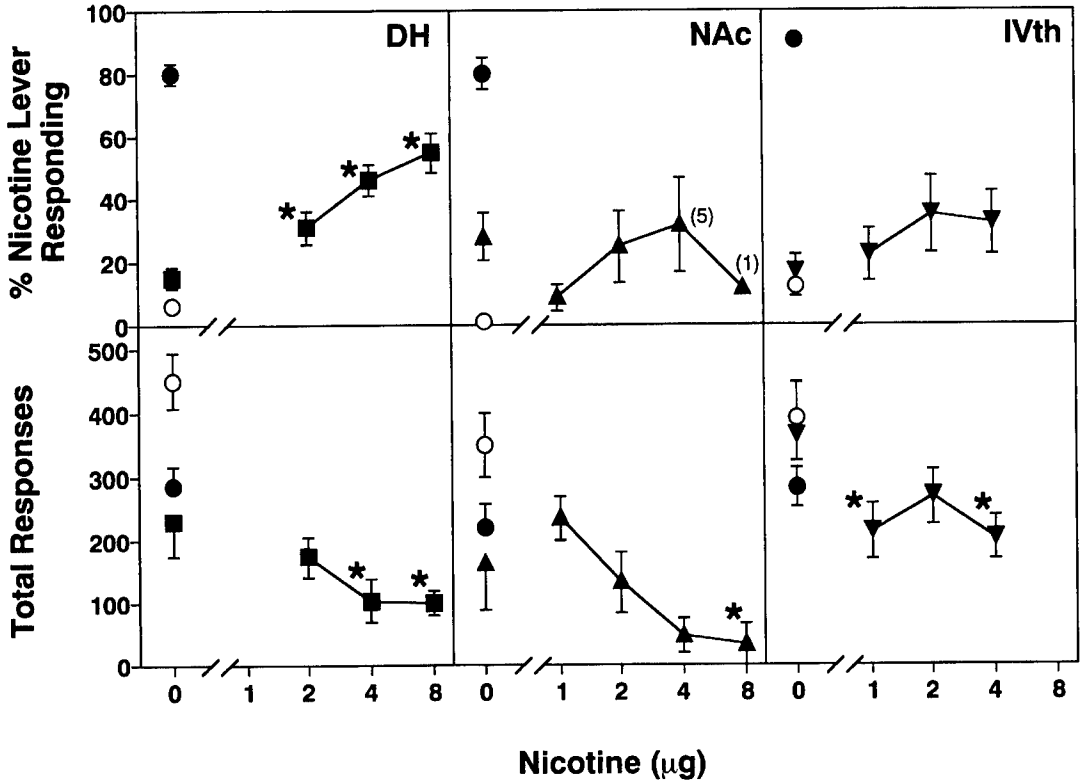


Figure 3. Effect of administering nicotine into the dorsal hippocampus (DH, ■), nucleus accumbens (NAc, ▲), and the fourth ventricle (IVth, ▼) on percent responding on nicotine-appropriate lever and on total number of responses for groups of 9–10 rats trained to discriminate nicotine (●) from saline (○). Points above 0 on the abscissae show results for the subcutaneous injections of saline and nicotine (0.2 mg/kg) and for intracerebral injections of vehicle. Each point represents the mean ± S.E.M. of observations made in 5-min extinction tests. Numbers in parentheses represent the mean number of animals in cases where suppression of responding precluded collection of data for discriminative effects for some rats. Asterisks denote significant increase in comparison with microinjection of vehicle or significant decreases in total responses as determined with Dunnett's *t*-test ($P < 0.05$). Reproduced with permission from Shoaib and Stolerman [17].

The neural substrate for this stimulus has yet to be fully elucidated. What is more puzzling is the observation that dopamine receptor agonists and antagonists can produce or modify the discriminative stimulus effects of nicotine, while no generalisation is observed following the local application of nicotine into dopaminergic nerve terminals. It would appear that dopamine may not be important in the aversive and cueing properties of nicotine. Such dissociation between stimulus properties presents a potential opportunity to harness in treating nicotine-based addictions.

Acknowledgments

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Brain choline has a typical precursor profile

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Abstract — Choline is product and precursor to both acetylcholine and membrane phospholipids, and, in the brain, is ultimately provided by the circulation. The brain is protected from excess choline and choline deprivation by a refined system of homeostatic mechanisms that maintain a level of extracellular choline that, for its role as precursor, meets saturation criteria under normal conditions. The kinetic and activity profiles of choline are typical for a biosynthetic precursor. (©Elsevier, Paris)

Résumé — La choline cérébrale a un profil typique de précurseur. La choline est à la fois le produit et le précurseur de l'acétylcholine et des phospholipides membranaires et, dans le cerveau, est finalement fourni par la circulation. Les profils cinétiques et d'activité de la choline sont typiques d'un précurseur de biosynthèse. (©Elsevier, Paris)

choline / acetylcholine / cholinergic mechanisms / brain

1. Brain choline: a precursor and an agonist?

Choline (Ch) is the biosynthetic precursor to phospholipids, platelet-activating factor and acetylcholine (ACh). As to the role of Ch in regulating cholinergic mechanisms, it has been discussed for almost a century whether endogenous choline not only serves as a precursor to ACh but also is an agonist at muscarinic and nicotinic receptors under in vivo conditions (see [28, 37]). Clear-cut evidence for agonist properties at both receptor types had been obtained even before Otto Loewi in 1921 detected the neuronal release of the 'Vagusstoff'. The question: "Is Ch an in vivo agonist?" is difficult to answer for two reasons: firstly, agonist effects have been observed only in vitro using high concentrations of Ch. Secondly, there is a lack of specific Ch antagonists that allow to discriminate between effects of Ch and ACh.

Muscarinic peripheral responses of heart, intestine and blood pressure have been described for Ch at concentrations between 40 and 8000 μM (see [28]). The frequently observed enhancement of brain ACh levels by Ch administration which is presumably due to a precursor-induced enhancement of the biosynthesis of ACh ([3]; see below) had been explained alternatively by a central muscarinic response due to the agonist properties of Ch [9].

Nicotinic effects of Ch on blood pressure, sympathetic ganglia and skeletal muscle preparations have been studied frequently in the past; effective concentrations were above 100 μM (see [28, 37]). Recently a specific activation of the α7-subtype of nicotinic receptor by Ch was detected in neuronal cells [1, 31], the EC_{50} being 1.58 mM. Almost the same values have been reported for displacement studies using L-^{3}H-nicotine binding in various brain regions (K_i = 1.3 mM) and for the Ch-induced release of the adrenal hormone (EC_{50} = 1.3 mM; [37]). Alkondon et al. [1] suggested that Ch may act in vivo as an endogenous specific ligand for these nicotinic receptors and thereby may contribute to the glutamatergic initiation of long-term-potentiation (LTP).

The present study summarizes the evidence that the kinetic profile of brain choline is typical for a nutrient precursor and does not lend support for a role as agonist.

2. Choline levels under resting conditions

The plasma level of free Ch under resting conditions is remarkably constant at around 10 μM in man and mammals. Ch-deficient diet lowers the plasma level to about 5 μM, whereas a Ch-rich diet leads to plasma levels of about 15 μM [16, 42]. Excessive intake of egg or soy lecithin caused a transient increase of maximally up to 40 μM [42]. These figures are remarkably constant at a low level considering the large reservoir of intracellular Ch (≥ 50 μM) and of Ch bound to phospholipids in the plasma (> 1 mM) and in cell membranes. The efficiency of homeostatic mechanisms regulating the concentration of free Ch in the plasma is obvious, but still allows dietary fluctuations ranging from 5 to 15 μM (under extreme conditions up to 40 μM). Such dietary fluctuations of the plasma level are typical for essential nutrients such as Ch.

In the brain, homeostatic mechanisms (see below) protect the tissue even from the above dietary fluctuations of plasma Ch levels [11, 27]. In the phy-

siological range of 5–15 μM the average extracellular concentration of Ch reflected by the CSF choline was kept constant at approximately 4–6 μM (Tucek [35], estimated 3 μM). The threshold plasma level leading to a rise in free brain Ch was almost 100 μM (after i.p. injection of 60 mg/kg). Ch-deficient diet had no effect on the CSF Ch, although the plasma level was reduced to about 5 μM. Only chronic Ch-supplemented diet led to a significant, but small increase in the CSF Ch level (15.3 μM [16].

The assumption that the extracellular Ch level is basically similar throughout the brain irrespective of a cholinergic innervation is evident from a large number of studies (e.g., [19, 20, 34].

3. Homeostatic control of brain choline

Ch is transported through the blood-brain barrier by a choline transport system (K_m = 442 μM; V_{max} = 10 nmol/g/min [4]) which is unsaturated at physiological plasma levels (10 μM). An increase of brain Ch uptake due to acute dietary elevation of the plasma level does not enhance extracellular Ch because newly taken up Ch is rapidly removed by brain cells [10, 11] preferentially by glial cells [41]. It deserves mention that glial cells in the brain are about 10 times more numerous than neurons. Koshimura et al. [20] injected 10 μmol Ch intracerebroventricularly and found a drastic increase of the rat striatal Ch content from 60 to 500 nmol/g, whereas the extracellular free Ch level as determined by the microdialysis technique showed a 50% increase only. After cellular uptake, surplus Ch is phosphorylated and slowly incorporated into phospholipids [11]. Uptake with subsequent phosphorylation is rapid and efficacious and protects the brain from increases in extracellular and intracellular free Ch levels.

Surplus bound Ch is eliminated from the brain as free Ch by a protracted release into the venous blood resulting in a negative arterio-venous difference [10, 11, 16, 27]. Ch net-release from the brain and net-uptake into the brain are in a dynamic equilibrium. Thus the negative arterio-venous difference is reversed into the positive (net-uptake of choline) when the plasma level rises due, for example, to acute dietary intake; the 'reversal point' was found to be about 15 μM in the rat [10]. Chronic Ch supplementation, which only slightly elevated the plasma level from 10 to 14 μM, drastically shifted the reversal point from 15 to 40 μM [16]. This result demonstrates the remarkable adaptation of the brain Ch homeostasis to chronic Ch load.

Knowledge about the regulation of uptake and release of free Ch on a cellular level is essential for the understanding of the brain homeostatic control of free extracellular Ch inasmuch as there is at least 10 times more intracellular Ch [36] and about 600 times more bound Ch than extracellular Ch. This holds true even more so for the control of the synaptic concentration of free Ch.

4. Cellular uptake and release of choline in the brain

The transmembrane gradient of free Ch (> 10) is dependent on the membrane potential and the high-affinity Ch uptake (HACU; K_m = 1–2 μM) present in cholinergic nerve terminals [36]. Tuček [36] suggested that the transmembrane gradient is due to the positive charge of Ch and the electronegativity on the inside of the plasma membrane. For example, HACU is reduced during K^+-depolarization, but is markedly enhanced at a time when the ACh store is depleted and the terminal is allowed to repolarize; this enhancement may be due to after-hyperpolarization and/or to mass action [38]. Protein kinases phosphorylating the transporter may regulate the V_{max} of the HACU [39]. Whatever mechanism controls the transmembrane gradient, there are various important mechanisms of cellular uptake and release of Ch.

In nervous tissue, extracellular Ch is taken up into neurons and glial cells by specific cellular uptake mechanisms. Cholinergic nerve endings possess the hemicholinium (HC-3)-sensitive HACU and a low-affinity uptake. It seems, that essentially the HACU is associated with ACh synthesis under normal conditions [39]. Also glial cells are equipped with some kind of a HACU [29]. In the leech central nervous system, electrophysiological and autoradiographic studies provided good evidence that glial cells more effectively accumulate Ch than neurons; ACh itself was not taken up but was metabolized to Ch which was subsequently removed by glial cells [41].

As mentioned above, surplus bound Ch (e.g., phosphatidylcholine) is eliminated by release of free Ch into the extracellular space and back into the blood via the CSF compartment [16, 27]. There are many conditions, such as hypoxia, elevation of Ca^{2+}, receptor-activation by transmitters and hormones, which enhance the extracellular Ch level by mobilizing free intracellular Ch or bound Ch. Receptor-activation of phospholipases (PLs), such as PLA_2 and PLD, enhance the release of choline from phospholipids. For the first time, a receptor-mediated release of Ch was observed in the isolated chicken heart and in the rat cortex in vivo [5, 23]. Unexpectedly physostigmine, bethanechol and oxotremorine enhanced the release of Ch from rat cortex and brain in vivo in an atropine-sensitive manner [2, 5, 14]; these observations were confirmed using the mi-

crodialysis technique in frontal cortex, hippocampus and striatum [34]. The muscarinic increase of the extracellular Ch concentration was apparently due to activation of PLD [13, 25]. Likewise glutamatergic activation of PLD and the release of Ch were recently observed in rat hippocampal slices [17]. In primary cultures of rat astrocytes, we observed a muscarinic and adrenergic stimulation of PLD activity [8]. All manipulations that cause a rise in the cytoplasmic Ca^{2+} level, e.g., hypoxia and receptor activation (adrenoceptors, NMDA receptor), are expected to enhance the release of Ch via PLA_2 activation [12, 15, 22].

5. Free choline and ACh synthesis

ACh synthesis is dependent on neuronal uptake of Ch from the extracellular space. The extracellular Ch represents a general pool that is maintained by a variety of sources, mainly plasma Ch, free intracellular Ch, Ch-containing phospholipids (hydrolyzed by PLD and PLA_2; see above) and ACh (hydrolyzed by ChE). The relative importance of these sources varies depending on the location. Thus ACh hydrolysis and muscarinic mobilization of Ch from phospholipids (see above) are exclusively operating in the cholinergic synapse.

Under normal conditions, the HACU into the cholinergic nerve ending is the rate-limiting step for the synthesis of ACh; inhibition of the HACU by hemicholinium-3 blocks ACh synthesis [26, 28] and release [20]. The extracellular Ch concentration (approximately 4–6 μM), which is above the K_m (1–2 μM) of the HACU, is not rate-limiting unless the synaptic Ch concentration is reduced. On the first glance, it may appear paradoxical that stimulation of ACh release, e.g., by scopolamine [34] or electrical nerve stimulation [24] lowers the extracellular Ch concentration and the ACh tissue content. Stimulation-induced activation of the HACU leads to a decrease in the synaptic Ch level which then may become rate-limiting for the ACh synthesis (see below). In this case, the subsequent reduction of the ACh tissue content can be prevented by exogenous Ch or lecithin (see [35, 36]. Therefore it is plausible that the stimulated, but not the basal ACh release in the rat hippocampus in vivo could be enhanced by exogenous Ch and by nicotinamide [18, 19]; nicotinamide mobilizes endogenous Ch from phospholipids [7]. Likewise it was shown that a phospholipase D-catalyzed hydrolysis of phosphatidylcholine can provide Ch as precursor to ACh synthesis [21].

According to the above described mechanisms of brain Ch homeostasis and to previous data [40], exogenous Ch increases the ACh release by enlarging the pool of surplus bound Ch and the availability of bound Ch as precursor of ACh synthesis. The availability of Ch from phosphatidylcholine can be enhanced by ACh itself through muscarinic receptor activation in the sense of a positive feedback control of ACh synthesis [13].

In the absence of a cholinesterase inhibitor, the extracellular level of Ch showed a biphasic change in response to cholinergic nerve stimulation, namely an initial peak due to hydrolysis of the released ACh followed by a depletion caused by activation of the HACU (see [24, 28]; see above). The response of the extracellular Ch level to cholinesterase inhibition is difficult to predict, especially for a long-lasting neuronal activity: stimulation of the HACU is opposed by the muscarinic release of Ch from phospholipids (see above).

6. Concluding remarks on the synaptic concentrations of choline

Although absolute concentrations of Ch and ACh within the synaptic cholinergic compartment are difficult to monitor, the following conclusions seem justified (for details see above).

1. Under resting conditions, the synaptic (extracellular) concentration of Ch is approximately 4–6 μM, i.e., when ACh is not released to a significant extent and the general homeostatic mechanisms determine the extracellular Ch concentration. In this situation the source of the extracellular Ch is mainly phosphatidylcholine, which represents an almost inexhaustible pool for free Ch. This Ch pool is subject to a variety of receptor-regulated mechanisms. In the absence of ChE inhibition, the efflux of ACh from rat cortex into the microdialysate in vivo was 0.28 fmol/min and that of Ch 330 fmol/min [33]. Inhibition of the ChE activity by 80% enhanced the ACh efflux to 1.3 fmol/min (> 700%), which corresponds to a small fraction of the extracellular Ch.

2. Stimulation of cholinergic nerves for a short period leads to a biphasic change of the synaptic Ch level, a brief increase (parallel to the rise of ACh) and a longer-lasting decrease due to neuronal uptake. The decline occurs at the sites of the HACU which are located within the synaptic compartment. During prolonged neuronal activation the decrease of synaptic Ch is dominating, apparently because the Ch originating from ACh hydrolysis is only partially recycled and is eliminated by the homeostatic mechanisms of brain Ch to a significant extent.

3. The idea that Ch may serve as a muscarinic and/or nicotinic agonist has been proposed since several decades. The above kinetic considerations make such a physiological role for the weak agonist

Ch unlikely. It is obvious that Ch cannot cumulate within the synaptic or extrasynaptic compartments. However, it cannot be totally excluded that the synaptic concentration of Ch rises for milliseconds at the ACh release sites to a significant extent and stimulates receptors located in the immediate vicinity [1]. The assumption that ChE inhibition may worsen the symptoms of cholinergic dysfunctions present in Alzheimer's disease, because allegedly the synaptic concentration of Ch is reduced [32], is not compatible with the homeostatic control of brain Ch and the beneficial effects of the ChE inhibitors used for the treatment of this disease.

4. The kinetic and activity profiles of Ch are typical for a biosynthetic nutrient precursor to a neurotransmitter. The brain is protected from a precursor excess by homeostatic mechanisms. However, under certain experimental, pathophysiological and pharmacological conditions Ch supplementation may enhance the release of ACh and may have discrete effects on certain membrane properties such as the density of cholinergic or non-cholinergic receptors [6, 30].

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Positive effects of allosteric modulators on the binding properties and the function of muscarinic acetylcholine receptors

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Abstract — Data are reviewed indicating that allosteric modulators can enhance the affinities of muscarinic receptors for their antagonists and agonists, that the enhancement of the affinity for agonists is relevant functionally, and that the allosterically induced conformational change also affects the interaction between the receptors and the G proteins. (©Elsevier, Paris)

Résumé — Les effets positifs des modulateurs allostériques sur les propriétés de liaison et la fonction des récepteurs muscariniques de l'acétylcholine. Les modulateurs allostériques peuvent augmenter l'affinité des récepteurs muscariniques pour leurs antagonistes et leurs agonistes. L'augmentation d'affinité pour les agonistes est fonctionnellement significative. Le changement conformationel, induit allostériquement, modifie l'interaction entre les récepteurs et les protéines G. (©Elsevier, Paris)

muscarinic receptors / presynaptic receptors / allosteric regulation / alcuronium / acetylcholine

1. Introduction

It has been known for more than two decades that certain neuromuscular blockers act as antagonists at muscarinic receptors. In a now classical study, Clark and Mitchelson [2] have shown that the antagonistic action of gallamine on muscarinic receptors in guinea-pig heart atria is allosteric, and Stockton et al. [25] demonstrated the allosteric nature of the action of gallamine by analyzing its effects on the binding of N-[^{3}H]metylscopolamine ([^{3}H]NMS) to cell membranes. Data on the inhibition of muscarinic receptors by neuromuscular blockers have been reviewed by Lee and El-Fakahany [16].

We have discovered that a neuromuscular blocker (alcuronium) can enhance (rather than diminish) the binding of a classical muscarinic ligand ([^{3}H]NMS) to muscarinic receptors in the heart, cerebellum and ileum, and that it does so by increasing the affinity of the receptors for the classical ligand [21, 28]. Our observations demonstrated unequivocally that the allosteric ligand binds to the receptor simultaneously with the classical ligand, and suggested that a new way may exist of how to enhance signal transmission at muscarinic synapses. Subsequent work in which we became involved developed in four main directions: i) allosteric enhancement of the affinity of muscarinic receptors for their antagonists; ii) location of the allosteric binding site; iii) allosteric enhancement of the affinity of muscarinic receptors for their agonists and its functional relevance; and iv) allosteric modulation of the interaction between the receptors and the G proteins (allosteric activation of the receptors). The following is a short overview of our findings.

2. Allosteric enhancement of the affinity for antagonists

Alcuronium-induced enhancement of the affinity of muscarinic receptors for [^{3}H]NMS is subtype specific and only occurs at the M_2 and M_4 receptor subtypes. The affinities of the M_1, M_3 and M_5 receptor subtypes for [^{3}H]NMS are diminished by alcuronium, and so are the affinities of all receptor subtypes for [^{3}H]quinuclidinyl benzilate ([^{3}H]QNB) [9, 11, 28]. The difference between the effects of alcuronium on the affinities for [^{3}H]NMS and [^{3}H]QNB cannot be explained by the presence of quarternary nitrogen in the former and its absence from the latter antagonist because alcuronium also enhances the affinity of the M_2 receptors for [^{3}H]atropine (a compound without a quarternary nitrogen) and diminishes their affinity for N-[^{3}H]methyl-QNB (a compound with a quarternary nitrogen) [6]. Cellular components other than the muscarinic receptors are probably not necessary for the enhancement of the affinity for [^{3}H]NMS since the phenomenon can also be observed on solubilized receptors uncoupled from G proteins [19].

Other compounds with the potential to allosterically enhance the binding of ligands to the classical (orthosteric) binding sites of muscarinic receptors include strychnine [15, 23], brucine [1, 13], eburnamonine [13, 24], vincamine [13], fangchinoline and tetrandrine [4] and 9-methoxy-α-lapachone [5]. It

has not been clarified which features of the molecule of the allosteric modulator are important for its positive cooperative effect with regard to the binding of orthosteric ligands. In fact, no allosteric modulator of muscarinic receptors is known which would produce only positive effects. The direction of the interaction (positive or negative) varies depending on the modulator, the orthosteric ligand, and the subtype of the receptor.

3. Location of the allosteric binding site

Alcuronium and other allosteric modulators of muscarinic receptors slow down both the association of ligands with and their dissociation from the orthosteric binding sites [7, 20]. Data on the effects of alcuronium on the kinetics of [^{3}H]NMS binding can be explained on the assumption that alcuronium (when attached to the allosteric binding site) hinders both the access of [^{3}H]NMS to and its departure from the orthosteric binding site [22]. This led to the proposal that the binding site for alcuronium is located close to but more extracellularly than the orthosteric site, perhaps at the entry to the binding pocket of muscarinic receptors [22]. The proposed location of the allosteric binding site received support from experiments with site-directed mutations of muscarinic receptors [17, 18] and with chemical modification of their functionally important amino acid residues [8, 10]. Allosteric modifiers protect the tyrosine and aspartate residues in the area of the orthosteric binding site against chemical modification, apparently by hindering the access of the modifiers to the orthosteric site [8]. By using chemical modifications, it proved possible to separate the action of alcuronium on the kinetics of [^{3}H]NMS binding and on the affinity of receptors for [^{3}H]NMS, which indicates a degree of independence of these two actions of alcuronium and other allosteric ligands [10]. Allosteric modulators with positive and negative effects on the affinity for [^{3}H]NMS compete for the same binding domain [23].

4. Allosteric enhancement of the affinity for agonists and its functional relevance

The effects of allosteric ligands on the binding of muscarinic receptor agonists were little studied because the affinity for agonists is generally lower than that for the antagonists and it is difficult to utilize the conventional radioligand binding methods. We applied an indirect approach and investigated the effects of five allosteric modulators on the affinities of the M_1-M_4 muscarinic receptors for twelve muscarinic agonists [13]. We have found that the affinity for acetylcholine is enhanced by brucine at the M_1 and M_3 receptors and by eburnamonine at the M_2 and M_4 receptors. In general, the allosteric enhancement of the affinity for various agonists was low. It was the highest for the combination of eburnamonine and pilocarpine at the M_2 receptors (25-fold). Allosteric enhancement of the affinity for acetylcholine by brucine and its derivatives was also reported by Birdsall et al. [1].

To detect whether the increase in the affinity for muscarinic agonists detected in radioligand binding experiments is of functional relevance, we performed experiments in which we measured the inhibitory effect of muscarinic receptor agonists (acting on the presynaptic muscarinic receptors) on the release of acetylcholine from rat striatal slices. We discovered that the inhibitory actions of furmethide, oxotremorine-M and bethanechol were potentiated by brucine, in accordance with what could be expected from data on the allosteric interaction between the binding of brucine and the three agonists to the M_4 receptors [3].

5. Allosteric activation of muscarinic receptors

Allosteric modulators induce a change in the conformation of the orthosteric binding site, which results in the change of the affinity for the orthosteric ligands. It is a question whether the change of conformation only concerns the area of the orthosteric site, or other receptor domains just as well. We have found in experiments on CHO cells expressing individual subtypes of muscarinic receptors that the allosteric modulators, acting in the absence of muscarinic agonists, had agonist-like effects (inhibition of the synthesis of cyclic AMP in cells expressing the M_2 and M_4 receptors, and stimulation of the production of inositol phosphates in cells expressing the M_1 and M_3 receptors) [12]. These effects were not observed in cells which had not been transfected with the genes for muscarinic receptors, and were not blocked by QNB (indicating that the modulators did not bind to the orthosteric binding site). It is thus apparent that muscarinic receptors can be activated not only from their orthosteric, but also from their allosteric binding sites. The activating effect could be also observed in a reconstituted system containing purified M_2 receptors and purified Go proteins. Data obtained in the reconstituted system indicate that receptor activation induced by the allosteric ligands differs from that induced by the orthosteric ligands [14].

6. Conclusion

The discovery of positive cooperativity between the allosteric and orthosteric ligands of muscarinic receptors opens new ways to pharmacological modulation of neurotransmission on muscarinic synapses and adds to the understanding of the molecular physiology of muscarinic receptors [26, 27].

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On the transcriptional regulation of neuronal nAChR genes

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Abstract — The promoters driving transcription of the neuronal nicotinic genes α7 and β3 have been characterized in the chicken. Although their regulatory modalities are thoroughly different, they nevertheless lead to co-expression in the same neurons. (©Elsevier, Paris)

Résumé — La régulation transcriptionnelle des gènes nicotiniques neuronaux. Les promoteurs qui contrôlent la transcription des gènes nicotiniques neuronaux α7 et β3 ont été caractérisés chez le Poulet. Quoique leurs modalités régulatrices diffèrent profondément, elles aboutissent cependant à leur co-expression dans les mêmes neurones. (©Elsevier, Paris)

nicotinic receptors / genetics / physiology / gene expression regulation

1. Introduction

Eleven genes encoding neuronal nicotinic acetylcholine receptors (nAChR) subunits have been identified in vertebrates (for review see [14, 16], and closely related neuronal nAChR genes have been characterized in invertebrate model systems such as *Drosophila melanogaster* (for review see [6] and *Caenorhabditis elegans* [1, 5, 18]. They fall into homology classes that predate the divergence of vertebrates and invertebrates, demonstrating that they have been present as families of related genes since a very early stage in nervous system evolution [10, 13]. Although the availability of cloned neuronal nAChRs has led to a massive improvement in our understanding of their physiology and structure, very little is known about their functions in the central nervous system, and the reason for their multiplicity remains a puzzle. There is evidence that they are mostly, but not exclusively, effectors in the presynaptic control of neurotransmitter release [19]. Their role in nociception [2, 8] and their implication in at least one form of familial epilepsy [17] suggest that they play varied and diversified parts in neural function.

One plausible view has it that the ancestral nAChRs were functional homopentamers, as exemplified by the α7–α9 series of present-day subunits. Such structures are inherently limited in the number of different regulatory interactions they can accommodate, so that evolution retained variants of duplicated genes whose products were able to assemble into heteropentamers of various, non-equivalent compositions. Heteropentamers are much richer in potential regulatory sites than homopentamers because of the vastly increased repertoire of distinct subunit interfaces they provide. In addition to increasing the range of ligands capable of modulatory activities on an increased spectrum of receptor types, heteropentamery also allowed for more modulators to interact with a single receptor species, thereby increasing the range of possible coincidence detectors, a most desirable property in signaling integration [3].

As the repertoire of nAChR subunits increased by gene duplication and by selection of variants capable of novel interactions with ligands, it is likely that the promoters governing the transcription of individual nAChR genes were also submitted to evolutionary pressure tending to select and retain a variety of different transcriptional modalities. Such a process would have the decisive advantage of ear-marking subsets of nAChR genes for particular uses in different tissues (e.g., muscle vs. neuronal receptors), or at different developmental stages (e.g., embryonic vs. adult muscle receptors).

In our laboratory, we have recently examined the promoter regions and associated transcription factors that regulate the activity of the neuronal homomeric α7 and heteromeric β3 nAChR genes. We were prompted to select these genes not only because they are respective examples of the homomeric and heteromeric nAChRs, but also because whereas α7 is widely expressed very early on in neuronal and non-neural precursors [4, 9, 12], later to acquire a more restricted expression domain, β3 has a narrowly restricted expression pattern at all stages of development [7, 11]. We have found that although the two genes are regulated by thoroughly different *cis-trans* interactions, their expression domains overlap in the ganglion cells of the developing retina. These

observations illustrate the diversity and versatility of the regulatory mechanisms that have evolved in parallel with an increasing nAChR gene repertoire.

2. Materials and methods

To determine which region of a cloned nAChR gene does contain all or most of the information required for proper spatial and temporal expression, we examined the DNA sequences located immediately 5′ of the transcription initiation sites. This was achieved by subcloning upstream of reporter genes such as the bacterial β-galactosidase (with added nuclear localization signal) and chloramphenicol acetyltransferase (CAT) genes, transfecting into a range of freshly dissociated neural and non-neural cells from tissues isolated at various developmental stages, and scoring for reporter gene activity. The β-galactosidase reporter enabled us to detect which cell types were capable of activating the promoter, whereas the CAT reporter afforded an easy quantitative assay of the overall activity of a given tissue. This experimental approach is dependent on the standardized, efficient and reproducible lipofectin-mediated transfection procedure we have developed in the laboratory. Properly used, the technique permits efficient transfection of many types of neural (neuroretina, optic tectum, telencephalon, spinal cord, peripheral ganglia, etc.) and non-neural tissues at all developmental stages [12]. Transfection efficiency reaches 30%, and transfection-induced neuronal death appears minimal.

3. Results and discussion

3.1. The α7 promoter

A short DNA fragment, 177 base pairs (bp) in length and encompassing the two major transcription cap sites, contains essentially all the information required for tissue and stage specific control [12]. In the retina, the 177-bp fragment drives reporter gene transcription in the same cells (i.e., the ganglion cells) and at the same developmental stages as the endogenous α7 gene.

The α7 promoter sequence is extremely rich in the bases G and C and is therefore a candidate for multiple regulatory interactions with transcription factors such as SP1, AP2 and GCF, whose consensus binding sites are GC-rich (*figure 1A*). Site-directed mutagenesis either to enhance or to abolish promoter activity is in progress and preliminary results indicate that multiple point mutations are required for promoter inactivation, suggesting that multiple, overlapping binding sites intervene to provide a degree of redundancy (S. Couturier, unpublished results). We do not know as yet if the tissue and stage-specific regulation of transcription is achieved by dedicated, nervous system specific transcription factors or by a cell-type specific combination and/or ratio of ubiquitous transcription factors.

3.2. The β3 promoter

Analysis of the regulatory sequence directing β3 gene transcription has enabled us to isolate a DNA fragment, 143 bp in length and located immediately upstream of the transcription cap site, containing all the cis-elements required for specifically targeting reporter gene expression to certain neurons in the retina, including ganglion cells and a subset of amacrine neurons (figure 1B). In addition, the 143-bp DNA fragment has proper temporal and tissue resolution, being able to promote stronger reporter trans-

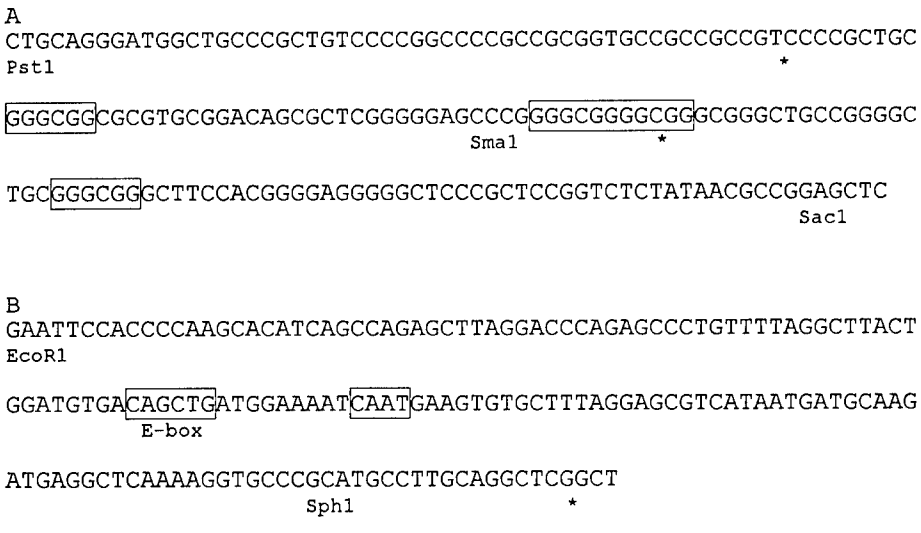


Figure 1. Sequences of the α7 (**A**) and β3 (**B**) promoters. The major sites of transcription initiation are marked by an asterisk. The boxed motifs are SP1 consensus sequences in **A** and E- and CAAT-boxes in **B**.

cription in E5 than in E13 retina, and failing to sustain transcriptional activity in optic tectum neurons or in glia [7, 11].

Mutagenesis of the several potential consensus binding sites for transcriptional factors within the 143-bp control element has revealed that two of them, an E-box and a nearby CAAT-box, are critical for function. E-boxes are the binding sites for basic helix-loop-helix (bHLH) transcription factors, many of which participate in neural determination and differentiation. Accordingly, we attempted to transactivate the transfected β3 promoter by supplying cloned neural bHLH proteins to cells that normally do not express β3.

We found that although none of the tested neural bHLH proteins (ash1, CTF4, neuroD and neuroM) were able to activate the β3 promoter, the muscle bHLH factor myoD did turn on transcription at the transfected (and endogenous) β3 promoter in a range of different neurons, but not in non-neural cell types. Moreover, we demonstrated that the ability of the β3 promoter to discriminate between such related bHLH factors as myoD and neuroD is due to the short 70 bp sequence extending 3′ of the E-box, whereas the elements placed in 5′ of the E-box are entirely dispensable. In marked contrast, the promoter of the muscle nAChR receptor α1 subunit was found to be activatable by all tested bHLH proteins in neurons, and exclusively by myoD in non-neural cells [15].

One possible interpretation of these findings is that, whereas myoD is able to activate the α1 promoter as a homodimer (or perhaps as a heterodimer with one of the ubiquitous bHLH proteins), it must form a heterodimer with a neural bHLH to activate the β3 promoter. In contrast, none of the tested neural bHLH factors are capable of activating β3, either as homodimers or as heterodimers with resident bHLH factors. Thus, we have obtained rigorous proof that a neural bHLH factor of limited host-range is responsible for tissue-specific expression of the β3 gene, but that factor has yet to be isolated and identified.

In addition, a CAAT-box located 9 bp downstream of the E-box is also required for promoter activation, and a minimal spacing between the CAAT- and E-boxes must be maintained for proper function. This suggests that activation of the β3 promoter requires protein-protein interactions between factors bound at the two sites [15]. Thus, the limited expression domain of the β3 gene in the nervous system may simply result from the coincident expression of a bHLH protein and of a CAAT-interacting factor. Both these proteins may have quite large expression domains, as it suffices that they rarely overlap to ensure stringent transcriptional control of the genes they coregulate.

Acknowledgments

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Position effect variegations and brain-specific silencing in transgenic mice overexpressing human acetylcholinesterase variants

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Abstract — Position effect variegations as well as brain-specific silencing were observed in novel transgenic mouse pedigrees expressing human acetylcholinesterase (AChE) variants. Muscle AChE activities varied between 1.6- and 350-fold of control in these lines, one carrying insertion-inactivated InE6-AChE and two with 'readthrough' I4/E5 AChE, all under control of the ubiquitous CMV promoter. In contrast, brain AChE levels remained within a range of 1.5-fold over control, suggesting an upper limit of brain AChE which is compatible with life. (©Elsevier, Paris)

Résumé — Effets positionnels de transgènes de variants d'acétylcholinestérase humaine et inhibition spécifique de leur expression dans le cerveau de souris transgéniques. Les effets positionnels de transgènes constitués de variants du gène de l'acétylcholinestérase humaine, ainsi que l'inhibition spécifique de leur expression ont été observés dans de nouvelles lignées de souris transgéniques. Les activités de l'acétylcholinestérase (AChE) dans les muscles de ces animaux varient dans un rapport de 1,6 à 350 selon la lignée. Une des lignées comporte une forme inactive de l'AChE (InE6-AChE), deux autres comportent une forme de l'AChE contenant l'intron 4 'readthrough' (I4/E5). Tous les variants de l'AChE utilisés dans ces expériences sont sous le contrôle du promoteur ubiquiste du cytomégalovirus. Par contre, l'activité de l'AChE ne varie que dans un rapport de 1 à 2 dans le cerveau, démontrant qu'il existe une limite supérieure du niveau d'expression de l'AChE compatible avec la survie. (©Elsevier, Paris)

acetylcholinesterase / alternative splicing variants / gene dosage effects / muscle / transgenic pedigrees

1. Introduction

Many studies of gene regulation in mammals in vivo have been made possible by transgenic technology (for review see [10]). Early transgenes were constructed with several promoter and enhancer sequences, however, these were rarely sufficient to ensure native levels or tissue-specific expression [19]. Thereafter, the site of genomic integration of mammalian transgenes was found to play a major role in their control [6]. Therefore, individual *Drosophila* or mouse lines with multicopy transgenes exhibited expression levels that were partially or completely unrelated to the number of the integrated transgenes [20]. These phenomena may be especially pronounced when the overexpressed transgene changes a vital process such as cholinergic neurotransmission, for example in transgenic mice carrying the human transgene for the acetycholine (ACh) hydrolysing enzyme acetylcholinesterase (acetylcholine acetylhydrolase, EC3.1.1.7, AChE). In adult transgenic mice overexpressing synaptic AChE in brain neurons [3] we observed structural and functional abnormalities in neuromuscular junctions [1] as well as attenuation of dendrite branching and depletion of dendritic spines harboring synapses of cortical neurons [4]. However, the specific contribution of the transgenic AChE toward each of these phenomena remained unknown.

In addition to its function in cholinergic neurotransmission, compelling evidence demonstrates a neurite growth-promoting activity of AChE. This activity does not depend on the capacity of AChE to hydrolyse ACh, as it persists in the presence of active site inhibitors [11, 17, 24] and is sustained in insertion-inactivated human recombinant AChE (InE6-AChE) [25]. However, the neuritogenic activity of AChE was found to be limited to the synapse-characteristic and membrane-associated E6-AChE isoform which includes the exon 6-encoded amphipathic C-terminus. In contrast, the non-synaptic secretory I4-AChE isoform, which terminates the pseudointron 4-derived hydrophylic peptide, did not affect neurite growth from *Xenopus* motoneurons, nor did it promote process extension from rat glioma cells [12].

Another developmental activity of AChE relates to synapse growth and maintenance. This function

*Correspondence and reprints
Abbreviations: AChE, acetylcholinesterase; BChE, butyrylcholinesterase; CMV, cytomegalovirus; Hp, human acetylcholinesterase gene proximal promoter (586 bp upstream from the transcription initiation site); PCR, polymerase chain reaction; SV40, simian virus 40; ATCh, acetylthiocholine; BTCh, butyrylthiocholine.

depends on increasing the enzyme's hydrolytic capacity within the synapse. Evidence supporting this notion is that both E6 AChE [21–23] and a non-natural C-terminally truncated form, E4 AChE, promote the enlargement of *Xenopus* neuromuscular junctions, but the catalytically inactive InE6-AChE and the nonsynaptic AChE isoform I4 AChE do not [25]. However, it remained uncertain whether I4 AChE is also inert during mammalian development.

Two recent developments call for reconsideration of these two questions. First, several active site inhibitors of AChE were approved for chronic therapeutic use in Alzheimer's disease [16]. This implies the continued presence of the catalytically inactive E6-AChE protein, with potential effects on synapse maintenance and plasticity, in brain neurons of treated patients. Second, acute psychological stress and exposure to active-site AChE inhibitors were both shown to induce massive long-term accumulation of I4-AChE in the mammalian brain [13, 14]. Altogether, these called for testing the in vivo effects of excess InE6-AChE or I4-AChE on the mammalian brain and muscle. Towards these goals, we now report the creation of transgenic mouse lines carrying these two transgenes and demonstrate position effects of their insertion into the host mouse genome on the extent and tissue specificity of their expression in brain and muscle.

2. Materials and methods

Construction of vectors and the corresponding PCR primers were recently described [25]. To create founder transgenic mice, the I4-AChE and InE6-AChE inserts were both excised from the corresponding plasmids together with the CMV minimal promoter-enhancer sequence and the SV40 polyadenylation signal, using enzymatic restriction with *Kpn*I and *Spe*I. Purified insert DNA was then injected into 70 pronuclei of fertilized mouse eggs for each insert, essentially as previously described [3]. Transgene-carrying mice were identified by PCR analysis of tail DNA and transgene penetrance determined at each generation. To evaluate copy numbers, we performed a semi-quantitative PCR analysis as described previously [13] and in comparison to parallel reactions with plasmid DNA of known concentration.

Xenopus oocyte microinjection and homogenization were as detailed elsewhere [25]. To determine the thermal stability of the variant AChEs, homogenates of *Xenopus* oocytes were incubated at the noted temperatures and times.

Mouse tissues (brain or muscle) were isolated as described elsewhere [3]. Hydrolysis rate of acetylthiocholine (ATCh) was measured in *Xenopus* oocyte or mouse tissue homogenates using an automated multiwell colorimetric assay as described [1, 25]. Protein concentrations were measured using the Bio-Rad DC Protein Assay kit (Bio-Rad Laboratories, 2000 Alfred Nobel Dr., Hercules, CA 94547, USA).

3. Results

To study the regulation of AChE expression in the mammalian brain and muscle, we created a series of transgenic mouse pedigrees overexpressing several variants of human AChE. Four human ACHEcDNA constructs were employed for this study. These all included the potent, ubiquitous minimal CMV enhancer-promoter, except for one construct where the proximal 586 base pair fragment from the human ACHE gene was used [3]. The coding sequences included two natural variants and one engineered construct. These encode the brain and muscle-abundant E6-AChE isoform carrying the exon 6-derived amphipathic C-terminal peptide [22]; the 'readthrough' I4-AChE isoform C-terminated with the pseudointron I4-derived hydrophylic peptide [12]; and the insertion-inactivated InE6-AChE isoform C-terminated with the exon 6-derived peptide but with no capacity to hydrolyze ACh [25]. *Figure 1* presents these different vectors and the distinct properties predicted for their protein products.

We have used plasmid DNA-microinjected *Xenopus* oocytes as an analytical system for the assessment of the expression efficacy of the above vectors. Gel electrophoresis under non-denaturing conditions revealed production and E6-AChE characteristic migration properties for the catalytically inactive but immunopositive InE6-AChE variant [25]. The two catalytically active E6 and I4 AChE variants both retained over 90 and 50% of their hydrolytic capacity in oocyte homogenates when incubated for 5 h at 37 °C and 42 °C, respectively (*figure 2*). This demonstrated that the variant C-terminal peptides do not contribute significantly toward the heat stability of AChE. That the C-terminally truncated E4-AChE isoform presented similar heat stability, confirmed this conclusion [25].

Novel transgenic FVB/N mouse lines were created with the CMV I4-AChE and the CMV InE6-AChE vectors and their properties compared to those of the HpE6-AChE pedigree [1, 3, 4] and of control FVB/N mice. Each of these transgenes displayed unimpaired Mendelian inheritance patterns for over five generations, suggesting compatibility with survival. *Figure 3* presents these pedigrees, one for HpE6-AChE, two with CMV I4-AChE and one with CMV InE6-AChE. Kinetic follow-up of PCR amplifications, using host mouse DNA and primers selective for each of the transgenes revealed variable first cycle of appearance (27, 27, 30 and 30 for 500 ng samples of genomic DNA) for the two independent pedigrees with CMV I4-AChE transgenes, lines 45 and 70, one with HpE6-AChE and one with CMV InE6-AChE, respectively.

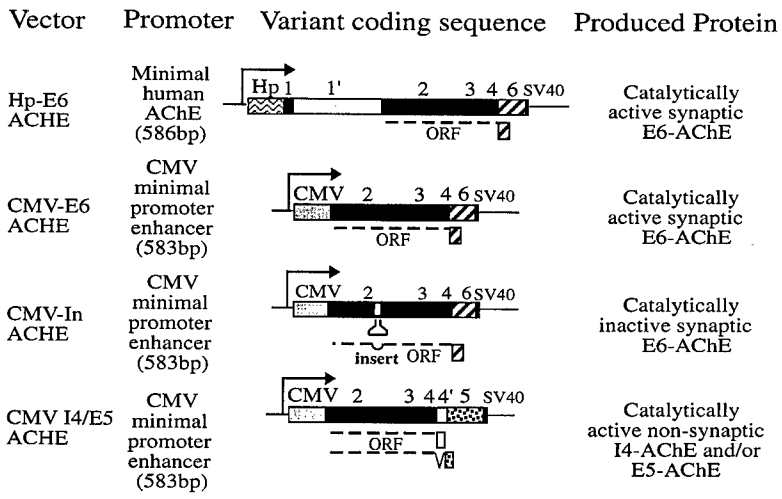


Figure 1. Human AChE variants overexpressed in transgenic model systems. Presented are recombinant DNA inserts from plasmids constructed to perform this transgenic study. Hp, human ACHE minimal promoter; CMV, cytomegalovirus minimal promoter-enhancer; SV40, consensus polyadenylation site. Arrows indicate the orientation of transcription and exons 1–6 are numbered. Intron I1 and pseudointron I4 are hyphenated and the open reading frame of each coding region is noted by a dashed line below it, with the naturally variable C-terminal peptides and the engineered inactivating insert highlighted. The major characteristics of the AChE proteins produced from each of these vectors are also noted. See Beeri et al. [3], Seidman et al. [22] and Sternfeld et al. [25] for further details of each of these vectors.

The efficacy of AChE expression in brain and muscle tissues from these transgenic mice was evaluated by ATCh hydrolysis rates in tissue homogenates in the presence and absence of the specific BChE inhibitor IsoOMPA. These measurements demonstrated drastic differences in the specific activity of muscle AChE (*table I*). Hydrolysis capacity reached levels of 190 ± 22 (S.E.M.) and 2700 ± 330 (S.E.M.) nmol ATCh hydrolyzed/min/mg protein in the two I4 AChE lines as compared to 7.6 ± 0.5 (S.E.M.), and 12 ± 2.3 (S.E.M.) nmol/min/mg in muscle from control FVB/N mice and transgenics carrying InE6-AChE, respectively.

In contrast to the drastic variabilities in muscle AChE levels, brain AChE specific activities remained grossly similar in all of these transgenic lines. These ranged from 160 ± 5.0 (S.E.M.) and 170 ± 15 (S.E.M.) nmol/min/mg for the mice carrying InE6-AChE and control FVB/N mice and reached 240 ± 21 (S.E.M.) and 230 ± 25 (S.E.M.) nmol/min/mg for the two I4 AChE pedigrees with muscle activities 25- and 350-fold higher than control. HpE6-AChE transgenics revealed the highest values, reaching 300 nmol/min/mg levels in brain, 50% higher than the brain activity in control FVB/N mice [3]. *Table I* presents these comperative data for

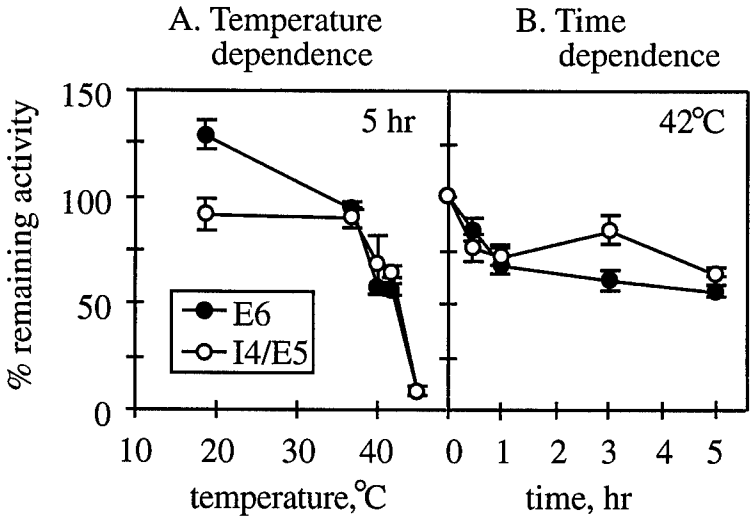


Figure 2. Heat stability of the recombinant AChE variants. Presented are relative ATCh hydrolysis rates of recombinant E6-AChE and I4-AChE variants. Enzymes were produced in microinjected *Xenopus* oocytes from the corresponding vectors including the CMV promoter (see *figure 1*). Twenty-four h post-injection, 1:10 w/v oocyte homogenates in high salt-detergent buffer [25] were incubated at various temperatures for 5 h (**A**) or at 42 °C for the noted time periods (**B**). Endogenous *Xenopus* AChE activity was measured in homogenates from non-injected oocytes and the corresponding values subtracted. Shown are average results of 2–3 experiments and standard errors of three measurements for each sample. Note that 'readthrough' I4 AChE of human origin is at least as stable as the synaptic E6-AChE variant.

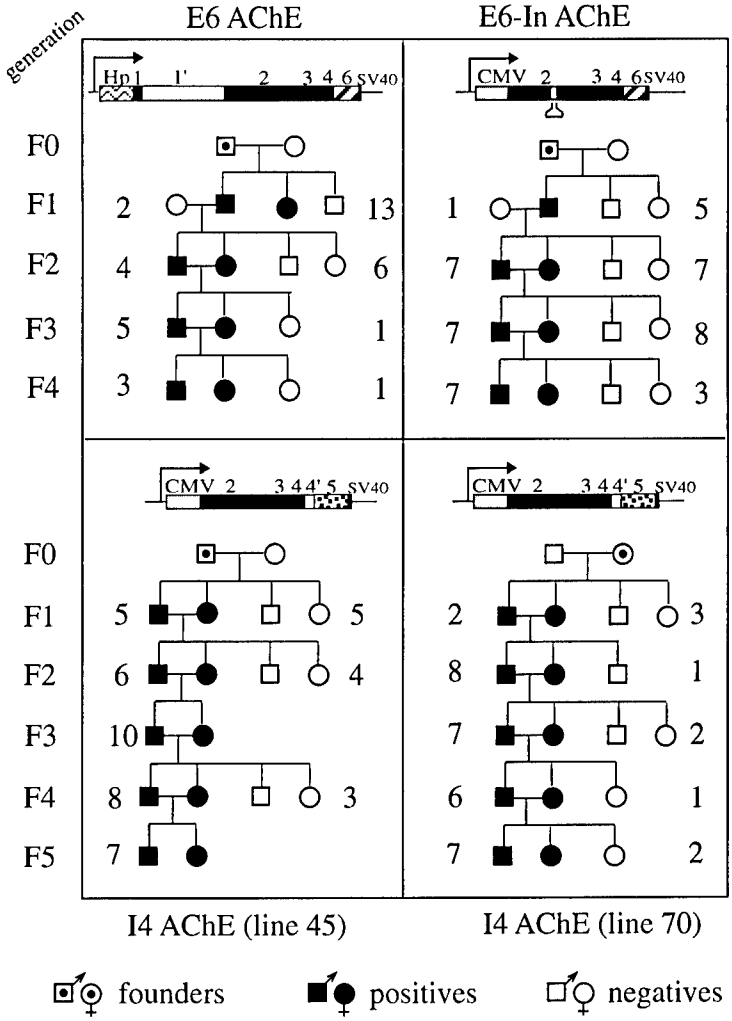


Figure 3. Mendelian inheritance of transgenic AChE variants in mouse pedigrees. Presented are pedigree data for four distinct lines of mice carrying the noted transgenic AChE constructs. Transgene presence was determined by PCR amplification from tail DNA samples as previously detailed [3]. Numbers of positive and negative mice in each litter are noted on the left and right, respectively. Note the increase in positive litter mates within advanced generations, reflecting unimpaired Mendelian inheritance.

brain and muscle AChE levels, clearly demonstrating the distinct expression patterns of these transgenes in brain and muscle.

To test whether overexpression of AChE affects endogenous cholinesterase activity, butyrylcholinesterase (BChE) activity was determined in these homogenates by measurements of butyrylthiocholine hydrolysis in the presence and absence of the specific AChE inhibitor BW284C51 (*table I*). These analyses showed an increase of 1.8 over control in muscle BChE activity in the I4-AChE transgenic line that showed a 350-fold increase of AChE activity. In contrast, decreased brain BChE activities to levels 0.46 and 0.58 of control were observed both in the InE6-AChE transgenic line and the I4-AChE transgenic line with the 25-fold increase in muscle AChE activity (line 45). The changes in BChE activities in both muscle and brain were thus much lower than those in AChE activities in these tissues.

4. Discussion

Creation of three novel transgenic mouse pedigrees revealed position effect variegations in muscle expression as well as brain-specific silencing of the CMV I4-AChE transgene encoding the secretory, soluble non-synaptic 'readthrough' form of AChE. When compared to transgenic mice carrying the InE6-AChE transgene and the corresponding catalytically active E6-AChE transgene, a pattern emerges which testifies to considerable permissivity of muscle AChE variations but maintains brain AChE levels within a very narrow activity window.

We found that two mouse pedigrees, both carrying apparently similar copy numbers of the I4 AChE transgene under control of the CMV promoter, express 25- and 350-fold excesses of AChE in muscle. This clearly reflects position effects of the insertion of these transgenes into the host mouse genome. In

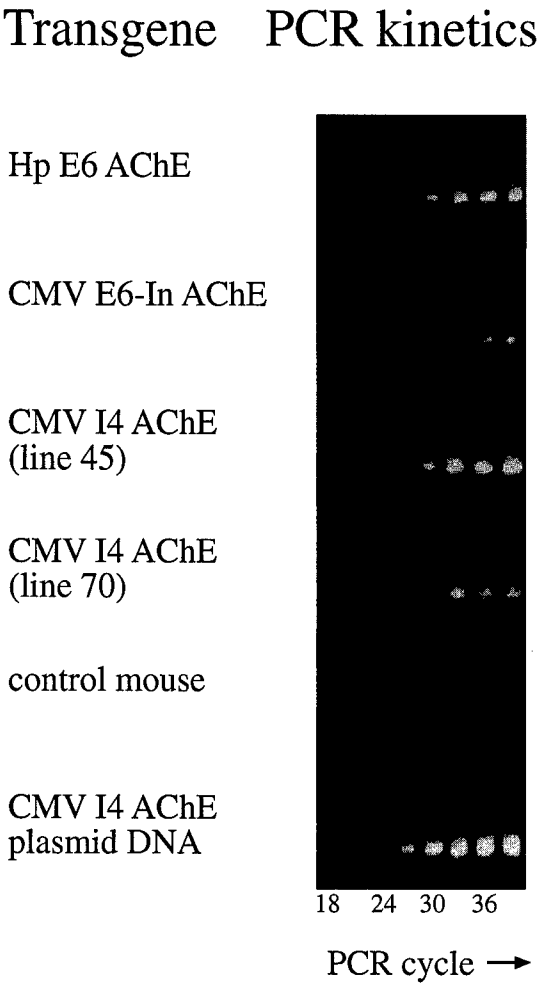


Figure 4. PCR indications for differential gene dosage. Shown are kinetic accumulations of PCR products drawn every third cycle, from 500 ng tail DNA of positive F4-F7 generation mice from each of the noted transgenic pedigrees. Genomic DNA from a control mouse was incubated with primers selective for the AChE coding sequence to exclude the possibility of false positives. CMV I4-AChE plasmid DNA (10 pg) was used with the same primers to calibrate copy numbers. Note the parallel kinetics, indicating similar gene dosage, for lines 45 and 70, both of which carry CMV I4-AChE transgenes. One out of three reproducible analyses is presented for each transgene. PCR cycles in which samples were taken are noted under the gel panels.

both *Xenopus* embryos and transfected mammalian cells, the CMV promoter was found to be 20-fold more potent than the endogenous 586 bp HpACHE promoter [5, 12]. This, in turn, suggests enhancement of the CMV promoter activity in muscle by neighboring sequences at the transgene integration site in at least one of the I4 AChE pedigrees. Moreover, in several other tissues of these transgenic lines, a pattern of overexpression similar to that of muscle was observed (data not shown). Our I4 AChE pedigrees are therefore a good example of positive effects imposed by the chromosomal integration sites, unlike many other cases where the *cis*-acting elements included in transgenes were insufficient to overcome the negative effects imposed by the integration site. This phenomenon of repressed expression of genes translocated to abnormal integration sites was much earlier termed 'chromosomal positioning effect' [7, 18, 26]. The observation of enhanced I4-AChE production in our transgenic animals therefore suggests integration at sites favorable for transcription.

Selective activation or silencing of mammalian genes in specific cells and tissues is regulated by locus control regions that act in *cis* to ensure correct functioning of adjacent transcription units in all cells of a certain lineage [15]. This operates through the establishment of an open chromatin configuration and requires several domains within the locus control regions [2, 8]. The massive overexpression of muscle AChE in the analyzed transgenic mice may therefore reflect integration in the proximity of such region(s). That this overexpression apparently does not interfere with neuromuscular properties is compatible with the non-synaptic localization of I4 AChE as well as with its minimal effect on neuromuscular junction development [22, 25].

Within the same mouse pedigrees with massive muscle AChE increases, variations in brain AChE activity were remarkably limited. It is unlikely that this silencing was due to the known phenomenon of transgene repeats [9], as it was limited to brain and did not affect muscle expression. Rather, it is likely that the viability of these transgenic pedigrees depended on minimal changes in their brain AChE levels, so that the only lineages that survived were those where the transgene's expression in the brain was effectively silenced. This suggests that the upstream domains adjacent to the two I4 AChE transgenes should include effective brain silencing in addition to muscle enhancing elements and calls for testing their composition and the mechanisms of their function in comparison with other regulators [27].

The reduced brain AChE levels in the InE6-AChE transgenics suggest active expression of this transgene as well as competition of its protein product with host E6-AChE on its translation, post-translational processing and/or deposition sites. That muscle AChE activity was somewhat increased in these mice may further reflect feedback responses of those neuromuscular structures where integration of inactive AChE caused an artificial increase in ACh levels. These newly created transgenic models should therefore be most valuable for testing the biological

Table I. Cholinesterase activities in brain and muscle of transgenic mice expressing AChE variants. Presented are ATCh and BTCh hydrolysis rates ± SEM for three measurements in homogenates (1:10 w/v) from brain and muscle of the noted numbers (*n*) of transgenic and control mice from the inbred pedigrees presented in *figures 3* and *4*. Measurements were performed essentially as described [25] in the absence or presence of 10^{-5} M of the selective AChE inhibitor BW284C51 or the selective BChE inhibitor, Iso OMPA. Note the large changes in muscle AChE activity. Spontaneous hydrolysis rates were subtracted from all values.

	AChE activity in nmol ATCh hydrolysed/min/mg protein						*BChE activity in nmol BTCh hydrolysed/min/mg protein*						
	Whole brain			*Muscle*			*Whole brain*			*Muscle*			
Transgenic pedigree	*Total*	*+10^{-5} M Iso OMPA*	*Control (%)*	*Total*	*+10^{-5} M Iso OMPA*	*Control (%)*	*Total*	*+10^{-5} M BW*	*Control (%)*	*Total*	*+10^{-5} M BW*	*Control (%)*	*n*
CMV E6-In-AChE	180 ± 9.5	160 ± 5.0	95	12 ± 2.3	12 ± 2.4	160	2.0 ± 0.1	1.0 ± 0.3	46	2.1 ± 0.7	1.5 ± 0.1	78	1
CMV I4-AChE (line 45)	240 ± 17	240 ± 21	140	190 ± 22	190 ± 23	2500	4.0 ± 0.4	1.3 ± 0.2	58	11 ± 2.0	1.8 ± 0.3	97	4
CMV I4-AChE (line 70)	230 ± 22	230 ± 25	131	2700 ± 330	2700 ± 250	35 000	3.8 ± 0.5	1.7 ± 0.4	75	120 ± 11	3.5 ± 0.7	180	2
Control mice	180 ± 12	170 ± 15	100	7.3 ± 0.5	7.6 ± 0.6	100	3.0 ± 0.4	2.3 ± 0.4	100	2.2 ± 0.3	1.9 ± 0.2	100	3

effects of AChE overproduction and sustained inhibition and for exploring the molecular mechanisms controlling both the expression patterns of these transgenes and the ability of the host mice to accommodate these changes.

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Muscarinic acetylcholine receptors activate the acetylcholinesterase gene promoter

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Abstract – The acetylcholinesterase (AChE) gene promoter contains several overlapping binding sites for Sp1 and Egr-1 transcription factors. Cotransfection experiments and promoter assays showed that Egr-1 can potently activate transcription from the human AChE promoter. Muscarinic acetylcholine receptors (mAChR) rapidly activate, via protein kinase C-mediated signaling, expression of the *Egr-1* gene, leading to dramatically increased nuclear concentrations of Egr-1 protein, and to increased binding of Egr-1 to specific DNA recognition sequences. These mAChR-induced increases are followed by increased transcription from the human AChE promoter. In vivo studies with intraventricular infusions of the cholinergic immunotoxin 192 IgG saporin showed more than 80% decrease of AChE activity in cholinergic target areas of the hippocampus and brain cortex. The results are compatible with a combination of decreased AChE activity in degenerating subcortical cholinergic projections, and additional decreases in postsynaptic AChE gene expression. Together our data show that mAChR can activate transcription from the AChE promoter via increased synthesis of Egr-1. The results suggest a feedback mechanism by which the AChE gene is activated by cholinergic neurotransmission, possibly leading to increased formation of AChE protein and accelerated degradation of acetylcholine at cholinergic synapses. This possibility suggests testing of cholinomimetic compounds currently in development for the treatment of Alzheimer's disease for their potential ability to increase AChE gene expression. (©Elsevier, Paris)

Résumé — Les récepteurs muscariniques activent le promoteur de l'acétylcholinestérase. Le promoteur du gène de l'acétylcholinestérase (AChE) contient plusieurs sites chevauchant pour les facteurs de transcription Sp1 et Egr-1. Des expériences de co-transfection ont montré que le facteur Egr-1 active puissamment le promoteur de l'AChE humaine. Les récepteurs muscariniques (mAChR) induisent une expression rapide du gène Egr-1, par la voie de la protéine kinase C, augmentant considérablement le niveau de la protéine Egr-1 dans le noyau et sa liaison aux éléments de réponse sur l'ADN. L'activation des récepteurs muscariniques augmente ainsi la transcription contrôlée par le promoteur du gène humain d'AChE. L'injection intraventriculaire in vivo de l'immunotoxine cholinergique saporine-IgG192 provoque une diminution de plus de 80 % de l'activité de l'AChE dans les cibles cholinergiques de l'hippocampe et dans le cortex. Ces effets peuvent s'expliquer par une diminution de l'activité de l'AChE dans les projections cholinergiques subcorticales en dégénérescence, accompagnée d'une diminution de l'expression post-synaptique de l'expression d'AChE. L'ensemble de nos résultats montre que les récepteurs muscariniques peuvent activer la transcription du gène de l'AChE en stimulant la synthèse d'Egr-1. Ces données suggèrent un mécanisme de rétro-contrôle, dans lequel le gène de l'AChE est activé par la neurotransmission cholinergique, ce qui peut augmenter la production d'AChE et accélérer l'hydrolyse de l'acétylcholine dans les synapses cholinergiques. Il serait donc intéressant de voir si les composés cholinomimétiques actuellement en cours de développement pour le traitement de la maladie d'Alzheimer peuvent augmenter l'expression de l'AChE. (©Elsevier, Paris)

muscarinic acetylcholine receptors / acetylcholinesterase / Egr-1 / APP / amyloid / Alzheimer's disease / AChE inhibitors / muscarinic agonists

1. Introduction

Muscarinic acetylcholine receptors (mAChR) are a family of cell surface receptors with seven transmembrane domain topology. They couple to G protein-mediated regulation of a complex variety of intracellular signaling pathways that involve the phospholipases A_2, C, and D, protein kinase C (PKC), mitogen activated protein (MAP) kinase, Jun kinase (JNK), tyrosine kinases, adenylyl cyclase, intracellular Ca^{2+} levels, as well as the regulation of K^+ and Ca^{2+} channels (for reviews, see [26, 81–83]). Among the many cellular responses induced by mAChR is the regulation of posttranslational modification of two proteins with central roles in the pathophysiology of Alzheimer's disease: presenilin 1 (PS1) and β-amyloid precursor protein (APP). Stimulated mAChR increases the phosphorylation of the PS1 C-terminus, and it increases proteolytic α-secretase processing of APP within its β-amyloid (Aβ) domain, thus reducing the amount of Aβ peptides secreted into extracellular compartments [19, 32, 38, 57–61, 79]. In mammalian brain, muscarinic neurotransmission is involved in several aspects of neuronal plasticity including learning and memory [14, 21, 22, 56, 84]. Muscarinic transmission is im-

paired in several CNS disorders associated with memory loss, including AD [25] (for review see [40]), as well as in the memory decline associated with old age [20]. Together, these observations promoted the development of such cholinomimetic drugs as acetylcholinesterase (AChE) inhibitors and muscarinic agonists for the treatment of AD. Several AChE inhibitors and muscarinic agonists drugs are tested currently in clinical trials [5, 13, 36, 52, 72]. Some of these compounds received FDA-approval for the treatment of AD.

AChE is a serine hydrolase that terminates the action of acetylcholine (ACh) at cholinergic synapses by hydrolyzing it [65, 73] (for reviews, see [45, 46, 76]). The human AChE gene promoter contains consensus binding sites for Sp1, Egr-1 (*zif/268, Krox24, NGFI-A* [7, 39, 48]) and AP2 transcription factors, and deletion experiments demonstrated that the overlapping Sp1 and Egr-1 sites are essential for activation of AChE gene expression, whereas AP2 repressed it [24, 29]. Importantly, the activities of AChE gene promoters may be regulated differently in mouse and man. In contrast to the possible repression by Egr-1 of the murine AChE gene promoter [41, 53], the human AChE gene promoter is strongly activated by Egr-1, as indicated by cotransfection experiments with *Egr-1* expression constructs [78]. *Egr-1* is a member of the early growth response (*Egr*) gene family of nuclear transcription factors with immediate-early gene induction kinetics, and with zinc-finger DNA binding domain structure [74, 75]. *Egr-1* expression is rapidly inducible by a broad variety of external stimuli including transsynaptic neuronal excitation, differentiation cues, mitogens and tissue injury (for review, see [23]). In the central nervous system, electrical high-frequency stimulation of the perforant path, for example, induces *Egr-1* expression in postsynaptic target cells of the dentate gyrus [9]. This increase is blocked by NMDA agonists suggesting that it is mediated by glutamate via activation of NMDA receptors [7]. Moreover, serotonin, noradrenalin, dopamine, bradykinin, vasopressin, nicotine and muscarinic agonists can stimulate *Egr-1* expression in a variety of cell types suggesting that Egr-1 mediates long-term transsynaptic responses triggered by a broad variety of neurotransmitters and neuromodulators [1, 8, 10, 18, 30, 34, 50, 62, 69, 70, 78, 80, 85].

Because the AChE gene is a target for Egr-1-mediated transcriptional regulation, and because *Egr-1* expression can be stimulated by mAChR, we tested, by using cotransfection experiments, whether mAChR can regulate the AChE gene promoter via expression of *Egr-1*. In addition, we used the 192 IgG-saporin lesion model of cholinergic differentiation to test whether AChE expression in cholinergic target areas is under the control of subcortical cholinergic projection neurons.

2. Materials and methods

Cell culture experiments, Northern blots, Western blots, mobility shift assays, cotransfections and promoter analyses were done as described [78].

2.1. Immunotoxin lesions

Cholinergic immunolesions were performed according to Rossner et al. [66–68]. Rats were anesthetized with 60 mg ketamine i.m. and 40 mg nembutal i.p./kg body weight, and placed in a stereotaxic apparatus (Stoelting, Wood Dale, IL, USA). The skull was opened at bregma, 0.8 mm and +1.2 mm lateral to the longitudinal suture by placing a small hole. Stereotaxic infusion of 4.0 µg 192 IgG-saporin (Chemicon Int., Temecula, CA, USA) in 10 µL PBS, pH 7.4, was performed at a depth of 3.4 mm below the cortical surface at a rate of 1.0 µL per minute into the left lateral ventricle using a Hamilton syringe with a 31 gage needle (Hamilton, Bonaduz, Switzerland). After injection the syringe was kept for an additional 5 min at the injection site to allow complete diffusion. Brains were analyzed 7 and 14 days after 192 IgG-saporin infusion. Vehicle controls were processed identically, but received PBS only. Untreated controls were used for normalizations of both 192 IgG-saporin-treated animals and vehicle controls. These experiments were approved by the Committee for Animal Experimentation at Regierungspräsidium Leipzig.

2.2. AChE histochemistry

Frozen tissue sections were processed for AChE histochemistry according to the protocol of Andrä and Lojda [2]. Briefly, the sections were preincubated with 0.1 M Tris-maleate buffer, pH 5.6, for 30 min. Slices were incubated in reaction medium that contained 0.4 M sodium citrate, 0.12 M cupric sulfate, 0.16 M potassium ferricyanide, and 50 mg acetylthiocholine iodide in 0.1 M Tris-maleate buffer in the presence of the cholinesterase inhibitor tetraisopropylpyro- phosphoramide (10 µM, Sigma, St. Louis, USA). Incubations were done at 37 °C for 30 min. Sections from the individual treatment groups were processed in parallel, and they were treated identically. Sections were digitized with a CCD camera, and reaction intensities were quantitated by densitometry.

3. Results

The results of these studies clearly showed that stimulation of m1AChR with the muscarinic agonist carbachol rapidly increased transcription of the *Egr*-family of transcription factors Egr-1, Egr-2, Egr-3 and Egr-4 [78]. Western blots showed that nuclear levels of Egr-1 protein were undetectable in the basal, unstimulated conditions, and that m1AChR stimulation dramatically increased nuclear levels of Egr-1 protein. We also demonstrated, by using mo-

bility shift assays, that Egr-1 synthesized in response to m1AChR stimulation bound to an Egr-specific DNA recognition sequence, and that this interaction was blocked by anti-Egr-1 antibodies. Pharmacological experiments indicated that the m1AChR-induced mobility shift of the Egr recognition site was blocked by atropine, strongly supporting a specific, mAChR-mediated effect of carbachol. Similar to m1AChR activation, stimulation of PKC with the phorbol ester phorbol myristate acetate (PMA) led to increased Egr-1 binding in the mobility shift assay, and down-regulation of PKC by chronic PMA treatment reduced it. Together, these results indicated a role of PKC in the coupling of Egr-1-mediated transcriptional regulation to m1AChR activity. We next demonstrated, by co-transfection, that carbachol can increase transcription from a minimal Egr-1-responsive promoter fused to a luciferase reporter construct. Additional co-transfection experiments with the human AChE gene promoter fused to a luciferase reporter and a CMV-driven expression plasmid for *Egr-1* demonstrated that Egr-1 dramatically increased transcription from the AChE gene promoter. Co-expression of the human AChE gene promoter construct with m1AChR demonstrated that carbachol increased transcription from the promoter. Again, this increase was blocked by atropine indicating that m1AChR can increase the expression of the AChE gene, and the increase was mimicked by PMA, but not by 8-bromo-cAMP [78].

In order to test the relevance of these observations in vivo, we reduced the cholinergic input from the subcortical projection system into the target areas in brain cortex and hippocampus by infusing the cholinergic immunotoxin 192 IgG-saporin into the lateral ventricles of adult rats. 192 IgG-saporin dramatically reduced histochemical staining of AChE activity in cortex and hippocampus within 7 and 14 days. In addition, AChE activity dropped significantly, after 14 days, in the cholinergic cell bodies in the medial septum (*figure 2*). Densitometric analyses showed that magnitudes of these decreases were in the range of 80% as compared both to the untreated control condition and to the injected vehicle controls that underwent identical stereotaxic infusion procedures (*figure 3*).

4. Discussion

Our study showed that muscarinic m1AChR can activate the promoter of the AChE gene. Internal signaling pathways that coupled mAChR to the AChE gene promoter included protein kinase C and increased transcription of the *Egr-1* gene, leading to increased cellular concentrations of *Egr-1* mRNA within 10 min of stimulation. This increase is followed by more than 100-fold increases in nuclear concentrations of Egr-1 protein within 2 h of m1AChR stimulation, and the increase was blocked by atropine, supporting a specific mAChR-mediated mechanism. Egr-1 proteins synthesized in response to m1AChR stimulation bound to Egr-specific DNA recognition sequences as evidenced by electrophoretic mobility shift assays, and antibodies against EGR-1 blocked this interaction, demonstrating its specificity. Promoter assays showed that stimulation of m1AChR increased transcription either from a minimal Egr-1-responsive promoter, or from the AChE gene promoter. In addition, expression of Egr-1 readily increased transcription of the AChE gene promoter. Increased transcription from both promoters was blocked by atropine and it was mimicked by PMA, but not by 8-bromo-cAMP, suggesting a role for PKC, but not PKA, in the coupling of the AChE gene promoter to mAChR. If confirmed in brain, these data suggest a feedback mechanism in the regulation of muscarinic neurotransmission by which cells stimulated with ACh increase the formation of AChE that, in turn accelerates the termination of ACh action at cholinergic synapses (*figure 1*).

In order to test whether muscarinic regulation of AChE gene expression is relevant in vivo, we reduced the cholinergic input into the cortex and the hippocampus of rats by 192 IgG-saporin immunolesion. Presynaptic terminals of the basal forebrain cholinergic projection neurons selectively take up this immunotoxin, and it kills these cells. Other brain cells, including striatal cholinergic interneurons, for example, remain unaffected by this treatment. The

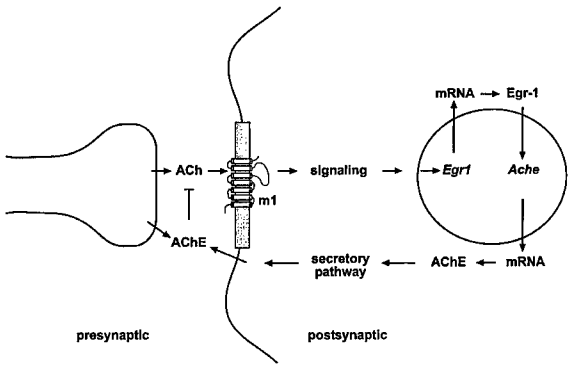


Figure 1. Proposed mechanism of the cholinergic control of AChE expression. We have shown that stimulated m1AChR increases *Egr-1* mRNA and Egr-1 protein that binds to, and activates Egr-1 responsive promoters including the AChE gene promoter. The proposed increase in AChE mRNA, protein and secretion is hypothetical, but supported by independent recent observations [35] (A. Nordberg, personal communication; see *Discussion*).

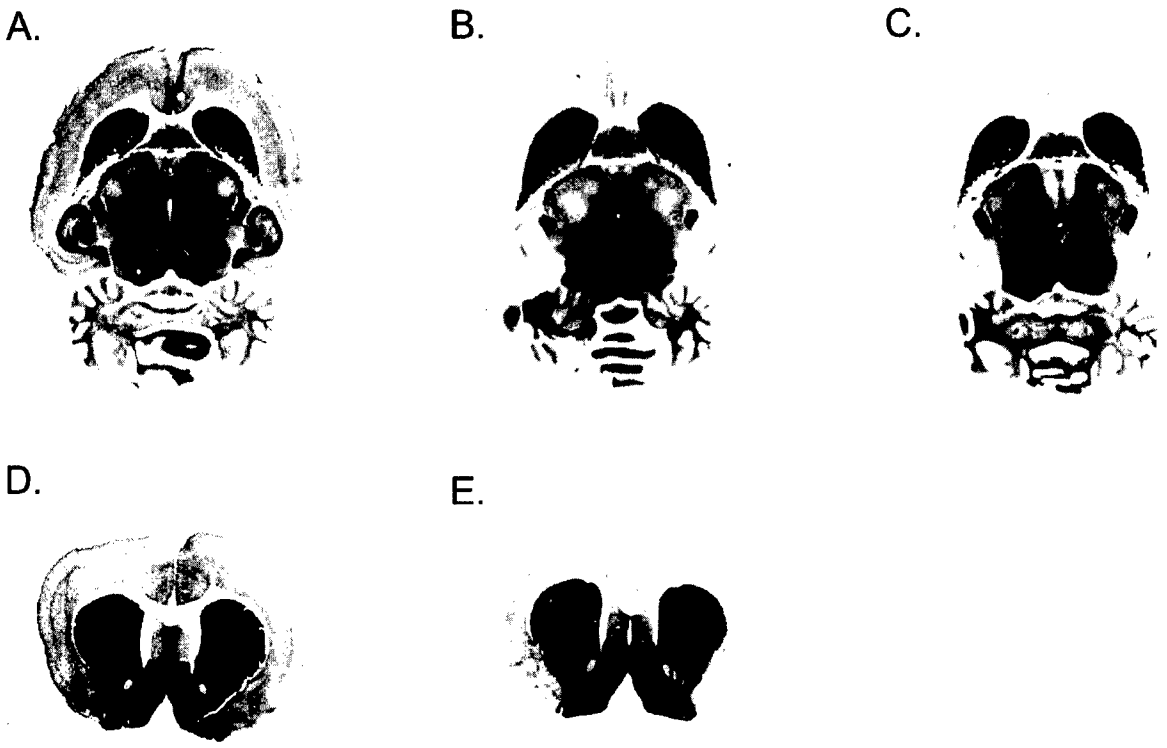


Figure 2. 192 IgG-saporin reduced AChE activity in cholinergic target areas. 192 IgG-saporin or vehicle (**A, D**) was infused into the lateral ventricles of adult rats and AChE histochemistry was done 7 days (**B, E**) or 14 days (**C**) following the infusion. 192 IgG-saporin reduced AChE activity in the hippocampus and throughout the cerebral cortex within 7 days, and AChE activity also decreased in the medial septum 14 days after infusion (**C**). In contrast, AChE activity in the cholinergic interneurons of the striatum and the thalamus remained unaffected by the immunotoxin.

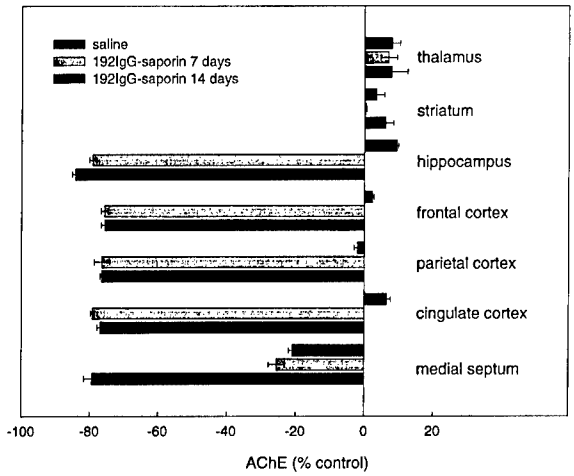


Figure 3. Densitometric analyses of AChE reductions induced by 192 IgG-saporin. Densitometry was done in the brain regions indicated, and they were normalized to untreated normal controls. In comparison to the vehicle controls, 192 IgG-saporin decreased AChE activity in hippocampus, frontal cortex and cingulate cortex by more than 80%.

192 IgG-saporin immunolesion causes cognitive deficits in the Morris water maze [42] consistent with the known role of the subcortical cholinergic projection neurons for learning and memory in rats [21, 84]. In our experiments, 192 IgG-saporin caused a dramatic reduction in AChE activity throughout the brain cortex and the hippocampus within 7 days, and low AChE activities were maintained for at least 14 days (*figure 2*). AChE in the medial septum decreased within 14 days of the immunolesion, but AChE remained unchanged both in the thalamus and in the striatum (*figure 2C*). Densitometric analyses showed that the magnitudes of these decreases were in the order of 80 to 90% as compared to either the vehicle controls of the untreated controls (*figure 3*).

These data are consistent with the concept that AChE gene expression in postsynaptic cholinergic target cells is under the control of presynaptic cholinergic input. In the mammalian brain, the AChE gene is normally expressed by pyramidal neurons throughout the cortex, in addition to high levels of expression in the striatum and the basal forebrain

[6, 11, 27, 28]. Therefore, AChE activities in cholinergic target areas are derived from two sources: first from axonal transport by the subcortical cholinergic projection neurons, and second, from endogenous synthesis of AChE in cortical and hippocampal neurons. It is thus likely that the decrease in AChE activity caused by the 192 IgG-saporin lesion reflects the sum of decreases associated with degenerating presynaptic cholinergic afferences and decreased activity-dependent AChE gene expression in cortical and hippocampal cholinergic target cells (see *figure 1*).

The concept of cholinergic regulation of AChE gene expression is supported by three additional independent observations: First, the AChE inhibitor pyridostigmine dramatically increased AChE gene expression in hippocampal brain slices in vitro [35]. These data strongly suggest that higher tissue levels of ACh in hippocampal cholinergic target regions can increase AChE gene expression in hippocampal cells. Second, clinical observations showed that treatments of AD patients with the cholinesterase inhibitor tacrine for 12 months significantly increased AChE activity in CSF by 50%, as compared to baseline. In contrast, AChE activities in CSF of untreated matched AD patients remained stable over a time period of 17 months (Agneta Nordberg, personal communication). Third, early studies from the mid eighties demonstrated that the systemic application of the AChE inhibitor physostigmine increased AChE activities in CSF in an atropine-sensitive manner, suggesting the involvement of muscarinic receptors [47]. Together, these findings underscore the concept that the regulation of AChE gene expression in brain is activity-dependent, and that it is under the control of external signals including muscarinic receptors. Muscarinic receptor subtype analyses showed that, in addition to m1AChR, the m2, m3, and m4AChR can also stimulate the transcription of the *egr-1* gene, although to different extents [78]. It is therefore possible that AChE gene expression in presynaptic cholinergic neurons is also under the control of ACh produced and secreted by the same neurons.

In addition to Egr-mediated signaling, mAChR are known to increase the expression of the immediate early genes *c-jun, jun D, c-fos, fos B, fra-1* and *nur-77* [3, 4, 12, 16, 31, 34, 77]. Because these transcription factors regulate the expression of a broad variety of target genes, it is possible that muscarinic receptor activity is involved in the activity-dependent regulation of these target genes as well, possibly contributing to activity-dependent plastic responses of postsynaptic cells. Known target genes of Egr-1 regulation include the growth factors PDGF, βFGF, and TGF-β1 [23, 44, 71, 80], the prohormone convertase 2 [33] as well as the interleukin-2 receptor beta-chain [43], suggesting roles in mitogenenic responses and in the regulation of cell growth and differentiation. An additional example for an Egr-1-dependent target is the phenylethanolamine *N*-methyltransferase gene in the adrenal gland [49]. It encodes the enzyme that converts norepinephrine to epinephrine, and the expression of this gene is known to be under the control of muscarinic stimulation [17, 50, 51]. In addition, Egr-1 can be involved in apoptosis, either activating it via a p53-mediated cell death pathway, or suppressing it, depending on the cell biological context [54, 55].

4.1. Possible clinical implications

AD is associated with decreased cholinergic neurotransmission due to the early degeneration and loss of the long cholinergic afferences to the cerebral cortex and hippocampus [40, 86]. This deafferentation is associated with decreased AChE activities in brain cortex and hippocampus [15, 63, 64]. In the cerebral cortex of AD patients, AChE activity is significantly reduced in pyramidal neurons, despite unchanged densities of neurons visualized by Nissl stains of adjacent sections [28]. Our observation of muscarinic regulation of AChE gene expression suggests that this loss of AChE in cortical pyramidal cells could reflect decreased muscarinic stimulation of AChE gene expression, rather than loss of AChE positive neurons.

The activity-dependent expression of Egr-1 mediates important neuronal functions related to stimulus-induced plasticity, cell growth, long-term potentiation and synaptic reorganization. These functions may be impaired in post-synaptic neurons as a result of the cholinergic deafferentation. Because many of these functions are regulated by the activity-dependent expression of Egr-1-dependent target genes that may be also under cholinergic control, it is possible that expression of these genes in AD brain is decreased as well. Postmortem studies are required to test this hypothesis.

It is well established that, besides the degeneration of subcortical cholinergic projection neurons in AD, postsynaptic mAChR expressed by cholinergic target cells are preserved in AD cortex and hippocampus. Thus, cholinomimetic compounds were designed to replace the ACh deficit, and to stimulate postsynaptic cholinergic target neurons. As a result, several AChE inhibitors as well as muscarinic agonists are currently under development and tested in clinical trials for the treatment of AD. Consistently, the AChE inhibitors appear to reduce the rate of progression of clinical and neuropsychological measures of dementia severity, but the magnitudes of these ef-

fects are limited, and dementia continues to progress, after an initial halt, within several months of treatment. If confirmed in human brain, limited efficacy of these compounds may be related to the possibility that they increase AChE gene expression via Egr-1-mediated transcriptional activation. Thus, in vivo studies are required to test whether pharmacological treatments designed to stimulate mAChRs are associated with increased AChE gene expression, increased AChE protein levels, and increased enzyme activity with accelerated breakdown of acetylcholine. If so, additional pharmacological concepts need to be developed that aim at maintaining the beneficial effects of mAChR stimulation without the possible unwanted side effect of increased AChE gene transcription.

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Structure-function analysis of muscarinic acetylcholine receptors

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Abstract — The structural basis underlying the G protein coupling selectivity of different muscarinic receptor subtypes was analyzed by using a combined molecular genetic/biochemical approach. These studies led to the identification of key residues on the receptors as well as the associated G proteins that are critically involved in determining proper receptor/G protein recognition. Mutational analysis of the m3 muscarinic receptor showed that most native cysteine residues are not required for productive receptor/G protein coupling. The putative extracellular disulfide bond was found to be essential for efficient trafficking of the receptor protein to the cell surface but not for receptor-mediated G protein activation. (©Elsevier, Paris)

Résume — Analyse structure-fonction des récepteurs muscariniques à l'acétylcholine. La base structurale sous-jacente des différents sous-types de récepteurs muscariniques, liés sélectivement aux protéines G, est analysée par une approche combinée de génétique moléculaire et de biochimie. Ces études ont mené à l'identification de résidus clefs sur les récepteurs ainsi que sur les protéines G associées impliquées de façon critique dans la reconnaissance appropriée (ou particulière) récepteur/protéine G. (©Elsevier, Paris)

muscarinic receptors / mutagenesis studies / receptor-G protein coupling / receptor structure / cysteine mutagenesis

1. Molecular basis of G protein coupling selectivity

The five muscarinic receptors (m1–m5) are prototypical members of the superfamily of G protein-coupled receptors (GPCRs). Based on their differential G protein-coupling properties, these receptors can be classed into two major functional categories (for a review, see Wess [21]): the odd-numbered muscarinic receptors, m1, m3, and m5, preferentially couple to G proteins of the G_q/G_{11} family, resulting in the hydrolysis of phosphoinositide lipids mediated by activation of different phospholipase Cb isoforms. The m2 and m4 receptors, on the other hand, are primarily linked to G proteins of the G_i/G_o class, which, at a biochemical level, mediate the inhibition of adenylyl cyclase.

1.1. Functionally critical residues on the receptor protein

Recently, mutational analysis of different muscarinic receptors has led to the identification of a series of single amino acids that are critical for proper G protein recognition [21]. These residues are predicted to be located within the second (i2) and third (i3) intracellular loops. Different molecular genetic approaches, including insertion [7] and random mutagenesis strategies [12], indicate that a limited number of primarily hydrophobic residues at the N-terminus of the i3 loop play a key role in determining receptor/G protein coupling selectivity. These residues are predicted to form a surface that maps to the hydrophobic side of an amphiphilic α-helix [1, 7, 20].

Analogously, systematic mutational analysis of the C-terminal portion of the i3 loop of different muscarinic receptors has identified a set of four mostly hydrophobic amino acids (corresponding to Ala488, Ala489, Leu492, and Ser493 in the rat m3 muscarinic receptor; *figure 1*) that also play a significant role in dictating coupling selectivity [6, 8, 14]. Since these residues are thought to be located in a region predicted to be α-helically arranged [1, 20], they define a second hydrophobic surface that is critical for G protein recognition (*figure 1*).

In addition to residues at the i3 loop/membrane junctions, amino acids in the i2 loop have also been implicated in determining receptor/G protein coupling selectivity. For example, studies with hybrid m2/m3 muscarinic receptors have identified four hydrophilic m3 muscarinic receptor residues (Ser168, Arg171, Arg176, and Arg183; *figure 1*) that are critical for selective recognition of $G_{q/11}$ proteins [6]. Since little is known about the conformation of the i2 loop, the spatial arrangement of these amino acids remains to be elucidated.

According to the molecular model of the transmembrane receptor core proposed by Baldwin [2, 3], the two functionally critical receptor surfaces present at the N- and C-terminus of the i3 loop are predicted

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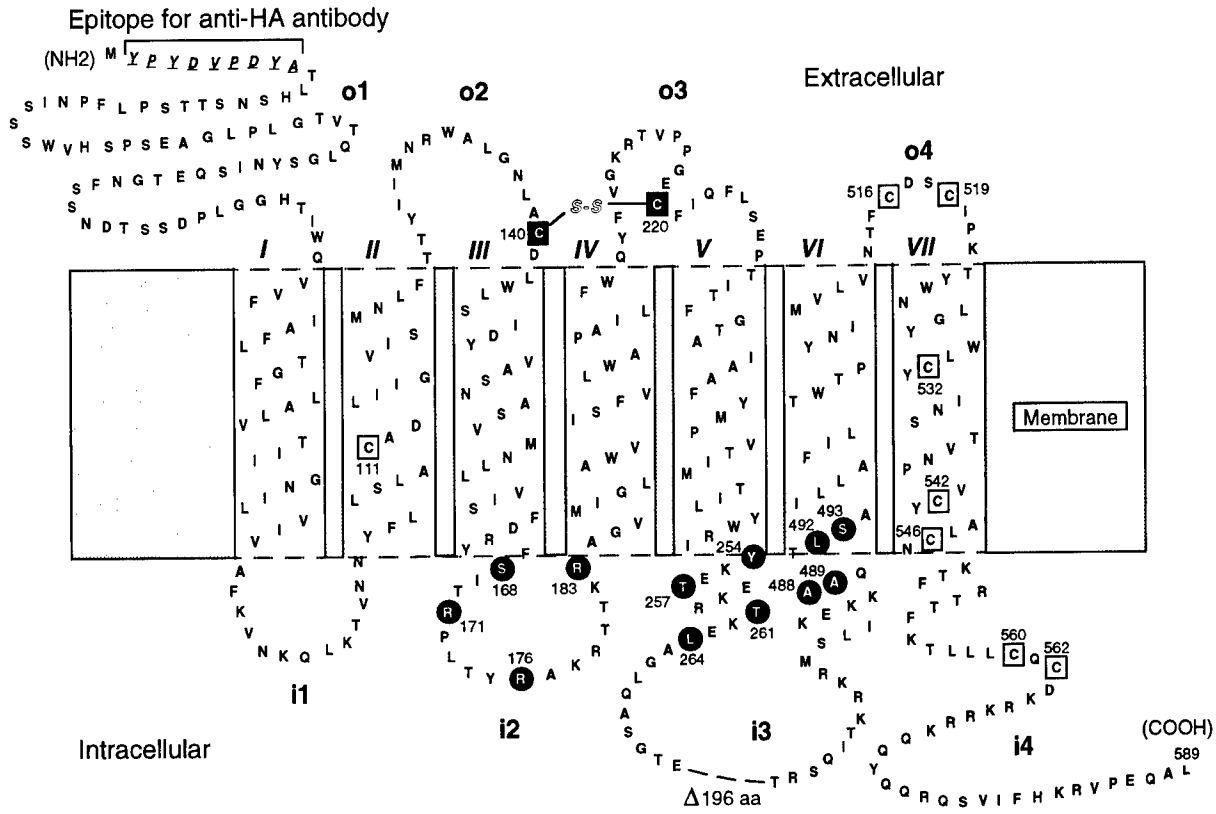


Figure 1. Transmembrane topology of the rat m3 muscarinic receptor. An N-terminal hemagglutinin (HA) epitope tag was introduced into mutant receptor constructs that were studied via immunological techniques (ELISA, confocal microscopy, etc.). Amino acids highlighted by filled circles have been shown to determine the G protein coupling selectivity of the m3 muscarinic receptor [21, 22]. Cysteine residues are boxed. The two extracellular cysteine residues that are conserved among most GPCRs and are predicted to be linked via a disulfide bridge are highlighted in black. Most of the i3 loop (196 amino acids), except for the N- and C-terminal 22 amino acids, can be deleted without affecting m3 receptor function (F.-Y. Zeng and J. Wess, unpublished results). Numbers refer to amino acid positions in the rat m3 muscarinic receptor sequence [4].

to project into the interior of the helical bundle, where they may be engaged in tertiary interactions with residues in adjacent receptor domains [22]. Since site-directed spin-labeling studies suggest that receptor activation is accompanied by an 'outward' movement of TM III and TM VI [10, 11], the resulting 'opening' of the intracellular receptor surface may allow G proteins to access the functionally important residues at the i3 loop/membrane junctions.

1.2. Functionally critical residues on the G protein α subunits

To better understand the molecular mechanisms governing receptor/G protein interactions, it is also important to identify specific regions/amino acids on the G protein(s) that are in contact with the receptor protein. A region that has been implicated most consistently in receptor/G protein coupling is the extreme C-terminus of the G protein α subunit (Gα) [5, 19]. Consistent with this notion, we recently showed that the receptor selectivity of Gα subunits can be changed by single C-terminal point mutations.

We initially employed a gain-of-function mutagenesis strategy to identify individual amino acids within the C-terminal tail of $\alpha_{i/o}$ subunits that are critical for receptor coupling selectivity [15]. Coexpression (in COS-7 cells) of a series of C-terminally modified mutant α_q subunits with the G_i-coupled m2 muscarinic receptor led to the identification of two mutant α_q subunits containing single $\alpha_q \rightarrow \alpha_i$ point mutations at their C-terminus which, in contrast to wild type α_q, were able to productively interact with the m2 receptor. This study provided the first example that the receptor coupling selectivity of G protein α subunits can be altered by single amino

acid substitutions [15]. The two single α_i residues present in the two mutant α_q constructs capable of efficiently interacting with the m2 muscarinic receptor were a –4 cysteine and a –3 glycine, respectively. These two residues are conserved among all members of the α_i/α_o protein family (with the exception of α_z which lacks the –4 cysteine).

In a subsequent study, Kostenis et al. [17] used a similar approach to map functionally important C-terminal α_q residues. In this case, amino acids at the C-terminus of α_s were replaced with the corresponding α_q residues, and the resulting mutant G proteins were studied (in cotransfected COS-7 cells) for their ability to gain coupling to the $G_{q/11}$-linked m3 muscarinic receptor. This analysis led to the identification of two $\alpha_s \rightarrow \alpha_q$ single point mutants which gained the ability to functionally interact with the m3 muscarinic and other $G_{q/11}$-coupled receptors [17]. The single α_q residues present in these constructs were a –5 glutamate and a –3 asparagine, respectively, which are found in all members of the $\alpha_{q/11}$ protein family.

Although these studies demonstrate that the C-terminus of Gα is critically involved in receptor/G protein coupling, other regions of Gα may also contribute to receptor binding and the selectivity of receptor/G protein interactions. Specifically, several lines of evidence suggest that the N-terminal portion of Gα may be in contact with the receptor protein [5, 19].

Functional studies with an N-terminally truncated α_q subunit indicated that the N-terminal six amino acid extension characteristic for α_q and α_{11} subunits (MTLESI(M)) is critical for constraining the receptor coupling selectivity of these proteins [16]. When this sequence was removed by deletion mutagenesis, the resulting mutant α_q subunit gained the ability to be activated by the m2 muscarinic as well as other $G_{i/o}$-coupled receptors which normally do not couple efficiently to wild type (full-length) α_q.

To study which specific amino acids within the N-terminal segment of $\alpha_{q/11}$ are critical for constraining the receptor coupling selectivity of these subunits, this region of α_q was subjected to systematic deletion and alanine scanning mutagenesis [18]. Surprisingly, coexpression studies in COS-7 cells showed that all 14 mutant G proteins studied (but not wild type α_q) gained the ability to productively interact with the $G_{i/o}$-linked m2 muscarinic receptor. Similar results were obtained when we examined the ability of selected mutant α_q subunits to couple to the $G_{i/o}$-coupled D2 dopamine and the G_s-coupled β2-adrenergic receptors. Additional experiments indicated that the functional promiscuity displayed by all investigated mutant α_q constructs was not due to overexpression (as compared with wild type α_q) or initiation of translation at a downstream ATG codon (codon seven) [18]. These findings are consistent with the notion that the six-amino acid extension characteristic for $\alpha_q/_{11}$ subunits forms a tightly folded protein subdomain that is critical for regulating the receptor coupling selectivity of these subunits. One possibility is that this subdomain has a gate function by selectively preventing access of $G_{i/o}$- and G_s-coupled receptors. It is also conceivable that the six-amino acid extension exerts indirect conformational effects on the structure of $G\alpha_{q/11}$ that are crucial for maintaining the receptor selectivity of these subunits.

Interestingly, while all mutant α_q subunits incorporated significant amounts of [^{3}H]palmitate, we noted that the strength of the palmitoylation signal was generally weaker, by about 40–75%, in the case of the mutant α_q subunits as compared to wild type α_q [18]. This observation raises the possibility that palmitoylation may play a role in regulating receptor/G protein coupling selectivity.

2. Cysteine mutagenesis as an approach to study muscarinic receptor structure and function

To elucidate the molecular mechanisms involved in muscarinic receptor activation, it would be highly desirable to obtain more direct structural information about the conformational differences between the resting and the activated receptor state. Currently, however, no high-resolution structural data are available for any GPCR, primarily due to difficulties in studying membrane proteins by X-ray crystallography or NMR.

To gain insight into muscarinic receptor structure, we are currently in the process of creating mutant receptors lacking native cysteine residues. Single cysteine residues will then be introduced into defined positions of the Cys-free mutant receptors, followed by their covalent modification by SH-group specific fluorescence agents, spin markers, or radiolabeled compounds with differing physicochemical properties. Such studies will allow the determination of the localization of specific amino acids with respect to the membrane/cytoplasm interface, the relative orientation of transmembrane helices, and the identity and orientation of secondary structure of targeted receptor regions.

We recently created three mutant m3 muscarinic receptors which contained no, two (Cys140 and Cys220), or three (Cys140, Cys220, and Cys532) remaining cysteine residues (resulting in m3 (C-less), m3 (C2), and m3 (C3), respectively) (*figure 1*). Surprisingly, both the m3 (C2) and m3 (C3) mutant receptors still retained the ability to stimulate

carbachol-dependent inositol phosphate production to the same maximum extent as the wild type receptor (F.-Y. Zeng, A. Soldner, and J. Wess, unpublished results), whereas the completely Cys-free receptor (m3 (C-less)) was functionally inactive. This observation indicated that the majority of native cysteine residues are not critical for m3 muscarinic receptor function.

Cys140 and Cys220 are highly conserved among most GPCRs of the rhodopsin family and are predicted to be linked via a disulfide bond [9, 13]. Interestingly, replacement of these residues by alanine in the m3 muscarinic receptor showed that this putative disulfide bond is not essential for protein stability (as determined via immunoblotting) and receptor-mediated G protein activation (studied in second messenger assays) (F.-Y. Zeng and J. Wess, unpublished results). However, ELISA and immunofluorescence studies demonstrated that the presence of both conserved cysteine residues is required for efficient trafficking of the m3 muscarinic receptor to the cell surface.

Notably, a mutant m3 receptor in which Cys140 and Cys220 were the only remaining cysteine residues was able to react with SH group-specific crosslinking agents as well as [^{14}C]N-ethylmaleimide (F.-Y. Zeng and J. Wess, unpublished results), suggesting that disulfide bond formation between Cys140 and Cys220 may not be quantitative. These results shed new light on the functional role of the two extracellular cysteine residues present in most GPCRs.

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Scanning mutagenesis of transmembrane domain 3 of the M1 muscarinic acetylcholine receptor

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Abstract — Scanning mutagenesis of transmembrane domain 3 of the M1 muscarinic acetylcholine receptor has revealed a highly-differentiated α-helical structure. Lipid-facing residues are distinguished from a patch of residues which selectively stabilise the ground state of the receptor, and from a band of amino acids extending the full length of the helix, which contribute to the active agonist-receptor-G protein complex. The most important residues are strongly conserved in the GPCR superfamily. (©Elsevier, Paris)

Résumé — Mutagenèse du domaine transmembranaire 3 du récepteur à l'ACh muscarinique M_1. La mutagenèse montre que le TM3 possède une structure en hélice α comportant une bande d'AA correspondant à la zone de liaison avec les protéines G. (©Elsevier, Paris)

muscarinic / acetylcholine / G protein / mutagenesis / transmembrane

1. Introduction

Even when the structure of a protein is known at atomic resolution, site-directed mutagenesis studies are needed to understand the contribution of individual amino acids to its function. In the case of the G protein-coupled receptors (GPCRs), structural models are based on the analysis of sequence variations, in the context of a 6–8 Å resolution projection map of rhodopsin, determined by cryo-electron microscopy [1, 2]. Thus for the GPCRs, site-directed mutagenesis and chemical modification provide essential experimental information to refine the models, and to begin to understand receptor structure-function relationships.

Scanning mutagenesis, in which the side-chain interactions made by each amino acid in a given sequence are simplified, or eliminated, as a result of their sequential replacement by a smaller amino acid, commonly alanine [14], has provided a useful tool for the location of functional epitopes within receptors, and the analysis of their function [5]. Here we outline the application of this approach to transmembrane domain 3 (TMD 3) of the M1 muscarinic acetylcholine receptor (M1 mAChR). We show how the functional characterisation of the mutant proteins predicts the structure of the target sequence, gives information about its orientation within the three-dimensional structure of the receptor, and suggests possible functions for individual amino acids.

2. Materials and methods

Mutations were made by a PCR method as previously described [10]. Mutant receptors were expressed in COS-7 cells under the control of an SV40 promoter. Ligand binding assays to measure the affinity of the antagonists N-[^{3}H]-methyl-scopolamine (NMS), [^{3}H]-quinuclidinyl benzilate (QNB) and agonists such as acetylcholine (ACh) were performed as described [10]. Assays of receptor function were performed by measuring ACh-stimulated phosphoinositide (PI) turnover [10]. Results were analysed by means of the extended ternary complex model of receptor function, as outlined below.

3. Results and discussion

3.1. The extended ternary complex model as a tool for the analysis of mutagenesis data

Mutation of amino acids in the M1 mAChR leads to a range of effects. One of the most striking is an increase in agonist affinity correlated with an enhancement of the basal functional activity. This phenotype is consistent with the operation of a two-state mechanism of agonist action, in which there is a pre-existing equilibrium, defined by an equilibrium constant K, between the active (R*) and inactive (R) states of the receptor. Agonists bind with higher affinity to the R* state than to the R state. A mutation which displaces the equilibrium in favour of the activated state, through an increase in the value of K, simultaneously increases both basal activity and agonist affinity.

The simplest receptor-response model to predict this behaviour is the extended ternary complex (EXTC) model of agonist action shown in *figure 1*. In this model, pre-existing (R*) and agonist-induced (AR*) complexes bind the G-protein, leading to the formation of catalytically active R*G or AR*G com-

plexes. These undergo GDP-GTP exchange, resulting in activation of the G protein. The EXTC model was first used to analyse the properties of a constitutively activated β-adrenergic receptor [12] and is widely applicable to GPCR mechanisms.

In using this model, we have assumed that the PI response, which is the functional response measured in our experiments, is directly proportional to the ratio of the concentration of activated-receptor G protein complex to total G protein, ie,

$$PI/PI_{max} = ([AR^*G]+ [R^*G])/[GT] = [\underline{AR^*G}] +[\underline{R^*G}] \quad (1)$$

In equation 1, and the following equations, the underline is used to denote normalisation either by division by GT for the concentrations of complexes involving the receptor, or multiplication by GT in the case of the affinity constant KG.

For the wild-type M1 receptor expressed in COS-7 cells, measurement of the ACh dose-response curves after receptor blockade has shown that $\underline{RT}$ is about 20 for the wild-type receptor [10]. If $\underline{RT} >> 1$, it can be shown that the solution to the EXTC model reduces to:

$$[\underline{AR^*G}] + [\underline{R^*G}] = (P2 + P4[A])[\underline{RT}]/(1 + P2[\underline{RT}] + (P4[\underline{RT}] + K_{Bin})[A])$$

where $P2 = K\underline{KG}$; $P4 = \alpha K_A K\underline{KG}$, and $K_{Bin} = K_A (1+\alpha K)$.

This is an extension of equation 22 of Black and Leff [4].

There is usually no detectable GTP-shift in the agonist binding curves in membrane preparations from COS-7 cells, so K_{Bin} has been determined using the Hill equation:

$$([AR]+[AR^*])/[RT] = (K_{Bin}[A])^{nH}/ (1 + (K_{Bin}[A])^{nH}) \quad (2)$$

In general, dose-response data and binding data have been analysed by simultaneous fitting to equations (1) and (2).

By setting [A] = 0, we see that:

$$Basal = P2[\underline{RT}]/ (1+P2[\underline{RT}]) \quad (3)$$

while the apparent activation constant, K_{Act}, defined as $1/EC_{50}$, is:

$$K_{Act} = (P4[\underline{RT}] + K_{Bin})/ (1 + P2[\underline{RT}])$$

It follows that:

$$P4 = (K_{Act}/ (1\text{-}Basal) - K_{Bin})/[\underline{RT}] \quad (4)$$

Thus the parameters P2, P4 and K_{Bin} derived by fitting are well-determined by the measurable quantities evoked by the expression of the receptor in the cell, namely: i) the concentration of binding sites, $[\underline{RT}]$; ii) the basal activity; iii) the EC_{50}, derived from the PI assay; and iv) the binding constant, derived from an agonist binding experiment performed

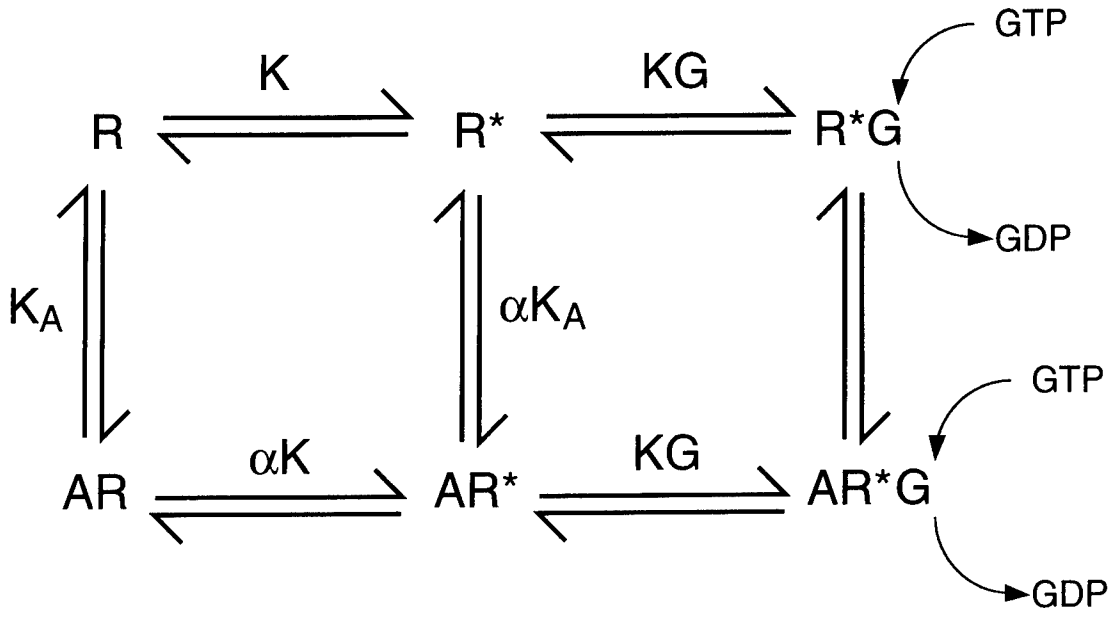


Figure 1. Extended ternary complex model of agonist-receptor-G protein interaction.

in parallel. Furthermore, it is possible, by combining them in various ways, to obtain information about the underlying molecular affinity constants of the model, thus:

$$P4/P2 = \alpha K_A \quad (5)$$

gives the affinity of the agonist for the R*G complex and:

$$P4/K_{Bin} = \underline{KG}\alpha K/ (1 + \alpha K) \quad (6)$$

is a measure of the affinity of the G protein for the ensemble of [AR] and [AR*]. If an estimate of KG can be obtained, the values of αK for the various mutants can be calculated, and then used to estimate the value of K_A from the equation:

$$K_A = K_{Bin}/ (1 + \alpha K) \quad (7)$$

Thus, under favourable circumstances and subject to certain explicit assumptions, the effects of mutations on the free energy associated with the ground-state binding of agonists (and antagonists), measured by K_A, can be separated from effects on the free energy of the activated state(s) relative to the ground state, i.e., of R* relative to R (measured by K), of AR* relative to AR (measured by αK), and of AR*G relative to AR (measured by αKKG).

3.2. Scanning mutagenesis of transmembrane helix 3 of the M1 mAChR

Models of GPCRs suggest that TMD 3 forms the nucleus of the seven-fold helical bundle thought to make up the transmembrane structure [1, 2]. In the the M1 mAChR, TMD 3 is initiated by a disulfide-bonded Cys [9]. It contains the binding site aspartate [9, 13], and terminates in amino acids which are important in G protein recognition, and in stabilising the receptor's tertiary structure [8, 10]. In rhodopsin, a change in the packing relationships between TMD 3 and the surrounding TMDs accompanies G protein activation [6], but the roles of most of the amino acids of TMD 3 in these processes are unknown.

We have carried out scanning mutagenesis on all of the amino acids between Leu100 and Tyr124 of the M1 mAChR, replacing each residue by Ala (or by Gly if the parent sequence itself contained Ala). The PI dose-response curves, and ACh binding curves measured after expression of the mutant receptors in COS-7 cells were analysed by the use of the EXTC model of receptor activation.

Two of the mutants provided a key to analyse this data set. These were S120A, and L116A.

S120A was expressed at 81% of the wild-type level. Unlike the wild-type receptor it gave significant basal activity, equivalent to 6.6% of the ACh-stimulated maximum signal. The basal signal was inhibited by atropine with an IC_{50} of 10 nM.

In contrast, L116A was expressed at 2% of the wild-type level, and at this level gave no detectable basal PI signal. However, the ACh-stimulated maximum signal was equivalent to 40% of the wild-type maximum.

Postulating that the L116A mutation might destabilise the receptor structure, we attempted to counteract this effect by culturing the cells in the presence of 10^{-6} M atropine. This increased the expression level 50-fold, to 100% of the wild-type level. At this level, the L116A mutant gave high basal activity, equivalent to 70% of the maximum signal.

The L116A and S120A mutations increased basal activity, and agonist affinity, and decreased the slope of the agonist binding curve. This suggested that their primary effect is to increase the isomerisation constant, K, between the ground and activated states of the receptor, without changing KG. By combining the increase in the value of K, calculated from the basal activity (eqn. 3), with the values of $P4/K_{Bin}$ (eqn. 6), we estimated a value of 14.1 for KG. This led to values of αK of 0.83, 1.66 and 46 for the wild-type, S120A and L116A variants.

From equation (5), the values of αK_A calculated for S120A and L116A were 1.0×10^9 M^{-1} and 4.8×10^8 M^{-1} respectively. These values agree well, and suggest that the binding constant K_A and the cooperativity α of ACh binding are very similar for the two mutants. A cooperativity of about 8000 is compatible with the difference in affinity of high efficacy muscarinic agonists for the G protein-coupled and uncoupled states of the mAChRs [3]. The calculations predict that the affinity of ACh for the G protein-coupled state of the receptor is about 10^9 M^{-1}.

Utilising a value of 8000 for α gives values of K of about 10^{-4}, 2×10^{-4} and 5×10^{-3} for the wild-type, S120A and L116A receptors. These suggest that only a small proportion of the receptor naturally exists in the activated state (0.01%, wild-type, 0.02% S120A, 0.5% L116A) but that the fraction becomes substantial in the presence of ACh (45% wild-type, 62% S120A, 98% L116A).

It is interesting that the apparent 50-fold increase in the isomerisation constant of the L116A mutant was accompanied by a 50-fold decrease in its expression level. One possible explanation is that the mutation decreases the stability of the ground-state of the receptor, but without affecting the stability of the activated state. This would be expected to diminish the probability of successful receptor folding within the cell, and simultaneously to increase the probability of isomerisation to the activated state of those molecules which did fold sucessfully.

The value of 14.1 for KG has been applied to estimate (subject to certain assumptions) the changes in K_A, and in the product αKKG for all of the mutants studied.

Figure 2 shows a helical net representation of the effects of the mutations on three parameters characterising the receptor. In each case, the change is shown relative to the wild-type receptor. The three parameters are: i) the receptor expression level, which may give an indication of the effect of the mutation on the stability of the ground state structure; ii) the formation of the AR binary complex relative to R; and iii) the formation of the ARG ternary complex relative to AR. Left-pointing arrows indicate a decrease and right-pointing arrows an increase in the stability of the AR binary complex relative to R (filled arrows), or the ARG ternary complex relative to AR (open arrows), while the diameter of the circle representing each amino acid is equal to the Log of the ratio of wild-type expression to mutant expression plus one. Reductions in the expression ratio or the K_A of less than three-fold, or in AR*G of less than six-fold are taken to be insignificant.

This representation gives insight into the probable roles of the various residues. The nine null residues, by the above criterion, are segregated on one face of the helix. Six occur in the outer half, between L100 and A111, but only three in the inner half, between S112 and Y124. In a Cys-substitution mutagenesis study of TMD 3 of the D2 dopamine receptor [7], nine of the twelve residues found to be unreactive with a polar thiol reagent correspond to the null residues in the M1 mAChR, and, with the exception of F121, are predicted to face towards the lipid bilayer in Baldwin's original model [1]. The two mutagenesis studies thus confirm the prediction of the model.

In contrast, only one of the residues whose mutation strongly affected receptor expression, D105, is found in the outer half, but six are found in the inner half of the helix. This is consistent with closer packing around the inner (more intracellular) than the outer (more extracellular) part of the helix.

On the inward-facing surface of the outer part of the helix, facing away from the lipid bilayer towards the core of the receptor, there is a patch of residues, centred on D105 and Y106 whose mutation strongly affected the formation of the ACh-receptor binary complex. With the exception of S109A, these mutations also reduced the affinity of the antagonists NMS and QNB. A subset of the mutations, W101A, D105A, Y106A and S109A also strongly reduced the formation of the ternary complex. However, mutation of two residues on the periphery of this patch, L102 and N110 primarily reduced ACh binary complex formation, but had little further effect on ternary complex formation.

These findings reinforce the primacy of D105, supported by Y106, in ACh binding, and signal transduction [11, 13,15]. Notably, D105 is the only residue whose mutation completely abolished signalling. However, D105 appears to have a complex function. It may contribute to the stability of the

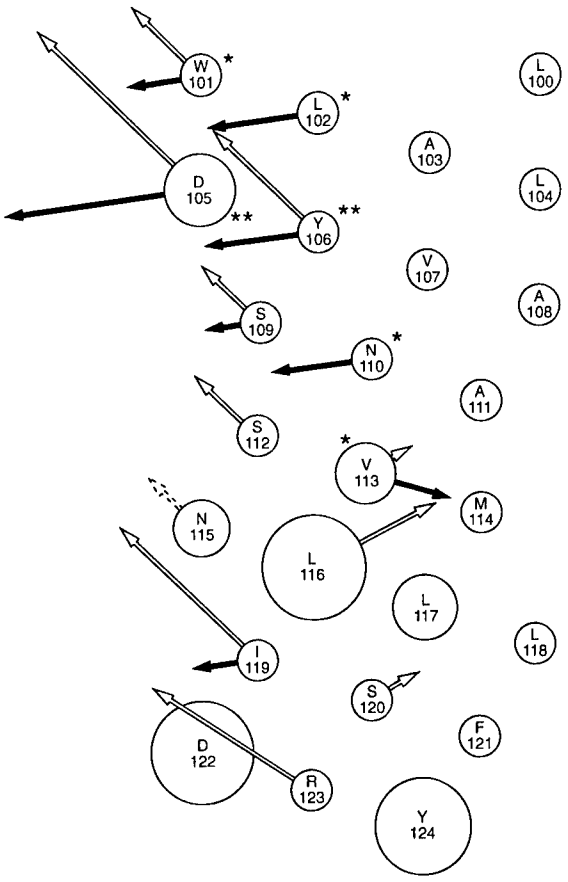


Figure 2. Scanning mutagenesis of transmembrane domain 3 of the M1 muscarinic acetylcholine receptor. The figure shows the amino acids mutated, from Leu 100 to Tyr 124. Mutations are from X to A, or from A to G. The diameter of each circle represents the effect of the mutation on the expression level relative to the wild-type; Log ([R_T,wt]/[R_T,mutant]) + 1. Filled arrows represent the effect on ACh-receptor binary complex formation; Log (K_A, mutant) –Log (K_A, wild-type). Open arrows represent effects on ARG ternary complex formation; Log (αKKG, mutant) –Log (αKKG, wild-type). Left-pointing arrows represent a decrease relative to the wild-type, and right-pointing arrows an increase relative to the wild-type. Asterisks show a reduction in NMS affinity: *10–100-fold; **> 100-fold. The dotted arrow shows a 25% reduction in catalysis of GDP-GTP exchange by the N115A mutant.

ground-state receptor structure as well as being vital for formation of the binding site.

These observations strongly support the view that this patch of amino acids is the primary agonist (and antagonist) contact site in TM3. However, they do not allow us to decide whether additional contributions to the stability of the ternary complex arise from the strengthening of their ground-state interactions with the agonist (effect on α), or from the formation of new intramolecular contacts within the receptor structure itself (effect on K) promoted by agonist and G protein binding.

Interestingly, the tightly-delimited segment of TMD 3 whose mutation affects ternary complex stablity extends beyond the binding site residues to the cytoplasmic end of the helix. Mutation of S112, and most particularly, I119 and R123 all had large effects on the calculated stability of the ternary complex. Furthermore, mutation of I119 increased the Hill slope of the binding curve, and decreased the agonist K_A. Mutation of R123 also slightly steepens the ACh binding curve [8], but does not affect ACh affinity. These mutations did not affect receptor expression levels.

The results do not allow us to distinguish completely between possible intramolecular (effects on K) and intermolecular (effects on αKG) of these three mutations. Effects on α seem relatively unlikely because of their distance from the agonist binding site. On balance, it seems likely that I119 makes an intramolecular contact which is primarily important for the stability of the AR* state and the formation of the G protein binding pocket, although it probably exists in a primordial form in the AR complex. R123 may have a dual role, with potential for direct contact with the G protein, as well as intramolecular interactions.

N115 is an interesting variation on this theme, in which mutation may have slightly reduced the catalytic efficiency, rather than the stability of the AR*G complex.

Finally, there is a group containing V113, L116 and S120 whose mutation increased basal signalling, and ternary complex formation. This patch is displaced by one residue (100 degrees) from the segment which diminished signalling. Two of the mutations, V113A and L116A, also reduced receptor expression. We have argued that the primary contribution of L116 is an intramolecular interaction which promotes the stability of the ground state, but not the activated state of the receptor. A supporting part may be played by S120, whilst V113 may also have an indirect effect on the conformation of the primary binding site itself, since its mutation decreased NMS affinity while increasing ACh affinity. We postulate that these residues may help to constrain the receptor to the inactive state in the absence of agonists.

In summary, scanning mutagenesis has revealed strong functional differentiation of the surface of TMD 3 of the M1 mAChR. It has confirmed the α-helical nature of the domain, and determined its orientation with respect to the lipid bilayer. It has shown that one corner of the helix is devoted to the formation of intermolecular (ACh-receptor, receptor-G protein) or intramolecular bonds which are essential for formation of the active AR*G ternary complex, whilst within the inner, more buried part of the domain, an adjacent compact patch of amino acids probably forms intramolecular contacts which selectively stabilise the ground state but not the activated state of the receptor. The key residues are very highly conserved in the GPCR superfamily, so these results may have wide implications for understanding the activation mechanism of these receptors.

Acknowledgments

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Molecular mechanisms for the regulation of the expression and function of muscarinic acetylcholine receptors

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Abstract — The regulation of muscarinic acetylcholine receptor expression and function was investigated in cultured cells and in knockout mice. Muscarinic agonist exposure causes m2 receptor desensitization and sequestration and decreases the expression of cardiac potassium channels. The expression of m2 receptors in chick retina is regulated by a developmentally regulated secreted factor. Mice lacking the m1 receptor exhibit a loss of muscarinic regulation of M-current potassium channel activity and pilocarpine-induced seizures. (©Elsevier, Paris)

Résumé — Mécanismes moléculaires de la régulation, de l'expression et de la fonction des récepteurs muscariniques à l'acétylcholine. La régulation de l'expression et de la fonction des récepteurs muscariniques a été étudiée dans des cultures de cellules et par inactivation de gènes chez la souris. Les agonistes muscariniques entraînent une désensibilisation et une séquestration des récepteurs m2 et réduisent l'expression des canaux potassium cardiaques. L'expression des récepteurs m2 dans la rétine du poulet est régulée par un facteur sécrété, lui-même contrôlé au cours du développement. Les souris 'knock out' montrent une perte de la régulation muscarinique de l'activité des canaux potassium (courant M) et présentent des arrêts cardiaques induits par la pilocarpine. (©Elsevier, Paris)

muscarinic receptor / knockout mouse / seizures / desensitization / retina / potassium channel

1. Introduction

Muscarinic acetylcholine receptors (mAChR) are expressed in neurons of the central and peripheral nervous systems, cardiac and smooth muscle, and a variety of exocrine glands. Muscarinic receptors are members of the superfamily of receptors which produce their physiological responses by interacting with members of the GTP-binding regulatory protein (G-protein) superfamily. Five subtypes of mammalian mAChR, termed m1–m5, have been identified and shown to be encoded by distinct genes. The five subtypes of mAChR can be grouped into two broad functional categories, with the m2 and m4 receptors preferentially coupling to the Gi/o family of G-proteins, and the m1, m3, and m5 receptors preferentially coupling to the Gq family of G-proteins [15]. Our laboratory has been interested in determining the molecular and cellular mechanisms involved in the regulation of the expression and function of the mAChR. In this article, we will describe work directed at clarifying the molecular mechanisms involved in the regulation of muscarinic receptor expression at the transcriptional and post-translational levels and genetic approaches to determine the function of mAChR in vivo.

2. Regulation of mAChR by G-protein coupled receptor kinases and β-arrestin

There are both long-term and short-term mechanisms for the regulation of mAChR expression and function in response to the continued presence of acetylcholine or other agonists [11]. Phosphorylation by members of the G-protein coupled receptor kinase (GRK) family of an agonist-occupied receptor is thought to induce the binding of one of the members of the arrestin family of proteins to the activated receptor, blocking further receptor-G-protein interactions and thus causing a rapid desensitization of receptor signal transduction. In addition, the binding of arrestins to phosphorylated receptors can promote receptor internalization [3, 6].

We have described a reporter gene assay using a luciferase reporter gene under the transcriptional control of a cAMP response element (CRE) as a sensitive detector for changes in the levels of intracellular cAMP [9, 10]. This assay allows measurement of changes in physiologically relevant intracellular cAMP levels without requiring the addition of phosphodiesterase inhibitors (which are usually added

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when one is measuring cAMP levels biochemically) or supraphysiological concentrations of forskolin. In addition, it allows measurement of responses following cotransfection of clones encoding receptors and G-proteins in transiently transfected cells without confounding effects due to the presence of untransfected cells in the cultures. The CRE-reporter genes used in these assays when expressed in JEG-3 cells respond only to changes in cAMP and not to intracellular calcium. Furthermore, the changes in CRE-luciferase expression require the presence of a functional cAMP-dependent protein kinase. This system allows transient transfection experiments to be carried out using a virtually unlimited combination of receptors, G-proteins, kinases, etc. Our studies have shown that this approach can be used to study the function of both muscarinic receptors and G-proteins. [9, 10]. We have now used this system to determine the ability of G protein-coupled receptor kinase-2 (GRK2) and β-arrestin-1 to regulate the phosphorylation, desensitization, and sequestration of the m2 mAChR [12]. Treatment of JEG-3 cells transiently expressing an epitope-tagged m2 mAChR with the muscarinic agonist carbachol induced an approximately 4-fold increase in receptor phosphorylation in the absence and an 8-fold increase in receptor phosphorylation in the presence of cotransfected GRK2, when compared to cells transfected with receptor alone in the absence of agonist. Using the expression of a cAMP-regulated reporter gene to measure receptor function, in the absence of cotransfected GRK2 and β-arrestin, the m2 mAChR exhibited modest functional desensitization of its ability to regulate CRE-luciferase expression following exposure to agonist. While transfection of β-arrestin-1 by itself had no effect on m2 signaling, transfection of GRK2 enhanced agonist-induced desensitization, and cotransfection of GRK2 and β-arrestin-1 acted synergistically to promote functional desensitization. GRK-2 and β-arrestin-1 did not affect signaling by a receptor deletion mutant lacking the GRK phosphorylation sites in the third cytoplasmic loop. GRK2 and β-arrestin-1 also synergistically increased both the rate and magnitude of agonist-induced sequestration of the m2 mAChR. These results demonstrate that GRK2 and β-arrestin-1 act in concert to cause agonist-induced desensitization and sequestration of the m2 mAChR and show that these proteins have multiple actions on the m2 mAChR.

3. Regulation of GIRK potassium channels by mAChR activation

Muscarinic receptors decrease the rate of contraction of the heart in part by activating a G-protein coupled inwardly rectifying potassium channel, composed of two subunits, GIRK1 and GIRK4 [4]. Muscarinic receptors activate GIRK channels in atrial cells but not in ventricular cells. In chick heart, this tissue-specific functional response is due to the fact that, while atrial cells express both GIRK1 and GIRK4, ventricular cells only express significant levels of GIRK1 but not GIRK4, resulting in an inability to form a heteromultimeric functional channel [14]. We have previously demonstrated that long-term (i.e., over several hours) exposure of chick heart cells to muscarinic agonists causes large decreases in the number of muscarinic receptor binding sites and in the levels of mRNA encoding the mAChR [1]. We have recently investigated the effects of mAChR activation on the expression of GIRK subunits [14]. Treatment of chick embryos in ovo with the muscarinic agonist carbachol resulted in a time- and concentration-dependent decrease in the level of GIRK1 and GIRK4 mRNAs in the atria. Carbachol treatment also decreased the level of GIRK1 polypeptide expression as detected by immunoblot analysis. The decrease in mRNAs could be prevented by concomitant administration of the antagonist atropine, and subsequent administration of atropine after treatment with carbachol alone resulted in a gradual return of GIRK1 and GIRK4 mRNAs back to control levels. These results demonstrate that persistent activation of the mAChR can decrease the expression of an effector protein required for receptor-mediated signaling. Because a variety of G-protein coupled receptors can regulate GIRK activity in the nervous system, these results raise the possibility that this could represent a novel mechanism for the regulation of neurotransmitter receptor responsiveness by both homologous and heterologous receptor activation.

While agonist treatment decreased the levels of GIRK1 and GIRK4 mRNAs in atria, there was no effect on the level of GIRK1 mRNA in the ventricles. In contrast, carbachol treatment causes similar decreases in the levels of mAChR mRNAs and mAChR binding sites in atria and ventricles. These results suggest that the signaling mechanisms for the regulation of mAChR mRNAs levels following mAChR activation are different from those responsible for the regulation of GIRK mRNA levels.

4. Regulation of retinal mAChR during embryonic development

Previous working using affinity alkylation and SDS gel electrophoresis suggested that the pattern of expression of mAChR in the chick retina changes dramatically during embryonic development [5, 13].

We used solution hybridization, immunoprecipitation, and immunoblot analyses to examine the expression of chick m2 (cm2), cm3, and cm4 mAChR mRNA and protein in embryonic and post hatched chick retina. While the cm4 receptor is the predominant receptor early in development, the cm3 receptor increases somewhat and the cm2 receptor increases particularly dramatically during the second week of embryonic development [8]. A similar increase in cm2 expression occurs in cultured embryonic chick retinal cells. Treatment of fresh cultured retinal cells with conditioned medium from retinal cells cultured for at least 5 days, but not from retinal cells cultured for 1–2 days, results in a selective increase in expression of cm2 but not cm3 or cm4 receptors due to the action of a protease-sensitive secreted factor [7]. The cm2-inducing factor stimulates expression of a luciferase reporter gene under the transcriptional control of the cm2 promoter, showing that the increase in cm2 expression results from increased gene transcription. Addition of 14 known neurotrophic and growth factors to cultured retinal cells did not increase the expression of the cm2 receptor. The induction of the cm2 receptor gene in the retina may be due to the action of a novel developmentally regulated secreted factor.

5. Genetic dissection of mammalian mAChR function

We have begun to use gene targeting by homologous recombination to determine the function of individual mAChR subtypes in the mammalian nervous system [2]. A targeting vector was designed to delete the translational start site and the region encoding the amino terminal, first transmembrane domain, and a portion of the first cytoplasmic loop from the mouse m1 receptor gene. Embryonic stem cell clones in which the mutant gene was incorporated into the genome by homologous recombination were used to obtain m1 knockout mice. Homozygous m1 mutant mice arose from crosses of heterozygotes with a Mendelian ratio of approximately 1:4, and exhibited no differences in body weight, longevity, fertility, or overt behavior compared to wild type mice.

There was an approximately 50% decrease in the total number of mAChR binding sites from the forebrains of knockout compared to wild type mice, while there was no significant decrease in the level of mAChR sites in the cerebellum (where there is little m1 receptor expressed). Immunoprecipitation analyses demonstrated that there was no detectable expression of m1 receptor in knockout mice, while the levels of m2, m3, and m4 receptors were not affected. Immunocytochemical analyses showed that there was also no significant difference in the overall morphology of the brain or in the pattern of expression of the m2, m3, and m4 receptors.

Muscarinic agonists cause robust suppression of the M-current potassium channel in sympathetic neurons derived from wild type mice, while there was no effect of muscarinic agonists on the M-current in sympathetic neurons from knockout mice. In contrast, angiotensin II was able to suppress the M-current in both wild type and mutant neurons. Thus, the m1 receptor is the only muscarinic receptor which regulates M-current activity in sympathetic neurons.

Systemic administration of the muscarinic agonist pilocarpine causes seizures that resemble those observed in patients with temporal lobe epilepsy. Homozygous mutant mice are totally resistant to the administration of doses of pilocarpine which result in multiple tonic-clonic seizures in wild type mice. Interestingly, heterozygous mutant mice are almost as resistant as knockout mice to the seizures produced by administration of high levels of pilocarpine. These results suggest that the m1 receptor plays a crucial role in the initiation of seizures in the pilocarpine model of epilepsy. The m1 mutant mice may be useful in delineating the pathways involved in seizure initiation and in the roles of the m1 receptor in mediating the many behavioral responses elicited by acetylcholine in the nervous system.

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Role of neurotrophic factors in motoneuron development

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Abstract — More than 10 factors from different gene families are now known to enhance motoneuron survival, and to be expressed in a manner consistent with a role in regulating motoneuron numbers during development. We provide evidence that: a) different factors may act on different sub-populations of motoneurons; b) different factors may act in synergy on a given motoneuron. Thus, the functional diversity of motoneurons, and the cellular complexity of their environment, may be reflected in the mechanisms that have evolved to keep them alive. ©Elsevier, France)

Résumé — Rôle des facteurs neurotrophiques au cours du développement des motoneurones. Plus de 10 facteurs de familles géniques différentes sont maintenant connus pour leur capacité à favoriser la survie des motoneurones. Le profil d'expression de ces facteurs est cohérent avec l'idée qu'ils sont impliqués dans la régulation du nombre de motoneurones au cours du développement. Nos résultats suggèrent que : a) différents facteurs peuvent agir sur des sous-populations de motoneurones différentes; b) différents facteurs peuvent agir en synergie sur un motoneurone donné. Donc, la diversité fonctionnelle des motoneurones, ainsi que la complexité cellulaire de leur environnement, se retrouvent dans les mécanismes qui ont évolué pour assurer leur survie. ©Elsevier, France)

motoneuron / neurotrophic / survival / neurogenerative disease

1. Introduction

Synapses formed on muscle by motoneurons are of vital importance for the survival of the organism. This means that the survival of these neurons and the maintenance of their synapses are the object of multiple controls [2]. Another consequence is that diseases that affect the survival of motoneurons are rapidly fatal. Molecules that keep motoneurons alive during development may therefore be useful in man to slow down pathological neuronal degeneration [4].

Neurotrophic factors are a diverse group of polypeptides with multiple biological roles; their common feature is that they promote survival of certain neuronal populations [5]. Certain of these factors act during development to regulate neuron numbers in the peripheral nervous system. Since the classical limb ablation experiments of Viktor Hamburger in the chick embryo, it has been known that motoneurons too require neurotrophic factors from the limb for their normal survival, but only recently have some of the molecules potentially involved been identified [2, 5]. However, although many candidate factors are known to keep motoneurons alive in experimental situations, the physiological survival factors for motoneurons have not yet been unambiguously identified. Furthermore, the number and diversity of the candidate factors identified are still not well understood in biological terms.

2. Identification of motoneuron survival factors

Our approach to the identification of molecules potentially involved in keeping motoneurons alive in vivo has been to develop highly purified preparations of spinal motoneurons from chicken and rat embryos [6]. When cultured in complete media, these neurons will nevertheless undergo apoptosis unless provided with exogenous trophic support, either in the form of tissue extracts or conditioned media or, more importantly, in the form of recombinant neurotrophic factors. Although this culture system is obviously in some ways distant from the in vivo situation, it has clear advantages: molecules thus identified must be acting directly on motoneurons, and their potency and efficacy can be directly and precisely quantified. Indeed, many of the factors identified in this system act extremely potent, and can provide long-term support for a significant fraction of the motoneurons initially seeded.

The factors identified belong to several different gene families: i) neurotrophin family: BDNF (brain-derived neurotrophic factor), NT-3 (neurotrophin-3), NT-4/5 [7]; ii) transforming growth factor-beta family: GDNF (glial cell line-derived neurotrophic factor), NTN (neurturin), PSP (persephin), TGFβ3 [3, 8–10]; iii) cytokines of the interleukin-6 family: CNTF (ciliary neurotrophic factor), LIF (leukaemia inhibitory factor), CT-1 (cardiotrophin-1) [1, 8, 11]; and iv) other growth factors: members of the FGF

(fibroblast growth factor) family, HGF (hepatocyte growth factor) [3, 13].

Nearly all of these factors have also been shown by others to be active in preventing motoneuron cell death in vivo, either during programmed cell death in the chicken or mouse embryo, or following axotomy in neonatal rat pups, or in mouse mutant strains that show motoneuron degeneration. Thus, cultured purified motoneurons have provided a reliable testing system, and there is a general consensus that many factors can indeed act to promote motoneuron survival. One consequence of this is that they are all potential candidates for therapeutic applications involving motoneuron degeneration. However, much remains to be learned about their biological roles. Are all indeed required for normal motoneuron survival? If so, why are so many different factors involved? And at what stages? We and others have been addressing different aspects of this problem.

3. Trophic requirements of motoneuron sub-populations

In terms of their early development, embryonic motoneurons have often been considered as a homogeneous group of cells. However, different classes of motoneuron (alpha, gamma, fast, slow, etc.) fulfill very different functions in the adult. Furthermore, it is known that different sub-populations of embryonic motoneurons express different molecular markers (e.g., transcription factors). It is therefore likely that the development of these sub-populations may be differentially regulated.

For instance, heterogeneity is apparent in terms of the action of neurotrophic factors on the survival of developing motoneurons. In knockout mice for the neurotrophic factor GDNF or for the cytokine receptors CNTFRα or LIFRβ, motoneuron loss is reproducible but partial. This is mirrored by the observation that no individual neurotrophic factor has been shown to be capable of keeping all motoneurons alive for long periods, and that combinations of factors are more efficient [1, 11]. These results may be compared with well-established data on the development of spinal sensory ganglia, in which sub-populations of neurons with different functions (proprioceptive, nociceptive, etc.) respond to, and require, specific neurotrophic factors for their survival. What is lacking in the case of motoneurons is a suitable repertoire of molecular markers for motoneurons responding to different factors, and a direct link between molecular diversity in the embryo and functional diversity in the adult.

One obvious potential marker for responsiveness to a given neurotrophic factor is the presence of the appropriate receptor. Although certain receptors for neurotrophic factors are expressed by virtually all motoneurons, this is not the case for all of them. Probably the best example is provided by c-Met, the receptor for hepatocyte growth factor (HGF/SF). HGF/SF is an important component of myotube-conditioned media, as antibodies to HGF block 65% of the trophic activity of such media for cultured motoneurons [13]. However, although its actions on cultured motoneurons are quite potent, HGF is unable to keep all of the motoneurons alive, even at short survival times. This is mirrored by the fact that at a given rostro-caudal level, only a fraction of the motoneurons express c-Met. Thus, although its role in vivo remains to be demonstrated, HGF may play a role in maintaining a specific sub-population of motoneurons.

4. Synergistic actions of neurotrophic factors

Another possibility is that each cellular partner of a given motoneuron may interact with the motoneuron using a different factor. For example, we have shown that GDNF is produced by Schwann cells, whereas the cytokine CT-1 is synthesized by muscle [8, 11]. Blockade of GDNF activity reduces the levels of trophic support produced by Schwann cells by 75%, while antibodies to CT-1 inhibit muscle cell line-conditioned media by 45%. We recently showed that these factors can act in synergy on motoneuron survival, providing a potential mechanism for selective preservation during development of those motoneurons that have contacted both the target muscle and Schwann cells [1]. This research may have direct repercussions for the use of neurotrophic factors in the clinic, and indeed there are several reports in animal models of increased effectiveness of the administration of combinations of neurotrophic factors.

5. Conclusions

The field of study of survival factors for motoneurons has seen rapid progress over the past 4 years. Many of the potential actors (neurotrophic factors, receptors, etc.) have now been identified, and new model systems have been developed to test their activities. Nevertheless, we are only just beginning to fit them into the puzzle represented by the development over time of the different components of the neuromuscular system [2, 12]. In particular, we believe that it is important to appreciate the cellular complexity of the motoneuron population in order to understand the action of neurotrophic factors in

this system. This may be of importance not only for developmental studies, but also in evaluating candidate trophic factors for potential clinical use in specific pathological situations.

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For brevity, only recent work and reviews from our laboratory are given. Many important articles by others in the field are cited in the listed references.

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Cholinergic foundations of Alzheimer's disease therapy

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Abstract — Cholinesterase inhibitors (ChEI) represent the drug of choice for Alzheimer's disease (AD) treatment. They produce significant improvement on cognitive as well as non-cognitive function for a period up to 1 year during the first 3 years following clinical diagnosis. The magnitude of cognitive improvements is similar for different ChEI, however, differences are seen with regard to incidence and severity of side effects, optimal ChE inhibition, pharmacokinetic properties and mode of administration. (©Elsevier, Paris)

Résumé — Fondement cholinergique du traitement de la maladie d' Alzheimer. Les inhibiteurs de la cholinestérase constituent le traitement de choix de la maladie d'Alzheimer. Prescrits dans les trois premières années suivant le diagnostic clinique, ils améliorent de façon significative jusqu'à un an les fonctions cognitives et non cognitives. L'ampleur des améliorations cognitives est semblable pour les divers inhibiteurs de la cholinestérase, mais des différences sont observées au niveau de l'incidence et de la gravité des effets secondaires, de l'inhibition optimale de la cholinestérase, de la pharmacocinétique et du mode d'administration. (©Elsevier, Paris)

cholinesterase inhibitors / Alzheimer's disease

1. Past and future of cholinesterase inhibitors in Alzheimer's therapy

Cholinesterase inhibitors (ChEI) represent the drugs of choice for Alzheimer's disease (AD) treatment. Following the first generation of non-specific drugs such as physostigmine, a second generation of more selective products has been developed. These compounds show less side effects in humans at effective doses. The data originating from recent clinical trials demonstrate that optimization of effect and maintenance of clinical gains for 1 year is presently a feasible target. Future studies will demonstrate whether or not: i) if it is possible to extend the benefit for a period longer than 12 months; and ii) if there is a protective effect for minimal cognitive impairment (MCI) subjects at risk for conversion to AD. The considerable amount of basic knowledge on the cholinergic system's physiology and pharmacology accumulated during the last 50 years has greatly contributed the therapeutic success of ChEI. Physostigmine was first isolated and used in therapy more than half a century before the discovery of acetylcholine as a neurotransmitter [9] (*table I*). Cholinesterases were isolated from the mammalian brain 20 years after the discovery of ACh as a neurotransmitter (*table I*) The utilization of ChEI as anti-Alzheimer drugs represents the most recent acquisition of the CNS drugs development which started in the fifties with neuroleptics and continued into the sixties with the discovery of anti-Parkinson agents. The first ChEI to be used against AD was physostigmine followed by tacrine (*table I*). Subsequently, metrifonate and galanthamine were tested simultaneously with other ChEIs (*table I*). Cholinesterase inhibitors, particularly s.c. second generation, (post-physostigmine and post-tacrine compounds), affect cortical as well as sub-cortical neurotransmitters other than ACh [14]. Their effects on NE and DA are of particular clinical interest for AD therapy. Another feature of ChEI is their ability to enhance the release of non-amyloidogenic soluble derivatives of APP from cortex both in vitro and in vivo and possibly

Table I. Cholinesterase inhibitors. a long history.

	Ref
Physostigmine	Isolation 1864 (Jobst and Hesse)[a] Therapeutical use 1877 (Laquer)[a]
Acetylcholine	Synthesis 1867 (Baeyer) A neurotransmitter 1914 [9])[a]
Cholinesterases	Isolation and name 1932 (Stedman)[a] Found in the brain 1935 (Stedman and Stedman)[a]
Cholinesterase inhibitors as anti-Alzheimer drugs	1979 Physostigmine i.v. [10] 1983 Physostigmine [33] 1981 Tacrine [32] 1988 Metrifonate [3] 1989 Galanthamine [28]

[a]Goodman and Gilman, The Pharmacological Basis of Therapeutics, McGraw Hill Publ., Ninth Edition.

to slow down formation of amyloidogenic compounds in AD brain [24]. This process could explain the stabilization effect on cognitive deterioration seen during ChEI therapy. Cholinergic alternatives other than use of ChEI are being investigated such as the utilization of nicotinic and muscarinic agonists. Non-selectivity and presence of severe side effects has so far prevented therapeutic application of these compounds as complementary cholinergic approaches.

2. Second generation cholinesterase inhibitors

Cholinesterase inhibitors presently in clinical trials or in current use (1998) in Japan, USA and Europe include less than 10 drugs. Most of these compounds have advanced to clinical phase III and IV and two (tacrine, and donepezil) are registered in USA and in Europe [16]. Two other compounds (rivastigmine and metrifonate) are close to registration both in USA and Europe. This second generation ChEI, in order to replace tacrine, has to fulfill specific requirements such as lower toxicity and easier administration [16]. Based on current selection criteria and occurrence of complications a number of ChEIs (particularly carbamates) has been discontinued. Are there major differences among various compounds with regard to efficacy, percentage of treatable patients, drop-out and incidence of side effects? *Table II* compares the effect of six ChEIs on ADAS-cog test using intention to treat (ITT) criteria. The duration of these clinical trials varied from 24 to 30 weeks and the total number of patients was above 4000. All six ChEIs (tacrine, eptastigmine, donepezil, rivastigmine, metrifonate and galanthamine) produced statistically significant improvements using standardized and internationally validated measures of both cognitive and non-cognitive function. The magnitude of cognitive effects measured with the ADAS-cog scale for all six drugs either expressed as a difference between drug- and placebo-treated patients or as the difference between drug-treated patients and baseline is similar (*table II*). This similarity in size of cognitive improvement suggests a ceiling effect at approximately five ADAS-cog points average for ChEI tested in mild to moderate (CDR 1–1.5) stages of the disease at present dosage. These results suggest that a maximal effect has not yet been reached with all drugs tested. The high percentage of drop-outs and more severe side-effects seen with some particular drugs such as tacrine (hepatotoxicity and general cholinergic toxicity) suggest a limit in achievable levels of ChE inhibition and demonstrable drug effect. Similarity in clinical efficacy is also underlined by the practically identical effect on global scales such as the clinicians interview-based impression of change-plus (CIBIC-plus) of a 0.4 points difference seen for tacrine, rivastigmine and metrifonate, drugs having very different chemical structure and pharmacological profile. The percentage of improved patients varies from 25% (rivastigmine low dose) to 50% (tacrine and donepezil high dose) with an average of 34%. This indicates that one-third of treated patients shows a positive response, however, one should keep in mind that a small percentage (about 10%) of patients do not improve on the ADAS-cog with any of the used drugs while other patients demonstrate a response significantly higher than five points. Difference between responders versus non-responders may reflect the level of cholinergic damage present in the brain and/or a genetic factor (e.g., APOE-4 alleles). From the 6-month data presented in *table II* one can also observe that patients treated with the active compound change little cognitively from baseline at the beginning of the study to the end. This suggest a stabilization effect on patient deterioration which could be clinically more significant than the expected symptomatic improvement.

Table II. The effect of six cholinesterase inhibitors on ADAS-cog test (ITT).

Drug (ref)	*Dose (mg/day)*	*Duration of study (weeks)*	*Treatment difference from*		*Improved patients (%)*	*Drop-out (%)*	*Side effects (%)*
			*Placebo**	*Baseline***			
Tacrine [12, 21]	120–160	30	4.0–5.3	0.8–2.8	30–50	55–73	40–58
Eptastigmine [7, 20]	45	25	4.7	1.8	30	12	35
Donepezil [11, 29, 30]	5–10	24	2.8–4.6	0.7–1	58	5–13	6–13
Rivastigmine [1]	6–12	24	1.9–4.9	0.7	25	15–36	28
Metrifon [5, 6, 23, 25]	25–75–80	12–26	2.8–3.1–3.2	0.75–0.5	35	2–21–8	2–12
Galanthamine [36]	30	12	3.3	1.8	–	–33	–

ADAS-cog, AD assessment scale-cognitive subscale; ITT, intention to treat;*study end point vs. placebo; **study end point vs. baseline, donepezil E 2020; ENA 713, rivastigmine; metrifon, metrifonate.

3. Differences in effects among cholinesterase inhibitors

The relation between percentage of peripheral ChE inhibition and cognitive (ADAS-cog) or global impression of change rated by the clinician (CGIC) effect is reported in *table III*. These data support the fundamental principle of cholinergic pharmacology that brain ChE inhibition relates directly to an increase of synaptic ACh levels leading to cognitive and memory improvement. This relationship might vary for each drug as each compound may produce cognitive improvement and therapeutic effect at different levels of ChE inhibition [13, 16, 18]. This concept is in agreement with pharmacological data in animals [16, 22] and humans [13]. The level of peripheral enzyme inhibition measured in the patient (AChE activity in erythrocytes or plasma BuChE activity) varies between 30% and 80% (*table III*) depending on dose and pharmacological characteristics of the compound. The ChE inhibition measured in the periphery (plasma or RBC) does not accurately reflect the level of CNS inhibition. For some drugs (donepezil and metrifonate) the mean level of peripheral ChE inhibition is 65–70% and could be brought to be as high as 90%. For other drugs, inhibition is as low as 30% (physostigmine, tacrine, eptastigmine). As predicted by pharmacological and behavioral data, there is a direct correlation between ChE inhibition (or drug plasma concentration) and cognitive effect [14, 15]. Also, as expected from animal models, there is no direct correlation between ChE inhibition and severity of side effects [2, 29]. As an example, degree of improvement in ADAS-cog and CIBIC-plus was directly proportional with the degree of AChE (RBC) inhibition following metrifonate treatment while side effects were very mild even at high level of inhibition (70%) [8]. A direct clinical implication of this relationship is that drugs producing only mild cholinergic side effects at high dosage (resulting in high level of brain ChE inhibition) may be tested in the patient within their full range of therapeutic potential. The predicted U-shaped curve of the relation ChE inhibition/cognitive effect can be observed for some ChEI at a certain dose range (*table III*).This is explained by the fact that increasing the dose of the inhibitor, efficacy is progressively increased until adverse effects become a limiting factor. A second explanation is the specific inhibition kinetic of ChEI and the substrate-induced saturation effect of ChEs (substrate inhibition effect) Under normal conditions, the level of ACh brain elevation varies according to brain AChE inhibition [16–18]. With increased brain concentrations of ACh, substrate inhibition kinetics of enzyme activity become a limiting factor. This relation is observed in brain tissue in vitro and is probably present also in vivo [17]. Plotting velocity of enzymatic reaction against substrate (ACh) concentration, a bell-shaped curve with a defined peak in case the of AChE activity in brain and erythrocytes and a sigmoid curve in case of BuChE (butyrylcholinesterase) activity in serum are observed. Thus, AChE is inhibited by a large excess of ACh such as it can be produced by a high level of inhibition of brain AChE. Substrate elevation decreases the catalytic potency of the enzyme and subsequently its pharmacological effect. From this relationship it can be predicted that high ChE inhibition reached too rapidly in time during treatment because of a rapid passage of the drug into the brain and its accumulation in CNS will not further increase efficacy but only augment CNS dependent side effects (drowsiness, nausea, vomit, etc.). Therefore, therapeutically, it is an advantage to use a slow-release type of ChEI inhibiting both brain enzymes (AChE and BuChE) at a slow pace (using escalating dose followed by a maintenance dose) in order to reach gradually steady-state levels of brain ACh. Such a procedure may also lower the risk of cholinergic receptor down-regulation and tolerance-producing enzyme induction. There is an excellent agreement between clinical and animal data for both physostigmine and tacrine with regard to dose/behavioral effects relationships. Rupniak et al. [31] using two primate models in rhesus monkeys found that both tacrine and physostigmine improved

Table III. Relation between percent ChE inhibition and effect on ADAS-cog or CGIC.

Drug (ref)	*Dose (mg/day)*	*Steady state (% inhibition)*	*Optimal ChE (% inhibition)*	*Correlation ChEI/ADAS-cog or CGIC*
Physostigmine [34]	3–16	40–60 (BuChE)	30–40	U-shaped
Eptastigmine [7, 19]	30–60	13–54 (AChE)	30–35	U-shaped
Metrifonate [4]	30	35–75 (AChE)	65–80	U-shaped
Donepezil [29]	5	64 (AChE)	60	Linear
Tacrine [12, 21]	160	40 (BuChE) 60 (AChE)	30	Linear
Galanthamine [36]	20–50	50–60 (AChE)	50	U-shaped

ADAS-cog, A D assessment scale-cognitive subscale; CGIC, clinician global impresssion of change.

Table IV. Comparison of pharmacokinetic properties of five cholinesterase inhibitors used in Alzheimer therapy.

Drug ref.	*Plasma conc. (μg/L) C-max*	*Time to peak (h) T-max*	*Elim. half-life (h) T ½*	*Metabolism type*	*Plasma prot. % abs.*	*Elimination (%)*	*Bio-availability (%)*
Tacrine	–	1–2	2–4	Hepatic (P450)	55	–	17
Donepezil [30]	30–60	3–4	73	Hepatic (P450)	96	60 renal	100
Rivastigmine [1]	–	0.3	5	Non-hepatic	40	Renal	36
Metrifonate [27]	500	0.5	2	Non-hepatic	20	Renal	90
Galanthamine [35]	543	0.5	4.4–5–7	Hepatic (P450)	10	Renal	100

visual recognition memory significantly. Both drugs showed a clear inverse U-shaped relationship with a maximal effect at around 0010–0.02 mg/kg i.m. for physostigmine and 0.8–1 mg/kg for tacrine. Lower or higher doses did not improve performance but only side effects. Central cholinergic side effects which may develop early in the treatment are not related directly to brain AChE inhibition but mainly to elevation of ACh levels [17, 18]. In the rat, this increase in ACh is not directly correlated to AChE inhibition. Peripheral side-effects may occur depending on a rapid redistribution of the drug (or of its metabolites) between non-CNS (peripheral organs such as bronchi, gastrointestinal tract, heart and muscles) and CNS compartments and peripheral (BuChE) inhibition. The occurrence of both peripheral and central side effects seen with all compounds tested so far demonstrates that none of the presently available inhibitors is truly 'brain specific'. With very long-acting ChEI (such as methanesulfonyls and organphosphates) the rate of enzyme activity recovery is limited by the rates of synthesis of new enzyme. This rate of synthesis is different for each tissue. In rat brain, the synthesis of new enzyme occurs with a mean half-life of approximately 11 days compared to 1, 3, and 6 days for ileum, heart and muscles [26]. This ten-fold difference between slow regeneration of brain AChE and rapid resynthesis of peripheral enzyme together with high brain selectivity of these compounds favors therapeutical effect over occurrence of side effects. This mechanism explains the low toxicity of metrifonate even at high (80–90%) AChE inhibition as compared to other ChEIs (*table II*).

4. Comparison of pharmacokinetic properties of cholinesterase inhibitors

A comparison of pharmacokinetic properties of five ChEI used in Alzheimer therapy is presented in *table IV*. Several important differences are apparent with regard to metabolism as well as other characteristics. While tacrine, galanthamine and donepezil are metabolized through the hepatic route (P-450), rivastigmine and metrifonate are not. Metrifonate, in particular, is transformed non-enzymatically into the active compound which is a potent inhibitor of ChE. This metabolic difference is clinically important since elderly patients show a decrease of hepatic metabolism.

Another important characteristic is the difference in drug elimination reaching a long $T_{1/2}$ (73 h for donepezil) and a short $T_{1/2}$ (2 h for metrifonate). Bioavailability is maximal for galanthamine and metrifonate (100% and 90% respectively). Plasma protein binding is lowest (10 and 20% respectively) for galanthamine and metrifonate and highest (96%) for donezepil. Since elderly patients are generally treated with several drugs simultaneously, this is of particular interest in relation to drug interactions.

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The role of Aβ42 in Alzheimer's disease

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Abstract — Our recent studies of plasma, fibroblasts, transfected cells and transgenic mice show that a fundamental effect of the mutations linked to familial Alzheimer's disease (FAD) is to increase the extracellular concentration of Aβ42. This effect of the FAD-linked mutations is likely to be directly related to the pathogenesis of Alzheimer's disease (AD) because Aβ42 is deposited early and selectively in the senile plaques that are an invariant feature of all forms of AD. Thus our results provide strong evidence that the FAD-linked mutations all cause AD by increasing the extracellular concentration of Aβ42 (43), thereby fostering Aβ deposition, and they support the hypothesis that cerebral Aβ deposition is an essential early event in the pathogenesis of all forms of AD. Interactions between the basal forebrain cholinergic system and Aβ that could influence AD pathogenesis are discussed. (©Elsevier, Paris)

Résumé — Rôle de l'Aβ42 dans la maladie d'Alzheimer. Des études récentes effectuées sur le plasma, des fibroblastes, des cellules transfectées et des souris transgéniques montrent que les mutations liées à la maladie d'Alzheimer familial (FAD) augmentent la concentration extracellulaire d'Aβ42. Cet effet des mutations liées à la FAD est probablement directement lié à la pathogénocité de la maladie d'Alzheimer puisque Aβ42 est présent plus tôt et plus sélectivement dans les plaques séniles par rapport aux autres formes d'AD. Ces résultats confortent l'hypothèse que le dépôt d'Aβ est un événement essentiel dans toutes les formes d'AD. (©Elsevier, Paris)

amyloid / senile plaques / cholinergic system / familial Alzheimer's disease linked mutations

1. Amyloid β protein deposition in Alzheimer's disease

In patients with Alzheimer's disease (AD), large numbers of senile plaques are found throughout the cerebral neocortex and hippocampus. These senile plaques, which are present in small numbers in the brains of aged mammals and normal elderly individuals, are observed in large numbers only in AD and thus are specific for this disorder. 'Classic' neuritic senile plaques consist of a spherical cluster of altered neurites surrounding an amyloid core composed of 5–10 nm wide fibrils that can be visualized on light microscopy by staining with Congo Red or thioflavin S [30]. In these plaques, microglia are intimately associated with the amyloid cores, and there are surrounding astrocytes with processes that project through the altered neurites toward the amyloid core. In many cases of AD, amyloid fibrils are also found in the walls of cerebral blood vessels [10].

The principal proteinaceous component of the amyloid deposited in AD is an ~4 kDa peptide, referred to as the amyloid β protein (Aβ), that has been isolated both from plaque cores and meningeal vessels of AD brain [11, 17, 20, 21, 23]. Immunocytochemical studies with antisera to Aβ have established that Aβ is deposited not only in the neuritic plaques described above but also in large numbers of diffuse plaques, which are poorly circumscribed, immunoreactive lesions that are distinguished from neuritic plaques in that they show minimal neuritic change or glial reaction [19, 32].

Aβ is produced by a single copy gene on chromosome 21, where it is encoded as an internal peptide within a large precursor protein referred to as the amyloid β protein precursor (βAPP). In each of the various Aβ-containing βAPP isoforms produced through alternative splicing of the βAPP gene, the 42 amino acid Aβ peptide begins 99 residues from the carboxyl terminus of the βAPP and it extends from the extracellular/intraluminal region (~28 amino acids) into the single membrane spanning domain (~14 amino acids) of the βAPP [9, 17]. In 1992, my group and several others showed that normal processing of the βAPP results in secretion of 4 kDa Aβ [13, 26, 28] that is readily detected in human cerebrospinal fluid, plasma, and medium conditioned by cultured cells. This soluble, secreted Aβ is primarily Aβ1–40 (~90%), but minor amounts of Aβ1–42 (~5–10%) and other Aβ species are also secreted [7, 29, 31].

Secreted Aβ is produced through the action of proteolytic activities referred to as β and γ secretase. Cleavage of full length βAPP by β secretase produces a large secreted derivative, sAPPβ, and an ~12 kDa cell-associated, C-terminal fragment with Aβ at its amino end. This ~12 kDa C-terminal fragment is then cleaved on the carboxyl side of Aβ by γ secretase to release Aβ. A third proteolytic activity, α secretase, cleaves full length βAPP between Aβ16

and Aβ17 producing a large secreted derivative ending at Aβ16, sAPPα, and an ~ 9 kDa cell-associated, C-terminal fragment that begins at Aβ17.

Subsequent cleavage of this ~ 9 kDa derivative by γ secretase produces p3, a small, secreted C-terminal fragment of Aβ that begins at Aβ17. Because it precludes production of full length Aβ, cleavage of the βAPP holoprotein by α secretase is generally regarded as anti amyloidogenic.

Over the past decade, strong evidence has been obtained that the deposition of Aβ in senile plaques plays a seminal role in AD pathogenesis. Several lines of investigation indicate that the cholinergic system may influence Aβ deposition in ways that could significantly affect AD pathogenesis. First, there is excellent evidence that the activation of M1 and M3 receptors by acetylcholine can enhance cleavage of βAPP by α secretase, an effect that appears to be mediated at least in part by activation of protein kinase C. Since this will tend to favor p3 production and reduce Aβ production, it could reduce extracellular Aβ concentration thereby slowing Aβ deposition. Thus, in principal, treatment of AD with anti-cholinesterases could enhance activation of M1 and M3 receptors thereby reducing Aβ deposition. In addition, the loss of basal forebrain cholinergic neurons that occurs as a prominent feature of AD, could, by reducing cholinergic transmission, set up positive feedback that tends to foster Aβ deposition increasingly as cholinergic neurons are lost. Second, Inestrosa and his colleagues have presented evidence that acetylcholinesterase interacts with Aβ, at least in vitro, in a way that tends to foster the formation of amyloid fibrils. The purpose of this brief review, which is excerpted in large part from a recent book chapter by the author, is to present the emerging evidence that Aβ deposition plays a seminal role in AD so that the potential importance of the interactions between Aβ and the cholinergic system (see other reports) is clear.

2. Effect of mutations that cause early onset familial Alzheimer's disease

It is now well established that early onset familial AD (FAD) can be caused by dominant mutations in the amyloid β protein precursor gene (APP) on chromosome 21[3, 12, 22], the presenilin 1 gene (PS1) on chromosome 14 [27], and the presenilin 2 gene (PS2) on chromosome 1 [18, 24]. If Aβ deposition is an essential early event in the pathologic process that causes AD, then each of the genetic changes known to cause AD must foster Aβ deposition. Thus one good way to test the Aβ deposition hypothesis is to determine if all the FAD linked mutations do, in fact, cause changes that foster Aβ deposition. There are many ways that these mutations might cause Aβ deposition. Our laboratory and several others have examined the hypothesis that the FAD-linked mutations act by increasing the extracellular concentration of Aβ.

To evaluate the APP mutations known to cause FAD, we and others examined fibroblasts [5] from subjects carrying these mutations or transfected cells expressing the FAD-linked βAPP mutations [2, 4, 29]. These studies established that the FAD-linked mutations on the amino (βAPP 670N/671L) and carboxyl (βAPP 717I, F, or G) sides of Aβ do, in fact, alter βAPP processing in a way that fosters amyloid deposition either by coordinately increasing the extracellular concentration of Aβ1–40 and Aβ1–42 (43) (βAPP 670N/671L) [2, 4, 29] or by selectively increasing the extracellular concentration of Aβ1–42 (43) (βAPP 717 mutations) [29], a peptide that forms insoluble amyloid fibrils more rapidly than Aβ1–40 in vitro [16].

To determine if the FAD-linked APP mutations increase the extracellular concentration of Aβ in vivo and to assess the PS1 and PS2 mutations, we performed blinded comparisons of plasma Aβ1–40 and Aβ1–42 from carriers and controls [25]. Two studies were performed, the first compared 12 carriers with 31 non-carriers from the Swedish APPK670N,M671L kindred, and the second compared nine subjects with PS1G209V, PS1M146V, PS1H163R, or PS1E120D mutations; three subjects with PS2N141I mutations; and one subject with an APPV717I mutation with 14 controls. In addition, we analyzed plasma Aβ in 71 elderly patients with sporadic AD and 75 controls well matched for age, sex, and ethnicity. Remarkably, Aβ1–42 (43) was significantly increased in essentially identical fashion in the plasma of subjects with each type of mutated gene known to cause early onset familial AD. In the 12 subjects with PS1/2 mutations, there was an unequivocal selective increase in the mean concentration of Aβ1–42 (43). As expected from previous studies, there was a coordinate increase in Aβ1–40 and Aβ1–42 (43) in subjects with APPK670N,M671L mutations but a selective increase in Aβ1–42 (43) in the subject with an APPV717I mutation. Plasma Aβ42 (43) was increased in all of the presymptomatic carriers that were examined and it was not increased in the vast majority of symptomatic sporadic AD subjects that we examined. Thus, the elevated Aβ42 (43) observed in subjects with FAD-linked mutations is not a secondary phenomenon of the AD state.

To establish that PS mutants increase brain Aβ, we analyzed the brains of transgenic mice expressing mutant (PS1M146L or PS1M146V) or wild type PS1 [8]. This analysis showed unequivocally that overex-

pression of mutant PS1 selectively increases the endogenous Aβ42 in mouse brain. Thus, it established that these mutations increase Aβ42 not only in plasma and peripheral cells but also in brain, the target organ for AD pathology. In addition, this study showed that PS1 mutations act in a truly dominant fashion because the normal endogenous PS1 genes present in the mice that were expressing mutant PS1 did not prevent the increase in Aβ42. In another study carried out collaboratively with D. Borchelt, G. Thinakaran, S. Sisodia and their colleagues at Johns Hopkins University [1], we analyzed the brains of transgenic mice co-expressing βAPP K670N,M671L and either wild type PS1 or mutant PS1A246E. In this experiment, the PS1A246E mutation significantly increased the relative amount of brain Aβ42, even though the amount of total brain Aβ was substantially increased by the overexpression of the APP transgene. In this same study, N2a cells co-expressing human βAPP and either wild type or mutant PS1 (PS1M146L, PS1A246E, PS1ΔE9) were examined. Again, each of the mutations increased the relative amount of A42 found in the medium. In their study of transfected cells and transgenic mice expressing wild type or mutant presenilins, similar results were obtained by Citron et al. [6].

3. Discussion

Collectively, these studies of plasma, fibroblasts, transfected cells and transgenic mice show that a fundamental, generalized effect of the FAD-linked APP, PS1 and PS2 mutations is to increase the extracellular concentration of Aβ42 (43). The plasma data are particularly important because they establish that these mutations increase extracellular Aβ42 (43) in vivo. This effect of the FAD-linked mutations is likely to be directly related to the pathogenesis of AD because Aβ42 (43) is deposited early and selectively in the senile plaques that are an invariant feature of all forms of AD [14, 15]. Thus our results provide strong evidence that the FAD-linked mutations all cause AD by increasing the extracellular concentration of Aβ42 (43), thereby fostering Aβ deposition, and they support the hypothesis that cerebral Aβ deposition is an essential early event in the pathogenesis of all forms of AD. Other observations that provide additional support for a critical role for Aβ deposition in AD include: i) the finding that aggregated synthetic Aβ is toxic to cultured neurons in vitro; ii) the observation that Aβ can trigger the classic complement cascade in vitro; and iii) the finding that the Aβ deposited in neuritic plaques is intimately associated with proteins of the classic complement cascade and with reactive microglia likely to be releasing cytokines and reactive free radicals that could have neurotoxic effects.

Based on these observations, it is reasonable to propose that extracellular Aβ deposition initiates a pathologic cascade that involves the formation of senile plaques, neurofibrillary tangles, and ultimately neuron and/or synapse loss resulting in dementia. The available data are consistent with this model and strongly support it. Thus it is important to develop drugs that lower Aβ42 or that prevent the aggregation and deposition of Aβ in other ways, as there is an excellent chance that such drugs will provide effective therapy for AD, if compounds can be identified that enter the brain in sufficient concentration to be effective without causing unacceptable toxicity. As mentioned above, one way to do this may be by modulating effects that the basal forebrain cholinergic system has on Aβ metabolism.

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Some cholinergic themes related to Alzheimer's disease: Synaptology of the nucleus basalis, location of m2 receptors, interactions with amyloid metabolism, and perturbations of cortical plasticity

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Abstract — Cholinergic neurons in the nucleus basalis of Meynert (nbM) receive cholinergic, GABAergic and monoaminergic synapses. Only few of these neurons display the sort of intense m2 immunoreactivity that would be expected if they were expressing m2 as their presynaptic autoreceptor. The depletion of cortical m2 in Alzheimer's disease (AD) appears to reflect the loss of presynaptic autoreceptors located on incoming axons from the nucleus basalis of Meynert (nbM) and also the loss of postsynaptic receptors located on a novel group of nitric oxide producing interstitial neurons in the cerebral cortex. The defect of cholinergic transmission in AD may enhance the neurotoxicity of amyloid β, leading to a vicious cycle which can potentially accelerate the pathological process. Because acetylcholine plays a critical role in regulating axonal growth and synaptic remodeling, the cholinergic loss in AD can perturb cortical plasticity so as to undermine the already fragile compensatory reserve of the aging cerebral cortex. (©Elsevier, Paris)

Résumé — Quelques aspects cholinergiques de la maladie d'Alzheimer : synaptologie du noyau de Meynert, localisation des récepteurs M_2, interactions avec le métabolisme de la protéine amyloïde, perturbations de la plasticité corticale. Les neurones cholinergiques du noyau basal de Meynert (nb M) reçoivent des synapses cholinergiques, GABAergiques et mono-aminergiques. Une petite fraction de ces neurones présente une forte immunoréactivité pour les récepteurs m2, telle qu'on l'attendrait pour des auto-récepteurs présynaptiques. La diminution des récepteurs m2 corticaux dans la maladie d'Alzheimer (AD) semble refléter la perte d'auto-récepteurs présynaptiques localisés sur des axones provenant du nbM et aussi la perte de récepteurs postsynaptiques localisés sur un nouveau groupe de neurones interstitiels du cortex, produisant NO. Le défaut de transmission cholinergique dans la maladie d'Alzheimer peut augmenter la neurotoxicité de l'amyloïde β, dans un cercle vicieux d'accélération du processus pathologique. Etant donné le rôle critique de l'acétylcholine pour la croissance axonale et pour le remodelage des synapses, la perte cholinergique dans l'AD peut perturber la plasticité corticale et compromettre ainsi la réserve déjà fragile de compensation fonctionnelle du cortex cérébral vieillissant. (©Elsevier, Paris)

Ch4 / nitric oxide / cerebral cortex / dementia / memory

1. Introduction

The connections of the cerebral cortex can be divided into three broad categories: 1) Local-circuit projections; 2) long-distance projections interconnecting remote cortical areas with each other and with subcortical structures such as the thalamus, striatum and claustrum; and 3) diffuse modulatory projections which arise from relatively small regions of the basal forebrain and brainstem, which bypass the thalamus, and which are widely distributed throughout the cerebral cortex. These diffuse projections include cholinergic inputs from the basal forebrain, histaminergic inputs from the hypothalamus, dopaminergic inputs from the ventral tegmental area-substantia nigra, serotonergic inputs from the brainstem raphe nuclei, and noradrenergic inputs from the nucleus locus coeruleus [34].

The cholinergic projection from the nucleus basalis of Meynert (nbM) to the cerebral cortex constitutes the most massive of these diffuse modulatory pathways. The nbM and its cholinergic projections continue to attract a great deal of attention because of their importance to many aspects of brain physiology and because of their severe degeneration in Alzheimer's disease (AD). In this very selective review, four related themes will be addressed: the emerging chemical synaptology of the nbM, the compartmentalization of m2 receptors, the interactions between the cholinergic system and beta amyloid (Aβ) metabolism, and the role of acetylcholine (ACh) in cortical synaptic plasticity.

2. Chemical synaptology of the primate nbM

The nbM contains cholinergic neurons (also known as the Ch4 group) intermingled with non-cholinergic neurons, most of which are GABAergic. Some of these GABAergic neurons are interneurons, others project to the cerebral cortex and innervate cortical inhibitory interneurons [14, 18]. The cell bodies (perikarya) of the Ch4 neurons contain indented nuclei, prominent nucleoli and cytoplasm rich in or-

ganelles [20, 42, 51]. Synaptic input to the perikaryon and proximal dendrites is sparse but increases distally.

The nbM has a complex neurotransmitter circuitry, much of which is still in the process of being unraveled. The nbM in the monkey and rat projects to the entire cerebral cortex but receives substantial cortical projections predominantly, if not exclusively, from limbic and paralimbic regions of the brain, including the amygdala [35, 52]. The relative paucity of reciprocal feedback projections from the neocortex is a property shared by the other ascending diffuse modulatory projections to the cerebral cortex. This aspect of connectivity may be essential for allowing these modulatory projections to rapidly shift information processing states throughout the cerebral cortex without the intervention of local feedback loops and in a way that is responsive primarily to the demands of the limbic system and the internal milieu [33]. The cortical inputs into the nbM are mostly glutamatergic but can also be GABAergic. At least in the rat, projections from the amygdala synapse directly onto cholinergic Ch4 neurons [54].

Dissociated cell cultures of nbM neurons show that they are responsive to acetylcholine, neurotensin, substance P, and L-glutamate [13, 39]. In the monkey, electron microscopic analyses of tissue labeled for the concurrent demonstration of the cholinergic marker choline acetyltransferase (ChAT) and the GABAergic marker glutamic acid decarboxylase (GAD) showed the presence of GABAergic symmetrical synapses onto the dendrites of both cholinergic and non-cholinergic nbM neurons [47]. Some of these synapses may represent the local axonal collaterals of GABAergic neurons, others may have an extrinsic origin in the cerebral cortex, amygdala, or nucleus accumbens.

The nbM also receives cholinergic innervation. Electron microscopic investigations of ChAT-immunolabeled nbM tissue in the rat shows that the vast majority of cholinergic terminals contact non-cholinergic neurons [30]. In the monkey, we find that cholinergic synapses onto cholinergic Ch4 neurons are somewhat more frequent and that they take the form of large asymmetrical synapses [47]. The precise source of the cholinergic input to the nbM is unknown but could include collaterals from cholinergic neurons of the basal forebrain or ascending projections from the Ch5–Ch6 group of pontomesencephalic cholinergic nuclei [21].

The monoaminergic innervation of the nucleus basalis has been investigated using autoradiography and immunoreactivity for individual receptor subtypes, 5HT immunoreactivity as a marker of serotonergic pathways, dopamine beta hydroxylase (DBH) immunoreactivity as a marker for noradrenergic pathways, and tyrosine hydroxylase (TH) immunoreactivity as a marker for dopaminergic pathways. Such experiments showed that the nucleus basalis contains receptor sites for 5HT, dopamine, and norepinephrine [56]. In the rat, projections from dopaminergic ventral tegmental neurons, serotenergic raphe neurons, and noradrenergic locus coeruleus neurons have been identified in the nbM and have been shown to make direct synaptic contact with cholinergic Ch4 neurons [16, 21, 53].

In the human, TH and DBH immunoreactivity has been described in the septal area [15]. Our observations with the light microscope in the human brain show intense, preterminal-like TH and DBH immunoreactive axonal profiles in the nbM [46]. Reliable serotonin immunoreactivity has been difficult to obtain in the human brain. In the monkey brain, we detected a dense plexus of serotonin-immunoreactive as well as TH and DBH-positive axons within the nucleus basalis [46]. Electron microscopic analyses of tissue prepared for the concurrent visualization of ChAT and TH in the monkey showed the presence of moderately asymmetric TH-positive (presumably dopaminergic) synapses onto the dendrites of cholinergic (ChAT-positive) as well as non-cholinergic nbM neurons [47].

These observations show that the nbM provides a site for extensive cholinergic-monoaminergic interactions. In fact, $\alpha 2$ noradrenergic receptors can enhance the cortical release of acetylcholine, and the influence of noradrenergic stimulation upon the activity of cortical neurons appears to be mediated, at least in part, through the effect of such stimulation on the cholinergic neurons of the nbM [11, 49]. According to preliminary investigations, the nbM continues to receive dopaminergic and noradrenergic inputs in AD, suggesting that monoaminergic substances could be used in AD patients to stimulate the residual Ch4 neurons and their projections to the cerebral cortex [46].

3. Location of m2 receptors in the nbM and cerebral cortex

The investigation of cholinergic receptors entered a very productive phase with the molecular identification and cloning of five subtypes (m1–m5) of muscarinic receptors [6, 7, 26, 43]. All five of these receptors are G protein coupled: m1, m3, and m5 preferentially activate phospholipase C whereas m2 and m4 inhibit adenylyl cyclase activity [7]. Immunocytochemical experiments and in situ hybridization studies show that the m1 receptor subtype is found in the majority of cortical neurons, a distribution which is consistent with its role as the major

postsynaptic cholinergic receptor of the cerebral cortex [8, 29].

The m2 receptors are much less numerous and tend to have a different distribution in the cerebral cortex [32]. In AD, the severe loss of cortical cholinergic innervation is accompanied by a depletion of m2 and nicotinic receptors but a relative stability of m1 receptors [31, 48]. It has been customary to assume that m2 is a presynaptic autoreceptor on all cholinergic axons that extend from the mbM to the cerebral cortex and that these axons provide the major source of cortical m2 receptors. The depletion of m2 was therefore thought to represent the automatic consequence of the cholinergic axonal loss in AD. Recent observations, however, indicate that this view needs to be modified for two reasons. First, the presynaptic cholinergic axons coming from the nbM do not seem to make a substantial contribution to the overall pool of m2 receptors in the cerebral cortex. Secondly, the cerebral cortex contains m2 expressing neurons, the degeneration of which could provide an alternative source of m2 loss in AD.

The evidence for the presence of m2 receptors in the nbM is extensive. In dissociated nbM neurons, patch clamp techniques reveal currents associated with nicotinic as well as m2 muscarinic receptors [19]. The m2 receptor also appears to be the dominant species of cholinergic receptor in the nucleus basalis [27, 29, 50]. However, this receptor subtype is expressed at immunohistochemically detectable levels by only a third of Ch4 neurons in the rat [28]. In the monkey and human nbM, only 10–25% of Ch4 neurons express intense m2 immunoreactivity [38, 44]. In fact, the experimental elimination of Ch4 neurons in the monkey does not appear to alter the density or distribution of m2 receptors in the cerebral cortex [36]. Athough the possibility that immunohistochemically undetectable levels of m2 receptors may act as autoreceptors for a large number of of Ch4 neurons cannot be ruled out, these results are not compatible with the traditional view which attributes the m2 depletion in AD predominantly, if not exclusively, to the loss of axons ascending from the nbM to the cerebral cortex.

Cortical m2 immunostaining is located mostly in the neuropil but also in some perikarya, suggesting that this receptor subtype may function both as a pre-synaptic autoreceptor and also as a post-synaptic perikaryal receptor [29]. The m2 receptor subtype can also be associated with non-cholinergic terminals, suggesting that it may additionally act as a presynaptic heteroceptor through which ACh may modulate the release of other transmitters [37]. In the cerebral cortex of the monkey, m2 receptors are expressed by two different classes of neuronal perikarya: 1) intracortical neurons which are mostly pyramidal in shape; and 2) polymorphic interstitial neurons most of which are located at the junction of the cerebral cortex with the underlying U-fibers of the white matter [36, 45].

Some of the m2-expressing pyramidal neurons may become the targets of degenerative processes in AD and can thus contribute to the overall loss of m2 receptors in these patients. In the primate brain, the interstitial polymorphic m2 neurons become quite conspicuous. They are present in all cortical areas and display particularly interesting properties. Nearly all of these neurons in the human and monkey brains express acetylcholinesterase (AChE) and nitric oxide synthase (NOS) activity [45]. Their neuronal cytoplasm is rich in endoplasmic reticulum and the nucleoli are quite prominent. The proximal dendrites are sparsely spiny. In the human brain, these neurons have dendritic spans of up to 1250 microns. Their processes reach the overlying cerebral cortex where they seem to come in potential contact with neurons and blood vessels.

Nitric oxide (NO) has been implicated in the regulation of vasodilation and synaptic plasticity. These interstitial neurons may thus provide a cholinoceptive relay through which m2-mediated cholinergic stimulation may influence the release of NO and therefore cortical blood flow and plasticity. Alzheimer's disease does not alter the number of the NOS and m2-immunoreactive neurons but leads to a severe loss of their processes in the cerebral cortex [25]. The loss of these processes may make an additional contribution to the depletion of m2 receptors. The m2 depletion in AD may thus reflect degenerative changes sustained predominantly by neurons in the cerebral cortex rather than by neurons in the nbM.

4. Cholinergic-Aβ interactions in AD

The possible linkage between the cholinergic depletion in AD and the metabolism of Aβ has attracted considerable interest. One group of investigators had reported that lesions in the nbM of rats induced the formation of plaque-like deposits in the cerebral cortex [1], and another that neurons of the nbM overexpressed amyloid precursor protein (APP) in a way that could promote the formation of plaques in AD [9]. Neither result has yet received widespread confirmation and there is currently little support for the hypothesis that Aβ plaques are 'caused' either by the degeneration or dysfunction of nbM neurons.

Cholinergic axons of the cerebral cortex are lost early in the course of AD at a time when many other neuronal systems remain relatively spared [17]. The biological mechanisms that regulate this selective vulnerability remain poorly understood. There are no

local correlations between the density of Aβ plaques and the severity of the cholinergic depletion [17]. Furthermore, experimental injections of Aβ in the rat cerebral cortex are no more toxic to cholinergic axons than to other types of axonal systems [12]. There is, thus, no support for the hypothesis that the selective vulnerability of cholinergic innervation reflects an unusual susceptibility to Aβ neurotoxicity.

Although cholinergic degeneration does not cause Aβ plaques, and Aβ plaques do not cause the preferential vulnerability of cortical cholinergic axons, complex interactions of potentially profound pathophysiological significance are being identified between cholinergic neurotransmission and Aβ metabolism. For example, m1- and m3-mediated muscarinic stimulation of cortical neurons promotes the processing of APP by the α secretase pathway [40]. This pathway splits APP in the middle of the Aβ domain and therefore precludes the subsequent release of insoluble Aβ. Additional in vitro experiments have shown that nicotine-mediated cholinergic neurotransmission may protect neurons from Aβ neurotoxicity [23]. These two sets of experiments imply that a cholinergic depletion may not only increase the deposition of Aβ plaques but also their local neurotoxic effects.

Numerous experiments have also shown that Aβ disrupts ACh synthesis and the signal transduction events associated with cholinergic neurotransmission [2, 22]. It appears, therefore, that AD may be associated with a vicious cycle wherein the cholinergic depletion intensifies both the production and neurotoxicity of Aβ which, in turn, further reduces the effectiveness of cholinergic neurotransmission (*figure 1*). Interfering with this vicious cycle through the use of m1, m3 and nicotinic agonists may have potentially beneficial effects in AD.

5. Acetylcholine and plasticity

The role of ACh in plasticity has been investigated for many years [4]. Time lapse photography in cultured neurons, for example, show that acetylcholine inhibits neurite outgrowth and that this effect is mediated through nicotinic receptors [41]. Furthermore, the selective lesioning of cortical cholinergic innervation in the rat was shown to interfere with experience-dependent plasticity in the barrel fields. In one experiment, all whiskers except for D2 and D3 were trimmed. This led to an experience-dependent pairing between the D2 and D3 barrel fields in the cerebral cortex so that the D2 neurons started to show a greater responsivity to D3 that to the adjacent D1 which had been trimmed. This pairing, indicative of experience-induced synaptic plasticity, could not be obtained in rats with selective immunotoxic lesions of the cholinergic neurons in the nbM [3]. In another experiment on newborn rat pups, barrels representing intact whiskers failed to show the expected expansion into the territory of barrels representing trimmed whiskers in animals with nbM lesions [55]. Furthermore, pairing auditory stimuli with the electrical stimulation of the nbM in adult rats caused a long-lasting reorganization of primary auditory cortex so that the area optimally responsive to the paired tone increased substantially. This plasticity was not observed in rats with nbM lesions [24].

The impact of cholinergic neurotransmission has traditionally been attributed to its immediate effect on the ionic channels of cholinoceptive neurons. The observations listed above suggest that acetylcholine may also have an equally important and much more prolonged impact as a promoter of synaptic plasticity. Synaptic plasticity is not confined to early development. It persists throughout adulthood, serving multiple purposes ranging from the experience-induced reorganization of synaptic circuits to the reactive synaptogenesis necessitated by physiological synapse turnover [5, 10]. The apparently pivotal role of acetylcholine in the regulation of neuroplasticity suggests that its depletion in AD may accelerate the pathological process by undermining the capacity for compensatory reorganization.

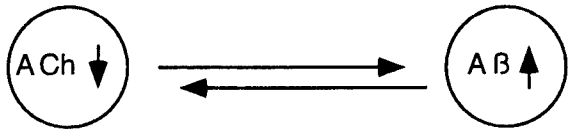


Figure 1. A schematic representation of the cholinergic 'vicious cycle' in AD. The cholinergic depletion promotes the deposition and toxicity of Aβ and Aβ decreases the effectiveness of cholinergic neurotransmission.

6. Overview

The initial hope, based on the relationship between dopamine and Parkinson's disease, that AD would turn out to be a cholinergic disease and that it would yield to cholinergic therapy has not materialized. There is currently no comprehensive 'cholinergic theory' of AD pathogenesis. However, there is a 'cholinergic lesion' in AD, with potentially vast implications for pathophysiology and pharmacotherapy. The depletion of cortical cholinergic innervation in AD is early and severe. This depletion may disrupt neural circuits involved in attention and memory, promote Aβ deposition and neurotoxicity, and

perturb neural plasticity. Each of these areas can become the target of specifically tailored cholinergic therapies.

Acknowledgments

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The effects of the acetylcholinesterase inhibitor ENA713 and the M1 agonist AF150(S) on apolipoprotein E deficient mice

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Abstract — Apolipoprotein E (apoE)-deficient and control mice were treated chronically with either the acetylcholinesterase (AChE) inhibitor ENA713, or the M1 muscarinic agonist AF150(S). Both treatments reversed the spatial working memory impairment of apoE-deficient mice but they differed in their effects on the levels of brain AChE activity. AF150(S) enhanced the brain AChE activity of apoE-deficient mice and rendered it similar to that of the untreated controls, whereas ENA713 reduced the brain AChE activity of control mice but had no effect on that of apoE-deficient mice. These findings suggest that AChE inhibition and M1 muscarinic activation have similar beneficial cognitive effects on apoE-deficient mice, but that the cellular and molecular mechanisms underlying these effects differ. (©Elsevier, Paris)

Résumé — Effets de l'inhibiteur d'AChE, ENA 713 et de l'agoniste AF 150(S) du récepteur M1 chez les souris déficientes en apolipoprotéine E. Le traitement de souris déficientes en apo E par l'ENA 713 (inhibiteur d'AChE) ou le AF150(S), agoniste M_1, améliorent la mémoire spatiale de ces souris mais par des mécanismes moléculaires différents. (©Elsevier, Paris)

apolipoprotein E / acetylcholinesterase / muscarinic animal model

1. Introduction

Genetic studies suggest that apolipoprotein E (apoE) genotype is a major risk factor for Alzheimer's disease (AD) [11]. Furthermore, neuropathological studies revealed that brain cholinergic deficits are more pronounced in AD patients which carry the allele E4 (apoE4), which is the risk factor for AD, than in patients which carry other apoE alleles. This led to the suggestion that apoE plays an important role in brain cholinergic function [10].

ApoE-deficient mice [9], provide a useful model system for studying the role of apoE in cholinergic and in other brain neuronal functions. Indeed, we have previously shown that apoE-deficient mice have memory impairments which are associated with synaptic loss of distinct neuronal pathways the magnitude of which is most pronounced in the basal forebrain cholinergic system [6]. The findings that apoE-deficient mice have impairments both in their cognitive function and cholinergic systems, renders them an attractive model system for examining the efficacy of distinct cholinergic therapeutic strategies. Centrally acting acetylcholinesterase (AChE) inhibitors [7] and M1-selective muscarinic agonists [4], have been reported to have some beneficial effect in AD patients.

In the present study we compared the effects of a prototype of each of these agents in apoE-deficient and control mice. Two groups of mice were treated for 3 weeks with either the brain specific AChE inhibitor ENA713 [2, 16], which is the S-isomer of RA7, originally developed by Weinstock et al. [15] or with the M1 muscarinic agonist 1-methyl-piperidine-4-spiro(2′-methylthiazoline) (AF150(S)) [3] and by comparing the resulting cognitive and neurochemical effects compared.

2. Materials and methods

2.1. Behavioral studies

ApoE-deficient mice (4 months old, $n = 10$ in each group) and age-matched controls of the same parent line (C57BL/6J), provided by Dr. J.L. Breslow [9], were subjected to the Morris water maze-working memory test (delayed matching to sample) for 5 successive days as previously described [5]. The drugs (0.5 mg/kg AF150(S) p.o. and 1.5 mg/kg ENA713 s.c.) were administered daily for 1 week prior to the initiation of the behavioral experiment. Administration was continued during the 5 days of the behavioral test, in which AF150(S) was administered 1 h before testing and ENA713 3–4 h after testing. Results were analyzed for each mouse as the mean path length of the second trial, divided by the mean path length of the first trial, as previously described [5]. The ratios obtained for each mouse represent the relative gain in performance from the first to the second trial and are thus a measure of working memory function.

2.2. AChE histochemistry

Four to five of the ten mice of each group which were tested in the Morris water maze were randomly selected for histochemical evaluation of their brains. In order to enable a comparison to be made of the results of the present study to those of previous experiments [5], the mice were treated with the drugs for an additional week. The AF150(S) and ENA713 treated mice were then killed at 4 h and 20 h respectively following the last administration of the drug. Their brains were excised sectioned and processed as previously described [1] and 3–5 consecutive frozen sections of each brain area were stained histochemically for AChE activity utilizing the Karnovsky method [8]. The resulting intensities of AChE staining of the indicated brain areas were determined by computerized image analysis [1]. Results, from the stained sections of each mouse brain at the level of the septum and the hippocampus, are presented as the mean optical density score for each brain area of each mouse. The intensities of staining of the different mice groups were compared using a two-way ANOVA, followed by simple main effects contrast analysis.

3. Results

The effects of the AChE inhibitor ENA713 and of the M1 agonist AF150(S) on the working memory of apoE-deficient and control mice were determined utilizing the Morris water maze and a paradigm in which the extent of improvement in performance of the mice was monitored by measurements of the ratio between the second and first of two identical daily trials. The results thus obtained are depicted in *figure 1*. As can be seen, the sham-treated apoE-deficient mice performed in both the AF150(S) and ENA713 experiments less well (i.e., path length ratios of 1.25 ± 0.34 and 1.25 ± 0.2 respectively), than the corresponding controls (i.e., path length ratios of 0.80 ± 0.18 and 0.82 ± 0.03 respectively). This is in accordance with previous reports [5, 6] and suggests that the working memory of apoE-deficient mice is impaired. Treatment of the two mice groups with either AF150(S) or ENA713 resulted in significant improvements of the performances of the apoE-deficient mice and rendered their working memory performance similar to those of control mice (*figure 1*). Accordingly, the path lengths ratios of the AF150(S) treated apoE-deficient and control mice were respectively 0.74 ± 0.1 and 0.78 ± 0.08, and the corresponding ratios of the ENA713 treated apoE-deficient and control mice were 0.75 ± 0.07 and 0.89 ± 0.1. Statistical analysis of this data revealed for both drugs a main effect of group (control versus apoE $P < 0.05$), and an interaction of group by treatment ($P < 0.05$).

The extent to which the AF150(S) and ENA713 mediated memory improvements are associated with neurochemical changes was monitored by histochemical measurements of brain AChE activity. As can be seen (*figure 2*) the two drugs had different effects on the hippocampal and cortical AChE activities of the two mice groups. Whereas AF150(S) reversed

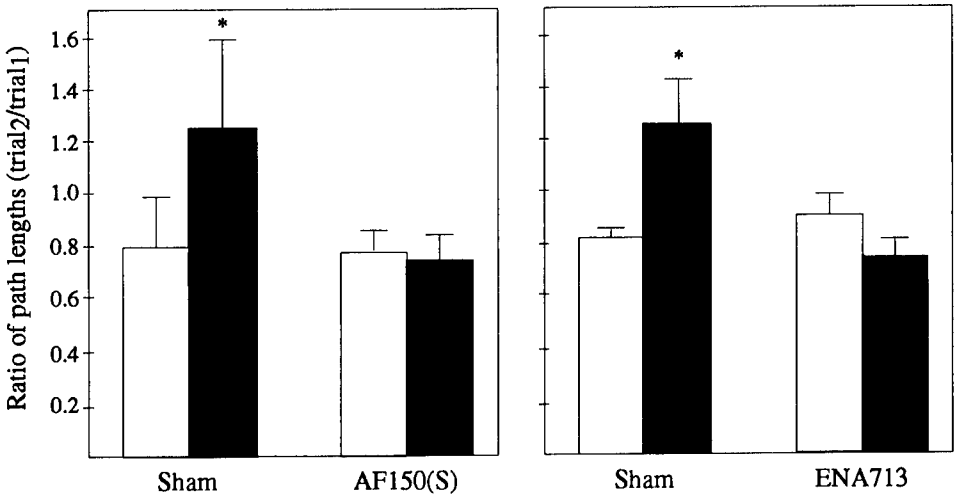


Figure 1. The effects of AF150(S) and ENA713 on the working memory of apoE-deficient and control mice. Spatial working memory was tested in the Morris water maze by measuring the ratio between the escape latency in the second trial to that of the first trial as described in *Materials and methods*. Results presented are the mean ± SEM of 9–10 mice in each group. The black bars correspond to apoE-deficient mice whereas the white bars correspond to controls. The drugs were administered once a day for 1 week prior to the initiation of the behavioral testing and then during the experiment. Mice which did not receive the drug were sham-treated with PBS. *$P < 0.05$ relative to sham-treated control.

the AChE deficit of the apoE-deficient mice by elevating their AChE activities, ENA713 had no effect on the AChE activities of these mice. Furthermore, AF150(S) induced a small and non-significant decrease in the cortical and-hippocampal AChE activities of the controls, whereas ENA713 caused marked decreases in the corresponding control AChE activities. Thus, AF150(S) abolished the difference in AChE activity between the two mice groups by enhancing the AChE activities of the apoE-deficient mice while having no effect on the corresponding control AChE activities (group by treatment $P < 0.001$). In contrast, ENA713 which also abolished the difference in AChE activities between the two mice groups, causes this effect by lowering the AChE activities of control mice while having no effect on those of the apoE-deficient mice (group by treatment $P < 0.03$).

The extent of a correlation between the levels of either cortical or hippocampal AChE activity and so the ratio of path lengths of individual treated and untreated mice was investigated. The results obtained are depicted in *figure 3*. As can be seen the AF150(S) treated and non-treated apoE-deficient and control mice showed a significant correlation between the levels of AChE activity and the cognitive performance of the individual mice. The higher the AChE activity the better the working memory performance. This correlation was statistically significant with regards to both cortical AChE activities ($r = -0.717$, $P < 0.01$), and to hippocampal ($r = -0.558$, $P < 0.05$) AChE activities. In contrast, ENA713 had the opposite effect to that of AF150(S),

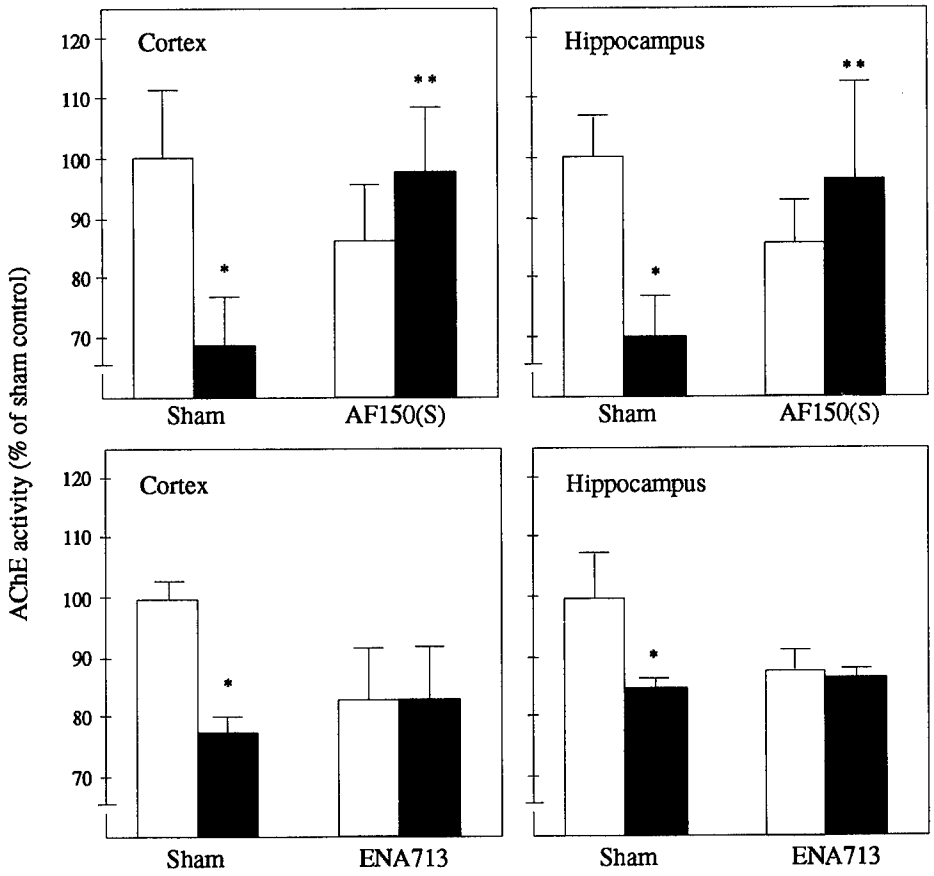


Figure 2. The effects of AF150(S) and ENA713 on AChE activities in the cortex and the hippocampus of apoE-deficient (dark) and control (white) mice. Mice were treated with the drugs for 3 weeks. The mice were killed 4 h following the last treatment with AF150(S) and 20 h following the last treatment with ENA713, after which their brains were processed and stained as described in *Materials and methods*. Stained sections of 4–5 mice in each group were scanned for each brain area and the intensities of the staining were averaged for each area of each mouse. Results presented are the means ± S.E.M. of each group. $*P < 0.03$ relative to sham-treated control. $**P < 0.05$ relative to sham-treated apoE-deficient mice.

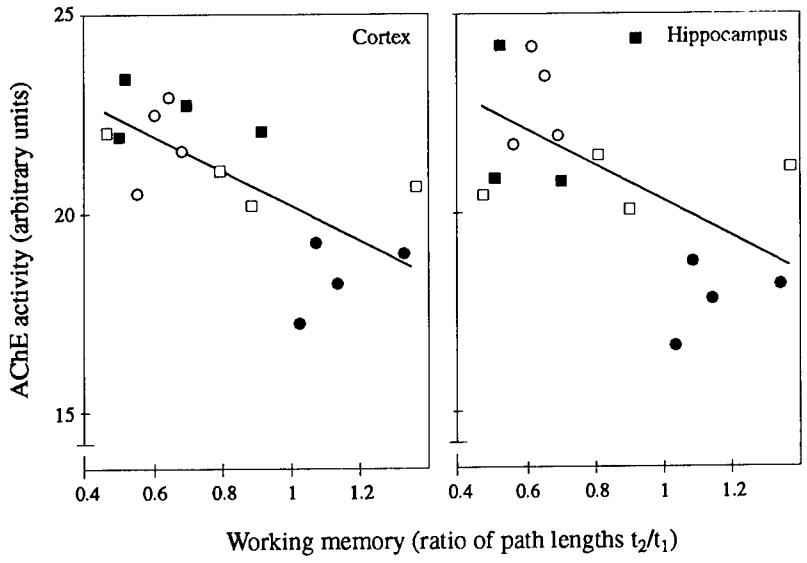


Figure 3. Correlation between working memory and AChE activities in the hippocampus and cortex of AF150(S) treated and non-treated apoE-deficient and control mice. Working memory and AChE activities were assessed as described in *Materials and methods*. Results presented are the individual values of each apoE-deficient mouse treated with AF150(S) (black squares) or sham treated (black circles) and of each control mouse treated with AF150(S) (white squares) or sham-treated (white circles). Correlation analysis of the results presented revealed R = –0.717 ($P < 0.01$) for the cortical AChE activity and R = –0.558 ($P < 0.05$) for the hippocampal AChE activity.

on AChE activity and thus there was no significant correlation between the cognitive performance and AChE activity of the ENA713 treated mice (not shown).

4. Discussion

The results of this study show that the memory impairments associated with apoE deficiency can be ameliorated by two cholinomimetic treatments, the M1 agonist AF150(S) and the AChE inhibitor ENA713. The two treatments however, seem to differ in their mechanisms of action. Treatment with AF150(S) induced an increase in cortical and hippocampal AChE activity in the apoE-deficient mice while having almost no effect on that of control mice. On the other hand, treatment with ENA713 reduced AChE activity in control mice but that of the apoE-deficient mice was unaffected.

The behavioral experiments with the AChE inhibitor were performed each day about 20 h after treating the mice. Thus, since the effect of ENA713 in rodents lasts only 6 h with this dose [2, 16], it is unlikely that the observed decreases in the levels of cortical and hippocampal AChE activities are due to inhibition of AChE by residual levels of ENA713. This assertion is also supported by the finding that AChE levels in other brain areas such as the striatum are not affected. This then suggests that the histological, as well as the behavioral effects of ENA713 result from the prolonged daily treatments. The AF150(S) behavioral experiments were performed shortly after the daily administration of the drug. However, in view of the pronounced effects of AF150(S) on both brain AChE activities and choline acetyltransferase (ChAT) levels of the apoE-deficient mice [5], it is likely that the effects of AF150(S) are also due to chronically activated mechanisms.

The biochemical mechanisms by which AF150(S) and ENA713, reverse the working memory deficits in the apoE-deficient mice, are not yet known. We have previously shown that AF150(S) elevates the brain ChAT and AChE levels of apoE-deficient mice back to those of the control mice [5]. One interpretation is that the effect of AF150(S) on working memory is due to restoration of brain cholinergic synaptic efficacy through activation of M1 muscarinic receptors. The effect of ENA713 on ChAT levels in apoE-deficient mice have not yet been determined. However, it has been shown in other models of cholinergic deficiency that ChAT levels are restored by prolonged treatment with ENA713 [12, 13]. It is thus likely that ENA713, like AF150(S) increases the efficacy of brain cholinergic synapses. However, as the effects of ENA713 and AF150(S) on brain AChE activity are different, we may conclude that the detailed cellular mechanisms by which these compounds reverse brain cholinergic deficiencies are not the same. This could be due in part to the finding that M1 muscarinic activation stimulates the expression of the AChE gene [14], and that apoE-deficient mice seem to have raised levels of M1 muscarinic receptors (in preparation).

In conclusion, this study revealed that cholinomimetic treatment of apoE-deficient mice either by an M1 muscarinic agonist or by an AChE inhibitor, ameliorates their memory deficits and suggests that

these effects are mediated by different cellular mechanisms.

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Neuromuscular responses to pyridostigmine bromide in organotypic spinal cord-muscle culture

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Abstract — Pyridostigmine bromide (PB) promotes and then silences cholinergic muscle activity, and disrupts the junctional regions of muscle fibers and associated nerve terminals, in organotypic mouse spinal cord-muscle cultures continuously treated with low concentrations of the drug for up to 14 days. Spontaneous muscle activity is restored within 1 week of drug removal. (©Elsevier, Paris)

Résumé — Effets neuromusculaires de la pyridostigmine sur des cultures organotypiques de moëlle épinière et de muscle. Dans des cultures organotypiques de muscle et de moëlle épinière de souris, traitées par de faibles doses de bromure de pyridostigmine (PB) jusqu'à 14 jours, on observe une stimulation puis un blocage de l'activité musculaire et une désorganisation des régions jonctionnelles des fibres musculaires et des terminaisons nerveuses associées. L'activité musculaire spontanée se rétablit une semaine après suppression de la drogue. (©Elsevier, Paris)

pyridostigmine bromide / neurotoxicity / muscle contractility / junctional myopathy / organotypic tissue culture / Gulf War Unexplained Illness

1. Introduction

Pyridostigmine bromide is a carbamate inhibitor of acetylcholinesterase (AChE), the enzyme responsible for the hydrolysis of neurotransmitter acetylcholine (ACh) at mammalian central and peripheral nerve terminals, including the neuromuscular junction. PB has been used in the civilian setting for the treatment of myasthenia gravis (a muscle disorder featured by serum ACh receptor antibodies) and, for military use, as an organophosphate nerve agent antidote enhancer. PB inhibits a percentage of peripheral cholinesterases and prevents access of soman to the inhibited (protected) enzyme, which subsequently decarbamylates to yield active enzyme [8, 10, 13]. During the 1991 Gulf War, US and UK troops were issued a blister pack containing 21 30-mg tablets of PB to be self-administered every 8 h for 1–7 days while under threat of nerve agent attack. A large majority of ground personnel received at least one dose and probably up to the full 21 tablets dispensed [23]. A study of 41 650 US soldiers, 34 000 of whom had taken PB for 6–7 days, revealed that pathophysiological changes were reported by about one half of this population [17]. Symptoms in this group were more frequent than would be expected from clinical experience with myasthenia gravis, in which larger therapeutic doses (30–60 mg every 4–6 h) of PB are used for prolonged periods. Moreover, only minor drug effects were reported in a 2-week, double-blind, placebo-controlled crossover study of seven 18- to 24-year-old healthy males subjected to heat stress while receiving PB 30 mg orally t.i.d. for 7 consecutive days [4]. Recent murine studies suggest that both PB and acute stress (forced swimming) initially increase ACh synthesis [16].

Toxic side effects are recognized in patients who receive PB for the treatment of myasthenia gravis; these are usually attributable to the reversible peripheral anti-cholinesterase actions of the pyridostigmine of PB, a quaternary moiety that fails readily to traverse the blood-brain regulatory interface except in animals subjected to acute stress [9]. The bromide moiety, which competes with chloride ions especially in states of dehydration, should readily cross the blood-brain barrier and is an established cause of neurological and neuropsychiatric disease (bromism), including fatigue and dimunition of muscle power that probably have a central origin [22]. There is neurophysiological evidence of a progressive terminal axonal dysfunction in motor nerves of myasthenic patients chronically treated with PB [3], but the etiology is unknown. There is also evidence from experimental animal studies that repeated treatment with large doses of PB, as well as long-term anticholinesterase therapy, results in pathological changes of muscle and motor nerve terminals at motor end plates [7, 11, 14, 15, 21].

The action of PB at the neuromuscular junction can be studied under controlled conditions using spinal cord explants which, when co-cultured with

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striated muscle explants, develop into a structurally and functionally coupled organotypic array that closely resembles tissue organization in vivo. Organotypic cultures have proved useful in studies of neuromuscular function and in dissecting neuromuscular responses to a wide range of chemicals that have dose- and time-dependent neurotoxic potential [6, 28]. The present study employs mouse cord-muscle cultures to examine the neurotoxic actions of PB at the nerve-muscle interface after short-term and continuous exposure to drug concentrations of therapeutic relevance.

2. Materials and methods

Experiments used organotypic mouse cord-muscle cultures that were allowed to mature in roller tubes. Mature cultures were treated for up to 14 days with PB in low concentration (3.8×10^{-6} M) or high concentration (38×10^{-6} M), sodium bromide (3.8×10^{-6} M), or maintained in PB-free nutrient fluid (control). The lower PB concentration produced 30–60% inhibition of human and canine erythrocyte AChE [18, 29], a level of enzyme inhibition comparable to that produced in human volunteers given oral PB 30 mg/8 h for 1 week [4].

Electrophysiological examination of cultures was performed weekly. The recording medium consisted of (in mM): 137 NaCl, 2.7 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 0.15 NaH_2PO_4, 1.34 Na_2HPO_4, 5.9 $NaHCO_3$, 5.5 glucose and 10 HEPES, pH 7.4, at room temperature. Drugs were dissolved and incorporated into the medium or applied directly over muscle by microinjection; final calculated concentrations are given. Intracellular microelectrodes were filled with 4 M potassium acetate, pH 4.0 (Aldrich, Milwaukee, WI, USA).

Data were evaluated by multivariate analysis of variance and logistic regression (Systat, SPSS, Chicago, IL and S-Plus, StatSCi, Inc., Seattle, WA, USA).

3. Results

Acute application of PB (3 μg/mL) to the bath solution rapidly (seconds) and reversibly enhanced the number and frequency of spontaneous muscle contractions. Continuous treatment with PB decreased muscle fiber activity as a function of calculated drug concentration and time (*figure 1*). Differences in recorded spontaneous muscle contractions of cultures treated with high calculated concentrations (10 μg/mL) versus low concentrations (1 μg/mL) were evident as early as 24 h of drug treatment. After 1 week of treatment, during which spontaneous muscle contractions progressively decreased, muscle fibers became less responsive to exogenous application of ACh (10^{-4} M). By 2 weeks, tested muscle fibers were largely silent (*figure 2*). However, direct stimulation through the recording electrode elicited muscle fiber contraction. Control cultures displayed spontaneous muscle contractions throughout the test

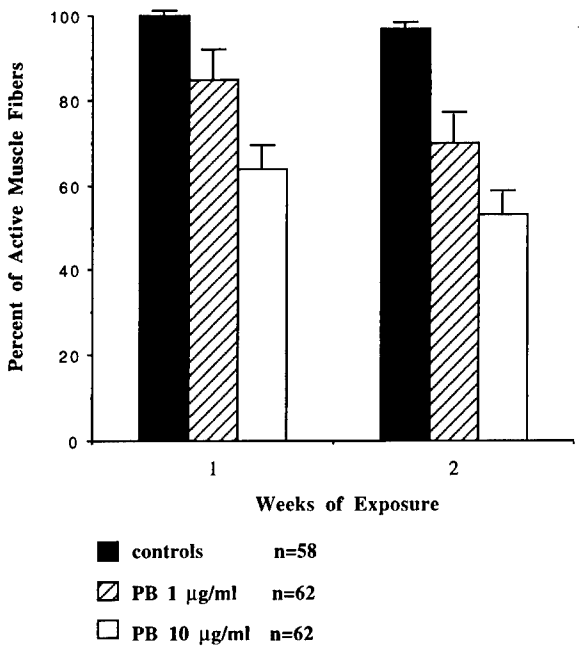


Figure 1. Relationship between percent of active muscle fibers, the dosage of pyridostigmine bromide (PB) and the drug treatment period.

period (*figure 2*). Removal of PB after a 2-week exposure resulted in a resumption of muscle activity. Within 1 week following withdrawal of PB, muscle fibers became functional, indicating that the neuromuscular effects of PB were reversible.

4. Discussion

Treatment with PB (1–10 μg/mL) induced the following sequential changes: i) an acutely reversible increase in muscle fiber activity consistent with drug-induced AChE inhibition; higher PB concentrations (200 mM–1 mM) are also reported to exert weak agonist effects on ACh receptors [1, 24]; ii) a reduction of sensitivity to exogenous acetylcholine consistent with an early desensitization of ACh receptors; iii) a progressive reduction of spontaneous neuromuscular activity without loss of muscle function; and iv) eventual recovery of spontaneous muscle activity following drug withdrawal. Since comparable pathophysiological changes fail to develop in cultures treated with sodium bromide, these neurotoxic phenomena are most likely direct anticholinesterase actions of the pyridostigmine moiety of PB at the neuromuscular junction. Recent studies of central cholinoceptors suggest that PB-induced blockade of AChE produces an initial increase in ACh followed by a long-lasting decrease, an effect mediated by trans-

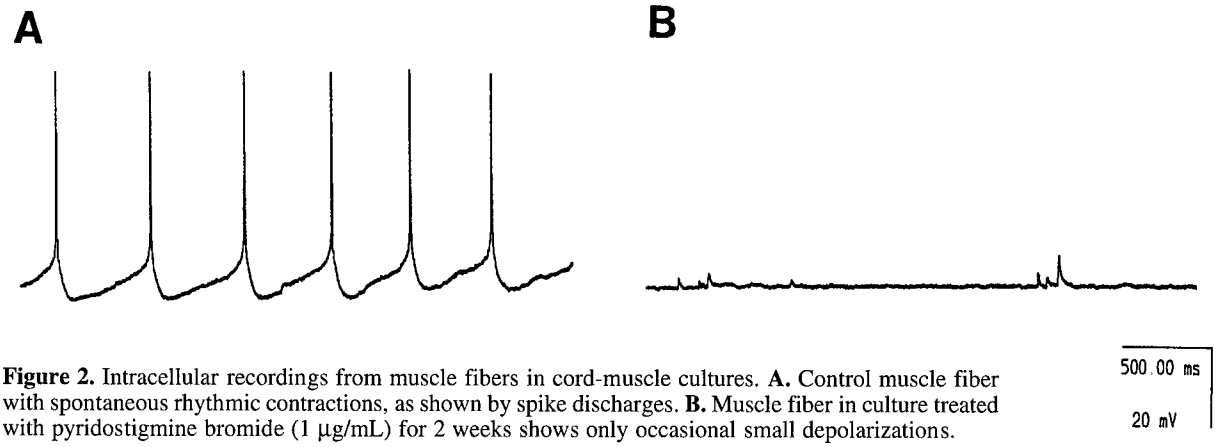


Figure 2. Intracellular recordings from muscle fibers in cord-muscle cultures. **A.** Control muscle fiber with spontaneous rhythmic contractions, as shown by spike discharges. **B.** Muscle fiber in culture treated with pyridostigmine bromide (1 μg/mL) for 2 weeks shows only occasional small depolarizations.

mitter-stimulated induction of mRNA levels encoding the early immediate transcription factor c-fos [16].

Organotypic cord-muscle cultures faithfully reproduce the timing, pattern, and spatial-temporal sequence of functional and structural changes induced by a variety of chemicals which display neurotoxic potential in animals and humans [28]. For example, continuous treatment of cord-muscle cultures with 2,5-hexanedione (2,5-HD), the agent responsible for induction of sensori-motor peripheral neuropathy in humans and animals repeatedly exposed to *n*-hexane, causes retrograde giant axonal degeneration of motor axons with consequent muscle denervation and silencing of spontaneous muscle fiber activity in a time course that matches similar changes in 2,5-HD-exposed animals [30]. In similar vein, characteristic changes would be expected at the human neuromuscular junction upon continuous exposure to a concentration of PB comparable to that achieved in cultures treated for 14 days with 1 μg/mL PB.

Neuropathological changes localized to neuromuscular junctions have been observed with a number of carbamate (PB, physostigmine) and organophosphate (tabun, sarin, soman) AChE inhibitors; this seems primarily to involve the muscle fiber and only secondarily affects the motor nerve terminal [7, 12]. This 'junctional myopathy' appears to be initiated by sustained accumulation of ACh at the motor endplate; this causes an increase in sarcoplasmic reticulum [Ca^{2+}] that triggers events leading to muscle fiber necrosis [5, 19, 20, 22, 25, 27]. Neuromuscular junctions in the diaphragm (used here) are among the most severely affected in animals treated with anticholinesterase agents, and pathological changes are also prominent in soleus, gastrocnemius, and quadriceps muscles. Morphological alterations include presynaptic mitochondrial swelling, withdrawal of nerve terminals from the postjunctional muscle membrane, and invasion of the synaptic cleft by Schwann cell processes. These characteristic ultrastructural changes may appear within hours of high-dose anticholinesterase drug administration, progress over a few days, and resolve within a couple of weeks [5]; they have also been observed after 7–14 days of continuous PB infusion, even at dosage rates as low as 9 μg/h [15]. Development of junctional myopathy is blocked by curare [31] and seems to depend on the degree and duration of AChase inhibition.

Junctional myopathy is likely to underly a life-threatening but spontaneously reversible (so-called intermediate) syndrome of proximal limb and intercostal muscle weakness that follows within days or longer of the successful treatment and resolution of a severe chemically-induced cholinergic crisis [26]. Since delayed-onset proximal muscle weakness was not reported by Gulf War veterans who experienced acute cholinergic effects from use of PB as a nerve-gas-antidote enhancer [17], it seems unlikely that cholinergic neuroexcitation in this setting was sufficient to induce a clinically significant junctional myopathy. Additionally, while muscle fatigue would predictably be associated with PB-induced neuromuscular damage, and fatigue is a prominent component of the symptom cluster reported by Gulf War veterans with unexplained illness, neurological examination has failed to reveal evidence of proximal muscle weakness typical of myopathy [2]. The reversibility of the PB-induced neuromuscular lesion in vitro as in vivo would also suggest that symptoms of fatigue which persist in Gulf War veterans years after exposure to PB are causally unrelated to the phenomenon observed in cord-muscle cultures continuously treated with PB. Involvement of motor nerve terminals at PB-affected neuromuscular junctions might explain neurophysiological evidence

of temporally progressive terminal axonal dysfunction in motor nerves of myasthenic patients chronically treated with PB [3].

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Contribution of nicotinic receptors to the function of synapses in the central nervous system: The action of choline as a selective agonist of α7 receptors

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Abstract — The α7-nicotinic receptor (nAChR)-selective agonist choline and nAChR-subtype-selective antagonists led to the discovery that activation of both α7 and α4β2 nAChRs located in CA1 interneurons in slices taken from the rat hippocampus facilitates the tetrodotoxin (TTX)-sensitive release of γ-aminobutyric acid (GABA). Experiments carried out in cultured hippocampal neurons not only confirmed that preterminal α7 and α4β2 nAChRs modulate the TTX-sensitive release of GABA, but also demonstrated that evoked release of GABA is reduced by rapid exposure of the neurons to acetylcholine (ACh, 10 μM-1 mM) in the presence of the muscarinic receptor antagonist atropine (1 μM). This effect of ACh, which is fully reversible and concentration-dependent, is partially blocked by superfusion of the cultured neurons with external solution containing either the α7-nAChR-selective antagonist methyllycaconitine (MLA, 1 nM) or the α4β2-nAChR-selective antagonist dihydro-β-erythroidine (DHβE, 100 nM). A complete blockade of ACh-induced reduction of evoked release of GABA was achieved only when the neurons were perfused with external solution containing both MLA and DHβE, suggesting that activation of both α7 and α4β2 nAChRs modulates the evoked release of GABA from hippocampal neurons. Such mechanisms may account for the apparent involvement of nAChRs in the psychological effects of tobacco smoking, in brain disorders (e.g., schizophrenia and epilepsy), and in physiological processes, including cognition and nociception. (©Elsevier, Paris)

Résumé — Contribution des récepteurs nicotiniques à la fonction des synapses dans le système nerveux central.: action de la choline comme agoniste des récepteurs α7. En utilisant la choline comme agoniste sélectif du récepteur nicotinique (nAChR) de type α7 et des antagonistes sélectifs des différents sous-types de nAChR, nous avons découvert que l'activation de récepteurs α7 et α4β2 localisés sur des interneurones de CA1 dans des tranches d'hippocampe de rat facilite la libération d'acide γ-aminobutyrique (GABA) sensible à la tétrodotoxine (TTX). Des expériences effectuées sur des neurones d'hippocampe en culture ont confirmé que des récepteurs α7 et α4β2 localisés sur des interneurones de CA1 dans des tranches d'hippocampe de rat facilite la libération d'acide γ-aminobutyrique (GABA) sensible à la tétrodotoxine (TTX). modulent la libération de GABA sensible à la TTX, et ont aussi démontré que la libération évoquée du GABA est réduite par une exposition rapide des neurones à l'acétylcholine (ACh, 10 μM–1 mM), en présence d'atropine, un antagoniste muscarinique (1 μM). Cet effet de l'ACh, qui est entièrement réversible et dépend de la concentration, est bloqué par superfusion des neurones avec une solution de méthyllycaconitine (MLA, 1 mM), un antagoniste sélectif d'α7, ou de dihydro-β-érythroïdine (DHbE, 100 nM), un antagoniste sélectif d'α4β2. On n'obtient la suppression complète de la réduction de libération de GABA induite par l'ACh que lorsque les neurones sont perfusés à la fois avec MLA et DHbE, ce qui suggère que l'activation des deux types de récepteurs, α4β2, module la libération des GABA par les neurones d'hippocampe. De tels mécanismes peuvent expliquer le fait que des récepteurs nicotiniques paraissent impliqués dans les effets psychologiques de l'action de fumer du tabac, dans des désordres cérébraux (par exemple le schizophrénie et l'épilepsie) et dans des processus physiologiques comme la cognition et la nociception. (©Elsevier, Paris)

choline / nicotinic receptors / slices / hippocampus / GABA

Abbreviations: α-BGT, α-bungarotoxin; ACh, acetylcholine; ACSF, artificial cerebrospinal fluid; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; CNQX, 6-cyano-7-nitroquinoline-2,3-dione; CNS, central nervous system; DHβE, dihydro-β-erythroidine; EGTA, ethyleneglycol bis(β-amino-ethyl ether)-N,N-tetraacetic acid; GABA, γ-aminobutyric acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IPSCs, inhibitory postsynaptic currents; MLA, methyllycaconitine; nAChR, nicotinic acetylcholine receptor; OPs, organophosphates; PSCs, postsynaptic currents; TTX, tetrodotoxin.

1. Introduction

It is generally acknowledged that the hippocampus, an area of the central nervous system (CNS) that is involved in processing cognitive functions [20], is very sensitive to cholinergic modulation, and that the density of neuronal nicotinic acetylcholine receptors (nAChRs) in the hippocampus is severely diminished in diseases characterized by learning and memory impairment (e.g., Alzheimer's disease) [21].

Thus, the developing hippocampus has become the focus of our research interest with regard to characterization of the nAChR function. One of the major hindrances to addressing nAChR function in the brain was the lack of specific agonists and antagonists for each of the receptor subtypes. This problem was further aggravated by the fast kinetics of inactivation of some of the neuronal nicotinic receptors, particularly of those composed of the α7 subunit. The field has now advanced immensely, and many of these problems have been overcome (reviewed in [2]).

In many areas of the brain, nAChRs have been shown to facilitate the release of neurotransmitters [4, 12, 13, 17, 18] (see also review [24] and references cited therein). In general terms, modulation of transmitter release by presynaptic nAChRs (i.e., nAChRs present in synaptic terminals) is insensitive to blockade by the Na^+-channel blocker tetrodotoxin (TTX), whereas modulation of transmitter release by preterminal nAChRs (i.e., nAChRs present in axons) depends on propagation of action potentials and is, therefore, sensitive to TTX. Identifying the neuronal nAChR subtype involved in modulating the release of a given neurotransmitter has been a more difficult task.

The introduction of a set of pharmacological and kinetic 'fingerprints' has been very useful in identifying the nAChR subtype that subserves a given nicotinic response [3, 7]. It has been suggested that fast-desensitizing nicotinic responses sensitive to blockade by α-bungarotoxin (α-BGT) or methyllycaconitine (MLA) are mediated by α7-containing nAChRs, and that slowly desensitizing nicotinic responses sensitive to blockade by dihydro-β-erythroidine (DHβE) and mecamylamine are mediated by α4β2 and α3β4 nAChRs, respectively [3, 7]. The recent discovery that choline modulates the function and expression of α7 nAChRs also introduced a key pharmacological tool to distinguish these from other nAChR subtypes [5, 19]. In cultured hippocampal neurons, choline fully activates α7 nAChR-mediated currents with an apparent EC_{50} of 1.6 mM, does not evoke α4β2 nAChR-mediated currents, and induces α3β4 nAChR-mediated currents with 20% of the apparent efficacy of acetylcholine (ACh). In addition, when continuously applied to these neurons, choline, like other nicotinic agonists, desensitizes the α7 nAChRs subserving type IA currents with an IC_{50} of 37 μM [5]. Therefore, choline as a nicotinic agonist has the unique capability of providing substantial clues regarding the nAChR subtype subserving a nicotinic response.

Initial studies from our laboratory provided evidence that activation of nAChRs in CA1 interneurons results in a variety of responses, including facilitation of γ-amino butyric acid (GABA) release [4]. In this paper, evidence is provided that the TTX-sensitive release of GABA from CA1 interneurons and from cultured hippocampal neurons is facilitated by activation of both α7 and α4β2 nAChRs, and that field stimulation-induced release of GABA from hippocampal neurons in culture is inhibited by the activation of both nAChR subtypes. Choline, by acting as a selective α7-nAChR-agonist, may have a role in controlling synaptic function in the mammalian CNS.

2. Materials and methods

2.1. Hippocampal slices

Slices of 250–300-μm thickness were obtained from the hippocampus of 12–24-day-old Sprague-Dawley rats according to the procedure described earlier [4]. Slices were stored at room temperature in artificial cerebrospinal fluid (ACSF), which was bubbled with 95% O_2 and 5% CO_2 and had the following composition (in mM): NaCl 125; $NaHCO_3$ 25; KCl 2.5; NaH_2PO_4 1.25; $CaCl_2$ 2, $MgCl_2$ 1; and glucose 25.

2.2. Cell culture

Primary cultures of hippocampal neurons were done as described previously [3].

2.3. Electrophysiological recordings

Whole-cell currents were recorded from the soma of CA1 interneurons in slices and from the soma of cultured hippocampal neurons according to the standard patch-clamp technique [15], using an LM-EPC7 patch-clamp system (List Electronic, Darmstadt, Germany). The signals were filtered at 2 kHz and either recorded on a video tape recorder for later analysis or directly sampled by a microcomputer using the pCLAMP6 program (Axon Instruments, Foster City, CA, USA). Neurons in slices were superfused with ACSF at 2 mL /min, and cultured neurons were superfused with external solution, which had the following composition (in mM): NaCl, 165; KCl, 5; $CaCl_2$ 2; glucose 10; and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) 5 (pH adjusted to 7.3 with NaOH; 340 mOsm). Atropine (1 μM) was added to the ACSF and to the external solution to block the muscarinic receptors. Patch pipettes were pulled from borosilicate glass capillary (1.2-mm outer diameter), and when filled with the internal solution had resistances between 2 and 6 MΩ. The internal solution consisted of (in mM): ethyleneglycol bis (β-amino-ethyl ether)-N,N-tetraacetic acid (EGTA) 10; HEPES 10; CsCl 80; CsF 80 (pH was adjusted to 7.3 with CsOH; 340 mOsm). All recordings were performed at room temperature. Data analysis was done using the pCLAMP6 program.

2.4. Field stimulation of hippocampal neurons in culture

A glass-mounted electropolished bipolar platinum microelectrode system (specially designed and assembled in our laboratory) was used to stimulate neurons synaptically connected to the neuron under study in culture. Evoked GABA-mediated inhibitory postsynaptic currents (IPSCs) were recorded in the presence of the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor antagonist 6-cyano-7-nitroquinoline-2,3-dione (CNQX; 10 μM). To avoid the Na^+ current transients that interfere with the synaptic currents, all experiments were carried out at positive membrane potentials. A supramaximal (~11 volt), 20-μs pulse was applied at a rate of 1 Hz via the bipolar electrode. Stable responses could be obtained during 30 to 60 min of recording.

2.5. Drugs used

Acetylcholine chloride, choline chloride, dimethylsulfoxide (DMSO), tetrodotoxin, γ-aminobutyric acid, picrotoxin, and atropine sulfate were obtained from Sigma Chemical Co. (St. Louis, MO, USA). α-Bungarotoxin was purchased from Biotoxins Inc. Methyllycaconitine citrate was a gift from Professor M.H. Benn (Department of Chemistry, University of Calgary, Alberta, Canada). Dihydro-β-erythroidine.HBr was a gift from Merck, Sharp & Dohme (Rahway, NJ, USA). A 250 mM stock solution of picrotoxin was made in DMSO. Stock solutions of all other drugs were made in distilled water. Further dilutions were made in either ACSF (for experiments in slices) or external solution (for experiments in cultured neurons).

3. Results

3.1. TTX-sensitive GABA release from CA1 interneurons is facilitated by activation of α7 and α4β2 nAChRs

Three components could be identified in nicotinic responses recorded from CA1 interneurons rapidly exposed to equipotent, nearly saturating concentrations of ACh and of the α7-nAChR-selective agonist choline. Two of these components, referred to as postsynaptic currents (PSCs) and fast current transients, depended on the propagation of action potentials, and, were, therefore sensitive to blockade by the Na^+-channel blocker TTX (*figure 1*). The third component had the slowest rising and decaying phases and consisted of a TTX-insensitive event, which is referred to as the nicotinic current. The PSCs were the consequence of agonist-induced facilitation of GABA release, because they could be blocked by the $GABA_A$-receptor antagonist picrotoxin (100 μM). Also, evidence has been provided that nicotinic currents and fast current transients were triggered by activation of nAChRs present on the neuron under study, whereas PSCs were triggered by activation of nAChRs present in presynaptic neurons [4, 6].

Fast-decaying nicotinic currents accompanied by GABA-mediated PSCs and fast current transients were observed in 68% of the neurons exposed to choline (10 mM); only approximately 17% of such neurons showed similar responses to ACh (1 mM). These responses, being evoked by the α7 nAChR-selective agonist choline and blocked by the selective α7 nAChR antagonists MLA (50 nM) and α-BGT (100 nM) (*figure 1*), were likely to be subserved by α7 nAChRs. It should be pointed out that the concentration of MLA needed to block these responses was considerably higher than that used to block α7 nAChR-mediated responses recorded from fetal hippocampal neurons in culture. Such a difference in the sensitivity of α7 nAChRs to MLA has been reported in previous studies [9] and suggests that the α7 nAChRs may exist in the hippocampus in multiple heteromeric and/or homomeric isoforms that change along with development. The question of whether these isoforms would also show differential sensitivity to the α7-nAChR-selective agonist choline remains open.

Nicotinic responses apparently resulting from summation of large numbers of PSCs, alone or accompanied by a small slowly decaying nicotinic current, were observed in response to ACh in about 77% of the interneurons, and were sensitive to blockade by the α4β2-nAChR-selective antagonist DHβE (*figure 1*), but not by MLA or α-BGT. These DHβE-sensitive responses were likely to be subserved by α4β2 nAChRs. Our findings, therefore, not only confirm those of a recent study showing that functional α7 nAChRs are present in CA1 interneurons [10], but extend the results by demonstrating that α4β2 nAChRs are also present in preterminal sites of these neurons, and that activation of both receptor subtypes can facilitate the TTX-sensitive release of GABA.

3.2. TTX-sensitive and field-stimulation-evoked release of GABA from cultured hippocampal neurons is modulated by activation of α7 and α4β2 nAChRs

A 1-s pulse application of ACh (1–1000 μM) to hippocampal neurons in culture increased the frequency of PSCs, which were not sensitive to blockade by the AMPA-receptor antagonist CNQX (10 μM), but were blocked by the $GABA_A$-receptor antagonist picrotoxin (100 μM). In agreement with the results obtained from hippocampal slices, the blockade of the ACh-induced, GABA-mediated PSCs by TTX (150 nM) indicated that these responses resulted from activation of nAChRs located in preterminal sites or on the somato-dendritic region of presynaptic GABAergic neurons.

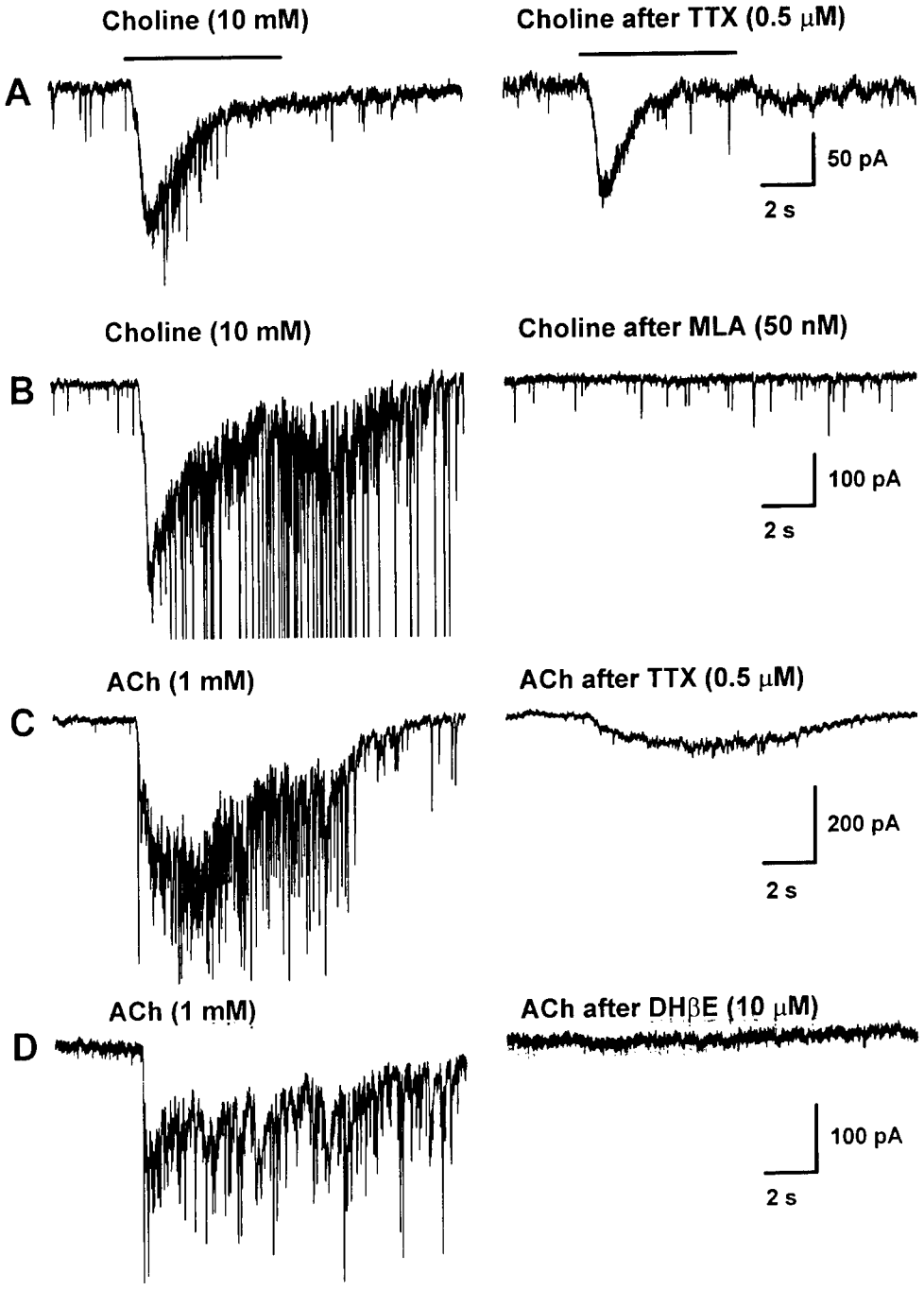


Figure 1. Nicotinic responses of CA1 interneurons. Responses recorded from four interneurons (labeled **A–D**) are shown. In **A**, choline induced a rapidly decaying whole-cell inward current accompanied by PSCs. Superfusion of the neurons with TTX (0.5 μM) abolished choline-induced PSCs, but not the nicotinic current. In **B**, choline evoked nicotinic inward current accompanied by PSCs and fast current transients. The fast current transients are purposely clipped to unmask the other components. Superfusion with MLA (50 nM) abolished all three components of the choline-evoked response. In **C**, ACh induced a response that consists mainly of PSCs. Superfusion of this neuron with TTX-containing solution blocked most of the response, leaving a small slowly decaying nicotinic current, which was due to the activation of nAChRs on the neuron from which the recordings were obtained. In **D**, ACh induced a similar response as in **C**, and this response was completely blocked by DHβE (10 μM). The duration of the agonist pulse is indicated by the solid line above the top traces. The interneurons studied were located at 100 μm (**A**), 150 μm (**B** and **C**) and 125 μm (**D**) from the midline of the pyramidal cell layer. Age of rats = 24 days (**A**, **C**, **D**) and 19 days (**B**). In all experiments the membrane potential was held at –60 mV.

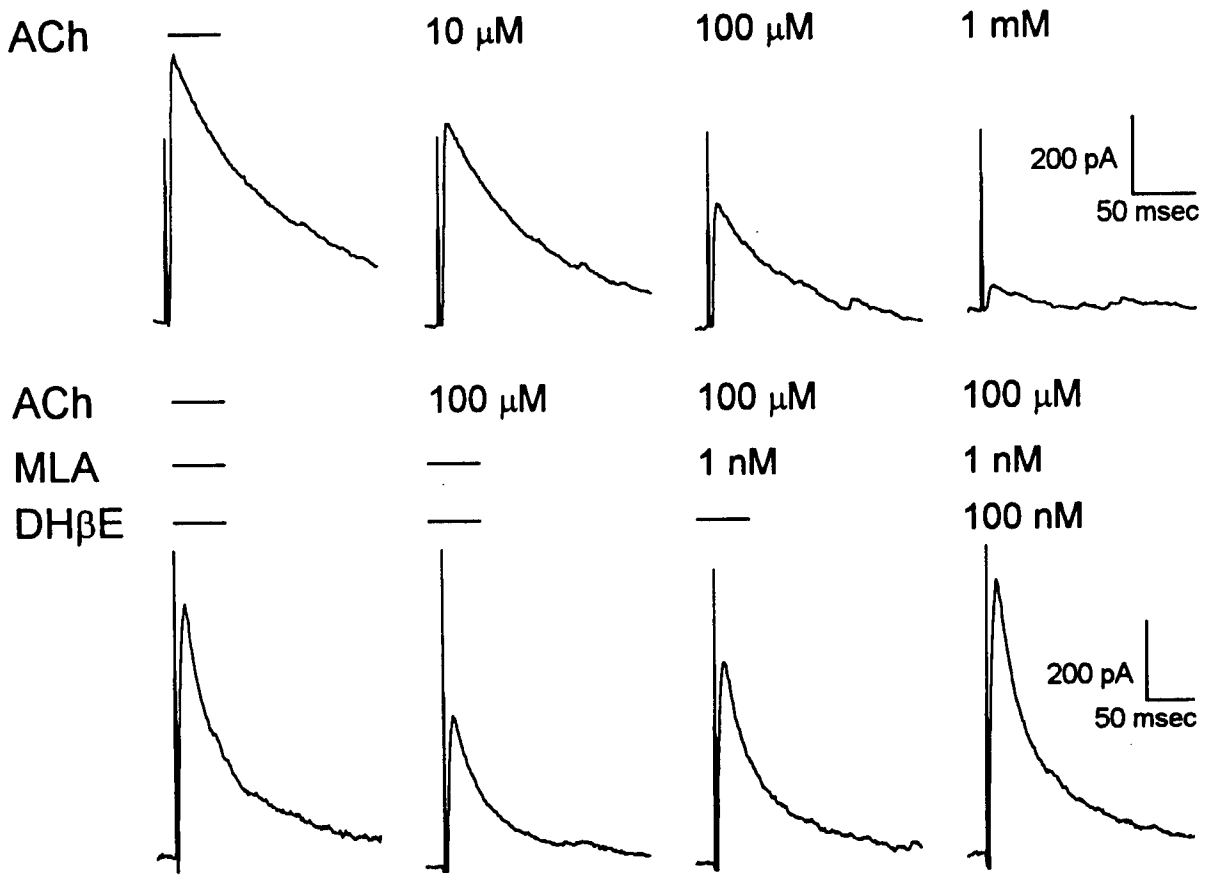


Figure 2. Acetylcholine reduces the amplitude of evoked IPSCs via activation of α7 and α4β2 nAChRs. In 1-s intervals, neurons synaptically connected to the neuron under study in culture were stimulated with a supramaximal (~11 volt), 20-μs pulse applied via the bipolar electroplated platinum electrode. After at least 10 control IPSCs were recorded from a single neuron, 1-s pulses of ACh (10 μM–1 mM) were delivered to the neurons simultaneously with the electrical stimulus sent to the bipolar electrode. Notice that ACh reduces the amplitude of the evoked IPSCs. This effect of ACh, which was concentration dependent, was completely reversible. In the presence of MLA (1 nM), the ACh-induced reduction of the IPSC amplitude was partially blocked, whereas in the presence of both MLA and DHβE (100 nM) a complete blockade of the ACh effect was achieved.

In the presence of the muscarinic receptor antagonist atropine (1 μM), rapid application (1–5 s) of ACh (10 μM–1 mM) to the cultured hippocampal neurons, in addition to increasing the frequency of GABA-mediated PSCs (data not shown), reduced the amplitude of GABA-mediated IPSCs evoked by field stimulation of a neuron synaptically connected to the neuron under study (*figure 2*). It is likely that depolarization resulting from ACh-induced activation of nAChRs in presynaptic neurons generates areas of blockade of conduction of action potentials, and, ultimately, leads to the reduction of the evoked release of GABA. The effect of ACh on the evoked release was only partially blocked when the neurons were perfused with physiological solution containing either MLA (1 nM) or DHβE (100 nM). A full blockade of the ability of ACh to reduce the evoked release of GABA was achieved by perfusing the neurons with physiological solution containing an admixture of MLA (1 nM) and DHβE (100 nM) (*figure 2*).

4. Discussion and conclusions

The α7 nAChR-selective agonist and nAChR-subtype-selective antagonists provided strong evidence that interneurons in the CA1 field of hippocampal

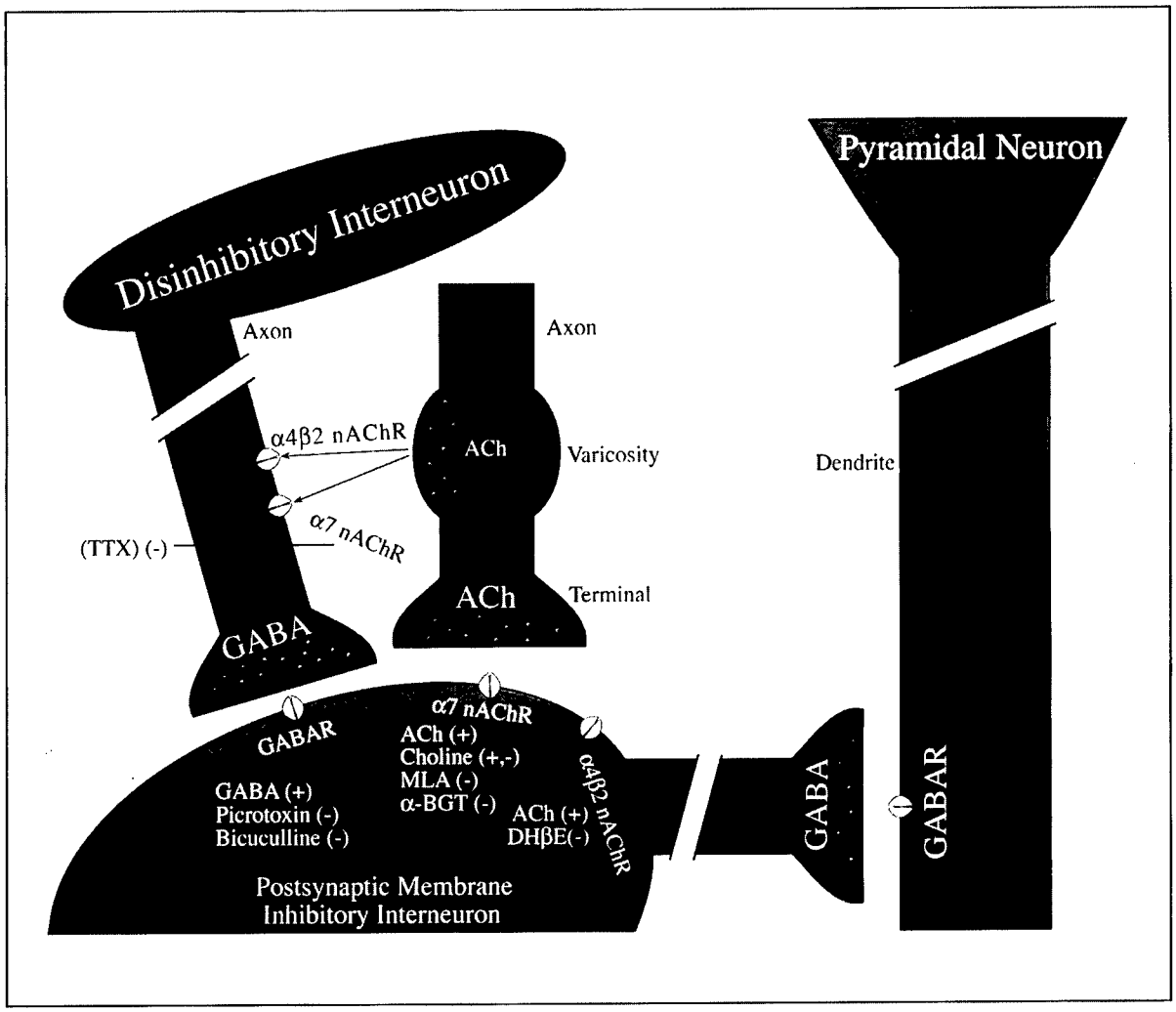


Figure 3. Scheme showing the possible sites of expression of functional α7 and α4β2 nAChRs in CA1 interneurons. This hypothetical model is based on the results presented in this paper and in Alkondon et al. [6]. The cholinergic axon is likely to come from the septum, and GABAergic axons are likely to originate from other interneurons in different layers of the CA1 region. As depicted in the far left side of the scheme, activation of α7 and α4β2 nAChRs located in presynaptic neurons can facilitate the TTX-sensitive release of GABA. Activation by GABA of $GABA_A$ receptors present in the interneuron being studied would result in elicitation of GABA-mediated PSCs, which are sensitive to blockade by $GABA_A$-receptor antagonists. The nicotinic currents recorded from the interneuron under study would have been the consequence of the activation of nAChRs present in that neuron. Currents resulting from activation of α7 nAChRs would decay within the agonist pulse and would be equally sensitive to choline and ACh as agonists and to MLA and α-BGT as antagonists. On the other hand, currents resulting from activation of α4β2 nAChRs would last longer than the agonist pulse, would be sensitive to ACh, but not to choline as an agonist, and would be blocked selectively by DHβE. By inference, as depicted in the far right side of the scheme, it is likely that modulation by nAChRs of the ongoing activity in the interneurons ultimately controls the excitability of the pyramidal neurons in the CA1 region. In this model, the sign (–) indicates that a given compound is capable of blocking the receptor activity, and the sign (+) indicates that the compound can activate a given receptor.

slices and cultured hippocampal neurons can express functional preterminal α7 and α4β2 nAChRs and that activation of both nAChR subtypes facilitates the TTX-sensitive and blocks the evoked release of GABA (see *figure 3*) [6]. It is, thus, possible that during cholinergic activation, ACh shuts off the GABAergic neuronal activity driven by presynaptic (glutamatergic) neurons and by itself modulates the release of GABA. This may be a unique mechanism by which cholinergic neurons take full control of ti-

ming of inhibitory inputs to hippocampal pyramidal neurons. It is noteworthy that the net result of activation of nAChRs appears to depend on the location of the receptor on the neuronal surface, because there is evidence that activation of α7 nAChRs present in glutamatergic terminals of hippocampal neurons facilitates the release of glutamate evoked by field stimulation of presynaptic neurons (Radcliffe and Dani, personal communication).

The neuronal circuitry in the hippocampus is highly complex, and interneurons are capable of innervating pyramidal neurons and neighboring interneurons [1, 14]. Thus, cholinergic nicotinic activation can produce both a direct inhibition and an indirect disinhibition of a pyramidal neuron, and timing of the two events could control the firing rate of a pyramidal neuron. This may represent a cholinergic mechanism that adjusts the activity of CA1 pyramidal neurons, and, consequently, the neuronal output from the hippocampus to higher brain centers. Disinhibition of CA3 pyramidal neuron activity by stimulation of septal GABAergic afferents has been recently demonstrated, and the importance of disinhibitory mechanisms in the hippocampal function has been stressed [23]. In the CA1 region, local GABAergic interneurons that are likely to be driven by various glutamatergic afferents have also been shown to have a disinhibitory function [1, 14]. Our results indicate that both α7 or α4β2 nAChRs may be involved in controlling the overall activity of these disinhibitory cells, and provide direct support to the concept that a GABAergic mechanism could explain the relationship between α7 nAChR gene locus and attentional deficits in schizophrenia [11], and that between cholinergic deafferentation and kindling epileptogenesis [16].

Our results also indicate that choline, by controlling the function of α7 nAChRs in the CNS may have a role in modulating the activity of the CA1 interneurons, and, ultimately, the excitability of the CA1 pyramidal neurons. This finding calls for a review of our understanding of the mechanisms underlying the neurotoxic effects of compounds that inhibit cholinesterase, including organophosphates (OPs) used as pesticides or nerve agents. It has long been accepted that accumulation of non-hydrolyzed ACh in the synaptic cleft could lead to hyperactivation of the cholinergic systems in OP-induced intoxication. With the discovery of the selective agonist action of choline on α7 nAChRs [5] and of the involvement of these in modulation of transmitter release [4, 12, 13, 17, 18], it could be postulated that the neurotoxic effects of cholinesterase inhibitors arise from an imbalance between ACh and choline concentrations at the synaptic cleft.

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Chronic neurobehavioral and central and autonomic nervous system effects of Tokyo subway sarin poisoning

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Abstract — To evaluate delayed (prolonged) neurobehavioral and neurophysiological effects of acute sarin poisoning, nine male and nine female patients of the Tokyo subway sarin poisoning in Japan were examined by neurobehavioral tests, posttraumatic stress disorder (PTSD) checklist, brain evoked potentials, computerized static posturography, and electrocardiographic R-R interval variability, 6–8 months after the poisoning. Their serum cholinesterase activities on the day of the poisoning (March 20, 1995) were 13–131 (mean 72.1) IU/L. The results suggested delayed effects on psychomotor performance, the higher and visual nervous system and the vestibulo-cerebellar system with psychiatric symptoms resulting from PTSD. (©Elsevier, Paris)

Résumé — Effets chroniques comportementaux des systèmes nerveux central et autonome des empoisonnements au sarin du métro de Tokyo. Pour évaluer les effets comportementaux et neurophysiologiques à long terme ou à retardement d'un empoisonnement au sarin, 9 hommes et 9 femmes qui ont été intoxiqués dans le métro de Tokyo le 20 mars 1995 ont été suivis pendant 6 à 8 mois par des tests neurologiques, une évaluation de stress post-traumatique ("post traumatic stress disorder", PTSD), des études de potentiel évoqué cérébral, une posturographie statique suivie par ordinateur, et une étude de la variabilité d'intervalle R-R électrocardiographique. Le jour de l'empoisonnement, l'activité de la cholinestérase sérique était de 13 à 131 UI/L (moyenne de 72,1 UI/L). Les résultats suggèrent que ces patients souffrent d'effets retardés sur leurs performances psychomotrices, leur système nerveux central et visuel et leur système vestibulaire, avec des symptômes psychiatriques révélés par le test de PTSD. (©Elsevier, Paris)

sarin / subway / Tokyo / neurobehavioral tests / posttraumatic stress disorder / computerized posturography / P300 / visual evoked potential / electrocardiographic R-R interval variability

1. Introduction

On June 27, 1994, about 600 residents in Matsumoto city, located approximately 150 km northwest of Tokyo, Japan, were poisoned due to a presumed terrorist attack with sarin (methyl phosphonofluoridic acid 1-methylethyl ester); all affected people recovered without abnormal findings on routine neurological and laboratory examinations except one patient who had severe anoxic encephalopathy due to respiratory arrest [24]. On March 20, 1995, a larger number of people (i.e., approximately 5500) were also poisoned with sarin in subways in Tokyo (Tokyo subway sarin poisoning). According to a follow-up study on the 640 cases, two cases died just after admission to hospital; no clinical abnormalities were found 3 months after the poisoning [29]. On the other hand, electroencephalogram (EEG) abnormalities in chemical plant workers have been reported more than 1 year after accidental exposure to sarin [10]. EEG changes in monkeys also have been observed 1 year after experimental sarin poisoning [7].

As a chronic neurological sequelae with decreased performance on neurobehavioral tests after acute organophosphate pesticide poisoning has been reported [30, 32, 37], we conducted a series of studies on delayed (long-term) effects of Tokyo subway sarin poisoning [27, 50, 51]. The paper presented here summarizes the results of our studies, indicating delayed neurobehavioral and neurophysiological effects of sarin poisoning together with posttraumatic stress disorder (PTSD).

2. Outline of the incidence

On March 20, 1995, 11 plastic bags containing odorous chemical agent were placed and poisonous vapors were released from the bags in five cars on three separate subway lines (Hibiya, Chiyoda and Marunouchi lines), which were scheduled to converge from the north and west on the Kasumigaseki Station (located tin the government district) between 8:09 and 8:13 a.m. in Tokyo. As a result, approximately 5500 people were poisoned in the subway cars and stations at around 8:00 a.m.; nine passengers and two station officers died. The chemical agent was quickly identified as sarin by the Metropolitan Police Department and the Self-Defense Force.

The victims were transported to nearby hospitals to receive emergency medical evaluation and treatment. St. Luke's International Hospital, located within 3 km from the affected subway stations, accepted a total of 640 patients including the subjects examined in the present study, which was the largest number of the cases from the subway poisoning. The Metropolitan Police Department and the Tokyo District Public Prosecutors have suspected that the Aum Sinri Kyo cult released sarin at rush hour to create massive confusion in the Tokyo area, aiming at disturbing the police investigation into murder crimes including the poisoning in Matsumoto city [41].

3. Subjects

The nature of the procedure in the present study was fully explained to all subjects, and the study was conducted with their informed consent in September to November 1995, i.e., 6–8 months after the poisoning. Hospital staff could contact 150 victims of the poisoning and advise them to receive a health checkup on possible neurological sequelae during this period, nine males and nine females consented readily to undergo the study. Thus, a total of 18 patients (sarin cases) were examined: one plasterer, one kitchen maid and 16 office clerks, who had been exposed to sarin vapor accidentally in the subway attack in Tokyo on March 20, 1995, on the way to their work.

On admission to the hospital, plasma cholinesterase (ChE) activities ranged from 13 to 95 (mean 68.2) IU/L for females and from 19 to 131 (mean 75.9) IU/L for males (13 to 131 IU/L with a mean of 72.1 for all cases); all except for three males showed the values lower than 'normal' (100–250 IU/L). There was no significant difference in the ChE activities between female and male sarin cases ($P > 0.05$). Their symptoms and signs on admission are shown in *table I*. They were treated by injection of atropine and pralidoxim for 1–2 days. Age, level of education, amount and frequency of drinking, number of cigarettes smoked per day, past and present illness, and use of drugs were surveyed through an interview; amount of ethanol consumption per week was calculated using ethanol contents in beverages, i.e., 15% for sake, 43% for whiskey and 5% for beer.

Control subjects were three groups of healthy Japanese without history of occupational or environmental exposure to toxic chemicals including sarin, viz.: 1) eight males and seven females, whose age, male-female ratio, education level, alcohol consumption or smoking (cigarettes per day) were not significantly different from the sarin cases, for neurobehavioral tests; 2) 18 sex- and age-matched controls without significant differences in age, alcohol consumption or smoking from the cases for brain evoked potentials and electrocardiographic R-R interval variability (CVRR); and 3) 18 females and 35 males, whose age, height, body weight, alcohol consumption or smoking were not significantly different from the cases, for posturography.

Table I. Major symptoms and signs in 18 sarin cases on admission

	Number of cases		
	Male	*Female*	*Total (%)*
Dyspnea	5	6	11 (61)
Headache	5	6	11 (61)
Nausea	5	4	9 (50)
Feeling of asthenia	4	4	8 (44)
Diplopia	3	4	7 (39)
Ocular pain	3	4	7 (39)
Diarrhea	3		3 (17)
Sneeze	2	1	3 (17)
Sore throat	2	1	3 (17)
Paresthesia	2	1	3 (17)
Vomiting	1	2	3 (17)
Darkness of visual field	1	2	3 (17)
Decreased visual field	2		2 (11)
Anxiety	2		2 (11)
Cough	1	1	2 (11)
Loss of consciousness	1	1	2 (11)
Cardiac arrest		1	1 (6)
Lacrimation		1	1 (6)

4. Methods

4.1. Neurobehavioral test

Subjects underwent nine tests: 1) digit symbol (psychomotor performance); 2) picture completion (visual perception); 3) digit span (attention and memory); 4) finger tapping (psychomotor performance); 5) reaction time (psychomotor performance); 6) continuous performance test (sustained visual attention); 7) paired-associate learning (learning and memory); 8) General Health Questionnaires (GHQ) (psychiatric symptoms); and 9) Profile of Mood States (POMS) (mood), in a quiet room during day time of weekdays. The first three tests (1–3) are subtests of the Japanese edition of the Wechsler Adult Intelligence Scale (WAIS) [17]; the second three (4–6) are subtests of Japanese version of computer-administered testing (Neurobehavioral Evaluation System) [19, 45]; test 7 is a subtest of the Japanese version of Wechsler Memory Scale (WMS) [15, 43]; and the last two (8, 9) were self-rating questionnaires [13, 16, 23, 46, 47].

4.2. Posttraumatic stress disorder (PTSD) checklist

A self-rating questionnaire consisting of 17 problems and complaints related to PTSD (*Appendix*) was administered on all subjects just before neurobehavioral testing. This was originally developed for PTSD based on DSM-III-R in veterans with military experience [42]; in the present study the list was translated into Japanese in which the term 'military experience' in the original form was substituted by subway sarin incident. The subject was asked to indicate how much he (she) had been bothered by that problem or complaints in the past month, using a rating from 1 (not at all) to 5 (extremely); the sum of the numbers was used as the score on PTSD.

4.3. Brain evoked potentials

The event-related evoked potentials (ERP) were measured as described previously [2, 3, 12]. A random sequence of two distinguishable tones (90 dB SPL) was presented binaurally through earphones at a rate of 0.5 Hz. Eighty percent of the 320 tones had a frequency of 1000 Hz (non-target tones) and 20% had a frequency of 2000 Hz (target tones). The subject was instructed to count the target tone mentally. In all cases, the reported total of the target tone was correct within three stimuli of the actual tones presented. Cerebral responses to the two stimuli were recorded using disc electrodes at the vertex, mastoids and forehead, and were averaged separately. The P300 component elicited by the target tone was defined as the first maximal positive wave between 250 and 500 ms; the N100 component by the non-target tone was also recorded to examine if the change in P300 was independent of the early auditory responses.

The pattern-reversal visual evoked-potential (VEP) was measured in a darkened room [1, 3, 36]. The subject sat in front of TV screen and was given a checkerboard pattern on the screen consisting of white and black squares, reversing at a rate of 2 Hz. One positive peek (P100) was recorded using standard EEG electrodes fixed to the occipital cortex, forehead and left mastoid (grand). Responses were averaged over 128 times.

The brainstem auditory evoked potential (BAEP) was measured by the method reported previously [3, 38]. Click signals were presented to the right ear through earphones at rates of 20 Hz. The BAEP was recorded using three standard EEG electrodes on the vertex, right mastoid and left mastoid (grand). The responses were averaged 1024 times with one replication.

4.4. Computerized static posturography

Postural balance was quantitatively measured by the method reported previously by us [4, 5, 48–50]. Measurements were carried out in a quiet room with flat floor using a strain-gauge-type force platform (Static Sensograph 1G06, NEC Sanei, Tokyo) connected to a microcomputer (PC9801, NEC, Tokyo) via an analog-to-digital converter (Analog Pro, Canopus, Tokyo). Subjects were instructed to refrain from alcohol or drugs the day before the study (12 h or more prior to the study).

Subjects were asked to stand quietly on the platform for 60 s with eyes open and then for 60 s with eyes closed. Strain gauges at the three corners of the platform measured vertical forces and converted them to electric voltages; the medio-lateral (right-left) and anterior-posterior location of the body's center of pressure (CP) in the horizontal plane was calculated from the voltages sampled at a frequency of 20 Hz by the microcomputer and recorded on a disk.

Displacements of the body's CP in the both directions were then subjected into the Fast Fourier Transformation analysis [5, 9, 48–50]. The total duration of sampling was 51.2 s (1024 points). The span of frequencies analyzed ranged from 0 to 4 Hz (0.02-Hz steps). Sum of the root squares of the power calculated from the power spectrum between: 1) 0 and 1 Hz; 2) 1 and 2 Hz; and 3) 2 and 4 Hz served as measures of the amount of postural sway for each frequency range with eyes open and closed.

Lengths of displacement of the CP in the medio-lateral and anterior-posterior directions within each sampling time were summed up for each direction and defined as the length of sway path of the body's CP in the medio-lateral (Dx) and anterior-posterior (Dy) directions, respectively; the area included within the sway path traveled by the CP during 60 s (Area) was also calculated. Romberg quotients, i.e., the ratio of the value with eyes closed to that with eyes open [5, 9, 48–50], for the power of the sway for each frequency band, Dx, Dy and Area, were calculated.

Some studies [6, 8, 9, 11, 21, 44] have suggested that: 1) the lesion of the vestibulo-cerebellum (lower vermis), which contains the vestibulo-cerebellar pathway, shows a sway of non-specific frequencies with eyes open (low Romberg quotient); 2) the lesion of the anterior cerebellar lobe is related to a sway of high frequency (2–4 Hz) predominantly in anterior-posterior direction with eyes closed (high Romberg quotient); and 3) the lesion in the spinocerebellar afferent pathway (including the spinocerebellar tract and proprioceptive nerve fibers in the legs) is associated with an omnidirectional sway

of low frequency (1 Hz or less) with eyes closed (high Romberg quotient).

4.5. Electrocardiographic R-R interval variability

Three-hundred R-R intervals on electrocardiogram were measured after the subject had been supine for 10 min; 100 consecutive R-R intervals with the minimal standard deviation (S.D.) were extracted [5, 25, 26]. The CVRR was defined as the ratio of S.D. to the mean (%). The spectrum of R-R intervals was calculated by autoregressive spectral analysis, and separated for the low frequency (LF) at the center frequency of 0.04–0.15 HZ and the high frequency (HF) component at 0.15–0.4 Hz by component analysis. Each component coefficient of variation (C-CVLF and C-CVHF,%) was defined as the ratio of the square root of each component to the mean R-R interval. The C-CVLF and C-CVHF are thought to reflect sympathetic and parasympathetic activities, respectively [40].

5. Results

The score on digit symbol test in the sarin cases was significantly lower than that in the controls; the scores on GHQ, fatigue (POMS) and PTSD checklist were significantly higher in the former group than in the latter (*table II*). When the score on PTSD was added to the covariates, only the score on the digit symbol test in the sarin cases was significantly lower than in the controls (*table II*). Scores on GHQ and fatigue (POMS) were significantly correlated with PTSD scores in 18 sarin cases ($r = 0.747$ and 0.471, respectively, $P < 0.05$). The P300 (ERP) and P100 (VEP) latencies in the sarin cases were significantly prolonged when compared with the controls (*table II*). In females, the power of postural sway of 0–1 Hz in the sarin cases was significantly larger than that in the controls in the anterior-posterior direction when eyes were open (*table II*); similarly, the Area with eyes open was significantly larger in these cases (*table II*). The Romberg quotient for the power of 0–1 Hz of sway in the anterior-posterior direction was significantly correlated with log ChE in the females ($r = 0.673$, $P < 0.05$).

No other measures in the present study showed significant differences between the cases and controls ($P > 0.05$).

6. Discussion

A significant decrease in the score on digit symbol test was observed in the cases 6–8 months after acute sarin poisoning. This decrease was also significant when the effect of PTSD was controlled in the analysis

Table II. Significant differences in neurobehavioral tests, posttraumatic stress disorder (PTSD) checklist, brain evoked potentials and computerized posturography between sarin cases and controls.

	Cases		*Controls*		*Significance level (P)*
	Mean	*Range*	*Mean*	*Range*	
Neurobehavioral tests[a]					
Digit symbol	15.9	11–19	17.5	14–19	< 0.05[b]
General Health Questionnaires	5.6	0–13	2.5	0–9	< 0.01[c]
Profile of Mood States (fatigue)	11.6	2–23	6.7	0–17	< 0.05[c]
PTSD[a]	25.9	18–45	21.2	17–38	< 0.05
Brain evoked potentials (ms)[d]					
P300 (event-related potential)	321	284–348	289	248–325	< 0.001
P100 (visual-evoked potential)	102	91–114	97	82–118	< 0.05
Posturography (eyes open)[e]					
Power of 0–1 Hz sway, anterior-posterior direction (cm)	3.47	2.1–4.9	2.72	1.7–3.4	< 0.05
Area (cm^2)	2.74	1.1–5.0	2.12	0.7–3.2	< 0.05

[a] 18 sarin cases and 15 controls; analysis of covariance where factors were group (sarin case or control) and gender and covariates were age, education level, alcohol consumption and smoking.
[b] $P < 0.01$ when PTSD scores were added to covariates.
[c] $P > 0.05$ when PTSD scores were added to covariates.
[d] 18 sarin cases and the same number of age-and sex-matched controls; paired *t*-test.
[e] Nine females sarin cases and 18 female controls; analysis of covariance where factor was group (sarin case or control) and covariates were age, height, body weight and alcohol consumption.

of covariance. It is thus suggested that a chronic effect on psychomotor performance (motor persistence, sustained attention, response speed, and visuomotor coordination) as measured by digit symbol test was caused directly by acute sarin poisoning. Similarly, the P300 (ERP) and P100 (VEP), which are considered to reflect cognitive function and the conduction time from the retinal to the visual cortex, respectively [12, 38], were prolonged in the sarin cases, indicating the delayed effects of sarin on the higher and visual system. These findings essentially coincide with the observations of chronic neurological sequelae after acute organophosphate poisoning including sarin [7, 10, 30, 32, 37].

In the posturography, the sway of low (0–1 Hz) frequency and the area of sway with eyes open were significantly increased in the female sarin cases; the Romberg quotient for the former measure was significantly related to log ChE in the cases. Therefore, it is suggested that a delayed (long-term) effect on the vestibulo-cerebellar type of sway was caused in females. This may coincide with the observations of neurological sequelae after acute organophosphate poisoning. However, in contrast to the present study, pesticide applicators with concurrent exposure to organophosphate showed that a total length of the sway path was affected more with eyes closed than with eyes open [31]; the discrepancy between the two studies remains unexplained. The result that the posturographic effects were observed only in females in the present study agrees with the finding that the effects of organophosphate (maloxon) are more severe in female than in male rats [33].

The posturographic change in females was significantly related to log ChE, agreeing with the findings that acetylcholine mediates the vestibulo-cerebellar function [20, 34]. In contrast, the relationship of the score on digit symbol test, P300 or P100 to ChE was not significant. This may coincide with the observations in rats that ChE in the brain just after poisoning by sarin did not predict signs of encephalopathy 1–2 days after the poisoning [18] and that behavioral abnormalities were not correlated with concurrent brain acetylcholinesterase activities 6 and 24 h after poisoning by sarin, soman or tabun (N-dimethylphosphoramide- cyanidate, a toxic organophosphate compound) [14]. Thus, the mechanism of the central nervous system toxicity of sarin and related organophosphate compounds other than the inhibition of acetylcholinesterase activity should be investigated.

Lesions in the brain, i.e., neuronal degeneration and necrosis, have been observed in rats surviving single subcutaneous injection of sarin or soman (0-(1,2,2-trimethylpropyl)-methylfluorophosphate, an organophosphate compound with potential for use in war) [18, 22, 35]. One of these studies [35] examined the histopathological changes up to 35 days after exposure and revealed that the changes were still present, suggesting that toxic organophosphate compounds such as sarin and soman can produce irreversible (or long-term) damage to the central nervous system. It is, therefore, possible that the significantly reduced scores on the digit symbol test in the cases in the present study can be attributed to irreversible changes in the central nervous system caused by sarin.

The score on PTSD in the sarin cases was significantly higher than in controls, agreeing with the observation of PTSD in the cases of Tokyo subway sarin poisoning [28, 29]. When PTSD score was not used as a covariate in the analysis of covariance, the scores on GHQ and fatigue (POMS) were significantly increased in the cases whereas the increases were not significant after PTSD was added to the covariates. Also, the scores on GHQ and fatigue were positively related to PTSD in sarin cases. Therefore, psychiatric (GHQ) and mood (POMS) changes are considered to result from PTSD. As psychological symptoms, such as complaints of physical distress and psychopathological profiles of Minesota Multiphasic Personality Inventory, related to PTSD have been observed in Korean war veterans 30 years after the war [39], the psychiatric and mood changes might have had persisted in the cases since the Tokyo subway sarin poisoning.

Finally, because of small sample size and low recruitment of sarin cases in the present study, a further study on a larger number of victims of the Tokyo subway sarin poisoning will be necessary to confirm the observations of chronic neurobehavioral effects of sarin and of PTSD. Also, imaging techniques such as magnetic resonance imaging may be useful for examining structural changes in brain to related to sarin exposure and/or PTSD.

Appendix

PTSD checklist

1. Repeated, disturbing memories of the subway sarin incident?
2. Repeated, disturbing dreams of the subway sarin incident?
3. Suddenly acting or feeling as if the subway sarin incident happens again?
4. Feeling very upset when something happened that reminded you of the subway sarin incident?
5. Trouble remembering important parts of the subway sarin incident?
6. Loss of you interest in activities that you used to enjoy?

7. Feeling distant or cut off from other people?
8. Feeling emotionally numb, or being unable to have loving feelings for those close to you?
9. Feeling as if your future will somehow be cut short?
10. Trouble falling or staying asleep?
11. Feeling irritable or having angry outbursts?
12. Having difficulty concentrating?
13. Being 'superalert', or watchful or on guard?
14. Feeling jumpy or easily startled?
15. Having physical reactions when something reminds you of the subway sarin incident?
16. Avoid thinking about the subway sarin incident, or avoid having feeling about it?
17. Avoid activities or situations because they reminded you of the subway sarin incident?

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Long term health effects of low dose exposure to nerve agent

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Abstract — Possible long-term toxic effcts of nerve agents have been investigated using sensitive toxicological screens and extensive toxicity studies in various animal models. Data on humans have been obtained from controlled studies and accidental exposures. Studies in the area of 'low dose' exposure to nerve agents are currently being performed. (©Elsevier, Paris)

Résumé — Effets à long terme de faibles doses d'agents neurotoxiques. Les éventuels effets toxiques à long terme d'agents neurotoxiques ont été analysés grâce à des tests toxicologiques sensibles, et à des études de toxicité dans divers modèles animaux. Les données concernant les humains ont été obtenues à partir d'études expérimentales contrôlées et d'intoxications accidentelles. L'étude de faibles doses d'agents neurotoxiques est actuellement en cours. (©Elsevier, Paris)

nerve agent / soman / sarin / tabun / VX / acetylcholinesterase / EEG / delayed neurotoxicity / subchronic

1. Introduction

Nerve agents exert their effects by inhibition of the enzyme acetylcholinesterase (AChE), leading to accumulation of excess levels of the neurotransmitter acetylcholine (ACh) at cholinergic synapses. Enzyme inhibition is both rapid and irreversible, thus making organophosphorus (OP) nerve agents such as tabun (GA), sarin (GB), soman (GD), cyclosarin (GF) and VX highly toxic and extremely dangerous chemicals designed to kill or incapacitate enemy forces, disrupt military operations and deny terrain to the adversary. Additionally, nerve agents have been used recently as weapons of terror. In June 1994 and again in March 1995, sarin was released in public places by a Japanese cult. In the latter incident, 5500 people on Tokyo subways sought medical care; about 4000 suffered no effects from the agent, but 12 people died [1].

Nerve agents gain entry by apsorption through the lungs or skin and impair the activity of cholinergic synapses, including those of smooth and skeletal muscle, autonomic ganglia and the central nervous system (CNS). Acute toxic effects of nerve agents can be elicited at very low concentrations while lethal effects are observed at somewhat higher concentrations. Threshold symptoms for vapor exposure are commonly stated to be miosis, rhinorrhea and airway constriction, generally appearing at a Ct (vapor concentration × exposure time) of 2–3 mg/min/m^3 [2]. Low to moderate exposure of skin to liquid nerve agent causes localized sweating, nausea, vomiting and a feeling of weakness [3]. Lethal amounts of vapor or liquid cause a rapid cascade of events culminating within a minute or two in convulsions, loss of consciousness, apnea, flaccid paralysis and death [3]. Toxicity is thus concentration-dependent, requiring a defined minimal concentration of agent; recovery generally occurs by synthesis of new AChE (See *tables I* and *II* for toxicity of nerve agents).

Concerns regarding Persian Gulf Illness as well as ongoing concerns about low-level chemical warfare exposures and potential exposures to low-levels of sarin at Khamisiyah, Iraq in March, 1991 have led to much discussion about the consequences of low dose exposure to nerve agent, especially long term effects of such exposure on human health.

2. Low-level nerve agent exposure and long term health effects

Low levels of exposure to nerve agent can be considered as those levels that result in minimal reduc-

Table I. Vapor toxicity (mg/min/m^3).

Agent	LCt_{50}	ECt_{50} *(miosis)*
GA	400	2–3
GB	100	3
GD	70	< 1
GF	UNK	< 1
VX	50	0.04

Table II. LD_{50} on skin.

Agent	*Amount (mg)*
GA	1000
GB	1700
GD	50
GF	30
VX	10

tion of AChE with no observable clinical signs and no subjective symptoms. Three levels of low dose exposure can be described: level 1 is an exposure that results in no clinical signs or symptoms and an AChE inhibition of < 20%; level 2 is an exposure that results in no clinical signs or symptoms but a moderate inhibition of AChE (> 20%); and level 3 is an exposure that results in mild clinical signs such as salivation, miosis and tachycardia and subjective symptoms such as shortness of breath. Exposure values in humans have been established for GB (*table III*) and are explained below.

The LCt_{50} is the dose expected to kill 50% of the exposed population. No neuromuscular effects (NNM) are expected at doses below 4 mg min/m^3. No observable effects (NOEL) are seen with levels at or below 0.5 mg min/m^3. An 8-h exposure to 0.05 mg min/m^3 will remain below NOEL. The remaining values in *table III* represent occupational exposure limits as established by the U.S. Department of Health and Human Services. Significant safety factors have been applied.

Table III. Human exposure values for GB.

Agent GB
LCt_{50} = 100 mg min/m^3
No death dose = 10 mg min/m^3
NNM effect dose = 4 mg min/m^3
ECt_{50} (miosis) = 2–4 mg min/m^3
NOEL = 0.5 mg min/m^3
MSC (1 h) = 0.001 mg/m^3
MSC (8 h) = 0.0003 mg/m^3
Safety factor of 0.1 for general population
0.0001 mg/m^3 (1 h)
0.00003 mg/m^3 (8 h)
0.000003 mg/m^3 (72 h)

Data available on humans exposed to nerve agents comes from two sources: human experiments and accidental exposures.

2.1. *Controlled studies on volunteers*

Between 1958 and 1975, the U.S. Army conducted research involving 6000 soldier volunteers to learn how specific chemicals may affect humans. Of this group, over eleven hundred subjects were exposed to low and moderate levels of nerve agents under controlled conditions. The doses used in these experiments were equal to or less than 1.5 ID_{50} units. The ID_{50} is the dose of agent that causes early signs of incapacitation (miosis) in 50% of exposed individuals. In 1982, the U.S. Army requested that the Committee on Toxicology of the National Research Council conduct a study of the possible chronic adverse health effects on the above group of servicemen who had been exposed to chemical agents under experimental conditions. The first report found no evidence to support a finding of adverse long-term or delayed health effects following exposure to nerve agents [4]. However, this report was unable to rule out the possibility that some anti-AChE agents produced long-term adverse health effects in some individuals. The report deferred to the outcome of a follow-on morbidity study to 'shed further light' on this issue. In the follow-on study, a questionnaire was sent to subjects of the earlier studies in order to assess the current health status of over 4000 subjects voluntarily exposed to chemical agents between 1958–1975 [5]. The long-term health effects of greatest interest included: 1) increased cancer risk; and 2) adverse mental, neurological, hepatic and reproductive effects. Results indicated that subjects who received nerve agents, as a group, did not differ from controls who had received no chemical treatment.

2.2. *Accidental exposures and electroencephalographic changes*

Over the past 50 years, hundreds of industrial and laboratory workers have been accidentally exposed to both asymptomatic and symptomatic levels of nerve agents. The strongest evidence in humans of a possible long-term effect of exposure to nerve agents is from studies reporting changes in electroencephalograms (EEGs). There is no single study or set of studies that exactly addresses the acute and long-term changes in EEG activity produced by nerve agents. Also, virtually all the animal EEG studies of nerve agent exposure have focused on the effects of high dose exposure and the mechanisms and treatment of these more serious toxic effects. Estimated ranges of red blood cell cholinesterase inhibition are correlated to observed EEG effects.

Chronologically, the first study to suggest long-term EEG effects following nerve agent exposures was by Metcalf and Holmes, who described their findings from a group of workers at a sarin production plant [6]. Exposed workers had higher voltage EEGs with more pronounced alpha rhythm and bursts of slow waves during drowsiness. The individuals also had a high incidence of 'narcoleptic' sleep patterns, corresponding presumably to early REM sleep.

The widely cited work of Duffy et al. [7] describes EEG changes found in a group of 77 workers accidentally exposed to sarin showing signs and symptoms of exposure and AChE inhibition > 25%. Forty-one of the 77 workers had multiple (> 3) exposures. EEGs from this group were compared to those of a control group from the general population. Subtle, but statistically significant increases in EEG

beta-band activity over controls were observed for 1 to 6 years after exposure in the sarin group. Individuals exposed to sarin were reported to have more and longer periods of REM sleep than the general population. This was also true, however, of the control group in this study. These 77 workers reported no adverse health effects and no behavioral changes. The significance of the alterations in EEG patterns is uncertain because no behavioral effects can be attributed to them. The panel analyzing the results from the U.S. Army study [5] could neither confirm nor exclude the findings of Duffy et al. [7]. To summarize what is presently known, long-term changes in EEGs have been observed with symptomatic exposures; multiple exposures produce more prominent effects. Increases in beta activity are seen during drowsiness or hyperventilation. Increases in REM sleep occur after OP exposure. Records are also available on workers with exposures to OP pesticides [8].

2.3. Animal studies using primates

Results are available from a non-human primate study using sarin [9]. Rhesus monkeys were implanted with cortical and depth electrodes and injected with sarin in one of two dosage schedules: a single high dose (5 μg/kg, i.v.) that elicited seizures, or low doses (1 μg/kg, i.m.) once a week for 10 weeks that caused no clinical effects. EEGs were recorded prior to exposure, 24 h and 1 year after exposure. The animals from both dose schedules had increases in high frequency beta activity, but were otherwise healthy. No long-term behavioral effects were noted.

2.4. Other animal studies

There are few data on asymptomatic doses in animals since high doses are generally selected by investigators to elicit observable effects. Studies have been performed using chronic exposures to symptomatic non-lethal doses of OP nerve agents (GA, GB, GD and VX). With the exception of one study using GA, the seven studies reported no persistent changes in histopathology, hematology, clinical chemistry or other biochemical parameters [10]. Another group of five studies used subchronic, symptomatic doses of GD, GB or VX up to two times the ID_{50}. Myopathy was consistently produced with cardiomyopathy prominent in animals experiencing seizures [11].

Attenuation of hormonal responses to physiological or pharmacological challenge was observed in a single study 2 weeks after an acute symptomatic dose of GD, possibly attributable to suppression of diurnal hormonal cycles [12].

2.5. Toxicological studies

The majority of closely controlled toxicological studies have been conducted in animals at the National Center for Toxicological Research or the University of California, Davis following strict guidelines. The G-agents have been screened for mutagenicity or clastogenicity using in vitro and in vivo assays. GB and GD have been found not to be mutagenic [13]. GA was found to be weakly mutagenic in three different assays but not teratogenic in two animal species, rabbits and rats [14]. Essentially, investigation of long-term toxic effects of nerve agents using sensitive toxicological screens and extensive toxicity studies in various animal models has yielded negative results over a wide range of parameters.

2.6. Delayed neurotoxicity

Evidence for neurotoxicity and neuropathology has been sought in a number of studies using nerve agents. Since it is widely known that certain OP pesticides produce a delayed neuropathy, nerve agents have been tested for the same phenomenon: OP-induced delayed neurotoxicity (OPIDN), using the white leghorn hen, an animal model sensitive to delayed neurotoxicity. Wide ranging doses of nerve agents were employed in 11 studies: no neuropathy or neurotoxic esterase inhibition was seen with VX or with G-agents at doses < 11 LD_{50} in animals treated with atropine and pralidoxime chloride to ensure survival [15]. According to Sidell and Hurst [16]: 'the syndrome (delayed neurotoxicity) has not been noted in the handful of humans severely exposed to nerve agents or in the hundreds of humans with mild to moderate effects from nerve agents.' Thus, in this respect, nerve agents differ significantly from some OP pesticides.

OP pesticides and OP nerve agents have much in common, but differ significantly in their acute toxicity and physical properties. Some of their targets and some effects also differ. Nerve agents do not produce OPIDN at concentrations achievable with vapor exposure. Therefore, extrapolation from pesticide effects as they relate to neurotoxicity may be inappropriate.

3. Conclusion

Controlled studies of human exposures, reports of accidental exposures, and animal studies collectively indicate that exposures to low level OP nerve agents do not produce chronic illness. None of the nerve agents have been shown to be mutagenic or carcinogenic. Nerve degeneration is considered an unlik-

ely outcome either from acute or long-term exposure to any nerve agent. Long-term EEG changes are known, but they are of uncertain significance. The majority of the data supports the conclusion that there are no observable long-term adverse health effects following exposure to asymptomatic levels of nerve agents. In fact, the final recommendation of the Department of Health and Human Services, published in the Federal Register on March 15, 1988 stated: 'Questions related to the nerve agents proved relatively easy to resolve. The information bases are fairly complete, and there appears to be little risk either of adverse health effects from long-term exposure to low doses or of delayed health effects from acute exposures.'

Because there are gaps of knowledge regarding the potential health consequences of exposure to low (asymptomatic) concentrations of nerve agents, the Department of Veteran Affairs in cooperation with the Departments of Defense and Health and Human Services has initiated research projects in this area. These research projects are being conducted in laboratories almost exclusively outside the U.S. Government by both U.S. and international academic and industrial research organizations. It is hoped that a more complete picture of the health consequences of low-level OP nerve agent exposure will result from these efforts over the next 3 years.

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Cholinergic excitation induces activity-dependent electrophysiological and transcriptional responses in hippocampal slices

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Abstract — To explore the correlations between short-term neurophysiological events initiated by over-activation of acetylcholine receptors, and long-lasting changes in brain function, we combined electrophysiology and PCR-based measurements in hippocampal slices or live mice subjected to stress or drug-induced cholinergic activation. Our findings reveal a common cascade of neuronal events resulting in delayed suppression of cholinergic transmission. (©Elsevier, Paris)

Résumé — Réponses électrophysiologiques et transcriptionnelles induites par l'activation cholinergique dans l'hippocampe. Pour explorer les corrélations entre des événements électrophysiologiques à court terme, initiés par une stimulation des récepteurs cholinergiques, et des modifications à long terme des fonctions cérébrales, nous avons utilisé des mesures électrophysiologiques et des mesures d'expression de gènes basées sur la PCR. Les modèles étaient des tranches perfusées d'hippocampe ou des souris vivantes exposées à des drogues. Nos résultats indiquent que le stress psychologique, tout comme les drogues anticholinergiques, produisent une cascade d'événements identiques, entraînant la suppression différée de la transmission cholinergique. (©Elsevier, Paris)

acetylcholinesterase inhibitors / hippocampal slices / molecular neurophysiology / muscarinic responses

1. Introduction

The importance of acetylcholine (ACh) in higher mammalian cognitive functions and behavioral responses is well documented [4, 8, 9]. Brief abnormalities in cholinergic transmission are sometimes associated with long-term changes in memory, mood and behavior; prolonged changes are notably associated with several neurologic diseases (e.g., Alzheimer's dementia). However, the molecular mechanisms which lead from transiently disturbed cholinergic transmission to long-lasting alterations in brain functions are not well understood. For example, acute psychological stress entails immediate activation of central nervous system (CNS) cholinergic pathways which results in excessive ACh release [16]. Likewise, experimental, therapeutic or occupational exposure to inhibitors of the ACh hydrolyzing enzyme, acetylcholinesterase (AChE), induces instantaneous elevation of ACh levels due to suppressed ACh hydrolysis. Thus, both these conditions elicit short-term hyper-activation of cholinergic receptors and also cause delayed and persistent abnormalities in CNS structure and function [3, 26]. In line with these earlier reports, we have recently observed both rapid and long-lasting changes in the expression of key cholinergic proteins in the cortex and hippocampus of mice, following either acute stress or exposure to cholinesterase inhibitors [17]. The transcriptional responses which initiated these changes were prevented by Na^+ channel blockers as well as by intracellular Ca^{2+} chelators, suggesting that they were preceded by modulation in neuronal electrical activity. Receptor-ligand interactions may therefore activate signal transduction pathways, modulating the extent and duration of the cascade of events which connect hyperactivation to the long-term changes.

To explore this hypothesis, we studied the dose and time-dependent electrophysiological responses to various AChE inhibitors in vitro and investigated the potential role of these events in modifying CNS gene expression. To this end, we subjected hippocampal slices to controlled levels of anticholinesterases and characterized the resultant neurophysiological responses as well as the corresponding changes in mRNA transcripts of key cholinergic genes. Our findings reveal electrophysiological and molecular neuronal responses which are mediated by muscarinic receptors. These rapid, yet long-lasting changes may lead to permanent neurophysiological impairments.

Abbreviations: ACh, acetylcholine; AChE, acetylcholinesterase; CCh, carbamylcholine; CNS, central nervous system; PCR, polymerase chain reaction; RT-PCR, reverse transcriptase PCR.

2. Materials and methods

2.1. Brain slices

Mice were anesthetized and killed by decapitation, with brains quickly removed into ice-cold artificial cerebrospinal fluid (aCSF) containing (in mM): NaCl, 124; KCl, 3; $MgSO_4$, 2; NaH_2PO_4, 1.25; $NaHCO_3$, 26; D-glucose, 10; and $CaCl_2$, 2 (pH 7.4), saturated with 95% O_2, 5% CO_2. Corticohippocampal slices (400 μm thick) were cut in the sagittal plane using a vibratome (Vibroslice, Campden Instruments, Loughborough, UK), and were placed in a humidified holding chamber, continuously perfused with oxygenated aCSF at 37 °C. Drugs were added to the aCSF at the concentrations noted in the text.

2.2. Forced swim stress

Forced swim stress protocol was adapted for use in adult FVB/N mice as described [13]. Briefly, naive animals were subjected to two 4-min swim sessions separated by a 4-min interval in a 60 × 60 cm water bath (12 cm deep) at 21 ± 1 °C.

2.3. Electrophysiological recordings

Electrophysiological recordings were made using standard techniques [11, 12]. Extracellular recording electrodes were filled with a solution containing (in mM): NaCl, 140; KCl, 3; $MgCl_2$, 2; D-glucose, 10; $CaCl_2$, 2, and HEPES, 10 (pH 7.4). Electrodes (tip diameter 4–6 μm) were placed in the CA1 cell-body layer. Synaptic responses were evoked by delivering 20 μs stimuli through bipolar tungsten electrodes. Electrophysiological data were digitized on-line using Axotape (Axon Instruments, Inc.).

2.4. RNA extraction

Cerebral cortex and hippocampus from mouse brains were quickly dissected and placed in liquid nitrogen. Tissue was homogenized using a Thomas glass homogenizer and RNA extracted by the guanidinium thiocyanate RNA-Clean method (AGS, Heidelberg,Germany). For kinetic follow up of RT-PCR, PCR products were sampled every third cycle as detailed elsewhere [17]. Densitometric analysis was performed on ethidium-bromide stained agarose gels using the Adobe Photoshop software.

2.5. AChE activity measurements

Acetylthiocholine (ATCh) hydrolysis levels in brain homogenates were determined spectrophotometrically in triplicate and were expressed as nanomol ATCh per min per mg protein. The rate of values representing spontaneous ATCh hydrolysis was determined and subtracted.

3. Results

The time course and drug dependence of transcriptional responses associated with cholinergic activation were detected by reverse-transcriptase PCR

Table I. RT-PCR density from stressed mice and slices exposed to anti-AChEs[a].

Gene	*Treatment*	*Post insult time (min)*	*mRNA level (% of control)*	*PCR cycle of first appearance (mean)*	*n*	*P (t-test) (compared to control)*
c-Fos	Control	10	100 ± 33	28 ± 1.6	7	
	Anti-ChEs	10	380 ± 275	25 ± 1.7	4	0.003
	Stress	10	401 ± 151	25 ± 1.5	5	0.011
ChAT	Control	30	100 ± 43	28 ± 2.0	15	
	Anti-ChEs	30	30 ± 1933	31 ± 1.6	5	0.019
	Stress	30	19 ± 9	32 ± 1.3	6	0.027
VAChT	Control	30	100 ± 37	30 ± 0.0	2	
	Anti-ChEs	30	46 ± 0	33 ± 0.0	2	
	Stress	30	37 ± 8	33 ± 0.0	2	
AChE	Control	30	100 ± 4	31 ± 1.7	3	
	Anti-ChEs	30	877 ± 97	22 ± 1.7	2	0.001
	Stress	30	275 ± 93	24 ± 0.0	5	0.029
Synaptophysin	Control	30	100 ± 5	25 ± 1.5	13	
	Anti-ChEs	30	87 ± 8	25 ± 1.6	5	n.s.
	Stress	30	87 ± 15	25 ± 1.6	6	n.s.

[a]RT-PCR was performed on RNA extracted from cortico-hippocampal slices incubated with 1 μM DFP or 1 mM pyridostigmine or from cortices of control or stressed mice. Control cortices were indistinguishable from mouse slice values. Products were sampled every third PCR cycle, electrophoresed, and stained with ethidium bromide. Presented are the relative band intensities as percent of control levels ± S.D. Values were calculated from three densitometric analyses of first visible PCR cycle of appearance. *n* equals the number of animals in each experimental group. *P* values were calculated using Student's *t*-test, for each treatment compared to its own control gorup.

kinetics (RT-PCR). Earlier cycle of product first appearance in this test reflects higher levels of the corresponding mRNA; within the linear phase of product accumulation, each cycle represents a two-fold increase in mRNA amounts [17]. *Table I* summarizes these RT-PCR analyses as referring to the mRNA level of several key genes under experimental conditions (stress in vivo or anticholinesterase exposure in slices) compared to control in vivo or slice conditions. In agreement with the definition of c-fos as an early immediate gene [14], we observed four-fold increases in c-fos mRNA levels as soon as 10 min post insult, the insult being either stress or exposure to an anti-AChE. This increase potentially elevates the amount of the resultant c-fos transcription factor, and thus changes the transcriptional efficacy of promoters. The choline acetyl transferase promoter, which directs expression of the ACh synthesizing enzyme, ChAT [1], and the co-regulated vesicular acetylcholine transporter, VAChT [5] and the AChE promoter [2, 19] both belong to this class of c-fos responders. This suggested that ACh availability might be subject to c-fos regulation, but we could not predict in what direction, since c-fos may either enhance or suppress gene expression depending on its modular interactions with various Jun proteins.

RT-PCR analyses demonstrated 2–3- and 3–5-fold reductions for ChAT and VAChT mRNAs under anticholinesterase exposure or stress, respectively. In contrast, AChE mRNA revealed an opposite trend of nine- and three-fold increases for anti-cholinesterase and stress exposure, respectively. Similar analyses demonstrated no significant change for synaptophysin mRNA, indicating a selectivity of the observed modulations for cholinergic elements (*table I*). Intraperitoneal injection of a variety of AChE inhibitors induced changes in brain mRNA levels which were parallel to those observed in brain slices (data not shown). These findings suggested that the c-fos mediated transcriptional responses reflected a consensus consequence to the CNS increase in ACh levels following blockade of AChE's catalytic activity and demonstrated the validity of the in vitro brain slices system for parallel electrophysiological and molecular analyses of the direct effects of AChE inhibitors (see Kaufer et al. [18] for further details of this model system).

To explore the physiological consequences associated with the above transcriptional modulations, we recorded evoked potentials in the pyramidal cell layer of the hippocampal CA1 region. Brief stimulus to the stratum oriens, containing septo-hippocampal afferent cholinergic fibers [6, 25, 27], under normal conditions evoked a typical synaptic potential and population spike; amplitudes of both were proportional to stimulus intensity. Exposure of slices to pyridostigmine resulted in a dose-dependent increase in population spike amplitude (*figure 1A,B*). Increased spike amplitudes were measured over a wide range of stimulus intensities, without affecting the maximal amplitude (*figure 1B*). This increase in population spike amplitude plateaued at pyridostigmine doses above 10 μM, which paralleled exactly the extent of AChE inhibition by pyridostigmine in cortex homogenates: catalytic activity measurements over the same ranges of pyridostigmine concentrations revealed inhibition of 25% to > 95% of AChE activity, with plateau over 10 μM (*figure 1B,* inset and Friedman et al. [13]). These findings support the notion that the electrophysiological effects of pyridostigmine were associated with decreased AChE catalytic activity. That the observed response to this AChE inhibitor was indeed due to increased concentrations of unhydrolyzed, released ACh, was further supported by the dose-dependent increases in population spike amplitude with increasing concentrations of the cholinergic agonist carbamylcholine (CCh) (*figure 1C*). Similar responses were observed using other AChE-inhibitors, including physostigmine (10 μM) (*figure 1D*) or diisopropyl fluorophosphate (DFP) (1 μM) ([18] and data not shown). In all cases, the augmented response was reversed when a muscarinic antagonist (atropine, 5 μM) was added to the aCSF (*figure 1D*).

Acetylcholine is known to modify membrane properties of neurons via slow second-messenger dependent processes [7, 21, 23]. Thus, prevention of ACh hydrolysis in the synaptic cleft is expected to alter incoming stimuli in a frequency-dependent manner. Therefore, we tested the effects of anti-AChEs on responses to repeated stimulation. Under low stimulus intensities, evoked synaptic responses did not evoke population spikes either in normal solution or following pyridostigmine (10^{-4} M) application (*figure 2A*). However, under these low stimulation levels, pyridostigmine application resulted in a prominent facilitation of the second stimulus at inter-stimulus intervals of 30–200 ms (*figure 2A*). The expected duration of up to 200 ms for these paired-pulse facilitation responses under normal conditions characterizes afferent excitatory connections to the CA1 region [10, 20]. High doses of AChE inhibitors extended this duration to over 500 ms (data not shown and see Kaufer et al. [18]). With 10 repetitive stimuli at 40 Hz, physostigmine further induced a large and prolonged negative deflection in the extracellular recording (*figure 2B*), indication of influx of positive ions into nearby neurons.

The transcriptional responses following AChE inhibition preceded an increase in AChE activity (see [17]). Therefore, we expected that anticholinesterase-induced increased excitability will be followed

by a delayed phase of suppressed neuronal excitability. *Figure 2C* shows that exposure to physostigmine prompted, within 1 hr, increased population spike amplitudes. When we extended AChE inhibition to 3 h, the augmented synaptic response as well as action potentials were significantly muted. These observations demonstrated that AChE inhibitors mediate a transient, early phase of enhanced excitability that is followed by a secondary phase of suppressed neuronal activity. Both phases were prevented by the presence of atropine (*figure 1D* and see Kaufer et al. [17]), confirming their dependence on muscarinic receptors activation.

4. Discussion and conclusion

Using hippocampal mouse brain slices, we performed an in-depth analysis of the short-term changes in the evoked electrophysiological events following exposure to various anti-cholinesterases or ACh analogues. In parallel, we examined in these slices the levels of mRNA transcripts encoding key cholinergic elements, and compared the changes in these transcript levels to those occurring in vivo under acute exposure to anti-AChEs or psychological stress. Together, these experiments suggest a convergent pathway leading from immediate elevation in

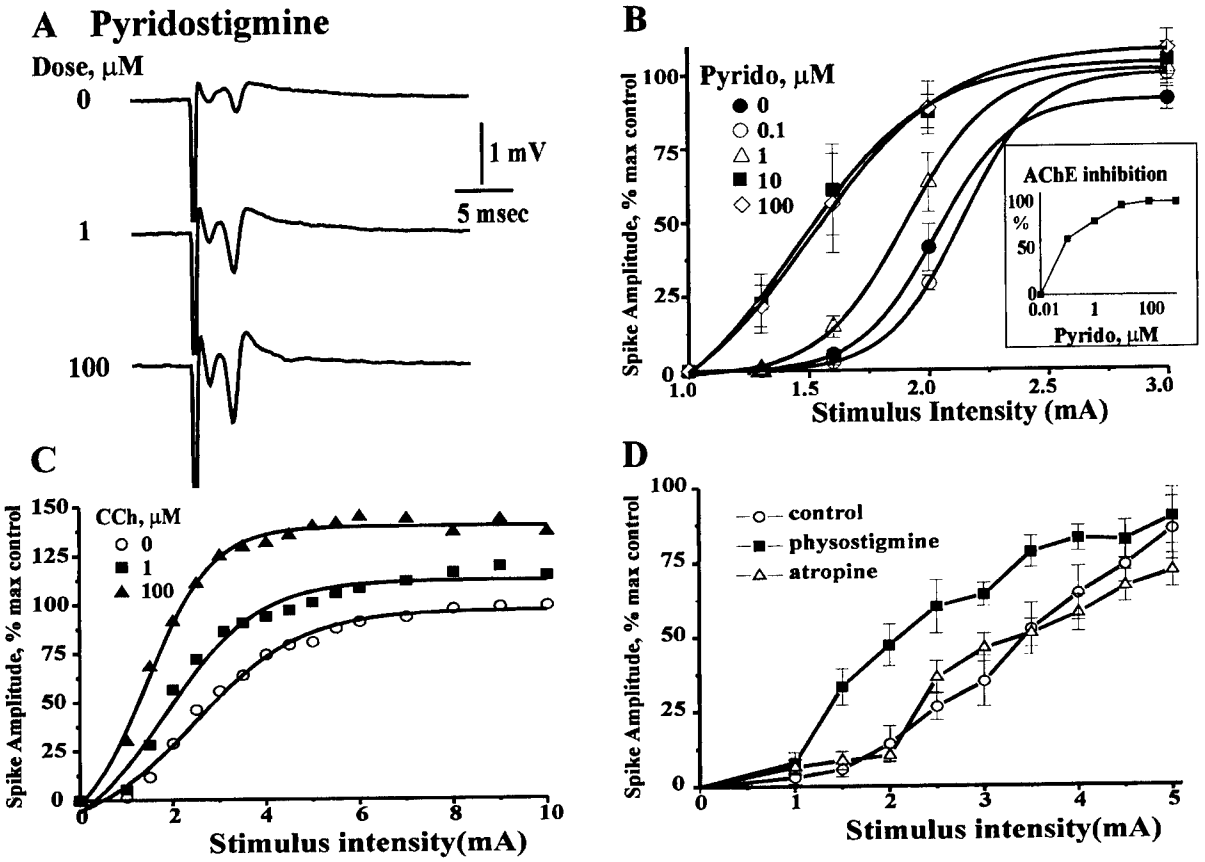


Figure 1. Muscarinic-dependent increases in neuronal excitability in sagittal hippocampal slices maintained in vitro. **A.** Dose-dependent increases in evoked population spikes. Recordings represent activities in hippocampal slices exposed to the noted doses of pyridostigmine for one hour. **B.** Spike amplitude increases under pyridostigmine. Spike amplitudes were measured under the noted stimulus intensities for pyridostigmine concentrations over the range of 0.1–100 μM. Inset, measurements of AChE activities in homogenates reveal inhibition in the same concentration range of pyridostigmine. **C.** Response to ACh analogue. Dose-dependent increases in population spike amplitudes are presented for slices exposed to the noted concentrations of the cholinergic agonist carbamylcholine (CCh) **D.** Muscarinic-dependence of anti-AChE effect. The increase in evoked response observed following 10 μM physostigmine (filled squares), as compared to control conditions (open circles) was reversible upon the administration of the muscarinic antagonist, atropine (open triangles).

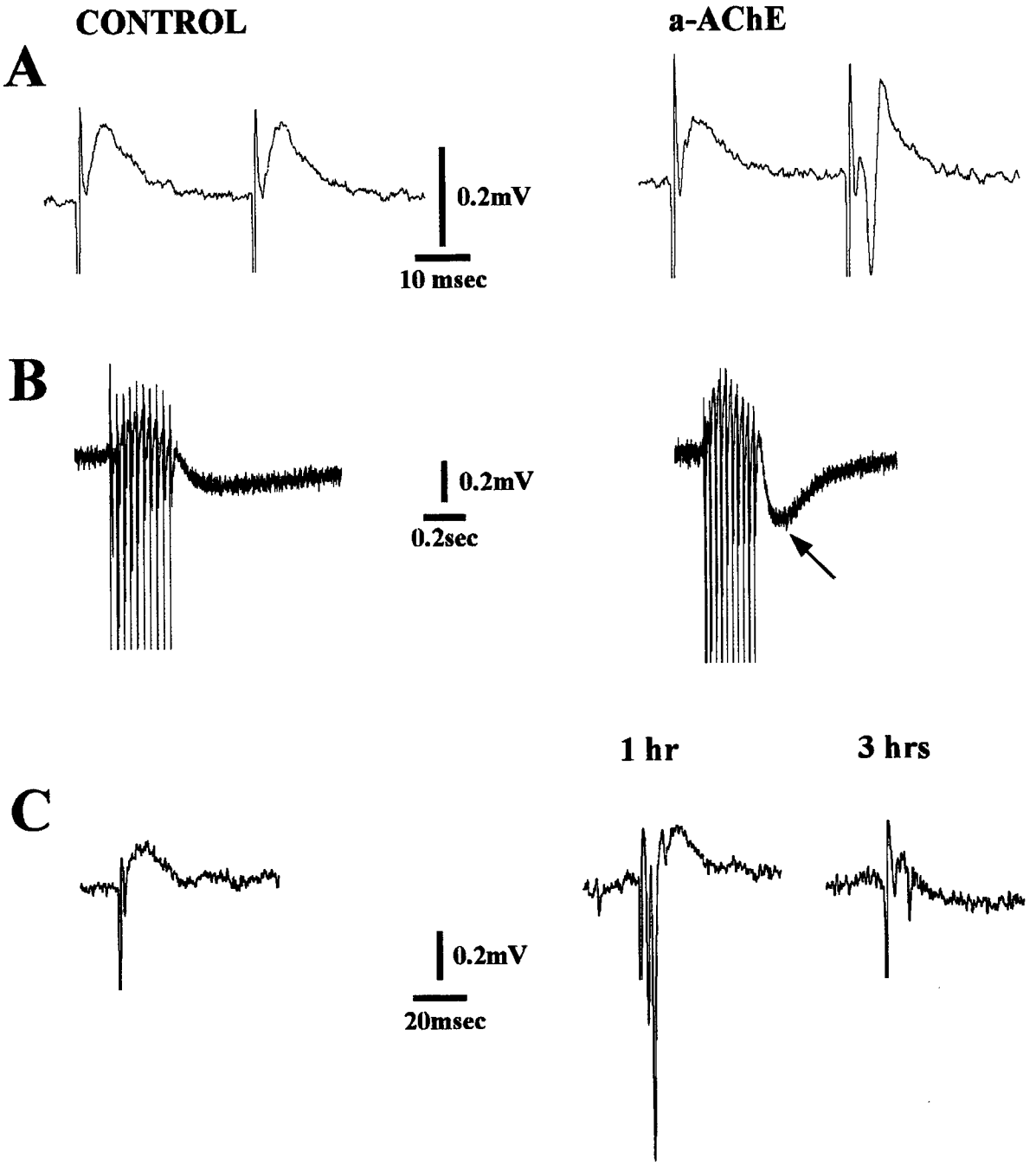


Figure 2. Effect of anti-cholinesterases is time-and stimulus-dependent. **A.** Paired pulse responses to low-intensity stimuli. Normal responses were recorded at 30 ms interval, 0.3 mA, under control conditions or following 1 h perfusion with pyridostigmine. Note the lack of facilitation of the synaptic currents under control conditions, and the prominent facilitation following pyridostigmine (a-AChE), observed as stimulus triggered population spike. Plotted traces are average of six consecutive stimuli. **B.** Modulated response to repetitive stimulation. Ten repetitive stimuli of the stratum-oriens at 40 Hz evoked responses which were first facilitated, then depressed and followed by a slow negative potential. Following 1 h of exposure to 10 μM physostigmine (a-AChE), population spike and facilitation increased, depression decreased, and the following slow current was prominently enhanced in amplitude and duration (arrow). **C.** Time dependent changes in population spike amplitudes. Increases were observed 1 h following exposure but not at 3 h (see text).

ACh levels to delayed suppression of cholinergic neurotransmission.

Analysis of our electrophysiological measurements reveals several intriguing points. First, we noted that both the increase in population spike amplitudes and the inhibition of AChE's catalytic activity reached plateau levels at similar pyridostigmine concentrations (around 10^{-5} M). This strongly supports the notion that the inhibition of AChE-mediated ACh hydrolysis was the cause of these physiological responses. Moreover, a non-hydrolyzable ACh analogue caused similar augmentation in excitability, and the augmented synaptic responses were reversible upon atropine administration. Therefore, we conclude that released ACh enhances CA1 output.

It is noteworthy that a difference between the effect of cholinesterase inhibitors (e.g., pyridostigmine or physostigmine) and that of the ACh analogue, CCh, on population spike amplitudes was observed. Anticholinesterases increased spike amplitudes without exceeding the maximal amplitude under control conditions, whereas CCh elevated spike amplitudes in a dose-dependent manner to levels above those measured for controls. This suggests that perfused CCh, but not stimulus-dependent released ACh, recruits more neurons to fire action potentials in response to the maximal employed stimulus. Cutting the long septo-hippocampal afferents in the preparation of our slices may have decreased cholinergic input on CA1 neurons and be the basis for this observed difference. In support of this, preliminary experiments showed hippocampal slices cut on the coronal plane to respond poorly to anti-AChEs, compared to sagittal slices, with no difference between preparations in their response to CCh.

Different mechanisms have been proposed for the action of ACh on CA1 pyramidal neurons. The observed enhancement of stimulus evoked response could be the result of the following cholinergic-dependent mechanisms: 1) increased glutamate release [15]; 2) increased post-synaptic response to the released glutamate [22]; 3) modulation of local synaptic inhibition [24]; and/or 4) decreased threshold of post-synaptic action potentials. This last option is particularly attractive, since under low-intensity stimuli, released ACh did not significantly alter the synaptic current. Moreover, the stimulus-dependent enhancement of evoked responses following exposure to anti-AChEs probably reflects the slow, second messenger-dependent actions of ACh on membrane conductances via activation of muscarinic receptors (see for example [7, 21].

Reduced firing threshold following anti-AChE exposure is often associated with additional spikes as well as with late inward currents following repetitive stimulation (*figure 2B*). This indicates prolonged depolarization, which is expected to be associated with increased Ca^{2+} influx through NMDA- and voltage-dependent channels. Pharmacological manipulation demonstrated that changes in transcription following anti-AChEs are preventible by the intracellular Ca^{2+} chelator, BAPTA-AM. This points to the importance of Ca^{2+} as an intracellular second messenger, bridging rapid modulaions in electrical activity to long-term changes in the structure and function of the cholinergic synapse.

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Novel m1 muscarinic agonists in treatment and delaying the progression of Alzheimer's disease: An unifying hypothesis

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Abstract — M1 selective agonists from the AF series (e.g. AF102B, AF150(S)), via m1 muscarinic receptors, activate distinct signal transductions, enhance amyloid precursors proteins secretion from transfected cells and primary cell cultures, show neurotrophic effects and are beneficial in a variety of animal models for Alzheimer's disease. Such m1 agonists may be effective in the treatment and therapy of Alzheimer's disease. (©Elsevier, Paris)

Résumé — Les agonistes sélectifs de type M1 appartenant à la série AF (par exemple AF102B, AF150(S)) activent de manière sélective certaines routes de transduction du signal via les récepteurs muscariniques de type m1, augmentent la sécrétion de la protéine amyloide par les cellules transfectées et les cultures primaires de cellules, produisent des effets neurotrophiques et se montrent bénéfiques pour une variété de modèles animaux de la maladie d'Alzheimer. De tels agonistes peuvent être efficaces pour traiter et guérir la maladie d'Alzheimer. (©Elsevier, Paris).

AF series / m1 agonists / Alzheimer's disease

1. Introduction

An m1 selective muscarinic agonist may be effective in treatment of Alzheimer's disease (AD), regardless of the extent of degeneration of presynaptic cholinergic projections to the frontal cortex and hippocampus (for review see [4]).

Mismetabolism of amyloid precursor proteins (APP) may induce AD via increased formation of β-amyloids (Aβ). However, activation of the non-amyloidogenic pathway may be beneficial in the treatment of AD, since upon activation of this pathway Aβ formation is reduced [3, 17]. This non-amyloidogenic pathway can be enhanced via activation of m1 mAChR [3, 9, 10, 17, 18]. The cholinergic hypofunction in AD can be tied with formation of Aβ and these can impair the coupling of m1 muscarinic receptors (m1 mAChR) with G-proteins [15]. This uncoupling leads to decreased signal transductions, impairments in cognition, a reduction in levels of trophic secreted APP, generation of more neurotoxic β-amyloids and a further decrease in acetylcholine release. This 'vicious cycle' may be prevented in principle by m1 selective agonists. Such m1 agonists are found in the AF series (e.g., project drugs: AF102B, AF150(S)) [4, 5]. The following text is an overview of recent findings with these compounds.

2. Select signal transduction by the AF series

The AF series are functionally selective m1 agonists (e.g., on $[Ca^{2+}]_i$ elevation, phosphoinositides hydrolysis or arachidonic acid release), yet antagonists in elevating cAMP levels in cell cultures transfected with m1 mAChR [5]. These m1 agonists may activate distinct sets of G-proteins and extend drug selectivity beyond the ligand recognition site. This property might be beneficial clinically due to altered signal transductions in AD. In this context, both mRNA for Gsα (which mediates activation of adenylate cyclase and enhanced release of cAMP) and m1 mAChR were reported to be elevated in hippocampi of AD patients [13, 14]. We propose that AD treatement by m1 agonists should not stimulate adenylyl cyclase via m1 mAChR but should activate PI hydrolysis [5, 7]. In the event that this hypothesis is valid, concerns over the long-term use of highly efficacious muscarinic agonists, which activate all m1 mAChR-mediated signal transductions, including m1 mAChR coupling with Gs, leading eventually to an increased cAMP level in the brain, will be raised. The same caution should be applied to acetylcholinesterase (AChE) inhibitors for the long-term treatment of AD since in this case elevated acetylcholine levels, due to AChE inhibition, can again enhance brain cAMP levels.

3. Processing of APP and elevation of secreted APPs

We used PC12M1 cells, which express both kinase tyrosine receptors and m1 mAChR in order to investigate the effects of m1 agonists on levels of secreted APPs and the role of various neurotrophines on m1 mAChR-mediated effects. We found that m1 selective muscarinic agonists, such as AF102B [9] and AF150(S) (unpublished results) increase APPs secretion via the non-amyloidogenic pathway, without desensitizing the m1 mAChR. In addition, nerve growth factor (NGF) augments muscarinic-induced APPs release and reduces membrane-associated APP [10]. This activation is also augmented by other neurotrophins such as basic fibroblast growth factor (bFGF). Some signal-transduction pathways activated by muscarinic agonists and by growth factors, respectively, may cross-react, resulting in an augmentation of the response at their convergence point. The muscarinic-induced APPs secretion is mediated by several transduction pathways which operate in parallel and involve PKC-dependent and *ras*-dependent cascades. Additional stimulation of m1 mAChR by muscarinic agonists in these cells results in activation of mitogen activated protein kinases (MAPK) which is *ras*-dependent and PKC-independent and is synergistically enhanced by neurotrophins [11, 12].

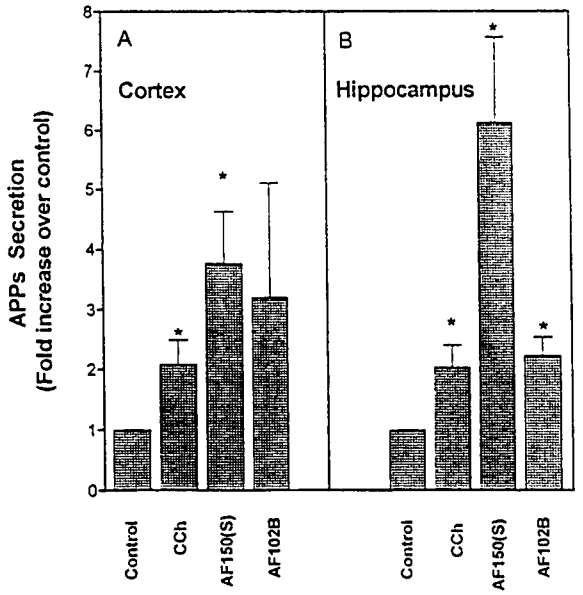


Figure 1. APPs secretion from rat cortical (**A**) and hippocampal (**B**) primary cell cultures by 100 μM carbachol (CCh), AF102B and AF150(S). Data were normalized according to control values which were determined in absence of muscarinic agonists. Data represent 2–6 individual experiments and are presented as means ± SEM. $*P < 0.05$ relative to control evaluated by paired Student *t*-test.

4. Effect of cholinergic stimulation on APPs level in embryonic rat brain – primary cell cultures

Primary cell cultures of hippocampus and cerebral cortex derived from 18–19 days old fetuses were used in this study. The effect of 100 μM of carbachol, a non-selective muscarinic agonist and two m1 functionally selective muscarinic agonists AF102B and AF150(S) on APPs secretion was evaluated. Blockade of their effects were tested in presence of 10 μM pirenzepine, an m1 selective antagonist or 50 μM gallamine, an m2 antagonist. We found that these muscarinic agonists significantly increased (2–6-fold over control) APPs secretion after 1 h of incubation (*figure 1*). These effects were completely blocked by pirenzepine, indicating that activation of m1 mAChR subtype may be responsible for the elevated APPs secretion. Notably, these data corroborate our results from rat cortical brain slices [18].

5. Neurotrophic and neuroprotective effects

The AF series induce neurotrophic-like effects, which are synergistic with a variety of neurotrophines (NGF [8]), bFGF, epidermal growth factor [11]) and can promote survival of cultured primary CNS neurons [1]. Like in case of the non-amyloidogenic APP processing pathways, these m1 agonists promote and exert neurotrophic activities in conjunction with some neurotrophine-dependent and -independent signal(s) [11].

6. Effects of the AF series in animal models for AD

AF102B and AF150(S) restored memory and learning deficits in a variety of animal models that mimic cholinergic deficits in AD, without producing adverse central and peripheral side-effects at effective doses and showing a relatively wide-safety margin (for AF150(S) see *table I*; for other compounds from this series see [4, 5]). Notably, AF150(S) restored working memory impairments to normal in apolipoprotein E-deficient mice [6]. Furthermore, this cognitive improvement was associated with a parallel recovery of brain cholinergic markers (choline acetyltransferase (ChAT), AChE and m1 mAChR) [6], as well as a reduction of tau protein hyperphosphorylation [16]. Thus, AF150(S) emerges as a promising candidate drug for the treatment of AD.

Table I. AF150(S), a compilation of pharmacodynamic and behavioral profiles.

	Dose (mg/kg, p.o.)	*Effects (mAChR subtype mediated)*
	Peripheral nervous system effects [2]	
Redness around mouth	50	(?)
Salivation	100	(M1, M3)
Chromodacryorrhea	200	(M3?)
Diarrhea	≥ 500	no effect (M3)
	Central nervous system effects	
Sedation	200	(?)
Tremors	500	(M2, M4)
Hypothermia	500	(M2)
Open field	1–5	no effect
	Memory and learning	
Social memory (naive rats)[a]	1, 5	positive effect
AF64A-rats [2]		
Passive avoidance	0.3, 0.5, 1	positive effect
MWM-RM	0.5, 1, 5	positive effects
MWM-WM	0.2 (chronic)	tendency for positive effects
RAM	0.5, 5	no effects
	1	positive effects
Social memory[a]	1	positive effects
Ischemia-rats[a]		
MWM (WM)	0.5 (chronic)	positive effects
Apolipoprotein E-deficient mice [6, 17]		
MWM (WM)	0.5 (chronic)	positive effects on MWM-WM, ChAT,
Cholinergic markers		AChE, m1 mAChR, overphosphorylation
Tau phosphorylation		of *Tau*
Pigeons [19]		
Scopolamine (0.03 mg/kg, i.p.) induced deficits in DMTS	4 (i.p.)	positive effects

[a]Brandeis et al., unpublished results.
RAM, eight-armed radial maze; DMTS, delayed matching-to-sample; i.p., intraperitoneal; p.o., oral; MWM, Morris water maze; RM, reference memory; WM, working memory.

7. Conclusions

The m1 agonists from the AF series operate by several mechanisms acting in concert to positively affect cholinergic neurotransmission, neuronal survival and, ultimately, restore and preserve mnemonic processes in a variety of animal models which mimic the cholinergic hypofunction in AD. Apart from directly boosting the cholinergic signal, these novel compounds have the added advantage of positively acting, via m1 mAChR receptors, on other target systems which are central to the pathogenesis of several neurodegenerative diseases, including AD. For example, the three hallmarks of AD, cholinergic deficiency, Aβ and hyperphosphorylated *tau* proteins have been shown to be potential targets for these compounds (for review see [4]). AF150(S) and AF102B have the potential of reducing the formation of Aβ and preventing Aβ-induced neurotoxicity. Notably, AF150(S) and AF102B show neurotrophic effects and increase secretion of APPs which are both neuroprotective and neurotrophic. This may be important in compensating for possible dysfunctions of neurotrophines in AD. Due to their effects on *tau* dephosphorylation, such m1 agonists may impair paired helical filaments formation [16, 20]. Finally, due to the positive role of m1 mAChR on most of the identified 'villains' and risk factors in AD, including apoptosis (unpublished results), such m1 agonists may represent a unique therapeutic strategy in AD. Long term use of m1 agonists in early stage of AD patients and/or other populations at risk are needed to test this unifying hypothesis both in treatment and therapy of AD.

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Molecular interactions of acetylcholinesterase with senile plaques

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Abstract — Acetylcholinesterase (AChE) present in Alzheimer plaques is resistant to low pH, anti-ChE inhibitors and high substrate concentrations in comparison with the free enzyme. Kinetic and pharmacological studies of AChE-amyloid complexes indicate that steric hindrance by the amyloid over the gorge and the peripheral site of AChE is responsible for these effects. (©Elsevier, Paris)

Résumé — Interactions moléculaires de l'acetylcholinestérase avec les plaques séniles. L'acétylcholinestérase (AChE), présente dans les plaques séniles de la maladie d'Alzheimer, est résistante au bas pH, aux inhibiteurs et aux hautes concentrations de substrat. Les études cinétiques et pharmacologiques des fibres du complexe amyloide-AChE indiquent que l'encombrement stérique de l'amyloïde au niveau de la gorge et du site periphérique de l'AChE est responsable de ces effets. (©Elsevier, Paris)

AChE / amyloid plaques / AChE-Aβ-amyloid fibril complexes / Alzheimer's disease

1. Introduction

Although Alzheimer's disease (AD) is a complex disorder with mutations in many genes accounting for 15–20% of AD patients, the large majority of AD cases occur sporadically [14]. There is therefore a need to search for the mechanisms responsible for the progressive cognitive decline observed in these cases. One of the most relevant neuropathological characteristics of AD brain is the senile plaques which are formed by a core of amyloid-β-peptide (Aβ) fibrils. Numerous other proteins have been found associated with amyloid deposits, including apolipoprotein E, α_1-antichymotrypsin, heparan sulfate proteoglycans and acetylcholinesterase (AChE) among others [8, 13]. Most of the cortical AChE activity present in Alzheimers brain is predominantly associated with the amyloid core of senile plaques rather than with the neuritic component found at the periphery [9, 17]. Interestingly, histochemical studies indicate that the AChE associated with senile plaques differs enzymatically from the esterase associated with normal fibers and neurons in several respects [5].

A number of studies with synthetic Aβ in vitro have shown that this peptide aggregates and forms amyloid fibrils similar to the filaments found in the brains of AD patients [15]. The substitution of Glu22→Gln, found in hereditary cerebral hemorrhage with amyloidosis of the Dutch type, yields a peptide with increased ability to form amyloid fibrils [10]. On the other hand, the single mutation Val18→Ala induces a significant increment of the α-helical content of Aβ, and dramatically diminishes fibrillogenesis [16]. During the past years our laboratory has shown that AChE promotes the assembly of $A\beta_{1-40}$ peptide into amyloid fibrils, and that a hydrophobic environment close to the peripheral binding site of the enzyme is likely to be involved in this process [4, 7, 12]. Recently we found that stable AChE-Aβ complexes are formed during the growth of amyloid fibrils induced by AChE [1, 2, 11]. Here we examine the kinetic and pharmacological properties of AChE bound to amyloid fibrils formed with wild-type $A\beta_{1-40}$ and two mutant peptides that differ in their fibrillogenic capacity: $A\beta_{Val18\rightarrow Ala}$ and $A\beta_{Glu22\rightarrow Gln}$.

2. Enzymatic properties of the AChE present in AChE-Aβ complexes

Figure 1A, D, G shows the Lineweaver-Burk plots (1/v versus 1/S) obtained for free AChE and for the AChE bound in the following fibrillar complexes: AChE-$A\beta_{1-40}$, AChE-$A\beta_{Val18\rightarrow Ala}$ and AChE-$A\beta_{Glu22\rightarrow Gln}$. It is clear that the kinetic parameters of the enzyme change, as the K_m and V_{max} values for AChE associated to amyloid were higher than those for the free enzyme in all three cases (see also *table I*). Comparison of the kinetic parameters given in *table I* shows that the K_m value for the AChE-$A\beta_{1-40}$ complex was eight-fold higher than that for the enzyme alone. Similarly, for the AChE-$A\beta_{Glu22\rightarrow Gln}$ complex, the K_m value was elevated nine-fold and for AChE-$A\beta_{Val18\rightarrow Ala}$, 28-fold. When kinetic studies were carried out under varying pH conditions, AChE associated to either the wild-type or the mutant Aβ peptides was more resistant to inhibition by low pH

Table I. Comparison of the kinetic parameters of acetylthiocholine hydrolysis obtained for the AChE associated with amyloid-β fibrils and the free enzyme.

Parameter	*AChE*	*Aβ1–40+AChE*	*AβVal-18→Ala+AChE*	*AβGlu-22→Gln+AChE*
K_m (mM)	0.097 ± 0.007	0.764 ± 0.025*	2.680 ± 0.021*	0.856 ± 0.082*
V_{max} (μmol/mL/min)	0.129 ± 0.015	0.222 ± 0.029*	0.495 ± 0.018*	0.237 ± 0.025**

The AChE used in these studies was the hydrophobic G_4 form affinity purified from bovine caudate nucleus on an acridine-Sepharose column, and the AChE associated with amyloid-β fibrils was obtained from the aggregation of the $A\beta_{1-40}$ peptide induced by AChE, as described previously [1, 2, 7]. Values represent means ± S.D. of 4–6 separate assays made in duplicate for the AChE and 3–5 for the complex Aβ+AChE. The K_m and V_{max} were determined from Lineweaver-Burk plots (1/V vs. 1/S), with 0.03–1.0 mM acetylthiocholine. * Significantly different from control with $P < 0.001$ by non-paired Student's *t*-test; **$P < 0.01$.

(Figure 1B, E, H). Similarly, AChE associated in AChE-Aβ complexes was more resistant to incubation under high substrate concentrations *(figure 1C, F, I)*.

3. Pharmacological studies of the AChE present in AChE-Aβ complexes

The AChE associated to amyloid also appears more resistant to inhibition by anti-ChE agents as observed with both active site inhibitors such as tacrine, edrophonium and BW284c51, and with peripheral anionic site blockers, such as propidium and gallamine *(table II)*. In almost all cases, a higher inhibitor concentration was required to obtain the same level of inhibition observed with the free enzyme. Overall, the $A\beta_{1-40}$ analog containing the single amino acid substitution Val18 to Ala, showed the largest difference with respect to the free enzyme, suggesting that it has a greater degree of interaction with AChE than the other Aβ peptides. Contrastingly, the complex AChE-Dutch $A\beta_{Glu22\rightarrow Gln}$ mutant was the least affected of all the complexes studied, and in some cases, particularly for gallamine, almost no difference was found in the IC_{50} values. In summary, for most of the anti-cholinesterases tested the inhibition of AChE associated to Aβ complexes ranked in the order: AChE-$A\beta_{Val18\rightarrow Ala}$ > AChE-$A\beta_{1-40}$ > AChE-$A\beta_{Glu22\rightarrow Gln}$ > AChE.

These results are consistent with the idea that the association of AChE with Aβ fibrils leads to changes in its enzymatic properties, in the absence of any pathological alteration of the enzyme.

4. Discussion and conclusion

We have studied the kinetic and pharmacological properties of AChE incorporated into amyloid fibrils formed from the wild-type $A\beta_{1-40}$ peptide as well as the mutants $A\beta_{Val18\rightarrow Ala}$ and $A\beta_{Glu22\rightarrow Gln}$, and compared those characteristics with the free enzyme. Our results indicate that independently of the type of AChE-Aβ complex formed, the behavior of the bound enzyme differs to that of the free enzyme, and is reminiscent of the properties observed for the AChE present in the amyloid deposits of Alzheimer's brain. In previous work, the finding that the AChE associated to senile plaques presented lower sensitivity to excess substrate and to different anti-cholinesterase agents as well as higher resistance to inactivation by low pH than the normal neuronal enzyme [5], led to the suggestion that the enzyme bound to amyloid could correspond to a different molecular form of AChE induced during the disease [8]. Partial support for this hypothesis came from studies with the embryonic form of brain AChE, which also presents kinetic and pharmacological changes with respect to the adult enzyme [3]. These changes are similar to those observed for AChE bound to amyloid deposits. However, it is clear from the studies reported here that the same enzyme in two different environments can undergo kinetic and pharmacological modifications, and that the bound state of the enzyme is sufficient to induce these changes.

A plausible explanation for the differences observed is related to a physical phenomenon, i.e., the location or occlusion of the enzyme enmeshed within a fibrillar environment, in such a way as to produce

Figure 1. Change in the kinetic parameters of the AChE associated with amyloid-β fibrils. First row: Lineweaver-Burk (double-reciprocal) plots of AChE activity free and associated with amyloid-β fibrils over a range of substrate (ATCh) concentrations. **A.** Complex with wild-type Aβ. **D.** Complex with $A\beta_{Val18\rightarrow Ala}$. **G.** Complex with $A\beta_{Glu22\rightarrow Gln}$. Second row: pH dependence of the AChE free and associated with amyloid-β fibrils. Optimal pH of AChE and complexes was determined over a pH range of 4.0–9.0 in phosphate buffer. **B.** Complex with wild-type Aβ. **E.** Complex with $A\beta_{Val18\rightarrow Ala}$. **H.** Complex with $A\beta_{Glu22\rightarrow Gln}$. Third row. Activity of AChE as a function of substrate concentration. The rate of hydrolysis is plotted as a function of substrate concentration on a Log acetylthiocholine concentration. The bell-shaped curves show that AChE and the Aβ+AChE complex are subject to excess substrate inhibition but the free AChE is inhibited at low ATCh concentrations and the Aβ+AChE complex at higher ATCh concentrations. **C.** Complex with wild-type Aβ. **F.** Complex with $A\beta_{Val18\rightarrow Ala}$. **I.** Complex with $A\beta_{Glu22\rightarrow Gln}$.



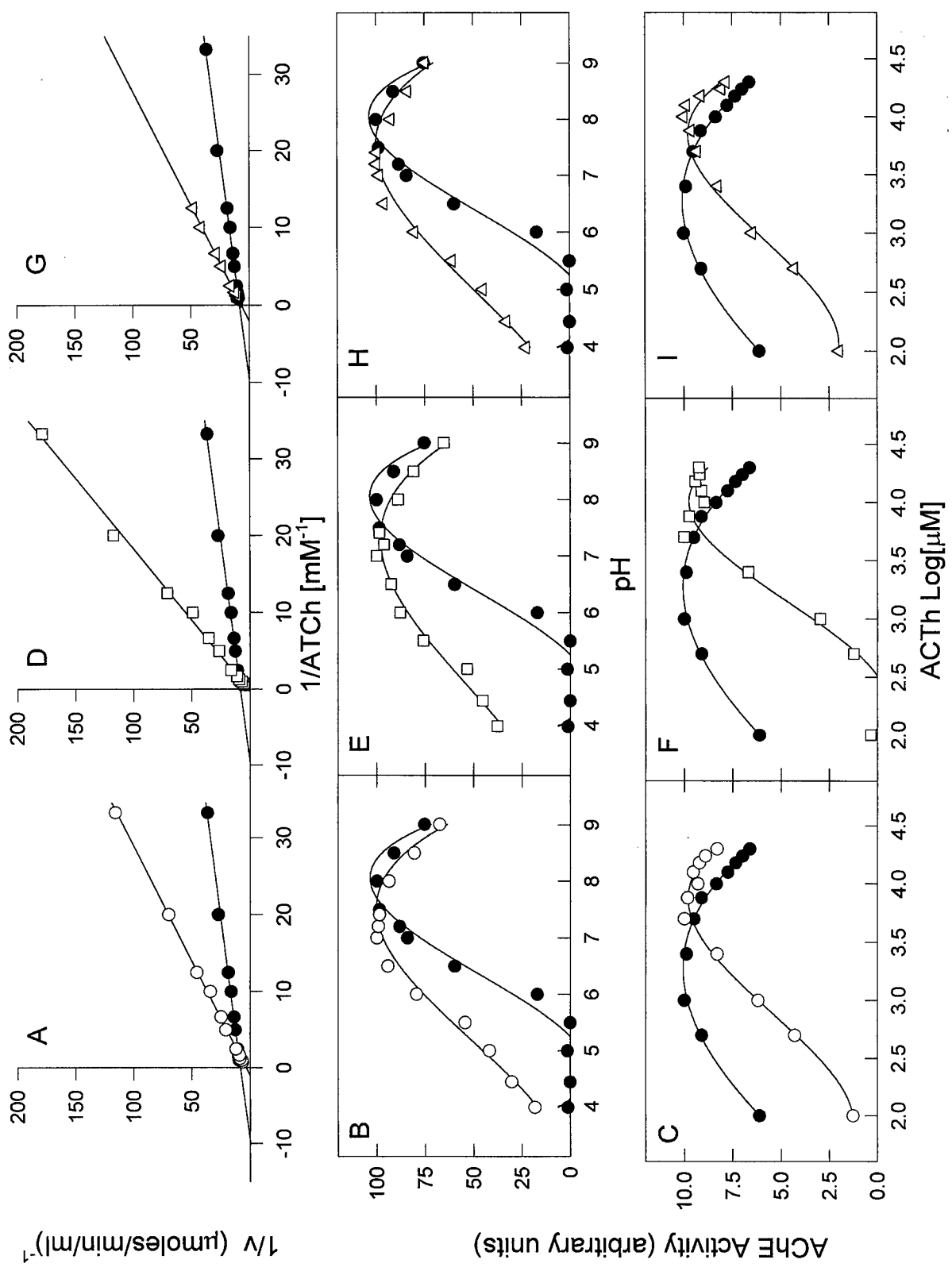

A
B
C
D
E
F
G
H
I
1/ATCh [mM^{-1}]
pH
ACTh Log[μM]
1/v (μmoles/min/ml)$^{-1}$
AChE Activity (arbitrary units)

Table II. Effects of anti-ChE agents (IC_{50}) on the AChE associated with amyloid-β fibrils and the free enzyme.

Inhibitor	*AChE*	*Aβ1–40+AChE*	*Aβ Val-18→Ala+AChE*	*AβGlu-22→Gln+AChE*
Active site IC_{50}				
Tacrine (10^{-9} M)	445.0 ± 17.6	1074.8 ± 73.3***	1422.6 ± 204.9***	625.8 ± 28.2***
Edrophonium (10^{-6} M)	5.36 ± 0.48	17.6 ± 4.6*	26.9 ± 0.2***	8.70 ± 0.92*
BW284c51 (10^{-9} M)	57.1 ± 1.3	116.9 ± 2.7***	193.2 ± 22.0***	91.5 ± 11.3**
Peripheral site IC_{50}				
Propidium (10^{-6} M)	34.6 ± 1.2	72.0 ± 4.8***	257.1 ± 24.0***	47.2 ± 2.1**
Gallamine (10^{-3} M)	8.76 ± 0.46	13.3 ± 0.45***	27.0 ± 2.5***	8.77 ± 0.97ns

For determination of IC_{50} values, samples were incubated with appropriate concentrations of the various inhibitors for 30 min at room temperature and the AChE activity was determined colorimetrically. Values represent means ± S.D. of 4–6 separate assay made in duplicate for the AChE and 3–5 for the complex Aβ+AChE. ns = not significant with $P > 0.05$ and significantly different from control with: *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ by non-paired Student's *t*-test.

some degree of steric hindrance over the catalytic gorge and the peripheral binding site of the enzyme. Under these conditions, accessibility to the corresponding sites increases only after a reasonable increase in the substrate or inhibitor concentrations thereby giving rise to the effects on AChE activity seen for each specific compound.

Acknowledgments

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Cholinergic modulation of extracellular ATP-induced cytoplasmic calcium concentrations in cochlear outer hair cells

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Abstract — Outer hair cells (OHC) of the mammalian cochlea modulate the inner hair cell (IHC) mechanoelectrical transduction of sound. They are contacted by synapsing efferent neurons from the CNS, their main efferent neurotransmitter being acetylcholine (ACh). OHC function and in particular their control of $[Ca^{2+}]_i$ is highly important and is modulated by ACh and also by other substances including extracellular (EC) ATP. OHC carry at their efferent synapse a not yet completely identified neuronal type of ionotropic ACh receptor (AChR), with an unusual pharmacology, which is, in vivo and in vitro, reversibly blocked by α-bungarotoxin (α-bgtx). The AChR mediates a fast influx of Ca^{2+} into OHC which, in turn, activates a closeby located outwardly-directed Ca^{2+}-dependent K^+-channel, thus shortly hyperpolarizing the cell. A cloned homomeric α9 nAChR mimicks the function and pharmacology of this receptor. We here report results from a study designed to observe only slower effects triggered by EC ATP and the ACh-AChR system. EC presence of ATP at OHC increases $[Ca^{2+}]_i$ by activating both P_{2x} and P_{2y} purinoceptors and also by indirect activation of OHC L-type Ca^{2+}-channels. The L-type channel activation is responsible for a large part of the $[Ca^{2+}]_i$ increase. Simultaneous EC presence of ACh and ATP at OHC was found to depress ATP-induced effects on OHC $[Ca^{2+}]_i$, an effect that is completely blocked in the presence of α-bgtx. Our observations suggest that the ACh-AChR system is involved in the modulation of the observed EC ATP-triggered events; possibly the OHC AChR is able to act both in its well known rapid ionotropic way, but also, perhaps after modification in a slower, metabotropic way interfering with the EC ATP-induced $[Ca^{2+}]_i$ increase. (©Elsevier, Paris)

Résumé — Modulation cholinergique de la concentration de Ca^{2+} cytoplasmique induite par l'ATP extracellulaire dans les cellules ciliées de la cochlee. Nos observations suggèrent que le système ACh-RACh est impliqué dans la modulation des événements induits par l'ATP extracellulaire ; il est possible que les RACh des cellules ciliées soient capables d'agir à la fois par la voie rapide ionotropique mais aussi, peut-être, par une modification d'une voie métabotropique plus lente interférant avec l'augmentation de $[Ca^{2+}]$ intracellulaire induite par l'ATP extracellulaire. (©Elsevier, Paris)

outer hair cell / purinergic / muscarinic-cholinergic / modulation / calcium

1. Introduction

The apparatus responsible for the transduction of sound energy into neural activity is the organ of Corti, located within the cochlear duct. The sensory cells, one row of inner haircells (IHC) and three rows of outer haircells (OHC) are attached at their basal end to the basilar membrane while only the stereocilia of the OHC apical end are embedded into the tectorial membrane; those of IHC reach but do not quite touch it [5, 9]. IHC act as mechanoelectrical transducers and release neurotransmitter onto neurons of the spiral ganglion inside the cochlea, thus initiating, via the cochlear component of the cranial nerve VIII (see e.g. [8]), the conduction of auditory information into the brain and the auditory cortex. Information about acoustic signals to the central nervous system (CNS) passes via nuclei in the medulla, pons, midbrain and thalamus to the auditory cortex. 95% of the afferent fibers contact IHC and only 5% OHC.

The role of OHCs is interesting; they are part of a feedback system from the CNS and believed to take part in the control of the sensitivity of IHC and the primary afferent nerve to a specific acoustic stimulus [4, 14]. OHC appear to be able to modulate their shape, i.e., to shorten when they depolarize and to lengthen when they hyperpolarize in response to sound stimulus, an ability that, by increasing or decreasing tension on the tectorial membrane, is believed to allow the cells to influence the transfer of sound energy to the IHC.

Efferent innervation of OHC mainly consists of medial olivocochlear fibers that use acetylcholine (ACh) as their main transmitter [8]. Other substances influence the signal from the nerve to OHC; adenosine 5′-triphosphate (ATP), believed to act as a modulator on OHC, γ-aminobutyric acid (relevant for more apical OHC) and, for example, enkephalins.

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It is well established that the efferent synapse of OHC carries a presynaptic muscarinic acetylcholine receptor (mAChR) subtype [3] probably involved in transmitter release, of which so far little is known. Postsynaptically, an ionotropic, reversibly α-bungarotoxin (α-bgtx)-binding [1, 10, 15, 17] neuronal type of AChR was described. This AChR allows a very rapid Ca^{2+}-influx into OHC which in turn mediates the activation of a closely located Ca^{2+}-dependent, outwardly directed K^+-channel which hyperpolarizes the cell [12, 13]. The OHC AChR is classified as a neuronal nicotinic nAChR (α9) subtype with unusual properties [6, 7], i.e., it has a mixed nicotinic-muscarinic pharmacology and is sensitive to strychnine [7, 13, 15, 17].

OHC also carry both ionotropic ($P_{2X}R$) and metabotropic ($P_{2Y}R$) purinergic receptors ([11, 16, 18] and others) and extracellular (EC) ATP was shown early this decennium to act as an important modulator on OHC [16]. The activation of these receptors strongly increases intracellular $[Ca^{2+}]_i$, from respectively extra- and intracellular sources [2] and so do L-type Ca^{2+}-channels seemingly activated as a secondary event after EC ATP-application [16].

The purinergic and the cholinergic systems appear to interact. Simultaneous EC presence of ACh and ATP depresses the $[Ca^{2+}]_i$ increases induced in OHC as compared to those induced by EC ATP alone (for review see [11]). Here we present results from a study of mechanisms involved in the control of OHC $[Ca^{2+}]_i$ by the two compounds, their receptors and the L-type Ca^{2+}-channel, including observations pointing to the AChR located at the OHC efferent synapse as being able to act both as an ionotropic and a metabotropic AChR.

2. Materials and methods

2.1. Preparation of outer hair cells

Pigmented guinea pigs of either sex (Duncin Hartley) were anaesthetized with CO_2 prior to decapitation. OHC were non-enzymatically microdissected as previously described in [15]. The temporal bones were removed and dissected in ice-cold Hanks balanced salt solution (HBSS) containing (in mM): NaCl 137, KCl 5.37, $MgSO_4$ 0.81, KH_2PO_4 0.4, $CaCl_2$ 1.25, Na_2HPO_4 0.35, $NaHCO_3$ 4.2, D-glucose 5.5, Hepes 5.0 (pH 7.4, osmolarity, 320 ± 3 mOsm/L). OHC's were then separated from membranes and supporting cells by gentle pipetting. Only OHC from the second and third turns of the cochlea were used for experiments.

2.2. Calcium imaging

Isolated OHC were loaded with fluo-3/AM (2 mM) for 30 min at 20–22 °C in HBSS. After incubation, 300 μL of the cell suspension was transferred onto concanavalin A-coated coverslips which formed the bottom of an open perfusion chamber (volume, 0.1 cm^3). The perfusion rate was approximately 100 μL /min and the chamber was placed on the stage of an inverted Leitz fluovert FU microscope.

A confocal laser scanning fluorescence microscope (CLSM-Leica, Germany) with an Argon-Krypton laser (Coherent) using the 488 nm line was used for measurements of the relative cytoplasmic calcium concentration ($[Ca^{2+}]_i$) in OHC's. The fluo-3 loaded cells were excited at 488 nm and emitted fluorescense was passed through a 515 nm long pass filter and displayed. The time resolution of the measurement was 3.0 s. Results were expressed as the ratio of fluorescence/resting fluorescence, i.e., F/F_0.

2.3. Electrophysiology

Conventional patch-clamp experiments in whole-cell and perforated patch configurations were carried out using an Axopatch 200 patch-clamp amplifier (Axon instruments, Foster City, USA). During experiments the cells were immersed in Leibkowitz medium (L-15, Gibco) containing (in mM): NaCl 135, KCl 4, $CaCl_2$ 1.26, $MgCl_2$ 2 and amino acids (pH 7.4, osmolarity, 325 ± 2 mOsm/L). In a few cases HBSS was used instead. Pipettes were pulled from borosilicate glass on a DMZ universal puller and had a resistance between 3–6 MΩ. Pipettes for whole-cell recordings were back-filled with an intracellular solution containing (in mM): KCl 144, $MgCl_2$ 4, EGTA 5, Na_2ATP 2, Na_2HPO_4 8, NaH_2PO_4 2, (pH 7.3) while in pipettes for perforated-patch experiments the solution used had a composition of (in mM): KCl 10, K_2SO_4 76, NaCl 10, $MgCl_2$ 1, Hepes 10 (pH 7.35) and amphotericin B final concentration 0.1%. Experiments were carried out in current-clamp mode to monitor changes in membrane potential.

2.4. Chemicals

Acetylcholine chloride (ACh), adenosine 5′ triphosphate (ATP), amphotericin B and concanavalin A (type IV) were obtained from Sigma (St. Louis, Missouri, USA); fluo-3/AM, D-600 and nifedipine were obtained from Molecular Probes (Europe BV, the Netherlands) and suramin monosodium salt was from Bayer (Leverkusen, Germany).

3. Results

Application of 10–100 μM EC ATP to OHC mediated a concentration-dependent and reversible increase in $[Ca^{2+}]_i$ (*figure 1*, n = 29) and a potent depolarization (n = 22). The increase of the $[Ca^{2+}]_i$ was blocked by application of the non-selective P_2 receptor antagonist suramin (50–100 μM) thereby demonstrating the presence of such receptors on OHC, confirming earlier results ([2] and others).

When OHC were immersed in Ca^{2+}-free extracellular solution, EC ATP (100 μM) was still able to elicit an increase of the $[Ca^{2+}]_i$ in most (*figure 2*, n = 5 of 8) cells although the amplitude was depressed. This demonstrates that the EC ATP-induced

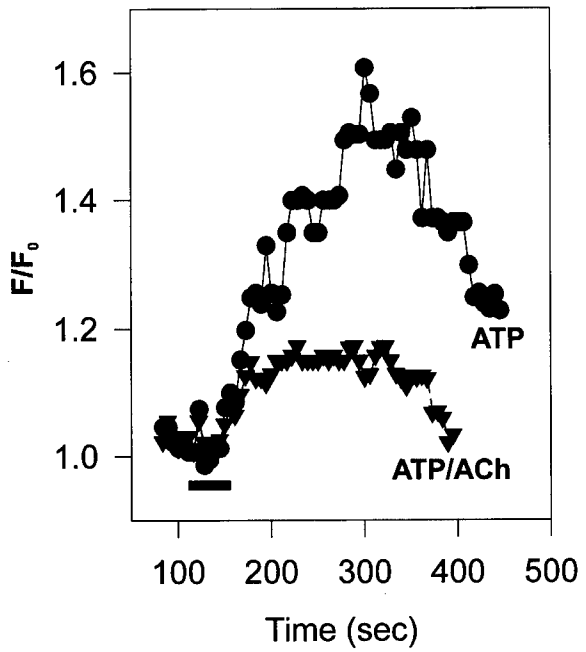


Figure 1. EC ATP (1–100 μM) induces an increase in OHC $[Ca^{2+}]_i$ in a concentration-dependent manner. When ACh (25–100 μM) is coapplied with ATP the amplitude of the $[Ca^{2+}]_i$ increase is markedly depressed. At even higher concentrations of ACh (up to 100 μM) the depression got even more pronounced.

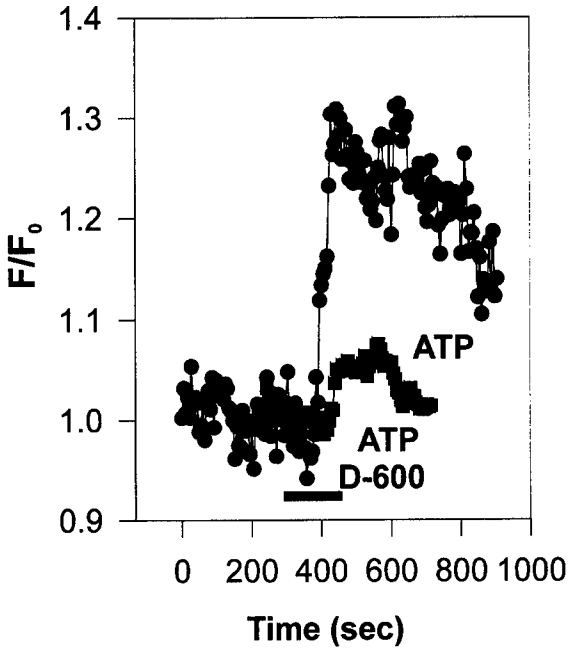


Figure 3. The increase in $[Ca^{2+}]_i$ induced by EC ATP (10–100 μM) was supressed in a concentration-dependent manner, when the L-type Ca^{2+} channel blocker D-600 (1–100 μM) was present.

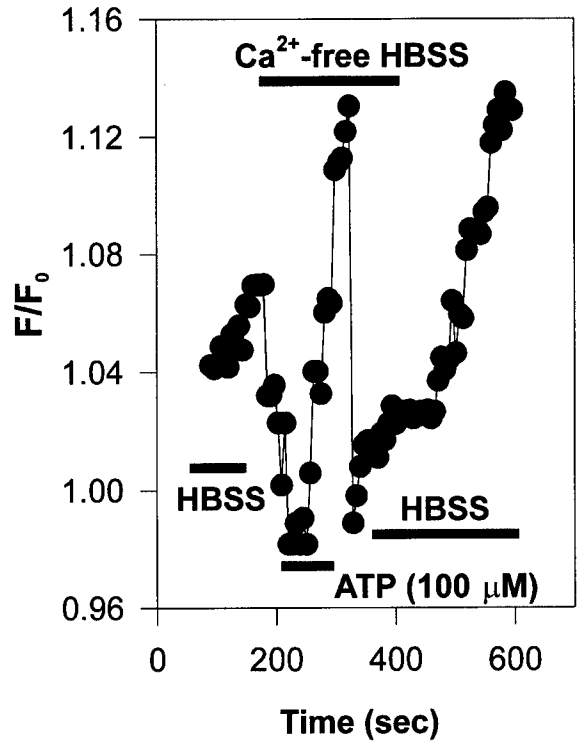


Figure 2. EC ATP (100 μM) is able to increase the $[Ca^{2+}]_i$ in Ca^{2+}-free extracellular solution although the amplitude is depressed as compared to control.

$[Ca^{2+}]_i$ increase is mediated both by influx of Ca^{2+} into OHC as well as by release of Ca^{2+} from intracellular stores.

To investigate if L-type Ca^{2+} channels contribute to the increase in $[Ca^{2+}]_i$ induced by EC ATP application, we exposed OHC to the L-type Ca^{2+} channel blockers D-600 (1–100 μM, n = 15), or nifedipine (2–5 μM, n = 4) during EC ATP application. The $[Ca^{2+}]_i$ increase caused by application of EC ATP was strongly depressed (*figure 3*) in a concentration-dependent manner, demonstrating that these channels are activated and contribute to the rise in $[Ca^{2+}]_i$.

ACh (25–50 μM) when applied to OHC before or during EC ATP-application (50 μM) mediated a clear depression of the increase in $[Ca^{2+}]_i$ (*figure 1*, n = 3) compared to that induced by EC ATP alone. ACh alone had no effect on $[Ca^{2+}]_i$ or membrane potential at the time resolution used in these experiments (n = 6).

To investigate if ACh application modulates the $[Ca^{2+}]_i$ increases due to EC ATP through an action on L-type Ca^{2+} channels, experiments were performed in which L-type channels were blocked by D-600 (50 μM, n = 3); the results show that even under these circumstances ACh was able to depress the increase in $[Ca^{2+}]_i$ induced by EC ATP.

During experiments performed in Ca^{2+}-free extracellular solution no increase in $[Ca^{2+}]_i$ was normally observed when ACh and ATP were co-applied (n = 9).

We furthermore investigated if α-bgtx, known to reversibly antagonize the fast AChR-induced Ca^{2+} influx into OHC and their subsequent hyperpolarization, also blocked the ACh-mediated depression of the EC ATP-induced $[Ca^{2+}]_i$ increase. When 50 nM of α-bgtx was present, application of 50 μM of EC ATP generated a large increase in the $[Ca^{2+}]_i$ also in the presence of 50–100 μM ACh (*figure 4*, n = 3). α-bgtx had no effect on the increase in $[Ca^{2+}]_i$ due to EC ATP only (n = 3).

4. Discussion

We have confirmed earlier observations that EC ATP activates P_2 receptors on the OHC membrane and that this results in a marked increase in $[Ca^{2+}]_i$. The cells were also potently depolarized by EC ATP.

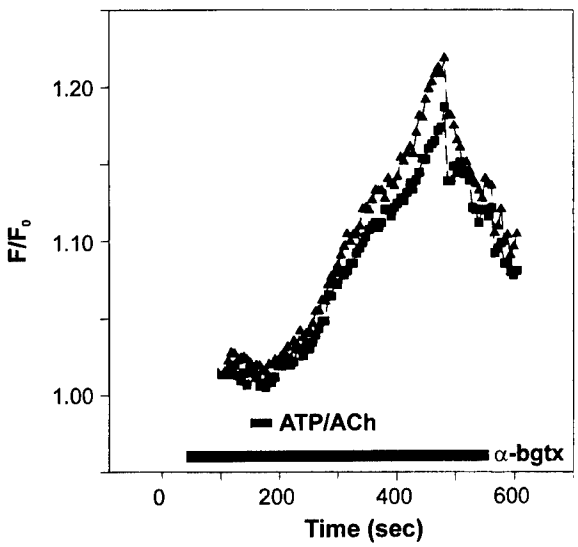


Figure 4. α-bgtx (50 nM) clearly antagonized the ACh mediated depression of the EC ATP-induced (100 mM) $[Ca^{2+}]_i$ increase. At ACh concentrations (100 μM) that normally almost blocked the $[Ca^{2+}]_i$ increase completely, a relatively large $[Ca^{2+}]_i$ was observed when α-bgtx was present. The two traces indicate different regions of the cell (triangles, middle; squares, basal).

The $[Ca^{2+}]_i$ increase induced by EC ATP was not abolished in Ca^{2+}-free extracellular solution which shows that the increase in $[Ca^{2+}]_i$ induced by EC ATP is due to both influx of Ca^{2+} into the cell via ionotropic P_{2x} receptors as well as release of Ca^{2+} from intracellular sources via the activation of P_{2y} receptors.

L-type Ca^{2+} channels have been shown to be present in OHC. When L-type channels were blocked with relevant blockers the EC ATP-induced $[Ca^{2+}]_i$ increase was strongly supressed in a concentration-dependent manner. This demonstrates that these voltage-dependent channels are activated during EC ATP application, probably as a result of the initial depolarization of the cell, and strongly contribute to the ATP-induced $[Ca^{2+}]_i$ increase.

ACh supresses the increase in $[Ca^{2+}]_i$ induced by EC ATP, a known but unexplained phenomenon reconfirmed here. We found that ACh depressed the remaining EC ATP-induced $[Ca^{2+}]_i$ increase in a similar proportion also when L-type Ca^{2+} channels were blocked. We therefore suggest that L-type Ca^{2+} channels are not directly involved in the mechanism for the ACh-induced depression of the EC ATP-induced $[Ca^{2+}]_i$ increase. Furthermore, in Ca^{2+}-free extracellular solution no increase in $[Ca^{2+}]_i$ was normally observed when ACh and ATP were co-applied, suggesting that ACh might modulate the EC ATP-induced $[Ca^{2+}]_i$ increase, at least partly, through an effect on the P_{2y} receptor mediated release on Ca^{2+} from intracellular stores.

α-bgtx, a reversible antagonist at the OHC ionotropic AChR, was also able to antagonize the ACh-mediated depression of the EC ATP-induced increase in $[Ca^{2+}]_i$, suggesting that this receptor might have a dual function. On a very short timescale the receptor acts ionotropically, allowing rapid influx of Ca^{2+} resulting in a short-lasting hyperpolarization of OHC due to the activation of the outwardly directed Ca^{2+}-activated K^+ channels. On a timescale of seconds to minutes the ACh-AChR system does not affect OHC $[Ca^{2+}]_i$ significantly but it modulates $[Ca^{2+}]_i$ increases mediated by EC ATP application. This latter phenomenon may be a metabotropic effect.

In summary the results suggest that the basolaterally located AChR of OHC may have both metabotropic and ionotropic properties. The mechanism for this is at present unknown. Possibly, a phosphorylation of the AChR on the external membrane is required for the receptor to function in a metabotropic manner. Alternatively the 'unmodified' AChR itself can act in this way. It's structure is still not completely known. The possibility that OHC instead express two different AChR remains, but this seems

unlikely as the pharmacology of the rapid and the slow AChR-induced actions seems to be identical.

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The acetylcholine, GABA, glutamate triangle in the rat forebrain

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Abstract — The present overview demonstrates that stress, fear, novelty, and learning processes are associated with arousal and increases of extracellular levels of cortical and hippocampal ACh, independently of increases of motor activity. Forebrain cholinergic systems appear to be regulated by GABAergic and glutamatergic inputs. However, several other neurotransmitter systems play a role. (©Elsevier, Paris)

Résumé — Le triangle acétylcholine, GABA, glutamate dans le cerveau antérieur du rat. Nous résumons ici un ensemble de résultats qui démontrent que le stress, la peur, la nouveauté, et les processus d'apprentissage sont associés a l'éveil et à une augmentation des niveaux d'acétylcholine extracellulaire dans l'hippocampe et le cortex, indépendamment de l'augmentation d'activité motrice. Le système cholinergique du cerveau antérieur semble être contrôlé par l'innervation GABAergique et glutamatergique. Cependant, plusieurs autres systèmes de neurotransmetteurs interviennent également. (©Elsevier, Paris)

acetylcholine / GABA / glutamate / microdialysis

1. Cognitive processes associated with a raise in extracellular ach levels

The roles of the forebrain cholinergic systems in the cognitive functions have been reviewed recently [16], and the following functions have been identified: a) the nucleus basalis magnocellularis (NBM)-cortical cholinergic system contributes greatly to visual attentional functions, but not to mnemonic processes per se; b) the septo-hippocampal cholinergic projection is involved in the modulation of spatial working memory processes; and c) the diagonal band-cingulate cortex cholinergic projection impacts on the ability to utilize response rules through conditional discrimination.

The activity of the cholinergic systems ascending from the NBM to the cortex, and from the septum to the hippocampus can be monitored in vivo measuring the extracellular levels of acetylcholine (ACh) by microdialysis. Rat exposure to novel environment evoked an exploratory behavior involving increase of attention and locomotor activity, and elicited large increases of extracellular level of cortical ACh [21, 29], which faded with habituation to the environment. Rats performing spontaneous alternation in a Y maze also displayed a large increase in extracellular level of cortical ACh [21]. This task requires attention, and involves spatial working memory and motor activity. Conversely, object exploration non-associated with increase of locomotor activity failed to alter extracellular levels of cortical ACh, although this task is known to depend on the intact cortical cholinergic system [4, 15]. Novelty appears to activate also the septo-hippocampal system as shown by the increase of ACh extracellular level in dorsal hippocampus of rats introduced in an open field, thus inducing an exploratory activity associated with increase of locomotion [3]. Indeed, the increase in ACh extracellular level strictly matched the intensity of locomotor/exploratory activity. Object exploration, on the other hand, elicited a much smaller increase of extracellular levels of ACh than novelty (Aloisi, personal communication). Although these experiments show a relationship between motor and cholinergic activities, it is not yet clear whether the increases of ACh release elicited by exposure to novel environment are associated with, or depend on locomotor activity. Indeed, locomotor activation correlated with increases of ACh release in the hippocampus, cortex and striatum of adult rats [12], and in the frontal cortex of young but not of old rats [34]. Moreover, Nilsson and Björklund [37] reported that a short period of swimming was associated with a sharp increase of hippocampal ACh extracellular levels. This increase, however, persisted for a long time after the swimming session, thus indicating that it was a consequence of the arousal induced by swimming more than that of the locomotor activity. Accordingly, novelty represented not by the environment but by unconditioned stimuli, such as a tone or a light, caused a large increase of ACh extracellular levels in the frontal cortex and the hippocampus but little motor activity [1], and fear related behaviors, among which freezing is predominant, are also characterized by large increases of extracellular ACh levels in both cortex and hippocampus.

Although both trained and untrained rat groups showed similar motor activity when placed in an operant chamber, only untrained rats exhibited increases of ACh extracellular levels of frontal cortex and hippocampus associated with a rise in the reinforced responses, i.e., when the rats were acquiring the procedure [38]. Thus, the performance of the trained rats did not activate the forebrain cholinergic systems. Other studies, however, reported that cortical ACh extracellular levels increased during reinforced response execution also in well trained rabbits [40], but the paradigm used involved response inhibition, which requires attention and arousal. A very large increase of ACh levels was reported in the frontal cortex of trained rats during anticipation of a palatable meal, a stimulus associated with strong arousal [43]. Finally, the increase of extracellular levels of rat hippocampal ACh was much larger during acquisition of a discriminative task than during that of a non-discriminative task [48].

Therefore, attention and arousal appear to be associated with the activation of both cortical and hippocampal cholinergic systems, independently from an increase of motor activity. Consistently, presentation of sensory stimuli elicited an increase in ACh levels of the frontal cortex and hippocampus without changes in motor activity [2, 28]. Ragozzino et al. [39] reported that intrahippocampal infusion of glucose failed to modify ACh spontaneous release, but enhanced hippocampal ACh output during spontaneous alternation, and improved the performance, thus confirming the role of hippocampal ACh in this task, which involves attention and spatial working memory.

Also, stress activates the forebrain cholinergic pathways, likely because of its association with arousal. Restraint stress induced in male rats a marked increase of ACh release from the hippocampus [26, 27, 37, 51]. A further increase of hippocampal ACh levels was observed immediately after the rats were set free [46]. Novelty represents a stressful condition for the rat, and the cholinergic activation caused by exposure to novel environment is attributable, at least partly, to stress. Handling, which is a mixture of stress and sensory stimulation, also caused an increase of hippocampal ACh levels [36, 37], but gentle handling used to move rats from a cage and to put them back immediately failed to alter cortical ACh levels [21]. Thus, it seems likely that while an increase in locomotor activity is associated with an increase in ACh release from the cerebral cortex and hippocampus, there are conditions, such as stress, fear, novelty, learning process, in which the activation of the cholinergic systems occurs independently from the increase in motor activity.

2. Which mechanisms activate the forebrain cholinergic neurons?

Regardless of whether the forebrain cholinergic neurons are activated by sensory stimuli, stress, novelty or learning, the question arises as to the nature of the signal triggering them, thus leading to increased firing and, consequently, increase of ACh release.

2.1. Septal-hippocampal pathway

GABA is largely present in the basal forebrain, septum, hippocampus, and cerebral cortex [45], where it appears to play an important role in the modulation of cholinergic neurones. GABAergic inputs from lateral to medial septum impinge on cholinergic neurons [32]. Intraseptal administration of muscimol, a $GABA_A$ receptor agonist, decreased both release [19, 24], and turnover [5] of hippocampal ACh. Intraseptal administration of bicuculline, a $GABA_A$ receptor antagonist, increased the hippocampal rate of high-affinity choline uptake [50], thus suggesting that GABA exerts a tonic inhibition of the cholinergic activity [14].

If septal cholinergic neurons are controlled by GABAergic neurons, the next question is: how are those latter regulated? Glutamate seems to play an important role. Glutamatergic inputs to the septum exerted a tonic excitatory influence on septal GABAergic activity [19], thus suggesting that they might inhibit indirectly septal-hippocampal cholinergic neurons. Indeed, intraseptal administration of 3-(2 carboxypiperazin-4-yl)propyl-1-phosphonic acid (CPP), an NMDA receptor antagonist, reduced GABA extracellular level in the septum, simultaneously increasing hippocampal ACh release [19]. There is no evidence that glutamate influence directly septal cholinergic neurons. The tonic regulation of septal-hippocampal cholinergic activity by dopamine appears also to be mediated by GABA, since dopamine effects on hippocampal cholinergic activity are reversed by bicuculline [13].

2.2. NBM-cortical pathway

GABA affects the cortical cholinergic activity at three levels: the NBM, the septum, and the cortex. Direct injection of muscimol into NBM decreased cortical ACh release. This effect was blocked by picrotoxin, a $GABA_A$ antagonist [8]. Moreover, cortical ACh output is decreased by the infusion into the basal forebrain of benzodiazepine receptor agonists, and is enhanced by its antagonists [36]. Finally, while systemic administration of picrotoxin increased ACh spontaneous release from the frontal cortex,

diazepam injected i.p. reduced the increase of ACh induced by tactile stimulation [2]. Local administration of CPP into the septum increased cortical ACh and GABA release, and the effect on ACh was prevented by muscimol [20]. In the medial septum the entire population of neurons is represented by cholinergic and GABAergic cells [30]. GABA neurons project from the medial septum to the cortex [20], thus suggesting that septal-cortical GABAergic neurons modulate cortical cholinergic activity. These GABA neurons, in turn, appear to be regulated by glutamatergic inputs to the GABAergic interneurons of the lateral septum. Indeed, the blockade of NMDA receptors located on these GABAergic interneurons increased the release of cortical GABA [20]. It is not yet clear by which mechanism GABA elicits an increase of ACh release from the cholinergic nerve endings. At least two explanations can be offered. First, the activation of a GABA transporter localized on cholinergic terminals, which appears to regulate also ACh release [7]. Alternatively, the increase in cortical GABA levels could inhibit GABAergic interneurons, which in turn inhibit ACh release. While local administration of GABA did not affect spontaneous ACh release from the cerebral cortex of freely moving rats, perfusion with bicuculline caused a concentration-dependent increase of ACh release. The effect of bicuculline is blocked by muscimol [17]. These findings confirm that the cortical cholinergic network is under a tonic inhibitory GABAergic control mediated by $GABA_A$ receptors. GABA also appears to regulate the release of cortical ACh through a pre-synaptic action, and some 5-HT/ACh interactions may be mediated by GABA [22]. Moreover, through the activation of cortical histamine H_3 receptors, histamine has been shown to inhibit ACh release from rat cortex in vivo [6] through cortical GABA interneurons [18]. Finally, the GABAergic fibers ascending from the septum could impinge on, and modulate the activity of cortical intrinsic cholinergic neurons. Their existence in the rat brain has been recently confirmed by immuno-histochemistry of the vesicular ACh transporter [44]. However, their functional role, and their contribution to cortical ACh extracellular levels need to be defined.

The glutamatergic pathways modulate the cortical cholinergic activity both indirectly, regulating the activity of the GABAergic neurons, and directly, impinging on the cholinergic neurons of the NBM. Indeed, the perfusion of NBM with CPP [20], or kynurenate [41] decreased cortical ACh spontaneous release. Moreover, the cortical ACh release induced by brain stem stimulation is inhibited also by kynurenate application to the NBM [41]. This observation indicates that a major glutamatergic pathway, stimulating the cholinergic neurons of the NBM through NMDA receptors, originates from the pedunculopontine tegmentum [42]. According to these authors, AMPA receptors are only involved in the desynchronization, presumably through a circuit including GABAergic projections to the thalamus.

3. Conclusions

The forebrain cholinergic systems are activated in conditions inducing arousal, whether or not associated with an increase in motor activity. The arousal may be induced by non-cognitive stimuli, such as stress or sensory stimulation, or by performance of tasks involving learning and working memory, such as exploration of a novel environment, spontaneous alternation, acquisition of a behavior. The neuronal circuits and neuro-transmitters which trigger the activation of the forebrain cholinergic systems during arousal are not entirely clear yet. The interactions of cholinergic neurons located in the septum and NBM with GABAergic and glutamatergic systems here reviewed, suggest that GABAergic interneurons exert an inhibitory tone on cholinergic neurons. However, this mechanisms is not universal, since in the cerebral cortex increases of GABA release are associated with those of ACh release [20]. In the dorsal hippocampus, the local stimulation of non-NMDA receptors stimulates the release of both GABA and ACh through a mechanism occurring at nerve ending level [23]. Further studies will be necessary to learn the behavioral implications of the interactions between cholinergic and GABAergic systems, including experiments correlating changes of ACh outflow in behaving rats with those of GABA extracellular levels. The GABAergic neurons are in turn modulated by many neurotransmitters, including glutamate. However, it has been shown that glutamatergic neurons affect the cholinergic neurons not only indirectly, modulating the GABAergic tone, but also directly. With the caveat of the dual role of neurotransmitter and metabolite of glutamate, investigations correlating changes in glutamate, GABA and ACh extracellular levels during specific behaviors could define the respective roles of the three neurotransmitters

Finally, although it is well beyond the scope of this review to describe exhaustively the regulation of the forebrain cholinergic systems, it must be mentioned that also other neurotransmitters contribute to this regulation. Dopaminergic neurons activate the forebrain cholinergic neurons [8, 10, 11]; serotonin modulates the cortical cholinergic system with various effects depending on the receptor subtype activated [9, 22, 25, 49]; noradrenaline exerts a tonic inhibitory regulation of frontal ACh release [47];

cholecistokinin stimulates cortical ACh release [31]; VIP increases hippocampal ACh release through a presynaptic mechanism [33]. This short and by no means complete list of neurotransmitters acting upon the forebrain cholinergic systems is enough to demonstrate the complexity of their regulation and raises the question of their respective involvement in different behavioral conditions. It also suggests that the forebrain cholinergic systems is the critical final pathway of a large number of inputs influencing the cognitive processes.

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Enzymes hydrolyzing organophosphates as potential catalytic scavengers against organophosphate poisoning

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Abstract — Enzymes hydrolyzing organophosphates could be used as catalytic scavengers for treatment of organophosphate poisoning and for decontamination. Two organophosphorus hydrolases (OPH) were selected: the *Flavobacterium* sp./*Pseudomonas diminuta* phosphotriesterase (PTE) and human paraoxonase (HuPON). Genes encoding these enzymes were cloned and functional recombinant enzymes expressed. PTE was expressed in *E. coli*. Natural HuPON was purified from human plasma; recombinant HuPON was expressed in human embryonic kidney 293 T cells. Although HuPON displays interesting catalytic properties, a site-directed mutagenesis program was undertaken to improve its catalytic efficiency. PTE has high efficiency in hydrolysis of organophosphates, including nerve agents. PTE injected in rat has a half-life of 100 min. However, to overcome pharmacokinetic problems of injected OPH and/or immunological incompatibility, the model enzyme (recombinant PTE) was immobilized onto a hollow-fiber reactor. This reactor designed for extracorporeal blood circulation is under experimentation for post-exposure detoxification. (©Elsevier, Paris)

Résumé — Enzymes hydrolysant les organophosphorés, épurateurs catalytiques potentiels contre l'intoxication par les organophosphorés. Les enzymes hydrolysant les organophosphorés (OPH) sont des épurateurs catalytiques potentiels qui pourraient être utilisés pour le traitement de l'intoxication organophosphorée, ainsi que pour la décontamination. Nous nous sommes intéressés à deux enzymes : la phosphotriestérase (PTE) de *Flavobacterium* sp./*Pseudomonas diminuta* et la paraoxonase humaine (HuPON). Les gènes codant pour ces enzymes ont été clonés et les enzymes recombinantes fonctionnelles exprimées. La PTE a été exprimée dans *E. coli*. La PON naturelle a été purifiée à partir du plasma humain ; l'HuPON recombinante a été produite dans des cellules embryonnaires de rein humain 293 T. Bien que HuPON possède des propriétés catalytiques intéressantes, nous avons entrepris d'augmenter son efficacité catalytique par mutagenèse dirigée. La PTE possède une activité catalytique élevée vis-à-vis de nombreux OPs, dont les neurotoxiques de guerre. La demi-vie biologique de cette enzyme injectée au rat par voie intraartérielle est de l'ordre de 100 min. Cependant, pour éviter les éventuels problèmes pharmacocinétiques et/ou immunologiques consécutifs à l'injection d' OPH, l'épuration du sang pourrait être réalisée dans un réacteur à OPH grâce à un système de circulation extracorporelle. Dans ce but, la PTE, enzyme modèle, a été immobilisée dans un réacteur à fibres creuses. (©Elsevier, Paris)

organophosphate poisoning / scavenger / organophosphorus hydrolase (OPH) / phosphotriesterase (PTE) / paraoxonase (PON)

1. Introduction

In the past 10 years, pretreatment and treatment of organophosphate poisoning have made progress. However, the persistence for long time after absorption of organophosphate molecules (OP) in depot sites from where they are slowly and continuously released into the blood can cause clinical complications as seen in patients severely poisoned by OP insecticides [29]. Indeed, from blood, OP rapidly reaches peripheral and central acetylcholinesterases that are maintained inhibited at a high level (*figure 1*). The presence of endogenous scavengers in plasma probably reduces the long-term toxicity of OPs. Thus, enzyme-catalyzed degradation or removal of OP in blood could greatly improve the efficiency of the classical treatment of organophosphate poisoning. In this connection, it should be remembered that hemodiafiltration was successfully applied in Tokyo to a patient with severe sarin poisoning resistant to standard drug therapy [30].

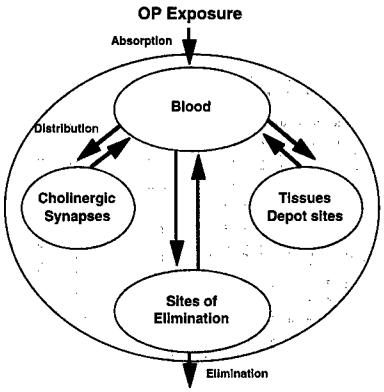


Figure 1. General scheme showing availability and metabolism of OP in human body.

Organophosphate-degrading enzymes and other catalytic and pseudocatalytic scavengers have gained in interest and the technology of biological scavengers is now emerging. Catalytic scavengers could be used as detoxifying drugs. Intravenous administration of catalytic scavengers is expected to reduce the risk of post-exposure OP poisoning due to the slow release in blood of OP from peripheral compartments. In the near future these catalysts could also be a part of the package of countermeasures for selective harmless decontamination of skin and eyes and for medical protection (prophylaxis) against nerve agents. Also, genetically engineered microorganisms could be used for OP detoxification, and environment/equipment decontamination [5].

2. OP-degrading enzymes

Numerous OP-degrading enzymes have been isolated from animal tissues and microorganisms. These enzymes are structurally different. *Alteromonas* prolidases (E.C. 3.4.13.9) are very active and have considerable potential for nerve agent decontamination [6]. Mutants of human butyrylcholinesterase (E.C. 3.1.1.8) capable of hydrolyzing OPs have been designed. These mutants hydrolyze various OPs, including the nerve agents soman, sarin and VX [18–20]; however, their catalytic activity is still too low to be of practical interest. To date, the most active organophosphate-degrading enzyme is the phosphotriesterase (E.C. 3.1.8.1; PTE) that has been isolated from *Pseudomonas diminuta* and *Flavobacterium* sp. *(table I)*. This enzyme is a dimeric zinc metalloenzyme of 72 kDa; the 3-D structure of both apo- and holo enzyme has been solved [1]. Functional recombinant PTE expressed in *E. coli* administered intravenously was found to protect mice against toxic doses of soman, sarin or paraoxon [2, 25, 26]. Pretreatment by PTE and carbamates was found to be very effective against DFP [27] or sarin [28] poisoning. Human paraoxonase (E.C. 3.1.8.1; HuPON) is a plasma 44 kDa hydrophobic monomeric glycoprotein associated with HDL apo A-I. Ca^{2+} is required for stability and catalytic activity. Two allelozymes (R191 and Q191) have been recognized; they exhibit differential activity toward OP substrates *(table I)*. Little information is available on the active site characteristics of PON [23], its sequence shows no similarity with any other proteins, and its 3-D structure has not yet been resolved. Although PON has not yet been used in protection trials against chemical warfare agents, the relative resistance of rabbit to OP correlated with high endogenous PON activity in serum (10 times higher than in human serum), suggesting that PON has good detoxifying capacity [13]. Therefore, we focused our attention on two enzymes that look promising: the bacterial PTE and HuPON.

3. Rationale of *in vivo* enzyme-catalyzed hydrolysis of OPs

Even in the most severe poisoning, the concentration of OP in blood is thought to be well-below the K_m of OP-degrading enzymes. For example, the concentration of paraoxon in blood of patients poisoned by parathion has been estimated to range between 0 and 400 nM [9]. K_m values for paraoxon were found to be 271 μM for paraoxonase B [22], and 68 μM for Zn-wild type PTE [15]. Paraoxon concentrations in blood are at least 175 times lower than the lowest K_m, supporting the contention that OPH-catalyzed hydrolysis of paraoxon in blood is first order. Thus, OPs circulating in blood of poisoned patients are expected to be hydrolyzed by OPHs according to first order kinetics:

$$v = k_{cat}/K_m . [E][OP]$$

where [E] is the OPH active site concentration, [OP] is the concentration of OP and $(k_{cat}/K_m)\cdot[E]$ is the first-order rate constant of hydrolysis. The amount

Table I. Bimolecular rate constant k_{cat}/K_m (M^{-1} min^{-1}) of two OP hydrolases.

OP	*HuPON*		*Pseudomonas diminuta PTE*
	A (Q191)	*B (R191)*	*(Zn–wt)*
Paraoxon	6.8×10^{5a}	2.4×10^{6a}	2.03×10^{9b}
DFP	3.7×10^{4c}	ND	5.8×10^{8e}
Sarin	9.1×10^{5d}	6.8×10^{4d}	4.8×10^{6f}
Soman	2.8×10^{6d}	2.1×10^{6d}	6.0×10^{5f}
VX	–	–	–

[a]pH 7.4 at 37 °C [22]; [b]pH 9.0 at 25 °C [15]; [c]pH 7.4, 1 mM $CaCl_2$ at 25 °C, Josse et al., unpublished; [d]pH 8.5, 1 M NaCl and 2 mM; [e]pH 7.2 at 37 °C [16] $CaCl_2$ at 25 °C [7]; [f]pH 7.2 at 37 °C [8].

of enzyme that should be injected in the bloodstream to reduce the organophosphate concentration by 50% per unit time is:

$$[E] = \left\{ \frac{Ln2}{k_{cat} / K_m} \right\} / t$$

Under steady state conditions, taking the volume of plasma as 40 mL per kg of body weight and the median time for a complete cycle of a molecule in the blood circulation as t = 1 min to decrease toxic plasma concentrations of paraoxon by half in 1 min, one should inject at least 75 µg of *Pseudomonas diminuta* PTE or 40 mg of human paraoxonase. However, the concentration in blood of injected enzyme [E] is decreasing with time due to the fact that enzyme is eliminated from the bloodstream. For example, the clearance of asialoglyproteins is very fast because these proteins are trapped by hepatocyte receptors which recognize galactosyl residues. The kinetics of elimination can be expressed by the general equation:

$$[E] = \sum_i C_i e^{-k_i t}$$

where i is the number of compartments, coefficients C_i depend on the dose and k_i are related to the actual rate constant of elimination. For a one-compartment system, the biological half-time is $t_{1/2}$ = ln2/k. All these parameters have to be taken into account to determine the dose to be administered and the interval times between repeated injections.

4. Phosphotriesterase

The gene of *Flavobacterium/Pseudomonas* PTE was cloned in pET17b and functional recombinant PTE was expressed in *E. coli* BL 21. The enzyme was purified to homogeneity by a three-step chromatography method (CM Sepharose, Green agarose and size exclusion on AcA54). Another enzyme was made in which the signal peptide was deleted and a 10 His tag was added at the N-terminus. This enzyme was expressed in *E. coli* HMS 174, and it was purified in a single step by metal-chelate affinity chromatography.

Pharmacokinetic study of recombinant PTE flash-injected in the femoral artery of rats showed a circulating half-life long enough ($t_{1/2}$ = 105 ± 5 min) to be of pharmacological interest [24]. However, engineering of human-compatible injectable PTE might be difficult to achieve. To circumvent this problem, we considered the possibility for post-exposure detoxification by circulating blood through a cartridge containing immobilized PTE. Several enzymes have been used for extracorporeal therapy for years [14]. Such an approach in emergency medicine is not unrealistic since artero-venous hemofiltration on hollow-fiber cartridges has been used by rescuers to avoid crush-syndrome in earthquake casualties for instance. Although the implementation of such devices needs skilled medical staff, this would pose no problem at field hospital level. Our first results showed that storage stability of recombinant PTE was considerably increased by immobilization on a Sepharose 4B matrix, but K_m for paraoxon of immobilized enzyme was increased at least 15 times. This corroborates results obtained with immobilized PTE in other types of reactors [3, 4, 17]. Since hollow fiber based reactors have been proven to be safe and efficient, e.g., for extracorporeal circulation across artificial organs, removal of toxins from blood, cancer therapy, we chose to immobilize PTE onto hollow fibers. The reactor is a bundle of cellulosic hollow fibers on which PTE was coupled by cyanogen bromide. Although preliminary results demonstrated the feasibility of this system, it must be optimized to be of medical interest.

5. Paraoxonase

We have undertaken both chemical and site-directed mutagenesis studies of PON to identify important active site residues, the ultimate goal of this work being to make PON mutants capable of degrading OPs with an efficiency similar to that of bacterial PTE. The natural enzyme was purified from human plasma by affinity chromatography on blue Cibacron gel according to [10]. The gene of HuPON was cloned in pGS vector and transiently expressed in 293T/17 cells. A 6 His tag was linked on the C-terminal position. Recombinant PON was purified by metal-chelate affinity chromatography. The His tag did not affect PON activity. Chemical modification of natural HuPON was performed to identify important amino acids. As for PTE, modification with diethylpyrocarbonate (DEPC) provided no clear evidence for the presence of histidine residues in the active site, but the pH dependence study of DEPC inactivation giving pKa of 6.28 and 6.98 for modified residues, argues for the presence of histidine (H) residues. PON inactivation induced at pH < 6 suggested that carboxylic residues (D, E) may be involved in Ca^{2+} binding to the active site. Lastly, chemical modification by N-bromosuccinimide (NBS) clearly indicated that tryptophan (W) residues play a role in activity. In addition, the luminescence

spectrum of the calcium analog terbium supported the contention that Ca^{2+} interacts with a tryptophan residue [11]. Tryptophan residue (s) may be involved in binding of aromatic substrates.

Conserved residues W, H, D and E among known sequences of PON were selected for mutagenesis. Site-directed mutagenesis of the HuPON gene was performed with PCR and Pfu polymerase. Mutagenesis was performed on the four tryptophan residues (W → A/F), on the histidine residues (H → N), on aspartate /glutamate residues (D/E → A) and on asparagine residues (N → A). Transient expression of mutated PON in human embryonic kidney 293 T/17 cells was used for screening catalytic activity of mu-

Table II. Kinetic parameters of selected HuPON mutants toward two OPs and phenylacetate.

OP	*Mutant*	*Relative* K_m [a]	*Relative* k_{cat} [a]
Chlorpyrifosoxon[b]	W193A/F	1.51/1.55	0.47/1.37
	W201A/F	1.08/1.18	0.34/2.11
	W253A/F	1.21/1.63	2.49/2.90
	W280A/F/L	ND/1.66/1.90	< 0.01/0.94/0.18
	H114N	ND	< 0.01
	H133N	ND	< 0.01
	H154N/Y	ND/1.10	< 0.01/0.52
	H160N	1.16	0.43
	H242N	ND	< 0.01
	H245N	1.02	0.28
	H250N	0.75	0.61
	H284N	ND	< 0.01
	H 347N	1.52	0.15
	D168A	ND	< 0.04
	D182A	ND	0.07
	D268A	ND	< 0.04
	D278A	2.23	< 0.04
	E194A	ND	< 0.04
Diazoxon[c]	W193A/F	1.38/1.10	0.39/0.88
	W201A/F	1.36/3.67	0.34/4.19
	W253A/F	0.93/1.64	1.99/3.60
	W280A/F/L	ND/0.81/1.25	< 0.01/0.56/0.06
	H114N	ND	< 0.01
	H133N	ND	< 0.01
	H154N/Y	ND/1.25	< 0.01/063
	H160N	1.37	0.44
	H242N	ND	< 0.01
	H245N	1.16	0.32
	H250N	0.71	0.58
	H284N	ND	< 0.01
	H347N	1.34	0.12
	D168A	ND	< 0.05
	D182A	ND	< 0.05
	D278A	ND	0.05
	E194A	3.32	< 0.05
	N132A	1.13	0.06
Phenylacetate[d]	W280L	0.59	0.15
	H154Y	0.93	0.48
	D53A	–	0
	D107A	ND	< 0.8
	D121A	ND	< 0.8
	D168	0.77	0.04
	D182A	1.56	0.04
	D268	ND	0.04
	D273A	ND	< 0.8
	D278A	1.14	0.08
	E32A	ND	< 0.8
	E48A	ND	< 0.8
	E52A	–	0
	E194A	1.57	0.12
	N132A	1.32	0.06

[a] K_m and k_{cat} relative to those of wild-type enzyme taken as 1.
[b, c, d] pH 8.5, 2 mM $CaCl_2$, 1 M NaCl at 25 °C.

tants toward phenylacetate and two OPs: diazoxon and chlorpyrifos oxon (*table II*). Mutagenesis results showed that eight residues are important: W280, H114, H133, H154, H242, H284, E52 and D53. These residues very likely play a key role in the active site machinery, e.g., W280 as a component of the substrate binding site and H residues as nucleophile poles, but we cannot rule out the possibility that some of them are involved in the Ca^{2+} binding sites which control both the enzyme structural stability and activity. E52 and D53 are very likely involved in Ca^{2+} binding [12]. Six residues are essential for activity: N132, D168, D182, E194, D268 and D278.

6. Future prospects

Mutants of HuPON having improved OPH activity and enzymes having catalytic or pseudocatalytic OPH activity have to be designed; they can be reasonably expected in the very near future. For that purpose, more research has to be done on the catalytic mechanisms of OPH and quantitative structure-activity relationships. In a very near future, resolution of the X-ray structure of rabbit and human PON should also stimulate research in the field. Lessons from nature will help to design mutants of cholinesterases and carboxylesterases having high OPH activity. It is, for instance, noteworthy that insecticide resistance of a blowfly is due to a single amino acid mutation G → D, in a position homologous to G117 in human butyrylcholinesterase, that converts a carboxylesterase to an OPH [2].

Controlling glycan heterogeneity of sialoglycoproteins by optimization of their biosynthesis in mammalian cells and carbohydrate engineering should slow down the clearance of therapeutic recombinant enzymes (e.g., HuPON) from blood circulation. In the case of recombinant bacterial enzymes, surface modifications (e.g., PEGylation) are expected to reduce immunogenicity. This should be an area of intensive effort in the next years. Moreover, new approaches such as the extracorporeal approach should be considered with particular interest.

Stability problems are also of major importance. Stable thermozymes for decontamination would have the advantage of storage at ambient temperature without loss of activity and to be usable under field conditions. Screening of bacteria that thrive in extreme environments (e.g., submarine hydrothermal vents, hot springs, very salty media) is underway. Some archaebacterial strains have already been found to be capable of degrading a variety of organophosphates, including chemical warfare nerve agents.

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Four acetylcholinesterase genes in the nematode *Caenorhabditis elegans*

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Abstract — Whereas a single gene encodes acetylcholinesterase (AChE) in vertebrates and most insect species, four distinct genes have been cloned and characterized in the nematode *Caenorhabditis elegans*. We found that *ace-1* (mapped to chromosome X) is prominently expressed in muscle cells whereas *ace-2* (located on chromosome I) is mainly expressed in neurons. *Ace-x* and *ace-y* genes are located in close proximity on chromosome II where they are separated by only a few hundred base pairs. The role of these two genes is still unknown. (©Elsevier, Paris)

Résumé — Quatre gènes d'acétylcholinestérase chez le nématode *Caenorhabditis elegans*. À l'inverse de la situation des vertébrés et de la majorité des insectes, chez qui un gène unique code pour l'acétylcholinestérase (AChE), quatre gènes d'AChE ont été clonés et caractérisés chez *Caenorhabditis elegans*. Le gène *ace–1* (localisé sur le chromosome X) et le gène *ace–2* (chromosome I) assurent respectivement l'expression de l'AChE dans les tissus musculaire (*ace–1*) et nerveux (*ace–2*). Les gènes *ace–x* et *ace–y* ne sont séparés que de quelques centaines de paires de bases sur le chromosome II et leur rôle est pour l'instant inconnu. (©Elsevier, Paris)

acetylcholinesterases / Ace genes / *Caenorhabditis elegans* / nematodes

1. Introduction

Plant parasitic nematodes are devastating pathogens, responsible for an estimated annual $100 billion loss in crops worldwide [5]. Chemical nematicide treatments include carbamates and organophosphates that inhibit acetylcholinesterase (AChE) and lead to inhibition of the neuromuscular transmission and death. *Caenorhabditis elegans* is a free living nematode which constitutes however an useful model of parasitic species [12]. Whereas a single gene encodes AChE in vertebrates [20] and in the majority of insects [11], the situation is more complex in *Caenorhabditis elegans*. Initial studies clearly identified two kinetically distinct major classes of AChE, A and B, encoded by two genes: *ace-1*, located on chromosome X, and *ace-2*, located on chromosome I [7, 14, 15]. Later, a third class of AChE (class C, accounting for only 5% of total AChE activity) was characterized by its very low K_m for ACh and its high resistance to eserine. Class C AChE was shown to be encoded by *ace-3*, a locus on chromosome II [16]. Finally, a careful examination of class C mutants suggested the existence of a fourth class (D) of AChE that represented less than 0.1% of the total AChE activity [21]. Since no mutation in class D AChE was isolated, the corresponding *ace-4* locus was neither identified nor chromosome-mapped.

The complete coding sequences of *ace-1* in *C. elegans* [2] and in the related nematode species *C. briggsae* [9] have been reported. We now present additional data on the sequence of all *ace* genes [10], the tissue-specific expression of *ace-1* and *ace-2*, and on the peculiar genomic organization of *ace-x* and *ace-y*, two *ace* genes located in close association on chromosome II. Because of the close proximity of these two genes, we cannot yet identify which of these two genes encodes the class C AChE (and should be named *ace-3*), the other being *ace-4*.

1.1. Ace-1

Ace-1 was initially cloned by PCR with degenerate oligonucleotides deduced from the sequences EDCLYLN and FGESAG that are conserved in the whole cholinesterase family. The deduced amino acid sequence possessed all characteristic features of an acetylcholinesterase. The cDNA was expressed in Sf9 cells transfected by a recombinant baculovirus: the secreted enzyme had catalytic properties compatible with those of class A AChE [2]. Finally, using the strain p1000 of *C. elegans* that presents a dramatic reduction of class A AChE, we found a single nucleotide change in *ace-1* introducing a stop codon TGA in place of the codon TGG encoding W(84) ([24], conventional numbering of amino acids [19]). We have compared the sequences of *ace-1* in *C. elegans* and in the closely-related species *C. briggsae*. The coding sequence of *ace-1* in the two species is highly conserved, but intronic sequences and

3'UTR are largely divergent (Culetto et al., submitted). We then cloned a 2.4 kb fragment of the 5'UTR of *ace-1* in the two *Caenorhabditis* species, and found that transfection of *ace-1* gene including this upstream region was sufficient to restore a normal movement to the uncoordinated double mutant *ace-1⁻/ace-2⁻* of *C. elegans,* and a normal distribution of histochemically-detectable AChE activity (Culetto et al., submitted). In order to study the tissue-specific expression of *ace-1* during development and in the adult, we transfected translational fusions of 2.4 kb of *ace-1* 5'UTR and the reporter gene of green fluorescent protein (GFP). This construct is expressed in all body-wall muscle cells, in the three pharyngeal pm5 muscle cells, and in a few neurons of the head, tail and retrovesicular ganglia (Culetto et al., submitted). A comparison of conserved sequences in the 5'UTR in *C. elegans* and *C. briggsae,* and GFP expression driven by different deletions of this region, shows that a distal conserved block is responsible for the expression of *ace-1* in body-wall muscle cells, and that another block is responsible for the expression in pharyngeal muscle cells (Culetto et al., submitted).

1.2. Ace-2

No additional *ace* sequences were obtained by PCR using the initial sense primer EDCLYLN used for *ace-1*. We then used RT-PCR and degenerate primers deduced from the sequence GSEMW(84)N (*Torpedo*) and GTEMW(84)N (mammalian AChEs) in combination with the reverse primer FGESAG. Total mRNAs from the strain p1000 (null mutation in *ace-1*) were used, in order to reduce the annealing of the primers to *ace-1* mRNAs [24]. Two different clones (40 and 48) with the expected size (370 nt) were isolated that presented 52% and 53% identity with *ace-1* at the amino acid level (55% identity between clones 40 and 48). Probes from these two clones were successively hybridized on a YAC grid. This indicated that clone 40 hybridized with two YACs on chromosome I (Y44E3 and Y52G11), in a region compatible with the previous genetic mapping of *ace-2* [7]. The deduced amino acid sequence showed all characteristic features of an acetylcholinesterase (see legend to *figure 1*). A 2.5 kb fragment of the 5' flanking region of *ace-2* was cloned in *C. elegans* and used in translational fusions with GFP. Transfection of these constructs led to stable transformants, in which GFP was expressed in a large number of neurons but not in body-wall nor in pharyngeal muscles. Thus *ace-1* and ace-2 have a very different pattern of tissue expression.

1.3. Ace-x and ace-y

Clone 48 isolated above hybridized to YACs Y48B6 and Y59C8 of chromosome II near the locus *unc-52*, a position expected for *ace-3*. BLAST analysis of *C. elegans* sequences data base revealed no identity with clone 48, indicating that this region of the *C. elegans* genome had not been sequenced yet. However, we found two sequences homologous to clone 48 in the data base of *C. briggsae*. These two sequences were located in a single phosmid of *C. briggsae* DNA (i.e., in close location on genomic DNA). One of these sequence presented 94% identity with clone 48, and was clearly its homologue in *C. briggsae*. The other sequence, which was neither *ace-1* nor *ace-2*, and presented 74% identity with clone 48, was thus a new *ace* sequence. We amplified its homologue in *C. elegans* using RT-PCR, and showed that it hybridized to the same two YACs Y48B6 and Y59C8 as clone 48. We thus have two *ace* genes, located in very close proximity on chromosome II, in *C. elegans* and *C. briggsae*. One of these two sequences corresponds to *ace-3*, the gene encoding class C AChE but it is not possible, at the moment, to decide which sequence is *ace-3* and which one corresponds to a new *ace* gene (*ace-4*). These sequences are thus called *ace-x* and *ace-y*, *ace-x* being located upstream of *ace-y*. RT-PCR indicated that the two genes *ace-x* and *ace-y* are transcribed in both species of *Caenorhabditis* [10]. Genomic sequencing in *C. elegans* showed that there are only 356 nt separating the stop codon of *ace-x* and the initiator ATG of *ace-y* and only 200 nt between the polyadenylation signal of *ace-x* and the *trans*-splicing site of *ace-y*. *Figure 2* shows the genomic organization of *ace-x* and *ace-y* in *C. elegans*. The alignment of the four *ace* sequences from the initiator Met to the residue G202 is shown in *figure 1* [10].

2. Conclusions and perspectives

2.1. Ace-1 and ace-2

The apparent lack of effect of a single null mutation in either *ace-1* or *ace-2*, and the uncoordinated movements of the double null mutant *ace-1⁻/ace-2⁻* led to the conclusion that the two enzymes (class A and class B) are largely redundant, at least for the control of locomotion [7, 15]. In addition, the histochemical localizations of both enzymes are very similar [7]. Thus, the distinct pattern of tissue expression of the two genes, revealed with the reporter gene GFP, was unexpected. A simple explanation for the pattern of histochemical staining could be that

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ace-1  -----MRNSLLFFIFLPSTILAVDLIHLHDG---------SPLFGEEVLS----QTGKPL
ace-2  MRAPVIGRHLTYHVFCQFALVTLFIVRRIEPRSIVRGDHVVHTPLGTIRGVGQTFDGAKV
ace-x  -----MKPKLAFFAFFIFITVFIDSVQAVHP-------VVLETKLGDIKGTEFFFLSKKI
ace-y  ----MRRRRVLLLLLSTTTFLRVSTAQQDEPK---ASVVEVQTKLGTVRGTESDHGNKRV
                 .          . .   .                    .          .
                                                  ▼ O70          ●84
ace-1  TRFQGIPFAEPPVGNLRFKKPKPKQPWRIPLNATTPPNSCIQSEDTYFGDFYGSTMWNAN
ace-2  SAFLGVPYAKPPIGSRRFKMAEMIDRWSGELEARTLAKTCYLTIDSAFPQFPGAEMWNPP
ace-x  RTFFGVPFAEPAVEDFRFRKPREKKQWRGLYDATKPANACFQTRDNYNTSFWGSEMWNAN
ace-y  RSYLEVPFAEPPINEHRFKKPTPARPWNGTISANTLSPACFQGRDSYDPTFWGSEMWNAN
         .  .*.* * .   **.       *     *     .*     *      * *. ***
          ▼                          ●114   ●121     ●130
ace-1  TKLSEDCLYLNVYVPGKVDPNKKLAVMVWVYGGGFWSGTATLDVYDGRILTVEENVILVA
ace-2  GAISEDCLNMNIWVPED----HDGSVMVWIYGGGFFSGTPSLDLYSGSVFAAKEHTIVVN
ace-x  TQISEDCLYLNIWAPADA---YNLTVMVWFFGGGFYSGSPSLSIYDGKALTSTQNVIVVN
ace-y  TPVSEDCLYVNIWAPADA---YNLTVLVWLFGGGFWYGSPSLLLYDGKELATRGNVIVVN
         .***** .*.  *          .*.** .**** *. .* .* *   .  . *.*
          ■                     ■                          ▽200
ace-1  MNYRVSIFGFLYMNRPE-APGNMGMWDQLLAMKWVHKNIDLFGGDLSRITLFGESAG
ace-2  VNYRLGPFGFLYFGDDSPIQGNMGLMDQQLALRWVHENIGAFGGDRSRVTLFGESAG
ace-x  INYRLGPFGFLYLGHPD-APGNMGLLDQQLALHWVRQNIVSFGGNPDKVAVFGQSAG ◄
ace-y  INYRVGPYGYLFLDQED-VPGNMGMLDQQLALYWIRDHIFSFGGNPARISLVGESAG ◄
         .***.  .*.*.        ****. ** **. *.. .*  ***   .... *.***
```

Figure 1. Alignment of the N-terminal amino acid sequences deduced from *ace* genes in *C. elegans*: *ace-1*, *ace-2*, *ace-x* and *ace-y*. The sequences extend from the initiator Met to G202. The numbering of amino acids is that of *Torpedo* AChE [19] where residue 1 is the mature N-terminus. In the region shown, the first S-S bond between C67 and C94 (arrowheads), W84 (the choline-binding site) and the active S200 are conserved, as well as R149 and D172 (squares) involved in a salt bridge. Out of the five aromatic residues lining the catalytic gorge in this region of *Torpedo* AChE, only four are conserved or semi-conserved (84, 114, 121, 130, filled circles). The Y70 residue of *Torpedo* AChE is replaced by a non-aromatic residue in all *ace* genes (open circle), as in *Drosophila* AChE. Horizontal arrows show the motif FG**Q**SAG in *ace-x* and **V**GESAG in *ace-y*.

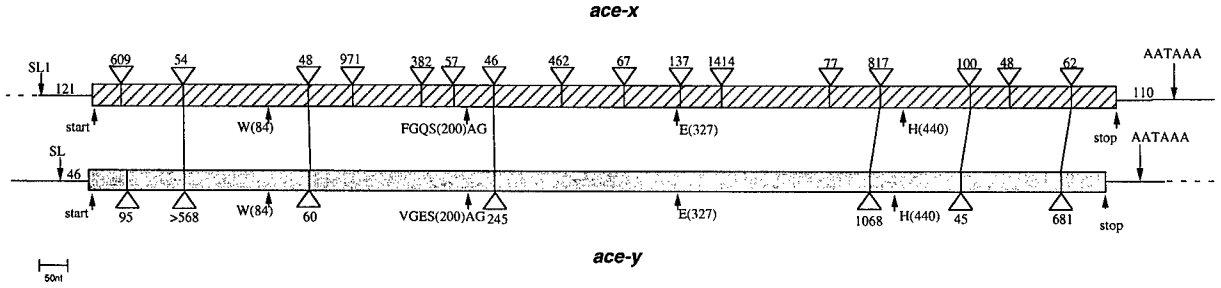


Figure 2. Genomic organization of *ace-x* and *ace-y* on chromosome II in *C. elegans*. *Ace-x* has 16 introns within the coding sequence against only 7 in *ace-y*. Six introns are in conserved position. A short insertion (8 amino acids) is present between introns 12 and 13 in *ace-x*. Note the short distance between the two contiguous genes: 356 nt between the stop codon of *ace-x* and the initiator ATG (start) of *ace-y*; 200 nt between the polyadenylation signal (AATAAA) and the site of *trans*-splicing (SL).

AChE is transported and preferentially accumulated at synapses, whether it is produced by muscle (class A) or nerve cells (class B).

2.2. *Ace-x and ace-y*

Another important result of our recent work is the identification of two additional *ace* genes (*ace-x* and *ace-y*), located close to each other on chromosome II, in both species of *Caenorhabditis*. The proximity of the two genes precludes, for the moment, the identification of *ace-x* or *ace-y* to *ace-3*, the only *ace* gene of chromosome II which has previously been defined in the literature. To allow a formal identification, sequencing of *ace-x* and *ace-y* in the known mutant strains of *ace-3* in *C. elegans* (alleles dc2, dc3, p1304, p1306 [7]) is currently under way. In vitro expression of *ace-x* and *ace-y* is also being performed for comparison of catalytic properties with those of class C [17]. The close arrangement of *ace-x* and *ace-y* in *C. elegans* genome is reminiscent of the organization of a number of polycistronic units (or operons) that have been described in this species [3]. In those units, a single promoter drives the coordinated transcription of several genes [3]. Whether this is the case for *ace-x* and *ace-y* is currently being studied.

2.3. *Features of ace genes in Caenorhabditis*

All *ace* sequences shared the following residues in conserved positions (numbering of *Torpedo* AChE, [19]): W84 as the choline-binding site; S200, E327 and H440 as the catalytic triad; three intrachain S-S bonds between C67-94, C254-265 and C402-521. Twelve (*ace-1*), eleven (*ace-2*) or thirteen (*ace-x* and *ace-y*) out of the fourteen aromatic residues lining the gorge in *Torpedo* AChE [2, 22] were conserved or semi-conserved. Interestingly, at the position of F288 in *Torpedo* AChE (a critical residue controlling the size of the acyl pocket and the substrate specificity of the enzyme), we found G in ACE-1 and M in ACE-2, whereas F288 was conserved in *ace-x* and *ace-y*. Both ACE-1 and ACE-2 enzymes hydrolyze significant amounts of butyrylthiocholine (see [14, 26]), and it will be interesting to test the substrate preference of the products of *ace-x* and *ace-y*. Also, the FGE(199)SAG motif present in *ace-1* and *ace-2*, as well as in all cholinesterases sequenced so far, is changed to FGQSAG in *ace-x* and VGESAG in *ace-y*. The directed mutation E199Q has been performed in vertebrate AChE (reviewed in [25]) and BChE [18]. In all cases, E199Q changes the inhibitor specificity, reduces the catalytic efficiency and the aging of the phosphorylated enzyme. Since *ace-3* is a major component of AChE in some phytoparasitic nematodes such as *Meloidogyne* [6], this possible resistance to aging could be important for managing the use of organophosphates against nematodes in agriculture.

The active serine of the four *Caenorhabditis ace* genes is encoded by the codon TCn as in *Drosophila* AChE, whereas it is encoded by AGy in all vertebrate and prochordate AChEs [4, 23]. This supports Brenner's hypothesis on the evolution of AChEs, that predicts the existence of two lines of descent that could have originated separately [4].

Class A AChE (encoded by *ace-1*) possesses a typical hydrophilic C-terminus, with regularly spaced aromatic residues, as in the T subunits of vertebrate AChE or BChE [9, 13, 20]. Such a C-terminal fragment might adopt the conformation of an amphiphilic alpha helix crucial for oligomerization [8, 20]. In contrast, *ace-2*, *ace-x* and *ace-y* encode hydrophobic C-termini, which however show no clear homology with those of vertebrate H subunits or *Drosophila* AChE. In vitro expression is needed to decide whether these enzymes possess a glycolipid anchor as it was shown for AChE of class B in the nematode *Steinernema* [1]. Finally a free cysteine residue is lacking in the C-terminal region of ACE-X and ACE-Y. Recent observations have shown that a C-terminal free cysteine is not a prerequisite for dimerization of AChE. Thus in vitro expression of the *ace-x* and *ace-y* cDNAs constitutes a valuable model to test the relative contribution of the four helix bundle (the only source of non-covalent interactions between subunits) and the interchain disulfide bond in the formation of dimers.

Acknowledgments

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Medical management of organophosphate-induced seizures

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Abstract — Recent studies concerning management of soman-induced seizures are reviewed. While drugs classically used against epilepsy in hospital appear ineffective against soman, muscarinic receptor blockers are shown to be able to prevent or stop seizures within the first 5 min after their onset. Benzodiazepine could also be considered as an emergency treatment useful during the first 10 min of seizure. Comparatively NMDA antagonists appear to be able to terminate soman-induced seizures even if the treatment is delayed after 40 min of epileptic activity. Drugs with both antimuscarinic and anti-NMDA properties may represent the most adequate pharmacological treatment to treat soman intoxication. However, the results obtained until now with these drugs must be completed in relation with their possible efficacy after i.m. administration. Propositions for future studies are reviewed. (©Elsevier, Paris)

Résumé — Traitement médical des crises d'épilepsie induites par les composés organophosphorés. Les recherches concernant le traitement médical des crises d'épilepsie induites par les composés organophosphorés sont passées en revue. Alors que les antiépileptiques classiquement utilisés en milieu hospitalier s'avèrent inefficaces face au soman, les antagonistes des récepteurs muscariniques sont capables de prévenir ou d'arrêter des crises épileptiques jusqu'à 5 min après leur déclenchement. Les dérivés benzodiazépiniques peuvent aussi être considérés comme un traitement d'urgence efficace durant les 10 premières min d'un état de mal. Comparativement, les antagonistes NMDA sont capables d'arrêter des crises même s'ils sont injectés 40 min apres leur déclenchement. Les composés présentant des propriétés mixtes anticholinergiques/antiNMDA pourraient donc s'avérer être les plus prometteurs pour le traitement d'une intoxication organophosphorée. Cependant, leur efficacité après injection intramusculaire, reste à être totalement évaluée. D'autres voies de traitement sont proposées. (©Elsevier, Paris)

soman-induced seizures / NMDA / epilepsy / organophosphate

1. Introduction

Centrally mediated seizures and convulsions are one of the toxic signs that occur following poisoning with organophosphorus (OP) anticholinesterase nerve agents such as soman (pinacolyl methylphosphonofluoridate). Originally, these convulsions were considered a factor that complicated definitive treatment of the more immediate life-threatening effects that nerve agents can have on respiratory function. In the past decade, however, it has become apparent that these seizures rapidly progress to status epilepticus, and contribute to the profound brain damage and cardiac pathology that can develop as a consequence of exposure to these highly toxic compounds. Effective management of nerve agent-induced seizures is critical for both immediate casualty treatment, and minimization of brain and cardiac damage, as well as a rapid and full recovery from the effects of agent exposure.

2. Non-specific management of seizures

In a first approach it seems logical to treat the epileptic syndrome induced by organophosphates like others epilepsies observed in hospitals. Tonic clonic status epilepticus can be defined as a condition in which prolonged or recurrent tonic clonic seizures persist for 30 min or more. Schematically, two main phenomena could be described in the genesis of neuronal hypersynchron activities with high frequencies: firstly, intrinsic neuronal phenomena leading to an hyperexcitability of neurons and to the generation of repetitive discharges of high frequency action potentials. This could be constituted by perturbations of the intracellular machinery and by changes of the properties or of the structure of neuronal membranes (particularly of complex proteins constituting ionic trans-membrane channels); secondly, inter-neuronal phenomena with a failure of the inhibitory GABAergic transmission and the stimulation of excitatory transmission.

2.1. Classical treatments

Classical treatments in hospital of status epilepticus is founded on the use of several drugs acting on neuronal membranes and modifying in a general manner the synaptic transmission. This leads to an attenuation of the formation and the propagation of abnormal excitations. These compounds have no di-

rect effect on the pathological processes underlying the epilepsy, i.e., in our case. no direct effect on the neurochemical mechanisms involved in the toxicity of soman. Classically used drugs to manage epilepsy are barbiturates, hydantoins, and valproate [1]. Benzodiazepines will not be included in this section since these products may be considered as acting on a specific target involved in the toxicity of organophosphates, i.e., the GABAergic system.

Phenobarbital, pentobarbital, diphenylhydantoin, carbamazepine, valoproate have been tested against soman [3, 4]. None of these drugs were effective in preventing soman-induced convulsions. Barbiturates were only effective at anesthetic doses (40 mg/kg and above). It thus appears that drugs classically used against epilepsy in hospital are totally ineffective against organophosphate intoxication in both preventive and curative treatments.

2.2. *Use of additional drugs*

Additional therapies are classically used during the treatment of generalized seizures in humans. Among these drugs only calcium channel blockers have been tested (flunarizine, nifedipine, verapamil) without success against convulsions induced by soman [2, 5, 6].

3. Specific management of soman-induced seizures

Due to the relative lack of activity of classical antiepileptic drugs (see above), it is important to use specific medications acting on a precise target involved in the development of seizures under soman. There is thus a need to firstly describe the precise mechanisms, known until now, underlying the toxicity of organophosphates (*figure 1*).

Nerve agent induced seizures are thought to be initiated by large increases in brain acetylcholine that occur after the inhibition of brain acetylcholinesterase by the agents [7, 8]. Increases in brain acetylcholine are capable of triggering recurrent seizure activity in susceptible brain circuits. If left unchecked, seizures rapidly recruit other neurotransmitter systems. The two neurotransmitter systems most critical to this second set of events appear to be the inhibitory GABA system and those involving the excitatory amino acids. Concerning the GABA system, there is a transient impairment of the $GABA_A$ receptor function at the beginning of seizures [9], suggesting that a temporary decrease of $GABA_A$ergic function may contribute to the early development of seizures (first 10 min). Recent work also emphasized the role of the glutamatergic system in the control of nerve agent-induced seizures. During soman poisoning ACh at a certain threshold triggers release of glutamate, since this release is under control of cholinoceptive cells [10, 11]. Glu concentration reached a maximal and sustained value in limbic areas after 70 min of seizure [12]. Glu release induces a rapid activation of AMPA receptors during the first 15 min of seizures leading then to a delayed, sustained activation of NMDA receptors in hippocampus [13]. In our opinion AMPA receptors are involved in the early propagation of seizures in combination with muscarinic cholinergic receptors (first 10 min of seizures). Our recent work suggests that AMPA and cholinergic receptors are activated firstly sequentially in the onset and the first 5 min of seizures and then in parallel during the propagation [14]. Then NMDA receptors are involved in the

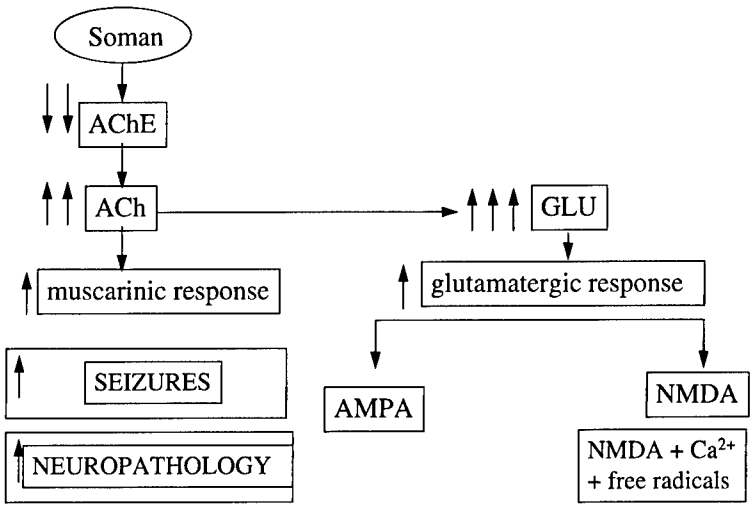


Figure 1. Neurochemical events underlying organophonosphate-induced seizures and neuropathology.

maintenance of epileptic activity and the apparition of subsequent neuropathology [14].

First of all, anticholinergic drugs will be presented. According to the sequence of events presented above, it seems logical to think that anticholinergic drugs will act during the genesis and the early propagation of seizures. Recent work with EEG recordings of McDonough and Shih [15] and Bodjarian et al. [16] demonstrate the ability of atropine to prevent or to stop seizures during the first 5 min of epileptic activity. Conversely, when given at a high dose after 10 min i.m., atropine fails to stop seizures [16, 17]. The anticonvulsant efficacy of atropine as measured by its ED_{50} against soman was evaluated in [15]. Administered 5 min after seizure onset the ED_{50} was about 3 mg/kg i.v. while it reaches 87 mg/kg after 20 min of seizures. Similar result were obtained with scopolamine. When scopolamine treatment was delayed for 20 min after seizure onset the ED_{50} for termination of epilepsy increased 100-fold compared to the value obtained 2–5 min after seizure onset. Based on these results, we assumed that 5 min of seizure activity is representative of the early cholinergic phase of seizure control. Management of seizures after 10 min of paroxystic activity with only anticholinergic drugs thus appears very difficult or impossible. The requirement for progressively higher drug doses, and the eventual failure of anticonvulsant activity by scopolamine and atropine at longer seizure durations could be due to several factors. First, greater drug doses may be required at longer seizure times to compete at the receptor with the progressively greater concentrations of endogenous acetylcholine that occurs after soman intoxication. Alternatively, or in conjunction with this first factor, it is possible that neuronal function has become critically compromised at the longer seizure times due to seizure-induced neuropathology and/or muscarinic receptor loss. A third alternative is that at the longer durations the seizure process is no longer maintained by cholinergic mechanisms.

In summary, muscarinic receptor blockers appears to be able to prevent or stop seizures within only the first 5 min of seizures. There is thus a necessity to develop other anticonvulsants able to counteract soman-induced seizures either during the genesis, the propagation or the maintenance of epileptic activity. Indeed, since the circumstances of poisoning cannot be predicted and may lead to delayed therapy, the search for drugs capable of arresting soman seizures after they are well-established still remains a crucial problem for the teams involved in medical management of OP intoxication.

The second purpose will focus on benzodiazepine products. Benzodiazepines are classically used in the treatment for status epilepticus in the hospital field. This type of molecule is used in first treatment before barbiturates, hydantdoin, carbamazepine. We know that the GABAergic system is involved during the first 10 min of seizures since a decrease of $GABA_A$ergic function has been observed [8]. It seemed thus logical to investigate the ability of benzodiazepine to counteract soman toxicity by enhancing GABA activity. Information from the reports of Lipp [18, 19], Rumps et al. [20, 21], Rumps and Grudzinska [22] and subsequently supported by the results of Lundy and co-workers [23, 24], Martin et al. [25] and others has indicated the efficacy of diazepam, a commercially available benzodiazepine anticonvulsant, in the treatment of OP poisoning, including soman. In 1991, Shih et al. demonstrated that benzodiazepines like diazepam, midazolam, clonazepam, loprazolam and alprazolam were ineffective in preventing soman-induced convulsions (observation of clinical signs) when used without adjunction of atropine [2]. A more recent study, done with EEG recording, demonstrated that diazepam injected alone i.v. was able to stop seizures induced by soman when used after 5–10 min of seizures [15]. However, for a large proportion of animals where diazepam was initially successful in stopping seizures, there was a reoccurrence of sustained seizure activity after varying periods of initial anticonvulsant effect. Globally the ability of benzodiazepines injected alone to counteract soman induced seizure activity seems limited to the first 5–10 min of status epilepticus thus confirming the transient participation the GABAergic system in the genesis of seizures. McDonough and Shih tested the ability of diazepam to stop seizures 20–40 min after their onset by using the benzodiazepine in conjunction with scopolamine i.v. [15]. Their results demonstrate that in these conditions diazepam continued to exert pronounced anticonvulsant effects at seizure durations of 20 min. However, at 40 min of seizure only four out of six animals stopped seizure and paroxystic activity reoccurred in one of these four animals. The anticonvulsant effectiveness of diazepam thus diminished significantly as a function of seizure duration. In an other study, Shih et al. also demonstrated a potentiation of the effect of diazepam by atropine sulfate. As the doses of atropine sulfate increased, the anticonvulsant ED_{50} for diazepam decreased [2].

Altogether, it thus appears that: a) benzodiazepine could be considered as an emergency treatment useful before or during the first 10 min of seizures in combination with atropine; and b) that there is an obvious necessity to develop new anticonvulsants able to stop seizures after 30–40 min. Indeed, it appears that the normal inhibitory function of the GABA system is overwhelmed by the massive stimulation and that the neurochemical events maintai-

ning seizure activity are not directly affected by the benzodiazepine products.

In this third part, the recent results obtained with drugs modulating the glutamatergic system will be given. Indeed, if the seizures are allowed to progress unchecked, epileptic process per se, via excessive neuronal depolarization, results in a high release of glutamate that activates AMPA and then NMDA receptors, which can respectively propagate and maintain seizure activity independent of the initial cholinergic activation [26].

In view of the development of a delayed treatment able to stop seizures 40–60 min in duration, EAA antagonists that act at the NMDA receptor, such as MK-801, TCP and procyclidine, have been shown to be effective anticonvulsants when given before or after initiation of nerve agent-induced seizures [2, 15, 27–30]. Studies over the past 6 years have focused on the use of non-competitive NMDA antagonists to counteract soman intoxication because several selective compounds were available which readily crossed the blood-brain barrier (BBB) and were shown to provide good anticonvulsant and neuronal protection (for example dizocilpine or TCP). Competitive antagonists were also chosen because their blocking action could not be overcome by high levels of glutamate released in synapses. The main results obtained with TCP were the following:

1) In the absence of previous atropinization, no clear protection against seizure and neuropathology produced by soman was obtained when a preventive single dose of TCP was administered in rodents. On the other hand, when administration of TCP was repeated once 1 h after soman injection, the anticonvulsant and neuroprotective activity of the anti-NMDA compound became maximal [31]. Taken together, this indicates that: a) neuroprotection by TCP is closely related to the anticonvulsive activity; b) a long, lasting blockade of the NMDA-receptor by the non-competitive inhibitor is necessary for suppressing soman-induced convulsions and related neuropathology; and c) that presence of atropine in the brain is not an absolute prerequisite for TCP to express its beneficial activity on soman poisoning, provided the NMDA-receptor blocker is maintained sufficiently long at suitable cerebral concentration.

2) The presence of a centrally acting muscarinic blocker, potentialized the anticonvulsant properties of TCP [27]. Thus, in conjunction with pretreatment consisting of pyridostigmine and anticholinergic therapy, TCP given 15 min prior to soman did not significantly prevent the onset of seizure activity. However, status epilepticus completely aborted within 30–40 min in all the animals tested. At the same time maximal beneficial effect on survival and clinical recovery were obtained. The inability of TCP to prevent the appearance of electrographic seizure manifestations before 30–40 min is not surprising if we consider the voltage-dependent mode of binding of PCP-like drugs on NMDA-receptor. TCP acts as an 'open channel blocker'. Its maximal binding is only obtained when PCP sites inside the associated ion channels are sufficiently accessible through depolarization, increase in concentration of glutamate and relief of Mg^{2+} blockade.

3) Due to the 'prerequisite' period of 30–40 min it is therefore not surprising to observe a better activity of TCP when used to terminate ongoing seizures than as a preventive treatment.

4) We have observed that soman-induced seizures became more difficult to arrest with TCP after 90 min of seizures suggesting action of unknown worsening factors.

Similar results were obtained by American groups showing that MK-801, another non-competitive NMDA antagonist, was effective in terminating seizures that had progressed for 20 or 40 min. This was observed in the presence of a pretreatment with scopolamine [15]. It is important to mention that the administration of an antimuscarinic drug is necessary to protect animals from the respiratory depressant effects produced by the interaction between soman intoxication and the non-competitive NMDA antagonist. Indeed, whatever the drug used, TCP or MK-801, an acute respiratory depression or a respiratory arrest, is observable in all intoxicated animals [15, 27]. This respiratory depression disappeared in the presence of atropine or scopolamine. In fact, that TCP and MK-801 may profoundly influence respiration is not surprising since the Glu system plays a pivotal role in central respiratory control and anti-NMDA drugs can elicit apneustic respiration through drastic changes in brainstem rhythmogenesis. However, how atropine sulfate or scopolamine may counteract the deleterious effect of TCP or MK-801 on central respiration. How co-administration of TCP or MK-801 and atropine sulfate may reverse the detrimental activity of soman on central and peripheral respiration remains to be understood.

In summary, NMDA antagonists are capable of terminating soman-induced seizures even if treatment is delayed 40 min after seizure onset. However, they must be used in combination with the anticholinergic drug both to assure a maximal antiepileptic capacity and to prevent a profound respiratory depression.

According to these data, research has also focused on several anticholinergic compounds blocking the toxic effect of NMDA in vitro and in vivo. These drugs thus possess NMDA antagonist activity as well as anticholinergic properties [32]. Such compounds potentially possess the ideal pharmacological proper-

ties for treatment of nerve agent seizures especially at long treatment delays. These agents including trihexyphenidyl, biperiden and benactyzine. Trihexyphenidyl and benactyzine have been tested by Usamricd in 1993 [15]. They were injected i.v. in intoxicated rats after 2–5 min to 40 min of seizure. Both compounds were capable of terminating soman-induced seizures even after 40 min of seizure activity. The termination of soman-induced seizures by benactyzine and trihexyphenidyl at the long seizure duration (20–40 min) in many respects resembled the anticonvulsant effects provided by the combination of scopolamine and MK-801 at the same time. The anticonvulsant activity of trihexyphenidyl and benactyzine against soman-induced seizures at these longer seizure durations may thus be ascribed to mixed anticholinergic and NMDA antagonistic properties of this drug. In 1991 [2], biperiden, trihexyphenidyl and benactyzine injected 30 min before soman were also shown to prevent the onset of soman-induced seizures with ED_{50} similar to that of scopolamine (0.10 to 0.2 mg/kg). It thus appears that the drugs with both antimuscarinic and anti-NMDA properties may represent the best adequate of a pharmacological treatment to treat soman-intoxication. However, the efficacy of these compounds injected i.m. and the neuroprotective effects of these drugs remain questionable. Indeed, in a recent study, McDonough et al. [3] investigated the ability of biperiden, benactyzine and trihexyphenidyl, injected in conjunction with atropine sulfate i.m., to counteract seizures when injected 5 or 40 min after their onset. It appears, for each drug, that, after 40 min of seizures, the ED_{50} for termination of epilepsy increased 20- to 40-fold compared to the value obtained 5 min after seizure onset. It is thus clear that the ability of mixed anticholinergic- anti-NMDA compounds, injected i.m., decreases as a duration of the epileptic activity.

4. Conclusion

Drugs classically used against epilepsy in hospital are totally ineffective against organophosphate intoxication.

Muscarinic receptor blockers appear to be able to prevent a stop seizures only within the first 5 min of seizures. There is thus a necessity to develop other anticonvulsants able to counteract soman-induced seizures either during the genesis, the propagation or the maintenance of epileptic activity.

Benzodiazepine could be considered as an emergency treatment useful before or during the first 10 min of seizures in combination with atropine.

NMDA antagonists are capable of terminating soman-induced seizures even if treatment is delayed 40 min after seizures onset. However, they must be used in combination with an anticholinergic drug both to assure a maximal antiepileptic capacity and to prevent a profound respiratory depression.

The development of an 'ideal' antiepileptic drug against soman must take in consideration two specific targets, i.e., muscarinic, NMDA receptors. In this view, drugs with both antimuscarinic and anti-NMDA properties may represent the most adequate pharmacological treatment to treat soman-intoxication. However, the efficacy of these compounds injected by i.m. route and the neuroprotective effects of these drugs remains questionable.

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Treatment of organophosphate poisoning. Experience of nerve agents and acute pesticide poisoning on the effects of oximes

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Abstract — Organophosphate (OP) compounds have been used as pesticides and in chemical warfare (nerve agents). Two nerve agents, tabun and sarine, were used by the Iraqi army against Iranian troops and innocent people. Hundreds of the exposed combatants died in the field. Atropine sulphate has been used successfully in large doses to counteract the muscarinic effects of OP poisoning. The effects of oximes in human OP poisoning have not been well studied. Our aim was to study the effects of obidoxime and pralidoxime in OP pesticide poisoning. The patients were divided into three groups: atropine (A), obidoxime + atropine (OA) and pralidoxime + atropine (PA). Sixty-three patients (33 males, 30 females) with a mean age of 25 years were studied in different groups (43 A, 22 OA and 8 PA). There were no statistical significant differences in major clinical findings and acetylcholinesterase (AChE) activity on admission between the groups. Significant changes were observed during the treatment. Notwithstanding the severity of intoxication – particularly respiratory complications were more observed in the OA and PA groups – there were no fatalities in the PA group, whereas 4 (9%) and 6 (50%) patients in the A and OA groups died, respectively. AChE reactivation was only observed in the PA group, although it was not statistically significant (r = 0.4747). There was a good relationship between the AChE reactivation and outcome of the patients. High doses of obidoxime (8 mg/kg followed by 2 mg/kg/h) were found to be hepatotoxic and should be avoided. High doses of pralidoxime (30 mg/kg followed by 8 mg/kg/h) did not induce serious side effects and may be effective in some OP pesticides poisoning. (©Elsevier, Paris)

Résumé — Traitement des intoxications par organophosphorés. Effet des oximes dans le cas d'intoxication par gaz de combat et pesticides. Les composés organophosphorés (OP) ont été utilisés comme pesticides et comme gaz de combat (agents neurotoxiques). Deux gaz de combat, appelés tabun et sarin, ont été utilisés par l'armée iraquienne contre les troupes iraniennes et des populations civiles. Des centaines de combattants ont péri sur le champ de bataille. Le sulfate d'atropine à fortes doses a été utilisé avec succès pour supprimer les effets muscariniques des organophosphorés. L'effet des oximes n'a pas été bien étudié dans le cas d'empoisonnements humains pour ces agents. Nous avons donc entrepris d'analyser des effets de l'obidoxime et de la pralidoxime. Les patients ont été divisés en trois groupes, recevant l'atropine seule (A), l'atropine avec l'obidoxime (OA) et la pralidoxime avec l'atropine (PA). Soixante trois patients (33 hommes et 30 femmes) d'âge moyen 25 ans, ont été traités (43 A, 12 OA et 8 PA). Il n'y avait aucune différence statistiquement significative entre les états cliniques et les niveaux d'acétylcholinestérase (AChE) entre les groupes lors de leur admission, mais des différences importantes sont apparues au cours du traitement. Bien que la sévérité de l'intoxication, en particulier les complications respiratoires, ait été plus marquée dans les groupes OA et PA, il n'y a pas eu de décès dans le groupe PA, alors qu'il y en a eu 4 dans le groupe A (9 %) et 6 dans le groupe OA (50%). Nous n'avons observé de réactivation de l'AChE que dans le groupe PA, et elle n'était pas statistiquement significative (r = 0,5). Nous avons observé une bonne corrélation entre la réactivation d'AChE et l'état des patients. De fortes doses d'obidoxime (8 mg/kg, suivi par 2 mg/kg/h) ont produit une hépatoxicité et doivent être évitées. De fortes doses de pralidoxime (30 mg/kg, suivi de 8 mg/kg/h) n'ont pas induit d'effets secondaires sérieux et peuvent être efficaces dans certains cas d'intoxication par les OPs. (©Elsevier, Paris)

organophosphate / oxime / nerve agent / pesticide / poisoning

1. Introduction

Organophosphate (OP) compounds have largely been used as pesticides in many parts of the world and were also applied in chemical warfare (nerve agents). During the Iraq-Iran war, several chemical attacks were made by the Iraqi army against the Iranian troops and even innocent people. Between August 1983 and March 1984, among the chemical warfare, a nerve agent called tabun was found in the environmental samples and in the patients who were severely intoxicated and died soon after exposure [5]. Toxicological analyses of the blood, urine, skin, hair and gastric juice in 1986 revealed sulfur mustard and tabun [7]. Later in 1987 and 1988, particularly during the Halabjah massacre, another nerve agent called sarine was also identified.

At the beginning of the chemical warfare attacks, particularly with tabun in early 1983 at Majnoon island, due to the lack of physical and chemical protections, hundreds of the Iranian combatants died in the field. Mortality was much lower later, after using protective clothing, masks and first-aid treatments including auto-injectors containing atropine sulphate.

OP pesticide poisoning has also been a health problem in Iran due to the lack of control on their importation, sale and safe use. Self-poisoning by OP pesticides has been a common cause of admission to the Mashhad Poison Treatment Centre [1] with high morbidity and mortality. Atropine sulphate has

been used successfully in large doses to counteract the muscarinic effects of OP poisoning. The effects of oximes in human OP poisoning have not been well studied. A retrospective study on the effects of oximes in OP pesticide poisoning [2] and a report from Sri Lanka [4] revealed no therapeutic effects. We therefore planned to carry out a prospective clinical trial on the effects of obidoxime and pralidoxime in OP pesticide poisoning.

2. Patients and methods

Patients (aged 14–60 years) with history of oral ingestion of a known OP pesticide who were admitted within 6 h and revealed clinical features of moderate to severe poisoning were investigated. Patients with previous severe organ disorders and those of mixed poisoning (except for the solvent of pesticide) were excluded.

Clinical features on admission and at regular intervals (2, 6, 12, 24 h and then daily) were recorded on a designed form for each patient. Following emergency decontamination and resuscitation on admission (if required), blood samples were taken for the estimation of acetylcholinesterase (AChE) and for the relevant biochemical and haematological investigations at certain intervals as clinically indicated. AChE was estimated in red blood cells (RBC) by a spectrophotometric method using the modified Ellman method [6].

The patients were divided into three groups. The first group received atropine and the other supportive and symptomatic standard treatment. The second and third groups were treated as the first plus oxime infusion as summarised in *table I*. Loading doses of oximes were given over 5 min followed by constant infusion (maintenance) in normal saline until atropine was discontinued. Atropine (IV) administration was given rapidly initially to induce mild to moderate atropinisation (dry mouth, tachycardia, flashing and midriasis within 30 min). It was then continued in constant infusion to maintain atropinisation and when possible in descending manner, based on clinical improvement until discharge. The patients who were severely intoxicated and required mechanical respiration were transferred to ICU for the period of critical care therapy. The other patients were treated in the ward.

The major clinical and paraclinical parameters including twitching and convulsions, respiratory arrest, required mechanical ventilation and ICU therapy, the rate of AChE reactivation, changes in ECG, total atropine dose used, hospitalisation days and mortality rate of the three groups were compared using standard statistical analyses (Chi-square and Student's *t*-tests) by the Statistical Package for Social Sciences (SPSS). The numerical results are expressed as mean ± standard deviation.

Table I. Treatment regimens of oximes (IV).

Oximes	*Loading dose (mg/kg)*	*Maintenance (mg/kg/h)*
Obidoxime (Toxogonon®)	8	2
Pralidoxime (Contrathion®)	30	8

3. Results

A total of 63 patients (33 male and 30 female) with ages of 25.1 ± 10.4 years were studied. More than 10 OP pesticides that had been taken by the patients were identified. The most common OP pesticides that were involved in different groups and the major clinical findings and AChE activity on admission in each study group are summarised in *tables II* and *III*.

There were no statistical significant differences in the major clinical findings and AChE activity on admission between the groups. However, there were statistically significant differences between the groups on recurrent twitching and convulsions, repeated respiratory arrest, required mechanical ventilation and ICU therapy, hospitalisation days and fatality (*table IV*).

Although the severity of intoxication, based on respiratory complications and hospitalisation days, was higher (< 0.05 to < 0.005) in the pralidoxime group, no mortality was found in this group whereas 4 (12%) and 6 (50%) patients died in the atropine and obidoxime groups, respectively (*table IV*). Three of the patients who received obidoxime developed hepatitis and two of them died due to the liver insufficiency. AChE activities of the atropine and obidoxime groups decreased from 1.7 ± 1.4 and 3.1 ± 2.7 U/mL of packed cells (PC) on admission to 0.8 ± 0.3 and 2.7 ± 1.3 U/mL of PC on the 10th day after admission, whereas it was increased from 1.7±1.6 to 2.6±1.1 U/mL of PC in the pralidoxime group over the same period. Although the AChE reactivation was not statistically significant in the pralidoxime group, there was a significant difference ($P < 0.05$) between the mean slopes of the AChE reactivation curves in the atropine and pralidoxime groups (*figure 1*).

Table II. The most common OP pesticides that had been taken intentionally by the patients.

Groups	*Atropine alone*	*Atropine + obidoxime*	*Atropine + pralidoxime*
Diazinon	+	+	+
Oxydemethon methyl	+	+	−
Malathion	+	+	−
Ethion	+	+	−
Azinphos methyl	+	−	+

Table III. Summary of major clinical findings and acetyl cholinesterase (AChE) activity on admission in different groups.

Groups / *Findings*	*Atropine alone*	*Atropine + obidoxime*	*Atropine + pralidoxime*
No. of Patients	43	12	8
Sex (M/F)	24/19	6/6	3/5
Age (mean ± S.D.)	25 ± 10.6	26.4 ± 12	23. ± 7.9
No. of Patient with:			
Twitching	20	5	3
Convulsion	1	2	1
Coma (grade 2–4)	11	6	4
Respiratory arrest	6	1	3
QTc prolongation (> 0.50 s)	12	3	3
AChE[a] (mean ± S.D., U/mL of PC)	1.7 ± 1.4	3.1 ± 2.7	1.7 ± 1.6

[a]Normal, > 4.2 u/mL of PC.

Table IV. Significant changes on the severity of OP intoxication during treatment and outcome of the patients.

Groups	*Atropine alone*	*Atropine + obidoxime*	*Atropine + pralidoxime*	*P-value*
No. of patients	43	12	8	–
Recurrent twitching and convulsions	4	2	3	0.005
Repeated respiratory arrest	2	3	4	< 0.005
Required mechanical respiration	2	4	2	< 0.05
Required I.C.U. therapy	7	4	5	< 0.05
Hospitalisation days (mean ± SD)	6.1 ± 3.7	7.5 ± 4.3	14 ± 10	< 0.05
Fatal cases	4	6	0	< 0.005

Atropine doses were given on admission to induce mild to moderate atropinisation (*table V*).Also, 24 h and total doses, together with the total administered oxime doses, are summarised in *table V*.

4. Discussion

Although the severity of OP intoxication on admission in the three groups was similar based on clinical findings and AChE activities (*table III*), significant changes were observed during the treatment (*table IV*). Due to the limited number of the cases studied so far in the pralidoxime group, it is difficult to make a good judgment. However, it is of interest that there was no fatality in the pralidoxime groups, whereas the mortality rate was very high (50%) in the patients receiving obidoxime. There is no doubt that the high dose of obidoxime used increased mortality, particularly due to the hepatotoxicity which was only observed in this group. Liver dysfunction and hepatotoxicity of obidoxime has already been considered by Bismuth et al. [3].

Unfortunately there has been no control on the importation, distribution and safe use of OP pesticides in Iran. Thus, a very wide range of them are almost freely used in the country. More than 10 different OPs were taken by the patients as self-poisoning in this study. Therefore, the number of patients who had taken one particular OP in each group was not enough for statistical comparison. However, the study is continuing and we may reach a conclusion in the future.

It seems that the reactivation of AChE is an important factor for the patient's recovery as judged by the 100% recovery in the pralidoxime group, in which the AChE reactivation occurred (*figure 1*). The anticonvulsant activity and the anticholinergic effects of oximes that were reported [9, 10] were not observed in this study. In fact the atropine doses required for atropinisation were higher rather than lower in the oxime groups (*table V*).

Table V. Administered atropine and oxime doses (mean ± S.D.).

Groups	*Atropine alone*	*Atropine + obidoxime*	*Atropine + pralidoxime*
Initial atropine dose (mg)	51 ± 39	74 ± 75	180 ± 335
24 h of doses of atropine (mg)	123 ± 109	343 ± 362	295 ± 425
Total atropine doses (mg)	504 ± 1553	701 ± 571	542 ± 525
Oxime doses (g)	–	14.0 ± 7.4	60.6 ± 24.3

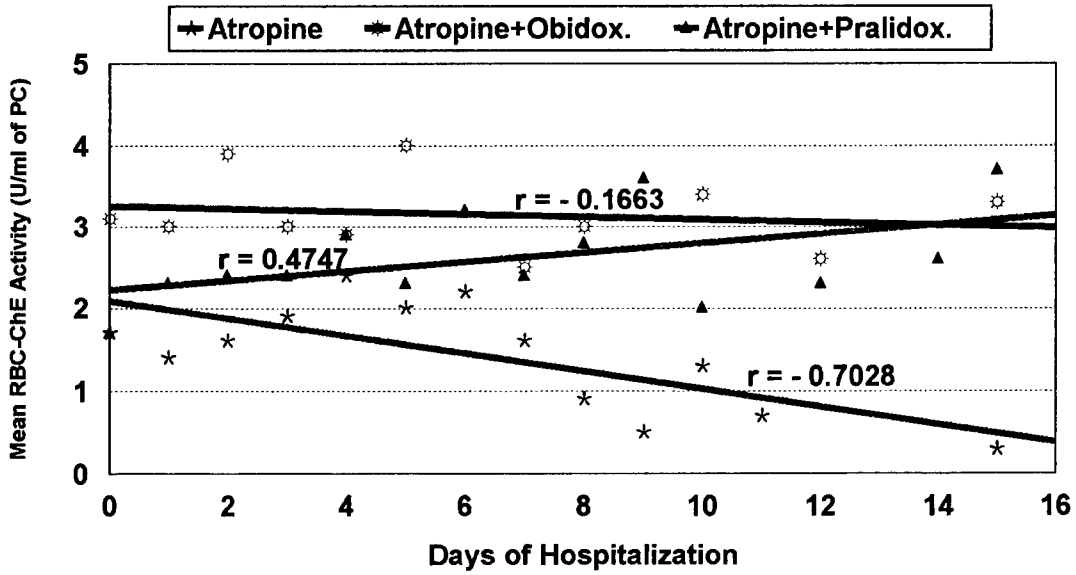


Figure 1. Mean acetyl cholinesterase (RBC) activity in the acute organophosphate poisoning.

High doses of pralidoxime revealed no serious side effects and significantly improved the patients outcome. It has also been used in children successfully without significant side effects in the Mashhad Poisons Treatment Centre and elsewhere [8]. Since it has been known that pralidoxime does not cross the blood-brain barrier (whereas obidoxime may do so) and thus can not reactivate the brain AChE, it can be concluded that the inhibition of central AChE is less important as suggested previously [3].

Based on the preliminary results of this study the following conclusions can be drawn: i) high doses of obidoxime may induce hepatotoxicity and can increase morbidity and mortality of OP poisoning. It is thus recommended to avoid using high doses of obidoxime; ii) high doses of pralidoxime revealed no serious side effects and since it reduces the morbidity and mortality, it could be used in OP poisoning; and iii) reactivation of AChE may play an important role for the outcome of OP poisoning.

However, further studies are required to investigate the effects of high doses of pralidoxime and low doses of obidoxime in common known OP poisoning. This is currently undertaken in the Mashhad Poison Treatment Centre of Iran.

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Influence of retinoic acid and of cyclic AMP on the expression of choline acetyltransferase and of vesicular acetylcholine transporter in NG108-15 cells

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Abstract — Treatment of the cholinergic cell line NG108-15 with retinoic acid or cAMP results in an increase of choline acetyltransferase activity (ChAT) whereas none of these agents influences the amount of the vesicular acetylcholine transporter (VAChT) as judged from vesamicol binding and immunoblot studies. We suggest that immaturity of posttranslational events controlling the expression of VAChT protein is responsible for the apparent absence of coregulation of ChAT and VAChT protein expression. (©Elsevier, Paris)

Résumé — Influence de l'acide rétinoïque et de l'AMP cyclique sur l'expression de la choline acétyltranférase et du transporteur vésiculaire d'acétylcholine dans les cellules NG108-15. Le traitement des cellules NG108-15 par l'acide rétinoïque ou l'AMP cyclique induit une augmentation de l'activité choline acétyltranférase (ChAT). Toutefois, la quantité de transporteur vésiculaire d'acétylcholine (VAChT), estimée par des mesures de liaison du vésamicol et par détection de la protéine sur immunoblot, n'est pas modulée par ces agents. Nous proposons l'hypothèse que l'absence apparente de corégulation des deux protéines ChAT et VAChT est due à l'immaturité de processus post translationnels régulant l'expression de VAChT. (©Elsevier, Paris)

choline acetyltransferase / vesicular acetylcholine transporter / NG108-15 / retinoic acid / dbcAMP

1. Introduction

A typical feature of cholinergic neurons is the presence of a set of specific proteins which is not found in other neuronal or non-neuronal cells. Two of these proteins, choline acetyltransferase (ChAT) and vesicular acetylcholine transporter (VAChT), i.e., the proteins which ensure the synthesis of acetylcholine (ACh) and the translocation and storage of ACh in synaptic vesicles, are essential traits of the cholinergic phenotype. A unique genomic organisation where the intronless VAChT gene is embedded within the first intron of the ChAT gene has recently been discovered in mammals [3, 12] and described as a 'cholinergic locus' [12]. This arrangement, which is conserved from the nematode [1] to man, offers an unusual template for a common regulation at the transcriptional level. Indeed, it has been reported that in primary cultures of sympathetic neurons, the activation of this locus by different treatments led to a phenotypic switch evidenced by a parallel increase in ChAT and VAChT mRNAs [5, 23] and functional ChAT and VAChT proteins [5]. Parallel upregulation of ChAT and VAChT mRNA expression by different factors has been described in cholinergic SN56 cells and parallel downregulation in PC12 cell line [6, 7, 25].

The cell line NG108-15 (mouse neuroblastoma N18TG-2 × rat glioma C6BU-1) displays traits regarded as characteristic for a cholinergic phenotype. Although the parental cells have no detectable ChAT activity, the hybrid cells were shown to be able to synthesise and release ACh [15] (for recent review, see Zhong et al. [32]) and thus may provide a useful model for studying basic aspects of the function of cholinergic neurons. However, these cells release only little of their endogenous ACh upon stimulation with a calcium ionophore bypassing voltage-operated calcium channels; the amount of ACh released becomes substantially higher after the cells had been loaded with exogenous ACh [18]. After differentiation of the cells with dibutyryl cyclicAMP (dbcAMP), the depolarization activated liberation of endogenous ACh increased [20, 21], whereas the release of loaded ACh activated by calcium ionophore remained unchanged [18]. Taken together it suggests that these cells have fully developed a releasing machinery but that the storage of ACh is defective. In line with this suggestion is the observation that the transfection of NG108-15 cells with cDNA for ChAT enhances ChAT activity and ACh content in the cells but does not increase either spontaneous or electrically evoked ACh release [31].

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In the present work we addressed the question whether differentiation factors known to increase the level of mRNAs for ChAT and VAChT in primary cultures and in cholinergic SN56 cell lines do induce the parallel appearance of functional ChAT and VAChT proteins in NG108-15 cells.

2. Results and discussion

2.1. Effect of retinoic acid and dbcAMP on ChAT activity

Several groups of agents have been shown to modulate ChAT expression on primary neuronal cell cultures and cell lines (for review see [30]). Among them, retinoids and cAMP were shown to enhance ChAT transcription [4, 7, 8, 16, 19, 22]. In the first set of experiments shown in *figure 1*, we tested the effect of retinoic acid and dbcAMP on the activity of ChAT and the synthesis of ACh from labelled choline. Consistent with previous reports on other cell lines, retinoic acid (1 μM) increased the activity of ChAT in NG108-15 cells (*figure 1A*). This increase was time dependent; it was already apparent after 48 h (2.5-fold increase) and it reached a plateau after 5 to 7 days (5-fold increase). Similarly, a dbcAMP treatment (1 mM) for 5 days also increased ChAT activity, although to a lesser extent (about 3-fold increase). Stimulation of ChAT activity by dbcAMP was concentration dependent (*figure 1C*). The concentration dependence curves showed that ChAT activity is increased at lower concentrations of dbcAMP than acetylcholinesterase (AChE) activity, which was only increased by 50% by 1 μM retinoic acid (*figure 1B*). Both treatments resulted in an augmentation of the accumulation of labelled ACh in the cells during a 30-min incubation in the presence of labelled choline (*figure 1D*). The extent of increase of labelled ACh content approximated that of ChAT activity. The accumulation of ACh was largely enhanced in controls and in treated cells as well, when an irreversible, permeable inhibitor of cholinesterases (paraoxon) was included in the incubation medium (*figure 1D*).

Our observation indicates that retinoic acid increases the expression of ChAT activity in NG108-15 to a larger extent than dbcAMP. Although we did not analyse the effects of these drugs on mRNAs levels, one can expect that the increased enzymatic activity is a consequence of enhanced translation, as it has been observed in the same cell line with cAMP [16, 22] and in other cell lines with retinoids [7].

2.2. Effect of retinoic acid and dbcAMP on VAChT

In the next experiments we followed the expression of VAChT protein in NG108-15 grown in basal medium or in the presence of retinoic acid or of dbcAMP. We first measured the level of VAChT using a binding assay with vesamicol as a specific ligand [2, 27]. Control cell membranes did bind vesamicol and the binding parameters did not appear to depend on the time in culture between 3–6 days. Pooled values of B_{max} and K_d in controls were 3.92 ± 0.36 pmol/mg protein and 128 ± 21 nM (n = 7), respectively. The dissociation constant of vesamicol binding is rather high compared to values found in synaptic vesicles isolated from *Torpedo* electric organ [9, 24] and in membranes of transfected cells expressing *Torpedo* or rat VAChT [12, 27]. Treatment of the cells with either retinoic acid for 3–6 days or with dbcAMP for 6 days had no significant effect on the binding of vesamicol (*figure 2A,B*). The values for B_{max} and K_d (*figure 2*C, D) were 91 ± 20% and 73 ± 12% (n = 5) of paired controls for retinoic acid treatment and 98 ± 42% and 90 ± 18% (n = 2) for dbcAMP treatment, respectively.

We next evaluated the level of VAChT expression using Western blot analysis. VAChT protein was detected with an immunopurified polyclonal antibody directed against the C-terminal part of the human VAChT [28]. The protein pattern recognised by the antibody was unexpected. Rat and human VAChT have a predicted molecular mass around 56 kDa and are highly glycosylated. They appear on immunoblot as a diffuse and heterogeneous band between 55 and 75 kDa [5, 13, 17, 28]. In NG108-15, the major band which is recognised by the antibody is a peptide with a smaller molecular mass of around 33 kDa, while only faint staining is found around 56 kDa. This small peptide is most probably a proteolytic fragment of the protein as it does not correspond to the N-deglycosylated form of VAChT which appears as a doublet around 39 kDa [29]. A similar peptide pattern was observed using an antibody directed against the C terminus part of the rat VAChT (data not shown). In line with vesamicol binding experiments, neither retinoic acid (*figure 3*) nor dbcAMP (data not shown) increased the level of the peptides which are detected by the antibody. *Figure 3* also shows that the level of synaptophysin, a synaptic vesicle marker, remained unchanged after retinoic acid treatment.

Immunofluorescence studies showed that VAChT staining appears as large dots completely different from the finely punctuate pattern of synaptophysin staining (*figure 4*) and from the pattern of VAChT staining which is observed in transfected N18 and AtT20 cells expressing Torpedo VAChT [29].

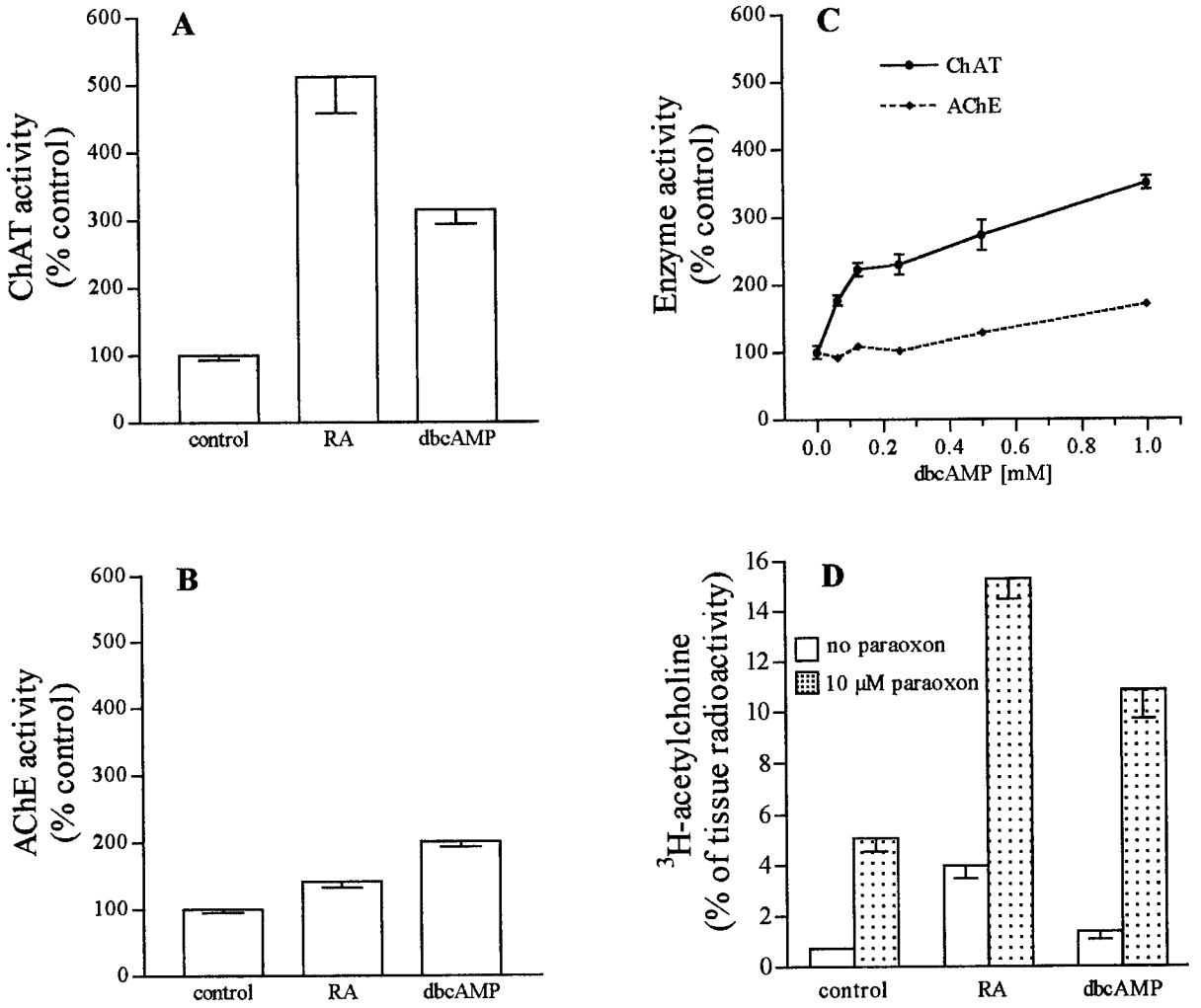


Figure 1. Effect of retinoic acid and dbcAMP on ChAT and AChE activities, and on ACh content. NG108-15 cells were grown for 5 days without addition (control) or in the presence of 1 µM retinoic acid (RA) or 1 mM dbcAMP. **A, B.** The cells were washed, disrupted in 10 mM sodium phosphate buffer, pH 7.4, and aliquots of this homogenate were used for the determination of ChAT activity (**A**) according to Berrard et al. [5] and AChE activity (**B**) as described by Ellman et al. [11]. **C.** The cells were grown for 5 days in the presence of increasing concentrations of dbcAMP and the activities of ChAT and AChE were assayed. The results in **A–C** are expressed in percentage of corresponding controls (n = 4–20). Control value of ChAT activity was 1.09 ± 0.09 nmol of ACh/mg protein × 15 min (n = 24) and that for AChE was 1.92 ± 0.08 of arbitrary units/mg protein × 20 min (n = 36). **D.** The cells were washed and then incubated for 30 min at 37°C in HEPES buffered saline containing tritiated choline either with or without 10 µM paraoxon. At the end of the incubation they were washed in fresh saline and extracted into 5% TCA. The content of labelled acetylcholine in TCA extract was determined as described by Doležal and Tuček [10] and is expressed in percentage of the total incorporated radioactivity (n = 2–8).

Figure 2. Effect of retinoic acid and dbcAMP on the binding of vesamicol. The cells were grown either in control conditions or in the presence of 1 µM retinoic acid (RA) for 3–6 days or in the presence of 1 mM dbcAMP for 6 days. The binding of vesamicol was measured on membrane preparation as described by Varoqui et al. [27]. **A.** Average curves of vesamicol binding from six independent experiments in controls and after treatment with RA. Kinetics parameters for control are B_{max} = 3.48 ± 0.10 pmol/mg protein and K_d = 99.3 ± 4.1 nM (mean ± S.E.M.). **B.** Average curves of vesamicol binding from two independent experiments in controls and after treatment with dbcAMP. Kinetics parameters for control are B_{max} = 3.51 ± 0.46 pmol/mg protein and K_d = 71.5 ± 0.5 nM (mean ± S.E.M.). **C, D.** Bmax and K_d values expressed in percentage of paired controls.

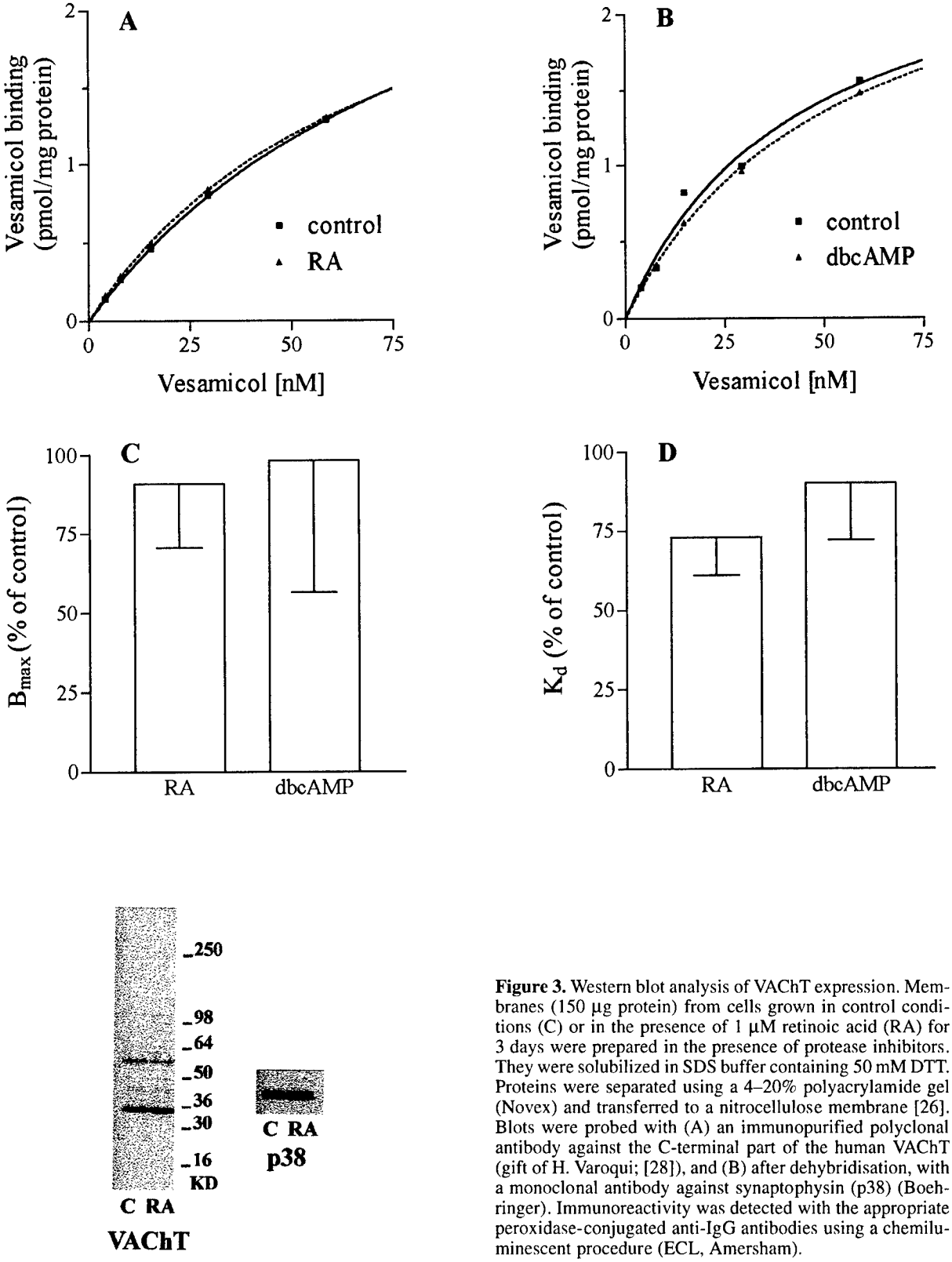


Figure 3. Western blot analysis of VAChT expression. Membranes (150 μg protein) from cells grown in control conditions (C) or in the presence of 1 μM retinoic acid (RA) for 3 days were prepared in the presence of protease inhibitors. They were solubilized in SDS buffer containing 50 mM DTT. Proteins were separated using a 4–20% polyacrylamide gel (Novex) and transferred to a nitrocellulose membrane [26]. Blots were probed with (A) an immunopurified polyclonal antibody against the C-terminal part of the human VAChT (gift of H. Varoqui; [28]), and (B) after dehybridisation, with a monoclonal antibody against synaptophysin (p38) (Boehringer). Immunoreactivity was detected with the appropriate peroxidase-conjugated anti-IgG antibodies using a chemiluminescent procedure (ECL, Amersham).

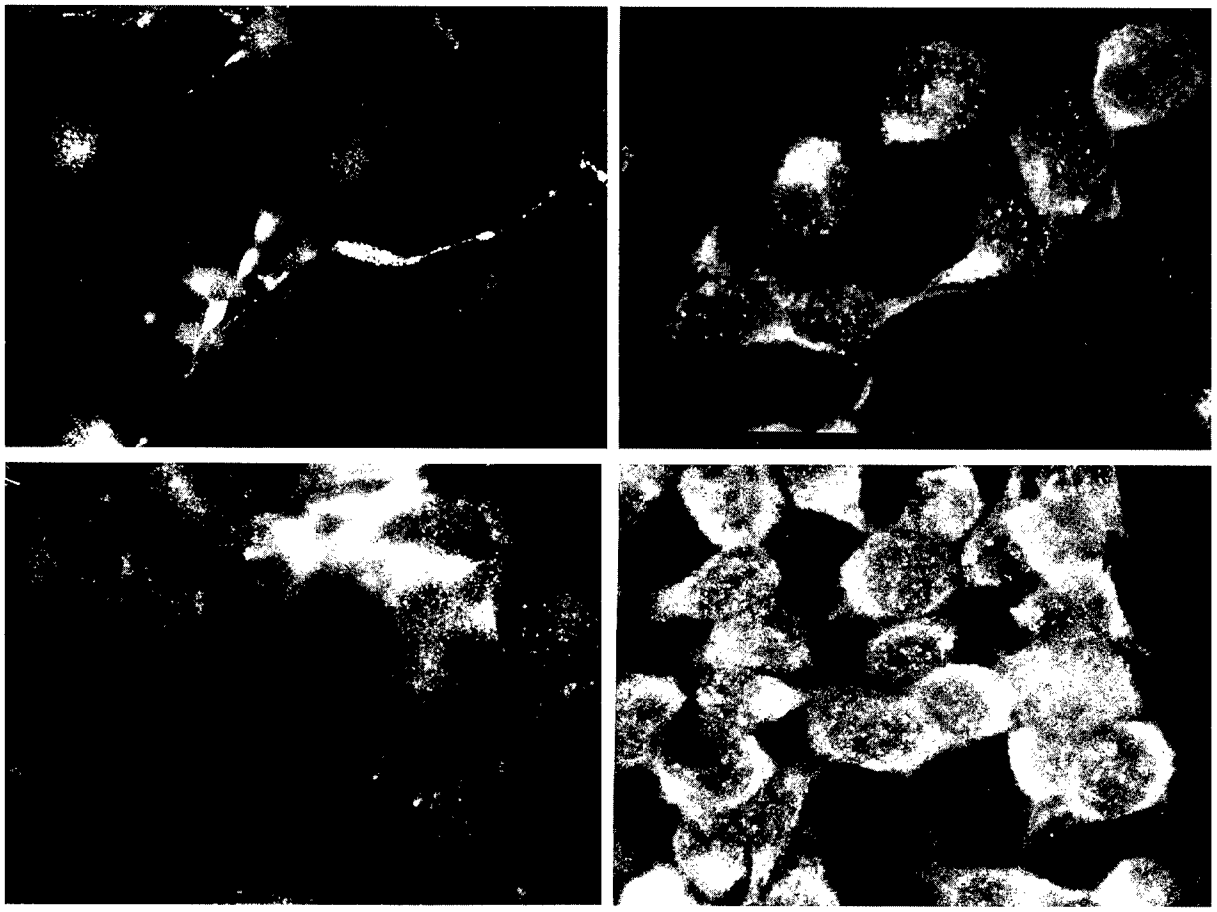


Figure 4. Immunocytohistochemistry of VAChT. Cells grown on coated glass coverslips in basal medium (upper part) or in the presence of 1 μM retinoic acid (lower part) were fixed and permeabilized with cold (–20°C) acetone. They were then incubated with a polyclonal rat VAChT antibody (right) or with an antisynaptophysin antibody (Boehringer) (left). Binding of primary antibodies was detected with suitable secondary antibodies. Immunofluorescence images were viewed with appropriate filters using an epifluorescence microscope.

3. Conclusions

The effects of cholinergic differentiation agents on cell lines have previously been estimated on the basis of ChAT and VAChT mRNA levels and ChAT activity. To our knowledge, their effect on the expression of the VAChT protein has not been reported. The present data indicate that in NG108-15 cells, the VAChT protein as recognised by antibodies and as judged from vesamicol binding, has unusual characteristics. Preliminary experiments revealed similar results on VAChT protein in SN56 (gift of B.Wainer; [14]) where transcription of VAChT is coregulated with that of ChAT [7]. These observations suggest that most of the protein is not correctly processed, either because the cells are unable to perform due posttransductional modifications such as N-glycosylations, and the protein is thus rapidly degraded, or that the targeting of the protein is compromised. Moreover, it has been reported that during the development of rat brain, there is a parallel increase of ChAT mRNA and ChAT activity whereas the appearance of a mature VAChT protein as detected on immunoblots lags behind VAChT mRNA transcription by several weeks [17]. Consistent with these observations, we suggest that hybrid cells as NG108-15 are incapable to undergo sufficient differentiation allowing a proper processing of VAChT and/or its incorporation into vesicle membrane. The mechanisms which participate in the posttranslational control of the expression of VAChT are still to be understood.

Acknowledgments

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From the cholinergic gene locus to the cholinergic neuron

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Abstract — The cholinergic gene locus (CGL) was first identified in 1994 as the site (human chromosome 10q11.2) at which choline acetyltransferase and a functional vesicular acetylcholine transporter are co-localized. Here, we present recent neuroanatomical, developmental, and evolutionary insights into the chemical coding of cholinergic neurotransmission that have been gleaned from the study of the CGL, and its protein products VAChT and ChAT, which comprise a synthesis-sequestration pathway that functionally defines the cholinergic phenotype. (©Elsevier, Paris)

Résumé — Du locus génétique cholinergique au neurone cholinergique. Le locus génétique cholinergique (cholinergic gene locus, CGL) a été identifié en 1994 et regroupe les gènes de la choline acétyltransférase et d'un récepteur vésiculaire d'acétylcholine (chromosome humain 10q11.2). Nous présentons ici des données sur la neuroanatomie, le développement et l'évolution de la neurotransmission cholinergique obtenus à partir de l'étude du CGL et de ses produits protéiques, VAChT et ChAT : ce système de synthèse-séquestration définit fonctionnellement le phénotype cholinergique. (©Elsevier, Paris)

synaptic vesicle / acetylcholine / VAChT / ChAT / peripheral and central nervous system / ontogeny / cholinergic differentiation

The cholinergic gene locus (CGL) comprises an approximately 80 kb region of the mammalian genome that encodes choline acetyltransferase (ChAT), the vesicular acetylcholine transporter (VAChT) and the regulatory sequences responsible for expression of VAChT and ChAT in cholinergic neurons. The CGL is so named because it encodes the two proteins that functionally define the cholinergic neuronal phenotype, within a single regulatory domain [16]. Our current view based on extensive analysis of the rat and primate nervous systems is that VAChT and ChAT mRNA and protein are invariably co-expressed, and thus are both adequate markers for the cholinergic phenotype (see [3] for review). VAChT immunohistochemistry offers certain advantages in surveying fixed tissue, and has allowed the unambiguous identification of peripheral muscular vasculature and the hypothalamus in the central nervous system, as targets of cholinergic innervation ([10, 11] and references therein). Most notably, VAChT immunohistochemistry has revealed that noradrenergic and cholinergic phenotypes are co-expressed in developing autonomic neurons of both the parasympathetic and sympathetic lineages. The former become exclusively cholinergic and the latter mainly noradrenergic through a process of phenotypic restriction, rather than noradrenergic/cholinergic 'switching', during development [12, 13]. Improved VAChT immunohistochemistry is perfectly suited to delineate peripheral cholinergic neurons and terminals and to characterize peptidergic cotransmitters. Our recent immunohistochemical and in situ hybridization studies indicate that virtually all enteric neurons of the upper gastrointestinal tract co-express VIP and acetylcholine, suggesting that the 'non-adrenergic/non-cholinergic (NANC)' component of the enteric nervous system is much less extensive than previously believed (Anlauf et al., unpublished observations). VAChT immunoreactivity is present in interneurons of both rodent and primate cerebral cortex ([10] and references therein). In addition, the patterns of cholinergic innervation of the primate cortex, now examined in detail in paraffin-embedded tissues, suggest a decidedly lamina- and area-specific cholinergic innervation. A detailed picture of the cholinergic innervation of the telencephalon provides the foundation for a clearer understanding of cholinergic function in cognition, arousal, and neurodegenerative diseases such as Alzheimer's disease. With respect to the suggested neuroinflammatory background of these diseases it is of interest that the prostaglandin synthesizing enzyme cyclooxygenase 2 (COX-2) is preferentially co-expressed with VAChT in primate cholinergic projection neurons of the basal forebrain (Depboylu et al., unpublished observations).

While expression of both VAChT and ChAT from the CGL is required for the cholinergic phenotype, non-concordance in VAChT and ChAT expression in vivo could function as a mechanism to fine-tune cho-

linergic function. For example, the ratio of VAChT to ChAT mRNA in at least some parts of the peripheral nervous system of the adult rat is higher than in the central nervous system (*figure 1*). This may be related to an early pattern of VAChT overexpression relative to ChAT both centrally and peripherally during development [6] (Schütz et al., unpublished observations). How might the CGL be regulated at the transcriptional level to allow differential expression of these two gene products, and to what purpose? In the nematode, VAChT and ChAT are transcribed from separate messenger RNAs that arise from a common primary transcript, with presumably a single transcriptional start site, via differential splicing [2]. This is also the case in *Drosophila* [7]. However, the ratio of VAChT to ChAT mRNA is quite different in the head and body of *Drosophila* [7], similar to the situation in peripheral versus central cholinergic neurons in mammals. Various laboratories have inventoried VAChT and ChAT transcripts of the central and peripheral nervous systems, or cell lines derived therefrom, and the segments of the CGL that are responsible for VAChT and/or ChAT expression in transgenic animals and cell lines (see [3, 18, 20] for reviews). At present, it appears that separate promoter/enhancers may drive VAChT and ChAT expression in a cell-specific manner, while a common exon for VAChT and ChAT (the 'R' exon) suggests that transcription from a single start site generating alternatively spliced VAChT and ChAT transcripts also occurs. Which is the dominant transcriptional mode in mammals? The answer may depend in part on physiological and anatomical context. In the brain, 'R' exon expression is much less than that for VAChT or ChAT orf-containing mRNAs (*figure 2*) This implies that 'R' may be a minor transcriptional pathway, and that separate promoters drive expression of VAChT and ChAT from the CGL, at least under normal physiological conditions in the adult mammalian central nervous

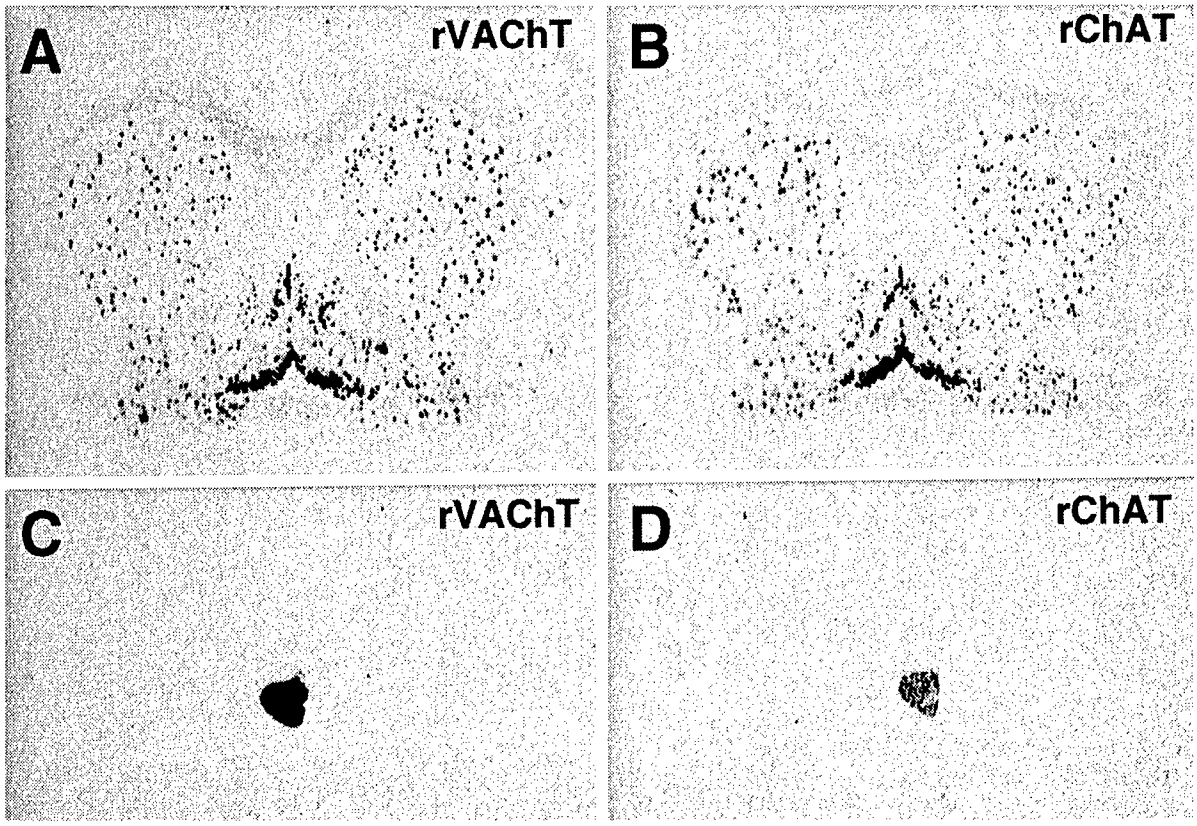


Figure 1. Comparative expression of VAChT and ChAT mRNA in brain and parasympathetic ganglion of adult rat. VAChT (**A**) and ChAT (**B**) mRNA levels are similar in adjacent brain sections. Note higher levels of VAChT mRNA (**C**) as compared to ChAT mRNA (**D**) in adjacent sections of the otic ganglion. Rat (r) VAChT and rChAT riboprobes of uniform size were synthesized and calibrated for hybridization efficiency as described by Hahm et al. [5].

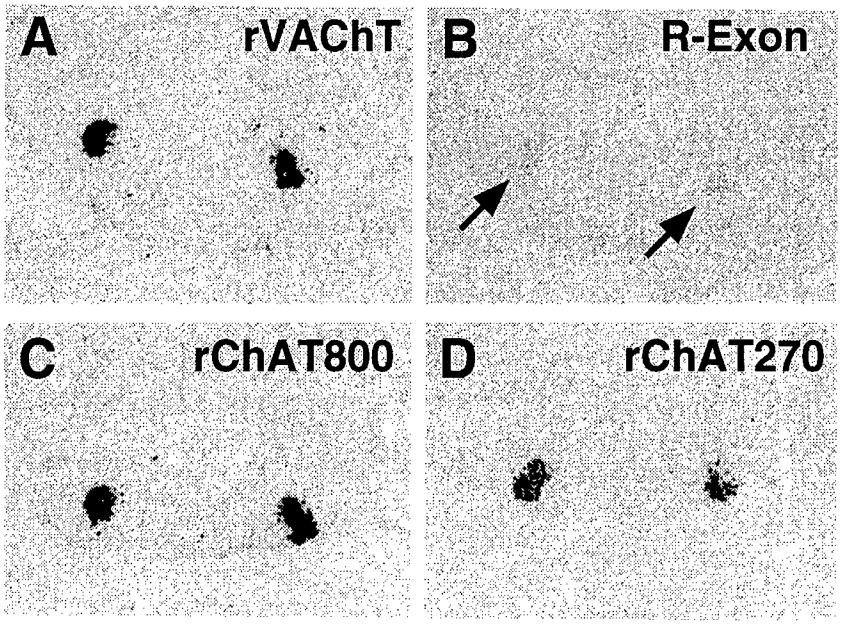


Figure 2. Relative expression of the R, VAChTorf, and ChAT first coding exon in the rat central nervous system. Sequential sections through rat brain stem hybridized with specific probes for RVAChT orf (**A**), R-exon (**B**) and rCHAT orf (**C, D**). Note the faint hybridization signal for the R-exon. Data from Hahm et al. [5].

system. Consistent with this possibility, Naciff et al. report that a genomic fragment that excludes the R exon and includes only about 600 bases of the CGL upstream of the ATG that initiates the VAChT orf is sufficient for correct cholinergic expression in vivo [8].

The catalytic properties of the VAChT and ChAT proteins themselves suggest that precise regulation of the CGL is required to maintain cholinergic function both centrally and peripherally. The affinity of acetylcholine for the transporter is reckoned to be approximately 1 mM [17, 18], close to the estimated concentration of acetylcholine synthesized in the cytoplasm of motor neurons by ChAT. Thus changes in the ratio of VAChT to ChAT mRNAs, if reflected in differential protein abundance, could directly affect the size of ACh quanta in cholinergic neurons. Increased cholinergic quantal size in *Xenopus* neurons expressing human VAChT support this assertion [14]. VAChT is targeted to small synaptic vesicles (SSVs) both in vivo and in cell lines [4, 19], yet appears to be transported to the nerve terminal via large dense-core vesicles [15]. The vesicular monoamine transporter type 2 (VMAT2) localizes preferentially to large dense-core vesicles (LDCVs) in PC12 cells, and appears to be transported to the nerve terminal in LDCVs as well. Since VMAT2 is localized to both small and large synaptic vesicles in brain [9], PC12 cells apparently produce 'cholinergic' but not 'noradrenergic' SSVs. This finding implies that there are special properties of each type of vesicle besides the mere presence of the appropriate transporter. Why so many different kind of vesicles? In cholinergic neurons, differential release of ACh and neuropeptides from two different vesicular compartments provides variation in the ratios of ACh and VIP, for example, released at different rates of stimulation (e.g., [1]). Differential targeting to subpopulations of small synaptic vesicles may also be physiologically relevant, for example at developmental stages in which autonomic neurons express both cholinergic and noradrenergic traits [13].

Expression of VAChT and ChAT from the CGL, which makes a neuron primordially cholinergic, also makes the cholinergic neuron amenable to genetic, ontogenic, pathophysiological, and neuroanatomical analysis. Understanding the transcriptional, translational and biochemical regulation of these two proteins ultimately will aid in unlocking the mechanisms by which chemically coded transmission arises during development, serves the needs of the organism, and perhaps goes awry in motor and cognitive neurodegenerative diseases.

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A syntaxin-SNAP 25-VAMP complex is formed without docking of synaptic vesicles

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Abstract — We show herein that syntaxin is already associated with SNAP 25 and VAMP during fast axonal transport, and in isolated synaptic vesicles, before docking of these secretory organelles at the active zones. (©Elsevier, Paris)

Résumé — Syntaxine, SNAP 25 et VAMP s'associent en absence d'amarrage des vésicules synaptiques à la membrane des terminaisons nerveuses. Nous montrons ici que la syntaxine est déjà associée à la SNAP 25 et la VAMP pendant le transport axonal rapide, et dans des vésicules synaptiques isolées, en absence d'amarrage de ces organites de stockage à la zone active. (©Elsevier, Paris)

axonal transport / SNARE complex / VAMP / SNAP 25

1. Introduction

In motor nerve endings, clusters of synaptic vesicles docked to specialized areas of the presynaptic plasma membrane, the active zones, can be observed [1]. According to the SNARE hypothesis [10], two proteins of the presynaptic membrane, syntaxin and SNAP 25, and a synaptic vesicle protein, VAMP, ensure the specificity of the docking and fusion of the neurotransmitter storing organelles to the presynaptic membrane through the formation of a multiprotein complex. Several cytosolic or membrane proteins will bind sequentially and reversibly to proteins of the SNARE complex during synaptic activity. Using a homogeneous population of cholinergic nerve endings prepared from *Torpedo* electric organ, we showed that syntaxin was recovered in the form of large 16S protein complexes, 65% of SNAP 25 and 15% of VAMP being associated with syntaxin [13].

Synaptic vesicle and presynaptic plasma membrane proteins are synthesized in the neuron cell body and transported to the nerve endings in intraaxonal vesicular membranes. VAMP, syntaxin and SNAP 25 are conveyed by the fast axonal anterograde flow [5, 11]. In order to know if the SNARE complex is assembled only in the nerve endings, we ligated the electric nerves which innervate the electric organs and looked for interactions between syntaxin, SNAP 25 and VAMP which have accumulated upstream from the axonal block. In addition, we looked in purified fractions of isolated cholinergic synaptic vesicles for the presence of syntaxin and SNAP 25, which participate in the formation of SNARE complex as has been described in brain synaptic vesicles [8].

2. Methods

The anti-syntaxin monoclonal antibodies 10H5 and HPC1 were obtained from Dr M. Takahashi and Sigma Ltd. respectively, anti-SNAP 25 antiserum from Alomone Ltd. (Israel). Anti-VAMP monoclonal antibodies (19K1, 19K2, 19K7) and anti-synaptophysin antiserum were prepared in our laboratory.

Synaptosomes were isolated from *Torpedo marmorata* electric organ as previously described [6]. After hypoosmotic lysis of synaptosomes in buffer A (10 mM Tris buffer, pH 8, 1 mM EDTA, 0.5 mM dithiothreitol, 1 mM PMSF), whole synaptosomal membranes were diluted (1 mg protein/mL) in buffer B (100 mM KCl, 10 mM Tris buffer, pH 7.2, 1 mM EDTA, 0.5 mM dithiothreitol, 10% glycerol).

Synaptic vesicles were purified according to Diebler and Lazereg [2]. In ligature experiments, *Torpedoes* were anesthetized (1 g MS 222 (Sandoz) in 3 L sea water). The first electric nerves were ligated where they run around a cartilaginous apophyse. The skin was stitched and the fish kept in sea water at 16 °C. Ligatured nerves were dissected out 48 h later. The fast axonal flow is about four times slower in the case of *Torpedo*, a cold blooded animal, than for mammals. Nerve pieces (1 cm long) were homogeneized (100 mg tissue/mL buffer B) in a glass/glass homogeneizer. Large debris was removed by a low speed centrifugation (700 $g_{max} \times 5$ min).

Membranes (from synaptosomes or electric nerves) were either directly immunoprecipitated by anti-VAMP magnetic immunobeads (antibody 19K2 bound to goat anti-mouse Ig antibodies linked to M450 Dynabeads) essentially according to manufacturer's protocol (Dynal). Or they were solubilized in buffer B (1 mg protein/mL) by addition of Chaps (30 mM final concentration). In some experiments, membranes (2 mg

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protein/mL in buffer A) were solubilized in SDS (0.5% final concentration) and then 10-fold diluted in 0.5% Triton X-100 in buffer B. Solubilized proteins were recovered in a high speed supernatant and subjected to immunoprecipitation by anti-syntaxin or anti-VAMP immunobeads or to velocity sedimentation through continuous sucrose gradients [11, 12].

After SDS-polyacrylamide gel electrophoresis and electrotransfer, blots were probed with various antibodies, whose binding was indirectly visualized using peroxidase conjugated anti-mouse or rabbit Ig antibodies (Institut Pasteur Production) and the Enhanced ChemiLuminescence system (Amersham).

3. Results and discussion

Recently, we have checked that several presynaptic antigens are transported by the fast axonal flow in *Torpedo* electric nerves and have measured their accumulation upstream from an axonal block [11]. The accumulation in the nerve segment just proximal to the ligature, expressed as percentage of the antigen content in the preceding 1 cm long nerve segment, ranged from five-fold for syntaxin (480 ± 170%, mean ± S.E.M. from seven independent experiments) and SNAP-25 (490 ± 80%, 8 experiments) to 10-fold for synaptotagmin (1110 ± 180%, nine experiments), VAMP being in between (665 ± 5%, two experiments). Therefore, syntaxin, SNAP 25 and VAMP in nerve segments proximal to the ligature correspond in majority to antigens transported by the fast anterograde axonal flow in *Torpedo* electric nerves.

In order to know if syntaxin, SNAP 25 and VAMP were transported separately or in association, we performed immunoprecipitation experiments on membrane proteins accumulating proximally to the electric nerve ligature, after solubilization by Chaps or SDS (*figure 1*). Synaptosomal (S) and nerve membranes proximal to the ligature (P) were solubilized and precipitated in parallel with anti-syntaxin or anti-VAMP immunobeads. An association between syntaxin, SNAP 25 and VAMP was demonstrated. Similar amounts of the syntaxin-SNAP 25-VAMP complexes were recovered, regardless of which detergent was used (either Chaps or SDS). Syntaxin-SNAP 25-VAMP complexes were also immunoprecipitated from solubilized control nerves, in the absence of the ligature (not shown).

The possibility that syntaxin and VAMP associate during solubilization was eliminated through the three following independent experimental arguments [11, 12]. First, similar amounts of syntaxin-SNAP 25-VAMP complexes were recovered both after Chaps or SDS solubilization (*figure 1*), both from synaptosomes and from ligated nerves. Hayashi and coworkers [3] have shown that the syntaxin-SNAP 25-VAMP complex was SDS-resistant, once formed, but that its constituents were unable to associate if

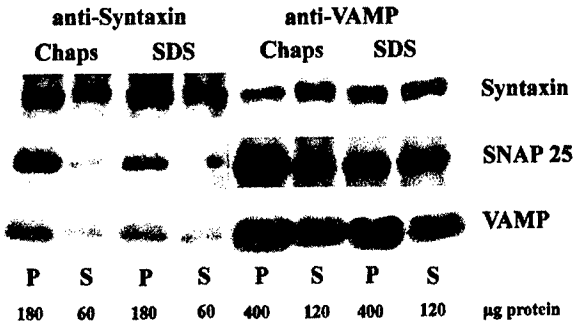


Figure 1. Immunoprecipitation of SNARE complexes solubilized in Chaps or SDS. Membrane proteins from synaptosomes (S) or nerve segments proximal to a ligature (P) were solubilized either by 30 mM Chaps (Chaps), or by 0.5% SDS and then diluted 10-fold in 0.5% Triton X-100 (SDS). Solubilized proteins were immunoprecipitated in parallel on anti-syntaxin (mAb 10H5) or anti-VAMP (mAb 19K1) immunobeads. Syntaxin-SNAP 25-VAMP complexes were detected in both conditions, regardless the detergent used or their synaptosomal or axonal origin.

solubilized in SDS. Second, the amount of syntaxin-VAMP complex was independent of the syntaxin and VAMP concentrations during solubilization of nerve membranes. This complex was recovered even when syntaxin and VAMP concentrations were around 5×10^{-9} M, far below concentrations required to study their association in vitro: EC_{50} in the micromolar range [3, 9]. Finally, using control nerve homogenates where most of syntaxin is not associated with VAMP, we have shown that exogenous VAMP, added at the beginning of the solubilization step, was unable to co-precipitate with syntaxin. Therefore, the axonal syntaxin-SNAP 25-VAMP complexes appear to be formed prior to the solubilization, to be SDS-resistant and extremely stable.

The size and abundance of SNARE complexes in solubilized control or ligated nerve segments were analysed after velocity sedimentation in continuous sucrose gradients (*figure 2*). Syntaxin distribution, a broad peak around 12-13 S without monomeric syntaxin, was similar in control and ligated nerves. A large proportion of SNAP 25 and VAMP was also recovered in intermediate fractions in ligated nerves, in contrast to control nerves. The syntaxin, SNAP 25 and VAMP peaks were broad, overlapping but not identical, suggesting the existence of several protein complexes containing these antigens. All of these complexes are accumulated in ligated nerves and therefore transported by the fast anterograde axonal flow. In control nerves, most of SNAP 25 and VAMP were recovered in the lightest fractions, probably as mono or dimers. These mono or dimers were also detected but not accu-

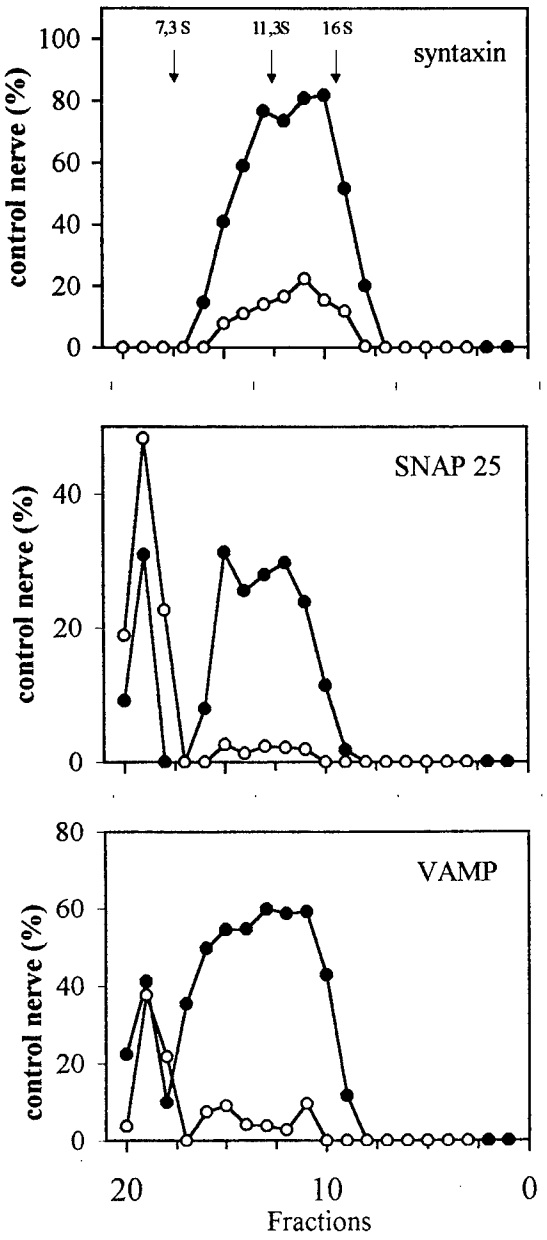


Figure 2. Velocity sedimentation of syntaxin, SNAP 25 and VAMP solubilized from ligated (●) or control (○) nerves. Proteins in electric nerve homogenates (2 mg/mL) were solubilized by 30 mM Chaps and centrifuged (0.4 mL /gradient) in 5-30% linear sucrose gradients in buffer B (40 000 rpm for 18 h in a Beckman SW40 rotor). Fractions were collected from the bottom of the gradients. The syntaxin, SNAP 25 and VAMP contents of each fraction were determined after dialysis and concentration, SDS gel electrophoresis and electrotransfer, and finally immunodetection with specific antibodies (mAb HPC1 and 19K7, anti-SNAP 25 antiserum). Antigen contents are expressed in percentage of the total content of control nerve gradient.

mulated in ligated nerves. In the case of SNAP 25, they correspond to soluble SNAP 25 and are detected in much lower amounts if nerve membranes rather than homogenates are used (not shown).

Purified cholinergic synaptic vesicles are highly enriched in VAMP and synaptophysin, as compared to synaptosomes, but also contain some SNAP 25 (*figure 3*) and syntaxin (not shown). This confirms previous biochemical [8, 15] and morphological [4] data on brain synaptic vesicles. Using anti syntaxin immunobeads, we have immunoprecipitated syntaxin-SNAP 25-VAMP complexes that are present in the membrane of isolated synaptic vesicles (not shown). It has been suggested that these complexes, involving proteins colocalized in the same synaptic vesicle membrane, are dissociated by NSF and α-SNAP in physiological conditions when cytoplasm is present [8].

If syntaxin, SNAP 25 and VAMP are associated side by side in the same membrane during rapid axonal flow, like in synaptic vesicles, they must be present in the same axonal organelles. Indeed, we have immunoprecipitated VAMP containing nerve vesicles and found that they also contained SNAP 25 (*figure 3*). Synaptophysin, which is abundant in synaptic vesicles, was not detected in the organelles immuno-isolated from nerves. The present observation should be compared with previous results [7] showing that synaptophysin was transported in organelles that did not contain syntaxin, SNAP 25, nor the vesicular antigen SV2. A better understanding of the sorting of nerve terminal proteins to synaptic vesicles or the presynaptic plasma membrane will require the characterization of the various membrane precursors transported by the fast axonal flow.

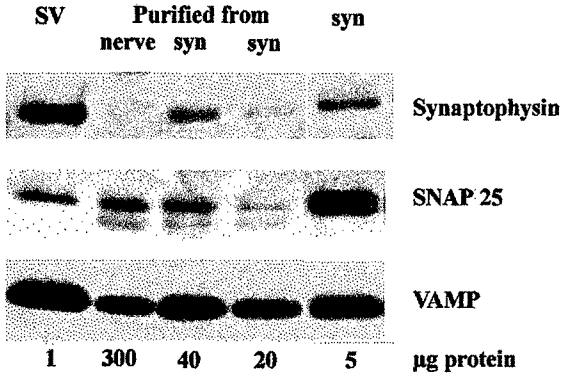


Figure 3. Immunoisolation of axonal or synaptosomal organelles on anti-VAMP magnetic beads. Vesicle membranes were immunoisolated on mAb 19K2 magnetic immunobeads from control nerve homogenates or disrupted synaptosomes. Their synaptophysin, SNAP 25 and VAMP contents were compared to that of purified synaptic vesicles (SV) and of whole synaptosomes (syn).

It was unexpected to find that complexes associating a synaptic vesicle protein, VAMP, and presynaptic membrane proteins, syntaxin and SNAP 25, could have formed outside the active zone, during axonal transport or in isolated synaptic vesicles. In the latter case, Otto and coworkers [8] have shown that these SNARE complexes involved proteins in the same membrane, interacting side by side. This contrasts to the active zone situation where SNARE complexes are believed to be formed by proteins in two opposing membranes. In yeast, side by side SNARE complexes are present in vacuolar membranes and must be dissociated by NSF and α-SNAP to allow homotypic intervacuolar fusion to proceed [14]. The present results suggest that VAMP and SNAP 25 are transported in the same axonal organelles and that axonal SNARE complexes would be similar to those of isolated synaptic vesicles. We still do not know if these SNARE complexes simply form because their three protein constituents are present in the same membranes. Alternatively, they could be required to direct properly presynaptic proteins to the nerve terminal or to avoid premature membrane fusion.

Acknowledgments

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Conclusions and comments. Xth International Symposium on Cholinergic Mechanisms

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Abstract — Ancient medicine men of Egypt and Arabia employed, under another name, the cholinergic agents, as did the hunters, warriors and shamans of Africa and South America. An explosion of cholinergic science occurred in the last and the current century, and the ISCMs witnessed and catalyzed this progress. The Xth ISCM emphasized the molecular characteristics of the receptors, cholinesterase and of the system engaged in liberation of Ach. (©Elsevier, Paris)

Résumé — Conclusions et commentaire. X^{ème} Symposium International sur les Meécanismes Cholinergiques. L'emploi des agents cholinergiques est ancien; il a commencé avec les guérisseurs Arabes et Egyptiens et les chasseurs, guerriers et shamans d'Afrique et d'Amérique du Sud. Le dernier siècle, ainsi que le nôtre, ont vu une explosion de la science cholinergique, dont les SICMs ont été les témoins et les catalyseurs. Le focus du X^{ème} SICM a été sur les caractéristiques moléculaires de récepteurs, de la cholinésterase et de la libération d'ACh. (©Elsevier, Paris)

cholinergic / central nervous system / Alzheimer's disease / transmission

1. Introduction

The progress and the success of the cholinergic field is, arguably, beyond that of any other field of biological knowledge. The field was named by Sir Henry Dale, but it antedates Dale by millennia, as it begins with the BC Egyptian and Arab medicine. The Calabar natives and the South American Indians followed with the hunt, rite and war use of curarimimetics and their antidotes [12]. And then, there was an outburst of modern studies which begun with the work of Scotch missionaries and Edinburgh pharmacognosists and doctors of the 1850s; then, there were the studies of German, French, English and American pharmacologists which lead to the discovery of the chemical transmission, at the periphery in the nineteen twenties and in the central nervous system 30 years later [18].

Since then, the past nine International Symposia on Cholinergic Mechanisms (ISCMs) initiated by Edith Heilbronn were the witnesses of and contributed to, the further, exponential progress of the cholinergic lore. Thus, the Ist 1970 ICSM presented data on biosynthesis and chemical analysis of acetylcholine (ACh), three or so muscarinic receptors and a few behavioral correlates of the cholinergic system, all departures from what was known in the 1960s, while the IXth, Mainz ISCM confronted us with many more muscarinic and nicotinic receptors, second and third messengers characteristics of their activation, molecular biology of the components of the cholinergic system, trophic factors and cholinergic correlates of dementia [21, 25]. And, we saw for ourselves, that the Xth ISCM was not a poor relative of the preceding ISCMs.

Of course, the early giants of cholinergicity, Dale, Loewi, Hunt, Dixon, Bovet, Koppanyi, Nachmansohn, MacIntosh passed away; the memory of Nachmansohn and of the Arcachon laboratories where he was so active were vividly depicted at this Symposium by Jean-Pierre Changeux and Victor Whittaker. I regret the absence of Sir William Feldberg, Bo Holmstedt, Israel Hanin, Peter Waser and Don Jenden at this Symposium. It is sad that the progress made since the last ISCM had to be marred by the passing away of their two worthy descendants, George Koelle and Sir John Eccles. George, who participated in several of ISCMs, was honored at this Symposium by Dr. Jack MacMahan. It was not possible to acknowledge similarly the more recent departure of Jack Eccles (May 2, 1997), and these few lines must suffice at present.

Sir John C. Eccles did for the central nervous system what Otto Loewi did for the peripheral nervous system: he demonstrated, via his Renshaw cell experiments [5, 6], the central presence of a chemical, cholinergic system, experiments that were responsible in part for his Nobel Prize award. But Eccles did much more than that, as he described, with Ben Libet and Rosamond Eccles, the muscarinic and nicotinic potentials of the sympathetic ganglia; explained, with Andrew Huxley and Alan Hodgkin, the ionic mechanisms of excitatory and inhibitory potentials; defined the circuitry, transmissive charac-

teristics and function of the cerebellum; and elaborated the mechanisms underlying the integrative character of the central nervous system. Accomplishments that merit Eccles his consideration as the century's premier neuroscientist! And, his attempts, with Karl Popper, at bridging the mind-consciousness gap constitute an intellectual peak [26]. It may be added that Eccles was a friend of and argued frequently with (see *figures 1* and 2), the other men, honored here, David Nachmansohn and George Koelle.



Figure 1. Sir John Eccles and David Nachmansohn at the 1959 Rio de Janeiro Symposium on Bioelectrogenesis, organized by Carlos Chagas and Antonio de Paez Carvalho. Eccles' and Nachmansohn's gesticulations are supposed to indicate that there is a small distance between their two positions (a small distance, but a major difference!).

2. Xth International Symposium on Cholinergic Mechanisms

2.1 General comment

The present Symposium emphasized the newest understanding of the molecular, genetic and developmental characteristics of the muscarinic and nicotinic receptors and of cholinesterases (ChEs) as they relate to function and pathology; it focused also on ACh release, expanding on and departing from, the classical image; and it dwelled on Alzheimer's disease (AD) and on the cholinergic aspects of the Persian Gulf War.

2.2. Receptors

Well beyond Dale's original image of muscarinic and nicotinic receptors, Patrick, Lindstrom, Karlin, Barnard, Raftery, Skok, Waser, Nordberg, Heine-



Figure 2. On the left, a lunch at the same Symposium. In the foreground, Sir John Eccles, George B. Koelle, Werner Loewenstein and a friend. In the background, David Nachmansohn (in profile) and Daniele Bovet. On the right, a 1962 diagram made by Eccles to explain the ionic currents underlying excitatory potentials.

mann, Changeux, Daniel Bertrand and Edson Albuquerque contributed to the study of the diversity of nicotinic receptors, their molecular biology and function, while Sir Arnold Burgen, Hammer, Heller-Brown, Hartzell, Dowdall, Ladinsky, Schimerlik, Bob Schwartz, and Ed Hulme focused similarly on the muscarinic receptors.

It is accepted today (see, for example, Galzi and Changeux [7] and Hucho and Tsetlin [13]) that the nicotinic receptors (NAChRS) are allosteric glycoproteins that are structured into pentameric combinations of alpha and beta subunits surrounding the cation-selective channel; in view of this allostericity these receptors may exist in several conformations. As shown at this Symposium, this organization is characterized by wide flexibility and lends itself to mutational and functional changes and to the generation of many heterologous and homo-oligomeric forms of the receptor. Thus, John Dani demonstrated that the α7 subunit plays the transmissive role as it mediates Ca^{2+} influx, Daniel Bertrand showed the importance of α4 and α7, and β2 oligomeric receptors for the NAChR sensitivity for agonists and desensitization processes (see also Corringer et al. [3]), Hucho and his collaborators defined the receptor conformation that exhibits high affinity/closed channel state and described the amino acid character of the accessible extramembrane of the receptor, while Steven Sine (see Watty et al. [36]) presented data indicating that the peptide loops that contribute to the non-alpha subunits regulate the agonist and antagonist affinities of the nicotinic receptors. Looking at the other side of the story Andre Menez investigated the cysteine spacing and the structural characteristics of toxins that explain their 'folding' properties (see also Drakopoulou et al. [4]) and thus may clarify certain ligand-binding properties of the NAChR. Marc Ballivet established a new family of NAChRs exhibiting a novel assembly α3 and α5 and β-subunits and described the significance of these and other subunits in the generation of currents of various conductance characteristics and in NAChR capacity for rapid desensitisation. In a different vein, he defined neurogenetic and evolutionary significance of genes controlling transcription factors of the helix-loop-helix proteins and of α-subunits (see also Roztocil et al. [34]).

The desensitization data presented by Marc Ballivet and Daniel Bertrand pertain to the story that begun with Steve Thesleff, Paul Greenagard and Edson Albuquerque [2]; it should be added that another allosteric change in the nicotinic receptor leads to an opposite process, that of sensitization [29], and I did not hear much about this particular phenomenon at the ISCMs!

Jean Cartaud, Fabrizia Bignami and their associates focused on the important area concerning relations between extrinsic and intrinsic proteins and the neuronal organelles. Their results indicate that in the *Torpedo* the intrinsic protein, NAChR communicates with the extrinsic protein, rapsyn, a NAChR clustering agent, via targeting intracellular organelles, such as those present in the Golgi apparatus or microtubules; this system is a part of the secretory, ACh release, pathway. Other, axon derived proteins, such as agrin and neuroregulin, and those derived from muscle, such as laminin 11 participate in the differentiation of the postsynaptic membrane, nerve terminals and terminal Schwann cells of the neuromyal junction, as explained by Josh Sanes and Steve Burden.

Continuing his long-time work on muscle disease, Andrew Engel concentrated on the genetic character of the myopathies resulting from deficiency of the lysosomal acid α-glucose (glycogen storage diseases); furthermore, Engel characterized the receptor modifications and the resulting transmission change in several forms of myasthenia gravis.

Now, as to the muscarinic receptors (MAChRs). Juergen Wess and his collaborators suggested that multiple intracellular receptor loops or domains within the α-subunits regulate the specificity of the G protein links with several, including the muscarinic receptors. Ed Hulme, continuing his work on the transmembrane helices of M receptors [14] found that within the α-helix of the domain 3 of the M1 receptor a specific band of amino acids that include an aspartate-arginine-tyrosine triad is critical for the G protein links as well as for 'efficient' receptor folding and thus for agonist and antagonist affinities.

As to the developmental aspects of the MAChRs Neil Nathanson demonstrated the significance of the GATA family of transcription factors for transactivation of the cardiac cm2 receptor gene in the chick; he also discussed the chick retinal trophic (secretory) factor that increases selectively expression of some MAChR receptors but not of others. Of special interest is his finding that certain trophic and related factors that were previously linked with the regulation of choline acetyltransferase transcription also regulate the transcription of the muscarinic receptor gene (see also Nathanson [31]).

2.3. Cholinesterases

This family of enzymes constitutes another, important component of the cholinergic transmission. Recent work of Jean Massoulie, Israel Silman and Hermona Soreq (see Heilbronn [10]) yielded an image of AChE as having a 'gorge' on the esteratic

site and a flatter anionic site; their work also defined amino acid composition of these sites and the multitude of molecular and physical forms that AChE may assume.

At this Symposium, Mona Soreq and her associates continued their life-long investigations of the genetics, the structure, and the function of AChE. Using transgenic and antisense models particularly concerning neurexin gene family-4, they described genetic, functional and circuital regulation of the generation of AChE variants. They also stressed their neuronal trophic effects, an effect described early by George Koelle, and antitrophic action of their overexpression.

The capacity of AChE to assume various physical and structural forms was emphasized by several investigators. Thus, Jean Massoulie described the genetics and the expression of physical forms of AChEs that relate to congenital conditions such as myasthenia and to deficits of junctional AChE, Dick Rotundo defined the developmental mechanisms involved in organization and structurization of AChE of the endplate, while Israel Silman referred to various folding and unfolding states of AChE that exhibit several 'molten molecule' states and hydrophobic cores. He also defined the structures of several OP-AChE conjugates and of their dealkylation products that result in 'aging' or non-reactivatability; he speculated that the junctional membrane participates, via energy mechanisms, in insertion and translocation of these various AChE states and in their affinity regulation. Finally, Palmer Taylor employed crystalline forms of mouse and *Torpedo* AChE and peptide toxins to relate AChE templates to ligand, OP and toxin binding, as well as to the route of substrate entry and its contact points with AChE. He stressed particularly the importance of the dimensions and composition of the acyl pocket, the choline binding site, and of the vicinity of the active center gorge.

2.4. *Functions and behaviors*

The role of receptors is to elicit, beyond their synaptic function, organ activity and behavior. Steve Arneric and his associates showed that nicotinic agonists of 3-pirydyl ether series attenuate, in several species, pain evoked by a number of pain stimuli and neuropathic pain states; they demonstrated that these compounds do not induce dependence in animals. These are important findings in view of the well known – disputed by very few pharmacologists such as David Warburton – addictive action of nicotine. It should be added that both nicotinic and muscarinic substances, as well as anti-ChEs are analgetic, and that this effect may be – there is a controversy here, see Karczmar [19] – non-opioid in nature. As a counterpart of Arneric's presentation, Emilio Merlo-Pich, who previously studied ethanol addiction (see Koob et al. [30]), defined the genetics and the neural pathways regulating the nicotinic addiction as he found that reward-related activation of central nicotinic receptors via nicotine self-administration by 'addicted' rats results in c-fos expression in mesocorticolimbic dopaminergic system. As to other behavioral effects, Mohammed Shoaib and his associates hypothesized a cholinergic-glutaminergic interaction as they showed that certain NMDA receptor antagonists prevent the up-regulation of nicotinic receptors upon chronic nicotinization and antagonize the resulting increase in nicotine locomotor effect, while locomotor, learning and other dysfunctions were described by Steve Heinemann, a pioneer in the studies of molecular and functional aspects of NAChRs for mice which suffered a 'knock-out' of their α9-nicotinic subunit.

These presentations refer to a few, important behavioral aspects of the NAChRs. Yet, it must be remembered that there is no known behavior that does not exhibit cholinergic correlates, and that these include the muscarinic as well as the nicotinic system (see Karczmar [19, 21, 23]). Steve Heinemann expressed hope that molecular neurobiology will reveal the genetics of these behaviors as well as of many disease states.

2.5. *Cholinergic transmission*

The classical notion elaborated particularly by Eduardo di Robertis and Victor Whittaker was that cholinergic transmission depends, via specialized presynaptic elements, on vesicular release of ACh and its direct action on postsynaptic or postjunctional receptors. Important new understanding of this early model concerns the vesamicol-sensitive protein controlling the uptake of the synaptically released ACh, the transport proteins regulating the movement, fusion with the presynaptic membrane and recycling of the vesicles, and the protein regulators of the release of ACh [9, 15]. At this Symposium, Louis Hersh and Carlos Ibanez-Moliner discussed the cholinergic gene locus which comprises genes for choline acetyltransferase (ChAT) and for vesicular ACh transporter (VAChT), as they demonstrated the role of protein kinase A II and other molecular mechanisms in regulation of the expression of both these genes. Jacques Mallet, Heinrich Betz and Jeffrey Erickson identified the molecular mechanisms and the domains which regulate, in a structure/function relationship, ACh transport, targeting of the synaptic vesicles, and vesicular fusion; in this context, Jeffrey

Erickson developed a new assay for the analysis of the kinetics of the VAChT.

A revolutionary attempt at changing the classical view was made by Israel, Dunant and Descarries. Maurice Israel and Yves Dunant employed ultra-rapid, ultra-sensitive method for ACh measurement to describe in the 1980s a non-vesicular, cytoplasmic release of ACh (pace Victor!) that was regulated by a proteolipid, mediatophore, and which actually, may be, as described by Israel at this Symposium, quantal in nature. Then, Laurent Descarries demonstrated, first for the serotonergic and noradrenergic and then for the cholinergic transmission that the classical synaptic differentiation may be, in several brain parts, a *rara avis*! Thus, besides synaptic, 'diffused' transmission may be obtained. He feels that the presence of extra-synaptic NAChcRs and MAChRs and special species of AChE, such as the globular G4 form at these sites, and, particularly, persistent level of ACh in the extracellular spaces in several brain regions, all advocate the existence of diffuse transmission. As indicated by the ardent discussion at this Symposium of the views of Descarries, Dunant and Israel, the matter is still a subject of controversy!

2.6. Alzheimer's disease (AD) and its treatment

Involvement of the cholinergic system in and the facilitatory effect of cholinergic agonists on, memory and learning is known since the nineteen forties (see Karczmar [17]). Thus, when the Perrys and the McGeers and Whitehead (see Thal [35] and Giacobini [8]) demonstrated that significant damage to cholinergic neurons occurs in AD, the field was open for cholinergic therapy of AD. In the last 10 years, Marsel Mesulam confirmed and expanded our understanding of this damage in AD patients, and he continued defining it at this Symposium. While it is known that NAChRs are affected in AD, Mesulam explored further changes in the MAChRs. He demonstrated a decrease in the m2 presynaptic autoreceptors located on the cholinergic radiation originating in the nucleus basalis of Meynert as well as a diminution of the frequency of the m2 cortical postsynaptic receptors; as the latter express NO synthetase, Mesulam speculates that their loss contributes to the loss of cortical plasticity; he discussed also the important and controversial problem of the relation between the changes in the cholinergic system and the beta amyloid metabolism. It must be added that there is a controversy as to both qualitative and quantitative changes of other MAChR subtypes in AD. As stressed by Mesulam, the neuronal damage extends in AD beyond the cholinergic neurons. Ezio Giacobini was particularly active, after the early disappointments with the physostigmine treatment of AD, in the development of antiChEs that would penetrate well into the CNS, induce a protracted inhibition of AChE, possibly exert selective effects at strategic sites and receptors and, of course, cause minimal side effects, and he presented at this Symposium the current data on this matter.

Today, AD appears to be a complex, genetically controlled apoptotic process that involves the formation of abnormal proteins (β-amyloids, τ-proteins) and an interaction between such factors as presinillins, proteases, protein precursors and, possibly, active radicals (see Karczmar [27]). This general scheme was further analyzed at this Symposium. Danny Michaelson emphasized that the AD pathognomy includes an apolipoprotein E mutant in which the hyperphosphorylation of the microtubular tau protein proceeds unimpeded and he described a loss of cholinergic as well as serotonergic and adrenergic neurons and memory and learning deficits in congenital apolipoprotein E-deficient mice. Chris Henderson suggested that the loss of trophic factors may also be a component of AD. And Steven Younkin demonstrated the importance in the early-onset AD type of mutations in presenilin 1 (PS1) gene on chromosome 14 as they lead to elevation of highly amyloidogenic peptides; he also defined the gene locus of the most effective mutation.

Altogether, AD cannot be considered as a cholinergic disease; there are also other reasons for expecting only a limited success with antiChE or cholinergic agonist therapy of AD [8, 22]. Indeed, even the newest, FDA, USA-approved drug, donepezil (Aricept), while ameliorating scores of certain subtle cognitive tests, does not improve the quality of life and restore the independence of the AD patients [33]. However, Danny Michaelson found that in his apolipoprotein E deficient mouse model a synthetic MAChR-1 agonist abolished their working memory impairments.

Altogether, non-cholinergic treatments are of importance. Chris Henderson described a synergistic trophic action on the motoneurons of a glial factor and of cardiotrophin-1, as a model for such a treatment, while Anders Bjorklund demonstrated in rats facilitatory cognitive effects and prevention of learning deficits in the course of aging when NGF was administered to middle-aged rats in the form of transplants of NGF-secreting neural progenitor cells. It must be added that at the VIIIth and IXth ISCMs of Claudio Cuello and Konrad Loeffelholz additional, potentially useful in AD trophics were described. Other alternative treatments are being tested at this time, including hormonal and anti-inflammatory therapies [28].

2.7. Toxicological threat of anticholinesterases

Anti-ChEs are highly toxic agents, as certain OP agents kill experimental animals at fraction of μg/kg doses, and as a few cc of their vapors may be deadly to hundreds of exposed people. In man, the anti-ChE death is due primarily to the prolonged depolarization and then desensitization of the cholinoceptive neurons of the respiratory centers and, subsequently, to the paralysis of the respiratory muscles. In some subjects and in some animal species the death may be muscarinic and 'asthmatic' in nature [20].

In the 1930s, German investigators and their government became aware of this toxicity and of the war potential of OP anti-ChE nerve agents and conducted vigorous research concerning their development, use as nerve agents, and antidoting. Inevitably, this was followed by similar efforts in the UK, France, USA and USSR (see Holmstedt [11] and Karczmar [28]). This research continues, and Dave Moore presented at this Symposium effects of subtoxic doses of nerve agents in human volunteers and in animals.

While classically the toxicity of OP and other anti-ChEs relates to the inhibition of ChEs and accumulation of ACh, Dave Moore's studies concern certain non-classical aspects of anti-ChE toxicity. These include 'ginger-Jake' paralysis caused at the end of the XIXth Century by tetra-o-cresyl phosphate and described since that time for other OP agents as the organophosphorus ester-induced neurotoxicity (OPIDN), and the delayed cognitive toxicity (DCT), defined first by F. H. Duffy and J. L. Burchfiel (see Karczmar [28]). Actually, that OP drugs may cause neuronal damage and myopathy has been recognized for the last 20 years [20, 23]. The mechanism of this damage is not clear; in the case of the neuronal OPIDN it may be related either to inhibition of a somewhat hypothetical enzyme, the 'neurotoxic esterase' [16] or to neuronal hyperactivity [1]. Wolf Dettbarn, one of the brilliant past associates of Dave Nachmansohn who focused for many years on the mechanisms of OP-induced myopathy, presented at this Symposium convincing evidence concerning the contribution of the hyperactivity and the peroxidation processes to this pathology.

While these agents were not used in the Second World War, there was documented accidental toxicity in relation with their manufacture. Furthermore, they may have been employed subsequently by Iraq and in Yougoslavia; many rogue states are capable of their development, and there is a record of their use by terrorists, as described at this Symposium by Kazuhito Yokoyama with regard to the Sarin attack in the Tokyo subway. Exposure to these agents during the Persian Gulf War of 1990/1991 relates to the Persian Gulf War Syndrome (PGWS). The PGWS is an unfinished matter; it is controversial within the USA, as well as between the official USA sources and certain French and Czech investigators, as referred to at this Symposium by Guy Blanchet (see also Karczmar [21]).

Besides DCT, OPIDN and other pathological actions of OP drugs, other novel factors may contribute to the PGWS. Thus, at this Symposium, Alon Friedman and Mona Soreq suggested that stress may damage the blood-brain barrier and facilitate brain penetration of drugs such as pyridostigmine that are included in the drug 'cocktail' employed to prevent or antagonize OP toxicity. This finding expands on the 1940s and 1950s demonstration that anti-ChEs facilitate penetration into the CNS of a number of pharmacological agents [17].

Altogether, pyridostigmine is not an innocent member of the cocktail. Its neurotoxicity was described at this Symposium by Peter Spencer, while Edson Albuquerque and his associates demonstrated recently direct channel effects of very low doses of pyridostigmine and OP drugs that may contribute to their AChE inhibition-related toxicity; they also stressed, at this Symposium, the role of nicotinic receptors and channels, and of endogenous choline, in the regulation of behavior and cholinergic toxicity.

3. Envoi

This Xth ISCM was a testimonial to the progress of cholinergic lore as well as a further contribution to its status. It focused on the molecular aspects of the cholinergic system, as it outlined the genetic mechanisms underlying its components, their variety and neurogenesis, and as it described the various NAChRs and the MAChRs, ChEs, ChAT and the proteins regulating transport and release of ACh, whether vesicular, cytoplasmic or diffuse. The power of the methodology undergirding this progress and the elegance of our present understanding of these items is nothing short of astounding. Thus, the Xth ISCM leaves us with an image of molecular interaction between the subunits of the receptors and ChEs, and between the receptors and the regulatory extrinsic and intrinsic proteins and organelles which, while complex, makes functional sense and underlies the flexibility and plasticity of the cholinergic system.

If I may gripe, it would be only with regard to the emphasis of this Symposium on the microstate of this knowledge which does not directly translate into the macrostate, such as the overt behaviors. A few references were made to the behaviors that are

endowed with cholinergic correlates such as pain, attention and addiction, but a concerted effort at presenting the general significance of the multiplicity of cholinergic behaviors was not made at this or other recent ISCMs. Such significance was hinted at by Stephen Arneric as he stressed that the stimulation of NAChRs reduces the negative effect of distraction on learning (see also Prendergast et al. [32]). This demonstration adds support to the notion that cholinergic, both nicotinic and muscarinic, agonists induce a specific behavioral syndrome, the cholinergic alert non-mobile behavior (CANMB) as they promote organism-environment interaction [24].

It must be stressed that the exploitation of the cholinergic system represents not only a cornucopia of plenty, but also a Pandora's box of calamities, and this aspect of the matter was presented as well, as we dwelled on the use of the OP agents by terrorists and rogue states. An additional example of this mixed blessing status of cholinergic agents concerns the agricultural use of anti-ChEs as insecticides and pesticides. This use constitutes a difference between life and death for undeveloped nations, yet each year there are, worldwide, thousands of fatalities related to this use [21] – a matter that has not been not discussed at this or other ISCMs. Altogether, the exploitation of the cholinergic system is of immense benefit to mankind as well as of potential danger.

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1 Contribution of nAChRs to the function of CNS synapses: The action of choline as a selective agonist

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It is generally acknowledged that the hippocampus, an area of the central nervous system (CNS) that is involved in processing cognitive functions [10], is very sensitive to cholinergic modulation, and that the density of neuronal nicotinic acetylcholine receptors (nAChRs) in the hippocampus is severely diminished in diseases characterized by learning and memory impairment (e.g., Alzheimer's disease) [12]. Thus, the developing hippocampus has become the focus of our interest with regard to characterization of the nAChR function. One of the major hindrances to addressing nAChR function in the brain was the lack of specific agonists and antagonists for each of the receptor subtypes. This problem was further aggravated by the fast kinetics of inactivation of some of the neuronal nicotinic receptors, particularly of those composed of the α7 subunit. The field has now advanced immensely, and many of these problems have been overcome. Our initial work in this field demonstrated for the first time that cultured hippocampal neurons respond to nicotinic agonists with at least one of three types of whole-cell currents referred to as IA, II, and III [1]. These currents, which can be distinguished from one another on the basis of their kinetic and pharmacological properties, are subserved by α7, α4β2 and α3β4 nAChRs. Type IA currents, by far the predominant response of hippocampal neurons to nicotinic agonists, are fast-desensitizing currents sensitive to blockade by the α7 nAChR-selective antagonists α-bungarotoxin (α-BGT), methyllycaconitine (MLA), and α-conotoxin-ImI, and show an intracellular Mg^{2+}-dependent inward rectification and a rundown that is associated with the intracellular high-energy phosphate compounds. In contrast to type IA currents, type II and III currents, which desensitize very slowly, can be recorded from a small population of the hippocampal neurons and can be differentiated from one another on the basis of their sensitivity to nicotinic antagonists. Activation of type II currents is inhibited by DHβE (100 nM), and that of type III currents is inhibited by mecamylamine (1 μM) [2].

Although nAChR subtype-selective antagonists have been very helpful in assigning a receptor subtype to a nicotinic response, the recent discovery that choline modulates the function and expression of neuronal nAChRs made up of the α7 subunit introduced a key pharmacological tool to distinguish α7 nAChRs from other nAChR subtypes [4, 8]. In cultured hippocampal neurons, choline fully activates α7 nAChR-mediated type IA currents with an apparent EC_{50} of 1.6 mM, does not evoke α4β2 nAChR-mediated type II currents, and induces α3β4 nAChR-mediated type III currents with 20% of the apparent efficacy of acetylcholine (ACh). Also, when continuously applied to these neurons, choline, like other nicotinic agonists, desensitizes the α7 nAChRs subserving type IA currents with an IC_{50} of 37 μM [4]. Therefore, choline as a nicotinic agonist has the unique capability of providing substantial clues regarding the nAChR subtype subserving a given nicotinic response.

Using drug-delivery systems that allow for agonists to be rapidly applied to the vicinity of neurons and equally rapidly removed, and infrared-assisted videomicroscopy, which allows for visualization of individual neurons in slices, initial studies from our laboratory showed that neuronal nAChRs located in preterminal sites of CA1 interneurons facilitate γ-amino butyric acid (GABA) release [3]. Answering the question regarding the nAChR subtype involved in modulating the release of GABA from CA1 interneurons became of utmost importance given that synchronization of the hippocampal neuronal activity has been shown to be enforced by the ongoing activity in the interneurons [6]. Thus, using a number of nAChR-subtype-selective pharmacological tools, particularly choline, we demonstrated that in hippocampal slices: i) CA1 interneurons can express functional α7 and α4β2 nAChRs; ii) α7 nAChRs are located predominantly in the soma-to-dendritic region of the CA1 interneurons, whereas α4β2 nAChRs are presently mostly in preterminal regions of these interneurons; and iii) both α7 and α4β2 nAChRs can facilitate the release of GABA from the interneurons [5]. Such findings provide direct support to previous studies in which a GABAergic mechanism was suspected to explain the relationship between the α7 nAChR gene locus and attentional deficits in schizophrenia [7], and in which cholinergic deafferentation was linked to kindling epileptogenesis [10]. In addition, these results indicate that choline, by controlling the function and expression of α7 nAChRs in the CNS may have a critical role in modulating the activity of the CA1 interneurons, and, ultimately, the excitability of the CA1 pyramidal neurons.

The finding that choline (a metabolite of the ACh hydrolysis) can control the function and expression of neuronal nAChRs bearing α7 subunits and that these receptors are capable of modulating differently the release of a variety of neurotransmitters raised the question of whether anticholinesterases, including the organophosphate (OP) nerve agents VX, soman, and sarin, can alter transmitter release from CNS neurons simply by altering the levels of choline and ACh. By means of the patch-clamp technique, we have recently demonstrated that at toxicologically relevant concentrations (≥ 0.1 nM), VX and sarin, but not soman, increase both tetrodotoxin (TTX)-sensitive and -insensitive release of glutamate and GABA from hippocampal neurons. The effects of VX and sarin are unrelated to cholinesterase inhibition and to alterations of the function of presynaptic nAChRs. In fact, the effect of VX on TTX-sensitive transmitter release appears to be mediated by its direct interaction with voltage-gated Na^+ channels. These studies altogether demonstrate the physiological relevance of nAChRs and choline in controlling synaptic function in the CNS and may provide the basis for a better understanding of the actions underlying the neurotoxic effects of many OPs, including the nerve agent VX.

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2 Sensitivity of bovine retinal acetylcholinesterase to phenserine: A derivative of physostigmine

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Currently, there has been a keen interest in the cholinesterases inhibitors, because of their therapeutic importance in the treatment of dementia disorders, such as Alzheimer's and other cholinergic-related impairments (Messamore et al., 1993; Smith and Swash, 1979; Becker and Giacobini, 1988). These inhibitors enhance the cholinergic response by inhibition of acetylcholinesterase (AChE). Phenserine, a derivative of the AChE inhibitor physo-stigmine, acts as a long lasting inhibitor of the central nervous system and may be used for the treatment of Alzheimer's disease in the future (Greig et al., 1995).

Recently, we have evaluated the inhibition of human erythrocyte AChE activity by phenserine (Al-Jafari et al., 1998). In the present study, we have determined the sensitivity of bovine retinal AChE to this drug and compared it with human erythrocyte AChE inhibition. The phenserine has inhibited bovine retinal AChE activity in a concentration-dependent manner. The IC_{50} for retinal AChE was found to be 0.249 μM (*figure 1*) and for human erythrocyte it has been reported to be 0.045 μM. These values indicate that the retinal AChE is 5.53 times less sensitive than human erythrocyte AChE.

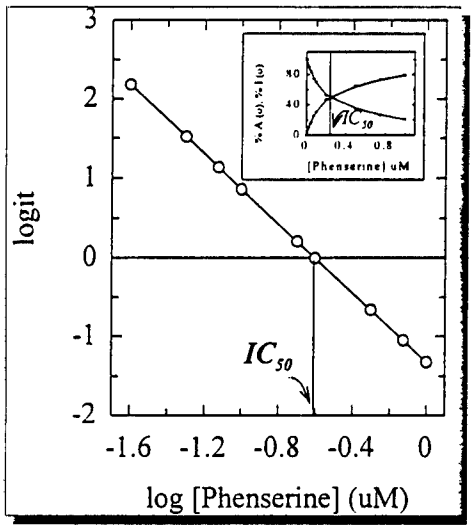


Figure 1. Inhibition of bovine retinal AChE by phenserine. Transformed data are presented in the form of a plot, where Logit = ln [% activity /% inhibition]. Correlation coefficient is 0.9988. Each point represents the mean of triplicate experiments. Inset represents a novel way of determination of IC_{50} by combination of two conventional methods in same plot, i.e., plot of percentage activity of AChE and percentage inhibition of AChE versus log phenserine concentrations.

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3 Determinants of channel gating located in the N-terminal extracellular domain of nicotinic α7 receptor

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The α7, α8, and α9 subunits of the AChR family form functional homomers when expressed in *Xenopus* oocytes (Couturier et al., 1990; Elgoyhen et al., 1994; Gerzanich et al., 1994; Schoepfer et al., 1990). We have taken advantage of the structural homogeneity of the two closely related homomeric α7 and α8 AChRs, in conjunction with their pharmacological differences, to identify regions that affect activation of these AChRs.

By single channel analysis of α7 AChR currents, we show that the difference in efficacy between the two agonists acetylcholine (ACh) and 1,1-dimethyl-4-phenylpiperazinium (DMPP) is due to a slower channel activation rate by DMPP. Using chimeras of the two closely related subunits α7 and α8, we map residues that affect channel activation rate and agonist affinity to two different regions of the extracellular domain. Residues that affect channel activation rate are within the sequence 1179, whereas residues that affect agonist affinity are within the sequence 180–208. Collectively, our results demonstrate that regions within the N-terminal extracellular domain of α7 and α8 AChRs (and possibly other ligand-gated channels) govern not only the affinity for agonists but also the transition rates between closed and open states.

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4 Rhythmically bursting activity in rat medial septum after selective lesion of the cholinergic medial septal neurons and during aging

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The medial septum and the diagonal band of Broca nuclei (MS-DBB) contain cholinergic and GABAergic neurons which project to the hippocampus. A significant proportion of these septohippocampal neurons (SHN) display a rhythmically bursting (RB) activity that 'paces' the hippocampal theta rhythm. RB activity in the septohippocampal pathway has attracted interest because there is evidence that it is involved in learning and memory. The neurochemical nature of septal RB neurons is not firmly established. Two populations of RB septal neurons have been identified: cells which lose RB activity after atropine might be cholinergic whereas cells which retain RB activity might be GABAergic (Stewart and Fox, 1989). One way to identify RB SHNs as cholinergic or GABAergic is to examine the bursting activity after selective lesion of one sub-population of the septal neurons. This was achieved in the first part of the this study by lesioning the cholinergic SHNs with the immunotoxin 192 IgG-saporin (Book et al., 1992).

Anatomical studies show that MS-DBB neurons are affected by aging (Armstrong et al., 1993) The number of cholinergic neurons and the immunoreactivity for the calcium binding protein parvalbumin (PARV) are reduced in aged rats (Krzywkowski et al., 1995). In the second part of the study, septal activity was recorded in 3-, 23- and 30-month-old unanesthetized rats to look for age-related alterations of RB activity.The question of the vulnerability of RB SHNs (cholinergic?) to aging was addressed by comparing 192 IgG-saporin lesioned and aged rats.

Methods

192 IgG-saporin-lesioned rats

Three-month-old Sprague-Dawley rats received bilateral injections of either 192 IgG-saporin or saline (control rats) in the lateral ventricle. Ten to 16 days after injection, experiences were performed in urethane anesthetized and unanesthetized rats. In the first case, rats were anesthetized and placed in a stereotaxic apparatus. The skull was opened over the MS-DBB. Recording electrodes for electroencephalogram (EEG) were placed in the dorsal hippocampus. SHN neurons were identified by their antidromic response to the fimbria-fornix stimulation. MS-DBB unitary activity was recorded from micropipettes filled with 1 M NaCl and 2% Pontamine Blue. Recording sites were marked by a local release of dye.

In the second case, MS-DBB neurons were recorded in the absence of anesthesia. A painless head-restraint system was implanted prior to the recording sessions. Electrodes were implanted in the hippocampus and in the neck muscles for recording elec-

tromyogram (EMG) Over a course of 7 days, the rat was gradually habituated to the restraint system. The recording procedure was similar to that described for urethane anesthetized rats. The state of arousal was monitored by EEG and EMG during the recording sessions. The neurons were subsequently classified in several groups according to the states of arousal (W, wakefulness; SWS, slow wave sleep; REM sleep, rapid eye movement sleep). Brain sections were stained with a 192-IgG monoclonal antibody for the detection of the low-affinity NGF receptors or with a PARV monoclonal antibody to identify the GABAergic neurons. A staining for AChE positive fibers in the region of the hippocampus was performed to check for the efficiency of the cholinergic lesion.

Aged rats

MS-DBB neurons were recorded in unanesthetized rats of 3-, 23- and 30-months of age using the method described above. In lesioned and aged rats, the analysis of the unitary activity included: 1) mean spontaneous activity; 2) temporal structure of the discharge (RB or non-RB) and frequency of the periodic events; 3) percentage of RB neurons; and 4) burst parameters.

Results

192 IgG-saporin-lesioned rats

Acetylcholinesterase (AChE) histochemistry revealed a near-complete loss of cholinergic septal neurons and of cholinergic fibers in the hippocampus. In urethane-anesthetized lesioned rats, the percentage of RB neurons decreased significantly as compared to controls (17% vs. 41% for SHNs, $P < 0.001$ and 5% vs. 19% for unidentified septal neurons, $P < 0.0001$). The axonal conduction velocity and the burst frequency of the SHNs which retained a RB activity were higher in lesioned as compared to control rats ($P < 0.001$ and 0.05). The number of spikes per burst was lower and the burst duration was shorter in lesioned rats ($P < 0.01$).The frequency and the amplitude of the urethane-resistant hippocampal theta were altered. In unanesthetized lesioned rats, no RB septal neurons were found during W, as compared to 25% in controls. Their number was also markedly reduced during REM sleep (9.7% vs. 38.5%, $P < 0.01$). Histochemistry in 192 IgG-saporin treated rats showed that neurons that still display RB activity were found in areas devoid of AChE-positive neurons but containing PARV-positive (presumably GABAergic) neurons (Apartis et al., 1998).

Aged rats

During W, the percentage of RB neurons was significantly lower in 23- and 30-month-old rats than in 3-month-old rats (25.6% and 27% vs. 43.8%, $P < 0.01$).This result is in agreement with data previously obtained in urethane anestetized rats (Lamour et al., 1989).The frequency of RB activity decreased at the age of 23 months (5.8 vs. 6 Hz, $P < 0.01$), this change being more pronounced at 30 months (5.3 Hz, $P < 0.0001$). The burst pattern was mildly affected by age: small increases in the number of spikes per burst and in interspike intervals (n.s.) caused a lengthening of the burst duration. During REM sleep, the decrease of RB activity occurred at a more advanced age than during W: the percentage of RB neurons and the frequency of the burst were unchanged at 23 months and decreased only at 30 months (47.6% vs. 80.5%, $P < 0.05$ and 6 vs. 6.8 Hz, $P < 0.01$). No RB activity was recorded during SWS irrespective of age. During W, the frequency of hippocampal theta decreased significantly in both 23- and 30-month-old rats ($P < 0.0001$). During REM sleep, the frequency remained unchanged in 23- but decreased in 30-month-old rats (6.3 vs. 7.0 Hz at 3 months, $P < 0.0001$). The age-related impairments of hippocampal theta and frequency of RB septal neurons followed the same tendency: they appeared earlier during W (23 months) as compared to REM sleep (30 months).

Conclusion

Our data show that RB activity is considerably reduced after selective lesion of the cholinergic MS-DBB neurons. They suggest that the large majority of the RB septal neurons are cholinergic and that the few neurons retaining RB activity in lesioned rats are GABAergic. Intracellular and whole cell patch-clamp recordings show that MS-DBB neurons display features that probably favour RB activity, among them are low-threshold Ca^{2+}conductances (Alvarez De Toledo and Lopez-Barnero, 1988) and inhibitory and excitatory currents with fast kinetics (Schneggenburger et al., 1992). If the majority of RB septal neurons are cholinergic then the decrease of RB activity in aged rats might be the consequence of the death of cholinergic neurons. Surviving neurons show morphological signs of progressive degeneration. It is conceivable that functional properties are impaired in damaged neurons which are no longer able to maintain a normal RB activity.

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5 An antibody specific for the neuronal nicotinic acetylcholine receptor alpha 4 subunit: High levels of expression in monoaminergic neurons

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In the brain, neuronal acetylcholine receptors (nAChRs) comprise two major types of subunits, α and β subunits. Current understanding of nAChR expression and distribution in the brain relies on localization of individual subunit transcripts or in the localization of protein based on immunohistochemistry. Different lines of evidence indicate that, in the central nervous system, nAChRs composed of α4 and β2 subunits are a majority among native nicotinic receptors.

We have attempted the immunohistochemical detection of the α4 subunit using a subunit-specific antibody. We have used a polyclonal antibody raised against a peptide sequence corresponding to the cytoplasmic loop region of the rat α4 neuronal nicotinic acetylcholine receptor, comprising residues 573–592 of the cDNA deduced sequence. First, we assessed the specificity of this antibody using different approaches. Western blot analysis of rat brain homogenates and membrane extracts of cells transfected with α4 and β4 cDNAs of nAChR subunits revealed a band of labelled protein with a molecular mass of 70 kDa.

Subsequently, we used this antibody to study the distribution of this subunit in the transfected cells and the rat brain tissue by light- and electron microscope immunohistochemistry.The immunoreaction product was mainly located in the cytoplasm and dendrites, with varying intensity. The distribution of α4 like-immunoreactive (α4-IR) cell bodies correlated well with previous in situ hybridisation results. Particularly strong labelling was detected in the monoaminergic cell groups in the brainstem. These included the dopaminergic cell areas such as the substantia nigra pars compacta and ventral tegmental area, the serotoninergic neurons of the raphe nuclei and the noradrenergic neurons of the locus coeruleus. Transmitter identity of the α4-positive neurons was tested by double-labelling sections of the rat midbrain both for α4 and for tyrosine hydroxylase, the rate-limiting enzyme responsible for the synthesis of dopamine, or for α4 and serotonin.

Using the DAB technique at the electron microscope level, α4-IR structures comprised the cytoplasm and, particularly, the rough endoplasmic reticulum. In some dendrites, the immunoreactivity was found for the first time in association with postsynaptic densities. Since we observed in co-localisation experiments that α4 coexists with tyrosine hydroxylase in the dopaminergic cells of the substantia nigra, we suggest that nAChRs containing the α4 subunit may play a functional role at the postsynaptic level of nAChRs in these cells.

6 Acetylcholine synthesis activation and neuronal differentiation

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Synaptogenesis has been a central problem in neurobiology for a long time; however, its mechanism is far from being clarified. Neuronal populations, in fact, utilise several transmitter molecules and receptors and follow different time-scales for the acquisition of their specific functions. Production of acetylcholine has been reported to occur in the early stages of the motor neuron development (Berg, 1978; Vaca, 1988); on the other hand a possible role of neurotransmitters in directing the early events of neuron/target interaction has also been postulated (Lipton and Kater, 1989).

Mouse neuroblastoma N18TG2 clone and the derived neuroblastoma × glioma hybrid 108CC15 line provide a useful experimental model to analyse the role of the activation of neurotransmitter synthesis in the progression along neuronal differentiation pathways. In fact, the inability of the former clone to establish synaptic contacts is overcome in the hybrid line and the accomplishment of this more advanced differentiation phenotype is accompanied by the induction of choline acetyltransferase (ChAT) expression, which is actively synthesised in the hybrid, while it is nearly undetectable in the parental line (Hamprecht, 1977). With the aim to establish whether induction of ChAT expression modifies the expression of other specific neuronal markers we have transfected N18TG2 cells with a cDNA construct for ChAT gene (Ishii et al., 1990) and characterised the isolated clones, with high ChAT activity, for their ability to express other neurospecific features (Bignami et al., 1997).

The isolated clones express different ChAT levels, as demonstrated by biochemical assay, Northern blot analysis and immunocytochemical staining. The ability of these clones to synthesize acetylcholine was also demonstrated by HPLC on cellular extract. Northern blot analysis shows increased expression in the transfected clones of mRNAs for neuronal proteins, associated to synaptic vesicle, such as sinapsin I. Fiber outgrowth of transfected clones has also been evaluated to establish whether there is any relation between ChAT levels and morphological differentiation. This analysis shows that the transfected clone 1/2, not expressing ChAT activity, displays a very immature morphology and its ability to extend fibers remains rather poor also in the presence of 'differentiation' agents, as retinoic acid. On the other hand clones 2/4, 3/1 and 3/2, exhibiting high ChAT levels, display higher fiber outgrowth as compared to clone 1/2 both in the absence or presence of differentiating agents (*figure 1*).

Over the last few years a number of data have become available suggesting that, even at early developmental stages, neurotransmitter molecules can be spontaneously released and may act as regulators

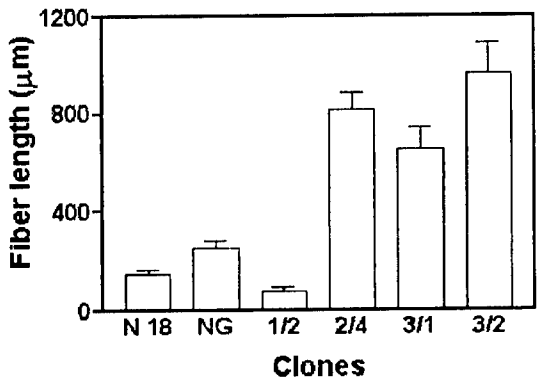


Figure 1. Quantification of process length per microscopic field in cultures maintained in the presence of 10^{-6} M retinoic acid for 4 days. N18, N18TG2 cells; NG, 108CC15 hybrid cells; 1/2, transfected clone not expressing ChAT activity; 2/4, 3/1 and 3/2, transfected clones expressing ChAT activity.

Table I. Secreted AChE activity in the culture medium.

Clones	*Secreted AChE*
aCS5	114.46 ± 9.83
CS37	31.5 ± 0.86
CS42	44.67 ± 1.46
CS43	63.15 ± 4.66

Comparison of AChE secretion in cultures of different clones. AChE release was measured over a 24-h period and was expressed as percent of the cellular content. Values are the mean ± SEM of at least 10 observations. aCS5: transfected clone not expressing ChAT activity; CS37, CS42 and CS43: transfected clones expressing ChAT activity.

of morphogenetic events (Lipton and Kater, 1989). AChE has also been reported as a regulator of neurite outgrowth, and various mechanisms for its action have been proposed (Small et al., 1995). Previous data showing changes of AChE cellular localization and secretion, related to developmental events (Biagioni et al., 1995; Coleman and Taylor, 1996), would be in favour of such hypothesis. It thus appeared of interest to perform a comparative study of AChE secretion in the different isolated clones (*table I*).

Comparison of AChE secretion in cultures of transfected clones showed that AChE release was significantly higher in aCS5 transfected clone not expressing ChAT activity as compared with the CS37, CS42 and CS43 transfected clones expressing ChAT activity. Sequential extraction of AChE activity revelead that the activity extracted in the detergent soluble fraction was considerably higher in the transfected clones expressing ChAT activity (50–55% of the total activity) than in the transfected clone not expressing ChAT activity (about 30%). The results obtained with the sequential extraction procedure support the hypothesis of a shift in the ChAT positive clones from a cytoplasmic to membrane association of the enzyme. Northern blot analysis of the different clones finally showed that the level of AChE mRNA was similar for all clones.

In conclusion these observations are in favour of a relation among activation of neurotransmitter synthesis and progression in the neuronal differentiation pathway. (This work was supported by funds from Fondazione Cenci-Bolognetti, MURST and CNR.)

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7 Kinetic and structural studies on the interaction of the anti-Alzheimer drug, ENA-713, with *Torpedo californica* acetylcholinesterase

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The anti-Alzheimer drug, (+)S-*N*-ethyl-3-[(1-dimethylamino)-ethyl]-*N*-methyl-phenylcarbamate (ENA-713; Exelon™) belongs to a series of miotine derivatives all displaying AChE inhibitory activity towards acetylcholinesterase (AChE) both in vitro and in vivo (Weinstock et al., 1986). Compared with other clinically useful carbamates, ENA-713 has a longer duration of action in vivo, and preferentially inhibits AChE of the brain cortex and hippocampus (Enz et al., 1992, 1993). In order to clarify the basic mechanism of inhibition of AChE by ENA-713, we studied its reactions with *Torpedo californica* (*Tc*) AChE in vitro. The ap-

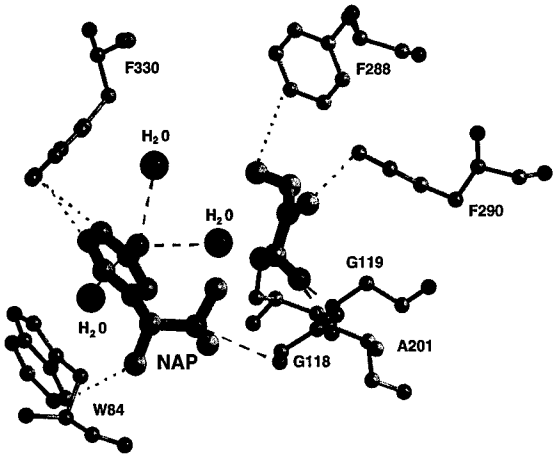


Figure 1. Close-up view of the active site of *Tc*AChE after inhibition with ENA-713, with the individual amino acid residues labelled for orientation. The leaving group, NAP, is bound by interactions with the protein and with three water molecules.

parent bimolecular rate constant for progressive inhibition was low: K_i = 6 $M^{-1}min^{-1}$ in 0.067 M Na/K phosphate buffer, pH 7.4, at 25 °C. The kinetics of reactivation were slow and complex, and displayed a substantial irreversible component. In contrast, *Tc*AChE inhibited by ethylmethylcarbamylchloride, in which the bulky aromatic leaving group, 3-[(1-dimethylamino)ethyl]phenol (NAP), is replaced by chloride, reactivated much faster and quantitatively upon dilution. The apparent reversible binding constant for *Tc*AChE with ENA-713 was 200 µM, whereas NAP had a K_i of 0.5 µM, at pH 7.4 and 25 °C. Thus, the product bound much more tightly to *Tc*AChE than did the intact carbamate in the reversible complex. Trigonal crystals of *Tc*AChE were soaked with ENA-713, and the structure was solved and refined to 2.2 Å resolution. The refinement showed that Ser200 of *Tc*AChE was ethylmethylcarbamylated, and NAP was bound non-covalently in the active site (*figure 1*). Significant contacts of NAP included hydrophobic interactions with Phe330 and Trp84, and its orientation was significantly different from that of edrophonium in the edrophonium-*Tc*AChE complex (Harel et al., 1993). We conclude that ENA-713 can inhibit AChE by two mechanisms: 1) covalent carbamylation followed by slow, partial decarbamylation; and 2) action of the carbamate as a pro-drug to deliver the leaving group, NAP, which is itself a good reversible inhibitor of AChE.

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8 Stimulation of muscarinic receptors in vivo induces dramatic modifications of the subcellular distribution of the m2 muscarinic receptor in striatal interneurons

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Experiments on cells transfected in vitro with G-protein coupled receptors demonstrated that their stimulation by agonists induces different events including phosphorylation, endocytosis of the receptor, dissociation of the ligand from the receptor, dephosphorylation and either degradation of the receptor or recycling to the plasma membrane (Koenig and Edwardson, 1997). However, the mechanisms regulating, in vivo, the compartmentalization and recycling of receptors in neurons and their control by neurotransmitters in physiological, experimental and pathological circumstances are still poorly understood (Dumartin et al., 1998).

The aim of our study was to determine how the cholinergic environment influences the subcellular localization of m2R in striatal interneurons in vivo by using immunohistochemical approaches at light and electron microscopic level (Levey et al., 1995; Bernard et al., 1997). 1) We have examined the cellular and subcellular distribution of m2R in striatal neurons of control animals. 2) We have studied the effect of the stimulation of muscarinic receptors with agonists on the localization of m2R at cellular and subcellular levels and we have determined the time course of this effect. In order to better understand the fate of the receptor after its own activation, we have quantified by using image analysis at electron microscopic level, the modifications of the distribution of m2R gold immunolabelling in the different subcellular organelles. 3) We have determined in these conditions if there is a specificity of internalization of a receptor by its own ligand or if a ligand is able to induce endocytosis of heteroreceptors. For this purpose, we have compared the distribution of the substance P receptor (SPR) and m2R after stimulation of muscarinic receptors.

We have shown that:

1) In control animals, m2R is expressed in cholinergic and NPY/somatostatin interneurons as auto- and heteroreceptors, respectively. In these neurons, m2R is located mostly at the plasma

membrane in perikarya and in dendrites. Presynaptic receptors have been detected in boutons. These data suggest that m2Rs are well-localized to modulate especially the activity of the cholinergic neuron including the electric activity and/or the release of Ach (Billard et al., 1995; Rouse et al., 1997). The m2Rs were usually detected at extrasynaptic sites, albeit they could be found rarely in association with symmetrical synapses, suggesting that the cholinergic transmission mediated by m2R occurs through synaptic and non-synaptic mechanisms.

2) The stimulation of muscarinic receptors with oxotremorine provokes a decrease of the density of m2R at the membrane (–63%) and an increase of those associated with endosomes (+86%) in perikarya. This suggests that the stimulation of m2R induces endocytosis of m2R. Internalization of m2R into endosomes has been shown in dendrites as well. The treatment induces a very strong increase of m2R immunoparticles associated with multivesicular bodies in perikarya and dendrites and thus could be the sign of an increase of the degradation of m2R. We have also shown a slight increase of m2R in the Golgi apparatus (+26%), but not in the endoplasmic reticulum, suggesting that the stimulation of muscarinic receptors may have an effect on the way of synthesis of m2R. We have demonstrated that internalization starts as soon as 3 min after injection and lasts for more than 3 h. Since our quantification has been performed 45 min after injection of oxotremorine, we cannot exclude that other phenomena (neosynthesis, etc.) occur with a different time course. In contrast, the stimulation of muscarinic receptors with pilocarpine has no effect on the distribution of m2R, probably due to the lower specificity for m2R of this drug compared to oxotremorine.

3) We have shown by double immunofluorescence that SPR, a G-protein coupled receptor, is co-localized with m2R in cholinergic and somatostatin/NPY striatal interneurons. In control rats, SPR and m2R are colocalized at the plasma membrane. In contrast, after oxotremorine, SPR stays at the membrane whereas m2R is internalized. This suggests that there is a specificity of internalization of a receptor by its own agonist.

Our data demonstrate for the first time that the cholinergic environment influences in vivo the subcellular distribution, the addressing and the receptor availability of m2R in striatal neurons as auto- and heteroreceptor. The different events following the stimulation of the receptors, including endocytosis, degradation and/or neosynthesis may have a key role in the function of striatal interneurons, especially in neurotransmitter release, and/or electric activity. Our data demonstrate also that the cholinergic transmission may occur through synaptic as well as non-synaptic mechanisms.

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9 Regulation of the expression of the gene encoding the rat vesicular acetylcholine transporter: Role of an upstream region

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Choline acetyltransferase (ChAT) and the vesicular acetylcholine transporter (VAChT) are encoded by two embedded genes, the intronless coding sequence of VAChT lying within the first intron of the ChAT gene. The structure of the ChAT and VAChT genes is conserved from worm to man and defines a so-called 'cholinergic gene locus' (Alfonso et al., 1994; Bejanin et al., 1994; Erickson et al., 1994; Naciff et al., 1997). In mammals, this organization is unusual for two genes whose products are both specifically required for the expression of the same neuronal phenotype. It suggests that transcription for both genes is coor-

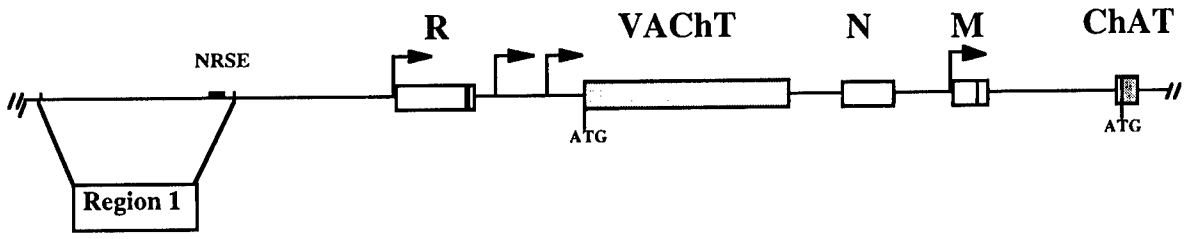


Figure 1. Schematic representation of the 5′ region of the rat ChAT gene and of the VAChT coding sequence. Open and grey filled boxes indicate non-coding and coding sequences, respectively. The black box represents the neuron-restrictive silencer element (NRSE). R, N and M are the three ChAT non-coding exons (Kengaku et al., 1993). Arrows represent the promoter regions identified in the cholinergic gene locus.

dinately regulated.

To dissect the regulatory mechanisms of ChAT and VAChT gene expression, the identification of the promoter regions of the cholinergic gene locus was required. In rodent, two promoters of the ChAT gene have been localized upstream from exons R and M respectively (figure 1; Ibanez and Persson, 1991; Bejanin et al., 1992; Misawa et al., 1992). We then identified rat VAChT transcripts containing the exon R, suggesting that ChAT and VAChT genes share a common upstream promoter (Bejanin et al., 1994). Moreover, we found two promoter regions of the VAChT gene downstream from exon R, which are active in cholinergic as well as in non-neuronal cells (figure 1; Cervini et al., 1995).

In the present study, we have investigated the regulatory mechanisms that restrict the activity of the rat VAChT promoters to cholinergic cells. We particularly focused on a genomic region of potential regulatory interest, designated as Region 1 and situated upstream from the R-type promoter (*figure 1*). This 2343-bp region has been shown to direct the expression in vivo of a heterologous promoter in cholinergic structures (Lönnerberg et al., 1995). Moreover, it contains a neuron-restrictive silencer element (NRSE) that interacts with a neuron-restrictive silencer factor to repress the activity of a downstream heterologous promoter in non-cholinergic cells (Lönnerberg et al., 1996). Transient transfection experiments of different types of cell lines reveal that Region 1 contributes to the cell specific regulation of the activity of the VAChT promoters, but is not sufficient to specifically restrict their activity to cholinergic cells. Experiments are in progress to determine whether the NRSE itself is required for the regulation of the VAChT gene expression.

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10 Effect of ciliary neurotrophic factor on cholinergic gene expression: Modulation by nerve growth factor and glucocorticoids.

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The two proteins essential for cholinergic neurotransmission, choline acetyltransferase (ChAT) and the vesicular acetylcholine transporter (VAChT), are encoded by the cholinergic locus, which exhibits unique genomic organization; the VAChT coding sequence is contained within the first intron of the ChAT gene. The two genes share some regulatory promoter sequences and noncoding exons. We and others had demonstrated that the expression of these genes is coordinately regulated (Berrard et al,. 1995; Berse and Blusztajn, 1995; Misawa et al., 1995; Berse and Blusztajn, 1997; Hill and Robertson, 1997; Matsuura et al., 1997; Sun et al., 1997), however, there are examples of physiological situations where only one is expressed, and not the other (Holler et al., 1996; Fujii et al., 1998; Kitamoto et al., 1998). Many extracellular factors, including neurotrophins, retinoids, glucocorticoids, and cytokines, have been shown to affect acetylcholine (ACh) production through the modulation of ChAT/VAChT expression. Here we demonstrate that the activation of cholinergic gene expression by ciliary neurotrophic factor (CNTF) is modulated by nerve growth factor (NGF) and glucocorticoids.

As a model, we use a hybrid cell line (SN56), generated by fusing septal primary neurons obtained from postnatal day 21 mice with murine neuroblastoma N18TG2 cells. SN56 cells have many features of septal cholinergic neurons, however they do not express the TrkA high affinity NGF receptor. For experiments involving NGF, we restored TrkA signaling in SN56 cells by stable transfection with the plasmid pDM115 (a gift from Dr. Moses Chao), carrying rat *trkA* cDNA under the control of the murine sarcoma virus long terminal repeat promoter. The plasmid contains the neomycin resistance gene under the control of the SV40 promoter, for selection with geneticin (G418). The transfected clones were maintained in medium with G418, and screened for TrkA expression by Western blotting. Two clones expressing high levels of TrkA, and one TrkA-negative transfectant were selected for further experiments.

Cells were grown in DME medium supplemented with 10% fetal bovine serum in the presence or absence of test compounds for varying time periods. For ACh measurements, the cells were incubated at 37 °C in medium supplemented with 5 μM choline and 15 μM neostigmine, for 1 h prior to collection. The content of ACh was determined by HPLC with an enzymatic reactor containing acetylcholinesterase and choline oxidase and an electrochemical detector. Northern blotting with mouse cDNA probes for ChAT and VAChT was performed as described previously (Berse and Blusztajn, 1995).

We found that the expression of ChAT and VAChT mRNA and ACh content are coordinately up-regulated by CNTF, glucocorticoids and NGF in SN56 cells. In addition, we showed that there is a synergistic effect of CNTF (20 ng/mL) and dexamethasone (a synthetic glucocorticoid, 1 μM) on ChAT and VAChT mRNA expression. CNTF treatment of SN56 cells caused rapid (within 5 min) translocation of the transcription factor Stat3 to the nucleus and activation of specific binding to the CNTF response element, as evidenced by electrophoretic mobility shift assay. Pre-treatment of the cells with dexamethasone for various periods of time did not affect Stat3 translocation evoked by CNTF. Several recent studies addressed the mechanisms of the synergy between glucocorticoids and the IL-6 family of cytokines (of which CNTF is a member). The proposed models include glucocorticoid-enhanced expression of IL-6 signaling components (Nesbitt and Fuller, 1992), as well as direct complex formation between Stat3 and the glucocorticoid receptor leading to transcription co-activation through both cytokine- and glucocorticoid-responsive elements (Zhang et al., 1997). Experiments are in progress to determine if a similar complex is formed upon co-stimulation of SN56 cells with CNTF and dexamethasone.

Stimulation of SN56 TrkA transfectants with NGF activated two signaling pathways; one inducing tyrosine phosphorylation of the adapter protein Shc and MAP kinases ERK1 p44 and ERK2 p42, and one modulating the cytosolic free calcium concentration. In the transfectants, NGF (100 ng/mL for 48 h) increased ChAT/VAChT mRNA expression and acetylcholine production up to two-fold. NGF treatment also induced better attachment to the substratum and neurite formation. These responses did not occur in the untransfected SN56 cells or in a TrkA-negative transfectant, which express only the low affinity p75 NGF receptor; therefore we conclude that these actions of NGF require TrkA signaling. Surprisingly, the combined treatment of SN56 cells expressing TrkA with CNTF and NGF revealed that NGF down-regulated the stimulatory effect of CNTF on the expression of the cholinergic locus. This is probably not due to interference with Stat3 signaling, since pre-treatment with NGF for various time periods between 15 min to 48 h did not inhibit rapid Stat3 translocation evoked by CNTF. The results suggest that NGF affects the induction of cholinergic gene expression by CNTF through mechanisms other than interference with the JAK/STAT pathway.

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11 Glutamate receptor-mediated synaptic responses in rat hippocampus after cholinergic deafferentation by the immunotoxin 192 IgG-saporin. Possible implications for the aging brain.

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Alterations in both cholinergic and glutamatergic systems have been suggested to contribute to the deficit in memory functions occurring during normal aging and related diseases (McEntee and Crook, 1993; Muir, 1997). Nevertheless, the relative role of each component remains an open issue. In fact, the effect of glutamate on acetylcholine (ACh)-mediated responses is well documented (see Giovannini et al., 1997 for review). On the contrary, less is known about a cholinergic influence on responses induced by glutamate receptor activation (Markram and Segal, 1990). In the present study, we studied the effects of lesioning the cholinergic septohippocampal fibres with the immunotoxin 192 IgG-saporin (Wiley et al., 1991) on the synaptic responses mediated by glu-

tamate in area CA1 of rat hippocampus. Concomitantly, these responses were recorded in aged animals to better assess the consequences of the cholinergic deficit in these animals.

Experiments were conducted in 3–4-month-old and 25–33-month-old male Sprague-Dawley rats, as well as in young rats treated with an intraventricular injection of 192 IgG-saporin (4 μg in 7.5 μL of saline). One animal was daily studied using extracellular recordings in the ex vivo slice (400 μm) preparation. The composition of the bath medium was (mM): NaCl, 119; KCl, 3; $MgSO_4$, 1.5; $CaCl_2$, 3; $NaHCO_3$, 26.2; NaH_2PO_4, 1.0; glucose, 11. Extracellular recordings were obtained from the apical dendritic layer of the CA1 hippocampal area using micropipettes filled with 2 M NaCl and having resistances of 2–6 MΩ. Presynaptic fibre volleys (PFVs) and field EPSPs (fEPSPs) were evoked by electrical stimulation (100 μs duration) of CA1 afferent fibres (Schaffer collaterals and commissural fibres) located in the stratum radiatum.

Input-output (I/O) curves were first studied in control medium in young (10 slices in five animals), 192 IgG-saporin pretreated (11 slices in three animals) and aged rats (seven slices in five animals). Stimulation of the stratum radiatum induced a PFV followed by a fEPSP, both increasing with the stimulus intensity. The fEPSPs were totally suppressed by CNQX (10 μM) thus reflecting the activation of non-N-methyl-D-aspartate receptors (non-NMDAr). The comparison of the I/O curves revealed that the PFV were not significantly altered in young and 192 IgG-saporin pretreated animals [$F(1,20) = 1.55$, $P = 0.23$] as well as in young and aged rats [$F(1,15) = 1.754$, $P = 0.2$]. However, when the fEPSP slopes were plotted against the slope of the PFVs, we found that the non-NMDAr-mediated responses were not statistically affected in rats with the cholinergic denervation of the hippocampus [$F(1,20) = 5.87$, $P = 0.17$]. On the contrary, fEPSPs were significantly depressed in aged animals [$F(1,15) = 6.229$, $P = 0.02$].

In another sets of experiments, I/O curves were constructed from slices perfused for at least 40 min with a Mg^{2+}-free medium and where CNQX was added 20 min before the recordings. In these conditions, a fEPSP was induced which differed from that recorded in control medium by its slow onset, its prolonged duration and its sensitivity to 2-APV (30 μM). Comparison of the NMDAr-mediated synaptic responses revealed that both the amplitude [$F(1,25) = 42.3$, $P < 0.0001$] and the duration [$F(1,25) = 5.87$, $P = 0.023$] of NMDAr-mediated fEPSPs were significantly increased in 192 IgG-saporin pretreated rats. In aged rats, we found that the duration of these responses was significantly enhanced [$F(1,28) = 5.08$, $P = 0.03$] whereas the amplitude was not statistically altered [$F(1,29) = 0.001$, $P = 0.97$].

The present study first did not reveal significant alterations of non-NMDAr-mediated synaptic responses of CA1 pyramidal cells after lesion of cholinergic septohippocampal fibres by the immunotoxin 192 IgG-saporin. These responses were significantly depressed in aged rats (Barnes et al., 1992; Papatheodoropoulus and Kostopoulos, 1996). These results therefore indicate that the cholinergic deficit occurring in the aged hippocampus (see Muir, 1997) do not significantly contribute to the impaired activation of these glutamate subtypes of receptors. Accordingly, this impairment was suggested to result mainly from a decreased number of synaptic contacts between glutamatergic afferents and pyramidal cells (Barnes, 1994). On the other hand, we found that synaptic responses mediated by activation of NMDAr were significantly increased both in amplitude and duration in rats pretreated by 192 IgG-saporin as we previously reported with the use of intracellular recordings (Jouvenceau et al., 1997). Because the presynaptic fibre volley was not altered in these animals, this enhanced NMDAr mediated responses might not be due to a sprouting of glutamatergic fibres in response to the loss of cholinergic afferences. In addition, it might result from an impaired activation of cholinergic heteroreceptors presynaptically localized on glutamatergic terminals inhibiting glutamate release (Marchi et al., 1989). However, this hypothesis predicts a parallel increase in non-NMDAr responses and we found that these responses were not affected. Finally, an increase in NMDAr binding sites seemed unlikely because it was reported to be unaffected or even decreased after cholinergic deafferentation by 192 IgG-saporin (Roβner et al., 1995; Nicolle et al., 1997). In view of these results, we suggest that the increase in NMDAr activation induced by the cholinergic denervation may rather reflect changes in the gating properties of associated channels as supported by the significant increase in the duration of these glutamatergic responses (see also Jouvenceau et al., 1997). Interestingly, we found that the amplitude of NMDAr mediated responses was not altered in aged animals despite the assumption of fewer synaptic contacts between glutamatergic afferent fibres and pyramidal cells in these animals (see also Billard et al., 1997) suggesting the occurrence of compensatory mechanisms. Considering the increased NMDAr activation recorded in the cholinergic deafferented hippocampus of 192 IgG-saporin pretreated rats, we may hypothesize that the age-related cholinergic deficit may be one possible mechanism of this compensation.

In conclusion, this study shows that in CA1 hippocampal area: i) the glutamatergic neurotransmission is differentially affected by the cholinergic denervation depending on the glutamate subtype of receptors; and ii) the age-related cholinergic deficit may facilitate NMDAr activation and consequently synaptic plasticity in aged animals.

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12 Acetylcholine synthesis and quantal release from mouse neuroblastoma after co-transfection of choline acetyltransferase and mediatophore cDNAs

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The non-cholinergic mouse neuroblastoma cells N18TG-2 do not express proteins such as choline acetyltransferase (ChAT) or vesicular acetylcholine transporter. Even when loaded with acetylcholine (ACh) they are not able to release the neurotransmitter (Falk-Vairant et al., 1996; Israël et al., 1994). They provide therefore an appropriate preparation for investigating the molecular requirements of calcium-dependent ACh release, and its relationship with ACh synthesis.

In the present work, we have studied the effect of N18TG-2 transfection with either rat ChAT cDNA or *Torpedo* mediatophore cDNA or both. The mediatophore is a proteolipid isolated from presynaptic membrane of *Torpedo* cholinergic nerve terminals. ACh release was determined by means of both a chemiluminescent assay and an electrophysiological assay using *Xenopus* myocytes as ACh detectors. When transfected with a plasmid coding for the rat ChAT, N18TG-2 cells accumulate endogenously synthesised ACh but, confirming previous results (Zhong et al., 1995), they are still unable to release the neurotransmitter. In contrast, upon transfection with a plasmid coding for the 16 kDa subunit of the *Torpedo* mediatophore, N18TG-2 acquire the ability to release artificially loaded ACh (Falk-Vairant et al., 1996). When co-transfected with both rat ChAT and *Torpedo* 16 kDa subunit cDNAs, N18TG-2 cells can then release endogenously synthesised ACh.

We further examined whether the release process supported by the mediatophore shows any difference depending on the source of neurotransmitter (loaded or synthesised). Results from real time detection of release using an ACh-sensitive *Xenopus* myocyte show no difference in the kinetic parameters of ACh secretion. The observed time constants are furthermore very close from values recorded in immature *Xenopus* neuro-muscular synapse (Girod et al., 1992). Quantal analysis, based on the failure ratio, also shows no difference in the quantal size, estimated in both cases at about 200 pA, i.e., about 500–1000 ACh molecules, which roughly corresponds to the size of a subminiature potential in the *Torpedo* electric organ or a neuromuscular junction. On the other hand, when the amount of ACh released is expressed as a function of cellular content, it is found that N18TG-2 cells transfected with mediatophore subunit alone and loaded with ACh release only 15.5% of their initial content, whereas this percentage is about 70% in the case of ChAT/16kDa co-transfected N18TG-2 cells. The co-transfected cells utilise thus a larger proportion of their initial transmitter store; in other words they seem more efficient for release than ACh-loaded cells. Different hypotheses can be put forward to explain this difference: 1) ChAT transfection could indirectly induce the expression of other synaptic proteins (Bignami et al., 1997), which could up-regulate the release process; 2) endogenously-synthesised ACh could be in a better position for fuelling the release process than externally-loaded ACh; and 3) the rate of ChAT activity could be boosted during release, supplying extra amounts of ACh, as previously observed in situ with a variety of preparations.

In conclusion, in N18TG-2 cells the mediatophore can be considered as the minimal molecular requirement for quantal ACh release. This process operates more efficiently when using ACh synthesised by the cells. This increased efficiency of mediatophore-supported ACh release by co-expression of ChAT must have a physiological significance and merits further investigation.

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13 Downregulation of muscarinic autoreceptor signaling in murine SN56 septal cholinergic cells by acetylcholinesterase inhibitors due to long-term exposure to released acetylcholine

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Acetylcholinesterase (AChE) inhibition elevates acetylcholine (ACh) concentrations within cholinergic neurons and in the synaptic cleft, causing prolonged interaction of ACh with its receptors. We describe the establishment and characterization of an in vitro model that resembles the cholinergic synapse, and the application of this model to the study of the effects of AChE inhibitors on the functional properties of the cholinergic synapse. The model employs a murine cholinergic neuronal cell line, SN56, that possesses both the capacity to synthesize, store, and release ACh, as well as to respond to ACh. The cellular responses to ACh are mediated by muscarinic receptors coupled to elevations of intracellular calcium concentrations ($[Ca^{2+}]_i$). Thus, SN56 cells can be used to study both the regulation of ACh synthesis and release as well as the regulation of muscarinic receptor function, i.e., the pre- and the postsynaptic cholinergic mechanisms. We studied the functional sequelae of the exposure of SN56 cells to AChE inhibitors by determining their ACh content and changes in intracellular free calcium concentrations ($\Delta[Ca^{2+}]_i$) in response to a muscarinic agonist, carbachol. Two inhibitors belonging to the class of organophosphorus (OP) compounds, DFP or paraoxon, caused time- and concentration-dependent elevations of intracellular ACh content by as much as nine-fold. The EC_{50} values for these OP were 0.1 μM and 2.5 μM, respectively. Similar elevations in ACh content were found in cells treated with the carbamates neostigmine and physostigmine. Moreover, in cells treated with AChE inhibitors, the spontaneously released ACh accumulated in the medium due to inactivation of surface AChE. Basal $[Ca^{2+}]_i$ in SN56 cells was 30–150 nM. Carbachol (1 mM) increased $[Ca^{2+}]_i$ to a maximum of 500 nM within 10 s. This effect was prevented by 1 μM atropine. In cells treated for 24 h with DFP (10 μM), paraoxon (10 μM) or physostigmine (100 μM), carbachol increased $[Ca^{2+}]_i$ to a maximum of only 250 nM, 120 nM and 160 nM, respectively. The effect of AChE inhibitors required long-term exposure (hours) as a 10-min preincubation of the cells with paraoxon or a 30 s exposure to physostigmine had no effect on carbachol-evoked $\Delta[Ca^{2+}]_i$. To determine if the OP specifically downregulated $\Delta[Ca^{2+}]_i$ evoked by muscarinic receptors, we tested the effects of these OP on bradykinin- and thapsigargin-evoked $\Delta[Ca^{2+}]_i$. The response to bradykinin is mediated by seven transmembrane domain receptors (Farmer and Burch, 1992) similar to muscarinic receptors, whereas thapsigargin causes elevations in $[Ca^{2+}]_i$ by inhibiting the endoplasmic reticulum Ca^{2+}-ATPase (Thastrup et al., 1990). Bradykinin (10 μM) or thapsigargin (1 μM) increased $[Ca^{2+}]_i$ in SN56 cells (by 250 and 300 nM, respectively). DFP (10 μM, 24 h) had no effect on these $\Delta[Ca^{2+}]_i$.

These data can be explained as follows. SN56 cells treated with AChE inhibitors accumulate large amounts of ACh because the newly-synthesized neurotransmitter cannot be hydrolyzed by the inactivated AChE. Some of this ACh is released spontaneously and accumulates in the medium because the membrane-associated AChE is also inactive. ACh in the medium binds to the muscarinic receptors, which desensitize upon long-term exposure to the agonist. As expected, this desensitization is specific for muscarinic receptors, as no desensitization of bradykinin receptors is seen and no changes in general cellular calcium homeostasis occur, as evidenced by the lack of changes in the basal $[Ca^{2+}]_i$ and by the absence of an effect of AChE inhibitors on the cellular responses to thapsigargin. This explanation is consistent with the demonstrations that muscarinic receptors desensitize rapidly upon exposure to an agonist in vitro (Tobin et al., 1992; Dell'Acqua et al., 1993; Robbins, 1993; Wojcikiewicz et al., 1994; Inoue et al., 1995; Schmidt et al., 1995; Coggan and Thompson, 1997) and with observations made in rats that treatment with DFP results in reductions of muscarinic receptor number (Schiller, 1979; Schwartz and Kellar, 1983; Aronstam et al., 1987; McDonald et al., 1988; Abdallah and El-Fakahany, 1991; Bushnell et al., 1991) accompanied by cognitive impairments (McDonald et al., 1988; Bushnell et al., 1991). Recent studies have shown that the latter action of DFP involves not only the reduction of muscarinic receptor numbers but also lowering of mRNA expression (Yagle and Costa, 1996).

Our in vitro model can be applied to the study of multiple compounds, including potentially toxic substances, that may affect any of the pre- or postsynaptic cholinergic mechanisms, because it permits the evaluation of multiple endpoints in a single experiment. AChE inhibitors are used clinically to treat certain diseases (e.g., the centrally-active AChE inhibitors tacrine or E2020 are used for the treatment of Alzheimer's disease). In addition humans may be exposed to AChE inhibitors (e.g., pesticides or chemical weapons). Indeed there is evidence that Gulf War Syndrome may be the result of exposure to low levels of OP. Recent studies have shown that exposure of rats to low doses of DFP causes impairment of spatial learning (Buccafusco et al., 1997; Prendergast et al., 1997) and downregulation of muscarinic and nicotinic receptors (Buccafusco et al., 1997; Wickelgren, 1997). These in vivo results point to a heretofore unappreciated adverse effect of AChE inhibitors: long-term cognitive impairment. The current use of AChE inhibitors (tacrine, E2020) to treat Alzheimer's disease may result in the downregulation of muscarinic and nicotinic receptors in the brains of patients taking these compounds. Receptors in brain areas targeted by the disease process (e.g., hippocampus and cortex) are probably not going to be adversely affected by AChE inhibitors because the cholinergic input to those areas is compromised, and the AChE inhibitors are likely at best to restore ACh levels, rather than to cause hyperphysiological concentrations of the neurotransmitter. However, in brain areas spared by Alzheimer's disease (e.g., striatum), ACh levels would be expected to increase, and result in side effects related to the downregulation of muscarinic receptors. Similarly, components of chemical weapons can be assessed in our model for their ability to downregulate muscarinic receptor function. Finally, the model is well suited as a tool for the development of pharmacological strategies to restore the function of muscarinic receptors downregulated by the use of AChE inhibitors. (Supported by US EPA CR821929-01-0.)

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14 Regulation of desensitization by beta subunit in neuronal nAchR

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The high diversity of neuronal nicotinic receptors generates a wide panel of ligand selectivity and desensitization kinetics.

Desensitization takes place upon long-lasting application of ligand, as a final step in the conformational changes of the molecule. It is a key issue for understanding the prolonged action of ligands and the physiological role of these receptors in nicotine dependence.

In heteropentameric receptors, the structures modulating desensitization may localize on the alpha as well as on the beta subunit. Here we report of molecular determinants carried by the beta subunit. Using a series of chimeras between the two pharmacolgically distinct receptors $\alpha3\beta2$ and $\alpha3\beta4$, we show that regions 1–121 and 129–229 of the N-terminal domain independently regulate the rate of desensitization. Each region can transfer the fast desensitizing rate of $\alpha3\beta2$ towards $\alpha3\beta4$. Both regions contain photoaffinity labeled residues and are likely to interact with the ligand.

Similarly, these two regions control the sensitivity to agonist cytisine, which selectively activates $\alpha3\beta4$.

15 Electrostatic homology modelling of a set of ChE-like neural adhesion proteins identifies a shared 'annular' motif with ChEs. Structural implications for a cell-recognition role of ChEs

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The concept of an electrostatic motif on the surface of biological macromolecules as a definite topographical pattern of electrostatic potentials in 3D space, provides a powerful tool for identification of functionally important regions on the surface of structurally related macromolecules [1]. We employ this tool to analyze the electrostatic properties of cholinesterases (ChEs) from various

species and show that all these structures reveal a negative external surface potential in an 'annular' area around the entrance to the active-site gorge that becomes more negative as one approaches the rim of the gorge. These potentials are highly correlated among the structures examined, down to a sequence identity as low as 35%, indicating that they are a conserved property of the cholinesterase family [2]. We further suggest that the coincidence between a shared topological arrangement of the macromolecular chain and a conserved electrostatic motif can be used to identify proteins sharing a common recognition mechanism. Using this approach, we identify a functional region common to ChEs and to gliotactin (GLI) [3], neurotactin (NRT) [4], and neuroligin-1 (NL-1) [5], a set of neural cell-adhesion proteins which have been suggested to be structurally related to ChEs due to their high sequence similarity, but lacking the key catalytically active serine [6].

In order to confirm the presence of shared structural motifs and to investigate the possibility that they might display similar electrostatic characteristics to ChEs, we have built homology models of the extracellular domains of GLI, NRT and NL-1 and compared them to the known structures of ChEs and of a series of lipases that have the same degree of sequence identity with ChEs and share the same 3D fold (*table I*). A quantitative analysis of the surface electric potential in the area around the entrance to the active site gorge for AChE, and in the analogous zone for the ChE-like domain of the adhesion proteins, shows that GLI, NRT and NL are all characterized by a high correlation coefficient with AChE and have an average potential in the 'annular' region very similar to that of AChE; in addition, their potential gradient closely follows that of AChE (*figure 1*). The same analysis performed on lipases with a ChE-like fold does not reveal the presence of a conserved electrostatic motif. These findings, taken together with the evidence for the involvement of ChEs in cell adhesion independent of their catalytic activity, suggests that the ChE-like domain of GLI, NRT and NL-1 may share a common recognition mechanism or a similar ligand in common with ChEs. Further evidence comes from the recent results of a study in which chimeric constructs of the C-terminal transmembrane domain of NRT fused to the extracellular cholinesterase-like domains of *Dm*AChE or *Tc*AChE, were shown to be endowed with the same

Table I.

Protein	*Percent identity to TcAChE (DmAChE)*	*Percent similarity to TcAChE (DmAChE)*
Cholinesterases		
*Tc*AChE	100.0	100.0
hAChE	57.6	85.0
mAChE	59.0	85.3
*Dm*AChE	36.3	65.8
7-hAChE	57.6	85.0
hBChE	52.4	83.1
Adhesion proteins		
NRT (*Dros.*)	31.1 (*25.1*)	57.7 (*50.4*)
GLI (*Dros.*)	30.6 (*23.5*)	61.0 (*55.7*)
NL (mouse)	32.3	60.1
Lipases		
*Gc*Lip	29.4	56.8
rBSSL	31.2	61.4
*Cr*CE	31.9	56.8

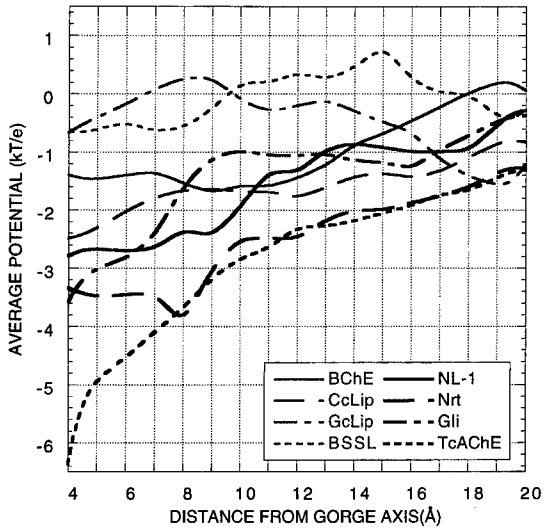


Figure 1.

hydrophilic properties as wild type NRT [7]. On the basis of these findings, we consider GLI, NRT, NL-1 and AChE to be members of a class of adhesion proteins which we have named 'electrotactins'. We hypothesize that a chimeric construct of the cytoplasmic domain of NRT with a mutant form of AChE, in which seven negative residues have been neutralized to abolish the electrostatic motif (7-hAChE) [8], will not display the same adhesive properties as a construct built with wild-type AChE. We further discuss these results in the light of the reported involvement of ChEs in the formation of β-amyloid fibrils [9], the major component of plaques in Alzheimer brain.

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16 Biotinylation of the tyrosine residues of fasciculin

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Fasciculins are 7 kDa peptides with four disulfide bridges; they belong to the family of three-fingered snake toxins that includes, among others, the selective nicotinic receptor blockers, α- and κ-neurotoxins [1]. Fasciculins are potent and selective inhibitors of acetycholinesterase (AChE); they bind to the peripheral site of the enzyme, a site located at the mouth of the structural gorge leading to the buried active center [2]. Kinetic studies have shown that the fasciculin-AChE complex, despite its high affinity and extremely slow rate of dissociation, possesses residual catalytic activity: in the complex in solution, both substrate and catalytic site inhibitors are able to access the active center of the enzyme (cf. [3] for references). However, the recently solved X-ray structure of the Fas2-mouse AChE complex suggested that fasciculin, bound at the peripheral anionic site of the enzyme, totally occludes substrate access to the active site, a departure from the kinetic data [4]. A functional map of the fasciculin molecule was established based on site-directed mutagenesis of several residues located at and near the complex interface [3]; however, the contribution of the tyrosine residues remained to be investigated.

Biotinylation of fasciculins Fas1 and Fas2, which respectively contain four and three tyrosine residues, was carried out using *p*-diazobenzoyl biocytin (DBB). Fas1 was found to be biotinylated on Tyr4, Tyr47 and C-terminal Tyr61, and Fas2, which contains the Tyr47Asn substitution, on Tyr4 and Tyr61. In both cases, Tyr22 was not reactive, consistent with absence of reactivity toward enzyme-catalysed iodination [5] and non-accessibility to solvent [6]. All the mono-, di- and tri-biotinylated derivatives were characterised structurally and functionally. Their respective inhibitory activities toward mouse AChE were established both kinetically and at equilibrium. Our results complement previous mutagenesis data [3] in providing new insight into the participation of the tyrosine residues of fasciculin in complex formation and stability. This work was supported by the Association Française contre les Myopathies (to PEB and PM).

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17 Human neuronal nicotinic acetylcholine receptors (nAChRs) may participate in the sexual dimorphism functionality of the brain

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Pathologies such as catamenial epilepsy and postnatal/postmenopausal depressions are associated with modification of women's hormonal status. These paroxismic situations, which are related to severe variations of circulating sex hormones, indicate that neuronal tissues are influenced by sex hormones (progesterone, 17β-oestradiol and testosterone). Thus, in non-pathological conditions, it is very likely that sex steroids may influence some of the central nervous system (CNS) functions. As an illustration, a recent positron-emission tomography (PET) study has revealed that progesterone and 17β-oestradiol modulate cognition-related activities in women[1]. From the endocrine point of view, sex steroids are synthesized in gonads (progesterone and 17β-oestradiol in ovaries, testosterone in testis). However, in the brain testosterone can be converted into 17β-oestradiol through P450 aromatase activity. To complicate matters further, some steroids are de novo synthesized by cells of the CNS and constitute endogenous sources of 'neurosteroids'[2]. As a correlate, women and men CNS are exposed to different steroids through their entire life. Thus, it is tempting to speculate that some brain functions may be differentially influenced by sex hormones.

Interestingly, sex differences related to the cholinergic system have been reported. In rodents, a sexual dimorphism of α-bungarotoxin binding in the brain has been observed[3]. Up-regulation of nAChRs by chronic administration of nicotine has also been shown to be sex related[4]. Functional studies indicate that 17β-oestradiol is able to potentiate the nicotine-evoked dopamine release in the striatum of female rats whereas it has an opposite effect in the striatum of males[5]. Moreover, the implication of the

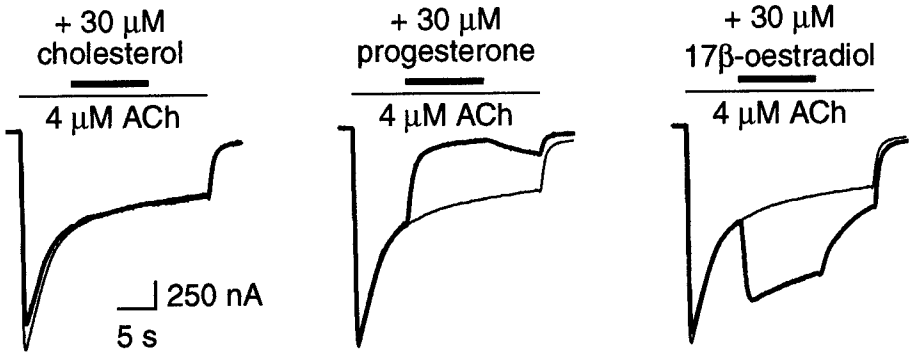


Figure 1. Inhibition and potentiaition by progesterone and 17β-oestradiol of human α4β2 nAChRs expressed in *Xenopus* oocytes.

cholinergic system and especially of neuronal nAChRs is strongly suspected in the sexual differences of rodents for learning task performances such as, for instance, the Morris water maze[6] or active avoidance paradigms[7]. In man, Alzheimer's disease (AD) affects women more frequently than men[8].

According to the literature, regulation of CNS functions by sex steroids may implicate two different pathways and time-courses. On one hand, sex steroids induce delayed and long-term effects following their binding to intracellular receptors[9]. On the other hand, these cholesterol-derived molecules have fast-mediated effects on proteins embedded in the cytoplasmic membrane such as G-protein coupled receptors[10], voltage-gated channels (VGCs)[11, 12] and ligand-gated channels (LGCs)[13–15].

Among the vertebrates, neuronal nAChRs constitute a large family of cationic ligand-gated channels (LGCs) encoded by eleven different genes, eight alphas (α2-α9) and three betas (β2-β4) that are broadly distributed within the brain but have also been identified in non-neuronal tissues[16]. These receptors result from the assembly of five subunits around a pseudo-axis of symmetry and form both the ionic channel and binding sites for their natural agonist, acetylcholine (ACh). Moreover, neuronal nAChRs present different regulatory binding sites for allosteric modulators such as calcium, arachidonic acid and steroids[17]. Although a progressive reduction of neuronal nAChRs is observed during aging, an important loss of nicotinic binding sites was found in patients suffering from AD or Parkinson's disease[18], a reduction that is thought to reflect a specific loss or down regulation of the α4 and/or β2 subunits[18]. Indeed, the α4β2 nAChR is believed to constitute the nicotine high-affinity binding sites in the brain[19] and to play a central role in smoking addiction[20–22]. The observation that nicotine as well as other nAChRs ligands can enhance cognitive performances triggered intensive research aiming to identify new molecules that could be of therapeutic use in the treatment of AD or Parkinson's disease[23].

To investigate the influence of sex hormones on human nAChRs, receptors were reconstituted with subunits expressed either in Xenopus oocytes or in stably transfected cells. Electrophysiological recordings confirmed that progesterone is a potent negative allosteric modulator of both homomeric and heteromeric nAChRs. In contrast, 17β-oestradiol at μM concentration potentiates α4β2 ACh-evoked currents, whereas this steroid still inhibits α3β2 and α7 nAChRs. Macroscopic effects were confirmed at the microscopic level by single channel recordings and demonstrated that 17β-oestradiol is a positive allosteric effector at the α4β2 nAChR. These results provide a molecular basis that may explain the lower success of women in smoking cessation trials as well as beneficial effects of estrogen replacement therapy for people suffering from AD. This work was supported by the Swiss National Science Foundation and the OFES to D.B.

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18 Expression of neuronal nicotinic acetylcholine receptor mRNA in rat dopaminergic neurons

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Numerous studies have demonstrated that activation of nicotinic acetylcholine receptors (nAChRs) can modulate dopaminergic neurotransmission. Nicotinic agonists have been shown to increase dopamine release both in vivo and in vitro[1] and several behavioural effects of nicotinic agonists can be directly attributed to this interaction with the dopaminergic system. Moreover, dopamine release in the mesolimbic system is thought to be responsible for the addictive properties of nicotine[2]. However, the composition of the native receptors which are implicated in these phenomena remains unclear.

Nicotinic receptors are pentameric ligand-gated ion channel proteins which can be composed of various subunit combinations. In the mammalian central nervous system six α subunits (α2–7) and three β subunits (β2–4) have been identified[3] and most of these (α3–6 and β2–3) have been detected in the substantia nigra (SN) and ventral tegmental area (VTA) by in situ hybridization[4]. Recent studies with β2 subunit knockout mice suggested that β2-containing nAChRs play a role in nicotine-induced dopamine release[5]. Pharmacological analyses also indicate that at least two different receptor subtypes, one containing a α3/β2 subunit interface, may be involved[5,6]. In addition, electrophysiological studies have shown the probable presence of α7 receptors in a minority of the VTA dopaminergic neurons[7]. However, these two brain regions also contain non-dopaminergic, mostly GABAergic, neurons and interneurons[8] and only the α6 subunit has been recently unambiguously localized in dopaminergic neurons[9].

We have used the RT-PCR method to determine which nAChR subunit's mRNA is specifically expressed in dopaminergic neurons of the rat SN and VTA after unilateral destruction of the ascending dopamine system by injection of 6-hydroxydopamine (6-OHDA).

The results obtained with sham-operated animals demonstrate the presence of α3–7 and β2–4 subunit mRNAs in the SN and of α2–7 and β2–4 subunit mRNAs in the VTA *(table I)*.

The results were identical for both hemispheres, showing that the injection procedure alone had not affected nAChR mRNA expression. Identical results were also obtained with contralateral (uninjected) hemispheres of 6-OHDA-treated animals *(table I)*. In situ hybridization studies have not previously demonstrated the presence of β4 and α7 subunit mRNA in the SN and VTA, nor of the α2 subunit in the VTA. This probably reflects the greater sensitivity of the RT-PCR technique used here and thus suggests that these subunits are expressed at a low level. Indeed, electrophysiological studies suggest the presence of α7 receptors only within a minority of VTA dopaminergic neurons[7] and pharmacological studies using the nicotinic agonist cytisine also implicate β4-containing nAChRs in the facilitation of dopamine release[1].

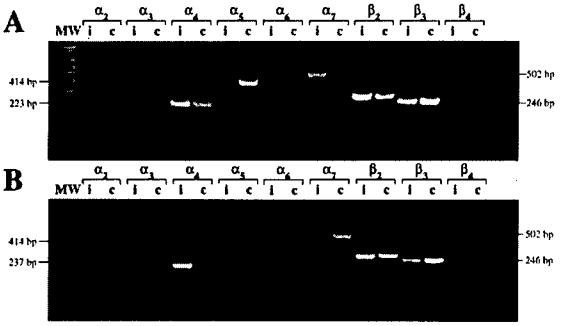


Figure 1. Analysis of PCR products obtained from SN (A) and VTA (B) RNA of a 6-OHDA-injected rat. Results from injected ipsilateral (i) and non-injected contralateral (c) hemispheres are presented. Subunit-specific primers used for each amplification are indicated above each lane. MW indicates molecular weight marker.

In contrast to the non-lesioned side, the 6-OHDA lesion resulted in a complete absence of amplification product for α3, α5, α6 and β4 subunits in the SN and for α2, α3, α5, α6, α7 and β4 subunits in the VTA *(table I)*. These results corroborate recent studies which have shown the probable presence of α3 and α6-containing nAChRs in dopaminergic neurons[6,9]. In addition, this is the first demonstration that the α5, α7 and β4 subunits are exclusively present in dopaminergic neurons of the SN and VTA

Table I. Results obtained by RT-PCR on the different regions studied. *n* = number of animals tested. +, positive amplifications (five samples out of five except for β3 in 6-OHDA-lesioned SN and VTA for which four positive amplifications out of five samples were detected) and (–) correspond to absence of signal for all samples tested as visualized on ethidium bromide-stained agarose gel after RT-PCR amplification.

	Brain region	*Localization*	*n*	*α2*	*α3*	*α4*	*α5*	*α6*	*α7*	*β2*	*β3*	*β4*
	SN	Ipsilateral	5	-	-	+	-	-	+	+	+	-
60HDA		Contralateral	5	-	+	+	+	+	+	+	+	+
	VTA	Ipsilateral	5	-	-	+	-	-	-	+	+	-
		Contralateral	5	+	+	+	+	+	+	+	+	+
	SN	Ipsilateral	5	-	+	+	+	+	+	+	+	+
SHAM		Contralateral	5	-	+	+	+	+	+	+	+	+
	VTA	Ipsilateral	5	+	+	+	+	+	+	+	+	+
		Contralateral	5	+	+	+	+	+	+	+	+	+

and therefore suggests that nAChRs mediating dopamine release also contain these subunits. The subunits still detectable following 6-OHDA-induced lesion may be expressed in non-dopaminergic neurons such as GABAergic neurons and interneurons of the SN and VTA. α4, α7 (expressed only in the SN), β2 and β3 subunits may be expressed in the large population of GABAergic neurons from the SN pars reticulata which is also sampled together with the SN pars compacta in our experiments, or in GABAergic interneurons present in both the SN and the VTA. However, we cannot exclude the possibility that these subunits are also present on dopaminergic neurons. Indeed, a recent study has demonstrated the probable presence of β2 subunit-containing nAChRs in such neurons[5].

The molecular nature of native nAChRs involved in the modulation of dopaminergic neurotransmission remains controversial1,[4–7,9] and is the object of considerable interest, particularly in relation to the known addictive properties of nicotine[2]. However, the large number of nAChR subunits expressed in the SN and VTA and the lack of subtype-selective drugs have hampered the characterization of nAChRs responsible for the effect of nicotine on the dopaminergic system. Here, using the RT-PCR methodology, we have identified the different neuronal nicotinic acetylcholine receptor subunit mRNAs expressed selectively in the dopaminergic neurons of the SN and VTA. Our data also provide direct evidence for the heterogeneity of nicotinic receptor subtypes in dopaminergic neurons. The molecular characterization of these receptors is an essential first step in understanding the relative contribution of different nAChRs in the modulation of physiological processes taking place in midbrain dopaminergic systems.

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19 Comparison of the effects of mutations and inhibition or activation by cholinergic compounds on kinetic parameters of Acetylcholinesterase: The ESTHER database and server

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ESTHER (for esterases, a/b hydrolase enzyme and relatives) is a database of sequences phylogenetically related to cholinesterases. These sequences define a homogeneous group of enzymes (carboxylesterases, lipases and hormone-sensitive lipases) sharing a similar structure of a central beta-sheet surrounded by alpha-helices (1). The purpose of ESTHER is to provide help with comparing structures and functions of members of the family. In this respect the compilation of compounds acting as inhibitor substrates or reactivators of acetylcholinesterase is valuable to compare kinetic data dispersed in the literature. This is a crucial point when therapeutic effects are sought as some of these compounds can act as inhibitors and slow substrates for cholineserase (Bambuterol), or act as agonists of acetylcholine receptors and bind acetylcholinesterase as well (Gallamine/flaxedil), or may interfere with choline reuptake and acetylcholine synthesis. Information on kinetic parameters of natural mutants of human butyrylcholinesterase is of utmost importance for monitoring pharmacokinetics of such compounds (2). The database is available through the Internet at: http://www.ensam.inra.fr/cholinesterase. The Database Managing System is ACeDB, initially developed for the *C. elegans* genome project (3). Information about this system is available at the USDA server (4). Short tutorials for searching the ESTHER database and building tables have been published (2, 5). Here we present examples of raw data as entered in ESTHER. Knowledge of the different tags or fields available helps formulating queries and building tables.

The maintenance of the system relies on the accuracy of information and up-to-date entering of data. Authors are encouraged to submit data. Example forms are available but any computer-readable files can be sent to us, as the conversion tools used to enter data can be adapted to many different formats. This is made possible by the simplicity of the format of entry of raw data in ACeDB.

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20 Relation between AChR and AChE expression and clustering during in vitro neuromuscular synaptogenesis

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The formation of the neuromuscular junction (NMJ) requires the clustering of both acetylcholine receptors (AChR) and acetylcholinesterase (AChE), respectively in the postsynaptic membrane and synaptic basal lamina (Hall and Sanes, 1993). We are seeking to elucidate some cellular and molecular mechanisms involved in the expression and clustering of AChR and AChE. Previous studies have suggested that accumulation of AChR is a prerequisite for the formation of AChE clusters (De La Porte et al, 1993, 1998). To clarify the underlying mechanisms we have used two variants of the C2 mouse muscle cell line (Yaffe and Saxel, 1977), S27 and C2R–. C2R– is defective in synthesis of the α-subunit of AChR (Black et al., 1987) and S27 is defective in glycosaminoglycan (GAG) synthesis (Gordon et al., 1993).

C2 wild-type myotubes spontaneously form AChE clusters that are always colocalised with AChR clusters. The S27 myotubes form AChR clusters only in the presence of nerve cells (soluble agrin is not sufficient). In these myotubes we observed no formation of AChE clusters, confirming that the lack of AChR clusters inhibits the accumulation of AChE in the basal lamina.

We analysed the specific activity in both variants. Compared with C2 wild type, the specific activity is increased 4.5-fold in C2R– (deficient in AChR synthesis) and decreased 3-fold in S27 (not deficient in AChR synthesis). This difference suggests that AChE and AChR expressions are related.

We are currently using an antisense strategy in C2 wild type and primary rat myotube culture to study directly the correlation between AChE and AChR expression and clustering. Preliminary results will be presented.

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21 Effects of atracurium, a neuromuscular blocking agent, on reconstituted human neuronal nicotinic acetylcholine receptors

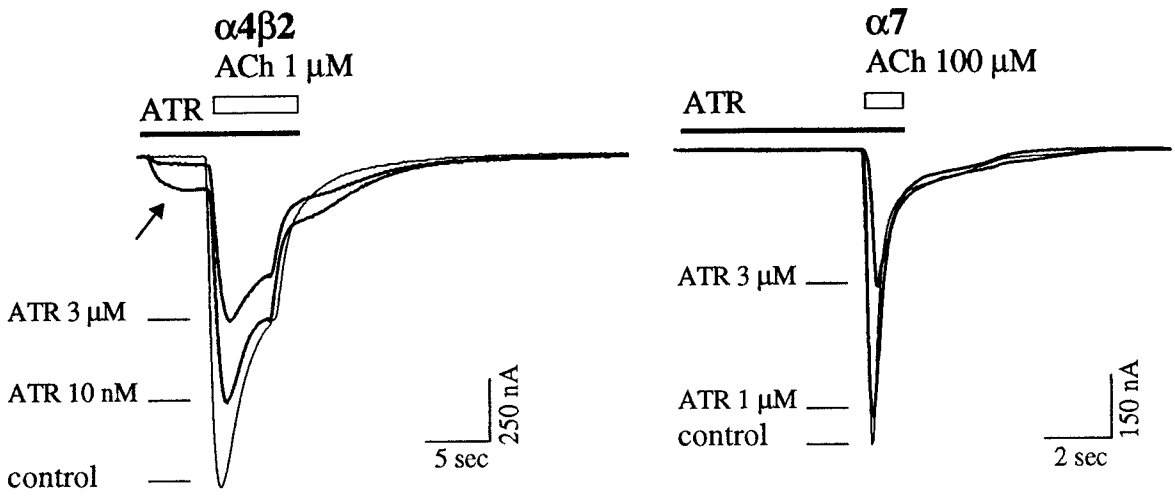
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(+)-Tubocurarine was the first compound pointing out the existence of a chemical synapse at the neuromuscular junction. It has been extensively documented that (+)-tubocurarine and curaromimetic agents exert several actions[1–3] on the nicotinic acetylcholine receptor (nAChR) including: a) a competitive inhibition of the receptor; b) an open channel blockade; and c) in some conditions, an agonist effect. Given their ability to reversibly inhibit transmission at the neuromuscular junction, neuromuscular blocking (NMB) agents are widely used in clinical practice. Although under normal circomstances NMB agents do not cross the blood-brain barrier, detectable amounts of NMB agents and their metabolites can be found in patient's cerebrospinal fluid[4, 5]. Moreover, we showed that NMB agents modify the synaptic transmission in rat hippocampus. When applied in the nanomolar range, atracurium (ATR) increased the excitatory transmission whereas applications in the micromolar range blocked the inhibitory transmission mediated by the γ-aminobutyric acid[6]. From these results, we hypothesized that NMB agents, and more specifically ATR, can interact with neuronal nAChRs.

To elucidate the mechanism of action of ATR on neuronal nAChRs, we have examined in the present study its effects on human α4β2 and α7 receptors reconstituted in *Xenopus* oocytes. We found that ATR exerts two effects on human receptors. First, ATR reversibly inhibits ACh-evoked currents on both α4β2 and α7 nAChRs with an IC_{50} in the micromolar range (respectively 1.5 and 4.3 μM, see *figure 1*). To further assess the effect of ATR, the peak of ACh-evoked currents on both α4β2 and α7 receptors was measured at several ACh concentrations in the absence or in the presence of ATR. Application of 10 μM ATR caused a displacement of the dose-response curve toward higher ACh concentrations corresponding to a reduction in apparent affinity, indicating that ATR acts as a competitive antagonist on both receptors. Secondly, when applied alone in the nanomolar range, ATR evoked small but reliable currents in α4β2 receptors (arrow). In contrast, no currents were detected in α7 receptors.

These data illustrate that ATR acts on the human α4β2 and α7 receptors and can inhibit nAChR-mediated neurotransmission



in the central nervous system. As it was shown that neuronal nAChRs can modulate neurotransmission release, it can be proposed that ATR can produce widespread effects[7–9]. Since NMB agents may reach the central nervous system in patients, these results contribute to the understanding of possible side effects observed in clinical conditions[10]. This work was supported by the OFES no 96.0295-1 to D.B. and the Swiss National Science Foundation no 3200.043626.95 to D.M., E.T. and D.B.

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22 Morphological alterations of motor nerve terminals after botulinum type-A poisoning or reinnervation of skeletal muscle in the tenascin-C deficient mouse

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Tenascin-C is an adhesion molecule of the extracellular matrix involved in many cellular processes during embryogenesis, particularly neurite extension and retraction during development (see review in Chiquet-Ehrismann, 1995). In the adult, tenascin-C is found in myelinating Schwann cells, at the nodes of Ranvier and at the neuromuscular junction (NMJ). In denervated muscles, its expression is up-regulated in endomysial spaces and in perisynaptic interstitial spaces. Its level and distribution return to normal after reinnervation. We have recently shown (Cifuentes-Diaz et al., 1998) that NMJ of tenascin-C deficient mice (Saga et al., 1992) exhibit abnormalities in peripheral nerves and in the NMJ structure. Many abnormalities of Schwann cells and axons were apparent. At the NMJ, preterminal disorganization was prevalent and pre- and post-synaptic abnormalities concerning the number

of axons in a gutter, the number of Schwann cell processes or the aspect of junctional infoldings were observed. Messenger RNA detection by RT-PCR confirmed the presence of low amounts of tenascine-C mRNA levels in skeletal muscle, suggesting that the mice deficient in tenascine-C are not complete knockouts of this gene, but low-expression mutants.

The aim of the present study was to determine the reactivity and sprouting capability of motor nerves from tenascin-C deficient mice under two experimental conditions: i) after a unique in vivo injection of *Clostridium* botulinum type-A toxin (BoTx/A) close to the mouse levator auris longus (LAL) muscle; and ii) after partial denervation of the triangularis sterni (TS) muscle by axotomy of intercostal nerves 3 and/or 4.

The local injection of BoTx/A is known to block acetylcholine release from motor nerve endings causing muscle paralysis and to trigger a profuse sprouting of nerve terminals in normal mice (see Juzans et al., 1996). To determine whether the ability of nerve terminals to expand their field of innervation over the muscle fibers was modified in tenascin-C deficient mice as compared to normal mice, the pattern of motor nerve outgrowth in whole-mount LAL muscles was analyzed and compared 20 days after BoTx/A injection. Sprouting was prominent in LAL muscles from normal mice *(figure 1A)* and the total nerve terminal length was markedly increased due to an increase in the number of terminal branches and to an increase in average branch length. In contrast, tenascin-C mutant LAL muscles injected with the same dose of BoTX/A exhibited a reduced capability of motor endings to overgrow in response to toxin injection *(figure 1B)*.

At different times after partial denervation of TS muscles, the animals were killed and muscles examined. Marked differences were detected in the reinnervation pattern between tenascin-C mutant and normal muscles. Reinnervation was delayed in the mutant muscles as compared to normals. Furthermore, in each mutant muscle investigated, many motor axons were observed growing beyond their denervated targets, reaching neighboring endplates. As a consequence, many endplates became polyinnervated. Polyinnervation was found to persist for many months after reinnervation *(figure 1C)*, which was never the case in normal mice (see Angaut-Petit et al., 1982).

Many proteins have been suggested to play a role in the formation, maturation and stability of the skeletal neuromuscular junction. Our data, in contrast to recent suggestions by Moscoso et al. (1998), indicate that tenascin-C is involved in nerve terminal outgrowth and in plasticity changes induced by presynaptic perturba-tions of the NMJ.

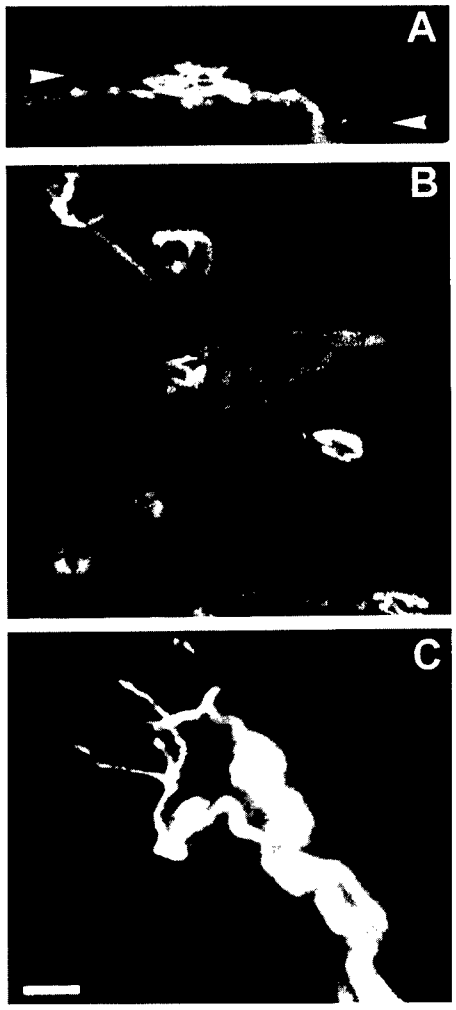


Figure 1. Whole mount preparations of LAL (**A, B**) and TS (**C**) muscles from normal (**A**) and tenascin-C mutant mice (**B, C**) double-stained with an anti-neurofilament antibody and rhodaminated α-bungarotoxin. **A, B.** The response of nerve terminals 18 days after a unique injection of BoTx/A to a normal (**A**) and a tenascin-C mutant muscle (**B**). Note the prominent sprouts in **A** (arrowheads) and their absence in **B. C.** A typical example of a reinnervated endplate 7 months after denervation. Notice that two axons supply the same synaptic site. Scale bar, 15 μm (**A**); 30 m (**B**); and 7.5 μm (**C**).

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23 Characterization of mice lacking the nicotinic receptor α4 subunit

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Nicotine, a drug of addiction, has been shown to have widespread effects in the central nervous system (CNS) by binding to one or more of the numerous subtypes of nicotinic acetylcholine receptors (nAChR). However, the involvement of a particular subunit in pharmacology and behaviour has been difficult to assess. We have made a mutant line of mice by gene targeting and homologous recombination that lacks the neuronal α4 subunit of the nAChR, one of the most widely expressed subunits in the CNS. Mutant mice develope normally, are capable of reproduction, and are indistinguishable from their wild-type littermates in a cage. By in situ hybridization, the α4 subunit mRNA is absent in mutant mice, however, no changes in the level of expression of other subunits are observed. ^{3}H-nicotine, and ^{3}H-cytisine binding sites are absent in most brain regions of mutant mice, however, some binding does remain in the interpeduncular nucleus, indicating that α4 is involved in most, but not all, of the high-affinity nicotine-binding sites. Furthermore, most ^{3}H-epibatidine binding sites disappear in mutant mice, however, some binding is still observed in the interpeduncular nucleus, the superior colliculus, the medial habenula, and the substantia nigra. Electrophysiological recordings from brain slices reveal a marked loss of nicotine-induced currents in the thalamus and substantia nigra pars compacta in mutant mice, but currents in the medial habenula are similar to those of wild type mice. These mice will be instrumental in examining the role of high affinity nicotinic receptors in the diverse behavioral effects of nicotine.

24 Neuropeptidergic facilitation of acetylcholine release from motor nerve terminals depends on tonic A2A-adenosine receptor activation

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It is generally accepted that at the neuromuscular junction co-existing classical neurotransmitters (acetylcholine, ACh) and neuromodulatory peptides (e.g., calcitonin gene-related peptide, CGRP) are released according to a pattern that depends on the stimulation conditions (e.g., Bartfai et al., 1988): neurotransmitter is released alone upon moderate conditions of stimulation, whereas, during high intensity or long term stimulation, neuropeptides are co-released with the classical neurotransmitters. Additionally, stimulation intensity significantly interferes with presynaptic receptor effectiveness (see Duckles and Budai, 1990). Adenosine modulates evoked ACh release by activating both A_1-inhibitory and A_{2A}-facilitatory receptors. The A_1/A_{2A}-receptor activation balance is highly dependent on the stimulation pattern, e.g., tonic A_{2A}-receptor-mediated facilitation was enhanced when either the stimulus frequency or pulse duration increased (Correia-de-Sá et al., 1996). It therefore seemed of interest to investigate whether activation of the presynaptic A_{2A}-receptor could influence the excitatory effect of two neuromodulatory peptides, CGRP and vasoactive intestinal peptide (VIP), on stimulation (5 Hz, 750 pulses, 0.04 and 1 ms duration) evoked ^{3}H-ACh release from the rat phrenic motor nerve terminals.

The experiments were performed at 37 °C on rat phrenic nerve-hemidiaphragm preparations loaded with [^{3}H]-choline (2.5 μCi/mL). The technique used was that described by Wessler and Kilbinger (1986). The preparations were superfused with Tyrode's solution and were continuously gassed with 95% O_2 + 5% CO_2. The phrenic nerve was electrically stimulated (5 Hz, 750 pulses, 0.04, 1 ms duration) two times at the 12th (S_1) and 39th (S_2) min after the end of the washout period. Test drugs were added 15 min before S_2 and were present up to the end of the experiments. Their effects on transmitter release were expressed by the ratios S_2/S_1 as compared to S_2/S_1 ratio in control experiments.

Both CGRP (100–800 nM) and VIP (30–300 nM) increased evoked ^{3}H-ACh release in a pulse duration-dependent manner. When 40 μs pulses were used, neither CGRP (200 nM) nor VIP (100 nM) significantly changed evoked transmitter release, however, when stimulus pulse duration increased from 40 μs to 1 ms, both CGRP (200 nM) and VIP (100 nM) facilitated evoked ^{3}H-ACh release by 36 ± 4% (n = 4) and 64 ± 33% (n = 4), respectively *(figure 1)*. As also shown in figure 1, increases in stimulation pulse duration decreased inhibition of ^{3}H-ACh release by the nicotinic antagonist, tubocurarine (TC, 1 μM), and favours tonic adenosine A_{2A} (facilitatory) receptor activation. Either inactivating endogenous adenosine, with adenosine deaminase (ADA, 2.5 U/mL), or blocking A_{2A}-receptors, with selective adenosine antagonists, PD 115,199 (25 nM) or DPMX (10 μM), prevented the facilitatory effects of both CGRP (400 nM, 69 ± 23%) and VIP (100 nM, 64 ± 33%). This effect was reversed by pre-incubating the preparations with the non-hydrolysable A2A agonist, CGS 21680C (2 nM). In contrast, no functional interactions between neuropeptides and nicotinic presynaptic receptors were detected.

It has been demonstrated that high intensity motor nerve stimulation: a) potentiates the tonic A_{2A}-receptor mediated facilitation of ACh release, while activation of A_1-receptors becomes less effective (Correia-de-Sá et al., 1996); b) reduces nicotinic ACh autofacilitation (Wessler, 1989; Correia-de-Sá and Ribeiro, 1994a); and c) increases the magnitude of both CGRP (Correia-de-Sá and Ribeiro, 1994b) and VIP excitation. The present results show that at the rat motor nerve endings presynaptic facilitatory actions of neuropeptides depend on the presence of endogenous adenosine which is tonically activating A_{2A}-receptors. Since both neuropeptides (Kobayashi et al., 1987) and A_{2A}-adenosine (Correia-de-Sá and Ribeiro, 1994c) receptors are positively coupled

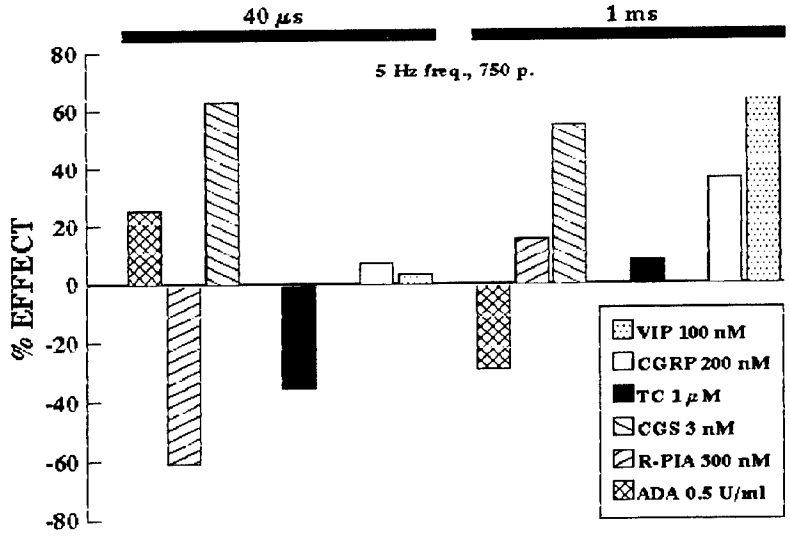


Figure 1. Increases in the stimulation pulse duration: i) decrease nicotinic acetylcholine autofacilitation; ii) potentiate CGRP- and VIP-facilitatory actions; and iii) favour tonic adenosine A_{2A} (facilitatory receptor activation).

to the adenylate cyclase/cyclic AMP system at the rat neuromuscular junction, cooperation between these receptors might occur at the second messenger transduction system level.

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25 Critical elements determining diversity in agonist binding and desensitization of neuronal nAChR

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Neuronal nicotinic acetylcholine receptors display different pharmacologies according to their subunit composition. In particular, the two putative main classes of brain receptors, $\alpha4\beta2$ and $\alpha7$, display respectively high and low apparent binding and desensitization affinity for agonists, and different specificities for acetylcholine versus nicotine. These features may account for the specific pharmacologies of physiological processes including nicotine addiction, and for the shape of the acetylcholine evoked currents in the brain.

To identify the molecular determinants underlying this diversity, sets of residues from the regions initially identified within the agonist binding site of the $\alpha4$ subunit were introduced into the $\alpha7$ agonist binding site, carried by the homooligomeric $\alpha7$-V201-5HT3 chimera. Introduction of the $\alpha4$ residues 183–191 into the $\alpha7$-V201-5HT3 selectively increased the apparent affinities for equilibrium binding and for ion channel activation by acetylcholine, resulting in a receptor that no longer displays differences in the responses to acetylcholine and nicotine. Intro-

duction of the α4 residues 151–155 into the α7-V201-5HT3 produced an approximately 100-fold increase in the apparent affinity for both acetylcholine and nicotine in equilibrium binding measurements. In both cases electrophysiological recordings revealed a much smaller increase (3- to 7-fold) in the apparent affinity for activation, but the concentrations required to desensitize the mutant chimeras parallel the shifts in apparent binding affinity. The data were fitted by a two-state concerted model and an alteration of the conformational isomerization constant leading to the desensitized state accounts for the 151–155 mutation phenotype, while alteration of the ligand binding site accounts for the 183–191 mutation phenotype.

Point mutation analysis revealed that several residues in both fragments contribute to the phenotypes, with a critical effect of the G152K and T183N mutations. Transfer of α4 amino acids 151–155 and 183–191 into α7 thus confers physiological and pharmacological properties typical of the α4β2 receptor.

26 Dissection of the heparin-binding domains present in the collagenic tail of mammalian acetylcholinesterase

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The asymmetric (A) forms of acetylcholinesterase (AChE) consist of one to three catalytic tetramers linked to a collagen-like tail (ColQ), characterized by Gly-X-Y repeats and a high content of imino acids. This collagenic subunit is responsible for the anchorage of the enzyme to the basal lamina, and for its very specific localization at the neuromuscular junction. One of the molecules that could explain the anchorage of the A forms of AChE in the basal lamina is heparan sulfate proteoglycan (HSPG). In this context, competition studies using synthetic peptides led us to describe the presence of two heparin-binding domains (HBDs) in the ColQ subunit of *Torpedo* (Deprez and Inestrosa, 1995). These HBDs are characterized by a central core of basic residues defined by a BBXB motif, and other surrounding basic residues (Cardin and Weintraub, 1989).

Here we present an analysis of the conserved HBDs present on the mammalian ColQ (Krejci et al., 1997) using site-directed mutagenesis. Wild type (wt) and mutated A forms were obtained by co-expression of AChET and rat ColQ mRNA in *Xenopus* oocytes and were then isolated by sucrose gradient velocity sedimentation. The relative heparin affinities were evaluated in terms of the concentration of NaCl required for elution of the bound enzyme from heparin-agarose columns using NaCl concentration steps, begining at 0.2 M and raising the concentration of the next elution step by 0.1 M NaCl.

Using this experimental approach, the wt enzyme presents two main elution peaks, at 0.5 and 0.6 M NaCl *(figure 1)*. The first set of ColQ mutations was designed to evaluate the behaviour of each HBD individually. In each case, the core of the domain was altered by replacing a doublet of basic residues with Asp-Pro (DP). We mutated either the N-terminal HBD domain (RKGR DPGR), the C-terminal one (KRGK DPGK) or both *(figure 1)*. The double mutant bound heparin poorly, demonstrating that the two identified HBDs are the main heparin-binding sites in ColQ. The enzyme that contains only the N-terminal HBD presents a first peak of elution at 0.3 M NaCl, while the enzyme that only contains the C-terminal HBD first dissociates at 0.4 M NaCl. Interestingly, we were surprised to find that the C-terminal HBD

	N-terminal HBD	C-terminal HDB	First elution peak
WT	GRPGRKGRPGPPGVP	GRPGRKGKQGQKGDS	0.5 M
KRGK→DPGK	GRPGRKGRPGPPGVP	GRPGDPGKQGQKGDS	0.3 M
RKGR→DPGR	GRPGDPGRPGPPGVP	GRPGRKGKQGQKGDS	0.4 M
Double Mutant	GRPGDPGRPGPPGVP	GRPGDPGKQGQKGDS	0.2 M*
C→N	GRPGKRGKPGPPGVP	GRPGDPGKQGQKGDS	0.3 M
N→C	GRPGDPGRPGPPGVP	GRPGRKGRQGQKGDS	0.4 M

Figure 1. Sequence and behaviour of the different mutant enzymes in heparin-agarose columns. For each mutant, the sequences at both the N-terminal and C-terminal domains are shown. In the last row, the NaCl concentration at which each enzyme is eluted is shown. *Only 60% of the double mutant enzyme is bound to the heparin-agarose column.

interacted more strongly with heparin than the N-terminal HBD *(figure 1)*, a fact consistent with the results obtained with triple-helical peptides derived from *Torpedo* ColQ (Deprez and Inestrosa, unpublished results).

To identify the origin of this difference, we replaced the core sequence of the N-terminal HBD by the C-terminal HBD, and vice versa. The results shows that this new C-terminal site (N C) behaves exactly as the wt C-terminal site. Likewise, the new N-terminal HBD (C N) presents the same elution pattern than the wt N-terminal domain *(figure 1)*. These results suggest that the difference between both domains does not depend on the core sequence (KRGK vs. RKGR), but probably on the surrounding residues, that differs on triple-helical stability, as shown by circular dichroism studies (Deprez, Brodsky and Inestrosa, unpublished results). Point mutations on different basic residues are also being presented. This work was supported by FONDECYT No 2970072 to P.D., CNRS and AFM to J.M., and a Presidential Chair in Science from the Chilean Government to N.C.I.

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27 Tacrine inhibits L-type calcium channels in the cholinergic SN56 cell line

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The inhibitor of cholinesterases tacrine has recently been introduced into clinical use as a drug for the treatment of Alzheimer's disease but its usefulness is controversial (Byrne and Arie, 1994). In addition to its desired effect, i.e., the preservation of the released acetylcholine, numerous additional effects potentially both beneficial and adverse have been observed in experimental work with tacrine (Freeman and Dawson, 1991; Kelly et al., 1991). We found in our previous work that tacrine and its analog methoxytacrine inhibited the release of acetylcholine from cortical slices and that this effect of both drugs was not related to their anticholinesterase activity (Tucek and Dolezal, 1991; Dolezal and Tucek, 1992). In this study we tested the possibility that tacrine inhibits calcium influx and in this way also the release of acetylcholine.

Using FURA 2 microfluorimetry (Dolezal et al., 1997), we investigated the effect of tacrine on the potassium depolarization-evoked increase of intracellular concentration of free calcium ions ($[Ca^{2+}]_i$) in the SN56 cells derived from mouse septal cholinergic neurons (Hammond et al., 1990). Tacrine inhibited the potassium-evoked increase of ($[Ca^{2+}]_i$) with an EC_{50} 3.7 μM. The inhibition of calcium influx by tacrine was not due to the anticholinesterase activity of the compound. Irreversible inhibitor of cholinesterases paraoxon (10 μM) had no such effect, and a combination of paraoxon and tacrine decreased the influx of calcium to the same extent as tacrine alone. Nifedipine (1 μM) inhibited the potassium depolarization induced increase of $[Ca^{2+}]_i$ by 53%, ω-conotoxin GVIA (0.1 μM) by 39%, and a combination of nifedipine and ω-conotoxin GVIA by 93%. The inhibitory effect of tacrine persisted in the presence of ω-conotoxin GVIA, but not when the L-type calcium channels had been blocked by nifedipine.

Apparently, even low concentrations of tacrine which are relevant to the situation in vivo (Nielsen et al., 1989) may inhibit L-type calcium channels. Many types of calcium channels can serve to carry calcium influx responsible for transmitter release (Turner et al., 1993). Although the electrical stimulation-evoked release of Ach from rat cerebral cortex depends mostly on the influx of calcium through the N-type calcium channels (Dolezal and Tucek, 1987; Wessler et al., 1990) we have shown that the dependency of transmitter release on the influx of calcium through specific types of calcium channels changes depending on the stimulus used (Dolezal et al., 1996). The inhibitory effect of tacrine on the L-type calcium channels can explain our observation that even 1 μM tacrine inhibited acetylcholine release from rat cerebrocortical slices evoked by 4-aminopyridine or potassium stimulation. Our data indicate that tacrine should be applied for strengthening of cholinergic transmission with utmost caution. By blocking the L-type calcium channels it can interfere with calcium-dependent events including the release of transmitters. This work was supported by the grant A7011506 of the Grant Agency of the Academy of Sciences of the Czech Republic.

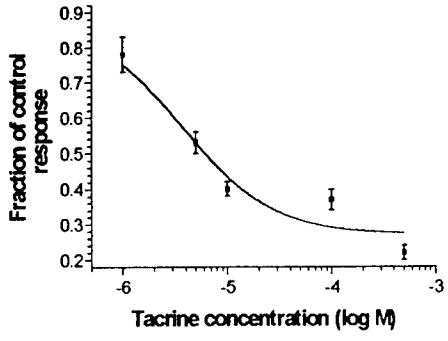


Figure 1. SN56 cells were stimulated twice by 71 mM K^+, with 20 min between stimulations. The first stimulation was in the absence of tacrine which was added at the indicated concentration (abscissa) 10 min before the second stimulation. The effect of tacrine (ordinate) is expressed as a ratio of peak increase of $[Ca^{2+}]_i$ during the second stimulation and during the first stimulation. Each point represents mean ± S.E.M. of 41–120 cells from at least two different seedings. Control ratio was 0.883 ± 0.027 (n = 120) and the calculated EC_{50} was 3.7 μmol/L.

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28 Do central nicotinic and muscarinic cholinergic receptors play differential roles in attentional and episodic memory mechanisms? A study using a new 5-arm maze protocol

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Introduction

The hypothesis that central cholinergic neurons play a key role in learning and memory processes remains controversial. Indeed, data from neuropathological investigations of Alzheimer's disease showed that the degree of dementia and recent memory deficits were correlated with the level of degeneration of ascending projections from the magnocellular forebrain cholinergic complex to the neocortex and hippocampus (Collerton, 1986). Attempts to produce an animal model of Alzheimer's disease have thus used more or less extensive and selective lesions of the cholinergic neurons of the magnocellular forebrain complex. It has often been reported that lesions of the septo-hippocampal pathway produces deficits in working memory whereas lesions of the cholinergic projections from the nucleus basalis to the neocortex produce deficits in reference memory (Wenk et al., 1987). However, data from studies using less non-selective lesion procedures have challenged this concept and have lead to the proposal (Muir et al., 1994) that the nucleus basalis-cortical cholinergic projections play a more selective role in attentional than in memory processes per se. In order to confront these two hypotheses and to study the roles of nicotinic and muscarinic receptors in cognitive function, we have developed a new 5-arm maze protocol which enables measures of both sustained visual attention (immediate response) and episodic memory (delayed response).

Materials and methods

The 5-arm maze is a hybrid of the 5 choice serial reaction task (Carli et al., 1983) which enables measures of sustained visual attention and the 8-arm radial maze which enables measures of spatial memory in rodents. The maze comprises a small start-box equipped with a transparent sliding door fixed opposite to 5 goal arms (35 cm long) which radiate in an angle of 120° from one segment of a circular platform (30 cm diameter). The arms, which contain a food pellet tray, can be lit by a 4 W light bulb housed in a PVC roof which covers the arms and closed by a vertical sliding door. Following 5 sessions of habituation during which C57/Bl6 mice (n = 16) were allowed to visit each of the 5 lit arms and to consume the food pellets, mice were trained on a task in which, on each trial, only one randomly selected arm among the five (chance level = 20%) is lit and baited. Using four trials per daily session mice attained the criterion of a minimum of 80% correct choices with a choice latency < 5 s on two consecutive sessions in 15–20 sessions. During acquisition of the basic task the arm was lit until the subject chose an arm. In the next phase mice were trained, over 5 further sessions, to the same criterion when the arm was lit for only 2 s. Mice then underwent 4 more sessions in which signal durations of 2, 1 and 0.5 s were randomly intermixed in order to construct a reference curve for attentional performance. To evaluate the roles of nicotinic and muscarinic receptors, respectively, in sustained visual attention mice were submitted to 4 more sessions, using the latter protocol 20 min after an acute i.p. injection of either the nicotinic antagonist, mecamylamine (4 mg/kg), the muscarinic antagonist, scopolamine HCl (0.8 mg/Kg), the combination of these doses of mecamylamine plus scopolamine or saline. A Latin square design was used in which 4 sub-groups of mice received, in rotation, one of the four different treatments on a given session.

Results

Results showed that each of these drug treatments produced significant effects during a period of 1 h following injection. At these doses, mecamylamine produced a moderate decrease in the percentage of correct choices whereas scopolamine, and in particular the combination of scopolamine plus mecamylamine produced highly significant decreases of correct responding which were very close to chance level. The decrease in the level of correct responding was, however, associated with large and significant increases in choice latencies for each of the drug treatments and which was entirely due to the fact that mice remained for long periods in the start-box before initiating a response which was then effected with unchanged running speed. Thus, while controls exhibited a mean choice latency of 2 s, treated mice exhibited choice latencies of around 7 s (mecamylamine), 12 s (scopolamine) and 19 s (mecamylamine plus scopolamine). All drug effects attenuated during the second hour following injection to values which were not significantly different from controls.

Discussion

The 5-arm radial maze protocol which we have developed is a useful test for measuring sustained visual attention performance in mice. In comparison to the 5 choice serial reaction task, which requires several thousand trials for rats to achieve the same criterion, the current test requires only 65–80 trials. Concerning the pharmacological study, the results suggest that nicotinic and muscarinic receptors are implicated in sustained visual attention since both sub-types of cholinergic antagonist produce decreases in the percentage of correct responding. The large concomitant increase in choice latency constitutes however, an obstacle as to an unequivocal conclusion of a selective effect on attention. The fact that the mice do not emit an immediate response entails that response accuracy no longer depends solely on the level of attention and now more heavily engages the neuronal mechanisms responsible for temporary maintenance of the memory trace or response selection and initiation.

Verification of the hypothesis that nicotinic and muscarinic receptors play differential roles in attentional and episodic memory processes will now have to await the outcome of the second phase in which mice will be tested for their ability to effect a delayed response (1 min–1 h). In this case it will be possible not only to replicate the pharmacological experiment using pre-test drug treatments, but also to use the post-acquisition protocol where mice are injected following signal presentation, thus avoiding any interference from drug-induced changes in the level of attention during acquisition and thus to evaluate their episodic memory performance in the same task. This use of the present 5-arm maze and this two-stage experimental strategy for dissociating attentional and episodic memory performance constitutes a powerful research tool for investigating changes in cognitive function which intervene during aging or which are produced in mice which have received a 'knock-out' mutation of genes responsible for the production of nicotinic or muscarinic receptors and will also enable studies permitting evaluation of nicotinic and muscarinic treatment strategies for ameliorating cognitive function.

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29 Elevated acetylcholine release in the hippocampus of transgenic mice expressing the human acetylcholinesterase

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Introduction

Transgenic mice expressing human acetylcholinesterase (AChE) show an up to two-fold higher AChE activity in various brain regions in comparison to control mice[1]. This change in cholinergic balance leads to a progressive impairment of learning and memory which is increasingly significant from the age of 2–3 months on. This learning impairment may be due to a change in cholinergic neurotransmission in the septo-hippocampal pathway which is intimately involved in cognitive processes. Therefore we investigated the level of acetylcholine in the hippocampus using the microdialysis technique.

Materials and methods

An I-shaped microdialysis probe was implanted into the dorsal hippocampus of 5–8-month-old transgenic and control FVB/N mice. On the following 2 days the probe was perfused with artificial cerebrospinal fluid (1 µL/min in neostigmine experiments, 2 µL/min in scopolamine experiments). Samples were collected in 15-min intervals and the concentration of ACh was measured by HPLC with electrochemical detection (BAS), as described previously[2]. The detection limit was 50 fmol. After the experiments we confirmed that the transgenic mice indeed carried the h-AChE transgene by PCR amplification of tail DNA, using primer specific for the human AChE gene.

Results and discussion

The basal hippocampal ACh release was measured in the presence of neostigmine in the perfusion fluid. At low neostigmine concentration (10^{-8} M) the ACh release in transgenic mice was two-fold increased in comparison to the control mice *(table I)*. Differences between transgenic and control mice were not significant at increasing neostigmine concentrations. In extension of previous observations that these mice display resistance to AChE inhibitors as well as enhanced choline uptake[3] we conclude that the higher hydrolysis of ACh in transgenic mice is compensated by an increase of the ACh release.

Autoreceptor function in transgenic mice.
The above described increase of the basal ACh release in transgenic mice may be caused by a change of presynaptic muscarinic autoreceptor inhibition. This possibility was tested by studying the responses to scopolamine (1 μM, in the presence of 10^{-5} M neostigmine) infused via the probe into the hippocampus. The effect of scopolamine on ACh release was similar in transgenic and control mice; the maximum effect of scopolamine in transgenic and control mice appeared after 90 min perfusion with an increase to 693 ± 127% (n = 6) and 592 ± 95% (n = 7) respectively. These results show that in the hippocampus the presynaptic control of ACh release via muscarinic autoreceptors was not changed in transgenic mice.

Table I. Basal efflux of ACh in presence of different neostigmine concentrations.

Neostigmine concentration in the perfusion fluid	*ACh efflux [fmol/min]*	
	Control mice (n = 4)	*Transgenic mice (n = 5)*
10^{-8} M	4.5 ± 0.76	9.8 ± 0.48**
10^{-7} M	16.9 ± 2.9	19.4 ± 3.1
10^{-6} M	45.4 ± 4.0	51.8 ± 7.7

**$P < 0.01$.

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30 Regulation of human choline acetyltransferase gene by inhibitors of histone deacetylase

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Choline acetyltransferase (ChAT) activity in sympathetic neuron cultures is increased up to 8-fold by a 2-day treatment with sodium butyrate (5 mM) or with trichostatin (1 μM) and trapoxin (30 nM), two highly specific inhibitors of histone deacetylase (HDAC) activity. Transfection of CHP126 human neuroepithelioma cells with ChAT-luciferase hybrid genes showed that the M promoter, but not R promoter, is activated up to 100-fold by HDAC inhibitors (Chireux et al., 1994, 1996). Transcriptional activation does not require ongoing protein synthesis, suggesting that HDAC inhibitors activate preexisting transcription factors, possibly through phosphorylation.

The stimulation of M promoter by butyrate and trapoxin is blocked by dominant negative mutants of ras and ERK2, and by PD980589, a highly specific inhibitor of MEK1. Constitutively active mutants of ras and MEK1 have little effects by themselves, but potentiate the effects of butyrate. Therefore, the functioning of the ERK/MEK MAP kinase cascade is required for the activation of ChAT M promoter by HDAC inhibitors (Espinos and Weber, 1998). In addition, H7, a serine/threonine protein kinase inhibitor, totally blocks the activation of ChAT promoter by butyrate or trapoxin, in both transient and stable transfection assay.

CBP/p300 protein is a co-activator for several transcription factors and display intrinsic histone acetylase transferase (HAT) activity. The overexpression of the p300 stimulates ChAT M promoter, and strongly synergizes with butyrate. The effects of p300 are suppressed by H7, as well as by adenovirus E1A protein.

These data thus show that transcription from the M promoter of human ChAT gene is modulated by the level of acetylation of nucleosomal core histones, and/or other chromatin associated proteins. HDAC inhibitors like butyrate and trapoxin acts by unmasking multiprotein complexes with HAT activity. Histone hyperacetylation is not sufficient for maximal transcription, that requires in addition the functioning of the MAP kinase cascade and of a H7-sensitive serine/threonine kinase.

Small permeant molecules capable of stimulating the transcription of human ChAT gene might help in stimulating acetylcholine synthesis in diseases characterized by cholinergic deficits.

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31 Two novel α-conotoxins isolated from the venom of *Conus consor*: isolation, synthesis and binding on the *Torpedo* nicotinic acetylcholine receptor

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Venom of the Conidae family is a major source of neuroactive compounds. Several classes of conotoxins have been characterized including α-, μ-, ω-, δ- and κ-conotoxins (Olivera et al., 1990). α-Conotoxins are the smallest conotoxins isolated from the venom of conus, comprising 12 to 19 amino acids with two or three disulfide bonds. This family of paralytic toxins target the nicotinic acetylcholine receptor. Two novel α-conotoxins, α-CnIA and α-CnIB, were isolated from the venom of Conus consor and characterized by binding experiments.

Conotoxin α-CnIA is a 14 amino acid peptide showing high homology with conotoxin MI (McIntosh, 1982). α-CnIA inhibits the fixation of [^{125}I]α-bungarotoxin on the *Torpedo* nicotinic acetylcholine receptor with an IC_{50} of 0.7 mM (figure 1). Binding experiments on the neuronal α7 receptor indicate no competition up to 10 μM.

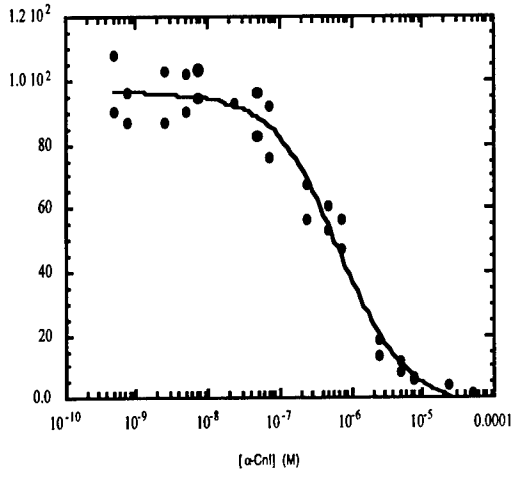


Figure 1. Inhibition of [^{125}I]-α-Bgtx binding to nicotinic receptor (*Torpedo* organ) by α-conotoxin CnIA.

Chemical synthesis of conotoxin α-CnIA was carried out using the Fmoc strategy with selective protection of the four cystine residues (Kamber et al., 1980). This led to correctly folded and biologically active product in reasonable amounts. Synthetic conotoxin compete with [^{125}I]α-bungarotoxin with no difference in binding affinity compared to natural toxin.

Synthetic α-CnIA was used for electrophysiological experiments. The toxin progressively diminishes the amplitude of evoked synaptic potentials in frog neuro-muscular junction (cutaneus pectoris). At 1 μM, complete blockade of the neuro-muscular junction occurs in 25 min. Concomitant disparition of spontaneous miniature end plate potentials clearly shows the post-synaptic effect of the toxin.

These new α-conotoxins are related to a large set of α-conotoxins including conotoxins GI, MI and SI. Thus, they provide novel probes for investigating further the functionality of the nicotinic receptor (Myers, 1991) and the synapse mechanisms.

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32 Muscarinic receptor-activated arachidonic acid release is enhanced by cytokines in A2058 human melanoma cells

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TNFα is a pleiotropic cytokine primarily secreted by activated and resident macrophages such as Kupffer cells and microglia (reviewed in 1). TNFα mediates a wide spectrum of biological activity including cell death, cell proliferation, and lymphocyte activation. Two receptors for TNFα have been identified, the p55 and p75, both of which have been cloned and sequenced. Most mammalian cells express at least one of these receptors. Previous studies have shown that TNFα can stimulate the release of arachidonic acid presumably through activation of phospholipase A2 (2). Neural degeneration of cholinergic neurons such as occurs during Alzheimer's disease, may involve cytokine-mediated inflammatory processes. A 85-kDa phospholipase A2, cPLA2, has been implicated in ischaemic neural loss (3) and is elevated in Alzheimer's brains (4). We investigated possible relationships between cytokine and muscarinic receptor signal transduction mediated by phospholipase A2.

We have previously demonstrated that the human melanoma cell line A2058 expresses only the m5 subtype of muscarinic receptor (5). In A2058 cells, treatment with the cholinergic agonist, carbachol (CC) induces a 2–4-fold increase in arachidonic acid release over basal levels. CC-induced arachidonic acid release is blocked by the muscarinic antagonist, atropine. The CC-induced arachidonic acid release is potentiated 2–3-fold by pretreatment of A2058 cells with either of the inflammatory cytokines, tumor necrosis factor-α (TNFα) or interleukin 1-β (IL-1β). Enhancement of CC-induced arachidonic acid release by TNFα pretreatment peaks near 1 h, consistent with a transcriptionally derived event. Western analysis of IkBα, the inhibitory subunit of the NFκB complex, suggests that both cytokines are capable of activating κB-driven transcription. Pretreatment of A2058 cells with either of the proteasome inhibitors, MG-115 or MG-132, blocks the cytokine-dependent degradation of IkBα but does not affect the enhancement of CC-induced arachidonic acid release. Western analysis demonstrates that both cytokines can trigger the phosphorylation (and activation) of p38 MAP kinase. Furthermore, pretreatment of A2058 cells with the p38 kinase inhibitor, SB202190, ablates cytokine-dependent augmentation without interfering with CC-induced AA release. Finally, no change in the phosphorylation state of the p44/42 MAP kinases follows cytokine treatment. Thus, inflammatory cytokines may regulate muscarinic signaling through the p38 MAP kinase pathway. Additionally, this study provides evidence that potentiation of muscarinic receptor-mediated signal transduction may be a direct result of inflammation.

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33 Modulation of dopamine release by the nicotinic agonist epibatidine in the frontal cortex and the nucleus accumbens of naive and chronic nicotine treated rats

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Accumulating data indicate that the neuronal nicotinic receptors (nAChR) are involved in memory, learning and dependence processes in brain (Newhouse, 1997; Lindstrom, 1997). The reduced number of nAChRs in neurodegenerative disorders, such as Alzheimer's disease (Nordberg, 1992), motivates further research to understand the properties of the various nAChRs, their distribution, physiological function and pharmacological properties.

The nAChRs belong to the family of ligand-gated ion channel receptors. The nAChRs consist of two type of homologous α and β subunits. To date, eight α (α2-α9) and three β (β2-β4) subunits have been cloned which can appear in different combinations (Gotti et al., 1997) with probably different functional properites. Three nAChRs binding sites have been found in brain using nicotinic receptor ligands such as nicotine, epibatidine and α-bungarotoxin with different affinities to α4, α3, α7 nAChRs subunits.

Interactive mechanisms between the cholinergic and dopaminergic systems seems to be important in learning, memory and dependence processes in which DA is suggested to play a role in attention and reward mechanisms (Wise, 1978; Beninger, 1983).

Nicotine has been shown to stimulate dopamine release in brain (Wonnacott, 1997). The involvement of nAChRs is not fully understood although both α4, α3 nAChRs have been suggested to be involved.

In this study we used the microdialysis technique to investigate the effect of the nicotinic agonist epibatidine (2.5 μg/kg subcutaneous) on nAChR-mediated dopamine release in the frontal cortex and nucleus accumbens of freely moving rats. We found that epibatidine significantly reduced the release of dopamine in both the nucleus accumbens and in the frontal cortex. The effect on DA release was more pronounced in the nucleuse accumbens compared to the frontal cortex. A single dose of epibatidine also caused a significant increase in DOPAC levels in the frontal cortex while no effect was observed in the nucleus accumbens. In addition, the HVA levels were significantly increased in both brain regions. Pretreatment with the nicotinic antagonist mecamylamine (2 mg/kg) partly inhibited the epibatidine-induced decreased release of dopamine in the nucleus accumbens.The findings suggest an opposite effect of epibatidine compared to nicotine on dopamine release in nucleues accumbens and frontal cortex. The finding suggests that different nAChR subtypes may mediate the effect of nicotine and epibatidine on dopamine release respectively.

Chronic treatment with nicotine is known to upregulate the nACRs in brain.We have earlier observed that the nAChRs differ in their magnitude of up-regulation and that the α4β2 nAChR is more readily up-regulated than the α3 nAChRs subunit (Warpman et al., 1998). Nisell et al. (1996) found that subchronic nicotine treatment enchanced the effect seen by nicotine on dopamine release in the frontal cortex but not in the nucleus accumbens. Marshall et al. (1997) however recently reported no significant effect of nicotine on dopamine release in the frontal cortex of rats subchronically treated with nicotine. In the present study we found that treatment with nicotine (0.45 mg/kg base s.c. twice daily) for 7 days counteracted the decrease in dopamine release seen following a single dose of epibatidine while no effect of subchronic nicotine treatment was oberved in the frontal cortex. The findings suggest changes in nAChR subtype properties following repeated nicotine treatment which may change the epibatidine-induced dopamine release. The studies are expected to provide further insight into the modulatory effect of nicotinic receptor on dopamine release in different brain regions and the particular nAChRs subtypes involved in these specific processes.

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34 The segregation of a lipid domain underlies structural and functional modulation of acetylcholine receptor in reconstituted membranes

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Purified acetylcholine receptor (AcChR) from *Torpedo* has been reconstituted into whole asolectin lipids or into defined multicomponent liposomes made of 50% phosphatidylcholine, 25% cholesterol plus 25% of one of the following phospholipids: phosphatidylcholine (PC), phosphatidyl-ethanolamine (PE), phosphatidylglycerol (PG) or phosphatidic acid (PA) (Avanti polar lipids). These phospholipids were all derivatives of egg PC and therefore, have the same fatty acid composition.

The ability of the reconstituted samples to promote cation translocation in response to agonist (carbamylcholine) binding was assessed by using a 'stopped-flow /fluorescence quenching' assay of Tl^+ influx. As expected from previously published data in similar lipid media, the samples reconstituted in vesicles made exclusively from zwitterionic PC/cholesterol mixtures completely lacked ion channel activity. On the contrary, the presence in the vesicles of phospholipids other than PC retains AcChR activity to an extent which varies depending upon the phospholipid. Thus, the largest responses to cholinergic agonists observed correspond to samples containing PA (about one half of the maximal response seen in the samples reconstituted in whole asolectin, used as a reference for full functional reconstitution), followed by those containing PG, the other anionic phospholipid used in these studies. Finally, the samples containing the zwitterionic PE exhibited a very low level of cation channel activity.

We are reporting here that specific alterations of the AcChR secondary structure accompany the reported lipid-induced effects on AcChR function. Fourier-transform infrared (FT-IR) studies of the reconstituted samples show that the amide I band in the infrared spectrum of the protein, when reconstituted in the more active, PA-containing matrix, resembles closely that observed in the fully-functional samples reconstituted in whole asolectin lipids, while it differed considerably from spectra obtained from inactive samples reconstituted in PC/cholesterol mixtures. Analysis of the overlapping secondary structure spectral components in the amide I band contour (Echabe et al., 1997) revealed that the maintenance of AcChR function by phospholipids such as PA, can be correlated with the preservation of a high percentage of α-helical components in the protein structure. On the other hand, the absence of such relevant lipids results in inactive reconstituted samples, in which the AcChR protein exhibits a significant loss

of α-helical structure and an increase in non-ordered structural components *(figure 1)*.

The question remains as to determining: i) what are the molecular events responsible for the effects of phospholipids in modulating AcChR structure and function?; and ii) why is PA the most effective phospholipid in this regard? In the former, the observation that lipid effects on AcChR structure and function occur correlatively and involve precisely lipids such as PA, with a higher affinity for binding to the AcChR protein (Fong and McNamee, 1987), suggests that the interaction of the lipids with the transmembrane portion of the AcChR is what causes that the lipid-bound protein adopts a functionally-competent structure, capable to respond properly to the agonists in the opening of the AcChR ion channel. To test such a hypothesis in terms of the involvement of possible changes in lipid organization and dynamics, we prepared samples of AcChR reconstituted in lipid mixtures containing 50% egg PC, 25% cholesterol and 25% of either perdeuterated DMPA (d-DMPA) or perdeuterated DMPC (d-DMPC). Such dimyristoyl derivatives were chosen for these studies because of their convenient phase transition temperatures. FT-IR spectroscopy and differential scanning calorimetric (DSC) studies on the effects of the protein on phospholipid dynamics within these reconstituted samples show that in the absence of protein, the complex population of membrane lipids remains ideally mixed. On the contrary, the presence of the AcChR protein in the reconstituted vesicles directs the formation of specific lipid domains that become segregated from the bulk lipid matrix *(figure 2)*. Furthermore, additional FT-IR and DSC studies indicate that such lipid domains are likely to be composed of monoanionic PA and cholesterol.

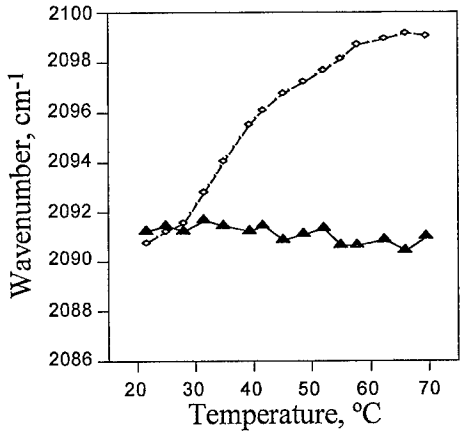


Figure 2. The AcChR protein induces temperature-dependent changes of the CD2 symmetric stretching band of vesicles made from 50% PC, 25% cholesterol and 25% of perdeuterated d-DMPA (diamonds). On the contrary, no temperature-dependent changes can be observed in the absence of protein, suggesting that under conditions, the lipid components are ideally mixed.

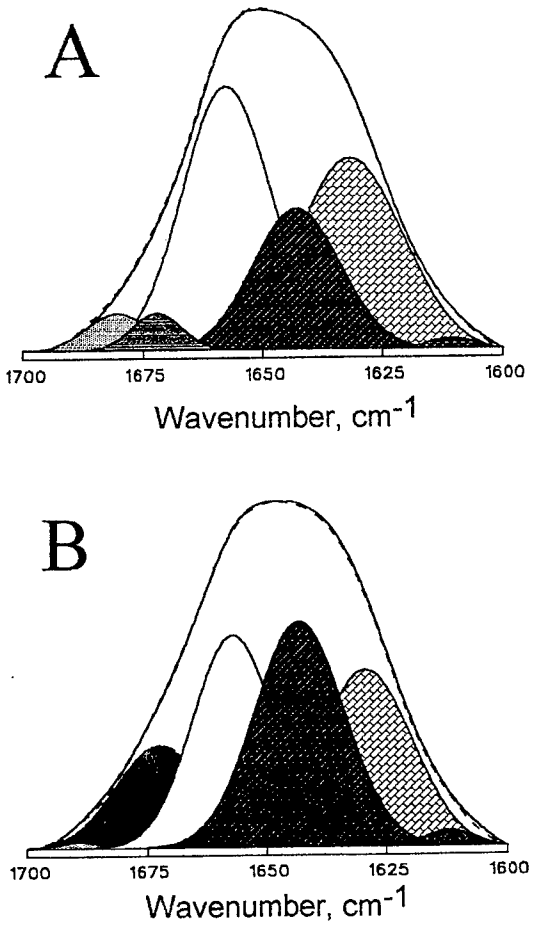


Figure 1. Secondary structure determination from curve-fitting the amide I band of 'functional' (PA + PC + Cho, A) and 'non-functional' (PC + Chol, B) reconstituted samples. Spectral components at 1652 and 1640 cm^{-1} are asigned to α-helical and non-ordered structures, respectively.

As to the selectivity exhibited for PA in this process, it has been reported that it can not be accounted for based on electrostatic interaction with the protein (Raines and Miller, 1993). This, along with the fact that all the phospholipids used in this work have identical acyl moieties, lead to the conclusion that the main contributing factor in the interaction with the lipids should be the recognition of the entire polar head group by the protein. This implies that the near to the lipid-water interphase ends of transmembrane protein segments in contact with the lipid bilayer such as the M4, are likely candidates to be involved in determining the observed selectivity.

As the main conclusion of this report, we propose that a protein-induced, lipid phase separation phenomenon, causing the segregation of specific lipid species, constitutes the basis for the lipid modulation of AcChR structure and function. Partly supported by grant PM95-0108 from the DGICYT of Spain.

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35 4-Oxystilbene compounds are selective antagonists for neuronal nicotinic α-bungarotoxin receptors

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As the pharmacological properties and physiological functions of nAChR subtypes are still largely unknown, great efforts have been made to investigate their structure in order to provide information that can be used to design more selective compounds. Much of the recent increase in nicotinic ligand research is due to the growing evidence that nAChRs are involved in brain function, and play a central role in the physiopathology of a number of disorders (1, 2)

It has long been known that 4-oxystilbene derivates have ganglioplegic activity (3) and are able to antagonise nicotine-induced tremors in rabbits. We therefore decided to test the selectivity of the five different 4-oxystilbene compounds on nAChR subtypes. Binding experiments showed that the most active was the old drug MG624 that specifically inhibits the binding of ^{125}IαBgtx to the α7-containing receptors and, although to a much lesser extent, the binding of ^{3}H-Epi to the β4 and β2-containing receptors in a heterogeneous receptor population. In order to clarify the pharmacological profile of this compound, we studied its capacity to bind the individual neuronal subtypes, and compared the results with the effects obtained in the same subtypes expressed in oocytes.It was found that MG624 inhibits the binding of ^{125}I-α-bungarotoxin (αBgtx) to the α7 subtype and that of ^{3}H-epibatidine (Epi) to the α4β2 subtype with K_i values of respectively 106 nM and 84 μM. The drug also inhibits ACh elicited currents (IACh) on the oocyte-expressed α7 subtype and the α4β2 subtype at half-inhibitory concentrations (IC_{50}) of respectively 109 nM and 3.2 μM.

In order to explore the interaction of MG624 with the α7 subtype, we took advantage of the expression in Xenopus oocytes of an α7 homomeric mutant receptor with a threonine-for-leucine 247 substitution (L247T α7) (4). Whereas MG624 did not induce any current on oocytes expressing the wild type α7 receptor, it did induce large currents on the oocyte-expressed L247T α7 receptor. The MG624 elicited current (IMG624) on the L247T α7 receptor has an EC_{50} of 0.2 nM and a Hill coefficient nH of 1.9, and is blocked by the nicotinic receptor antagonist methyllycaconitine (0.5 nM). These electrophysiological studies show that MG624 is a potent antagonist of neuronal α7 nAChR, which becomes a competitive agonist following the mutation of the highly conserved leucine residue 247 located in the M2 channel domain.

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36 Regulation of utrophin expression during development of skeletal muscle cells

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Utrophin is a large cytoskeletal protein of the spectrin superfamily that displays a high degree of sequence identity with dystrophin. In contrast to the latter which accumulates at the cytoplasmic face along the sarcolemma of normal muscle fibers, utrophin is preferentially expressed at the postsynaptic membrane of the neuromuscular junction in both normal and dystrophic muscle fibers. Since upregulation of utrophin levels into extrasynaptic compartments of muscle fibers is currently envisaged as a potential therapeutic strategy to counteract the effects of Duchenne muscular dystrophy (see 1 for example), it thus appears important to decipher the cellular and molecular mechanisms presiding over utrophin expression in muscle. We have recently begun a first series of studies aimed at identifying the regulatory events involved in maintaining the restricted expression of utrophin at the adult neuromuscular synapse. In these initial studies, we have shown that utrophin mRNAs accumulate within the postsynaptic sarcoplasm as a result of the local activation of the utrophin gene within the synaptic myonuclei (2). In addition, we demonstrated that the nerve exerts a profound influence on the local expression of utrophin through the release of nerve-derived trophic factors, which culminate in the transactivation of the utrophin gene via an N-box motif (3). Aside from identifying the signal transduction machinery responsible for maintaining expression of the utrophin gene within synaptic myonuclei, it appears also crucial to determine the factors leading to the repression of its expression in extrasynaptic compartments of muscle fibers (see 4). In this context, we have previously shown that, in contrast to the expression of the mRNAs encoding acetylcholinesterase and the various subunits of the acetylcholine receptor (AChR), expression of utrophin is largely insensitive to abolition of nerve-evoked electrical activity (5). In attempts to gain insights into the mechanisms preventing expression of significant amounts of utrophin in extrasynaptic compartments of muscle fibers, we have, in the present study, examined the cellular and molecular mechanisms regulating utrophin expression during the early stages of myogenesis and synaptogenesis using distinct yet, complementary approaches.

First, we examined utrophin mRNA expression in developing muscles to determine the time point at which these transcripts

become enriched within the postsynaptic sarcoplasm. To this end, we thus performed in situ hybridization experiments with synthetic oligonucleotides specific for utrophin transcripts on skeletal muscles obtained from embryonic to neo-natal mice. Results of these studies showed that compartmentalization of utrophin transcripts within the junctional region of muscle fibers occurs at early stages of muscle development and synapse formation.

Next, we studied the pattern of expression of utrophin during differentiation of C2 muscle cells grown in culture. In these experiments, we determined that utrophin levels increase by approximately 2-fold during fusion of myoblasts into multinucleated myotubes. This increase in protein expression was accompanied by a parallel elevation in the levels of utrophin transcripts. Interestingly, this increase in utrophin expression was blocked by treating the myoblasts with EGTA which prevents fusion thereby indicating that the changes in utrophin expression during myogenic differentiation are linked to alterations in the cellular architecture which occur upon fusion and are independent of the activation of the myogenic differentiation program. To determine the contribution of transcriptional versus post-transcriptional regulatory mechanisms in the control of utrophin mRNA levels during differentiation, we measured the transcriptional activity of the utrophin gene by nuclear run-on assays and observed that a modest increase in the rate of transcription in nuclei isolated from differentiated myotubes. We also determined that the half-life for utrophin mRNAs in myoblasts was 20 h and that it remained essentially unchanged in myotubes. These data indicate therefore that the increased expression of utrophin in myotubes is transcriptionally regulated and does not involve alterations in the stability of utrophin transcripts. In addition, these studies show that in contrast to several other synaptic and cytoskeletal proteins including AChR, AChE and dystrophin, expression of utrophin is only modestly increased during differentiation of myogenic cells. In separate experiments, we also determined that utrophin mRNAs preferentially associate with cytoskeleton-bound polysomes attached to the actin microfilament meshwork and that the extent of this association appears developmentally regulated since utrophin mRNAs are not found enriched within this polysomal fraction in myoblasts. These latter findings suggest that targeting of utrophin transcripts to the translational machinery is subject to developmental influences.

Finally, to determine whether the modest increase in utrophin mRNA expression seen during myogenic differentiation of cells grown in culture, also occurs in vivo, we injected soleus muscles with cardiotoxin which induced muscle fiber degeneration, and followed the pattern of expression of utrophin transcripts during the period of regeneration. In contrast to a large increase in dystrophin mRNA levels that occurred during regeneration, the abundance of utrophin transcripts remained relatively constant over this period as expected on the basis of the experiments described above. Since fetal and regenerating muscle fibers are known to contain high levels of utrophin, these data suggest therefore that post-translational regulatory mechanisms also play a predominant role in dictating utrophin levels in muscle. This work is supported by grants from the Association Française Contre les Myopathies, the Muscular Dystrophy Association of America and the Medical Research Council of Canada. A.O.G. is an Arthur Minden Predoctoral Fellow of the Muscular Dystrophy Association of Canada. B.J.J. is a Scholar of the Medical Research Council of Canada.

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37 3.5 kb AChE mRNA is more sensitive to degradation than 2.3 kb AChE mRNA in the denervated fast rat skeletal muscle

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Introduction

Acetylcholinesterase (EC. 3.1.1.7, AChE) is an essential functional component of the neuromuscular junction (reviewed by Hall and Sanes, 1993 and Massoulié et al., 1993). In the mammalian skeletal muscle, AChE mRNA is represented by two species (Rachinsky et al., 1990) originating from the alternate use of the two polyadenylation signals (Li et al., 1991). The 2.3 kb species encodes the hydrophilic form of AChE catalytic subunit; this size reflects the distance from the cap site to the first polyadenylation signal in the AChE gene. A second polyadenylation signal was found 1.2 kb in the 3' direction and its usage gives rise to the 3.5 kb species of AChE mRNA (Li et al., 1991). The role of divergency of 3' untranslated region of AChE mRNA is unknown. Additional sequence of the long transcript contains AU rich elements (Li et al., 1991; Fuentes and Taylor, 1993), which were found to influence the stability of several mRNAs (Greenberg and Belasco, 1993).

To elucidate the mechanisms underlying decreased AChE mRNA level in the denervated muscle we investigated in this study if the two AChE mRNA species differ in their sensitivity towards degradation under such conditions. We followed the time course of the AChE mRNA decrease after denervation of rat sternomastoideus muscle. We also studied degradation of both AChE mRNA species after their exposure to the subcellular fractions isolated from control and denervated muscles.

Experimental procedures

All experiments were carried out on the sternomastoideus muscles isolated from the female Wistar strain rats of about 190 g. AChE mRNA was determined by non-radioactive Northern blotting in the mechanically denervated muscle after selected time intervals. Digoxigenine labeled RNA, corresponding to the part of the second exon of the AChE gene (nt. 370–706) was used as a probe. In our in vitro model, total deproteinated RNA, isolated from the

Table I. Degradation of longer (3.5 kb) and shorter (2.3 kb) AChE mRNA species in the denervated rat sternomastoideus muscle. Each column represents means of densitometric analyses of two independent Northern blots. Results of densitometric analyses are expressed in relative units: control level of 2.3 kb AChE mRNA was taken as a reference (100 units).

AChE mRNA species	*Control*	*2 days after denervation*	*5 days after denervation*	*8 days after denervation*
3.5 kb AChE mRNA	96	15	13.5	0
2.3 kb AChE mRNA	100	20	34	20

sternomastoideus muscle, was exposed to subcellular muscular fractions prepared from another sternomastoideus muscle, which was either normal or denervated. Remaining AChE mRNA was determined after selected time intervals.

Results

As revealed by our Northern blot analyses, a longer AChE mRNA transcript (3.5 kb) practically disappears after 5–6 of denervation. On the other hand, the level of shorter species (2.3 kb) decreases during the first 2 days to about 20% of its control value, but then remains at this level in the subsequent six days *(table I)*. A small portion of AChE activity (< 30% of control activity) persisting in the denervated muscle 8 days after denervation seems therefore to be synthesized exclusively from the 2.3 species. Degradation of AChE mRNA was not specific, since α-actin mRNA was also affected. 3.5 kb AChE mRNA was degraded faster also under the in vitro conditions, suggesting that its reduced level in the denervated muscle indeed reflects higher degradation rate and not just its decreased synthesis.

Discussion

Unlike myoblast fusion of the C2 cells, during which AChE mRNA increases at the unchanged proportion of both AChE mRNA species (Fuentes and Taylor, 1993), 8 days of denervation of the fast rat muscle practically eliminates 3.5 kb AChE mRNA, but leaves a small portion of 2.3 kb species virtually intact. A functional meaning of expression of 2.3 kb species could therefore be to prevent complete blockade of AChE synthesis at the AChE mRNA level under the stressful conditions like denervation, which destabilizes several RNAs. On the other hand, expression of longer AChE transcript might serve to some yet unknown control of AChE synthesis in the skeletal muscle at the mRNA level.

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38 Photoaffinity labeling of nicotinic acetylcholine receptor with a tritiated agonist: A new tool for investigating the functional, activated state

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Upon agonist activation, the nicotinic acetylcholine receptor (AChR) undergoes allosteric transitions leading to channel opening and sodium ion influx. The molecular structure of *Torpedo marmorata* acetylcholine binding site has been investigated previously by photoaffinity labeling and delineated by three different loops on the α-subunit (loop A, Trp-86 and Tyr-93; loop B, Trp-149 and Tyr-151; and loop C, Tyr-190, Cys-192, Cys-193, and Tyr-198). However, most of the photosensitive probes used for this purpose interacted only with closed receptor states (1, 2). As the transition from the the resting (R) to the active state (A) takes place on a millisecond time scale, photosensitive agonists which generate highly reactive species are required in order to label the functional state (A) of AChR *(figure 1)*.

Thus, we have synthesized and developed a new photoactivatable agonist (PA) of AChR with functional activity on human TE 671 cell lines (3). In the absence of light, PA has micromolar affinity for ACh binding site on the D state (after proadifen preincubation) with a 10-fold decrease in affinity for the native form (without proadifen) as expected for cholinergic ligands. [^{3}H]PA was synthesized and used for photolabeling experiments *(figure 2)*. Upon UV irradiation (360 nm), [^{3}H]PA labels covalently AChR-rich membranes whereas less than 0.5% labeling occurs in the absence of light. The pattern of [^{3}H]PA labeling on the different subunits of the AChR in the D state shows a predominant incorporation into the α-subunit with minor incorporation into the γ-subunit, both in a protectable manner (~ 70 to 80% protection)

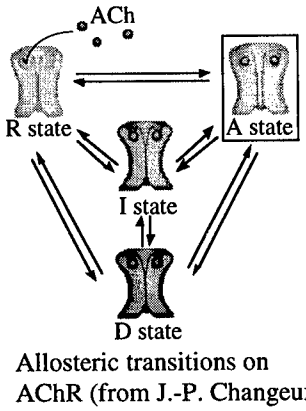


Figure 1. Allosteric transitions on AChR (from J.-P. Changeux).

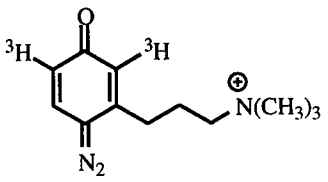


Figure 2. Structure of the photoactivatable agonist [^{3}H]PA.

with either agonist (carbamylcholine) or antagonists (d-tubocurarine, hexamethonium and α-bungarotoxin). The stoechiometry of photocoupling was measured: 1 mol of [^{3}H]PA is incorporated per mol of α-bungarotoxin binding site inactivated demonstrating the high efficiency of [^{3}H]PA as photoalkylating agent. The specific incorporation of [^{3}H]PA into the α and γ subunits is saturable for increasing probe concentration. Preparative photolabeling yields about 35 pmol of site-specific alkylated materiel per experiment (~ 7% of the total amount of ACh binding site into α-subunit) allowing us to prepare large scale of photolabeled AChR.

In a first step, large amounts of [^{3}H]PA alkylated polypeptides will lead us, after proteolytic cleavages, purification of tritiated peptides and microsequencing, to characterize the amino acids belonging to ACh binding sites in the D state.

In a second step, we plan to do time-resolved photolabeling with rapid mixing apparatus combined with laser photoirradiation. These experiments might permit us to label AChR with [^{3}H]PA on its functional activated state A.

A comparative study at the molecular level of the dynamic photolabeling experiments and those obtained at equilibrium for the D state may elucidate the topographical changes occurring at the ACh binding sites during agonist activation.

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39 Muscarinic receptors (M2, M3) in rat and guinea pig thoracic dorsal root ganglia

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Acetylcholine (ACh) excites mechanoreceptors and produces pain when applied to human skin. In the rat skin, a group of carbachol sensitive C-nociceptors can be excited by muscarine (7). Binding sites for ACh are present in rat dorsal root ganglia (DRG) and accumulate at the proximal site of a sciatic nerve ligature (8). Functional studies of cultured neurons from rat DRG show that stimulation of muscarinic receptors is followed by activation of the nitric oxide (NO)-cGMP signalling system (2), and muscarinic regulation of Ca^{2+}-currents has also been observed (9). For a better understanding of cholinergic regulation of sensory neuron function, the localization and distribution of muscarinic receptors, in particular its localization to defined subpopulations of sensory neurons, is of interest. Therefore, in the present study, thoracic DRG of rat and guinea pig were examined using histochemical and immunohistochemical methods for the presence of muscarinic M2- and M3-receptors (M2R, M3R) and markers for select neuronal populations. Sensory neurons of DRG can be subdivided in different groups including capsaicin-sensitive, non-peptidergic, small sensory neurons with binding sites for the alpha-D-galactose specific lectin, I-B4 (6), and small-sized primary sensory neurons containing the peptide, substance P (SP, 5). A group of SP-containing neurons also shows positive NADPH-diaphorase reaction (1) which indicates the presence of nitric oxide synthase (NOS, 3). Thus, I-B4, NOS/NADPH-diaphorase reaction, and SP were used as markers for further characterization of M2R- and M3R-immunoreactive (-IR) sensory neurons. These investigations were performed at formaldehyde-fixed tissues processed either as cryostat sections or as free floating sections of polyethylenglycol-embedded specimens.

Both rat and guinea pig DRG contained neurons with immunoreactivity for both subtypes of muscarinic receptors; the labelling densities of individual neurons varied over a wide range. In guinea pig thoracic DRG, M2R- and M3R-IR were present in nerve cell somata, axonal processes and in the endothelium and smooth muscle cells of intraganglionic blood vessels. Intense filamentous M3R-IR was observed in perikarya of medium-sized neurons (25–30 μm in diameter) which mostly expressed I-B4 binding sites. These cells contained neither NADPH-diaphorase activity nor SP-IR. Intense to slight M3R-IR was present in larger somata (40–50 μm in diameter) which exhibit IB-4 binding sites

and, occasionally, SP-IR and/or NADPH-diaphorase activity. M2R-IR was predominantly present in larger neurons, both with and without I-B4 binding sites. A minor population of medium-sized neurons contained also M2R-IR and I-B4 binding sites. None of the M2R-IR neurons expressed SP-IR. In rat thoracic DRG, M2R- and M3R-IR were present in medium-sized and larger neurons. Predominantly, they expressed I-B4 binding sites, but some perikarya without IB-4 staining were also seen. Medium-sized M2R-IR perikarya often showed SP-IR and positive NADPH-diaphorase reaction whereas medium-sized M2R-IR perikarya and all M3R-IR neurons were virtually devoid of SP-IR and NADPH-diaphorase activity.

The present demonstration of M2R-IR on SP-IR neurons is in accordance with previous pharmacological and electrophysiological experiments describing M2R or M4R dependent inhibition of peptide release from sensory nerve terminals (4, 9). On the other hand, perikarya with intense M3R-IR contained neither SP-IR nor NADPH-diaphorase activity, but expressed I-B4 binding sites. Thus, presumably capsaicin-sensitive, non-peptidergic nociceptive neurons bear M3R. Accordingly, the ACh-dependent NO/cGMP signalling pathway can be abolished by capsaicin treatment (2). This study was supported by SFB 547, project C2

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40 Expression of nicotinic receptor α and β subunits in the prenatal and aged human brain

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Neuronal nicotinic acetylcholine receptors (nAChRs) are ligand-gated ion channels comprised of α and β subunits. Presently six α (α2-α7) and three β (β2-β4) subunits have been identified and cloned from human brain (Mc Gehee and Role, 1995; Elliott et al, 1996; Gotti et al, 1997). Precise subunit composition of the various subtypes and their regional distribution in human brain is still unclear. The nAChRs appear to have a functional role during brain development, since periods of transient high receptor density have been reported in the frontal cortex, hippocampus, cerebellum and brain stem in humans during mid-gestation and neonatal periods (Kinney et al, 1993; Court and Clementi, 1995). The earliest reported binding of [^{3}H]-nicotine so far has been demonstrated in whole brain homogenates from 12-week-old fetuses (Cairns and Wonnacott, 1988) and choline acetyltransferase- and acetylcholine esterase activity has been detected in human forebrain from 8–9 gestational weeks (Candy et al, 1985; Perry et al, 1986). Few data are available concerning the ontogenesis of nAChRs in brain during the first trimester. Moreover, there are no reports on their regional distribution and the gene expression of the different nAChR subunits. In this study the development of nicotinic acetylcholine receptors (nAChRs) in brains from human fetuses of 4–12 weeks gestational age was investigated. By using RT-PCR technique the expression of α3, α4, α5, α7, β2, β3 and β4 mRNA subunits were all detected in the prenatal spinal cord, medulla oblongata, pons, cerebellum, mesencephalon, subcortical forebrain and cerebral cortex during first trimester development. Relative quantification of mRNA (Hellström-Lindahl et al., 1997) showed that highest levels for α3, α4 and α7 were expressed in spinal cord and α5 was most abundant in cortex. For the β-subunit mRNAs, β2 was high in cortex and cerebellum, β3 was highest in cerebellum whereas β4 seemed to be equally distributed in all regions. A comparison of expression of nAChR subunit mRNAs in the cortex and cerebellum of prenatal and aged (54–81 years) brain showed that mRNA levels for α4, α5, α7, β2 and β4 were all significantly higher in prenatal cortex and cerebellum than in aged brain, whereas the level of α3 transcript was similar, and β3 significantly higher in aged cortex.

Specific binding of [^{3}H]-epibatidine to prenatal brain membranes was detected as early as 4–5 weeks of gestation in many brain regions and the number of nAChRs increased with gestational age. The highest specific binding of [^{3}H]-epibatidine and [^{3}H]-cytisine was detected in spinal cord, pons and medulla oblongata and the lowest in cortex. Saturation analysis of binding data for both prenatal and aged brain revealed a single site for [^{3}H]-cytisine and two binding sites for [^{3}H]-epibatidine. The Bmax values obtained with [^{3}H]-cytisine and [^{3}H]-epibatidine were about five and ten times higher for prenatal whole brain compared to aged frontal cortex. The reduced levels of nAChR subunit

mRNAs in aged brain shown in the present study is consistent with the earlier reported loss of nAChRs in cerebral cortex and hippocampus during normal aging (Nordberg et al., 1992; Court and Clementi, 1995).The early presence of nAChR proteins and gene transcripts observed suggests an important role for nAChRs in modulating dendritic outgrowth, establishment of neuronal connections and synaptogenesis during development.

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41 Stimulatory influence of ethanol on the septohippocampal cholinergic pathway. A role for GABA receptors?

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The cholinergic septohippocampal pathway is involved in the processes of learning and memory and is a possible target for the cognitive dysfunction and amnesia observed after acute ethanol intake. Decreases of cortical cholinergic functions after high doses of ethanol have already been described using the push-pull technique (see ref. 2), measurements of ACh tissue levels (see ref. 2) and ACh release from brain slices (see ref. 4). An impairment of the hippocampal cholinergic system has so far only been observed after long-term ethanol administration (see ref. 1)

In the present study we used the microdialysis technique in combination with a sensitive HPLC system to monitor simultanenously changes of rat hippocampal acetylcholine (ACh) release and hippocampal ethanol levels upon acute ethanol administration. For this purpose, ethanol was administered either systemically (i.p.) or locally (via the probe). Samples were collected in 15-min intervals for estimation of acetylcholine and ethanol levels, respectively. Details of the microdialysis procedure and ACh determination were as described previously (see ref. 5).

In the present microdialysis study we report, to our knowledge for the first time, a stimulatory effect of ethanol upon the central cholinergic system in vivo. This was observed after systemic administration of low doses of ethanol as well as during local infusions into the hippocampus or the basal forebrain. The i.p. injection of low doses of ethanol (0.8 g/kg) led to a delayed stimulation of basal ACh release which occurred only 2 h past injection, i.e., at a time when hippocampal ethanol levels had already dropped to concentrations below 5 mM. The effect lasted for approximately 2 h (see ref. 3).

Similarly local infusion of ethanol via retrograde dialysis into the hippocampus (50 and 100 mM, respectively) led to a concentration-dependent increase of basal ACh release after 30 min of ethanol infusion. ACh levels remained elevated for up to 1 h after cessation of ethanol infusion when hippocampal ethanol levels had already dropped to zero.

In order to test whether GABAergic mechanisms may be involved in ethanol's actions, we used vigabatrin (GVG), an irreversible inhibitor of GABA transaminase. Vigabatrin, administered systemically (0.8 g/kg and 2.4 g/kg, respectively), led to a dose-dependent stimulation of hippocampal ACh release with maximum ACh levels occurring after 15 and 75 min, respectively (159 ± 9% and 173 ± 14% of basal efflux, respectively; n = 4; see *figure 1*). Local infusions (1 and 10 µM, respectively) also caused a concentration-dependent stimulation of hippocampal ACh release, but only after termination of vigabatrin-infusion. ACh levels returned to basal levels within 1 h.

The present study describes stimulatory effects of ethanol in low doses upon hippocampal ACh release which, seem to be due to a local effect of ethanol on the cholinergic septohippocampal pathway. The present data also indicate that these effects may be mediated by an involvement of a GABAergic mechanism, as both, ethanol and vigabatrin, lead to a stimulation of the hippocampal ACh outflow, possibly via stimulation of a disinhibitory GABA circuit.

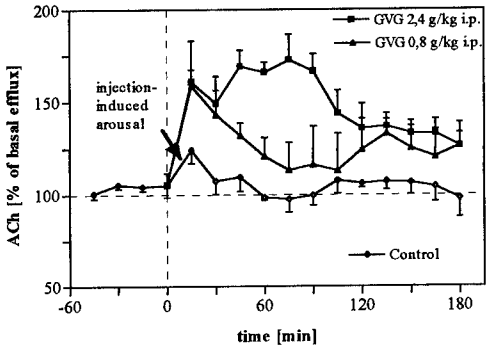


Figure 1. Effects of i.p. administration at t = 0 of 0.8 g/kg and 2.4 g/kg vigabatrin, respectively, on the basal outflow of ACh from the ventral rat hippocampus. ACh output is expressed as percent of basal efflux (1.57 ± 0.21 pmol/15 min); each data point represents the mean ± S.E.M. of four experiments. Open symbols represent controls (1.5 mL saline i.p.); filled triangles 0.8 g/kg vigabatrin and filled squares 2.4 g/kg vigabatrin.

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42 ADP-ribosyl cyclase coupled with muscarinic acetylcholine receptors via G proteins in NG108-15 cells

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Cyclic adenosine diphosphate ribose (cADPR) is synthesized from β-NAD^+ by ADP-ribosyl cyclase. Pharmacological studies suggest that cADPR is an endogenous modulator of Ca^{2+}-induced Ca^{2+} release from ryanodine-sensitive Ca^{2+} stores. However, the question whether or not cADPR mediates the intracellular action of conventional hormones and neurotransmitters in mammalian cells remains unsolved For cADPR to be accepted as an intracellular second messenger downstream of such receptors; in addition to its pharmacological effects, extracellular stimulation should activate (or inhibit) ADP-ribosyl cyclase. The typical pathway in mammalian cells consists of signals from receptors involved in forming intracellular second messengers, such as cyclic AMP or inositol-1,4,5-trisphosphate and diacylgrycerol, being transduced to effector enzymes of adenylate cyclase or phospholipase Cb via G proteins in the cell surface membrane. Therefore, I examined whether or not cADPR formation is regulated by ADP-ribosyl cyclase through the direct action of G proteins in the cell membrane.

Stimulation of muscarinic acetylcholine receptors (mAChRs) by carbamylcholine (CCh) changed the rate of cADPR formation in NG108-15 neuroblastoma × glioma hybrid cells, in which the enzyme activity was found in crude membrane fractions. Interestingly, activation of ADP-ribosyl cyclase by CCh was observed in membranes obtained from NG108-15 cells overexpressing m1 or m3 mAChRs, while inhibition was mediated by endogenous m4 mAChRs and exogenous m2 mAChRs (Higashida et al., 1997). These effects were mimicked by GTP in NG108-15 cells. The CCh-induced activation was inhibited by prior treatment of cells with cholera toxin, while the inhibition of ADP-ribosyl cyclase was sensitive to pertussis toxin. Although further validation is necessary to determine exactly which G proteins are involved in the stimulatory and inhibitory pathways to ADP-ribosyl cyclase, it can be hypothesized from these observations that the signal to ADP-ribosyl cyclase from mAChRs is mediated via G proteins (Higashida, 1997).

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43 Muscarinic acetylcholine m1–m4 receptors overexpressed in neuroblastoma-glioma hybrid NG108-15 cells as a cholinergic model

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Neuroblastoma × glioma hybrid NG108-15 cells possess endogenous m4 muscarinic acetylcholine receptors (mAChRs), which do not couple to phospholipase C but couple to adenylate cyclase and voltage-dependent Ca^{2+} channels (Higashida et al., 1990). Four genetic subtypes of mAChRs differ in the effects when stimulated by muscarinic agonists in NG108-15 cells overexpressing each mAChR (Noda et al., 1996). Broadly speaking, the principle falls into two categories: the odd-numbered receptors (m1 and m3) activate phospholipase C, increasecellular inositol trisphosphate/Ca^{2+} levels. Furthermore, recently, we found that formation of cyclic ADP-ribose (cADPR) is increased in a reaction mixture with crude membrane fractions obtained from NG108-15 cells overexpressing m1/m3 mAChRs by muscarinic stimulation (Higashida et al., 1997). This is associated with a transient decrease of the cellular NAD^+ level in an intact cell. In contrast, the stimulation of even-numbered receptors (m2 and m4) result in an inhibition of ADP-ribosyl cyclase as well as adenylate cyclase and an decrease of N-type Ca^{2+} currents. Signals from m2/m4 mAChRs are discriminated by their sensitivity for pertussis toxin, while those from m1/m3 mAChRs are not. Thus, coupling to adenylate cyclase, phospholipase C, ADP-ribosyl cyclase and Ca^{2+} ion channels from mAChRs shows a

dichotomy in NG108-15 cells, which may reflect mAChR-induced responses in intact neurons in vivo (Higashida, 1997).

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44 Acute and delayed effects of nitrogen oxide on metabolism of acetyl moiety of acetycholine in differentiated and non-diferentiated hybrid SN56 neurons.

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Loss of septal and cortical cholinergic neurons followed by development of mental deficitsis is a key feature of various degenerative brain diseases. This preferential vulnerability of brain cholinergic neurons to different pathogenic factors may be due to the fact that, in addition to energy production and structural lipid synthesis taking place in all types of neurons, they utilize acetyl-CoA for acetylcholine (ACh) synthesis (Szutowicz et al., 1996). Several pathologic conditions including hypoxia, excessive release of NO and glutamate that impair energy production and increase ACh release may contribute to this injury (Iadecola, 1997). On the other hand, various groups of cholinergic neurons were found to display a differential sensitivity to similar pathogenic inputs (Cooper and Sofroniew, 1996). The source of these differences is not known. Our studies have shown that in various brain regions, ratios of acetyl-CoA producing enzymes and choline acetyltransferase (ChAT) activities were significantly different (Szutowicz et al., 1982). Therefore, the aim of this work was to investigate if induction of cholinergic phenotype in SN56 septal hybrid cholinergic neurons may modify susceptiblity of their acetyl-CoA and ACh metabolism to increased NO levels.

SN56 cells (26–32 passages) were plated (2.5–2.7 ± 10^6 cells/30 cm^2) and grown to confluency for 3 days in Dulbecco's modified Eagle's medium with 10% (v/v) fetal bovine serum at 37 °C in an atmosphere of 95% air and 5%CO_2. In control conditions cells maintained stable levels of expression of puruvate dehydrogenase (PDH) and ChAT activities, equal to 7.7 ± 0.2 and 0.20 ± 0.007 nmol/min/mg protein, respectively. Addition of 1 mM dibutyryl cAMP (dbcAMP) inhibited PDH activity for 29% but increased that of ChAT 110%. On the other hand, 0.001 mM all-*trans*-retinoic acid (RA) did not affect PDH and simultaneously raised activity of ChAT 145% *(figure 1)*. Added together dbcAMP and RA suppressed PDH (24%) but exerted an additive activatory effect on ChAT activity (305%) *(figure 1)*. Protein content in control was equal to 2.95 ± 0.28 per plate. It was reduced in RA, dbcAMP and dbcAMP + RA treated neurons to 2.57 ± 0.36, 2.12 ± 0.25 and 1.39 ± 0.24 mg per plate, respectively. Drop of protein content in particular experimental group correlated with decreased cell density and increased neurite outgrowth.

Exposition of cells cultured for 2 days, to 1 mM sodium nitroprusside (SNP) for 10 min caused within the following 2 days a decrease of PDH activity in all groups (24–35%) *(figure 1A)*. Such treatment did not affect expression of ChAT activity in control and dbcAMP-treated cells *(figure 1)*. On the other hand, it suppressed by 35% activity of ChAT in neurons differentiated with RA or with dbcAMP + RA *(figure 1B)*. SNP did not affect protein content in control cells but decreased it in all experimental groups from 34 to 52%, without apparent changes in cell morphology (not shown).

Control and dbcAMP + RA-treated SN56 neurons when incubated in depolarizing medium (30 mM KCl), maintained stable level acetyl-CoA and steady rates of pyruvate utilization and ACh release, during the entire period of 30 min incubation at 37 °C. Addition of dbcAMP + RA to growth medium caused 35% inhibition of pyruvate utilization, 45% suppression of acetyl-CoA content and reduction of spontaneous and Ca-evoked ACh release for about 70% *(figure 2)*. In control neurons 0.2 mM SNP caused 35% inhibition of pyruvate utilization and slight activation of ACh release at unchanged level of acetyl-CoA. In dbcAMP + RA trea-

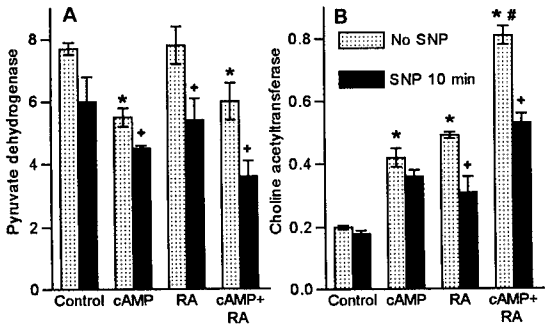


Figure 1. Effect of 10 min treatment with 1 mM SNP on postreatment expression of PDH (**A**) and ChAT (**B**) activities (nmol/min/mg of protein) in SN56 cells non-differentiated (Control) and differentiated with db-cAMP (cAMP) and retinoic acid (RA). Results are means ± S.E.M. of 5–10 experiments. $P < 0.001$ compared with respective control for ChAT and $P < 0.05$ for PDH; $^{+}P < 0.001$ compared with respective no SNP for ChAT and $P < 0.05$ for PDH; $^{\#}P < 0.001$ compared with respective cAMP or RA conditions.

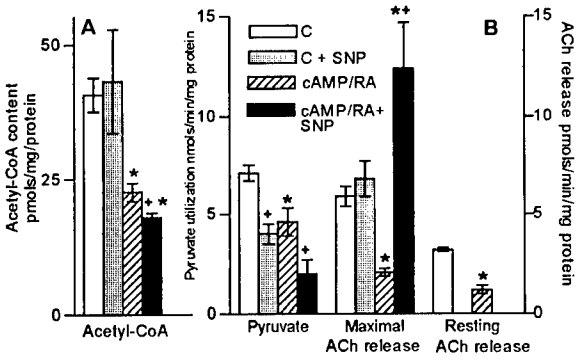


Figure 2. Effect of 0.2 mM SNP on (**A**) acetyl-CoA content, (**B**) pyruvate utilization, resting ACh release (no Ca in the medium) and maximal ACh release (1 mM Ca) in SN56 cells grown in basal conditions (**C**) or in the presence of 1 mM dbcAMP and 0.001 mM RA (cAMP/RA). Results are means ± S.E.M. of 4–5 duplicate experiments. $^{*}P < 0.01$ compared with respective control; $^{+}P < 0.05$ compared with respective no SNP conditions.

ted neurons, SNP brought about 50% inhibition of pyruvate utilization. However, unlike in controls, it caused a marked, six-fold inrease of ACh release and 21% decrease of acetyl-CoA content *(figure 2)*.

It is known that cholinergic differentiation of SN56 cells may proceed through the activation adenylate cyclase and directly through RA-activated transcription factors (Pedersen et al., 1995). Additive actions of dbcAMP and RA on ChAT activity indicate that expression of the cholinergic phenotype was mediated independently by these two intracellular signal transduction systems. It is possible that in vivo combined influences of multiple signaling pathways may provide highly variable levels of expression of cholinergic phenothype in particular groups of brain cholinergic neurons (Tomaszewicz et al., 1998). Decreased expression of PDH activity in differentiating neurons supports the hypothesis that rate of ACh synthesis is not tightly linked with the rate of acetyl-CoA generation in mitochondria (Szutowicz et al., 1996). The presumable efficacy of acetyl-CoA transport from mitochondria to cytoplasm is a rate-limiting factor for transmitter synthesis. However, in dbcAMP + RA treated cells rates of spontaneous and Ca-evoked ACh release were lower than in controls despite higher intracellular transmitter content. The source of this discrepancy remains to be found. One may conclude that it was not due to the low content of acetyl-CoA since activation of differentiated cells by SNP increased rates of ACh release/synthesis to much higher values than in non-differentiated ones. It might create increased demand for cytoplasmic acetyl-CoA and its shortage for mitochondria energy production as it was demonstrated here by the decrease of acetyl-CoA content in differentiated cells incubated with SNP *(figure 2)*. Also, a short time of exposure of differentiating SN56 to SNP may impair by such mechanism expression of PDH, resulting in development of cholinergic neurons more susceptible to various degenerative insults *(figure 2)*. These data may, at least in part, explain preferential susceptibility of septal cholinergic neurons to various neurotoxic inputs. This work was supported by K.B.N. project No 6P04A 013 10.

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45 Molecular mechanisms underlying the synapse-specific expression and activity-linked regulation of acetylcholinesterase at the neuromuscular junction: Role of intronic sequences

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We have previously shown that mRNAs encoding acetylcholinesterase (AChE) are highly concentrated within the postsynaptic sarcoplasm of adult muscle fibers (1, 2) where their expression is markedly influenced by nerve-evoked electrical activity (2, 3). In a first series of experiments, we have therefore examined the relative contribution of transcriptional versus post-transcriptional regulatory mechanisms in the synaptic accumulation of AChE transcripts in skeletal muscle fibers. To this end, we have cloned a 4.7 kb DNA fragment upstream of the translation start site in the rat AChE gene and generated multiple promoter-reporter gene constructs containing LacZ and a nuclear localization signal (nlsLacZ). These constructs were directly injected into tibialis anterior muscles of mice and 14 days later, muscles were excised and frozen. Muscles were subsequently cut in a cryostat and tissue sections were histochemically stained for the demonstration of β-galactosidase expression. The position of blue myonuclei indicative of promoter activity, was compared to that of neuromuscular junctions identified by AChE histochemistry. Injections of promoter-reporter gene constructs containing DNA fragments ranging from 1.5 to 4.7 kb led to a strong level of expression within muscle fibers. Surprisingly, quantitative analysis revealed that although expression of nlsLacZ was slightly higher in the postsynaptic sarcoplasm of muscle fibers, it was clearly not restricted to synaptic areas despite the presence of an N-box motif in the

promoter region shown recently by others (4, 5) and us (6, 7), to play a crucial role in directing the synapse-specific expression of AChR subunit genes and the utrophin gene, respectively. Nonetheless, deletion of 600 bp in intron 1 from the 1.5 kb promoter fragment completely abolished muscle expression. Transfection of motoneurons with these two latter constructs showed that both were effective in driving expression of LacZ in neuronal cells thereby indicating the presence of cis-acting elements essential for muscle expression in the first intron of the AChE gene. DNase 1 footprint analysis demonstrated that multiple regions in intron 1 were indeed protected by nuclear proteins extracted from skeletal muscles. Sequence analysis further showed that intron 1 contains a CArG box, as well as E- and N-box motifs. These results indicate that local transcriptional activation of the AChE gene is not the primary mechanism contributing to the maintenance of high levels of AChE transcripts at the neuromuscular junction. In addition, these results further highlight the role of DNA regulatory elements in the first intron of the AChE gene necessary for its expression in skeletal muscle.

In separate studies, we have examined the role of transcriptional and post-transcriptional regulatory mechanisms in the activity-linked regulation of AChE mRNA levels using the denervation model in both young (neo-natal to 2-week old) and mature rat skeletal muscles. Our results show that in comparison to the sharp increase (> 30-fold) in the levels of transcripts encoding the α-subunit of the acetylcholine receptor (AChR), denervation of adult muscle induced a rapid (within 2 days) and large (8-fold) decrease in the abundance of AChE mRNAs. Northern blot analysis also revealed that the two predominant species of AChE T transcripts expressed in adult muscle were reduced to a similar extent by muscle denervation. Furthermore, nuclear run-on assays showed that the transcriptional activity of the AChE gene remained essentially unchanged following denervation indicating that post-transcriptional events are responsible for the observed modifications in AChE transcript levels. In separate experiments, we examined whether denervation resulted in significant changes in the half-life of AChE and AChR α-subunit transcripts by injecting animals with actinomycin D. Quantitative analysis revealed that Egr-1 transcripts, used as a positive control in these experiments, were turning over rapidly (~1 h) in skeletal muscle. The estimated mRNA half-life in control muscle for AChE and the AChR α-subunit was ~ 9 and 6 h, respectively. Interestingly, denervation significantly increased the stability of both transcripts. In a final series of experiments, denervation of muscle from young rats induced a parallel increase (3-fold) in the expression of AChE and AChR α-subunit mRNAs. Both increases resulted from an activation in the transcriptional activity of these genes as assessed by run-on analysis. Based on these findings, we also performed electrophoretic mobility shift assays (EMSA) using oligonucleotides corresponding to the CArG and N boxes located in the first intron and observed specific binding activities with nuclear protein extracts isolated from both young and adult rat skeletal muscles. Interestingly, more binding activities were detected in nuclear extracts obtained from young muscles as compared to those from adult tissues. In agreement with our nuclear run-on assays, additional EMSA also showed that binding activities to these DNA regulatory elements were dependent on the state of innervation of the muscle fibers since they both increased with denervation of young muscles.

Taken together, these findings indicate that following short-term denervation, expression of AChE mRNAs is differentially regulated in developing versus adult skeletal muscles. In addition, our results suggest that transcriptional regulatory mechanisms are crucial in the control of AChE transcript levels in muscles from young animals subjected to altered levels of neuromuscular activation. By contrast, post-transcriptional events appear as key regulatory steps in dictating AChE mRNA levels in adult muscles. Since neuromuscular junctions in young muscles still undergo several maturational steps, we suggest that the activity-dependent transcriptional regulation of the AChE gene demonstrates a high level of plasticity only during synapse formation and maturation. This work is supported by the Medical Research Council of Canada (MRC). F.A.M. and A.M.K. are supported by studentships from the Ontario Graduate Scholarship Program and the Rick Hansen Man in Motion Foundation. B.J.J. is a Scholar of the MRC.

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46 Cholinesterase activity as a biomarker of exposure to temephos in *Chironomus* sp. and *Nereis* (Hediste) *diversicolor* in Morbihan (Brittany, France) coastal wetlands

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Cholinesterases (ChEs) have been widely used as biomarkers of exposure to organophosphates and carbamates in marine animals (Galgani and Bocquené, 1998). A pilot study has been undertaken to evaluate the ecological impact of Abate® (active ingredient temephos) used against mosquito larvae in Morbihan coastal wetlands. Midges *Chironomus* sp. and the marine worm *Nereis* (Hediste) *diversicolor* were used as sentinel species in three pilot sites. In situ exposure to temephos was monitored in these animals using ChE activity.

Control and treated areas were defined in each pilot site. Sediment samples (4.5 L) were collected in both areas 24 h and 3 days after treatment. Water temperature, pH, dissolved oxygen

concentration and salinity were measured in sampling zones. Animals were isolated from the sediments and immediately frozen in liquid nitrogen. They were stored at –80 °C until ChE activity measurement. Enzyme extracts were prepared from pools of 6 entire *Chironomus* sp. larvae and of the heads of 3 immature *N. diversicolor*. Individual measurements were performed on the head of mature *N. diversicolor*. Tissue samples were weighed and homogenized in 20 mM phosphate buffer pH 7.2, 0.1% Triton X-100, using an ice-cold glass homogenizer with a motor driven Teflon pestle. Homogenates were centrifuged at 10 000 *g* for 20 min at 4 °C. The supernatants were used for ChE assay. ChE activity was determined according to Ellman et al. (1961) and Galgani and Bocquené (1998) using acetylthiocholine iodide (ACTC) as the substrate. Absorbance at 412 nm was recorded for 2 min, using a Uvikon 943 double-beam spectrophotometer (Kontron Instruments). The activity was corrected for non-enzymatic hydrolysis of the substrate and calculated using least-square linear regression analysis over the first 30 s of the kinetics. Protein contents were determined according to Bradford (1976), using BSA (fraction V) as the standard, and a commercial Coomassie Blue G-250 reagent solution (Pierce Chemical Co.). ChE activity was expressed as nmol of ACTC hydrolysed/min/mg protein.

ChE activities in control animals were 37.4 nmol ACTC hydrolysed/min/mg protein for midge larvae extracts, and 64.4 and 62.9 nmol ACTC hydrolysed/min/mg protein for the head extracts of immature and mature *N. diversicolor*, respectively. Basal levels of cholinesterase activity will be used as references for comparison to the animals exposed to temephos during the 1998 mosquito control campaigns.

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47 Acetylcholinesterase inhibition by diazinon: A dual substrate model for kinetic study

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Acetylcholinesterase (AChE) is a hydrolytic enzyme which controls the transmission of nerve impulses by hydrolyzing the acetylcholine (a neuro-transmitter) in the nervous system (Al-Jafari, et al., 1997). It is the target enzyme for testing toxicity induced by organophosphate and carbamate insecticides (Kamal, 1997). In the current study we have determined various kinetic parameters of AChE inhibition by diazinon, an organophosphate insecticide (Worek et al., 1997). The kinetic parameters obtained from figures 3 and 4 are as follows: k_{oms}' = 0.821 min^{-1}; K_{sms}' = 1.0 (mM min); k_{oms} = 0.378 min^{-1}; K_{sms} = 0.40 (mM min)$^{-1}$; K_{asms}' = 0.175 $(mM/L)^2$; K_{asms} = 0.597 $(L/mM)^2$.

This is an unique, and a sensitive short approach. It is an promising approach for the estimation of various kinetic parameters for the inhibition of variety of enzymes by variety of chemicals, insecticides, herbicides and drugs.

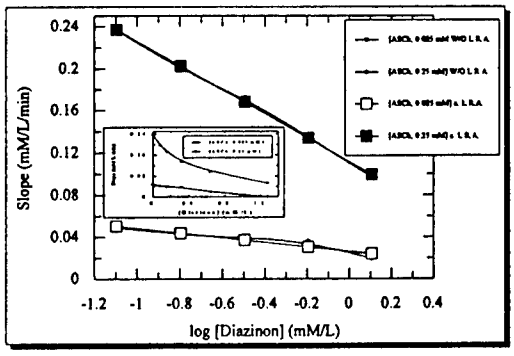


Figure 1. Secondary plot of figure: slope of each plot in figure 1 versus log concentration of diazinon at two concentrations of substrate (ASCh) as mentioned in legend box (W/O and a.L.R.A. means without and after linear regression analysis respectively). Inset: slope of each plot in figure 1 versus concentration of diazinon.

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48 Identification of inducible genes by activation of muscarinic acetylcholine receptors

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Muscarinic acetylcholine receptors (mAChR) are G protein-coupled cell surface receptors with classic seven transmembrane domain topology. Despite detailed knowledge of the molecular signaling pathways coupled to muscarinic receptors, little is known about genes and gene products that mediate the muscarinic receptor-associated functions. In brain, mAChRs are involved in long-term potentiation, synaptic plasticity, as well as in higher cognitive functions including learning and memory (Di Chiara et al., 1994). Such plastic alterations in neuronal structure and function are associated with rapid and transient transcription of activity-dependent genes like the immediate-early genes c-fos, jun-B, Egr-1, and Egr-2 on attention, learning, memory and cognition (Huerta and Lisman, 1993). In addition to their physiological function, muscarinic receptors are involved in the pathological cholinergic deafferentation in Alzheimer's disease as well as in the posttranslational modification of the amyloid precursor protein (Nitsch et al., 1992).

In order to identify genes that are induced by mAChR, we developed a screening approach based on the mRNA differential display technique (Liang et al., 1994). Human HEK293 cells stably transfected with the muscarinic acetylcholine receptor subtype m1 were stimulated by carbachol, and identical cells generated in parallel were used as unstimulated controls. Cell culture experiments were performed in triplicates. Total RNA was prepared from the cells and transcribed to cDNA by using one-base-anchor primers $HT_{11}A$, $HT_{11}G$ and $HT_{11}C$. The obtained cDNAs were subjected to polymerase chain reaction (PCR) employing the corresponding one-base-anchor oligonucleotides along with random primers by using PCR conditions as described (Zhao et al., 1995). PCR was performed in duplicates in the presence of $\alpha[^{35}S]$-dATP along with dCTP, dTTP and dGTP. The PCR-products were separated on sequencing gels, gels were dried, and X-ray films were exposed. Differential bands were excised, the contained cDNAs were eluted and re-amplified by high-stringency PCR employing the corresponding composite primer pairs. Reamplified cDNAs were purified and cloned into pBluescript IIKS+. Cloned cDNA fragments were sequenced.

A set of 64 different random primers was generated with a *Hind*III restriction site followed by 7 bp random sequences which were specifically designed to ensure the statistically comprehensive analysis of all mRNA species in one cell population of a defined cell status

After 68 of 192 possible PCR experiments, 34 differential bands were identified. Sequence analysis of 16 differential bands revealed that theses bands corresponded to 8 different genes *(table I)*.

By Northern blot analyses the expression of the immediate-early genes *egr-l*, *egr-2*, *egr-3*, NGFi-B, ETR101, and *c-jun* were proved to be upregulated by m1AChR activation. Time course analyses confirmed that mRNA levels of Egr-1, Egr-2, and Egr-3 increased readily after m1AChR stimulation, and that a maximum was attained within 50 min. At that time, Egr-4 mRNA was also detectable. Western blots and electromobility shift assays demonstrated synthesis of EGR-1 and EGR-3 proteins, as well as binding to DNA recognition sites in response to m1AChR activation. Activation of m1AChR increased transcription from EGR-dependent promoters including the acetylcholinesterase promoter. Activity-dependent regulation of Egr-1 mRNA expression and EGR-1 protein synthesis was also observed in cells expressing m2, m3, or m4AChR subtypes (von der Kammer et al., 1998).

In addition, we identified hcyr61 as well as the previously unknown human gene gig-2 (for G-protein coupled receptor induced gene), and verified muscarinic regulation of these genes by Northern blot analysis. hcyr61 encodes for a secretory protein with 381 amino acid residues. It is a member of the IGFBP (insulin-like growth factor binding protein) superfamily with possible roles in cellular interaction with the extracellular matrix. The cDNA of gig-2 codes for a 372 amino acid protein and is has partial homologies with serine/threonine kinase domains. Gig-2 is ubiquitously expressed, suggesting a housekeeping function that may be regulated in brain by neuronal activity.

Our results demonstrated that the developed screening system is useful to identify known genes as well as new genes that are under the control of m1AChR. First analyses revealed a high number of differential bands, with less than 10% false positive bands.

Table I. Characterisation of 16 analysed differential display bands of interest.

Gene product	*No. of DD-bands*	*Verified regulation by mAChRs*
Egr-1	3	+
Egr-2	1	+
Egr-3	3	+
Etr101	2	+
c-jun	3	+
NGFiB	1	+
gig-1 (hcyr61)	2	+
gig-2	1	+

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49 Long-term modulations of cholinergic neurotransmission following chronic stress

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Acute and chronic stress are known to induce alterations in hippocampal structure (McEwen, 1997; Gurvits et al., 1996) as well as specific electrophysiological responses (Xu et al., 1997). We have recently shown that both acute psychological stress and exposure to anticholinesterases induce concerted calcium-dependent modulation of genes regulating acetylcholine availability. These changes suggested long-term decrease in the availability of acetylcholine in distnict brain regions and coincided with muscarinic-dependent rapid enhancement and delayed depression of neuronal excitability in the CA1 area of the hippocampus (Kaufer et al., 1998).

In order to study the molecular and physiological mechanisms bridging between the acute alterations in cholinergic neurotransmission and long-lasting modifications in hippocampal functions we tested the delayed consequences of repeated stress or anticholinesterase treatments on mice up to 6 weeks following 4 consecutive daily exposures. The CA1 synaptic responses were significantly suppressed and the sensitivity to muscarinic antagonists drastically enhanced in the treated mice. Moreover, the 'readthrough' splice variant of acetylcholinesterase mRNA accumulated in perikarya and apical dendrites of pyramidal neurons in cortex and hippocampus. Together, these findings demonstrate weeks-long changes in cholinergic gene expression that are associated with long-lasting changes in cholinergic neurotransmission.

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50 Mechanisms regulating expression of nicotinic acetylcholine receptor subunits

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Our investigative aim is to identify mechanisms which regulate expression of acetylcholine receptors at the cell surface. Monoclonal antibodies and high affinity ligands are available to detect expression of receptor subunits, and to distinguish whether subunits are folded or assembled. Furthermore, an extensive collection of published studies on degradation and assembly of the receptor, along with the selective ligands with which it interacts, make this system well suited to delineate the factors regulating expression of multisubunit transmembrane proteins. Subunits of the receptor assemble intracellularly to enclose a centralized cation channel, which is exported to the cell surface in the pentameric arrangement of subunits as α-γ-α-b-δ. Monomers and subunit dimers, $\alpha\delta$ and $\alpha\gamma$, are retained intracellularly, and assembly with the β-subunit is a requirement for export of the complex to the cell surface. Unassembled subunits show little accumulation in cells, compared to assembled complexes, indicating that mechanisms for degradation depend on subunit assembly. Subunits which accumulate in cells eventually become components of the mature receptor, pointing out that cellular factors which protect subunits from degradation contribute to maturation of the ion channel.

Our studies, with transiently expressed receptor subunits in mammalian culture cells, have identified that the chaperone protein, calnexin, and the conjugation of polyubiquitin chains to the subunit have a significant impact on subunit degradation, stability and trafficking to the Golgi. Calnexin is a type-1 membrane protein, and it is thought to recognize oligosaccharide components in glycoproteins. By using the glycosidase inhibitor castanospermine, our experiments demonstrate that dissociating calnexin results in the enhanced polyubiquitination and degradation of receptor subunits in the proteasome. Our experiments also reveal that calnexin associates with unassembled subunits, and it dissociates when subunits assemble. In this manner, calnexin stabilizes subunits in preparation for assembly and expression at the surface. Since attachment of polyubiquitin molecules is thought to promote dislocation of proteins to the cytoplasm through the Sec61 translocon, one might hypothesize that attachment of polyubiquitin molecules acts to oppose transport of subunits into the secretory pathway leading to the Golgi. Therefore, calnexin attachment shows a role in the expression of subunits that are incorporated into the mature ion channel, by inhibiting polyubiquitination and dislocation to the cytoplasm.

Calnexin has an endoplasmic reticulum retention sequence in its cytoplasmic tail, and its attachment may also retain subunits in the endoplasmic reticulum. By using a cDNA which encodes for a truncated calnexin lacking the endoplasmic reticulum retention sequence, and the cell line, ts20, which expresses a temperature labile ubiquitin activating enzyme, retention in the endoplasmic reticulum and polyubiquitination can be inhibited.

Proteins expressed in ts20 cells, at 40 °C, are not polyubiquitinated. Alpha-subunits can be induced to be transported to the Golgi when coexpressed with truncated calnexin in ts20 cells at 40 °C, as detected by confocal microscopy. In the absence of these reagents, for example, when expression occurs in HEK cells, a-subunits remain in the endoplasmic reticulum. Alpha-subunits expressed in ts20 cells without coexpression of truncated calnexin, are also sequestered in the endoplasmic reticulum. Calnexin is not associated with assembled subunits, indicating that its role for retaining subunits in the endoplasmic reticulum is precluded when subunits assemble. These observations indicate that calnexin may have dual roles: along with inhibiting dislocation into the ubiquitin-proteasome pathway, calnexin may sequester receptor subunits in the endoplasmic reticulum prior to subunit assembly. The outcome of both of these roles is to stabilize unassembled subunits in place in the endoplasmic reticulum, thereby enhancing opportunities for assembly with other subunits of the receptor. In summary, our findings indicate that chaperone protein attachment and ubiqutination act together to regulate the fate of the unassembled subunit pool, a part of which is eventually incorporated into the mature ion channel. Observations that emerge from studies on the acetylcholine receptor should be applicable to the expression of other multisubunit transmembrane proteins. Supported by an American Heart Association postdoctoral fellowship to S.H.K. and NIH Grant GM 18360 to P.T.

51 nAChR and α-neurotoxin: New tools for old acquaintances

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Despite numerous studies (Oswald et al., 1982, Trémeau et al., 1995, Machold et al., 1995, Ackermann and Taylor, 1997), the structure of complexes between a snake curaremimetic toxin and nicotinic acetylcholine receptors (nAChR) remains unclear. This study, therefore, aimed at bringing new insights on this old question by exploiting today's techniques.

Using solid phase peptide synthesis, we prepared large amounts of various mutants of *Naja nigricollis* α-toxin. Two toxin mutants, R33C and K47C, were synthesized and characterized. These mutations caused moderate affinity decreases for *Torpedo* receptor.

Previous photoaffinity experiments done on nAChR showed that an aryldiazonium ion, DDF, as developed by Goeldner, Hirth and colleagues (Langenbuch-Cachat et al., 1988), is a well suited photoactivatable probe which allows both high coupling yields and labelling of several functionally important receptor residues. We therefore synthesized an analog of DDF, and coupled it to the free cysteine of R33C and K47C mutants, through a cleavable disulfide bond. Photoaffinity labelling experiments carried out with R33C-DDF toxin analogs (see *figure 1*), showed an efficient incorporation of the probe in the *Torpedo* a subunit only, indicating the proximity of the second loop of the toxin with this subunit. Synthesis of a radioactive DDF analog is in progress, to identify the residues labelled on the receptor.

Treatment of nAChR with low concentration of DTT is known to selectively reduce the α-subunit disulfide bond Cys192-Cys193, which is located in the acetylcholine binding site. Thus, the cysteines 192 or 193 can be labelled with small antagonists such as MBTA (Kao and Karlin, 1986). We explored the possibility that a maleimido analog introduced at an appropriate position of the toxin (see figure 1) could also label specifically Cys192 or Cys193. Using different R33C toxin analogs, we indeed observed an efficient labelling of the reduced cysteinyl residues. This result not only agrees with our photoaffinity experiments made with R33C-DDF that R33 interacts with the α-subunit but demonstrates its proximity with the acetylcholine binding site. By contrast, K47 is not in such a close proximity to the α-subunit, since K47 toxin analogs unexpectedly labelled the δ subunit. The only disulfide bond that can be reduced under our conditions in this subunit, involves the δ-δ bond that links two *Torpedo* receptor monomers. Consequently, we suggest that the K47 residue on the toxin third loop is in proximity to the penultimate C500 residue of the nAChR δ subunit.

These preliminary results demonstrate the interaction of the central toxic loop with the acetylcholine binding site on the α subunit, and of the third loop with the COOH terminus part of the δ subunit, in agreement with a location of the binding site between two subunits (as shown here for α and δ). If labelling of the d-C500 residue would be confirmed, this would imply an interaction of the toxin with the outside face of the extracellular part of the receptor.

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52 The role of charged transmembrane residues of rVAChT on ACh transport and vesamicol binding

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The neurotransmitter acetylcholine (ACh) is synthesized in the cytoplasm of cholinergic neurons and transported into synaptic vesicles by the vesicular acetylcholine transporter (VAChT). Rat VAChT has several charged amino acid residues within its predicted transmembrane domains (TM) which may play important roles in structure and function. In order to identify the functional charged residues of rVAChT involved in the transport of acetylcholine and in vesamicol binding, site directed mutagenesis of rVAChT was undertaken. Changing Asp46 (D46N) in TM2, Asp225 (D225N) in TM6, and His444 (H444R) in TM12 respectively, had no effect on ACh transport or vesamicol binding. However, replacement of His338 in TM8 with Arg or Asp398 in TM10 with Asn completely eliminated both ACh transport and vesamicol binding. Surprisingly, mutant D193N in TM4 did not affect ACh transport activity; however vesamicol binding was dramatically reduced. The same loss of vesamicol binding without affecting ACh transport was also seen with two other mutants; H413R mutant which is in the loop between TM10 and 11 and E449Q in TM12. Interestingly, with mutants K131A in TM2 or D425N in TM11, transport activity for ACh was completely blocked without any change in vesamicol binding. These mutants as well as mutant D193N, H413R, and E449Q clearly dissociate ACh binding and transport from vesamicol binding. These data suggest that Lys131 in TM2, His338 in TM8, Asp398 in TM10 and Asp425 in TM11 are important to ACh binding and transport. Asp193 and Asp398 in TM4 and TM10 respectively, Glu449 in TM12, His338 in TM8, and His413 in the cytoplasmic loop between TM10 and TM11 are involved in vesamicol binding. Together these studies begin to unravel the topology of the vesicular acetylcholine transporter.

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53 Ca^{2+}-dependent regulation by calcitonin gene-related peptide of neuronal type nicotinic acetylcholine receptor in neuromuscular pre- and postsynapse

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Calcitonin gene-related peptide (CGRP), the major neuropeptide, coexists with acetylcholine (ACh) at the motor nerve endings (Takami et al., 1985a, b; Matteoli et al., 1988), is released by electrical nerve stimulation or by cholinesterase inhibition in intact skeletal muscle, and the CGRP may negatively modulate nerve stimulation-evoked ACh release (Kimura et al., 1997) (*figure 1*). CGRP binds to its receptors (R) located at the neuromuscular postsynapse (Jennings and Mudge, 1989; Poper and Micevych, 1989; Roa and Changeux, 1991). This peptide prolongs open time of nicotinic AChR channel currents (Lu et al., 1993; Owens and Kullberg, 1993) in cultured myotubes and regulates neurmuscular transmission by modulation of nicotinic AChR phosphorylation and desensitization (Miles et al., 1989; Dezaki et al., 1996). CGRP prolongs the duration of nerve-stimulated non-contractile slow Ca^{2+} mobilization and $CGRP_{8-37}$, a CGRP antagonist, shortens its duration (Kimura et al., 1993). Confocal imaging of fexor digitorum brevis (FDB) muscle cells indicated that bath-application of CGRP after ACh application potentiated the ACh-elicited slow Ca^{2+} signal. Cotreatment of muscle cells with ACh plus CGRP led to a synergistic enhancement in the slow Ca^{2+} response compared with those elicited by ACh alone (*figure 1*). The first brief and localized rise in the $[Ca^{2+}]_i$ appeared to be due to the effect of CGRP alone. CGRP pretreatment, not after ACh application, has no effect on the ACh-elicited Ca^{2+} signals. The nerve-stimulated non-contractile Ca^{2+} mobilization is specially blocked by competitive nicotinic antagonists such as (+)-tubocurarine and pancuronium at low concentrations that have no effect on contractile Ca^{2+} transients (Kimura et al., 1990). ACh responses were completely depressed in both components and the potentiation by CGRP was greatly depresssed in the presence of (+)-tubocurarine. The involvement of the voltage-sensitive L-type Ca^{2+} channels in the CGRP potentiating mechanism may be ruled out because a Ca^{2+} channel blocker nitrendipine did affect neither ACh responses nor CGRP-induced potentiation. Therefore, CGRP may activate its own receptor, then indirectly enhance the neuronal type nicotinic AChR-operated slow Ca^{2+} signal because CGRP does

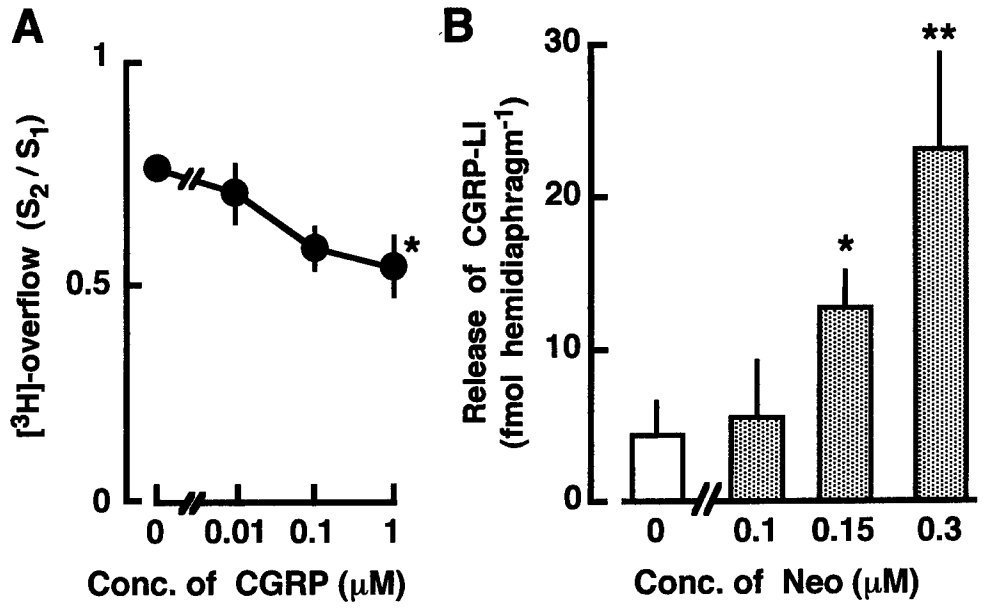


Figure 1. A. Concentration-response curve of CGRP for electrically evoked [^{3}H]ACh release (S2/S1, the ratio of the value at two stimulation periods) from mouse phrenic nerve-diaphragm muscle preparations. CGRP was applied in various concentrations 15 min before S2. Each point represents the mean value for relative ratios of ACh release (S2/S1) (n = 3–5) and vertical bars represent S.E.M. Significant difference from the control (without drug) was analyzed by one-way analysis of variance followed by Scheffe's test (*P < 0.05). **B**. Increasing effect of neostigmine on resting release of CGRP-like immunoreactivity (CGRP-LI) from rat phrenic nerve-hemidiaphragm muscle preparations. The tissues were incubated for 10 min with (0.1, 0.15 and 0.3 μM; stippled columns) or without (open column) neostigmine after equilibration. Significant differences from the control (without drug) were analyzed by one-way analysis of variance followed by Scheffe's test (*P < 0.05 and **P < 0.01). Values are means ± S.E.M. of 3 to 7 experiments.

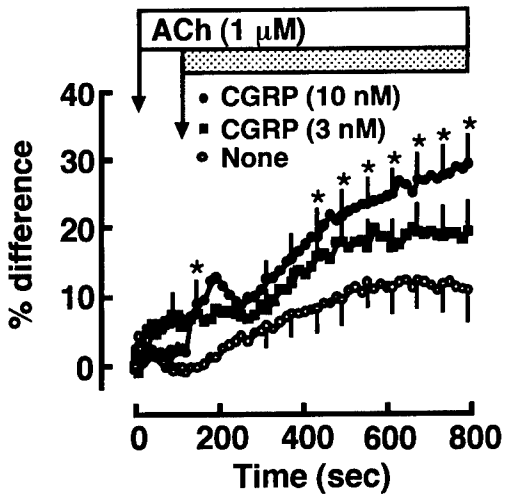


Figure 2. Potentiating effect of CGRP on ACh (1 μM)-elicited slow Ca^{2+} component in mouse FDB single skeletal muscle cells. The cells were exposed to CGRP 2 min after ACh application. The percentage difference of the fluorescence intensity between inside and outside the endplate region was estimated. Data are expressed as means ± S.E.M. (n = 27–30). *P < 0.05: significant differences from control response with ACh alone based on one-way ANOVA follwed by Scheffe's test.

not activate directly muscle type nicotinic AChR at the muscle surface (Takami et al., 1985b).

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54 The Schwann cell at the neuromuscular junction

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Schwann cells wrap around nerve axons to provide electrical insulation and are essential for the formation, maintenance and repair of the neuromuscular junction (NMJ) (Balice-Gordon, 1996). At the NMJ, they are juxtaposed with nerve terminal and muscle cell, with which they interact during synaptogenesis. We focused on their role in maturation of the NMJ.

Establishment of an NMJ involves a sequence of complex mechanisms. The progression of events, from the first neuromuscular contact (14–15 embryonic days) to the mature synapse (15 days post-natal), occurs over 3 weeks. As soon as the first nerve-muscle contact is established, acetylcholine receptors (AChR) and cholinesterases (acetylcholinesterase [AChE] and butyrylcholinesterase [BChE]) accumulate at synaptic sites. The accumulation of AChR in the muscular membrane is a prerequisite for normal accumulation of AChE in the basal lamina of the synaptic cleft (De La Porte et al., 1998). AChR mature up to 2 weeks after birth under the control of molecules synthesised by the motoneuron. AChR consists of α, β, γ and δ subunits at the embryonic stage, and the γ subunit is replaced by the ε subunit 2–3 days after innervation. This substitution affects the channel properties of the AChR (conductance and open time). Two molecules synthesised by the motoneuron are known to induce expression of the ε gene: acetylcholine receptor-inducing activity (ARIA)/heregulin and agrin. Another neuronal molecule, calcitonin-gene related peptide (CGRP), also increases transcription of the AChR α-subunit gene, but not of other subunits. Agrin also induces the clustering of AChR, AChE, BChE and heparan sulphate proteoglycan (HSPG), a component of the basal lamina.

Cholinesterases exist in several oligomeric molecular forms, distinguished by their sedimentation coefficient: the globular and the asymmetric forms. The catalytic subunits corresponding to AChE and BChE are encoded by distinct genes. The role of AChE

in cholinergic transmission is unambiguous, but the function of BChE is not clear except as an adhesion molecule. BChE, which is little expressed in the adult NMJ, also hydrolyses the neurotransmitter acetylcholine, and may thus participate in neurotransmission in vitro, and in vivo during development, and to a lesser degree in the adult NMJ. AChE and BChE are initially present in equal amounts at the NMJ, but as we demonstrated BChE levels diminish sharply between 7 and 15 days after birth, the stage at which the synaptic Schwann cell membrane becomes juxtaposed with the muscle membrane (Desaky and Uehara, 1987).

Synapses obtained in vitro, by conventional techniques of contact between myoblasts and spinal cord neurons, have the morphological, biochemical and electrophysiological characteristics of embryonic synapses. The events of postnatal maturation, such as disappearance of polyinnervation, changes in levels of AChE and BChE, and appearance of junctional folds, have never been observed, whatever the duration of culture or the age (embryonic or adult) of the cells used. In co-cultures of adult human muscles with explants from whole cord and dorsal root ganglia of 13- to 14-day-old rat embryos, Kobayashi et al. (1987) observed the formation of mature muscle fibres, as characterised by contractions and striations, well-organised AChE clusters, and the trend from multifocal to unifocal innervation. This result suggested to us that Schwann cells of dorsal root ganglia participate in synaptic maturation.

The factors involved in the morphological maturation (disappearance of polyinnervation, appearance of junctional folds) and changes of synaptic cholinesterases were not identified and we

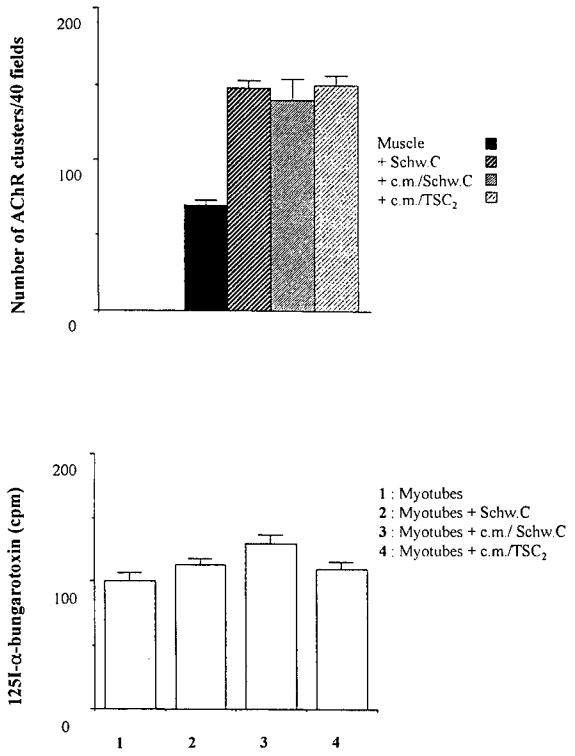


Figure 1. Number of AChR clusters (top) and ^{125}I-α-bungarotoxin binding sites (bottom) in 7-day-old myotubes cultured for 2 days with Schwann cells or with conditioned medium (c.m.) by Schwann or TSC2 cells.

postulated that Schwann cells were implicated in these events. We prepared mixed cultures, combining muscle cells (from the hindleg of 18-day-old rat embryos), spinal cord cells (from 14-day-old embryos) and Schwann cells (from the sciatic nerve of new-born animals or from the Schwann cell line TSC2, a gift from Prof. Tennekoon, Philadelphia, USA). Schwann cells and spinal cord cells were added to the muscle cells at day 5 of culture, the stage at which the myotubes were formed, contracting and morphologically differentiated. We used a monoclonal antibody (6.17) obtained in the laboratory, which recognises a molecule synthesised by Schwann cells and co-localised with the AChR at the NMJ. 6.17 antigen appears to be localised in the synaptic space, but not in the basal lamina. In vivo in the rat, 6.17 antigen is initially distributed diffusely and concentrates at the synapse 15 days after birth, and its maintenance at the NMJ depends on innervation (Koenig et al., 1988).

We have shown that during in vitro synaptogenesis, the addition of Schwann cells to muscle-neuron co-cultures: 1) allows visualisation of antigen 6.17 at the synapse and induces synaptic maturation (decrease in number of synapses and appearance of junctional folds (Chapron and Koenig, 1989); and 2) induces the disappearance of BChE at synaptic clusters, leaving only AChE activity, as in the adult neuromuscular junction (Chapron et al., 1997)). This maturation is inhibited by the presence of antibody 6.17. Antigen 6.17 is secreted by Schwann cells and TSC2, since medium conditioned by these cells reproduced the effect of the cells themselves. It may be wondered whether antigen 6.17 corresponds to a previously characterised molecule (cf. Chapron et al., 1997), and its molecular characterisation is in progress (in collaboration with H. Chneiweiss, INSERM U114, Paris). These results prompted us to postulate that Schwann cells could mediate changes in synaptic maturation during development. Heregulins and their specific receptors, members of the erbB family of tyrosine kinases, have been implicated in the control of growth and development of Schwann cells. Homozygous erbB3 mutant embryos lack Schwann-cell precursors and Schwann cells that accompany peripheral axons of motor (and sensory) neurons (Reithmacher et al., 1997). It will be of interest to look at the possible presence of high levels of BChE in such mutants.

AChR clustering may be induced by neuronal agrin, electric fields, latex beads, and basal lamina components (collagen, laminin). As the Schwann cell is closely associated with the nerve terminal, we investigated whether it plays a role in the formation of AChR clusters, and found that it does. When primary Schwann cells or medium conditioned by Schwann cells or by TSC2 cells, were added to muscle cells in culture, the number of AChR sites increased by only 10%, but the number of AChR clusters increased by 100% (figure 1). Antibody 6.17 does not inhibit the Schwann cell-mediated increase in the number of AChR clusters. This strengthens the idea that antigen 617 is specifically implicated in synaptic maturation. We have tested the possibility that agrin synthesised by the Schwann cell is the molecule responsible for this increase. But the agrin isoform expressed by the Schwann cell is the inactive one (B0) (in collaboration with G. Escher, Institut d'Embryologie, Lausanne, Switzerland). We are currently testing different factors synthesised and released by Schwann cells.

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55 Recovery of brain acetylcholinesterase activity in common carp *Cyprinus carpio* L. exposed to chlorfenvinphos and carbofuran insecticides

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Organophosphates and carbamates used in intensive agricultural production can reach naturel water either via sepage of chemicals from the soil or directly due to the spraying against pests. These compounds may become concentrated in organs of aquatic organisms, especially those at the top of the food chain. The main mechanism of action of these organophosphates and carbamate insecticides involves the inhibition of acetylcholinesterase (AChE). However, if exposure to the inhibitor is discontinued, AChE activities could recover the normal level.

In this work successive extraction of AChE of the common carp brain showed 85.6% of soluble form against 14.4% of membrane bound form.

After 96 h of exposure of 6–8 cm length carp to chlorfenvinphos (2.1×10^{-4} mg/L) and carbofuran 3×10^{-3} mg/L), the AChE activity decreases respectively to 10% and 70% compared to control group. Three times higher (15 days) the exposition time (96 h) is necessary, after transfer in clean water, to recover the AChE activity level identical to that of control.

56 Laser-assisted cell-picking and subsequent RT-PCR allows subtype analysis of mRNAs derived from the cholinergic gene locus in rat cardiac neurons

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Choline acetyltransferase (ChAT) and the vesicular acetylcholine transporter (VAChT) are encoded by the 'cholinergic gene locus' in a peculiar nested fashion, in that the entire coding region of VAChT mRNA is located between the first non-coding exons ('R' and 'N') of the ChAT gene (Usdin et al., 1995). A third non-coding exon ('M') of the ChAT gene follows between the N-exon and the coding ChAT exons. In the rat, a remarkable variety of mRNA species is derived from this cholinergic gene locus: transcription start initiated from three different promotors results in R-, M- and N-type ChAT mRNAs from which subtypes R1/R2 and N1/N2 are the result of differential splicing. The R-exon is also contained in V1a- and V1b-type VAChT mRNA whereas the V2-type VAChT mRNA is coded for in one exon. In cultured sympathetic postganglionic neurons all types of ChAT and VAChT mRNAs can be induced and their ChAT and VAChT expressions are coordinately but differentially regulated (Misawa et al., 1995). Extracts from whole rat spinal cord contain all five species of ChAT mRNAs (Kengaku et al., 1993), but it is not known whether individual mRNA species are cell-specifically expressed. A tool for subtype analysis of mRNA species derived from the cholinergic gene locus in select neuronal populations in situ is desirable for solving this and related questions, but yet has not been available. Focusing upon intrinsic cardiac neurons of the rat we addressed this issue by laser-assisted cell-picking (Schütze and Clement-Sengewald, 1994) and subsequent RT-PCR.

Rat heart atria were quick-frozen in melting isopentane, cryosections (6 µm) were collected on coverslips and stained with hematoxylin. Utilizing a nitrogen laser microbeam (337 nm wavelength; P.A.L.M., Wolfratshausen, Germany) connected to an inverted microscope, identified neuronal section profiles were dissected by photolysis from surrounding tissue under microscopic control and harvested with a micromanipulator. Cardiomyocytes obtained from the same sections served as control. From 1 to 11 neuronal section profiles were pooled and processed as a single specimen. They were transferred into 10 µL of a modified first strand buffer and snap frozen. Before cDNA synthesis, tubes were heated and the content divided into two aliquots of equal volume. One of them was subjected to complete RT-PCR, the other was processed identically except that reverse transcriptase was omitted. Thus, for each individual sample an internal control for un-

warranted amplification of genomic DNA fragments was run. Two primer pairs for amplification of ChAT mRNA were used: one of them was intron-spanning and located in the coding region of ChAT, the other was specific for that region of the non-coding R-exon which codes only for R1-type mRNA. Hence, the latter primer pair was not intron-spanning.

Using the intron-spanning primer pair, ChAT mRNA could be detected in neuronal samples by RT-PCR, and amplicons derived from cDNA were readily discriminated from those derived from genomic DNA in agarose gels simply by molecular size. Thus, the general feasibility of the technique was demonstrated, but these experiments did not allow subtype analysis of mRNA species since all diversity lies in the 5' non-coding region. Utilizing the R1-type specific primer pair, in initial experiments glial cells including their nuclei were not thoroughly separated from neuronal section profiles. From these samples an amplicon of expected size (121 bp) was generated by RT-PCR, but also when reverse transcriptase was omitted. Thus, in the present technique, which omits a DNase digestion step, contaminating DNA will also serve as a template in the following PCR. Therefore, in order to minimize load of genomic DNA in the sample, microscopically visible nuclei were photolyzed by the UV laser in subsequent experiments. Under these conditions, a single amplicon of expected size was generated by RT-PCR in 56% of neuronal samples, but only in 9% of samples when reverse transcriptase was omitted. Thus, distinction between cDNA and DNA was enabled even with primers located within the same exon, i.e., not intron-spanning. Using samples of cardiomyocytes with photolyzed nuclei, negative results were obtained, with the exception of a single positive case each by RT-PCR and when reverse transcriptase was omitted.

Laser-assisted cell-picking has previously been used to investigate genomic DNA by PCR, for example to analyze mutations in microscopically identified tumour cells (Becker et al., 1997). Here we demonstrate that it is also a useful tool for mRNA analysis by RT-PCR, and succeeded in identifying a specific mRNA variant (R1-type) from the cholinergic gene locus in a select neuronal population in situ. This R1-type mRNA is expressed in the majority of intrinsic cardiac neurons. Since the M-type is predominant in the rat spinal cord (Kengaku et al., 1993), there either exist differences between cardiac and spinal neurons with respect to preferred transcription start, or individual neurons transcribe multiple mRNA variants at different quantities. This study was supported by the DFG, "SFB Cardiopulmonary Vascular System", project C2.

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57 Heat stress, even extreme, does not induce penetration of pyridostigmine into the brain of guinea-pigs

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Stress situation due to forced swim test was recently shown to allow penetration of pyridostigmine (PYR) into the brain of mice. Accordingly, it was suggested that, in troops exposed to emotional stress under conditions of war, as during the Gulf War, the BBB may have unexpectedly become permeable to PYR thus leading to an increased frequency of CNS symptoms. In this study, the entry of PYR into the brain was investigated in guinea-pigs subjected to different heat stress levels. In a first group, guinea-pigs were maintained at room temperature for 2 h, their core temperature remaining stable at about 39.8 °C. In a second group, animals were placed in a climatic chamber in order to keep their core temperature at 41.5 °C for 2 h. In a third group, animals were subjected to a high ambient temperature (42.6 °C) for about 2 h and developed heatstroke symptoms, their core temperature progressively increasing and reaching around 44.3 °C. In each group, the stress of the animals was assessed by measuring the increase of plasma cortisol level and PYR (0.2 mg/kg, s.c.) was injected 90 min after beginning of the experiment. Penetration of the drug into the brain was examined by measurement of acetylcholinesterase (AChE) activity in the cortex, the striatum and the hippocampus of the animals after 30 min. A passage of this drug into the brain was also evaluated autoradiographically after i.v. injection of tritiated PYR 90 min after beginning of the experiment (100 µCi/animal). Whatever the group examined, no entry of PYR into the CNS could be detected. Exposure to an ambient temperature at 42.6 °C for 2 h resulted by itself in a partial inhibition of cerebral AChE activity. Our results, which agree with previous data obtained in humans exposed to heat stress, are opposite to the recent research showing a central passage of PYR in mice following a forced swim stress test. This demonstrated that the penetration of PYR into the brain of rodents under stress depends on the experimental conditions used (animal species, nature of the stressor, etc). Extrapolations to man, of results primarily obtained in rodents about central passage of a drug under stress, must thus be done very carefully.

58 Cytosolic phospholipase A2 (cPLA2) distribution in murine brain and functional studies indicate that cPLA2 does not participate in muscarinic receptor-mediated signaling in neurons

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Muscarinic receptors couple to a variety of signal transduction pathways including the activation of phospholipase A2 (PLA2) and subsequent release of arachidonic acid (reviewed in 1). The m2 and m4 receptor subtypes augment a previously stimulated arachidonic acid release while the m1, m3, and m5 receptors stimulate arachidonic acid release more directly. Arachidonic acid and some of its eicosanoid metabolites regulate neural function through modulation of ion channels, second messenger systems, gene expression, and neurotransmitter uptake and release. Relatively little is known about which PLA2 is involved in muscarinic receptor signaling or the physiologic consequences of the eicosanoid second messengers generated following muscarinic receptor stimulation.

The family of PLA2 enzymes are structurally diverse, yet all catalyze the hydrolysis of the acyl bond at the sn-2 position of membrane phospholipids to release arachidonic acid and lyso-lipids (reviewed in 2). These products are precursors of bioactive eicosanoids and platelet activating factor. Three distinct classes of PLA2 have been well characterized: a) the 14-kDa secreted PLA2 enzymes; b) the 80-kDa calcium-independent cytosolic PLA2 (iPLA2); and c) the 85-kDa calcium-dependent cytosolic PLA2 (cPLA2). The 85-kDa cPLA2 has been considered a potential effector in G protein-coupled receptor signaling for several reasons. cPLA2 is activated and translocates to cell membranes at submicromolar calcium concentrations achieved in the cytoplasm after stimulation of calcium mobilizing plasma membrane receptors. cPLA2 phosphorylation and presumably activation has been demonstrated for several ligands including ATP, thrombin, and bombesin. cPLA2 exhibits strong preference for deacylation of arachidonic acid over other fatty acids. These properties make cPLA2 a promising candidate as an effector involved in receptor-dependent signal transduction and neurotransmission. While ample evidence supports the role of cPLA2 in inflammatory processes, such direct evidence is lacking for the involvement cPLA2 in neurotransmitter function.

Muscarinic receptors couple to the release of arachidonic acid in several cell types including neurons (3) and fibroblasts (4), presumably through activation of an uncharacterized calcium-dependent phospholipase A2. The 85-kDa cPLA2 was recently implicated as an effector in muscarinic receptor-stimulated arachidonic acid release in 1321N1 astrocytoma cells (5). Therefore, we investigated the potential role of cPLA2 in muscarinic receptor-mediated signal transduction through expression studies in three different cell lines. U-373 MG cells were selected because they lacked cPLA2 and CHO and COS-1 cells because they express cPLA2 naturally at low levels therefore providing reasonable hosts for experiments designed to augment the endogenous levels of protein. PLA2 activity in extracts from CHO, U373, and COS cells transfected with cPLA2 cDNA were significantly increased over untransfected control cells. However, in none of the cells lines did the co-expression of muscarinic receptor and cPLA2 result in a significant increase in muscarinic receptor-mediated arachidonic acid release over cells expressing muscarinic receptor alone. These data suggest that muscarinic receptors do not couple through cPLA2 to release arachidonic acid.

Muscarinic receptor-dependent release of AA was previously shown in neurons in primary culture from various brain regions including spinal cord and hippocampus (3). The distribution of cPLA2 mRNA and cPLA2 immunoreactivity in murine brain were determined in order to investigate a potential role for cPLA2 in neurotransmission. cPLA2 mRNA was expressed in white matter, including cells contained within linear arrays characteristic of interfascicular oligodendrocytes. cPLA2 immunoreactivity in white matter was evident throughout the processes of fibrous astrocytes. cPLA2 expression in gray matter was confined to astrocytes at the pial surface of the brain. cPLA2 mRNA was detected in pia mater, both at the brain surface and inner core of the choroid plexus. The prevalence of cPLA2 expression in regions adjacent to fluid-containing compartments or vasculature suggests that cPLA2 may be involved in transport processes or in the maintenance of permeability barriers within the central nervous system. cPLA2 may not be directly linked to neurotransmission since cPLA2 mRNA and cPLA2 immunoreactivity were undetectable in neurons of murine brain. Support or regulation of neurotransmission may be provided through the activity of cPLA2 in glial cells.

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59 Multiparametric analysis of the trigger of soman induced epileptic seizures in rats. Correlation between enzymatic, neurochemical and electrophysiologic data

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Soman, an anticholinesterasic neurotoxic drug, induced epileptic seizure during severe intoxications. The neuropathological lesions are linked to the appearance of the seizures. But their trigger conditions remain still unknown and a great variability between animals is observed.

We have developed in the laboratory a new microdialysis technique allowing an 'in vivo' determination of cortical acetylcholinesterase activity in free moving rats. On the same rat, we have associated this measure with the determination of extracellular acetylcholine concentration by microdialysis and to the EEG recording and analysis.

We have follow these three sets of neurophysiological data during soman intoxication. We have measured in vivo the cortical acetylcholinesterase (AChE), we have followed in the cortex acetylcholine (ACh) level and we have recorded electro encephalographic (EEG) signal and analysed its power spectrum to extract energies especially in the gamma bands (30–40 Hz). We have correlated these data to distinguish three populations of rats, rats with no seizures and no spiking activity, rats with only spiking activity and rats with seizures.

We have determined the threshold where epileptic seizures occur after injection of soman. AChE inhibition must be over 70%, ACh extracellular level must be over 200-fold the baseline level. However, even with AChE inhibition over 70% and ACh increase over 200-fold, the animals which present an increase in gamma band over three-fold the baseline level did not start epileptic seizure. In the same conditions animals with no increase of gamma band started epileptic seizures with a delay from 10 to 49 min after soman injection.

This method offers a new way to develop medication against poisoning with anticholinesterasic neurotoxics. We have shown that the AChE inhibition and ACh release are necessary but not sufficient parameters to predict the appearance of epileptic seizure. After intoxication, a more integrated behaviour of the rat, like the response in gamma band, will at least decide if the animal will exhibit or not epileptic fit.

60 Cholinesterase (ChE) activity in normotensive rat (WKY) and spontaneous hypertensive rat (SHR)

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Intravenous (i.v.) or intracerebroventricular injection of cholinergic agents such as acetylcholine (ACh) and physostigmine (ChE inhibitor), can modify blood pressure in the rat (Krstic and Djurkovic, 1978; Brezenoff and Rusin, 1974). The injection i.v. of physostigmine induces a greater increase of the mean arterial pressure (MAP) in SHR when compared with WKY (Buccafusco and Spector, 1980). These results could be explained by different ChE and/or choline acetyltransferase (ChAT) activities. Actually, in the brain, specifically in the locus coeruleus, ACh concentration and the activity of ChAT, responsible for ACh synthesis, are increased in the SHR when compared with the WKY (Helke et al., 1980). Furthermore, in the SHR, the densities of the muscarinic receptors are markedly increased in the posterior hypothalamus (Hershkowitz et al., 1983) and decreased in the lung (De Michele et al., 1991). These results suggest that ACh may be involved in hypertension. The ACh regulation of blood pressure may also be due to the hydrolysis of this neurotransmitter by ChE, namely, acetylcholinesterase (AChE; EC 3.1.1.7) and butyrylcholinesterase (BChE; EC 3.1.1.8). The aim of this study was to determine AChE and BChE activities in plasma, erythrocytes, cerebral hemispheres, liver and lung preparations derived from WKY and SHR.

Male WKY and SHR (20 weeks old) were anaesthetised with pentobarbital sodium (60 mg/kg; intraperitoneal injection). The MAP measured with a Gould apparatus was 102 ± 9 mm Hg and 181 ± 6 mm Hg for WKY and SHR, respectively. After this measurement, blood samples were collected in heparinized vials and centrifugated at 3000 rpm/10 min/4 °C. Plasma was collected and erythrocytes were diluted (1/2 in water). Organs (cerebral hemispheres, liver, and lung) were removed. All samples were frozen (–80 °C).

The ChE activities were determined at room temperature using a microplate technique based on Ellman's colorimetric method (Ellman et al., 1961). Plasma and erythrocyte preparations were diluted with phosphate buffer pH 8 (1/54 and 1/540, respectively) whereas organs were pulverised and diluted (1/10) with phosphate buffer, pH 7.4. Tissue preparations were then homogenised by a polytron (400 W) at speed setting 7 (7 times 10 s). The lung and liver homogenates were diluted (1/54) for ChE activity measurement while cerebral hemisphere homogenates were diluted (AChE measurement: 1/162; BChE measurement: 1/54) with phosphate buffer, pH 8. To selectively inhibit either AChE or BChE activities, BW284c51 (1 µM) or iso-OMPA (10 µM) were used, respectively (Austin and Berry, 1953). Maximal velocity (Vmax) of ChE was measured for plasma and erythrocyte preparations with different concentrations of acetylthiocholine iodide (ACTI) whereas in the tissue preparations, ChE activities were measured in presence of a fixed concentration of ACTI (1 mM) *(table I)*.

A decrease of AChE activity (21% of total ChE activity calculated in WKY blood) was observed in blood derived from SHR. This decrease is principally due to a variation in erythrocyte AChE

Table I. ChE activities in WKY and SHR.

Samples	*AChE activity*		*BChE activity*	
	WKY	*SHR*	*WKY*	*SHR*
Plasma	132 ± 5	121 ± 2*	32 ± 1	54 ± 4*
Erythrocyte	1339 ± 127	1040 ± 74*	ND	ND
Liver	268 ± 17	233 ± 14	116 ± 4	126 ± 11
Lung	191 ± 22	293 ± 23*	304 ± 16	361 ± 12*
Cerebral hemisphere	3142 ± 76	2485 ± 66*	217 ± 13	239 ± 27

ChE activities are expressed as, U/L of plasma, U/L of erythrocyte, or mU/g of tissue. ND, not detected. *Data significantly different ($P < 0.05$) from corresponding values obtained in WKY. Values are means ± S.E.M., number of WKY used (6) and number of SHR used (4–15).

activity. An increase of blood BChE activity (1% of total ChE activity calculated in WKY blood) was observed in SHR.

AChE and BChE activities were increased (53% and 19%, respectively) in SHR lung when compared with WKY lung. In SHR cerebral hemispheres, a decrease of AChE activity (21%) was observed. No difference was determined in BChE and ChE activities in cerebral hemispheres and liver, respectively, when SHR and WKY were compared.

This study shows differences in ChE activities, in blood and lung, between SHR and WKY. In addition, the different brain cholinergic regulation of the systemic blood pressure, between SHR and WKY, may involve the AChE activity in cerebral hemispheres.

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61 Repetitive and spontaneous acetylcholine release from motor nerve terminals induced by a neurotoxin isolated from *Conus ermineus* venom

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Marine snails of the genus *Conus* are venomous animals classified according to their feeding behaviour into piscivorous, molluscivorous and vermivorous species (Kohn, 1983). Several peptide toxins have been isolated from the venom of fish-hunting cone snails: α-conotoxins active on nicotinic receptors (Myers and Cruz, 1993); μ-conotoxins which block voltage-dependant Na^+ channel in skeletal muscle (Cruz and Gray, 1986) and ω-conotoxins which interact with voltage-gated Ca^{2+} channels subtypes (Miljanich and Ramachandran, 1995). Each of these toxins potently block neuromuscular transmission in vertebrates producing flaccid muscle paralysis. Because of their specific actions on key elements of excitable cells and receptors, conotoxins have been widely used as tools for studying synaptic transmission mechanisms and for characterizing ion channels and receptor subtypes (for a recent review, see Olivera, 1997).

The observation that the venom of the fish-hunting cone snail *C. ermineus* elicits prominent muscle contraction in fish prompted us to purify the toxin(s) responsible for the observed action

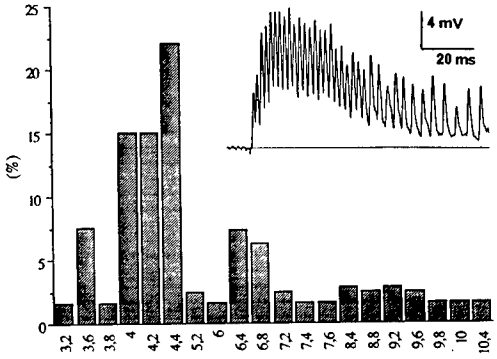


Figure 1. Interval distribution of repetitive EPPs. Recording made intracellularly at the junction fibers treated with δA-EVIA (200 nM) showed repetitive EPPs following a single nerve stimulus. The firing rate was initially at about 300 Hz and slowed with time (see insert). Measured intervals between the EPPs were averaged for 6 stimuli on the same fiber and plotted in the histogram.

of the venom and to characterize its mode of action on excitable membranes and neurotransmitter release from nerve terminals.

The present communication reports that *C. ermineus* venom contains a neuropeptide named δA-EVIA (M_r, 3300 D) which selectively increases synaptic efficacy at the vertebrate neuromuscular junction by increasing the number of synaptic responses evoked by a single nerve stimulus as well as the spontaneous quantal acetylcholine release from motor nerve terminals through a sodium dependent mechanism.

δA-EVIA, when applied to isolated frog nerve-muscle preparations (cutaneous pectoris) bathed in normal Ringer's solution causes a long-lasting spontaneous contraction of the muscle fibers which prevented stable intracellular recordings. In order to avoid muscle movements, excitation-contraction was uncoupled by formamide treatment (see del Castillo and Escalona de Motta, 1978). Intracellular recordings from formamide-treated junctions revealed that δA-EVIA (200 nM) induced repetitive action potentials in response to a single nerve stimulus. These action potentials were triggered by spontaneous and repetitive endplate potentials (EPPs) which could attain high frequencies *(figure 1)*. This repetitive synaptic activity had variable duration, could last tens of seconds and was not or little modified by increasing Mg^{2+} concentration in the extracellular medium or by addition of (+)-tubocurarine (3 μM).

Analysis of the quantal contents of EPPs, in preparations equilibrated with low Ca^{2+} (0.3 mM) and high Mg^{2+} (6 mM) Ringer's solution revealed that δA-EVIA (200 nM) did not modify the average number of quanta released during the first phasic EPP. Thus, it appears that the main effect of δA-EVIA is to alter the 1:1 input-output ratio of the neuromuscular junction. In other words, δA-EVIA induces a marked repetitive synchronous release of acetylcholine in response to a single nerve stimulus. Interestingly, the repetitive EPPs in a given train exhibited facilitation that could last tens of seconds.

In addition, δA-EVIA (200 nM) after a delay of about 40 min increased spontaneous quantal acetylcholine release, recorded as miniature endplate potential (MEPP) frequency, in a nominally Ca^{2+} free medium supplemented with EGTA (2 mM). Such an enhancement of MEPP frequency could be either prevented or reversed by the sodium channel blocker, tetrodotoxin (1 μM). These results indicate that the increase of MEPP frequency caused by δA-EVIA is related to sodium entry into motor nerve endings.

In conclusion, δA-EVIA isolated from *C. ermineus* venom constitutes a new excitotoxin that interacts with voltage-gated Na+ channels modifying nerve terminal excitability, neurotransmitter release and synaptic efficacy at the vertebrate neuromuscular junction.

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62 Developmental expression and localisation of acetylcholinesterase in rat muscles

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The localisation of acetylcholinesterase (AChE) is a key factor in the control of cholinergic transmission. It is determined by the AChE forms expressed by a cell: AChE exists under a multiplicity of forms generated by alternative splicing of a unique pre-mRNA (Massoulié et al., 1993). Two main types of subunits, T and H, contain the same catalytic domain but differ by their C-terminus. H subunits are glypiated and therefore anchored in cell membranes. T subunits may be associate with structural subunits such as a 20 kDa protein (G_4^a) or a specific collagen (ColQ) that anchor AChE respectively in membranes and in the basal lamina.

They may also be secreted as oligomeric forms or tethered by the T peptide in membranes. The regulation of alternative splicing and the availability of the associated proteins in a specific tissue determine the cellular targetting of the enzyme.

T mRNAs are the only mRNAs detected in adult brain and muscle whereas H mRNAs are the main transcripts detected in hematopoietic cells, together with variable amounts of T and R mRNAs. In R transcripts, the intron separating the last 3'constitutive exon from the H alternative exon is retained. This last transcript contains an in-frame stop codon and is at least partly polyadenylated. Its presence is generally associated with that of the H transcript. So far the protein has not been demonstrated in vivo although secreted monomeric AChE that could correspond to this form has been observed (Navaratnan et al., 1991). It remains that the R sequence could also be a regulator of splicing choices or simply leakage of splicing. At early stages of embryonic development (E13-E14), T, R and H transcripts are expressed in this decreasing quantitative order probably reflecting splicing choices in myoblasts and early differentiating myotubes. In fact, C2C12 myoblasts express H and T mRNAs whereas myotubes express mainly T transcripts together with a small proportion of R transcripts. The onset of innervation and the maturation of the synapse coincide with the progressive and exclusive expression of T mRNAs (Legay et al., 1995). However, this splicing choice could be dictated by the differentiation of the myotubes rather than by the presence of the nerve. C2C12 myotubes transiently transfected with a mini-gene containing the 3' alternative introns and exons produce only T subunits (Legay et al., in preparation).

The muscle synthetizes a collagen protein ColQ that interacts with the T peptide of AChE through its N-terminus and anchors the enzyme in the extracellular matrix (Krejci et al., 1997; Bon et al., 1997). Like the subunits of the acetylcholine receptor and other components associated with the cholinergic transmission, accumulation of both proteins are observed in slow and fast muscles at the neuromuscular junctions. Accumulation of the specific mRNAs is partly responsible for this phenomenon. T mRNAs are accumulated in all muscles under the synaptic contacts as soon as E14 (Legay et al., 1995) but although the ColQ gene is transcribed at that stage, ColQ mRNAs were not detected at specific zones. Synaptic accumulation of ColQ mRNAs could be visualized by this at a late post-natal stage of differentiation in the sternomastoid muscle, a fast muscle but not in slow muscles. These results suggest that the localisation of ColQ mRNAs depends on the pattern of muscle activity. RNAse protection assays corroborate these results in adult muscles showing that the level of ColQ mRNAs is equivalent in junctional and extrajunctional domains of soleus muscle whereas these mRNAs are only present in junctional areas of the sternomastoid muscle. However, in the soleus muscle, an increased density of the ColQ protein is detectable at the neuromuscular junction by immunohistochemistry. This suggests that, in this muscle, additional mechanisms ensure targetting and/or anchoring of ColQ. For example, if the ColQ protein associate with a target in the synaptic cleft, the localisation of ColQ would be linked to the distribution of the target, itself being subjected to the state of development and differentiation of the cell.

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63 Predicted secondary structure of a nicotinic acetylcholine receptor subunit. Incorporation of predicted solvent accessibility and experimental data into a two dimensional representation

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A refined prediction of the nicotinic acetylcholine receptor (nAChR) subunits' secondary structure was computed with third generation algorithms. The four selected programs, PHD, PREDATOR, DSC, NNSSP, based on different prediction approaches, were applied to each sequence of an alignment of nAChR and 5-HT3 receptor subunits, as well as of a larger alignment with additional related subunits sequences from glycine and GABA receptors. A consensus prediction was computed through a 'winner takes all' method. By integrating the probabilities obtained with PHD, DSC and NNSSP, this prediction was filtered in order to eliminate the singletons and to establish more precisely the structure limits (only 4% of residues were modified in this way). The final consensus secondary structure includes nine α-helices (24.2% of the residues, average length = 13.9) and 17 β-strands (22.5% of the residues, average length = 6.6). The large extracellular domain is predicted to be mainly composed of β-strands, with only two helices at the amino-terminal end. The transmembrane segments are predicted to be in a mixed α/β topology (with a predominance of α-helices), with no known equivalent in the current protein database. For the cytoplasmic domain only two amphipathic helices are predicted at both ends, with no periodic structures predicted for the remaining part (of variable length). The structured segments correspond to the more conserved regions, as defined by an analysis of sequence conservation per position performed on 152 superfamily members.

The solvent accessibility of each residue was also predicted from the multiple alignments with PHDacc. Each segment with more than three residues predicted exposed was considered to be external to the core protein. These data, along with a disulphide bond and a segment localised by electron diffraction, composed an envelop of structural constraints allowing to propose a partial folding of a nAChR subunit. Each subunit of the nAChR superfamily, polarised, possesses two different binding faces, oriented toward two other subunits within the oligomer. A 2D representation of the secondary structure is proposed which is fully compatible with experimentally identified residues known to belong to these two faces. In this representation, the segments of variable length are placed outside the core protein. This representation is not a tertiary structure and does not lead to prediction of any β- interaction. However, it provides a basic framework for further mutagenesis investigations, and for fold recognition (threading) searches.

64 Effect of continuous administration of pyridostigmine on the activity of functional acetylcholinesterase in guinea-pig muscle and brain.

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We have shown previously that repeated treatment in mice with the reversible anticholinesterase pyridostigmine caused increases in acetylcholinesterase (AChE) activity in skeletal muscle after the initial inhibition had worn off. Furthermore, pretreatment with pyridostigmine sensitised the muscle to a subsequent dose of the drug administered after the enzyme activity had returned to normal (Lintern et al. 1997a, b). In the present study the effect of continuous administration of pyridostigmine on the activity of AChE in muscle and brain, was studied in the guinea-pig. The drug was administered at a rate of 5.1 µg/h via an implanted osmotic pump. The pump was left in place for 6 days. Control animals received saline, administered in the same way. One group of animals was killed by cervical dislocation at 6 days, with the pump still in place, and other groups were killed at 4 h, 24 h, or 3, 7 or 14 days after removal of the pump. The activity of AChE was determined in the diaphragm and soleus muscles, and in the striatum and cerebellum.

The tissues were homogenised in high salt solution and the AChE molecular forms separated on a 5–20% sucrose density gradient (Haynes et al., 1984). The enzyme activity was determined by the method of Ellman (1961) modified for use with a plate reader. Major peaks of activity representing the G1, G4 and A12 forms were separated in muscle and major peaks representing the G1 and G4 forms were separated in the brain areas. The activity of each molecular form was determined by summing the activites of the fractions contributing to each peak.

Pyridostigmine administration for 6 days inhibited red cell AChE activity by 43 ± 2 (S.E.M.)% where n = 24. Figure 1 shows the results obtained for total and functional AChE activity in animals killed after 6 days of continuous administration of the drug (with the pump still in place). In both muscle types the total activity of AChE was significantly higher in the pyridostigmine-treated animals than in the saline treated controls. In general the activity of the predominant (G1, G4, and A12) molecular forms of the enzyme exhibited similar proportional increases. The effect of the drug on the functional A12 activity was particularly marked in the soleus muscles, causing an increase in activity of approximately 80%. After removal of the pump the changes in activity due to the effect of the drug exhibited a variable time course depending on muscle type.

In the striatum there was a slight increase in total, G1 and G4 activity compared to the saline-treated controls, although the changes were not significant. However in the cerebellum the total activity and the G1 and G4 activity was significantly reduced compared to the controls. After removal of the pump the changes in activity due to the effect of the drug exhibited a variable time course depending on the brain area being examined.

The biological half-life of pyridostigmine is approximately 50 min (Maxwell, 1995) but the homogenisation and separation procedures require approximately 24 h for completion. Therefore residual inhibition of the activity by the reversible inhibitor was probably negligible at the time the activity was determined. The decreases in AChE activity in the cerebellum were therefore probably due to down-regulation of the enzyme.

Pyridostigmine does not under normal circumstances cross the blood-brain barrier. However, effects of this drug on brain AChE activity have been reported after specific stress regimes (Friedman et al., 1996). It is not yet clear whether the changes in the enzyme activity reported here are a direct action of the drug, as a consequence of changes in the permeability of the blood brain barrier, or secondary to some peripheral action of the inhibitor. This work has been carried out with the support of CBD Porton Down. (c) British Crown copyright 1998/DERA. Published with permission of the Controller of Her Britannic Majesty's Stationary Office.

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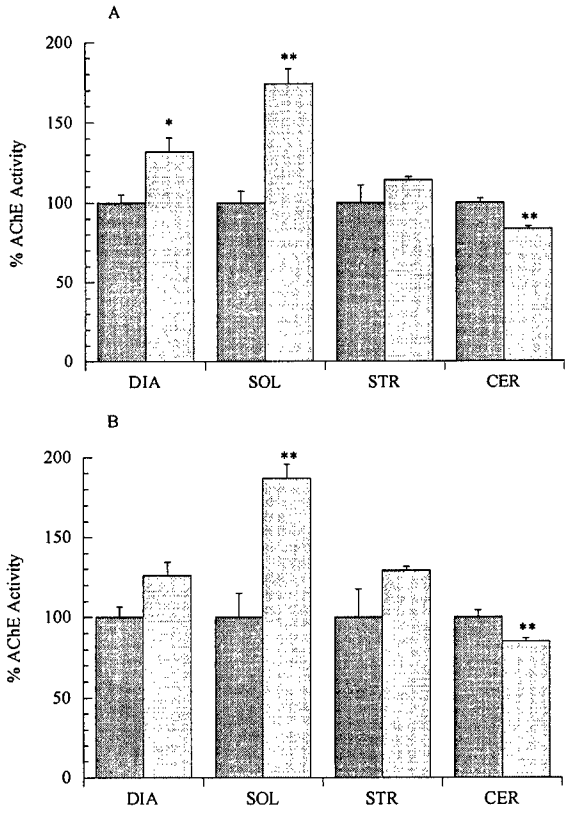


Figure 1. Activity of AChE after 6 days continuous administration of pyridostigmine in diaphragm (DIA) and soleus (SOL) muscles, and striatum (STR) and cerebellum (CER). **A**. Total activity. **B**. Functional activity (A12 in muscle and G4 in brain areas). Dark columns, saline-treated; light columns, pyridostigmine-treated. The values are means ± S.E.M. for four animals in each case. * $P < 0.05$ compared to saline-treated controls (for raw data).

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65 Bone morphogenetic protein-9 modulates acetylcholine content and choline acetyltransferase and vesicular acetylcholine transporter gene expression in the SN56 murine septal cell line

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Bone morphogenetic proteins (BMP) belong to the TGF-β family of cytokines that signal through receptor serine kinases. Their functions in the developing and adult nervous system are poorly understood, but include neurolation, apoptosis, neuronal and glial differentiation, cell survival, and possibly regulation of neurotransmitter phenotype (Ebendal et al., 1998; Mehler et al., 1997). We studied the effects of BMP-9 on acetylcholine (ACh) content and on choline acetyltransferase (ChAT) and vesicular acetylcholine transporter (VAChT) mRNA levels of SN56 cells.

SN56 cells were grown in DME medium (containing 4.5 g/L of glucose and 110 mg/L sodium pyruvate) supplemented with 10% fetal bovine serum in the presence or absence of human recombinant BMP-9 for varying time periods. One hour prior to cell collection, the cells were incubated at 37 °C in a physiological salt solution containing 50 μM neostigmine and 5 μM choline. The cells were washed once to remove extracellular choline and collected in methanol. Water-soluble choline-containing compounds were extracted and ACh content was measured by HPLC coupled to postcolumn enzymatic derivatization and electrochemical detection.

To determine ChAT and VAChT mRNA levels, total RNA from SN56 cells was extracted using the guanidinium thiocyanate-phenol/chloroform method (Chomczynski and Sacchi, 1987). Northern blot analysis of equalized RNA samples was performed with VAChT and ChAT cDNA probes as previously described (Berse et al., 1995).

Incubation of SN56 cells with BMP-9 (10 ng/mL) for varying periods of time caused initial increases in ACh content (by 45% and 60% after 12 and 24 h, respectively). However, by 48 h BMP-9 caused a reduction in the cellular ACh content (by 40%) which remained stable for up to 72 h *(figure 1)*. Consistent with this time-course, in cells treated for 48 h with varying concentrations of BMP-9, ACh content declined by as much as 60% in a concentration-dependent, saturable fashion with an EC50 for BMP-9 of 1.3 ng/mL.

Messenger RNA levels for ChAT and VAChT were reduced by 41% and 50%, respectively, 48 h after treatment with BMP-9. No significant changes were observed in the mRNA levels for these two cholinergic markers following shorter incubation periods with BMP-9 (i.e., at 12 and 24 h).

These data show that initial exposure of SN56 cells to BMP-9 results in increased ACh content, followed by a reduction in the levels of this neurotransmitter. This later reduction may be a consequence of down-regulation of VAChT and ChAT gene expression, since our results also show reduced levels of the mRNAs coding these two proteins. Whether this effect is entirely mediated by a direct BMP-9 signaling pathway through specific SMAD proteins, or results from the concomitant activation of signaling pathways involving other factors, remains to be determined.

These findings provide further evidence that neuronal cells respond to bone morphogenetic proteins, and suggest that BMP-9 can directly or indirectly affect the expression of the cholinergic phenotype. Supported by a National Institute on Aging grant AG09525.

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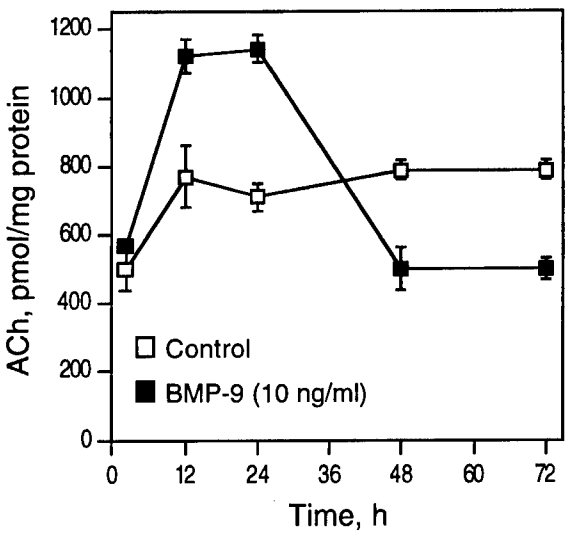


Figure 1. Time-course of the effect of BMP-9 on ACh content in SN56 cells. ACh content was measured by HPLC. Data are reported as means ± S.E.M.

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66 Acceleration of oxime-induced reactivation of acetylcholinesterase-organophosphate conjugate and ^{31}P NMR detection of phosporyl oxime from the conjugate

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The acute toxicity of organophosphorous compounds (OP) is usually attributed to the irreversible inhibition of AChE by these compounds. Reactivation of the OP-inhibited AChE by oximes is the primary reason for their effectiveness in the treatment of OP poisoning. Reactivation by oxime is accelerated by quaternary ligands such as decamethonium and SAD 128, which are devoid of nucleophilicity (1). We have shown that among the three different quaternary ligands examined, only edrophonium and decamethonium can accelerate the oxime-induced reactivation of the enzyme conjugate (2). The peripheral site ligand propidium slowed the reactivation process with all oximes tested. Determination of the kinetic reactivation rate constants with and without the accelerating ligands showed that in the presence of 50 μM edrophonium, 3.3- to 12.0-fold accelerations were obtained with 2-PAM, TMB4 and toxogonin as reactivators, and 1.6- to 3.0-fold enhancements were observed in the presence of 200 μM decamethonium with these oximes. However, the reactivation by HI-6 was not accelerated by either of the two ligands. Examination of edrophonium acceleration of oxime-induced reactivation with high (12.5 nM) and low (1.25 nM) enzyme:OP concaeration of the reactivation with 12.5 nM enzyme:OP was significantly lower than that with 1.25 nM enzyme:OP, indicating that acceleration may involve protection of the reactivated enzyme by the added ligand from re-inhibition by the putative product, the phosphoryl oxime (POX).

Acceleration of reactivation of DEPQ [7-(O,O-diethyl-phosphinyloxy)-1-methyl-quinulinium methylsulfate]-inhibited FBS and wild-type mouse AChE by edrophonium was the same with AChEs from these two sources. However, with DEPQ-inhibited AChE, only reactivations by toxogonin and TMB4 were accelerated by edrophonium. Reactivations by HI-6, 2-PAM and MMB4 were not significantly affected in the presence of edrophonium. Comparison of the initial reactivation rate constants and overall reactivation rate constants of DEPQ-inhibited wild-type mouse AChE by these five oximes supported the hypothesis that edrophonium prevents the re-inhibition of the reactivated enzyme by POX. If the POX is produced by reaction of oxime with the enzyme:OP and the re-inhibition of the reactivated enzyme is caused by the POX, the initial reactivation rate should be significantly faster than the overall reactivation rate, since re-inhibition occurs only after the initial stage when a sufficient amount of POX is accumulated in the reactivation medium. The initial reactivation rate constants of DEPQ-inhibited wild-type mouse AChE were 5.4- and 4.2-fold higher than those of the overall rate constants with toxogonin and TMB4. On the other hand, very little difference was observed between the initial and overall rate constants with the other three oximes. Approximately 2.5-fold increase in the reactivation rate constants with toxogonin and TMB4 was observed in the presence of 10 μM edrophonium. No effect was observed with the other three oximes. When initial and overall reactivation rate constants of wild-type mouse AChE inhibited by nerve agent sarin were compared, it was found that the reductions of the overall rate constants compared to the initial rate constants were greater than those observed with DEPQ-inhibited AChE with most of the oximes. With toxogonin and TMB4, 23.3- and 19.5-fold reductions of the overall rate constant were observed. Approximately 3-fold decreases in the overall rate constants were observed with MMB4 and 2-PAM. The initial and overall rate constants were essentially the same when HI-6 was used. Accelerations by edrophonium were more pronounced for the reactivation of sarin-inhibited AChE than DEPQ-inhibited AChE with most of the oximes. In the presence of 10 μM edrophonium, the overall reactivation rate constants of sarin-inhibited AChE by toxogonin and TMB4 were increased by the greatest amount, 4.7- and 4.3-fold, respectively. The increase was moderate with MMB4 and 2-PAM, 1.8- and 1.4-fold, respectively, and there was no change with HI-6. The results of experiments with sarin indicate that POX re-inhibition was more pronounced for the reactivation of sarin-inhibited AChE than DEPQ-inhibited AChE.

Recently it was shown that aspartate 74, a residue near the entrance of the active center gorge of AChE, is the primary determinant in governing specificity of the enzyme to cationic OP (3). The mutant D74N displayed 2–3 orders of magnitude decrease in the inhibition activity of cationic OPs. POXs are analogues of those cationic OPs and should demonstrate similar properties. Thus for this specific mutant, edrophonium-induced acceleration will not be evident if the acceleration is produced by preventing POX re-inhibition of the reactivated enzyme during reactivation. When mutant D74N was employed, no significant edrophonium-induced acceleration could be observed in reactivation of DEPQ-inhibited AChE by toxogonin and TMB4, and the time course of reactivation fitted to a mono-exponential phase association kinetic model. The data from mutant enzyme substantiated the involvement of edrophonium in protecting POX re-inhibition of reactivated enzyme formed during the reactivation of OP-inhibited AChE.

It was recently also shown that OP hydrolase can effectively hydrolyze quaternary ammonium-containing OPs (4). It is possible that POXs might be also hydrolyzed by it since they share common structural features. If the putative POXs formed during reactivation are hydrolyzed by OPH, reactivation of OP-AChE

conjugate may be accelerated by the presence of OP hydrolase. Parallel studies with edrophonium and rabbit serum OP hydrolase showed this to be the case. Reactivation of DEPQ- or sarin-inhibited AChE was accelerated in the presence of either edrophonium or OPH.

POXs are putative products during the reactivation of certain OP-AChE conjugates by oxime reactivators. No direct evidence had been obtained for the existence of POXs in the reactivation media, but they were indicated by kinetic studies to be present in some cases (5, 6). Several chemically synthesized POXs have been tested recently and shown to have a greater ability to inhibit AChE or BChE compared with the parent OPs (7). Therefore, isolation and identification of the POXs from the reactivation media is of importance. ^{31}P NMR measurements of the mixture of MEPQ and oximes showed that the POX between MEPQ and HI-6 may not exist for any significant length of time due to the extreme unstability of this POX in solution. However, POXs between MEPQ and toxogonin or TMB4 were found to be stable enough for ^{31}P NMR detection at pH 4.0. To isolate POX directly from the reactivation media of MEPQ-inhibited AChE, approximately 0.2 μmol of FBS AChE was absorbed to a small procainamide Sepharose 4B affinity gel-column to create an enzyme conjugate-reactivated enzyme cycling situation. Reactivation media contained 0.5 mM reversible inhibitor, edrophonium, and 1 mM oxime to keep the newly-formed POX from inhibiting the reactivated enzyme. After concentrating the reactivation media 100-fold, POXs formed during reactivation of MEPQ-inhibited AChE by toxogonin and TMB4, but not by HI-6, were confirmed for the first time by ^{31}P NMR.

In conclusion, results from this study using different AChEs and a variety of OP and oxime compounds confirmed that acceleration of oxime-induced reactivation of OP-inhibited AChE by some quaternary ligands, such as edrophonium and decamethonium, results from the prevention of POX re-inhibition of reactivated enzyme. OP hydrolase can hydrolyze the POX and also accelerate oxime-induced reactivation. POX re-inhibition during reactivation largely depends on the structure of the oximes and the OPs used. With some oximes like HI-6 and its analogues, POX re-inhibition will not occur either with phosphorylated AChE (DEPQ-inhibited AChE) or with phosphonylated AChE (MEPQ-, and sarin-inhibited AChE) due to the extremely poor stability of the POXs in solution. With 2-PAM, POX re-inhibition is found only with phosphonylated AChE. POXs formed by toxogonin and TMB4 with phosphonylated AChE are the most stable and can be isolated from the reactivation media. POX re-inhibitions are the most pronounced with toxogonin and TMB4 as reactivators during the reactivation of both the phosphorylated and phosphonylated AChE. Caution should be exercised in using toxogonin and TMB4 for treatment of poisoning with large dose of OP pesticide, since these two oximes may form POXs by direct reaction with free OP in vivo and produce more severe inhibition of AChE than the parent OP.

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67 Complementary binding studies between α-neurotoxin and the nicotinic acetylcholine receptor

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The nicotinic acetylcholine receptor (nAChR), which is composed of four homologous subunits with composition $\alpha 2\beta\delta\gamma$, binds agonist and competitive antagonists at the boundary between the $\alpha\gamma$ and $\alpha\delta$ interfaces. Recently Tsigelny et al.[1] have developed and refined a sequence homology model of the extracellular domains of individual subunits in nAChR. This model highlights three distinct regions of sequence in the α-subunit and four regions in the δ and γ subunits on the face opposing the α-subunit that contribute to ligand binding and specificity.

Conserved determinants in domain III of the α-subunit that influence affinity for α-toxin have previously been identified in our laboratory[2]. Binding sites between snake venom toxin *Naja mossambica mossambica* (Nmml), expressed from recombinant DNA bacterial systems, and mouse muscle nAChR, expressed transiently from transfected cDNAs on the surface of human embryonic kidney cells, were determined by utilizing site-directed mutagenesis and measuring equilibrium dissociation constants (K_d values) by competition against initial rates of ^{125}I-α-bungarotoxin[3]. The binding assays were conducted in the presence and absence of α-conotoxin MI, which shows significant site-selectivity (104 difference in K_d)[4], for the $\alpha\delta$ binding site over the $\alpha\gamma$ site. These α-subunit determinants include: 1) a conserved tyrosine (Y190) believed to contribute to stabilization of positive charges on the toxin; 2) a negative charge (D200) believed to affect binding at the $\alpha\gamma$ interface but not at the $\alpha\delta$ interface; and 3) residues (V188) and (P197), which when mutated to charged re-

sidues large decreases in affinity result presumably because of charge repulsion. The resulting decreases in K_d values varied from no change to 40-fold at the αδ site and 20- to 500-fold at the αγ site.

The three fingered α-neurotoxin Nmml shares ~60% residue identity with erabutoxin a for which the X-ray crystal structure has been solved[5]. Modification of cationic side chains K27E and R33E on loop II and K47A on loop III resulted in 1 to 2 orders of magnitude shift at the αδ-site and 1 to 4 orders of magnitude shift at the αγ-site[2]. Analysis of these combinations of mutations in both the receptor and the α-toxin suggest that key cationic side chains on the toxin bind in close proximity to this segment encompassing residues 180–200 of the receptor[6]. In addition, these studies have confirmed the ligand affinity distinctions between the αδ and αγ sites and have also suggested that the α-toxin interacts at a multipoint attachment with both α-subunit and δ/γ-subunit determinants.

This presentation will concentrate on results from continued studies involving the correlation of kinetic data for combinations of mutations in both the receptor and the α-toxin. The research focuses on the binding effects of point mutations on the α-subunit and on the γ and δ subunits defined based on the homology model. Analysis of complementary binding sites will further highlight the role of cation-τ and long range Coulombic interactions in binding the toxin as well as further characterize the orientation of the disulfide loops of the α-toxin with respect to the αγ and αδ interfaces on the nAChR.

NmmI mutations K47E and R36E were constructed from mutagenic oligonucleotides by subcloning cDNAs into the PEZZ18 expression vector. Introduction of a charge difference at positions 47 shows site selectivity between the αδ and αγ sites whereas K47A showed identical binding at the two sites. The mutation R36E, located on loop II and ~ 7 Å from R33, shows two orders of magnitude shift at the αδ-site and four orders of magnitude shift at the αγ-site.

Double mutant binding assays involving R36E and K47E and the α-subunit residues outlined above have been conducted. Thermodynamic mutant cycle analyses for the mutant pairs have assessed the effect of receptor mutations on the direct binding to the α-toxin versus conformation changes in the receptor or the ligand. Coupling energies were calculated from the difference in free energy of binding caused by the single mutation relative to its wild-type. The coupling energies for the mutant pair R36E–V188D showed simple additivity of free energy for the single mutations, which suggests that the residues are not linked or interacting. Whereas a decrease in affinity of 2.6 and > 1.5 kcal/mol at the αδ and αγ sites, respectively, was observed for the R33E–V188D mutant pair6. The R36E–V188K double mutant did show, however, an increase in affinity of ~3.0 kcal/mol at the αγ site, which is 1.5 kcal/mol greater increase in affinity than that observed for R33E–V188K6. These initial results suggest that interaction between positions 36 on the toxin and position 188 on the receptor is not a simple case of coulombic attraction and repulsion. This presentation will further analyze binding energetics for the pairwise interactions involving R36E and K47E with the α-subunit mutations at positions 188, 190, 197, and 200 as well as with δ- and δ-subunit mutations such as the pairs γSer111/δTyr113 and γPhe172/δIle178 located amino and carboxy-terminal to the conserved disulfide loop in the receptor subunits.

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68 Effect of differentiation on electrically-evoked transmitter release from NG108-15 cells loaded with exogenous acetylcholine

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NG108-15 cells synthesize acetylcholine (ACh) and form functional cholinergic synapses after cell differentiation by dibutyryl cyclic AMP (dbcAMP) (Nirenberg et al., 1983; Zhong et al., 1995). DbcAMP however has no effect on ACh release from NG108-15 cells preloaded with ACh and evoked by the calcium ionophore A23187 (Israël et al., 1994).

This lack of effect could be due to the fact that the calcium ionophore bypasses the activation of voltage activated calcium channels. Indeed, calcium channel expression in NG108-15 cells is modulated by cell differentiation: non-differentiated cells have only rapidly inactivating T-type channels, whereas slowly or non-inactivating N-and L-type channels appear after cell differentiation. (Eckert et al., 1990; Kasai and Neher, 1992).

The aim of this work was to compare ACh release from differentiated and non-differentiated NG108-15 cells preloaded with ACh, in a situation where release was evoked by an electrical stimulation. Under these conditions calcium influx should occur through the existing voltage-activated calcium channels. Thus, if calcium channels were implicated one would expect less release from non-differentiated cells.

Cell differentiation was obtained by treatment with dbcAMP or with prostaglandine (PGE1) and theophylline. Under our experimental conditions both treatments greatly enhance the expression of N-and L-type channels in NG108-15, the second treatment being more efficient.

For release expperiments, all cells where incubated with 40 mM ACh, in the presence of a cholinesterase inhibitor, for 24 h before the experiments. *Xenopus laevis* myocytes were used as ACh detector cells (Dan et al., 1994; Falk-Vairant et al., 1996;). NG108-15 cells were removed from their culture dishes, tranfer-

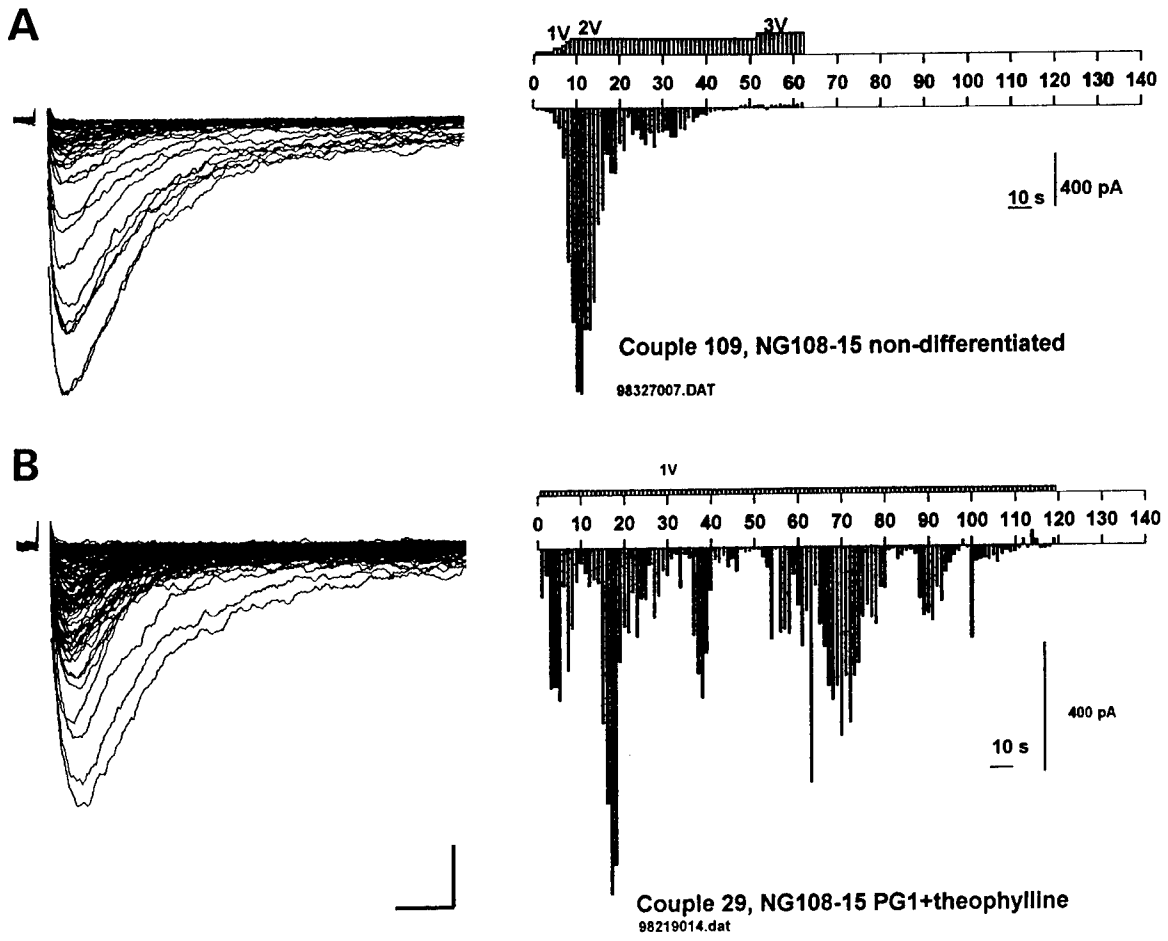


Figure 1. Electrically evoked ACh release by NG108-15 cells detected by *Xenopus* myocytes. **A**. Non-differentiated NG108-15. **B**. NG108-15 treated with prostaglandine PGE1 (10 µM) and theophylline (1 mM) for 3 days. Left column: superimposed responses of the myocyte during a train of stimulations (1–3 V, 2 ms, 0.4 Hz) Scale: vertical 500 pA (top), 250 pA (bottom), Horizontal 10 ms. Right column: Amplitude of individual responses shown on the left column, graphed in chronological order. Voltage applied to the stimulation pipette is indicated on the top. All cells were loaded with exogenous Ach. Please note the different scales in **A** and **B**.

red into the recording dish and placed in the vicinity of a myocyte, with the help of a suction electrode that was also used to stimulate the cell. Myocytes membrane currents were recorded under voltage clamp in whole cell configuration.

ACh-loaded non-differentiated NG108-15 cells where found to release ACh upon electrical stimulation, and several evoked responses could be observed from the same cell *(figure 1)*. When external calcium was replaced by 10 mM Mg^{2+} (in the presence of EGTA), release was no longer observed (not shown).

DbcAMP or PGE1 + theophylline treatments affected neither the amplitude nor the duration of electrically evoked release; these treatments, however, increase the total number of responses observed with a given cell *(figure 1)*.

The present results demonstrate that non-differentiated cells loaded with ACh are able to release it under electrical stimulation as they were under calcium ionophore challenge. Thus, calcium sources other than voltage-activated calcium channels must be involved. M. Malo was supported by an AFM fellowship.

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69 Tetrameric assembly and peripheral site-occluding loop of mouse acetylcholinesterase

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In mammals, the molecular forms of the cholinesterases are primary determinants of their tissue distribution and disposition within a cell; association of subunits also governs the turnover of the enzyme. The predominant form of acetylcholinesterase (AChE) in the central nervous system is an amphiphilic tetramer anchored to the membrane via a hydrophobic, non-catalytic subunit, whereas at the neuromuscular junction it is an asymmetric form containing one to three tetramers and associated with the basal lamina via a collagen-like subunit [1]. Abnormal associations of AChE arise in dementias of the Alzheimer type, where a significant and selective loss of the amphiphilic AChE tetramers is observed [2]. However, there is currently little structural information about the molecular determinants involved in association of subunits into tetramers and of tetramers with membrane-anchor subunits.

The crystal structure of a recombinant AChE from mouse (mAChE) in a complex with the peptidic inhibitor fasciculin (Fas2) provided the first template for the mammalian cholinesterases [3]. Slight differences in conformation were observed in the structure of Fas2-associated mAChE, compared with uncomplexed *Torpedo* californica AChE [4]. However, the limitations from comparing two different enzyme species may have precluded distinction of the species-related differences from the changes in conformation brought about by fasciculin binding. In addition, currently available crystallographic analyses of AChE reveal structures in which the entrance of the active site gorge is either occluded by a symmetry-related molecule or perfectly sealed by the inhibitor fasciculin [3–7], therefore precluding structural analysis of the peripheral anionic site region in its free state. A crystal structure of mAChE in the absence of bound fasciculin might therefore yield a conformational state resembling more closely that of the active enzyme in solution.

The crystal structure of mAChE was solved by molecular replacement using the mAChE coordinates extracted from the 3.2 Å resolution structure of the Fas2-mAChE complex as a search model; it was refined to 2.9 Å resolution with a R-factor and a R-free value of 21.5% and 24.5%, respectively, in the 15 Å to 2.9 Å resolution range.

This structure provides a significantly improved accuracy in the positions of the main and side chains of the mAChE molecule than the Fas2-mAChE structure, confirms some distinctive features of the mouse enzyme compared with *Torpedo* AChE, and permits direct comparison of a free and an occluded peripheral anionic site within the same crystal unit. Most importantly, this structure highlights certain determinants at the surface of mAChE that could participate in formation of oligomers upon assembly in normal and pathological states, allosteric modulation of catalysis, or surface interactions involved in non-cholinergic AChE functioning. This work was supported by USPHS and DAMD grants to P.T., a NSF-CNRS collaborative project to P.T. and P.M., the AFM to P.M. and P.E.B., and the European Union to Y.B. and P.M.

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70 Structural discrimination between the two agonist binding sites of the *Torpedo* nicotinic acetylcholine receptor using time resolved fluorescence

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Muscle type nicotinic acetylcholine receptors (nAChR) are heteropentamers $\alpha 2\beta\gamma\delta$. They carry two binding sites for acetylcholine and competitive effectors at the α/γ and α/δ subunit interfaces. To investigate for structural differences between the two binding sites, we used time resolved fluorescence (TRF).

TRF consists of exciting a fluorescent probe during an extremely short time, and measuring the time required by this molecule to reemit light (nanosecond time range). The re-emission speed depends on both the nature and chemical environment of the fluorescent probe. In the present work, we used the single photon counting method[1-3], which establishes the re-emission probability of a photon versus time with very high accuracy. Mathematical analysis was performed using the maximum entropy method[4,5] to give the fluorescence lifetime distribution, each lifetime putatively representing a particular state of the probe.

We studied the interactions between the fluorescent probe dansyl-C6-choline[6] (Dns-C6-Cho, excitation at 330 nm and emission at 520 nm) and the nAChR from *Torpedo marmorata*, solubilized in CHAPS. The lifetime distribution of free Dns-C6-Cho in buffer containing CHAPS is quasi monoexponential with a lifetime of 3.7 ns. In contrast, the distribution of Dns-C6-Cho in the presence of the receptor is multi-exponential, with lifetimes of 0.25, 1.5, 5 and 18 ns (mean lifetime of 9 ns). Preincubation of the receptor with α-bungarotoxin results in a distribution corresponding to free Dns-C6-Cho, showing that the signal is specific for the competitive agonist binding sites.

To detect putative differences in binding affinity and fluorescence fingerprint of the two binding sites, we measured the fluorescence decay at various occupation ratios of receptor by Dns-C6-Cho. No significant differences were found from 20 to 100% occupation of the binding sites, indicating that Dns-C6-Cho displays similar binding affinities for the two sites or that the microenvironment of Dns-C6-Cho in both sites is similar.

In order to discrimate between these two hypotheses, we used the α-conotoxin MI[7,8], which has a 20-fold higher affinity for the α/γ agonist binding site as compared to the α/δ binding site on our receptor preparation. We studied the fluorescence decay of bound Dns-C6-Cho at various occupation ratios of the α-conotoxin MI on the receptor. Preliminary experiments show different distributions at low and high occupation ratios, indicating that Dns-C6-Cho displays different fluorescent distributions depending on the binding sites. These data suggest that Dns-C6-Cho remains in different microenvironments when bound to the two sites, possibly due to the contributions of the γ and δ subunits.

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71 Is nicotine sharing common molecular and anatomical substrates with cocaine?

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Nicotine and cocaine are both psychostimulant drugs which activate dopamine (DA) neurons in the ventral mesencephalon. These neurons project massively to the striatal complex, the dorsal part (or striatum) which is involved in movement co-ordination and the ventral part (or nucleus accumbens, NAc) which is part of the limbic system. To investigate possible common molecular targets between these two drugs we have examined the effects produced by repeated injections (3 times a day during 15 days) of either nicotine or cocaine on peptide expression in the striatal complex and by a single or repeated injections on immediate early gene expression in various cerebral regions.

In the dorsal striatum, cocaine increased mRNAs encoding for substance P (PPT-A) and dynorphin (PPDYN) whereas nicotine did not change the levels of these mRNAs. The mRNA encoding for preproenkephalin (PPE), remained unchanged with both treatments. Similarly, in the ventral striatum (or NAc), nicotine did not affect the peptide mRNA expression except in the shell where it produced a decrease of PPDYN mRNA. In contrast, cocaine treatment induced increases of PPT-A mRNA in the core and PPDYN and PPE mRNAs in the rostral pole of the NAc. Thus in the striatal complex, peptides which are expressed by the great majority of neurons did appear to be common substrates for these

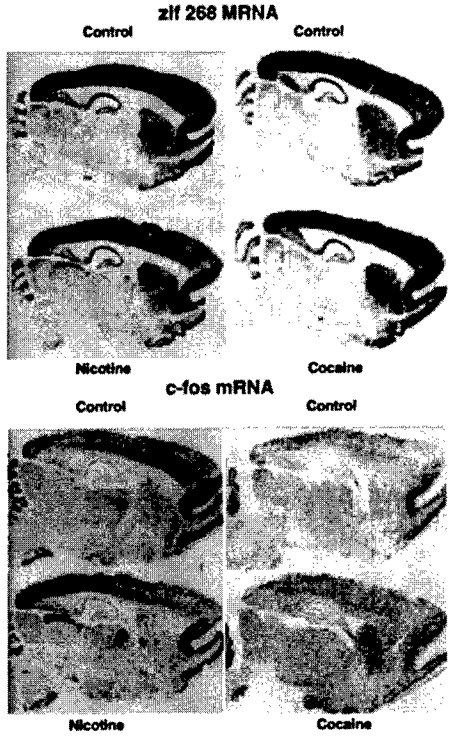


Figure 1. Rats were treated with nicotine (0.4 mg/kg, s.c.) or cocaine (12 mg/kg, i.p.) and killed 40 min later. Brains were cut sagitally and sections were processed for zif 268 and c-fos mRNA using 35S riboprobes. It can be noted that nicotine mainly induced zif 268 and c-fos mainly in the superior colliculus and associated structures as well as some thalamic nucleus (in particular the antero-ventral nucleus and the septum. In contrast, cocaine produced an induction of zif 268 and c-Fos mRNA mainly in the striatal complex.

two drugs; their expression was preferentially changed by cocaine (Mathieu-Kia et al., 1998).

The induction of immediate early genes (c-fos and zif 268) which can be considered as markers of changes in neuronal activity was also examined. Forty minutes after an acute injection of nicotine (0.4 mg/kg, s.c.) c-fos and zif 268 mRNA were increased in visual, visuo-motor and retino-limbic structures as well as in amygdala and septum but not in the dorsal striatum. In contrast, c-fos and zif 268 mRNAs were markedly increased but only in the striatal complex following an acute cocaine injection (12 mg/kg, i.p.) (see *figure 1*).

The c-Fos protein analysed 90 min after an acute (0.4 mg/kg) and the last injection of a chronic nicotine (0.4 mg/kg, 3 times a day for 15 days, s.c.) administration showed a pattern of induction similar to that found with c-fos mRNA analysis and in addition pointed to the involvement of various cortical areas. The acute versus chronic treatment comparison indicated a maintenance of c-Fos inducibility and in some structures a larger recruitment of c-Fos positive neurons after chronic nicotine injections (Mathieu-Kia et al., 1998). The persistence of a c-Fos induction was generally not observed after a chronic cocaine treatment which instead generates chronic fos related antigens (Chen et al., 1995; Nye et al., 1997).

While the striatal complex appears as a preferential target for cocaine, visual, retino-limbic and limbic areas are preferential targets for nicotine indicating that nicotine and cocaine share few common anatomical substrates.

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72 Artificial toxins to explore new receptors?

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Toxins from animals, plants and microorganisms are essential tools to discover and study receptors, and to investigate complex physiological pathways. Not surprisingly, therefore, numerous studies have been undertaken to understand how toxins work. As a result, some rules describing major properties of animal toxic proteins are now emerging which may serve as guidelines for future drug design. This presentation will essentially focus on snake toxins that block acetylcholine receptors.

Curaremimetic toxins from elapid and hydrophiid snakes are usually classified as short-chain toxins (60–62 residues and four disulphide bonds) and long-chain toxins (66 residues and 4/5 disulphide bonds). They all block acetylcholine receptors with high affinity and adopt a similar architecture, called the 'three-fingered' fold, which consists of three adjacent loops rich in β-pleated sheet protruding from a small globular core where four disulfide bonds are conserved. Using site-directed mutagenesis, we identified the functional residues by which a short-chain toxin binds to acetylcholine receptor from *Torpedo marmorata*. The site includes at least ten residues spread on the three loops. Approximately half of them, mostly located on the second and third loops, are highly conserved in both short-chain and long-chain toxins whereas the others, mostly located on the first loop are clearly variable. These observations suggest that the general propensity of curaremimetic toxins to bind to acetylcholine receptors may be associated with both a conserved functional core which may provide toxins with a minimal affinity for most receptor subtypes and with additional variable residues which may provide individual toxins with high affinity toward various receptor subtypes.

In this respect, we found it striking that only long-chain curaremimetic toxins, such as α-bungarotoxin or α-cobratoxin, possess five additional residues cyclized by a disulphide bond, located at the tip of the second loop. This additional small loop is unnecessary for a curaremimetic toxin to bind with high affinity to Torpedo acetylcholine receptor. Recently, however, we discovered a specific function associated with this intriguing loop. Thus, it was observed that long-chain toxins have the capacity to bind to the α7 neuronal receptor with high affinities ranging from 1 to 10 nM, whereas short-chain toxins bind to this receptor with much lower affinities ranging between 2 and 22 μM. To demonstrate that the small extra loop of long-chain toxins is directly associated with their high affinities for α7-type receptors, we synthesized three categories of derivatized curaremimetic toxins. First, we reduced selectively the additional disulphide bond of a long-chain toxin and modified its resulting free cysteines by specific reagents. The derivatized toxin displayed a considerably reduced affinity for α7 receptor. Second, we replaced the two extra half-cystines of the long-chain a-cobratoxin and α-bungarotoxin by serine residues and observed that their affinities for α7 decreased 25- and 35-fold, respectively. Third, we introduced an extra disulfide bond into a short-chain toxin having low affinity (K_d = 2 μM) for α7 and found that the resulting chimeric short-chain toxin displayed a 20-fold higher affinity for this receptor. Since all these derivatized toxins for the *Torpedo* receptor possess virtually unchanged affinities as compared to their parent toxins, we conclude that the extra loop is a key element, probably not the only one, that is responsible for the capacity of curaremimetic toxins to discriminate between muscular and neuronal receptors. This finding may open new perspectives to engineer artificial toxins with appropriate affinities for new acetylcholine receptor subtypes.

73 Neuronal nicotinic acetylcholine receptors: The importance of the host cell type in heterologous expression studies

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Several lines of evidence have led us to conclude that the folding, subunit assembly, intracellular distribution and functional properties of neuronal nicotinic acetylcholine receptors (nAChRs) can be influenced by the choice of host cell type used for heterologous expression studies.

We have found that the folding, assembly and cell-surface expression of the homo-oligomeric neuronal nAChR α7 and α8 subunits are strongly dependent upon the host cell type (Cooper and Millar, 1997; Cooper and Millar, 1998), an observation which has been supported by recent studies by other groups (Blumenthal et al., 1997; Kassner and Berg, 1997; Chen et al., 1998). Our evidence for subunit misfolding is based, in particular, upon the absence of binding of nicotinic radioligands, such as α-bungarotoxin, and conformation-sensitive monoclonal antibodies. We have now extended these studies by examining the distribution of the α7 subunit by fluorescent confocal microscopy in micro-injected cultured primary neuronal cells and polarized epithelial cells. These new studies have further strengthened our belief that the folding and efficient cell surface expression of this protein is critically dependent upon the host cell type.

Achimeric α7 $^{(V201)}$/5HT$_3$ subunit (which contains the N-terminal domain of α7 and C-terminal domain of the serotonin receptor 5-HT$_3$ subunit; Eiselé et al., 1993) is expressed very efficiently in cell lines in which α7 is misfolded (Cooper and Millar, 1998). Whereas the α7 (and α8) subunit appears to be misfolded when expressed in several cell types, the α7 $^{(V201)}$/5HT$_3$ (and α8 $^{(I201)}$/5HT$_3$) chimera is expressed efficiently on the surface of all cell types we have examined.

We believe that the cell-specific differences in the folding and transport of α7 (and α8) are not due simply to a requirement for these subunits to co-assemble with other nAChR subunits which may be expressed endogenously in cells such as primary SCG neurons or established cell lines such as SH-SY5Y and PC-12. This conclusion is supported by the observation that functional homo-oligomeric nAChRs are formed when α7 is expressed in several non-neuronal cell types, including the *Xenopus* oocyte (Couturier et al., 1990), rat pituitary GH4C1 cells (Quik et al., 1997), and some (Gopalakrishnan et al., 1995; Ragozzino et al., 1997), but not in other (Cooper and Millar, 1997), isolates of human kidney cells.

While the dramatic cell-specific differences in folding described above may be restricted to homo-oligomeric type nAChRs such as α7 and α8, we have also found recently that the functional properties of hetero-oligomeric recombinant neuronal nAChRs composed of α3β4 subunits differ in both single channel conductance and relative agonist potency when expressed in either *Xenopus* oocytes or a mammalian cell line (Lewis et al., 1997). Our data indicate that the functional properties of recombinant channels expressed in cultured mammalian cell lines resemble more closely native nAChR channels (e.g., those expressed in the rat superior cervical ganglion) than do recombinant channels expressed in *Xenopus* oocytes. Although it is not yet clear what is responsible for either these host cell-dependent differences in ion channel properties or the cell-specific folding of the α7 subunit (described above and in Cooper and Millar, 1997; Cooper and Millar, 1998), these findings help to illustrate the critical importance of the host cell environment.

We have also shown recently that the folding of Drosophila nAChRs is temperature-sensitive (Lansdell et al., 1997), as has been reported previously with the Torpedo nAChR (Paulson and Claudio, 1990). In contrast, we have shown that several Drosophila G-protein-coupled receptors, such as a muscarinic AChR (Millar et al., 1995), a dopamine receptor (Han et al., 1996)and an octopamine receptor (Han et al., 1998) form functional receptors when expressed in either mammalian cells (at 37 °C) or in a Drosophila cell line (at 25 °C). The functional expression of Drosophila GABA-gated ion channels in a Drosophila cell line (Millar et al., 1994; Buckingham et al., 1996) together with the absence of reports of the successful expression of this or other invertebrate ligand-gated channels in mammalian cell lines suggest that the temperature-sensitive folding seen with *Drosophila* nAChRs may reflect a phenomenon which is common to other ligand-gated ion channels.

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74 Probing the structure of the ligand binding site on the muscle nicotinic receptor with Waglerin peptides

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Waglerin-1 (Wtx-1) is a 22 amino acid peptide isolated from the venom of Wagler's Pit Viper, *Trimeresurus wagleri*. It acts via blockade of the nicotinic receptor at the adult mouse neuromuscular junction (nAchR); however, neonatal mice are resistant to the lethal effects of the toxin. In ^{125}I-α-bungarotoxin (^{125}I-α-Bgtx) competition experiments performed on HEK cells transfected with cDNAs for the adult form of the receptor (α, β, δ, and ε subunits), biphasic competition curves are produced with a K_D at the α-ε interface of 12.3 nM and at the α-δ interface of 21.3 μM. In experiments performed on the embryonic form of the receptor (α, β, δ, and γ subunits), a single phase curve with a K_D of 22.0 μM is produced, indicating that the Waglerin-1 is over 1700-fold selective for the α-ε interface over the α-δ or α-γ interfaces of the muscle nicotinic receptor. A series of chimeric and point-mutated γ and ε subunits were constructed in order to find the regions of the receptor responsible for the observed difference in affinity between the two sites and to determine which residues of the receptor were making a specific interaction with the toxin. Initial work was done on γ-ε chimeras in which the N-terminus from the γ-subunit was spliced to the C-terminus of the ε-subunit at a given junctional residue. In this series of experiments, the γ74ε chimera produced a 45-fold reduction in affinity, and the γ103ε chimera produced over 1000-fold change in affinity nearly to that found in the wild-type γ-subunit. These two experiments would indicate that there is an affinity determinant in the N-terminal 74 residues as well as between residues 74 and 103. No further decrease in affinity occurred in the γ165ε, γ173ε or γ177ε chimeras. Further smaller chimeras focusing on the region around residue 59 were constructed since this area has been found to be important for interactions with other ligands. Residues 59 and 61 of the ε-subunit, when mutated to the corresponding residues in γ, caused a reduction in affinity comparable to that found for the γ74ε chimera, with the individual point mutants contributing equally and additively to the affinity reduction. However, mutations at these residues in γ and δ to those found in ε did not reproduce a corresponding increase in affinity. The region between 74 and 103 has not yet been investigated.

In addition to the chimeras, experiments were performed on point mutants previously found to be important for determining the selectivity of the peptide toxin α-conotoxin MI. Of the three conotoxin determinants at residues 34, 111 and 173 (ε-subunit numbering) mutating residue Asp173 to either the Phe found in γ or the Ile found in δ caused a 500-fold and 200-fold reductions in affinity, respectively, nearly to that found in wild-type γ and δ subunits. Mutating the γ and δ subunits at the position corresponding to residue 173 to Asp caused only an 18-fold increase in affinity in δ and a four-fold increase in γ, similar to the results of the 59 and 61 position mutants. These data together indicate that residues 59, 61 and 173 are all necessary for high-affinity interaction of Waglerin-1 with the α-ε interface of the receptor, but not sufficient for high-affinity binding in general. Other residues that have not yet been found may be necessary for permitting Waglerin-1 to interact in the high-affinity orientation at the α-γ and α-δ interfaces.

Rats are also resistant to the in vivo effects of Waglerin-1. Although this could arise from a variety of factors, we sought to determine if the differences in the primary sequences of the rat receptor subunits versus the mouse subunits were mediating this difference in in vivo effect. Initial experiments on HEK-293 cells transfected with nAchR subunit cDNAs cloned from rat indicated that the adult rat receptors have an 84-fold lower affinity at the high-affinity α-ε site compared to mouse, and a ~four-fold lower affinity at the α-γ and α-δ sites. This 84-fold lower affinity was linked to the rat ε-subunit, since HEK transfected with mouse α, β and δ subunits and the rat ε subunit gave the same K_D as found at the α-ε site of HEKs transfected with all four subunits from rat. There are 10 residues in the extracellular domain of the rat ε subunit that are different from mouse. Of these 10, the entire 84-fold difference in affinity can be linked to residues 59 (Asp in mouse, Glu in rat) and 115 (Tyr in mouse, Ser in rat). Changing both residues in the ε subunit of either species changes the affinity to that found in the wild-type subunit of the other species. When residue 59 of the mouse ε is changed from an Asp to an Asn, there is no change in affinity. However, changing to a Gln causes a decrease in affinity comparable to changing to a Glu, indicating that the length of the sidechain, but not the negative charge, is of primary importance for mediating the interaction of position 59 of the ε-subunit with Wtx-1. At residue 115, changing the Tyr in the mouse ε to a Phe caused no change in affinity, whereas changing the rat residue from a Ser to a Phe caused the same increase in affinity as changing to Tyr. This indicates that Waglerin-1 is having a specific interaction with the aromatic group at this position.

We have also been studying the effects on affinity that occur when specific changes in the toxin are made. The wild-type sequence of Waglerin-1 has the following sequence:

NH_2—G-G-K-P-D-L-R-P-C-H-P-P-C-H-Y-I-P-R-P-K-P-R—COOH

There is an intramolecular disulfide between the two cysteines. Several experiments were aimed at delineating the minimum sequence of peptide required to retain the affinity and site-selectivity of the toxin. Omitting the N-terminal four residues or the C-terminal two residues produced less than a 3.5-fold change in affinity at both sites with little change in selectivity. Omitting the C-terminal three residues caused a five-fold decrease in affinity at the low affinity α-γ and α-δ sites only. Omitting both the N-terminal and C-terminal residues ([4–18]Wtx-1) caused a 5–6-fold decrease in affinity at both sites. [7–20]Wtx-1 caused a 17-fold decrease in affinity at the high affinity site only, and [7–15]Wtx-1 gave a 713-fold decrease in affinity at the α-ε site, making the two sites indistinguishable. Thus, residues mediating binding at the high affinity α-ε site reside on both arms of the toxin, in residues 5 or 6 and residues 16, 17 or 18.

Additional changes in the toxin were made by mutating the histidines at positions 10 and 14 of the full length toxin. Changing residue 10 to a Tyr or Arg increased affinity at the α-δ site by 12.3- and 6.7-fold, respectively. Changing His-14 to a Phe caused an 8.7-fold reduction in affinity, again only at the low affinity α-δ site. All of the data taken together indicate that Waglerin-1 may interact with the receptor in a different orientation at the α-ε site compared to the α-δ or α-γ site. The enhanced affinity at the α-ε site may depend primarily on interactions from the N- and C-terminal arms of the toxin, whereas the interaction with the α-γ and α-δ sites may be conferred primarily through residues found around the disulfide loop region.

75 In vitro evidence of peroxynitrite-dependent nitration of tyrosines in presynaptic proteins: Choline acetyltransferase is a sensitive target of ONOO-, and immunodetection of nitrotyrosine coincides with inhibition of the enzyme activity

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NO has recently been proposed as a second messenger modulating cholinergic neurotransmission. in mammalian brain[1], *Aplysia* ganglion[2], smooth and skeletal muscle[3,4] through prejunctional or retrograde actions. In order to study such a function at the presynaptic level, we showed previously[5] on a model of a peripheral cholinergic synapse, the nerve endings isolated from the electric organ of *Torpedo* marmorata, that NO and its derivative peroxynitrite (ONOO–) affect differently the release, the synthesis and the compartmentation of acetylcholine in synaptosomes. In the present study, we analysed further why ONOO- abolishes in

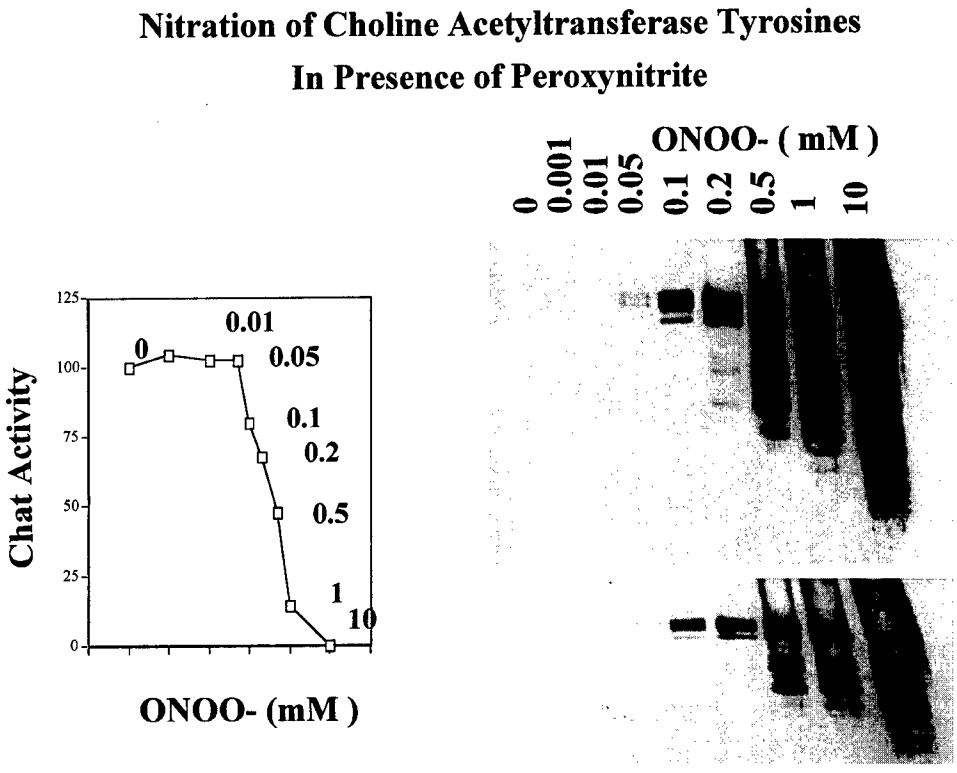


Figure 1. Left part. Dose-dependent effect of ONOO- on choline acetyltransferase activity. Choline acetyltransferase activity was measured[38] after 4 h preincubation time of the drug peroxynitrite (at the concentrations presented on the graph) applied directly on the enzyme. Samples were mixed with detergent (1% Triton X-100) 1 min before addition of 250 µM eserine, 0.05% bovine serum albumin and, the substrates (1-^{14}C)-acetyl-CoA (14 µM), 2.5 mM choline, and 75 mM. Results of abscissa are mean of duplicate of Chat activity expressed as % of controls and are from a representative experiment in ordinate a logarithmic scale of ONOO- concentrations was used. Right part. Immunolabeling detection of nitrotyrosine on bovine brain choline acetyltransferase. Using monoclonal anti-nitrotyrosine (UBI 1/1000), the immunoreactivities of nitrotyrosine were detected by ECL-peroxidase amplifier system after Western blotting. The two parts of the figures are photographs of the same blot after two times of exposure.

a concentration-dependent manner, the synthesis of acetylcholine and the choline acetyltransferase activity of synaptosomes. With the discovery of peroxynitrite-mediated oxidation of compounds and proteins[6, 7], our present effort was to highlight the effect of ONOO- (as a function of increasing drug concentrations) on the tyrosine of neuronal proteins and in particular of choline acetyltransferase (Chat). We looked for a covalent modification of tyrosine which converts it into nitrotyrosine[8,9]. We tried to link the changes in the protein with the enzymatic activity[10].

Results

Effect of ONOO– on bovine brain choline acetyltransferase activity (Chat)

Similarly to the previous experiments performed with *Torpedo* nerve endings[5], showing a blockade of the activity of choline acetyltransferase on aliquots of synaptosomal samples treated by peroxynitrite, we investigated the effect of ONOO– on a purified, and commercial source of bovine brain choline acetyltransferase. We show (*figure 1*, left part) that Chat activity is reduced under ONOO–. Inhibition starts in presence of 50 µM ONOO–. Half inhibition was obtained after 500 µM ONOO-, and complete inhibition was between 1 to 10 mM ONOO-.

Nitrotyrosine immunoreactivity detection in presynaptic proteins

Using a monoclonal anti-nitrotyrosine antibody, we then searched for immunoreactivity of proteins present in *Torpedo* synaptomes (incubated with 1 mM ONOO-, 4 h) after separation by SDS gel electrophoresis and transfer on nitrocellulose. With this technique of detection for nitration of tyrosines in the proteins, we observed an immunoreactivity obtained with monoclonal anti-nitrotyrosine antibody (1/1000^{e}) and we noted that only a few presynaptic proteins seem to be sensitive to peroxynitrite (not shown). Among these, an immunolabeling being detected at 67–70 kDa, the protein choline acetyltransferase could be one of them.

To support this, we decided to do an in vitro treatment (with increasing concentrations of ONOO-) of purified bovine choline acetyltransferase from commercial source. Immunodetection of nitrotyrosine is clearly obtained after exposure to ONOO-. Results obtained are presented in the right parts of *figure 1*, and correspond to photographs of a typical ECL-treated Western blot obtained after two different time expositions. Nitrotyrosine is obtained after treatment with the concentration of 50 µM ONOO-.The number of labelled bands increased progressively with higher concentrations of ONOO-, and a proteolysis developed further. We noted that some bands disappear at 10 mM ONOO-. Incubation of the blot with a polyclonal antibody against choline acetyltransferase led to a Chat immunoreactivity in presence of low concentrations of ONOO- (up to 500 µM). A loss of Chat immunoreactivity was obtained at the highest ONOO- concentrations.

We also investigated the peroxynitrite-dependent nitration of choline acetyltransferase purified from non-nervous origin (human placenta) and for other proteins: acetylcholinesterase from various sources, bovine erythrocyte Cu/Zn superoxyde dismutase and cyclophilin A, an ubiquitous peptidyl prolyl cis/trans isomerase, abundant in neurons. In general, we find that ONOO- (tested from 50 µM to 10 mM) induces less immunolabelling of nitrotyrosine than for bovine brain choline acetyltransferase. Different rates of nitration of tyrosines are obtained depending on the enzyme, the source and the peroxynitrite concentrations. For placental choline acetyltransferase, electric eel acetylcholine esterase and bovine thymus cyclophilin A, immunoreactivity of nitrotyrosine was evident only at 10 mM ONOO-.

The present results support the notion that peroxynitrite is, in vitro, a powerful modulator of cholinergic metabolism, leading to the total loss of choline acetyltransferase activity and to a proteolysis of the protein. Further studies are in progress to detail at the level of the protein Chat itself if other residues such as cysteine, and methionine are also involved in the effect of ONOO-. If such an biotransformation occurs in vivo when NO and O_2^- are generated in excess and simultaneously, the covalent change of the choline acetyltransferase protein should be important in pathologies due to a degenerescence of the cholinergic system, such as Alzheimer disease or amyotrophic lateral sclerosis in which a role for NO (and/or ONOO-) was previously proposed.

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76 Photoaffinity labeling with [^{3}H]DDF support the existence of two quaternary ammonium binding domains in human butyrylcholinesterase

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Two types of cholinesterases (ChE) can be distinguished in vertebrates: acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE). These very homologous enzymes rapidly hydrolyze the neurotransmitter acetylcholine but show distinct substrate and inhibitor selectivity. X-ray structures and affinity labeling of AChE as site-directed mutagenesis of both enzymes have shown that aromatic amino acids lining the active-site gorge of ChEs are behind this different specificity. Particularly, human BuChE lacks two important aromatic residues, Phe330 and Trp279 respectively belonging to the active site and the peripheral site of Torpedo AChE. As these two residues were photolabeled by *p*-(N,N-dimethylamino)benzenediazonium (DDF) (1), we used this probe to study the quaternary ammonium binding domains of BuChE.

Purified human BuChE was covalently photolabeled by [^{3}H]DDF. The photoincorporation of the probe could be fully prevented by cholinergic inhibitors such as tacrine. Following deglycosylation, alkylated BuChE was trypsinolyzed and digests were analyzed by LC/ES-MS. A comparison of tryptic fragments from labeled and unlabeled BuChE allowed to identify the tryptic peptide Y61-K103 as carrying the probe. A two-step purification procedure using respectively cation exchange chromatography and reversed phase HPLC led to highly purified labeled peptides. Microsequencing allowed to attribute radioactive signal to positions corresponding to Trp82 and Tyr332.

We propose that these labeling results reflect the occupancy of two distinct quaternary ammonium binding sites of BuChE. The photolabeling of Trp82 demonstrates that this residue shares the same feature as the homologous residue Trp84 in *Torpedo* AChE by contributing to the binding of cationic ligand through cation-p interactions. The labeling of Tyr332 supports the existence of a second quaternary ammonium binding domain in BuChE. According to the BuChE 3D-model (2) and mutagenesis results (3), we suggest that Tyr332 and Asp70 define a peripheral site of BuChE. However, the location and probably the function of this peripheral site are different from these of the AChE peripheral site.

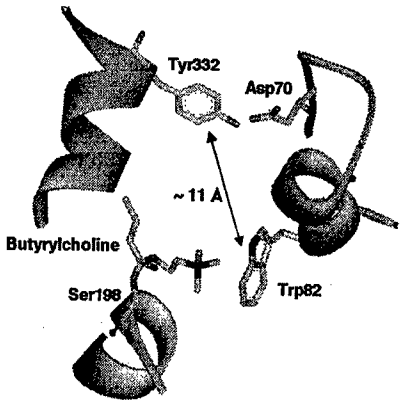


Figure 1. 3D-model of the active-site gorge of BuChE according to Harel and Sussman (2).

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77 Characterization of the 5'-flanking region of the mouse choline acetyltransferase gene in transgenic mice

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In order to identify a cholinergic tissue-specific promoter, we have analyzed the expression pattern of chimeric gene constructs consisting of 5'-sequences derived from the mouse ChAT gene and the bacterial β-galactosidase (LacZ) gene, in transgenic mice (Naciff et al., 1997, 1998). The analysis of transgenic mice bearing the β-galactosidase gene under the control of a 6417 bp segment from the 5'-untranslated region of the mouse ChAT gene (from -6371 to +46; +1 being the translation initiation site for ChAT), showed that this DNA fragment directed correct tissue-specific expression of the reporter gene to cholinergic cells. Within this 6.4 kb DNA fragment lies an uninterrupted open reading frame, in the same transcriptional orientation as ChAT, that encodes the mouse vesicular acetylcholine (ACh) transporter (VAChT). This transporter is responsible for the translocation of cytoplasmic ACh into synaptic vesicles (Song et al., 1997). Northern blot and reverse transcription-polymerase chain reaction analysis of total RNA showed that the mouse VAChT gene is transcribed into a single mRNA isoform of ~3.0 kb, and it is expressed in spinal

cord, brain and brain stem, tissues rich in cholinergic neurons, but not in peripheral tissues such as heart, liver and kidney. The 6.4 kb DNA fragment targeted LacZ expression only to cholinergic neurons located within the cholinergic system of the CNS of transgenic mice, and also promoted the overexpression of the VAChT gene (Naciff et al., 1997, 1998). The overexpression of VAChT in transgenic mice did not result in any detectable modification of motor behavior, general behavior, reproductive capacity, or life span, which suggests that the cholinergic system of those animals with elevated VAChT levels function as normally as in the wild type littermates.

We have expressed the VAChT gene contained within the 6.4 kb fragment of the 5'-flanking region of the mouse ChAT gene in transiently transfected HeLa and Cos-7 cells. These cells normally do not express VAChT or any other cholinergic marker. The immunolocalization of VAChT in transfected HeLa cells *(figure 1A)*, determined using a polyclonal antibody raised against the rat VAChT (Chemicon International Inc., Temecula, CA, USA), shows that the expressed VAChT is associated with vesicles located in the perinuclear region. These results demonstrate that the expressed transporter is inserted correctly in intracellular organelles delimited by membranes, where if coupled to a proton gradient could be functional. When the 6.4 kb segment from the 5'-untranslated VChAT gene was used as spacer between a CMV promoter and a nuclear targeted synthetic concatamer gene that functionally neutralizes calmodulin (CaM; Wang et al., 1995) in the nuclei of cultured transfected cells *(figure 1B)*, it did not interfere with the expression of this CaM inhibitor peptide.

Sequence analysis of the 6.4 kb DNA fragment from -6371 to +46, of the mouse ChAT gene (Misawa et al., 1992; Naciff et al., 1997, 1998), indicates that within the open reading frame of the VAChT gene, there are multiple potential regulatory sequences that, acting individually or cooperatively, are required to direct specific gene expression to cholinergic neurons. However, since the overexpression of the rat VAChT in developing *Xenopus* spinal neurons resulted in a considerable enhancement of ACh packaging at the developing synapses (Song et al., 1997), there is the possibility of modifications of the efficacy of synaptic transmission occurring in transgenic animals over-expressing the VAChT, and this issue has to be addressed.

The use of the 6.4 kb DNA segment from the 5'-flanking region of the mouse ChAT gene, to specifically target the expression of different gene products to the cholinergic system, may require the inactivation of the VAChT gene product, in order to eliminate possible undesired effects due to the over-expression of the VAChT. Eliminating the expression of extra VAChT can be accomplish by engineering a stop codon at the beginning of the coding sequence for VAChT in the 6.4 kb fragment.

The identification of this cholinergic-specific promoter will be useful to target the expression of different gene products to cholinergic neurons, to understand the mechanisms of diseases characterized by malfunction of cholinergic neurons, such as Alzheimer's disease or amyotrophic lateral sclerosis, and will be valuable in the design of strategies directed to treat those neurological disorders.

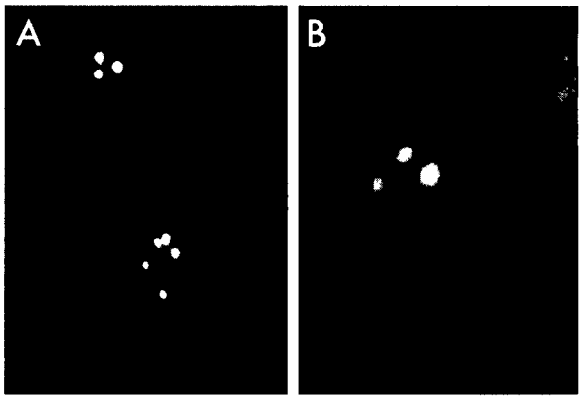


Figure 1. Immunolocalization of VAChT and CaM-inhibitor peptide in transfected HeLa cells. HeLa cells, which do not express VAChT or any other cholinergic marker, were transiently transfected with a chimeric gene construct consisting of a CMV promoter, the 6.4 kb DNA segment from the 5'-flanking region of the mouse ChAT gene containing the mouse VAChT gene, and a nuclear targeted synthetic concatamer gene that functionally neutralizes calmodulin (CaM). After 24 h of transfection, cells were fixed with para-formaldehyde, permeabilized with acetone, and incubated with goat polyclonal anti-rat VAChT (**A**) or rabbit anti-CaM inhibitor peptide (**B**) antibody, followed by fluorescein-conjugated secondary antibody.

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78 Involvement of protein kinases and protein phosphatases in the regulation of acetylcholine receptor gene expression

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Synapse formation at the neuromuscular junction (NMJ) proceeds through the enhancement of the synthesis of the acetylcholine receptor (AChR) at the NMJ and the repression of the synthesis of the extra-synaptic AChRs (outside the NMJ) by electrical activity (rev. in Changeux et al., 1990).

Protein kinases (Klarsfeld et al., 1989; Huang et al., 1992; Chahine et al., 1993) have been proposed to be involved in the repression process.

Myogenic transcription factors, which are essential for differentiation of the muscle lineage (rev. in Weintraub et al., 1991), bind to E boxes present in the AChR alpha-subunit promoter and activate transcription of the AChR gene (Piette et al., 1990).

The implication of serine/threonine protein kinases in the repression process, most probably via phosphorylation of the muscle transcription factor myogenin, has been demonstrated in chick cultures of myotubes. Myogenin (Wright et al., 1989) is phosphorylated in electrically active myotube fibers while at least partially dephosphorylated in electrically inactive (TTX-treated) myotubes (Mendelzon et al., 1994).

We further demonstrate the implication of the protein phosphatase metabolic pathway in the process of repression of the AChR gene expression. Inhibitors of protein phosphatases induce a decrease in the number of AChRs at the surface of chick myotubes and prevent the upregulation of surface AChRs induced by TTX. This repression is reversible and is not associated with a dedifferentiation of the myotubes.

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79 Complementation of a yeast choline transport mutant by *Torpedo* electric lobe cDNAs

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In 1992, the potassium transporter of *Arabidopsis thaliana* was cloned using an elegant paradigm based on heterologous complementation in a yeast mutant[1]. Since we also knew that the choline transporter of Saccharomyces cerevisiae had been cloned by homologous complementation[2], we decided to see whether it was possible to clone the neuronal choline transporter using a similar approach. To do so, it was first necessary to establish several prerequisite conditions.

The first task was to obtain a yeast strain compatible with a yeast expression vector and a system of selection for the transformants capable of choline uptake. This was done by crossing the choline transport mutant used for homologous complementation, D308-14D (α *ctr ise leu2 his4*, donated by Pr. S. Yamashita) with an a *Δura4* strain donated along with the yeast expression vector pFL61 by Dr. F. Lacroute. The offspring were then screened for a ctr ise *Δura3* phenotype that would allow the selection of transformants in the absence of uracil at a low concentration of myoinositol and the further selection of transformants capable of growth dependent on the uptake of extracellular choline at a high concentration of myoinositol[2]. The dependence of growth on choline uptake as a function of the myoinositol concentration is regulated by the *ise* mutation; yeasts that revert to the *ise*$^+$ phenotype grow well in high myoinositol whether or not choline is present in the medium. The locus of this mutation has not yet been characterized.

The resulting strain (S208) is not very robust and most methods of transformation gave few if any tranformants. Satisfactory transformation frequencies were finally achieved by using the LiAc/SS-DNA/PEG procedure[3].

The last prerequisite was a yeast expression library likely to contain clones coding for the neuronal choline transporter. The *Torpedo* electric lobe was chosen as a source of mRNAs since this tissue is highly specialized for presynaptic cholinergic function and has already proven to be a good source of cholinergic clones[4]. The cDNAs were prepared and inserted into pFL61.

The experiment could now begin. In order to screen the library which originally represented 400 000 different cDNAs, up to 500 000 transformants were tested for growth under selective conditions. After elimination of *ise*$^+$ revertants, about 100 clones were retained. A few of these transformed yeast were able to accumulate choline at a higher rate than the others. For these candidates, the plasmids were isolated and partially sequenced. While some of these clones could not be identified, others, such as enkephalin convertase, AKAP, p145, or cytochrome oxidase, were clearly un-

related to choline metabolism. These yeast were undoubtedly *ctr*$^+$ revertants, and as such needed to be eliminated.

The second phase of screening consisted of transforming individual aliquots of yeast with the plasmids isolated from the original positive colonies. Each batch of transformants was examined for choline-dependent growth under selective conditions. None of the clones were capable of strong complementation or supression of the *ctr* mutation. The strongest phenotype observed (about 50% of the transformants grew in selective medium) was associated with inorganic pyrophosphatase (clone 1.13) which intervenes in the incorporation of choline into phospholipids, and may be influencing choline-dependent growth in this way.

Taking into account the possibility that functional expression of a vertebrate transmembrane protein may not be optimal in the yeast, clones which had at least a weak phenotype (10–25% growth under selective conditions) were tested for choline uptake. This time large pools of tranformants could be used since each transformation was done with an isolated plasmid. The results of such an experiment are shown in *figure 1*. Of the 24 clones tested, only clone 4.17 conferred a notable increase in choline uptake on the transformed yeast. Furthermore, choline uptake by 4.17-transformed yeast was inhibited by 10 µM hemicholinium-3. Addition of 100 mM NaCl under hyperosmotic conditions tended to reduce choline uptake for all the clones tested and this was also seen with 4.17, although the HC-3 sensitive component was still apparent.

The sequence of clone 4.17 indicates a truncated ORF. The resulting C-terminal portion of the encoded protein has four hydrophobic regions likely to form transmembrane domains. Significant homology was found with the sequence of an unidentified *C. elegans* protein with 9–10 TMDs. This protein in turn shows a loose homology to various transporters.

The isolation of the full length clone will be necessary to further characterize the sequence and function of the expressed vertebrate protein. Studies on the distribution of the mRNA and

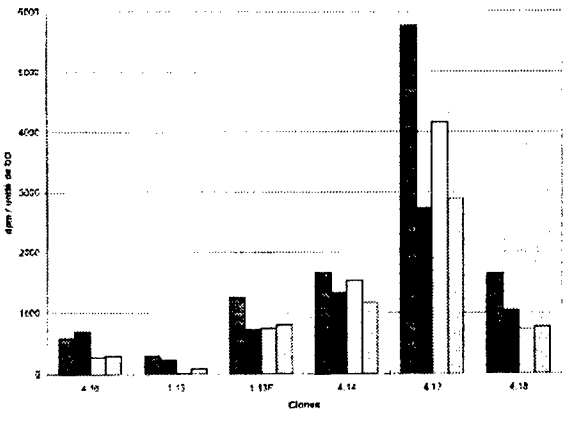


Figure 1.

of the native protein will also be needed to know if we are really on the tracks of the elusive neuronal choline transporter. We are greatly indebted to Maurice Israël for patiently allowing this story to unfold. We thank the Association Française contre les Myopathies for their financial support.

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80 Binding orientation of the α-neurotoxins with the nicotinic acetylcholine receptor

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α-Neurotoxins, members of three fingered toxin family, may be classified into short neurotoxins (60-62 residues and four disulfides bonds; i.e., erabutoxin, Nmm neurotoxin I) and long neurotoxins (66–74 residues and five disulfides bonds; i.e., cobratoxin, α-bungarotoxin). Both specifically bind to the muscle type nicotinic acetylcholine receptor (nAChR) and for the past decades these have been the critical tool used to purify initially and then characterize the nicotinic receptor. Several other toxins, notably fasciculin and the muscarinic receptor toxins which interact with cholinergic proteins, fall into this general family. In contrast to a acetylcholine esterase-fasciculin complex whose crystal structure has been solved [1], little is known about the orientation and precise receptor residues involved in the α-neurotoxin/nicotinic receptor interaction. Studies for a short neurotoxin, erabutoxin b, revealed that the tips of three loops are responsible for the nAChR binding [2]. Our study for Nmm neurotoxin I (NmmI) also found three residues at the tips of three loops, K27, R33, K47, at the proximity to the binding sites [3]. Mutagenesis, labeling studies, and homology modeling to structurally known proteins [4] predict that the N-terminal extracellular 200 amino acids, especially three regions from α-subunit and four regions from non three-subunits, form the surface at the subunit interface to which various ligands bind. By focusing on amino acid sequence differences between the neuronal and muscle type, and anionic residues at α-subunit of nAChR [5], we uncovered contributions of V188, Y190, P197, D200 to the NmmI binding and the coupling between R33/K47 of NmmI and α188–200 of nAChR by double mutant cycle analysis [6]. Because the binding

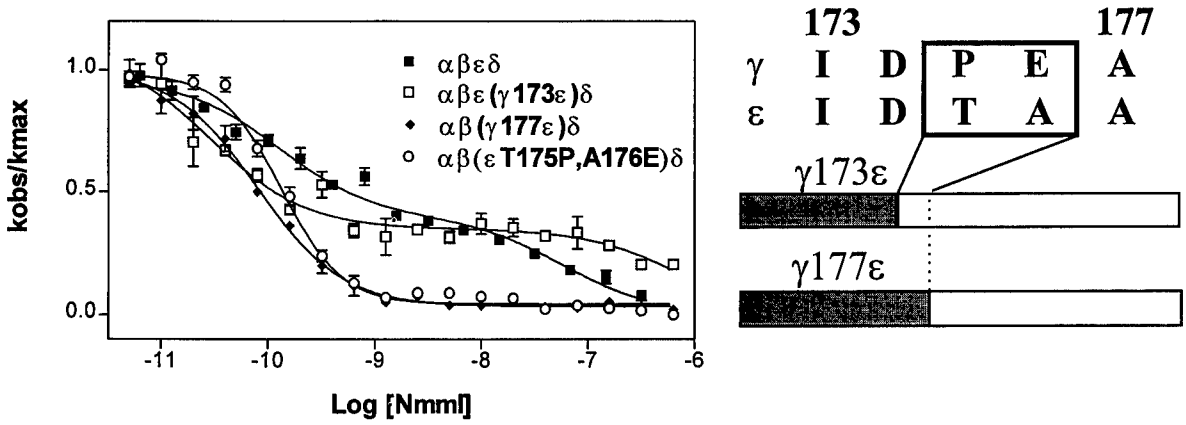


Figure 1.

surfaces are composed of the αδ and αγ or αε subunits interfaces, we extended our work to delineate the non-α subunits/toxin interactions.

Among the determinants on the γ and δ subunits, only the L119R [7] mutation at γ subunit gave over ten-fold decrease of affinity to NmmI. Secondly, we utilized the unique αε interface insensitivity (K_d for αε ~0.2 mM; K_d for αγ and αδ ~0.1 nM) to NmmI. Using subunit chimeras where the N-terminal portion of ε subunit is replaced with that of γ subunit, the origin of the αε insensitivity converged to two residues, εThr175 (Pro at γ and δ) and εAla176 (Glu at γ and δ) *(figure 1)*. Double mutants cycle analysis combining the receptor mutants and recombinantly generated NmmI mutants disclosed a tight linkage between γL119 at the receptor and R33 at the toxin with coupling energy of ~5 kcal/mol. From this linkage at γL119 and the unique ε subunit resistance, together with linkages established for the α-subunit [6, 8] and a model of folding in the receptor subunits [4], the spatial orientation of bound NmmI on the nAChR appears amenable to detailed study.

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81 Serotonin low affinity site in the nicotinic acetylcholine receptor

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Serotonin (5-hydroxytryptamine, 5HT) activates one type of ligand-gated receptor channel ($5HT_3$) and at least 14 G-protein-coupled receptors in the central nervous system and the periphery. In addition, electrophysiological studies suggest that 5HT agents block the nicotinic acetylcholine receptor (nAChR). We obtained the first biochemical evidence of ^{3}H-5HT binding to electric organ membranes and to the purified nicotinic re-

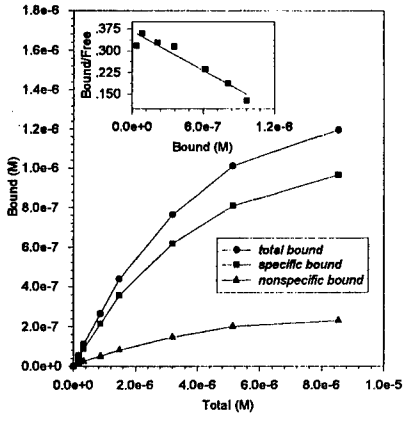


Figure 1. Saturation curves of the binding of ^{3}H-5HT to the purified nAChR. The nAChR (4.5 10–4 mg/mL) was incubated with different concentrations of 3H-5HT in the presence or absence of 5HT, in the dark, at 4 °C, for 1 h. Three μL of the samples were then dotted on a nitrocellulose membrane. The membrane was then incubated 30 min with 10 mM PBS, Triton X-100 1%, milk 5%, and washed 6 times with 100 mL of 10 mM PBS, Triton X-100 1%. The membrane was then cut in 1 1 cm squares which were laid on a vial with 3 mL of Xilene with 5% PPO and radioactivity was measured on a liquid scintillation counter. The figure includes data from a typical experiment. The insert shows the corresponding Scatchard analysis.

ceptor from Discopyge tschudii (Otero de Bengtsson and Biscoglio de Jiménez Bonino, 1997). Serotonin was used as a photoaffinity label and was irreversibly incorporated mainly in the alpha subunit. We then found that all subunits were labelled depending on the ligand/receptor concentration ratio. The Scatchard analysis of saturation curves (figure 1) obtained with the purified receptor showed a K_d value of (4.8 ± 1.9) µM and a B_{max} of (1.5 ± 0.3) µM (n = 3). Preliminary experiments, indicated an IC_{50} value of 4 mM and a K_i of (1.6 ± 0.3) µM (n = 2). The effect of carbamylcholine on the ^{3}H-5HT binding was studied. Competition experiments and autoradiographies suggested a competitive interaction in the millimolar range. The above results indicate that the nAChR possesses a low affinity site for serotonin. Further studies are being performed in order to better characterize such interaction.

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82 Microinjection of cultured sympathetic neurons: A method for studying the regulation of gene transcription

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Primary cultures of sympathetic neurons from superior cervical ganglia (SCG) of newborn rats constitute an attractive model system to investigate the mechanisms that govern the acquisition of the cholinergic phenotype. These neurons, initially noradrenergic, can become cholinergic in response to different extracellular factors, such as the cytokine CDF/LIF and retinoic acid (RA). In particular, these factors decrease both the activity and mRNA levels of enzymes involved in the synthesis of noradrenaline, tyrosine hydroxylase (TH) and dopamine β-hydroxylase (DBH). Simultaneously, they increase the activity of choline acetyltransferase (ChAT) and the number of molecules of the vesicular transporter of acetylcholine (VAChT), as well as the amount of ChAT and VAChT mRNA. These data suggest that CDF/LIF and RA may up-regulate the transcription of the ChAT and VAChT genes, which are organized as embedded genes, and concomitantly down-regulate the transcription of the genes encoding TH and DBH.

To study the mechanisms that regulate the expression of these genes, cultured SCG neurons need to be transiently transfected with reporter plasmids containing the promoter of the gene of interest. Classical methods (electroporation, calcium phosphate precipitation, lipofection, polyethylenimine-mediated transfection) were ineffective to transfect these neurons. Several studies have reported the use of microinjection to transfer genes into primary cultured neurons, but without any quantification of the expression of these genes (Garcia et al., 1992; Gagliardini et al., 1994; Chireux et al., 1994). In this study, we exploited the technique of microinjection to deliver plasmids into SCG neurons and to obtain quantifiable and reproducible reporter gene activities.

Neurons were grown on glass coverslips and about 1000 molecules of plasmid were injected into the nuclei of neurons. Injection of plasmids with reporter genes encoding b-galactosidase or the green fluorescent protein revealed a good survival of the injected neurons. A plasmid containing the gene of firefly luciferase under the control of the promoter sequences of the Rous sarcoma virus (RSV) was then injected in 100 neurons and a very high luciferase activity (more than 105 that of the assay background) could be detected 24 h after injection. The sensitivity of this technique allows the detection of activity in a single injected neuron. To assess injection efficiencies, a second plasmid was co-injected, in which a constitutively active promoter directs the expression of the Renilla luciferase gene. The activity of this latter luciferase is measured in the same cell homogenate as that used for the former, and is used to normalize it. In these conditions, the quantitative determination of the reporter gene activity was reproducible.

We then tested if a CDF/LIF responsive promoter is still regulated when microinjected in SCG neurons. CDF/LIF treated and untreated neurons were injected with a plasmid containing three cytokine-response elements of the peripherin gene inserted upstream from the thymidine kinase promoter linked to the firefly luciferase gene (Lecomte et al., 1998). A higher normalized reporter gene activity was detected in treated neurons, revealing that the up-regulation of a gene transcription by CDF/LIF can be measured in microinjected neurons.

A plasmid, in which about 5.2 kb of the rat VAChT gene promoter was linked to firefly luciferase gene, was then injected in the neurons. A high level of activity was detected after 24 h of expression, indicating that the activity of the VAChT gene promoters can be quantified using this method.

Taken together, these results reveal that microinjection of plasmid DNA is well suited to study gene regulation in cultured sympathetic neurons, in particular that of the ChAT and VAChT genes.

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83 Kinetic and thermodynamic effects of acetylcholinesterase on the solubility of Aβ peptide

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The principal hallmark of Alzheimer's disease is the presence of extracellular senile plaques, which consist of deposits of amyloid filaments. The main component of these compacted deposits is the amyloid-β-peptide (Aβ) of 40-42 amino acids, besides which the senile plaques also contain other proteins such as acetylcholinesterase (AChE). This enzyme directly promotes the assembly of Aβ peptide into amyloid fibrils (Inestrosa et al., 1996). The peptide has a critical concentration (Cr) below which it does not agrregates (thermodynamic solubility); over Cr, the Aβ peptide requires the formation of nuclei to trigger fibrillogenesis, thus determining the kinetic solubility of the peptide (Lansbury, 1997). However, neither the mechanism by which AChE promotes Aβ fibrillogenesis, nor the stage at which the enzyme exerts its effect, are known.

In this work, we dissect the effect of AChE on fibrillogenesis between its kinetic and thermodynamic components. In multipoint kinetic studies using turbidity, we observed a two-fold reduction in the lag time of amyloid fibril elongation when the enzyme was present in a molar ratio of 1:250 *(figure 1)*, two molecular forms of AChE was used, the amphiphilic G4 and the hidrophilic G1, we observed the same reduction in lag period with both of them. Once the system reached an equilibrium state, measured as a constant turbidity value, we determined the Cr of the Aβ aggregation process by two independent methods: densitometric analysis of SDS-PAGE and fluorimetric analysis. In the latter, extrapolation to zero of maximal fluorophor emission values, obtained using different Aβ concentrations, allowed us to estimate a Cr of 44.4 ± 2.8 mM. A similar determination of Cr was obtained in presence of varying AChE concentrations (10–100 nM), this results are summarized in *table I*. The solubility of Aβ was seen to decrease as the AChE concentration increased. Next, we calculated the apparent association constant the for Aβ polymerization (Kapp) using the relation Kapp = Cr^{-1} (Lee et al., 1977). The analysis of the association constants allow us to determine the true Cr for the Aβ peptide in presence of AChE, obtained a value of 24.04 ± 0.8 mM. Both aforementioned analytical methods showed the same decrease in Aβ solubility: 1.8-fold. Next we applied the Wyman analysis plotting ln Kapp v/s ln [AChE] (Lee et al., 1977); the slope of this plot revealed that the effect of AChE on the solubility of Aβ is due to the preferential binding of AChE to the polymeric form of the peptide rather than its monomeric state. Secondly, we were able to establish a stoichiometry of one molecule of AChE for every two molecules of Aβ peptide incorporated into the growing fibril. Finally, our results suggest that AChE promotes the Aβ aggregation by acting both as an heterologous seed (kinetic effect) and by reducing the solubility of the peptide (thermodynamic effect). This work was supported by a grant from FONDECYT N°1971240 and by a Presidential Chair in Science from the Chilean Government to NCI.

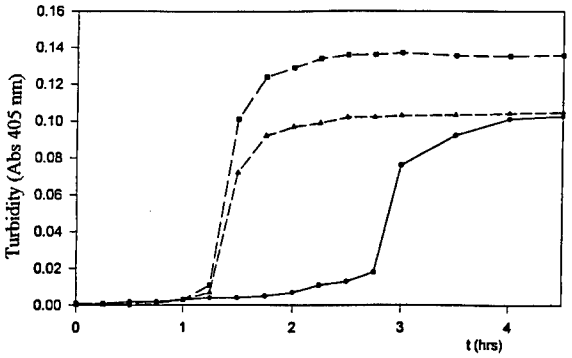


Figure 1. Turbidity of Aβ peptide aggregation The assay was carried out at 75 μM of Aβ alone (circles) or in presence of AChE G1 (squares) or AChE G4 (triangles) at 1: 250 MR (respect the catalytic subunits).

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Table I. Dependence of Aβ-amyloid solubility parameters on AChE concentration.The results are means ± S.D., $n = 3$, $P < 0.05$.

[AChE] Total (nM)	*Ccr (μM)*	*Kapp (L/mol)*	*G°app (kcal/mol)*
0	44.4 ± 2.8	2.26 ± 0.14 10^4	5.94 ± 0.04
10	40.7 ± 1.4	2.46 ± 0.08 10^4	5.99 ± 0.02
25	31.0 ± 1.1	3.23 ± 0.11 10^4	6.15 ± 0.02
50	23.7 ± 1.4	4.23 ± 0.25 10^4	6.31 ± 0.04
100	18.9 ± 3.5	5.42 ± 0.89 10^4	6.46 ± 0.11

ΔG°app = -RT ln Kapp.

84 SPOCK, a novel proteoglycan in a cholinergic synapse: The vertebrate neuromuscular junction

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SPOCK (Charbonnier et al., 1998), also known as 'testican' (Alliel et al., 1993), is a modular proteoglycan. SPOCK is the acronym of the peptidic modules defined within the proteoglycan's protein core. It designates motifs related to SPARC/Osteonectin and also depicts a 46 amino acid long domain centered around a CWCV (cysteine-tryptophan-cysteine-valine) tetrapeptide, and a Kazal-like protease inhibitory polypeptide, also present in the acetylcholine receptor (AChRs) clustering molecule agrin (Patty and Nikolics 1993), which has been characterized as a proteoglycan (Tsen et al., 1995). In addition to these structural features, SPOCK has been shown to be highly expressed in a subset of synapses within the mouse brain, in a postsynaptic location (Bonnet et al., 1996). These observations, and data dealing with the involvement of proteoglycans in AChRs clustering at the neuromuscular junction (NMJ) (Ferns et al. 1993), prompted us to evaluate the occurrence of SPOCK at the NMJ.

The present report deals with the immunohistochemical detection of SPOCK in normal murine skeletal muscle with the concomitant detection of AChRs. This study was carried out with antibodies raised in rabbits (719–7 and PTC9), directed against two chimeric proteins expressed in bacterial systems and corresponding to the SPOCK N- and C-terminal regions respectively *(figure 1)*.

SPOCK is found in the basal lamina of the muscle fibers. It appears highly concentrated at the NMJ and partially colocalized with the AChRs revealead by an a-bungarotoxin staining. A positive signal is also observed in interstitial nerves. Both antibodies gave rise to the same signals. *Figure 2* presents the SPOCK expression pattern obtained with the 719-7 immuneserum. The reaction was specifically inhibited by preincubation of the 719-7 immunserum with increasing amounts of the corresponding antigen.

The detection of SPOCK in intramuscular nervous branches led us to examine its distribution in peripheral isolated nerves. A strong signal was observed in Schwann cells, in axons, and at the node of Ranvier.

Thus, SPOCK is a new molecule, belonging to the expanding proteoglycan family, whose structural features and expression patterns are indicative of an involvement in the synaptic architecture at the NMJ. Experiments, currently in progress, to delineate its expression during development, are in agrement with a possible function of SPOCK in synapse formation or stabilization.

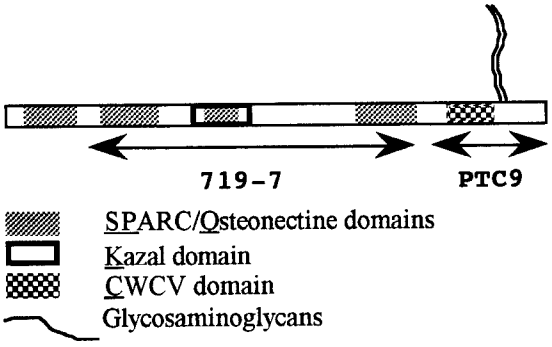


Figure 1. Localization, within the SPOCK protein core, of the expressed polypeptides used for the 719-7 and PTC-9 immunsera production. The modules are boxed.

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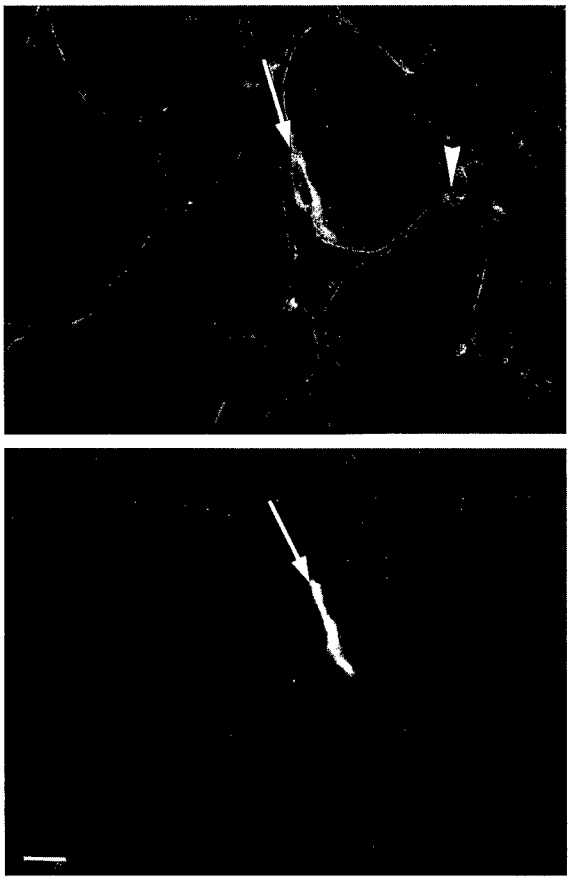


Figure 2. SPOCK expression at the neuromuscular junction. Transverse sections of adult mouse gastrocnemius muscle were doubled stained with the anti-recombinant SPOCK antibody 719-7 (top) and α-bungarotoxin (bottom). Immunoreactivity was present at the synaptic sites (arrows) identified by α-bungarotoxin binding as well as in interstitial nerve fibres (arrowheads). Scale bar 25 μm.

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85 Opposite cerebrovascular responses to physostigmine and tacrine in the cortex deafferented from the nucleus basalis magnocellularis suggest direct projections from this nucleus to intracortical inhibitory neurons and microvasculature

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Cholinesterase inhibitors used to treat Alzheimer's disease are thought to compensate for the functional impairment of the cholinergic basalocortical system. However, previous results obtained by measuring CBF in rats shortly after lesion of the nucleus basalis magnocellularis (NBM) did not support this contention (1, 2). In this study we investigated the cerebrovascular responses to physostigmine and tacrine late after the lesion. We sought to determine whether denervation supersensitivity occurred in the projection areas of the impaired NBM and enhanced the responsiveness to these cholinesterase inhibitors.

Regional CBF was measured using the [^{14}C]iodoantipyrine tissue sampling technique in conscious rats i.v. infused with physostigmine (0.1 or 0.2 mg/kg/h), tacrine (3 or 8 mg/kg/h), or saline, 3–5 weeks after unilateral lesioning of the NBM with ibotenic acid.

Physostigmine and tacrine dose-dependently increased blood flow in most cortical and subcortical regions compared to the control group. Unexpectedly, late after the lesion, interhemispheric comparisons showed that physostigmine caused dose-related smaller increases in blood flow in most cortical areas of the deafferented hemisphere. In contrast, tacrine caused greater blood flow increases in the frontal cortex of the lesioned hemisphere.

The results suggest that both physostigmine and tacrine stimulate a 'vasodilator' intracortical neuron and simultaneously another target responsible for the NBM-dependent component of their cerebrovascular effects. This component is of opposite nature according to the agonists used and presumably involves different postsynaptic targets. A reduced responsiveness to physostigmine occurring at 3–5 weeks postlesion can be explained by denerva-

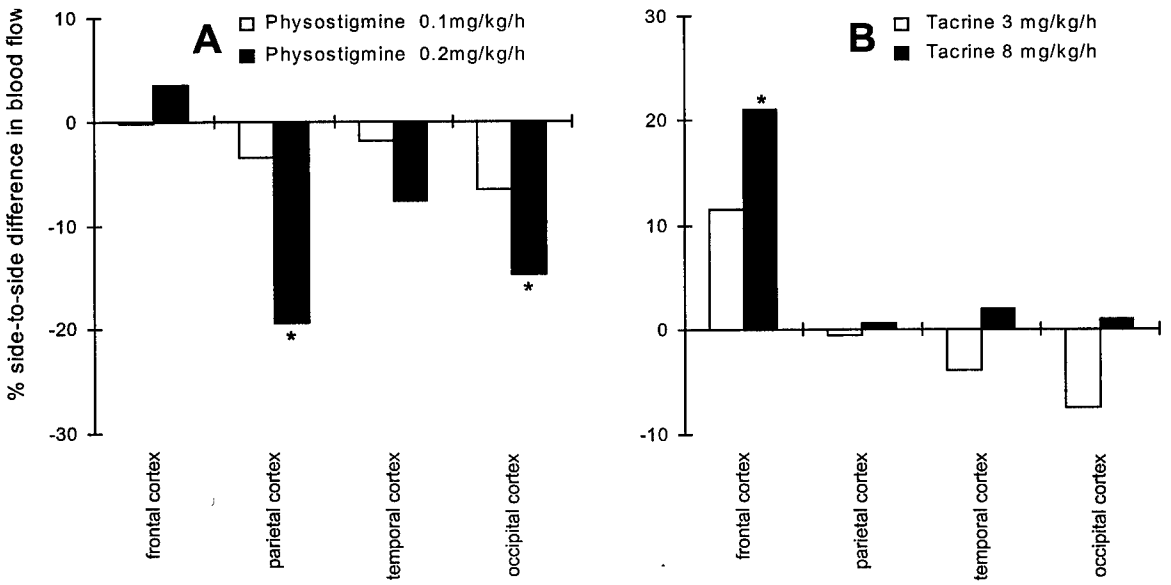


Figure 1. Cortical distribution of the side-to-side differences (with respect to the intact side) in blood flow in response to physostigmine (**A**) and tacrine (**B**), 3–5 weeks after unilateral NBM lesioning. The effects display the NBM-dependent component of the responses. Physostigmine induced dose-dependent smaller blood flow responses on the lesioned side, with the notable exception of the frontal cortex. In contrast, tacrine induced a dose-dependent greater blood flow response in the deafferented frontal cortex. *Significantly different from the intact side ($P < 0.05$, paired t-test).

tion supersensitivity of a synapse of NBM projections on to a cortical inhibitory interneuron. The facilitated increase in perfusion in response to tacrine in the deafferented hemisphere can probably be ascribed to a non-neuronal NBM target. This tacrine-specific effect occurs in the first week after the lesion (2) and then remains stable as long as 5 weeks after the lesion. It may involved the neurovascular junction of NBM terminals in the microvessel environment.

Altogether, we suggest that the cholinosensitive 'vasodilator' neuron of the cortical circuitry stimulated by physostigmine and tacrine is connected to an inhibitory interneuron. The latter could receive direct NBM projections and be brought into play mostly by physostigmine. The tacrine-specific effect could rely on a direct influence of NBM projections on the cortical microvasculature, which includes perivascular astrocytes (3).

Hence, physostigmine, tacrine, and perhaps all cholinesterase inhibitors, do not properly fulfill the principle of cholinergic replacement therapy for Alzheimer's disease, as they cannot compensate for a deficit of NBM functioning. However, the additional property of tacrine is beneficial to blood flow in a cortical area deafferented from the NBM. This tacrine-specific effect refers to brain perfusion in Alzheimer's disease. Cortical blood flow is highly sensitive to NBM activation (4), an effect which is doubled under physostigmine (5) and halved in aged rats (6). Consistent with studies showing that brain microvessels are the site of pathophysiological changes in Alzheimer's disease (6), our results support the idea that the clinical improvement obtained by prolonged tacrine treatment could be ascribed to such a circulatory effect. Moreover, the cerebrovascular reactivity to acutely administered tacrine might be a valuable index of its therapeutic efficacy.

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86 Some theoretical aspects of the allosteric interactions at muscarinic receptors

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Gallamine, the first allosteric ligand to be examined in detail, decreases the binding of muscarinic antagonist N-methylscopolamine (NMS) (Stockton et al., 1983). In contrast, alcuronium, another neuromuscular blocking agent, increases the binding of NMS (Tucek et al., 1990; Proska and Tucek, 1994).

Both above-mentioned allosteric ligands slow down dramatically the association and dissociation kinetics of NMS when it binds to muscarinic receptors. One possible interpretation is that these allosteric ligands either in a direct or indirect manner entirely prevent NMS dissociation and association (Proska and Tucek, 1994; Matsui et al., 1995).

Progress in the field is hampered by the unavailability of radiolabeled allosteric ligands with an affinity high enough to be used in the binding assay. The published methods of detection and quantitation of allosteric interactions at muscarinic receptors (Lazareno and Birdsall, 1995; Ellis and Seidenberg, 1992) postulate that allosteric ligand has fast kinetics, relative to those of NMS. The semiempirical methods cannot afford to estimate, by definition, the values of the allosteric ligand dissociation and association rate constants.

We have synthesized two apparently purely allosteric ligands: anatruxonium (we repeated the synthesis of it (Kharkevich and Skoldinov)) and URA 114 (Urbansky and Proska, 1997) are negative and positive respectively allosteric modulators of [3H]NMS binding to M_2 receptors. The magnitudes of their affinities were found to be comparable with those of [^{3}H]NMS. We have analysed, in detail, the effects of increasing concentrations of the allosteric ligands on the time course of [^{3}H]NMS binding. Our results were consistent with a ternary complex theory. The data were analyzed with Kinsim (Frieden, 1993), and Mathematica 3.0.1. (Wolfram Research Inc., 1997) software packages. We discuss the crucial difference between the kinetic behaviour of the systems described by the circular four-membered scheme or four-membered linear scheme. Supported by grant 203/97/0302 from the Grant Agency of the Czech Republic and from personal source (J.P.).

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87 Constitutive inhibitory action of muscarinic receptors on adenylate cyclase in cardiac membranes: effects of atropine, S-(–)-hyoscyamine, and R-(+)-hyoscyamine

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Muscarinic receptors in the heart have been shown to display agonist-independent spontaneous (constitutive) activity which causes changes in the opening of cardiac ion channels and in the activity of G proteins. We investigated whether an inhibition of the constitutive activity of muscarinic receptors induced by the binding of their antagonists brings about a change in the synthesis of cyclic AMP in rat cardiac membranes. Atropine and S-(–)-hyoscyamine were indeed found to enhance the forskolin-stimulated synthesis of cyclic AMP in rat ventricular membranes by up to 24%. The effect was stereospecific and the potency of R-(+)-hyoscyamine was more than 20-fold lower than that of the S-(–) enantiomer, confirming that the action of hyoscyamine is receptor-mediated. The effect did not depend on the presence of endogenous acetylcholine in the system used. The results suggest that the adenylate cyclase in the heart is exposed to continuous mild inhibition by the constitutively active muscarinic receptors in the membranes of cardiomyocytes.

88 Changes in gene expression in basal ganglia and associated structures following local fasciculin application in the striatum and in the substantia nigra

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The basal ganglia are structures involved in co-ordination of movements. There, acetylcholine (ACh) plays a critical role in the physiology of these structures. In the striatum ACh is synthesised in cholinergic inter-neurons which correspond to about 2% of the total neuronal population. Although a limited number of striatal neurons are able to synthesise ACh and its degradation enzyme, acetyl-cholinesterase (AChE), a high concentration of AChE is found in the striatum. In the substantia nigra (SN), AChE is also present and is synthesised in dopaminergic neurons. In both structures AChE can be released consecutively to changes in neuronal activity (Greenfield et al., 1980, 1983).

To examine potential roles played by AChE we analysed in the striatum and the SN the effects produced by a prolonged inhibition of AChE activity on striatal and nigral gene expression (peptides, enzymes and immediate early genes). This was investigated by locally applying fasciculin (10^{-5} M, 4 µL), a snake venom known to irreversibly block AChE activity (Karlsson et al., 1984).

In the striatum, AChE activity was inhibited by 80% for at least 72 h after fasciculin application and normal activity recovered in 1 week. At 24 h, the binding of ^{3}H-pirenzepine (a ligand which labels m1 muscarinic receptors) was reduced by 25% indicating that the toxin binds to this receptor subtype by mimicking some properties of the m1 toxin (Max et al., 1993). Although AChE activity was markedly inhibited, AChE mRNA expression in striatal cholinergic interneurons remained unchanged at 24 h. In striatal efferent neurons which are under a cholinergic control, the expression of glutamic acid decarboxylase (GAD67) and substance P mRNA expression remains unchanged in striato-nigral neurons. In striato-pallidal neurons, GAD67 mRNA expression remained unchanged but preproenkephalin (PPE) mRNA expression was slightly increased. Moreover, at distance, a marked increase in PPE mRNA expression was observed in the piriform cortex (see *figure 1*) ipsilateral to the injection suggesting the activation of a polysynaptic striato-cortical loop following striatal AChE inhibition.

In the SN, AChE activity was less affected by fasciculin since it was inhibited by 45% and 20% at 24 and 72 h, respectively, after fasciculin application. The differential toxin efficacy to inhibit AChE activity in the striatum and the SN could be attributed to a differential localisation (extra- versus intracellular) of AChE in these two structures. In the SN, the expression of AChE and tyrosine hydroxylase mRNAs was inhibited when fasciculin was

injected in the pars compacta where are localised dopaminergic neurons. Furthermore, the expression of the immediate early gene Zif 268 which has been used to visualise activated neurons (Sukhatme et al., 1988; Simonato et al., 1991) was examined following nigral fasciculin application. Whereas no local effect was observed, zif 268 mRNA levels were markedly increased at distance in the dentate gyrus (granular cells) and in the CA1 to CA4 regions (pyramidal cells) of the hippocampus. Of interest, the organo-phosphorus-induced convulsions could involve activation of such polysynaptic pathways.

In conclusion, irreversible inhibition of striatal and nigral AChE can modify locally the neuronal activity as evidenced by changes in peptide or enzyme expression as well as at distance as evidenced by distal changes in peptide and immediate early gene expression. Work supported by grant no 25-179/DRET/966ETCA/CEB/B.

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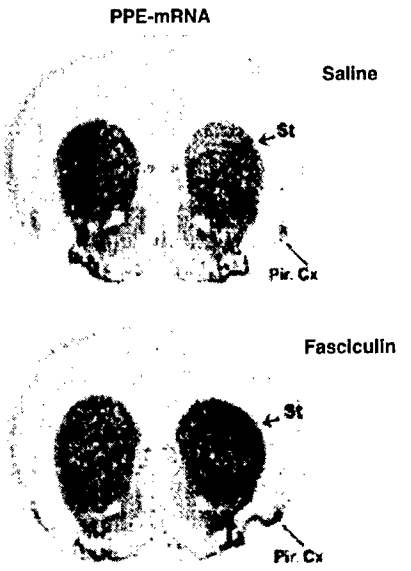


Figure 1. The expression of PPE mRNA was examined by in situ hybridisation 24 h after the injection of saline (upper part) or fasciculin (lower part) in the right striatum (St, short arrow). In fasciculin-treated rats, PPE mRNA levels were increased locally in the injected striatum and distally in the piriform cortex (Pir. Cx) ipsilateral to the fasciculin injection.

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89 Cholinergic mechanisms in plant fertilization

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Cholinergic mechanisms appear to exist in every living cell, not only belonging to vertebrata. Acetylcholine (ACH) and biogenic amines, neuromediators also found in plant cells, can play role of both signalling substances and growth regulators (Roshchina, 1991) ACH and the activity of cholinesterase was observed in the flowers: the pistil stigma of different species (Roshchina and Semenova, 1995) and in exine of pollen grains (Roshchina et al., 1994). To show a possible role of cholinergie mechanisms in a regulation of a fertilisation is the aim of the study. We analyzed: 1) the effects of ACH and its antagonists on the fertilization, in particular pollen germination in vivo and in vitro, as well as autofluorescence of pollen and pistil; 2) the cholinesterase activity in the reproductive organs during pollination and fertilization as well as the contribution of cholinesterase to the processes; 3) the action of cholinesterase inhibitors on the fruit and seed formation after flower organ treatment.

Objects of the study were pollen and pistils of *Hippeastrum hybridum* and of three clones of *Petunia hybrida* differing in functional activity of male gametophyte (self-compatible clone, self-incompatible clone and sterile clone) grown in greenhouse were investigated. Pollen and pistil stigma autofluorescence under 360–380 nm light excitation was studied (Roshchina and Melnikova, 1996). Pollen germination in vitro was estimated as the pollen tube formation on the artificial nutrient medium, including 10% sucrose (Roshchina and Melnikova, 1996) whereas the process in vivo was seen as the fruit and seed formation. The fruits and seeds are produced after both self- and crosspollination in *Hippeastrum hybridum* and *Petunia hybrida* of self-compatible clone whereas in *Petunia hybrida* of the self-incompatible clone (a male gametophyte inhibition) it occurs only alter cross-pollination, and in sterile clone (male sterility), the seeds are also formed only after cross-pollination. The cholinesterase activity was measured in extracts from pollen or pistils (Roshchina et al., 1994). After a month, the seed production was estimated.

As for *Hippeastrum*, ACH and their antagonists had effects on the pistil stigma autofluorescence, pollen germination in vitro and seed formation after the pistil treatment *(table I)*, whereas the pollen grain autofluorescence was unsensitive to them.

Table I. The effect of anticholinergic drugs, applied to the pistil stigma or pollen, on the formation of seeds of *Hippeastrum hybridum*. S, stimulation; NE, no effect; NS, no stimulation.

Substance	*Autofluorescence of pistil*	*Pollen germination in vitro*	*Seed formation*
ACH 10^{-5} M	S	S	Normal
Atropine 10^{-5} M	NE	NE	Absent
d-Tubocurarine 10^{-5} M	NE	NE	Absent
Atropine 10^{-5} M + ACH 10^{-4} M	NS	NS	Absent
d-Tubocurarine 10^{-5} M + ACH 10^{-4} M	NS	S	Absent
Neostigmine 10^{-5} M	S	S	S
Physostigmine 10^{-5} M	S	S	NE

ACH stimulates the fluorescence intensity of the stigma approximately two-fold, whereas after the preliminary treatment by d-tubocurarine or atropine the effects are not observed. It shows the receptors of acetylcholine exist on the stigma surface. Ihe ACH effects on pollen germination depend on the initial control level. When the index of germination is low acetylcholine stimulates the process ~two-fold above the control. Whereas, at high control germination rates there is little effect. Atropine also stimulated the pollen germination. When ACH was added after the treatment by d-tubocurarine, it is more stimulated than after the antagonist alone, in contrast to atropine, blocker of muscarinic type of animal cholinoreceptor. The sensitivity of a pistil stigma to ACH and their antagonists may be studied in vivo as a fertilization (treatrnent with anticholinesterase drugs, neostigmine and physostigmine, and with blocker of nicotinic cholinoreceptor, alkaloid d-tubocurarine. Moreover, the visible seed formation is seen later than in a control. In the case of tubocurarine and atropine, the seeds did not mature).

The hydrolysis of acetylcholine was observed in the extracts from both pollen and pistils *(table II)*. The participation of cholinesterase was evidenced from the significant decrease in rate of acetylthiocholine degradation by the inhibitors of cholinesterase, physostigmine and neostigmine *(table II)*. In the extracts from petunia pollen, the rate of choline ester hydrolysis was not inhibited by high substrate concentrations). The reaction rate was 3–4 times higher with acetylcholine than with butyrylthiocholine. The Michaelis constants (K_m) were 6×10^{-4} M with acetylthiocholine and 7×10^{-4} M with butyrylthiocholine, respectively, for the enzymatic reaction inhibited by hearing at 70 °C. High rate of acetylthiocholine hydrolysis in the pollen of two fertile clones was suppressed by 90% with the inhbitors of cholinesterase *(table II)*. Male sterility correlates with the cholinesterase lack or decrease of the enzyme activity. All the phenomena suggest the participation of cholinoreceptor and cholinesterase in fertilization. The study was supported by a grant of the Russian Fund for Fundamental Studies N 96-04-48091.

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Table II. The rate of acetylthiocholine hydrolyses by water extracts from pollen or pistils (in brackets) of various clones Petunia hybrida, differing in male fertility without and with the addition of the cholinesterase inhibitors. S.E. ± 2%.

	The rate of acetylthiocholine hydrolysis ($\mu mol\ kg^{-1}$ fresh $mass^{-1}\ s^{-1}$)		
Clone	*Control*	*+ Physostigmine 10^{-5} M*	*+ Neostigmine 10^{-5} M*
Self-compatible	1400 (492)	93 (307)	0 (330)
Self-incompatible	330 (50)	280 (22)	175 (0)
Sterile	25 (410)	300 (305)	230 (307)

90 Control of acetylcholinesterase gene expression in tissue-cultured skeletal muscle: Opposite regulation by protein kinases A and C

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Introduction

The expression of acetylcholinesterase (AChE) in nerves and muscle is regulated by cellular activity, such as the frequency of membrane depolarization, and by signals from pre-synaptic cells. The specific signalling pathways involved, however, are still poorly defined. We have used primary cultures of quail muscle cells to study the regulation of AChE because they express both the globular and collagen-tailed AChE forms, and because they organize a subset of these on their surface in the form of clusters associated with other extracellular matrix components. Using quail-mouse mosaic myotubes, derived from the fusion of quail and mouse C2/C12 myoblasts, and avian-specific anti-AChE antibodies, we showed that AChE molecules expressed by a given quail nucleus are selectively targeted to and retained on the region of the fiber surface overlying that nucleus (Rossi and Rotundo, 1992). Once secreted, collagen-tailed AChE molecules interact transiently with heparan sulfate protoglycans on the basal lamina prior to more permanent immobilization (Rossi and Rotundo, 1996). In vivo, specific binding sites exist at the adult neuromuscular junction for the localization of collagen-tailed AChE forms (Rotundo et al., 1997). Together, these studies indicated that synthesis, targeting, and localization of AChE is compartmentalized, and suggested that signals generated at the plasma membrane overlying individual nuclei may be responsible for locally regulating AChE expression. The questions then become: "what is the nature of the signals" and "at what level are they acting?".

Models for gene regulation at the neuromuscular junction have implicated protein kinase C (PKC), protein kinase A (PKA), and Ca^{2+} (reviewed in Duclert and Changeux, 1995), while stabilization of the acetylcholine receptors also may involve signaling through an adenylcyclase-linked pathway (Xu and Salpeter, 1995). In support of these hypotheses a specific isoform of PKC, PKC?, has been localized immunocytochemically to the NMJ (Hilgenberg et al., 1996), and in situ hybridization and immunocytochemical analysis have revealed that a regulatory subunit of cAMP-dependent protein kinase (PKA) accumulates at the neuromuscular junction (Imaizumi-Scherrer et al., 1996). Regulation of the AChE gene and/or protein expression through signaling pathways that convey information about membrane activity have been previously investigated in several laboratories (De la Porte et al., 1984; Fernandez-Valle and Rotundo, 1989; Valette and Massoulié, 1991; Godinho et al., 1994; Griffman et al., 1997).

The regulation of AChE is compartmentalized

To determine whether the signaling pathways in multinucleated skeletal muscle fibers are compartmentalized, we designed tissue-culture chambers capable of isolating the medium overlying small regions of individual myotubes. The myotubes were grown on scratched coverslips so that the fibers formed in paralleled arrays on the coverslip, and the chambers were sealed over them with silicone grease to creat separate chambers. Drugs that affected AChE expression were then added to one side of the chamber while the other remained as an untreated control (Rossi et al., abstract American Society for Neuroscience, 1994; and manuscript in preparation). In parallel, sets of conventional cultures were used to monitor the effects of the drugs on AChE expression. We observed a reduction in the number of cell surface AChE clusters per nucleus following muscle paralysis using tetrodotoxin (TTX) in both conventional cultures and chamber cultures, where TTX was applied on one side of the chamber and normal medium on the other side. In contrast, when scorpion venom was applied to cells in either the conventional or chamber cultures, there was an increase in AChE clusters per nucleus as a consequence of chronic membrane depolarization. To determine whether AChE mRNA regulation was also compartmentalized, myotubes in chamber cultures were treated with TTX or scorpion venom on one side of the chamber and normal medium on the other side. The AChE mRNA distribution was analyzed by in situ hybridization using a digoxigenin-labeled 760 nt cRNA probe complementary to a common region of the AChE catalytic subunit. The ratio of nuclei showing accumulated AChE mRNA to the total nuclei in myotubes was determined for each treatment condition. We observed 64% more AChE mRNA positive nuclei in the presence of TTX, and 30% less positive nuclei under scorpion venom treated regions of the myotubes compared to the control side, indicating that local membrane depolarization could locally regulate AChE transcript levels in the same myotube. The morphology of the myotubes was similar along the entire fiber length regardless of treatment, yet the propagation of spontaneous contraction was inhibited only in those fiber regions exposed to the drugs. We concluded that accumulation of AChE transcripts and protein in specific regions of the muscle fiber is due in part to regulation of transcription as a result of localized signal transduction pathways.

Transcriptional control of AChE in muscle

AChE transcripts first appear at the time of myoblast fusion andthen continue to increase for a short period of time. After reaching peak levels about 5 days after plating, AChE mRNA levels begin to decrease concommitantly with the increase in spontaneous muscle contraction. We analyzed whether the accumulation of AChE mRNA in quail muscle cultures during development and differentiation resulted from increased transcription or stabilization of the transcript. We observed that, in tissue cultured quail muscle, AChE mRNA is regulated at the transcriptional level during myoblast fusion and differentiation (Rossi and Rotundo, manuscript in preparation). The half life of AChE mRNA remained unchanged during myotube formation and maturation, using either the specific RNA polymerase II inhibitor DRB or actinomycin D to inhibit transcription. These studies also suggest major inter-species differences between mouse C2/C12 cells (Fuentes and Taylor, 1993) and avian cells with respect to AChE mRNA regulation. We observed that the rate of AChE mRNA transcription increased during differentiation of the muscle

fibers, but decreased after the onset of spontaneous contraction, with the second change being blocked by TTX.

Regulation of AChE by second messengers

Several reports have indicated the involvement of PKA and PKC in regulating proteins that accumulate at the neuromuscular junction, such as AChR (Fontaine et al., 1987; Chahine et al., 1993; O'Mally et al., 1997; reviewed in Duclert and Changeux, 1995) and NCAM (Rafuse and Landmesser, 1996), suggesting that nerve-induced changes in second messenger pathways can modulate muscle gene expression. In the present studies we investigated the role of PKA and PKC in modulating AChE expression using avian skeletal muscle cultures. Forskolin, a potent stimulator of adenylate cyclase that catalyzes the formation of cAMP, thereby activating cyclic-AMP-dependent protein kinases, decreased total AChE activity by 44% following overnight treatment. Forskolin reduced the expression of total AChE activity in a concentration-dependent manner, with a half-maximal inhibition near 30 μM. Similar results were obtained using 8-bromo cAMP and dibutyryl-cAMP, as well as inhibitors of phosphodiesterases. No effects of forskolin on total protein synthesis were observed. To determine whether the effects of forskolin and cAMP analogues could act at the level of AChE biosynthesis, cells were pre-treated overnight with these drugs. The total cell-associated AChE was then irreversible inhibited using diisopropylfluorophosphate (DFP), and cells were allowed to synthesize new AChE for 2 h in complete medium plus or minus drugs. Under these conditions, the rate of appearance of AChE activity in forskolin-treated cultures was 40% of controls, suggesting that forskolin affects the rate of AChE translation. Sucrose gradient analysis of AChE forms showed that, even after 24 h of recovery, all the forms are decreased in cells treated with forskolin. The collagen-tailed form in forskolin-treated cultures was only 15% of that in the control group. In addition, the rate of AChE secretion was decreased in forskolin-treated cultures. Interestingly, there was no effect on either total AChE activity or newly synthesized enzyme after only 3 h of treatment, suggesting that new synthesis is required. RNase protection assays were used to quantify the levels of AChE mRNA under the experimental different conditions and showed that AChE activity decreased in parallel with the decreasing levels of AChE mRNA. When phorbol-12,13-didecanoate (PDD), an activator of protein kinase C, was added to the cultures AChE activity was elevated.

In summary, activation of PKA inhibited the expression of AChE whereas activation of PKC increased enzyme activity, suggesting that the accumulation of AChE transcripts and enzyme at the neuromuscular junction involves localized regulation of AChE transcription. Moreover, these studies suggest a much more precise level of control than had been expected for an enzyme whose levels appear to be constitutively expressed at the synapse. This work was supported by a grant from the NIH to R.L.R.

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91 Effect of gestational nicotine exposure on hippocampal intrinsic cholinergic neurons

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Learning and memory deficits and disorders of motor activity have been reported in children exposed to in utero nicotine as a result of maternal smoking during pregnancy[1]. Neurobehavioral disturbances have been noticed in animal models of prenatal nicotine exposure[2]. In an earlier morphological study on hippocampal neurons, distinct morphological changes in the pyramidal neurons of Ammon's horn and granule cells of dentate gyrus were observed in postnatal period after gestational nicotine insult. There was a significant reduction in neuronal size and dendritic arbor, by light microscopy; dilatation and membrane disruption of Golgi cisternae along with microvacuolation of rough endoplasmic reticulum by electron microscopy[3]. These neurotoxic effects on hippocampal neurons are important as the hippocampus plays a major role in spatial learning and memory.

In order to explore effects of prenatal nicotine on hippocampus further, intrinsic cholinergic neurons of hippocampus were studied in timed pregnant Wistar female rats (125–150 g) who were subjected to intraperitoneal nicotine (2.5 mg/kg/day in two divided doses), from gestation day 6 to term. Pups were delivered normally and their brains were dissected after perfusion fixation. Coronal sections of the dorsal hippocampal region were studied for intrinsic cholinergic neurons, from postnatal (PD) 40 to 80, by immunohistochemical techniques using monoclonal antibody against choline acetyl transferase (ChAT), (Boehringer Mannheim) and localising neurons by the ABC method. Cholinergic neurons were identified in Ammons' horn CA1 region (in strata oriens, pyramidale, radiatum and lacunosum moleculare) and in hilar region of dentate gyrus. Stereologic analysis of ChAT immunoreactive neurons was done on a Leica QC 500 Image Analysis System. Count of cholinergic neurons and their area was significantly reduced ($P < 0.001$) in nicotine-exposed animals at PD 80. Cholinergic neurons of hippocampus modulate the activity of pyramidal neurons of Ammons' horn, which in turn influence other cortical regions; hence these morphological alterations observed in non-pyramidal ChAT immunopositive neurons are significant.

These morphological effects on hippocampal neurons correlate well with the Openfield motor activity of the animals recorded on Video path analyzer (Coulbourn Instruments). Total distance travelled, speed and duration of locomotion during a 30-min time period were significantly less in the experimental animals. Reduced hippocampal cholinergic activity as a result of gestational nicotine exposure during vulnerable period of neurogenesis is responsbile for motor dysfunctions observed in the present study.

These results provide morphological evidence of disruption of cholinergic activity in late developing region of brain, hippocampus, produced by gestational nicotine insult. It has been reported that nicotine interacts with nicotinic cholinergic receptors in developing brain producing discoordination of target cell differentiation and causes premature shift from replication to differentiation as a result of nicotinic receptor stimulation on target cells directly or via release of catecholamines indirectly[4]. No such morphological study has been reported correlating intrinsic cholinergic neurons of hippocampus with the neurobehavioral dysfunctions.

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92 Analysis of developmental evolution of synapses in CGRP knock-out mice

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Calcitonin gene-related peptide (CGRP) is a neuropeptide widely localized in central and peripheral nervous systems. Its different ascribed actions include modulations of the enteric autonomous system, cardiovascular effects, vasodilatation and inflammation phenomena, and stimulation of acetylcholine release particularly from motor nerve endings at neuromuscular junctions. Also CGRP is potentially involved in pain sensitivity to different stimuli. Taken together, these observations define the role of CGRP as a trophic or modulatory factor in neuron or neuronal-muscle cell interactions. To further precise the role of CGRP we made knock-out mice ($CGRP^{-/-}$) by exon 5 specific mutation of the calcitonin/CGRP gene.

Homozygous $CGRP^{-/-}$ mice live to adult-hood, reproduce normally and show no abnormalities when observed in their cage. However, preliminary behavioural analysis indicates some differences in base line nociception and morphine sensitivity between wild type and knock-out mice latencies when using heat tests.

93 Transgenic mammary gland expression of 'readthrough' human AChE: A model system for cholinesterase regulation in mammalian body fluids

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Apart from their well known association with cholinergic synapses, the acetylcholine hydrolyzing enzymes acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) appear in body fluids (e.g., plasma, amniotic and cerebrospinal fluids) where their function(s) are not fully understood. To investigate the molecualr mechanisms underlying AChE and BuChE secretion into body fluids, we studied mammary gland expression of cholinesterases in two lines of transgenic mice overexpressing the human (h) 3'-alternatively spliced 'readthrough' AChE mRNA transcript including the I4 pseudointron and the 3' terminal exon E5, (hAChE-I4 /E5) directed by the cytomegalovirus (CMV) efficient promoter. Two additional lines expressed catalytically active (Beeri et al., 1995, 1997) or insert-inactivated AChE terminated with the brain characteristic C-terminus translated from exon 6 (hAChE-E6 and hInAChE-E6; Sternfeld et al., 1998). Non-transgenic FVB/N mice served as controls. In mammary gland alveoli of hAChE-I4/E5 transgenic mice, in situ hybridization demonstrated pronounced labeling of hAChE-I4/E5 mRNA transcripts, considerably higher than the endogenous mouse (m) AChE-I4/E5 mRNA transcripts detected in both wild type FVB/N mice and the h-AChE-E6 transgenic mice. The capacity of milk cholinesterases to hydrolyse acetylthiocholine (ATCh) was tested in the absence or presence of the BuChE-specific inhibitor iso OMPA throughout the first 2 weeks after delivery. In both control mice and transgenics expressing active h-AChE-E6, BuChE was the predominant cholinesterase and hydrolysed ca. 35 nmol ATCh/min/μL milk. Iso OMPA (10^{-5} M) inhibited over 80% of this activity. Transgenics expressing h-InAChE-E6 displayed considerably lower levels of BuChE in milk, with activities increasing from virtually undetected amounts to 7.5 and 15 nmol/min/μL at days 7, 11 and 14 post-partum. This suggested competitive inhibition of host AChE production in a manner dependent on lactation time. Transgenic mice expressing 25-fold higher levels of h-AChE-I4/E5 in muscle displayed similarly higher AChE activities also in milk. Another line, with 350-fold transgenic h-AChE-I4/E5 levels in muscle, revealed increases of up to 160-fold higher than control amounts in milk, with two-fold post-delivery elevations from day 7 to 14. Non-denaturing gel electrophoresis followed by cytochemical staining demonstrated a complex migration pattern with a pronounced band co-migrating with h-AChE-I4/E5 produced in *Xenopus* oocytes. Altogether, the transgenic enzyme thus reached higher concentrations than the 50 nM cholinesterases present in normal human blood. Our findings present novel transgenic models for studying the secretion, control of production and biochemical properties of cholinesterases in mammalian milk.

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94 Implication of a multisubunit Ets related transcription factor in synaptic expression of the nicotinic acetylcholine receptor

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In adult muscle, transcription of the nicotinic acetylcholine receptor (AChR) is restricted to the nuclei located at the neuromuscular junction. The N-box, a new promoter element, was recently identified and was shown to contribute to this compartmentalized synaptic expression in the case of the AChR delta and epsilon subunits. Here, we first demonstrate that the N-box mediates transcriptional activation in cultured myotubes and identifies the transcription factor that binds to the N-box in myotubes and in adult muscle as an heterooligomer. The alpha subunit of GABP (GA binding protein) belongs to the Ets family of transcription factors, whereas the beta-subunit shares homology with IκB and *Drosophila* Notch protein. GABP binding specificity to mutated N-box in vitro strictly parallels the sequence requirement for beta galactosidase targetting to the endplate in vivo. In situ hybridization studies reveal that both GABP subunits mRNAs are abundant in mouse diaphragm, with a preferential expression of the alpha subunit at motor endplates. In addition, heregulin increases GABP alpha protein levels and regulates the phosphorylation of both GABP subunits in cultured chick myotubes. Finally, dominant negative mutants of either GABP alpha or GABP beta block heregulin elicited transcriptional activation of the AChR delta and epsilon genes. These findings establish the expected connection with a synaptic trophic factor which contributes to the accumulation of AChR subunits mRNAs at the motor endplate.

95 Mechanisms which regulate the cholinergic phenotype in sympathetic, central cholinergic and spinal motoneurons

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Besides their function in supporting neuronal survival, neurotrophic factors influence various functional properties of responsive neurons. In cell culture, neurotrophic factors of the CNTF/LIF family can switch the transmitter phenotype of sympathetic neurons from adrenergic to cholinergic (Schotzinger and Landis, 1990; Saadat et al., 1989; Ernsberger et al., 1997), and choline acetyl transferase expression both in central cholinergic neurons as well as in spinal motoneurons is influenced by neurotrophic factors (Gnahn et al., 1983; Magal et al., 1991; Williams et al., 1996; Wong et al., 1993; Alderson et al., 1996;). Although these mechanisms have been studied in detail in cell culture, it is not clear whether the same factors also regulate cholinergic transmitter production of these neurons in vivo. Ciliary neurotrophic factor and leukemia-inhibitory factor are not expressed within the innervated target region of cholinergic neurons such as sweat gland and skeletal muscle (Stöckli et al., 1991; Banner and Patterson, 1994). Instead, they are found in Schwann cells within the peripheral nerve and astrocytes within the central nervous system. In particular after peripheral nerve or mechanical brain lesion, the production of LIF is increased, and CNTF can be released in order to act on lesioned cholinergic neurons (Sendtner et al., 1997). Currently, mouse mutants lacking the genes for neurotrophic factors (DeChiara et al., 1995; Li et al., 1995; Sendtner et al., 1996; Francis et al., 1997) are analyzed to define the physiological endogenous role of these factors in regulating the cholinergic phenotype of these neurons.

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96 An engineered short-chain toxin with higher affinity for the neuronal α7 receptor

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Elapid and hydrophid snakes synthetize curaremimetic toxins which block nicotinic acetylcholine receptor from *Torpedo* with high affinities. These toxins are usually classified as short-chain (60–62 residues and 4 disulfide bonds) and long-chain (66–74 residues and 5 disulfide bonds) (Endo and Tamiya, 1991). On the basis of electrophysiological and binding experiments, we recently demonstrated that only long-chain toxins possessing 5 disulfide bonds are capable of blocking the α7 neuronal acetylcholine re-

Table I. Affinity constants of wild-type and modified curaremimetic toxins on the chimeric α7-5HT3 receptor expressed in HEK cells.

	Kd on the α7 receptor
	1 nM
α-Bgtx	5 nM
α-Cbtx	2 μM
Toxin-α	35 nM
Bgtx (C29S-C33S)	120 nM
Cbtx (C26S-C30S)	100 nM

ceptor with high affinities ranging between 1 to 10 nM (Servent et al., 1997). The short-chain toxins recognize this receptor with much lower affinities, their Kd values being at least three orders of magnitude higher than those of long-chain toxins. Selective reduction of the extra disulfide bond of the long-chain toxin, followed by a specific modification of the free cysteines with a bulky chemical reagent, further confirmed the critical role of this particular region for curaremimetic toxins to bind to the neuronal α7 receptor with high affinity.

We now tentatively investigated the binding contribution of this toxin region to the neuronal α7 receptor by synthetizing a number of toxin variants. At first, we synthetized genetically or chemically two derivatives of the long-chain α-cobratoxin and α-bungarotoxin, in which the extra two half-cystines were replaced by serine residues. The affinities of these derivatized toxins for the α7 receptor decreased by 25-fold and 35-fold respectively with K_d equal to 120 and 35 nM *(table I)*, whereas their affinity constants for the Torpedo receptor remained virtually unchanged. Then, we selected a short-chain toxin having low affinity (K_d = 2 μM) for the α7 receptor and by a chemical approach we introduced in it an extra disulfide and its associated loop, at a location homologous to that observed in long-chain toxins. The resulting chimeric toxin displayed a K_d equal to 100 nM *(table I)*, which corresponds to an affinity increase of 20-fold. Therefore, we have not only unambiguously localized a region that is associated with the capacity of toxins to recognize the neuronal α7 receptor but we also determined its contribution to this recognition.

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97 Nicotinic and muscarinic modulation of epileptic activities in the rat GABA withdrawal syndrome

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The γ-aminobutyric acid (GABA) withdrawal syndrome (GWS) is a focal epilepsy consecutive to the interruption of a chronic GABA infusion into the somatomotor cortex (Brailowsky et al., 1988). In neocortical slices obtained from rats presenting GWS, epileptic-like bursting activities are induced in pyramidal cells by intracellular depolarizing current injection (intrinsic bursting) and/or by white matter (WM) stimulation (synaptic bursting; Silva-Barrat et al., 1989, 1992). The aim of the present study is to analyse the role of cholinergic neurotransmission in this model of epilepsy. We tested the effects of acethylcholine and charbacol (Ach and Cch, acting on both muscarinic and nicotinic receptors) on intrinsic and synaptic bursting of pyramidal cells in slices obtained from GWS (epileptic) rats. These effects were compared to those observed on regular spiking cells from control (non-epileptic) rats. The respective contribution of muscarinic and nicotinic receptors was assessed using selective agonists methyl-acetylcholine (mAch, muscarinic), nicotine, and the muscarinic antagonist atropine. When applied alone, atropine did not significantly modify the membrane potential of the studied neurons.

All agonists in both epileptic and non-epileptic preparations caused a slow depolarization of the membrane potential associated with an increase of action potential (AP) firing induced by depolarizing current injection and, in most cells, with an increase of the input resistance. This effect which was stronger with nicotine inducing spontaneous AP firing, was considered unrelated to the epileptic behaviour. The excitatory effect of nicotine was associated with an increase of the afterdepolarization following synaptically induced APs. This generated additional APs even if the membrane potential was repolarized by current injection to control levels. In contrast, mAch decreased the afterdepolarization following APs.

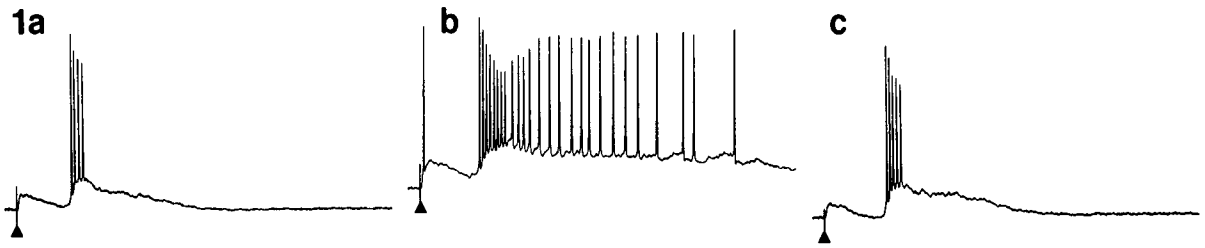


Figure 1. Potentiation by nicotine (**b**) of synaptically induced burst in an epileptic rat (control: **a**). The synaptic bursts persisted even when the membrane potential was brought back to resting level (**c**).

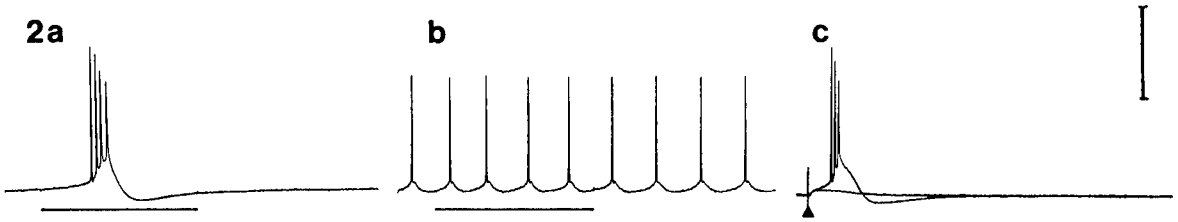


Figure 2. Transformation by mAch of intrinsic bursts induced by depolarizing current injection (**a**) into a tonic AP firing (**b**). **c.** Superimposed synaptically induced responses in control condition (burst) and during mAch application (depression of burst). Horizontal bar: pulse of depolarizing current (250 ms, 0.02 nA). Vertical bar: amplitude (50 mV). Triangles: synaptic stimulation.

In epileptic preparations, synaptic bursting was diminished or blocked by Ach and CCh. This effect was ascribed to muscarinic mechanisms because it was obtained with mAch (figure 2c) and reduced by atropine. When Cch was applied in combination with atropine, we observed a small hyperpolarization of the membrane potential associated with a diminished response to depolarizing current injection and to synaptic stimulation. Contrarily to the other cholinergic agonists, nicotine potentiated synaptic bursts *(figure 1a, b)*: under nicotine, the synaptic bursts persisted when the membrane potential was either depolarized by nicotine or brought back at resting level by current injection *(figure 1c)*.

In bursting pyramidal cells, Ach and Cch increased the number of bursts induced by depolarizing current injection. This effect was also induced by nicotine that favoured the appearance of spontaneous bursting activity. In contrast, mAch, when applied on bursting cells of GWS rats, provoked a transformation of bursts into a tonic AP firing associated with the slow depolarization *(figure 2a,b)*. When mAch was applied in combination with atropine, the membrane potential was not significantly modified and the epileptic bursts were activated instead of being trasformed in a tonic discharge of APs.

These results suggest that both cholinergic receptors are involved in the epileptogenic GWS with opposite effects. The activation of metabotropic muscarinic receptors (by mAch) depressed the 'epileptic like' discharges while the activation of ionotropic nicotinic receptors (by nicotine) facilitated them. The simultaneous activation of both types of receptors (by Ach or Cch) had a complex effect favouring intrinsic bursting and depressing synaptic bursts. Interestingly, Cch was more efficient than Ach and, in some cells, provoked oscillatory variations of the membrane potential associated with an important activation of AP firing. This observation might therefore provide a basis for a cholinergic modulation of the epileptic activity through the simultaneous activation of muscarinic and nicotinic receptors. Our results are also supported by recent findings showing that, in the GABA-injected cortical area, the number of choline acetyltransferase immunopositive neurones is significantly more important in GWS rats than in control rats (Araneda et al., 1994). Given that the cholinergic innervation of the cerebral cortex in normal rats mainly comes from the basal forebrain, the preceding observation suggests the appearance in epileptic rats of neurons neosynthetizing choline acetyltransferase. This work was supported by a grant from ECOS, U95E01.

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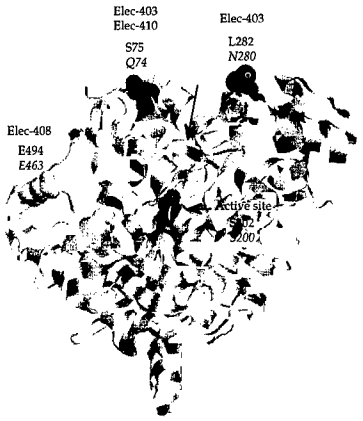
98 Inhibition of *Electrophorus* acetylcholinesterase by monoclonal antibodies

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In acetylcholinesterase (AChE), acetylcholine is guided along aromatic residues to the active site, located at the bottom of the catalytic gorge addin (Sussman et al., 1991). The way of exit of the products is unknown and this led to the hypothesis of a 'back door', opening at the bottom of the gorge (Gilson et al., 1994). However, this was not demonstrated experimentally and none of the known inhibitors binds to this region. AChE activity is irreversibly blocked by active site inhibitors or by ligands that bind to the peripheral site, located at the surface of the protein near the entrance of the gorge.

We studied *Electrophorus electricus* AChE for several reasons. Firstly, it has been used extensively for studies of acetylcholine hydrolysis. Secondly, its catalytic turn-over is the highest among studied AChEs. Thirdly, three monoclonal antibodies that inhibit specifically its catalytic activity have been isolated (Rémy et al., 1995). Two of them recognized overlapping but different epitopes



(Elec-403 and Elec-410); their binding is competitive with that of peripheral site ligands. The third antibody (Elec-408) binds to another site and its inhibitory effect is additive with the other two antibodies, thus defining a new regulatory site on the enzyme. In order to determine the binding sites of these antibodies, we constructed chimeric molecules composed of parts of *Electrophorus* AChE and rat AChE (unrecognized by the antibodies). The sensitivity of these chimeras to the antibodies allows us to define regions implicated in their recognition. By site directed mutagenesis, we have been able to abolish the inhibitory effects of each of the three antibodies. These experiments confirm that two antibodies bind to the peripheral site and recognize distinct epitopes with at least one common residue.The third antibody binds to another part of the enzyme, corresponding to the theoretical 'back door'. We hope that an analysis of the inhibition mechanism of this antibody will allow us to confirm the 'back door' hypothesis and to analyze its possible function during the catalytic cycle.

A ribbon three-dimensional model of Electrophorus AChE. The entrance of the catalytic gorge is indicated by an arrow; the active serine of the catalytic site and residues involved in the binding of the three antibodies are shown in space filling view.

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99 Integrin activation is required for protein tyrosine phosphorylation induced by activation of muscarinic m3 receptors

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Muscarinic receptors are characterized by seven transmembrane domains, and signal by coupling to heterotrimeric GTP binding (G) proteins. Of the five muscarinic receptor isoforms that have been cloned (Peralta et al., 1987; Bonner et al., 1988); three (m1, m3, and m5) stimulate phosphatidylinositol 4,5 bisphosphate (PIP2) breakdown, and two (m2 and m4) inhibit adenylyl cyclase (Peralta et al., 1988; Sandmann et al., 1991). Activation of PIP2-coupled receptors results in hydrolysis of PIP2 to form diacylglycerol and inositol-1,4,5-trisphosphate, with subsequent activation of protein kinase C (PKC). Muscarinic receptors activate other kinases as well, including mitogen-activated protein kinase (Crespo et al., 1994), and tyrosine kinases of the Src family (Wan et al., 1996; Tsai et al., 1997). A variety of proteins are tyrosine phosphorylated following muscarinic receptor activation, including the focal adhesion-associated proteins paxillin and focal adhesion kinase (FAK) (Gutkind and Robbins, 1992; Petryniak et al., 1996).

Focal adhesions are attachment sites found in cultured cells; at these sites the extracellular domains of cell-surface integrins bind to immobilized extracellular matrix (ECM) proteins such as fibronectin (Burridge et al., 1988). Focal adhesion formation is initiated experimentally by the plating of cell suspensions onto immobilized ECM proteins (Burridge et al., 1997), and results in clustering of the integrins, and the association of their intracellular domains with cytoskeletal proteins that anchor polymerized actin filaments (stress fibers) to these sites. A number of signaling proteins are recruited to focal adhesions, including the adapter protein paxillin, and the tyrosine kinase FAK (Clark and Brugge, 1995). Thus focal adhesions have both structural and signaling functions. Focal adhesions are also formed following the addition of growth factors or G protein-coupled receptor ligands to quiescent cells (Ridley and Hall, 1992; Seufferlein and Rozengurt, 1994). Exposure either to immobilized ECM proteins, or to soluble receptor ligands, results in transient tyrosine phosphorylation of similar sets of proteins, including tensin, $p130^{cas}$, paxillin and FAK (Burridge et al., 1997). In fibroblasts, G protein-coupled receptor-mediated tyrosine phosphorylation and focal adhesion formation are dependent on cytoskeletal integrity, on activation of the small molecular G-protein Rho, and on actomyosin contractility (Rankin et al., 1994; Chrzanowska-Wodnicka and Burridge, 1996; Sinnett-Smith et al., 1993).

The experiments described in this report were carried out in human embryonic kidney cells stably expressing muscarinic m3 receptors (HEK-m3). Treatment of serum-deprived monolayers of HEK-m3 cells with the muscarinic receptor ligand carbachol resulted in time- and concentration-dependent increases in tyrosine phosphorylation of the proteins paxillin and FAK (assessed by Western blot). The response approached maximal values within 10 min, and at a carbachol concentration of 1 μM. Although tyrosine phosphorylation of paxillin and FAK elicited by a variety of receptor ligands in Swiss 3T3 cells was reported to be independent of PKC (Sinnett-Smith et al., 1993), the response to carbachol of HEK-m3 cells appeared to be partially dependent on this enzyme, since it was reduced in the presence of 2.5 μM of

the specific PKC inhibitor GF109203X. Tyrosine phosphorylation of paxillin and FAK was also induced, although to a lesser extent, by phorbol 12-myristate 13-acetate, a direct activator of PKC; this response was abolished by the inhibitor.

An identical pattern of tyrosine phosphorylation was induced in serum-deprived HEK-m3 cells that were first placed in suspension, then allowed to attach to culture dishes coated with fibronectin. This response was not observed in cells adhering to dishes coated with poly-D-lysine. In agreement with results from other laboratories, tyrosine phosphorylation was inhibited when cells were allowed to adhere to fibronectin in the presence of soluble peptides containing the amino acid sequence arginine-glycine-aspartate (RGD), which mimics the integrin binding site on ECM proteins such as fibronectin. Interestingly, RGD peptides also reduced carbachol-mediated tyrosine phosphorylation in quiescent monolayers of HEK-m3 cells, suggesting that integrins might be direct downstream mediators of activated muscarinic receptors. Control peptides containing an inactive sequence (RGE) had no effect. The PKC inhibitor GF109203X did not alter tyrosine phosphorylation induced by adhesion to fibronectin, although it reduced the response to carbachol, suggesting that PKC lies upstream of integrins in the signaling pathway initiated by muscarinic receptor stimulation.

If integrin activation is necessary for muscarinic receptor-induced tyrosine phosphorylation, then the response should also require the presence of ECM proteins, which are necessary for integrin clustering and formation of focal adhesions. It is likely that ECM proteins were present in the monolayers used in the preceding experiments, since the cells were grown for several days in culture. Many cell types, including a number of kidney cell lines, secrete fibronectin and assemble it into a matrix; moreover, the $\alpha 1\beta 5$ integrin, which mediates cell adhesion to fibronectin, is expressed by HEK cells (Bodary and McLean, 1990). To test the dependence on ECM proteins of the response to carbachol, cells in suspension were plated on dishes coated with fibronectin or poly-D-lysine, then left undisturbed for 2.5 h, i.e., long-enough for the initial response to fibronectin to subside, but before substantial amounts of fibronectin could be secreted. The cells were then incubated in fresh medium in the absence or presence of carbachol for 20 min, and tyrosine phosphorylation was determined. As predicted by the earlier results, the response to carbachol was greatly attenuated in cells plated onto poly-D-lysine relative to those adherent to fibronectin.

As a final test of the hypothesis that integrins mediate muscarinic receptor signaling, the ability of muscarinic receptors to induce focal adhesion formation (an indicator of integrin activation) was examined using an immunofluorescence approach. Exposure to carbachol for 10 min induced the formation of stress fibers (visualized with fluorescent-conjugated phalloidin) and the appearance of focal adhesions (detected using an antibody to vinculin, a major component of these structures) in HEK-m3 cells.

In summary, the results suggest that stimulation of muscarinic m3 receptors elicits ECM-dependent clustering of integrins, with consequent formation of focal adhesions, and the recruitment and tyrosine phosphorylation of paxillin and FAK. PKC appears to mediate, at least in part, the activation of integrins by muscarinic receptor stimulation. Integrins thus represent novel intermediates in muscarinic receptor-coupled signaling pathways. Supported by NIH grant NS30791. HEK-m3 cells were a generous gift from E. Peralta.

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100 Effect of pyridostigmine administration in mice on the expression of motoneuron β-endorphin and muscle acetylcholinesterase

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There is considerable evidence that β-endorphin and its C-terminal dipeptide derivative, glycylglutamine, can control the expression of acetylcholinesterase (AChE) in cholinergic tissues, including skeletal muscle (Haynes et al., 1984; Haynes and Smith, 1985), heart (Nyquist-Battie and Millington, 1993), and sympathetic ganglion (Koelle, 1983). In previous work we have shown that repeated administration in mice of a low dose of pyridostigmine causes increases in the levels of AChE in skeletal muscle, after the acute inhibitory phase has worn off (Lintern et al, 1997; Lintern et al., 1997). We have also shown that this drug increases the expression of POMC-derived peptides, including β-endorphin, in motoneurons (Amos et al., 1996). Here we have compared the time course of the changes in AChE activity in the diaphragm muscle, and the expression of β-endorphin in motoneurons in the cervical spinal cord, following repeated administration of pyridostigmine, in the same animals.

In another study we showed that repeated treatment in mice with pyridostigmine sensitised the muscle to a subsequent low dose of the drug given 2 weeks after the repeated treatment was discontinued (Lintern et al., 1997). Therefore the effect of a booster dose of pyridostigmine on both muscle AChE and motoneuron β-endorphin was also studied.

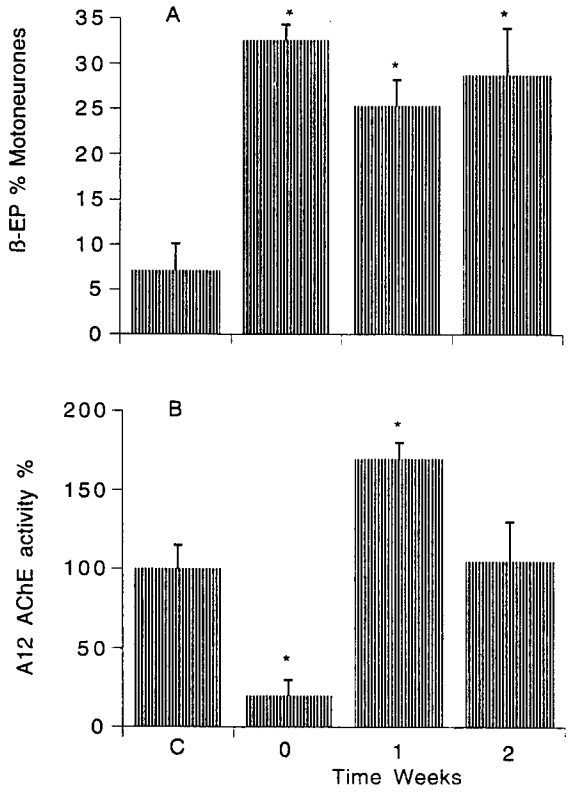


Figure 1. Time course of the changes in **A**, the proportion of β-endorphin immunoreactive motoneurone in cervical spinal cord, and **B**, functional A12 AChE activity in diaphragm muscle, at 24 h (0 weeks) and 1 and 2 weeks, after repeated treatment with pyridostigmine. *Significant compared to controls (**C**).

Pyridostigmine bromide (0.4 μmol/kg) was administered to male BKW (6 month old) mice, twice a day for 3 weeks. In one group a further single low dose (0.4 μmol/kg) of the drug was administered at 2 weeks after cessation of the repeated treatment, and the effects on the levels of AChE in the diaphragm muscle and the incidence of β-endorphin-immunoreactive motoneurons were compared with the effect of a single dose in animals which had had no previous treatment with the drug.

The molecular forms of AChE were separated on a sucrose density gradient as described before (Haynes et al., 1984) and the enzyme activity was determined using the method of Johnson and Russell (1975). Immunoreactivity for β-endorphin was detected in 20 μm histological sections of cervical spinal cord using the immunoperoxidase method as described before (Hughes and Smith, 1994).

At 24 h after the end of the 3-week repeated treatment there was a down-regulation of total AChE activity and the activity of the major component molecular forms, including the functional A12 form, in the diaphragm, followed 1 week later by an upregulation to levels significantly higher than normal *(figure 1)*. The incidence of motoneurons in the cervical spinal cord which were immunoreactive for β-endorphin was extremely low in untreated mice but there was a significant increase in the incidence after the pyridostigmine treatment. However, the increase in β-endorphin immunoreactivity preceded the increase in AChE activity.

In the animals which had been given a further low dose of the drug at 2 weeks after the treatment the AChE activity had initially returned to normal, although the β-endorphin immunoreactivity was still significantly (over four-fold) elevated *(figure 1)*. The effect of the 'booster' dose of the drug in these animals was compared with administration of the same dose of the drug to previously untreated animals. In the pretreated animals the booster dose caused a significant (approximately 130%) increase in the levels of the enzyme in the muscle at 3 h, but when the same single low dose of the drug was administered to untreated mice in which the levels of β-endorphin in the motoneurons was initially extremely low (*figure 1*, controls), the enzyme activity was unchanged at 3 h (although there was a three-fold increase in immunoreactive motoneurons at this time).

Thus after treatment with pyridostigmine when an increase in AChE levels in the muscle was seen, the increases were preceded by an increase in β-endorphin expression in the motoneurons. These findings are consistent with the increased expression of β-endorphin in the motoneuron being a prerequisite for the upregulation of AChE in skeletal muscle. This work has been carried out with the support of CBD Porton Down. (c) British Crown copyright 1998/DERA. Published with permission of the Controller of Her Britannic Majesty's Stationary Office.

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101 Metrifonate affects the peak interval performance in the rat

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Metrifonate is a member of the second generation of cholinesterase inhibitors (Giacobini, 1991) that has been found to be a promising therapeutic agent to combat Alzheimer's disease (Becker et al., 1990). In a number of studies metrifonate has been shown to improve the cognitive performance in various behavioural models (Schmidt et al., 1997). In this study the effect of metrifonate on timing behaviour was assessed. The rats were trained on a discrete-trial 20 s peak interval (PI) timing procedure in ten identical Skinnerboxes.

The PI procedure is a modification of the standard fixed interval procedure of reinforcement (Catania, 1970; Roberts, 1981). On a random half of the trials a lever is presented and the animal is free to respond at any time, but only the first lever-press response after 20 s is reinforced with a 45 mg food pellet. On the remaining trials, the peak trials, no food is given and the lever remains available until 50 s have elapsed. Then the lever is retracted for 10 seconds before the next trial starts. On the peak trials, response rate of trained animals increases to a peak, then the response rate decreases.

The effects of a single administration of metrifonate (10 mg/kg and 30 mg/kg) on responding on a 20 s peak procedure were assessed in Harlan Wistar rats. The animals had received six daily sessions on this schedule prior to drug administration. Metrifonate was administered orally 30 min before the seventh session started. Data were collected for each rat only on peak trials and sessions lasted 30 min.

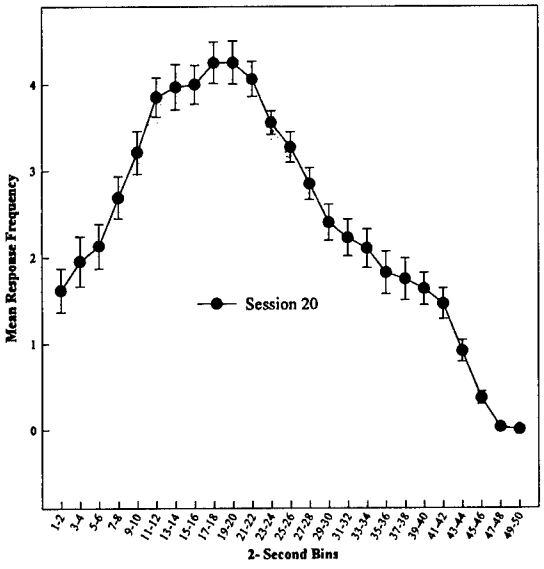


Figure 1. The effects of metrifonate in doses of 10 mg/kg, 30 mg/kg and vehicle (sodium citrate buffer) on response frequency during the 20 s peak procedure. Results are ± S.E.M., n = 9–10 per group.

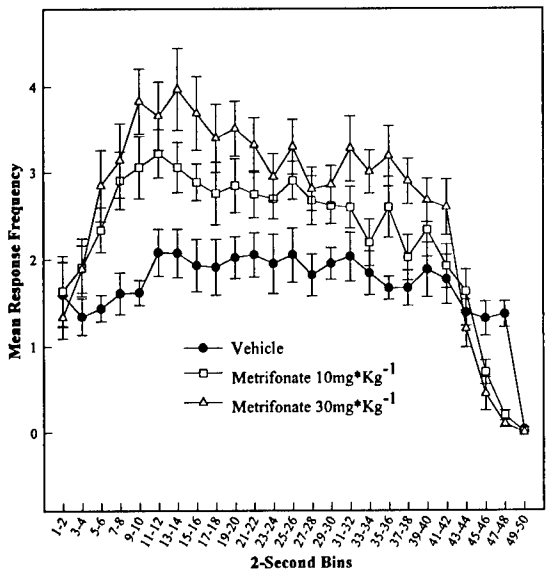


Figure 2. Mean response frequency ± S.E.M. (n = 30) during the 20 s peak procedure at session 20.

Metrifonate treatment increased the response frequency, averaged over all bins (general mean: F2, 26 = 8.98, $P < 0.01$). Post hoc comparison (LSD) revealed that the response frequencies of both the groups of rats treated with 10 and 30 mg/kg metrifonate differed from the vehicle-treated group. The distribution of responses was also affected by the metrifonate treatment (treatment by BIN interaction: F48,624 = 3.11, $P < 0.01$). Metrifonate-treated rats showed more of a peak, and this effect appeared to be dose-dependent.

The effects seen by acute metrifonate were achieved when the animals had not yet reached optimal level of performance (i.e., the maximum response rate was not at 20 s).

Training continued to demonstrate that the animals could reach an optimal level of performance. The animals achieved this after 20 sessions (see *figure 2*), the maximum response rate being at 19–20 s.

The behaviour of a rat in time discrimination procedures, such as the peak procedure is thought to be understood in the terms of an information processing model, which is to measure, compare and store the expected time of reinforcement. Shifts in the peak functions are thought to be used as evidence in the remembered time of reinforcement (Meck and Church, 1988). The present study suggests that metrifonate might improve acquisition of the timing task. Further study is required with subchronic metrifonate to determine whether metrifonate would also facilitate the rate of acquisition of the timing task in analogy with the previously reported improved acquisition of eye-blink conditioning in aging rabbits (Kronforst-Collins et al., 1997)

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102 Cholinergic/monoaminergic markers in the brain, and behavioral effects of cholinergic and serotonergic drugs in aged rats with cognitive deficits

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Compared to young adult rats, rats aged of 20 months or more show a more or less pronounced impairment in a variety of learning/memory tests (e.g., Ingram et al., 1994). Numerous studies suggest that cholinergic dysfunctions might be involved in such deficits (e.g., Collerton, 1986). In the present study, 3-month- and 24-month-old Long Evans female rats were used. In a water maze task, we first assessed spatial reference memory deficits (acquisition and probe trial) and constituted a population of aged rats showing moderate to severe impairment. Using HPLC detection, we then determined the concentration of acetylcholine (ACh), norepinephrine (NE), serotonin (5-HT) and a few metabolites (DOPAC, HVA and 5-HIAA), in the striatum, the hippocampus, the frontal, occipital and entorhinal cortex after killing by microwave irradiation (1.4 s, 4.5 kW). Finally, a separate sample of rats was tested in a water maze for working memory performance in order to assess the effects of the muscarinic antagonist scopolamine (0.2 and 0.5 mg/kg, i.p.), the muscarinic agonist pilocarpine (0.5 mg/kg, i.p.), the 5-HT_{1A} agonist 8-OH-DPAT (0.1, 0.3 and 1 mg/kg, i.p.), the α1 antagonist prazosin (1 mg/kg, i.p.) and the 5-HT_{1A} antagonist NAN 190 (2 mg/kg, i.p.). Drugs were also used in combination (e.g., scopolamine or NAN 190 with pilocarpine).

On the 2nd, 3rd, 4th and last testing days of the reference memory task, the average distance to reach the platform was significantly higher in aged as compared to young rats (data not shown). In the probe trial, young rats spent a signicantly longer time in the platform-quadrant than aged rats (25.8 ± 1.7 s and 18.3 ± 1.1 s, respectively, $P < 0.001$). The neurochemical results for which significant differences were found are shown in table I. In aged rats, significant correlations could be found between water maze performance (probe trial) and: ACh in the striatum (r = 0.45, $P = 0.05$), 5-HT in the dorsal hippocampus (r = 0.53, $P = 0.02$), 5-HIAA in the frontal cortex, dorsal hippocampus and striatum (r = 0.47 at least, $P = 0.039$ at most), 5-HIAA/5-HT ratios in the striatum and frontal cortex (r = 0.46, $P = 0.046$ and r = 0.51, $P = 0.034$, respectively), and NE in the dorsal hippocampus (r = 0.57, $P = 0.016$).

The psychopharmacological data are shown in *table II*. Briefly, 0.1 mg/kg scopolamine HBr, 0.5 mg/kg MBr, 0.5 mg/kg pilocarpine and 0.1 mg/kg 8-OH-DPAT induced no significant modification of performances. At 0.5 mg/kg, scopolamine HBr produced a significant deficit in young and aged rats, but the deficit found in young rats under scopolamine treatment did not exceed that found in aged rats given saline injections. NAN 190 and 0.3 mg/kg 8-OH-DPAT worsened performances, but only in aged rats. At 1.0 mg/kg, 8-OH-DPAT impaired performances in young and aged rats. Prazosin improved performances in young and

Table I. Neurochemical markers (values are ng/mg microwave-irradiated tissue).

Marker	*Brain region*	*Young*	*Aged*
ACh	Striatum	2.78 ± 0.40	1.72 ± 0.07
DA	Dorsal hippocampus	0.72 ± 0.09	0.39 ± 0.04
5-HT	Striatum	3.88 ± 0.35	3.10 ± 0.12
	Occipital cortex	2.04 ± 0.21	1.64 ± 0.08
5-HIAA/5-HT	Frontal cortex	0.68 ± 0.02	1.03 ± 0.09
	Dorsal hippocampus	1.03 ± 0.05	1.65 ± 0.17
	Ventral hippocampus	0.92 ± 0.03	1.51 ± 0.16
	Entorhinal cortex	0.45 ± 0.05	0.73 ± 0.08
HVA	Striatum	3.37 ± 0.16	2.61 ± 0.14
NE	Striatum	1.15 ± 0.12	0.84 ± 0.04
	Dorsal hippocampus	1.80 ± 0.12	1.40 ± 0.08
	Ventral hippocampus	1.82 ± 0.09	1.43 ± 0.06

Table II. Distances (in cm ± S.E.M.) to reach the platform in the working memory task (four daily trials with a new location of the platform each day) under various drug treatments (doses given in parentheses are in mg/kg, i.p.).

Drug condition	*Young (n = 11)*	*Aged (n = 41)*
Saline 0.9%	208.5 ± 19.1	413.1 ± 26.3
Scopolamine MBr (0.5)	206.6 ± 32.2	306.8 ± 35.9
Scopolamine HBr (0.2)	268.4 ± 36.8	451.2 ± 37.4
Scopolamine HBr (0.5)	394.0 ± 70.9	706.4 ± 39.1
Pilocarpine (0.5)	188.8 ± 16.1	350.8 ± 34.9
NAN 190 (2.0)	253.5 ± 11.8	638.3 ± 38.0
8-OH-DPAT (0.1)	178.3 ± 26.2	409.5 ± 33.0
8-OH-DPAT (0.3)	251.1 ± 31.0	519.3 ± 34.0
8-OH-DPAT (1.0)	496.3 ± 78.8	747.2 ± 44.3
Prazosin (1.0)	158.9 ± 8.2	315.8 ± 24.0
Scopolamine (0.5) + Pilocarpine (0.5)	375.6 ± 41.1	561.6 ± 29.5
Scopolamine (0.5) + NAN 190 (2)	634.7 ± 76.7	730.1 ± 38.0
Scopolamine (0.2) + 8-OH-DPAT (0.1)	410.3 ± 68.1	664.2 ± 36.9
Pilocarpine (0.5) + NAN 190 (2)	252.8 ± 31.1	381.4 ± 27.6

aged rats. Pilocarpine attenuated the scopolamine-induced impairments, but only in aged rats. In young rats, NAN 190 potentiated the scopolamine-induced deficits. In both groups, the combination of 8-OH-DPAT and scopolamine at ineffective doses (0.1 and 0.2 mg/kg, respectively) induced clear-cut impairments which were more pronounced in aged than in young rats. The combination of pilocarpine and NAN 190 had no significant effect.

In conclusion of these experiments, we confirm that aging results in (variable degrees of) cognitive impairment. Although the water maze task is particularly sensitive to disruption of spatial memory processes and despite a crucial involvement of the hippocampus in such mnesic processes, but also of cholinergic function in learning and memory, we failed to find any significant correlation between water maze performances and cholinergic (but also other) markers in the hippocampus (but also cortical regions). The only significant correlation found between memory performances and ACh concentration was in the striatum, a finding suggesting that the age-related cholinergic changes in the striatum might play an important role in the behavioral deficits observed. From our psychopharmacological data it is also clear that the cognitive deficits observed are not an exclusive matter of cholinergic dysfunction: injections of NAN 190 and 8-OH-DPAT were more efficient in aged as compared to young rats, as was the case for combined injections of scopolamine and 8-OH-DPAT at subamnestic doses.

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103 Human acetylcholinesterase transgenic mice show elevated muscle expression and brain specific silencing

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To study the regulation of AChE expression in the mammalian brain and muscle, novel transgenic FVB/N mouse lines were created with several variants of human AChE. The human ACHEcDNA constructs that were employed for this study were CMV I4-ACHE, encoding for the 'readthrough' AChE isoform C-terminated with the pseudointron I4-derived hydrophylic peptide (Karpel et al., 1996) and CMV InE6-ACHE which codes for the insertion-inactivated isoform C-terminated with the exon 6-derived peptide but with no capacity to hydrolyze ACh (Sternfeld et al., 1998). The properties of these transgenic lines were compared to those of the human promoter (Hp) E6-AChE transgenic line which overexpresses the brain and muscle-abundant isoform carrying the exon 6-derived amphipathic C-terminal peptide (Beeri et al., 1995) and of control FVB/N mice. Each of these lines displayed unimpaired Mendelian inheritance of the transgene for over 5 generations, showing that it is not lethal. Kinetic followup of PCR amplifications reflecting copy number of the transgene revealed similar copy number for the two independent pedigrees with CMV I4-AChE transgenes and a different copy number for the line with HpE6-AChE and CMV InE6-AChE line. The efficacy of AChE expression in brain and muscle tissues from these transgenic mice was evaluated by ATCh hydrolysis rates in tissue homogenates in the presence and absence of the specific BChE inhibitor IsoOMPA. These measurements demonstrated drastic differences in the specific activity of muscle AChE. While InE6-AChE transgenics revealed activities 1.6-fold higher than controls, the two I4 AChE lines displayed muscle AChE activities 25- and 350-fold of control mice. In contrast to the drastic variabilities in muscle AChE levels, brain AChE specific activities remained grossly similar in all of these transgenic lines. These ranged from 95% of control activity for InE6-AChE transgenics to 140% and 131% of control for the two I4 AChE transgenic lines. HpE6-AChE transgenics revealed the highest values, reaching activities 1.5-fold higher than the brain activity in control FVB/N mice (Beeri et al., 1995). These results demonstrate considerable permissivity of muscle AChE expression variations but maintainance of brain AChE levels within a very narrow activity window. The finding that two mouse pedigrees carrying apparently similar copy numbers of the I4 AChE transgene under control of the CMV promoter show such different levels of AChE overexpression may reflect position effects of the insertion of these transgenes into the host mouse genome. This overexpression apparently does not interfere with neuromuscular properties, which is compatible with the non-synaptic localization of I4 AChE as well as with its minimal effect on neuromuscular junction development (Seidman et al., 1995; Sternfeld et al., 1998). Within the same mouse pedigrees with massive muscle AChE increases, variations in brain AChE activity were remarkably limited. It is likely that the viability of these transgenic pedigrees depended on minimal changes in their brain AChE levels, so that the only lineages that survived were those where the transgene's expression in the brain was effectively silenced. This suggests that the upstream domains adjacent to the two I4 AChE transgenes should include effective brain silencing in addition to muscle enhancing elements. These newly created transgenic models should therefore be most valuable for testing the biological effects of AChE overproduction and sustained inhibition and for exploring the molecular mechanisms controlling both the expression patterns of these transgenes and the ability of the host mice to accommodate these changes.

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104 Role of Ca^{2+} and NO in differential effects of extra and intracellular aluminum on acetyl-CoA and acetylcholine metabolism in rat brain nerve terminals

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Excessive accumulation of aluminum (Al) and increased NO synthesis may coexist in brains affected by Alzheimer's and Parkinson's diseases, dialysis encephalopathy and by many other neurodegenerative brain diseases (Davson and Davson 1996; Meiri et al., 1993). Bulk Al concentrations in diseased brains were reported to be within 0.1–0.5 mM, whereas its intracellular level was likely to reach 10 mM range. Al forms slowly dissociating complexes with several extra- and intracellular sites that function

dependent on binding with Ca. Thereby, Al may interfere with several Ca-dependent steps of cellular metabolism. Also, extensive NO production was found to disrupt Ca^{2+} homeostasis in neurons (Brorson et al., 1997). Our studies have shown that Al inhibited, and NO activated acetyl-CoA and acetylcholine (ACh) metabolism in nerve terminals (Bielarczyk et al., 1998; Tomaszewicz et al., 1997). Therefore, in this work we aimed to find out how simultaneous action of both pathogens may affect metabolism of acetyl moiety of ACh in this neuronal compartment and if it may be linked with changes in Ca^{2+} homeostasis.

Synaptosomes isolated from rat brains were incubated in depolarizing medium (30 mM KCl) while mitochondria in sucrose-potassium medium using 2.5 mM pyruvate and 2.5 mM L-malate as energy substrates for 30 and 15 min, respectively (Bielarczyk et al., 1998). In such conditions, Al did not pass neuronal plasma membrane (Shi, Haug, 1990). In Ca-free medium 0.25 mM Al inhibited pyruvate utilization (28%), decreased acetyl-CoA content in synaptoplasm (25%), increased resting ACh release (50%) but did not change total content intrasynaptosomal Ca, which was equal to about 3 nmol/mg of protein. In the same conditions 0.2 mM sodium nitroprusside (SNP) had no effect on these metabolic parameters. Hence, extracellular Al itself may exert some Ca-like effects increasing nonquantal ACh release from terminals while NO apparently had no such capacity due to the lack of Ca.

Addition of 1 mM Ca increased intrasynaptosomal Ca content from 3.3 to 33 nmol/mg of protein and caused 40% inhibition of pyruvate utilization but simultaneously increased synaptoplasmic acetyl-CoA level (50%) and ACh release from synaptosomes (170%). In these conditions 0.25 mM Al decreased bulk Ca level in synaptosomes (40%) with no change in pyruvate utilization (not shown). Accordingly, it decreased synaptoplasmic acetyl-CoA (29%) and inhibited overall and Ca-evoked ACh release by 39 and 80%, respectively *(figure 1)*. On the other hand, 0.25 mM SNP increased synaptoplasmic acetyl-CoA (51%) as well as total and Ca-evoked ACh release (about 40%) *(figure 1)*. Al reversed SNP effects bringing synaptoplasmic acetyl-CoA and ACh release back to control values. Al-evoked decrease of synaptosomal Ca was likely to result from inhibition of voltage-dependent, verapamil sensitive Ca channels (Bielarczyk et al., 1998). It would inhibit Ca-dependent direct transport of acetyl-CoA from mitochondria yielding inhibition of ACh synthesis as well as directly by suppression of its quantal release. On the other hand, impairment of energy metabolism by NO led to the rise of synaptoplasmic Ca which activated efflux of acetyl-CoA from mitochondria and increased its utilization for ACh synthesis and subsequent release (Brorson et al., 1997, Tomaszewicz et al., 1997). Inhibition of energy production by NO along with increased utilization of acetyl-CoA for ACh synthesis in cholinergic synaptosomes can make them particularly susceptible to deletorious effects of this compound. Reversal NO effects by extracellular Al is in accord with reciprocal action of both compounds in changes of Ca homeostasis in nerve terminals (Bielarczyk et al., 1998; Brorson et al., 1997).

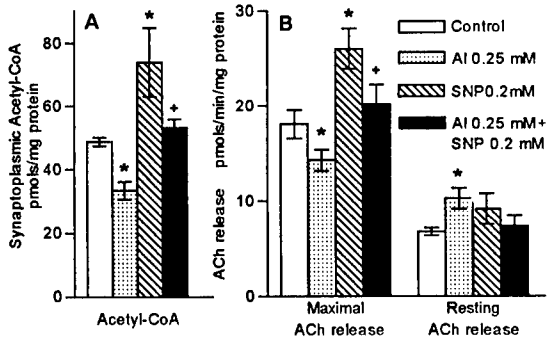


Figure 1. A. Effect of Al and SNP on acetyl-CoA content in synaptoplasm of depolarized synaptosomes incubated at 1 mM [Ca]. **B**. ACh release from depolarized synaptosomes incubated in the absence (resting release) and in the presence of 1 mM Ca (maximal release). Ca-evoked release was defined as a difference between respective maximal and resting ACh releases (not shown). Results are means ± SEM of five to 15 experiments performed in duplicate. $^{*}P < 0.05$, compared with respective control; $^{+}P < 0.05$, compared with respective 0.25 mM Al. All maximal ACh release values were different from respective resting release values.

Isolated mitochondria were used to study possible effects of intracellular Al. It has been found that Al may be transported through the plasma membrane as Al-transferrin complex (Shi and Haug, 1990). In Ca-free medium containing 5 mM Pi and 0.02 mM EDTA, 0.5 mM Al decreased pyruvate utilization (33%, not shown) and acetyl-CoA content (55%) but did not affect total CoA level. Addition of 0.01 mM Ca suppressed pyruvate utilization (36%) as well as acetyl-CoA (29%) and CoA (10%) contents *(figure 2)*. In these conditions Al increased $[Ca^{2+}]$ in the medium from 0.3 μM to 6.8 μM and intramitochondrial Ca content from. 21 to 29 nmol/mg of protein along with inhibition of pyruvate utilization (19%), decrease of acetyl-CoA (38%) and no change in CoA level. Addition of 0.2 mM SNP caused inhibition of pyruvate utilization as well as depression of acetyl-CoA (about 40%) and increase of CoA (about 50%) content irrespective of Ca presence in the medium *(figure 2)*. These data indicate that cytoplasmic Al itself, unlike Ca, did not increase permeability of the mitochondial membrane to acetyl-CoA/CoA. However, it was likely to increase cytoplasmic $[Ca^{2+}]$ due to competition with Ca-binding sites. This phenomenon together with inhibition by Al of Na/Ca antiporter might lead to overloading mitochondria with Ca and loss of CoA derivatives from this compartment. On the contrary, effects of NO on mitochondrial actetyl-CoA/CoA seem to be Ca-independent, although NO was also able to increase $[Ca^{2+}]$ in neuronal cytoplasm (Brorson et al., 1997).

Hence, in brain in vivo, extracellular Al is likely to exert Ca/NO-antagonistic effects on acetyl-CoA and ACh metabolism in nerve terminals, whereas intracellular Al would exacerbate symptoms of Ca and NO toxicity due its ability to increase $[Ca^{2+}]$ in neuronal cytoplasm. Therefore, final effect of Al accumulation on acetyl-CoA and Ach metabolism in the brain may depend on both, absolute Al concentrations and its ratios in extra and intracellular compartments.

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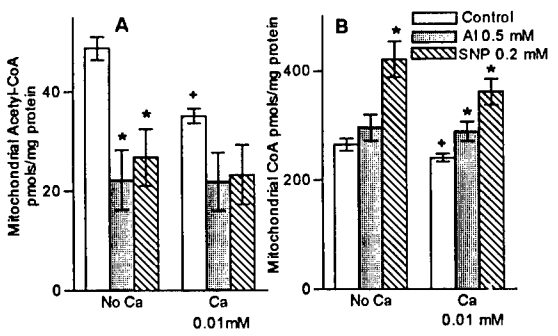


Figure 2. Effect of Al and SNP on acetyl-CoA (**A**) and CoA contents (**B**) in whole brain mitochondria. Results are means ± S.E.M. of five to 15 experiments performed in duplicate. $^{*}P < 0.05$, compared with respective control; $^{+}P < 0.05$, compared with respective no Ca conditions.

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105 Acetylcholinesterase of *S. mansoni* (Trematode). Interaction of globular species with glycosaminoglycans

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Vertebrate acetylcholinesterase (AChE, EC 3.1.1.7) occurs in multiple molecular forms, both asymmetric (A) and globular (G) (Massoulié et al., 1993). In invertebrates, the diversity of AChE molecular forms is more limited than in vertebrates (Massoulié et al., 1993; Talesa et al., 1995) and seems to be restricted to G forms. The existence of several molecular forms reflects primarily differences in their mode of attachment at the cell surface. The A forms of AChE have been reported to interact with heparin, a sulfated glycosaminoglycan (Brandan et al., 1984), and this interaction is responsible for their attachment to the basal lamina within the synaptic cleft (Brandan et al., 1986). However, several studies have reported interaction of G forms with heparin, although less strongly than the A forms (Ramirez et al., 1990; Sine et al., 1994; Talesa et al., 1995), and it has been suggested that they may also be associated with the basal lamina.

In this study the interaction of AChE (E C 3.1.1.7) and heparin was studied by affinity chromatography. In the parasite, *S. mansoni*, AChE is present throughout the life cycle (Arnon, 1991). As in vertebrates, it may play a role in termination of synaptic transmission at cholinergic synapses (Barker et al., 1966), but is also present on the tegument, where its biological role remains to be established (Tarrab-Hazdai et al., 1984; Camacho et al., 1994). At early stages of the life cycle, viz. in the cercaria and schistosomula, AChE appears as two principal molecular forms, both G forms, in approximately equal amounts, with sedimentation constants of 6.5 and 8 S (Espinoza et al., 1988; Camacho et al., 1994). The 6.5 S form, which is amphiphilic, is solubilized by bacterial phosphatidylinositol-specific phospholipase C (Espinoza et al., 1988, 1991), and, most likely, is homologous to the GPI-anchored G_2 form found in Torpedo electric organ and elsewhere (Silman and Futerman, 1987). Both forms are fully solubilized by the non-ionic detergent, Triton X-100. Upon passing such a detergent extract over a heparin-agarose column, only the 8 S form was retained on the column *(figure 1)*. The bound AChE could be progressively eluted by increasing the salt concentration, ca. 0.5 M NaCl being needed for complete elution. Monoclonal antibodies, raised against *S. mansoni* AChE (Espinoza et al., 1995), also distinguished between the two forms. Thus mAb SA7 bound the 6.5 S form selectively, whereas mAb SA57 recognized the 8 S form. Selective inhibition experiments, carried out on live parasites with the covalent AChE inhibitor, echothiophate (phospholine), which does not penetrate the tegument, selectively inhibited the 6.5 S form, thus suggesting an internal location for the 8 S form (Camacho et al., 1994). The selective binding of

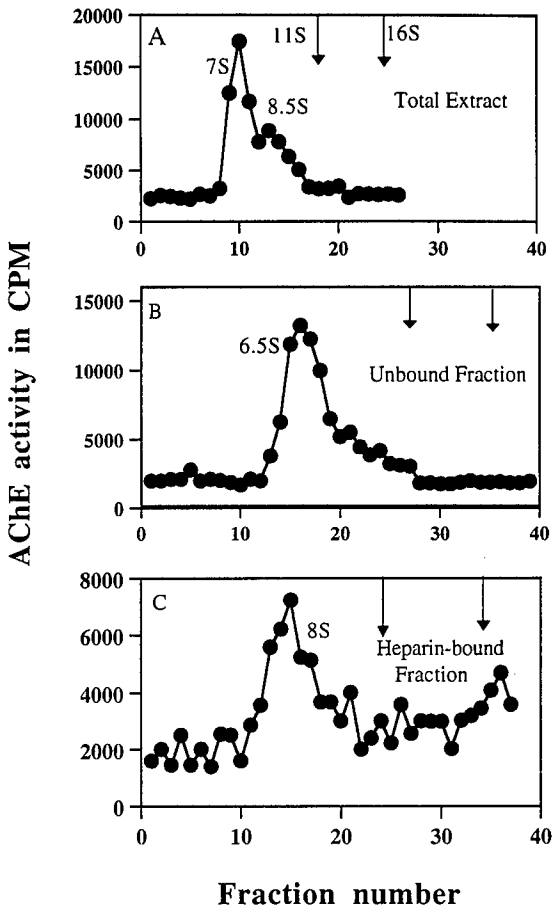


Figure 1. Differential binding of molecular forms of AChE from *S. mansoni* to an heparin-agarose column. A total extract of cercaria in 1% Triton X-100/0.05 M Tris hydrochloride, pH 7.5, was applied to a heparin-agarose column. Bound AChE activity was eluted with a 0-1 M NaCl gradient in the same buffer containing 0.1% Triton X-100 and pooled. Samples were analyzed by sucrose gradient centrifugation on 5–20% sucrose containing 0.1 % Triton X100-0.05 M Tris hydrochloride, pH 7.5. **A)** Total extract; **B)** Undbound fraction; **C)** Heparin-bound fraction. The arrows denote the positions of the markers: catalase (11 S) and β-galactosidase (16 S).

the 8 S form to heparin suggests that, within the parasite, it may be associated with the extracellular matrix at cholinergic synapses.

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106 A brain slice model of seizure activity induced by an organophosphorus anticholinesterase

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We are using an in vitro brain slice model to investigate the mechanisms of seizure activity induced by the organophosphorus anticholinesterase soman and to evaluate the effectiveness of potential anticonvulsant drugs against these seizures. Unlike previously-reported slice studies with nerve agents (Boyer et al., 1989; Lebeda and Rutecki, 1985; Sarvey and Williamson, 1985a; Williamson and Sarvey, 1985), this model contains the entorhinal cortex as well as the hippocampus. This allows the study of the spread of seizure discharges within the limbic system, and the development of prolonged, sustained discharges that are rarely seen in the simple hippocampal slice preparation (Nagao et al., 1996).

Male Dunkin-Hartley guinea pigs (180–240 g) were anaesthetised with halothane and killed by decapitation. The brain was removed and 400 μm thick horizontal slices were cut from a block of tissue containing the hippocampus and entorhinal cortex using a Vibratome. Experiments were performed in a Haas type interface chamber. The tissue was perfused with artificial cerebral spinal fluid (ACSF, composition in mM: NaCl 125; KCl 5; $MgCl_2$ 1; NaH_2PO_4 1.25; $NaHCO_3$ 26; $CaCl_2$ 2; glucose 10; pH 7.4, gassed with 95% O_2–5% CO_2) and maintained at 34 °C. Extracellular field potential responses were recorded using glass microelectrodes filled with 2 M NaCl (2–10 MΩ resistance).

Perfusion with soman (1 μM) induced a second population spike in the evoked field potential in CA1 or CA3 within 15–20 min. In almost all slices tested, this developed into spontaneous seizure activity within 30–40 min of soman application. As well as interictal bursts, many slices also showed longer periods of high frequency bursting analogous to ictal seizure activity (figure 1). This activity appeared similar to that induced by the muscarinic agonist pilocarpine in this preparation (Nagao et al., 1996). Both the second population spike and the spontaneous discharges could be blocked by diazepam, as previously reported (Boyer et

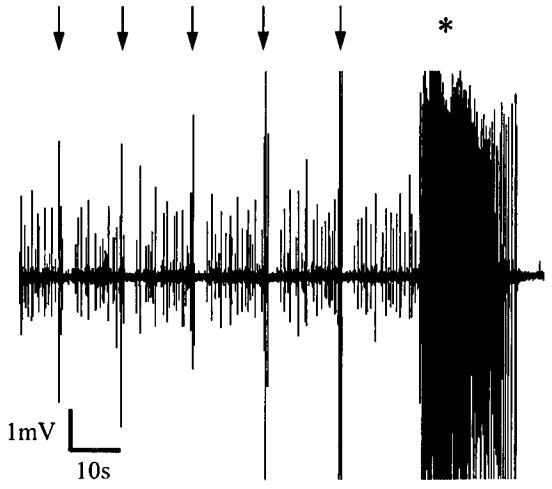


Figure 1. Epileptiform activity recorded in CA1 of a slice perfused with soman (1 μM). As well as simple interictal-like bursts, many slices also showed longer periods (1 s or more) of high frequency bursting (arrows), each followed by a period of inhibition of the simple discharges. This figure also shows a long-lasting episode (nearly 20 s) of high frequency bursting (asterisk), analogous to ictal seizure activity, which was followed by a silent period of 10s. The sustained activity was not seen in CA1 when a cut was made between CA3 and the entorhinal cortex (not shown).

al., 1989; Sarvey and Williamson, 1985; Williamson and Sarvey, 1985b), and by the AMPA/kainate antagonists CNQX and DNQX, but not by the non-competitive NMDA antagonist MK-801. We are now testing the effects of other anticonvulsant drugs on soman-induced epileptiform activity in this preparation. Supported by the Ministry of Defence. (c) British Crown Copyright 1998 / DERA. Published with the permission of the Controller of Her Britannic Majesty's Stationery Office.

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107 His 186 requires the presence of the α192–193 disulfide bridge to be able to interact with α-bungarotoxin

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Multiple binding sites participate in the interaction between the nicotinic acetylcholine receptor (nAChR) and α-bungarotoxin (α-BgTx) (Conti-Tronconi et al., 1994).

We have previously reported the involvement of two histidine residues in such interaction (Lacorazza et al., 1992, 1996a, 1996b) and that His 134, which belongs to the cysteinyl loop α124–127, being one of them (Venera et al., 1997).

In this work we study the possible role of His 186, included in the α185–200 toxin binding determinant. A peptide with the *Torpedo californica* α173–202 sequence (TcH) was synthesized and His 186 was ethoxiformylated (TcH-E) or substituted by Ala (TcA). Oxidation with thalium trifluoroacetate was performed in order to establish the α192–193 vecinal disulfide bridge. Effects of the modifications were evaluated by: a) a solid phase assay; and b) a competition assay between each peptide and the *Torpedo californica* receptor, in solution.

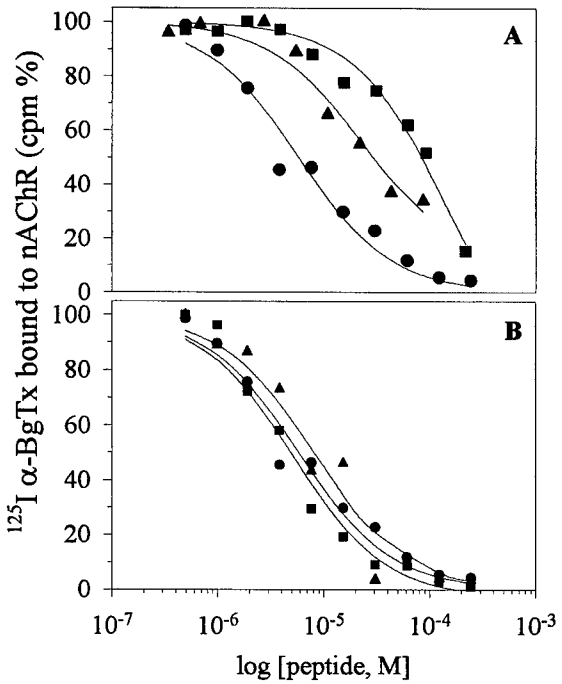


Figure 1. Competition of *Torpedo californica* peptides TcH (●), TcH-E (▲) and TcA (■) for ^{125}I α-BgTx. Peptides with (**A**) and without (**B**) the 192–193 disulfide bridge established, were incubated overnight at 4 °C with 2 pmol of ^{125}I α-BgTx prior to the addition of 1 pmol of *Torpedo californica* receptor.

Peptide ethoxiformylation and the substitution of His 186 by Ala led to an α-BgTx binding decrease of 60 and 90%, respectively, in dot assays. IC50 values, calculated from the competition assays, were (3.94 ± 0.82) 10^{-6} M, (2.25 ± 0.73) 10^{-5} M and (1.48 ± 0.41) 10^{-4} M, for TcH, TcH-E and TcA respectively. Fourth derivate spectra of native and modified peptides were superimposable thus indicating the absence of any conformational change responsible for the lack of toxin binding.

It should be pointed out that the α173–202 peptide binds the toxin even though the 192–193 disulfide bridge is reduced. However, the effect of both modifications occurs only when the bridge is present.

Results lead to the conclusion that peptide behaviour depends on the oxidation state of cysteines 192 and 193 and that participation of His 186 in the receptor-toxin interaction requires the existence of the disulfide bridge, as is the case in the native receptor.

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108 Effect of nerve growth factor and other diffrentiating agents on expression of enzymes of acetyl-CoA and acetylcholine metabolism in SN56 cholinergic cells

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Provision of acetyl-CoA from mitochondria to cytoplasm has been found to play a key role in regulation of acetylcholine (ACh) release and synthesis in depolarization-activated cholinergic neurons. We suppose that competition between demand for acetyl-CoA for energy production and for transmitter synthesis may be one of the sources of preferential impairement of cholinergic neurons in course of several encephalopathies (Szutowicz et al., 1996). However, various populations of cholinergic neurons in the brain display distinct individual differences in their vulnerability to particular degenerative conditions. It may be due to different ratios between their capacities to synthetize acetyl-CoA and to distribute it for energy production and ACh synthesis (Szutowicz et al., 1996). Therefore, in this study we aimed to demonstrate that treatment of one line of septal cholinergic neurons with various differentiating factors is capable to generate several cell phenotypes of different expression of enzymes of acetyl-CoA and ACh metabolism.

Hybrid cholinergic neurons SN56.B5.G4 from mouse septum were grown in culture for 3 days in Dulbecco's modified Eagle's medium containing fetal bovine serum supplemented with differentiating compounds at 37 °C in atmosphere 95% air and 5% CO_2.

Nerve growth factor (NGF) is known to exert differentiating and protective influence on brain cholinergic neurons through its high affinity trkA receptors (trkAR) (Lindsay et al., 1994). We did not find trkAR in SN56 cells. Immunofixation study revealed presence of low affinity p75 binding sites of yet unknown significance. Addition of 1 to 100 ng/mL NGF to culture medium caused concentration-independent inhibition of pyruvate dehydrogenase (PDH) and ATP-citrate lyase (ACL) activities up to 40% and 30%, respectively *(figure 1)*. Activities of choline acetyltransferase (ChAT) and acetylcholinesterase (AChE) were not affected *(figure 2)*. Adenylate cyclase system has been found to cause differentiation of cholinergic neurons (Berse and Blusztajn, 1997, Szutowicz et al., 1983). Addition of 1 mM dibutyryl cAMP (dbcAMP) resulted in 110% and 37% increases in ChAT and ACL as well as 29% inhibition of PDH activity, respectively. NGF (100 ng/mL) modified none of effects of dbcAMP *(figures 1, 2)*. Stimulation of transcription factor by 0.001 mM all-trans-retinoic acid (RA) (Berse and Blusztajn, 1997) brought about 145% increase of ChAT and 15 and 20% suppression of ACL and AChE activities, respectively. PDH activity did not change. In these conditions NGF caused 50% inhibition of PDH activity and partially reversed inhibitory influence of RA on ACL and its activatory effect on ChAT activity *(figures 1, 2)*. RA and dbcAMP used together had an additive effect on ChAT activity wich rose up to 405% of control value. On the other hand, RA partially reversed activatory effect of dbcAMP on ACL. RA did not affect inhibitory and activatory influences of dbcAMP on PDH and AChE activities, respectively. Addition of NGF to the medium containing RA and dbcAMP caused inhibition of ACL and PDH but no modification in expression of cholinergic phenotype *(figures 1, 2)*.

L-carnitine was found to enhance functions of cholinergic neurons both in vivo and in culture presumably by improvent of acetyl-CoA supply to cytoplasmic compartment (Nalecz and Nalecz, 1996). In SN56 cells, it caused concentration-dependent increases of PDH, ACL, ChAT and AChE activities. The highest 72% and 47% rises of ChAT and ACL activities took place at 0.1 mM L-carnitine. The increase of L-carnitine concentration caused gra-

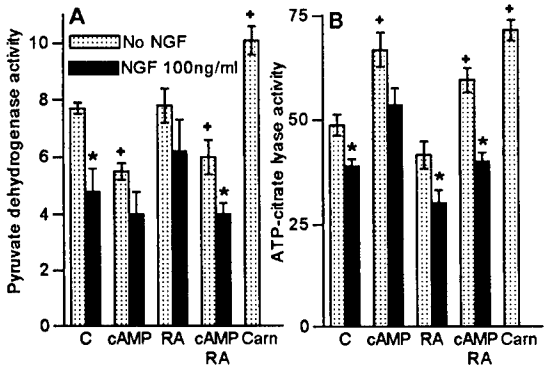


Figure 1. Effect of NGF on specific activities (nmol/min/mg of protein) of PDH (**A**) and ACL (**B**) in SN56 cells non-differentiated (C) or differentiated with 1 mM db-cAMP (cAMP) and 0.001 mM retinoic acid (RA). Effect of 0.1 mM (ACL) or 1.0 mM L-carnitine (Carn). Results are means ± S.E.M. of 6–12 experiments. $^{*}P < 0.05$ compared with respective no NGF conditions; $^{+}P < 0.01$ compared with respective control (C).

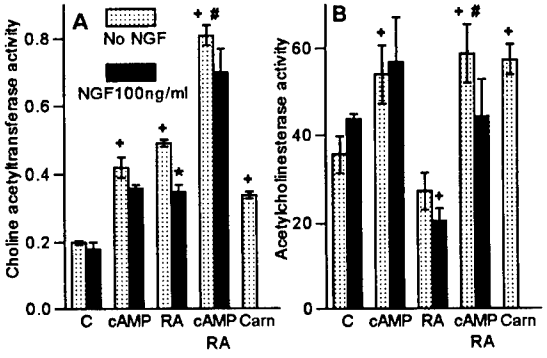


Figure 2. Effect of NGF on specific activities (nmol/min/mg of protein) of ChAT (**A**) and AChE (**B**) in SN56 cells non-differentiated (C) or differentiated with 1 mM db-cAMP (cAMP) and 0.001 mM retinoic acid (RA). Effect of 0.1 mM (ChAT) or 0.25 mM (AChE) L-carnitine (Carn). Results are means ± S.E.M. of 6–12 experiments. $^{*}P < 0.05$ compared with respective no NGF conditions; +P < 0.001 compared with respective control (C) for ChAT and $P < 0.05$ for AChE; $^{\#}P < 0.005$ compared with respective cAMP or RA conditions.

dual disappearance of this activatory effect. On the other hand, the highest activation of AChE (61%) was observed at 0.25 mM, whereas in the PDH (32%) it occurred at 1.0 mM L-carnitine concentration in the medium *(figures 1, 2)*.

Presented data demonstrate that one parental cholinergic cell may differentiate to various phenotypes with different ratios between acetyl-CoA and acetylcholine metabolism. It indicates that activation of p75 receptors by NGFdid not influence development of cholinergic phenotype in SN56 cells. On the other hand, NGF presumably reduced capacity of neurons to generate acetyl-CoA due to suppression of PDH activity. This finding is in agreement with data showing apoptotic effects of p75 receptor activation (Bunone et al., 1997). It may also explain increased vulnerability of NGF-treated cells to neurotoxic inputs. Data presented here demonstrate that development of cholinergic phenotype in cholinergic neurons may be regulated independently by different signal transduction pathways including adenylate cyclase and RA-dependent transcription factor and presumably by L-carnitine mediated stimuli. Changes in the expression of enzymes involved in acetyl-CoA metabolism were independent on cholinergic differentiation since the decrease, instead of expected rise, in PDH and ACL activities was observed after addition of dbcAMP and RA. These results are discrepant with observations made on cAMP-differentiated S-20 neuroblastoma cell and on developing brain where a rise in ChAT was accompanied by the increase in PDH and ACL activities (Szutowicz et al., 1980, 1983). It may be the specific property of SN56 cell line. Nevertheless, relatively low PDH activity in neurons with markedly activated cholinergic metabolism can make them particularly susceptible to different neurodegenerative conditions. On the other hand, effects of L-carnitine on SN56 are in accord with earlier findings showing correlated modifications of ACh and acetyl-CoA metabolism in cholinergic neurons (Nalecz and Nalecz, 1996). Thus, L-carnitine could exert a protective effect on highly differentiated cholinergic neurons by the increase of acetyl-CoA production from pyruvate. This work was supported by K.B.N. project No 4 P05A 04412.

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109 The signaling pathway of calcitonin gene-related peptide-induced acetylcholinesterase expression in muscle is mediated by cyclic AMP

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Calcitonin gene-related peptide (CGRP), a neuropeptide synthesized by motor neurons, is able to stimulate the expression of acetylcholine receptor (AChR) and acetylcholinesterase (AChE) that forms the post-synaptic apparatus at the vertebrate neuromuscular junctions (Fontaine et al., 1987; Massoulié et al., 1993). Several lines of evidence indicate that the CGRP-induced AChE expression in muscle is mediated by a G-protein and acted through a cAMP pathway (Choi et al., 1996, 1998). First, various drugs affecting the intracellular level of cAMP, such as N^6, $O^{2'}$-dibutyryladenosine 3', 5'-cyclic monophosphate, cholera toxin, and forskolin, could mimic the effect of CGRP in stimulating the expression of AChE in cultured myotubes. Second, when myotubes were transfected in vitro and in vivo with cDNA encoding constitutively active mutant $G\alpha_s$, the intracellular cAMP synthesis was increased. The increase in cAMP level was paralleled with an increase in the expression of AChE while the transfection of active mutant $G\alpha_i$ cDNA decreased the cAMP level as well as the AChE expression. Third, the intracellular cAMP-dependent protein kinase (PKA) activity was increased by the application of CGRP while the CGRP-induced AChE expression could be blocked by pre-incubating the myotubes with PKA inhibitors. Last, the chimeric constitutively active ATF1 cDNA when overexpressed in cultured myotubes could stimulate the expression of AChE. Although the mRNA and protein levels of AChE were increased by CGRP, the enzymatic activity of the CGRP-induced AChE remained relatively unchanged. These findings indicated that the CGRP-induced AChE regulation is mediated by a intracellular cAMP pathway and represented the first evidence to suggest the mRNA synthesis of AChR and AChE could be mediated by the same neuron-derived factor.

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110 'Vital' histoenzymatic reaction and electron microscopic observation of acetylcholinesterase activity in frog neuromuscular junction

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Acetylcholinesterase (AChE) hydrolyses acetylcholine released in the synaptic cleft. Fine localization of AChE contributes to understanding synaptic functions. In the first step of the present work, we analyse the reaction mechanism of Koelle's copperthiocholine (1949) and Karnovsky's 'direct coloring' thiocholine (1964) methods, the most widely used histochemical methods devised for localization of AChE activity. Based on this analysis we show that it became possible to perform a histochemical reaction derived from these methods, on living tissues in order to avoid artifacts due to postmortem degradation and fixation. Both methods used acetylthiocholine as substrate. In the cholinergic synapse, acetylthiocholine mimics the action of acetylcholine. Acetylthiocholine after enzymatic hydrolysis produces thiocholine, a strong reducing agent transforming cupric and ferric ions in the vicinity of the enzymatic site, respectively into cuprous and ferrous ions. The mechanisms of the chemical reactions taking place in the two methods were studied by Tsuji (1974). It was found that primary precipitate of Koelle's reaction was cuprous thiocholine (white precipitate), while that of Karnovsky's reaction was composed of cupric ferrocyanide (brown precipitate) and of unexpected cuprous thiocholine, chemically identical with Koelle's precipitate. Furthermore, it was observed that this cuprous thiocholine of Karnovsky's reaction was converted during incubation by potassium ferricyanide of the medium into cuprous ferricyanide (another brown precipitate). Thus, Karnovsky's brown precipitate is finally composed of cupric ferrocyanide and cuprous ferricyanide. Tsuji and Larabi (1983) proposed a hypothesis of intramolecular oxidoreduction of copper and iron ions between the two complex salts.

Hanker et al. (1973) observed that Karnovsky's histochemical precipitate was capable to oxidize diaminobenzidine and found that the precipitate is endowed with a catalytic activity. This catalytic activity was interpreteded as 'peroxidase-like' activity by Tago et al. (1986). These authors used highly diluted Karnovsky's medium and obtained a fine reaction product, invisible at the level of optical microscopy. The catalytic activity of the invisible precipitate was revealed in a second step by means of a sensitive diaminobenzidine (DAB) and H_2O_2 procedure. The oxidation of DAB was intensified by $NiSO_4$. Oxidizing catalytic activities of both cupric ferrocyanide and cuprous ferricyanide were demonstrated in vitro towards DAB alone and DAB plus H_2O_2 (Tsuji, 1998). These catalytic activities were explained by intramolecular oxidoreduction between the two complex salts of copper and iron. Therefore, this catalytic activity of Karnovsky's precipitate was qualified as 'oxidoreductase-like'. Furthermore, a modification of Tago's method, without intensification of DAB precipitate by $NiSO_4$, was proposed for fine electron microscopic cytochemistry.

To our knowledge, no vital histoenzymatic reaction has been devised for the nervous tissue. Indeed, metallic ions present in histochemical mediums appear to be highly toxic (Cooper et al., 1984), contrarily to organic dyes such as methylene blue (Tsuji and Yokoyama, 1981). The unexpected oxidoreductase-like activity described above permitted to dilute Karnovsky's medium. This gave us the possibility to adapt the histochemical medium for living tissues. In short, Karnovsky's histochemical medium was dissolved in Ringer solution containing citrate buffer and increased calcium chloride. Citrate buffer was necessary first, to stabilize cupric ions in order to hinder undesirable formation of non-histochemical precipitate of cupric ferricyanide and secondly, to reduce inhibitory effect of cupric ions. However, since citrate buffer captures free calcium ions in the synaptic region, an addition of calcium chloride was necessary. Thus, a compromise between the concentration of citrate buffer and that of calcium chloride is essential for vital histochemical reaction. The muscle activity in response to electrical stimulation of motor nerve was monitored by recording of muscular contraction. The transmission lasted 15 to 20 min until complete blockade. We ended 'vital' histochemical reaction at this moment. At this stage, however, the tissue was still alive. Indeed, the transmission was recovered after washing the preparation with Ringer solution containing citrate buffer and increased calcium chloride.

After the 'vital' histochemical reaction, the tissue was fixed by glutaraldehyde, washed and treated by DAB and hydrogen peroxide. Under optical microscope, staining of motor endplates by oxidized DAB precipitate was controlled. The staining was observed in most, if not all, the endplates in the depth of the tissue. After osmification, osmium black of diaminobenzidine gave fine localization of AChE activity in the synaptic cleft between pre- and postsynaptic membranes under the electron microscope. The present work was supported by a Grant-in-Aid for International Scientific Research (Joint Research) from Japan Ministry of Education. We are grateful to Dr. P. Anglade for critical reading of the manuscript.

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111 The NO-mediated effect of carbachol and glutamate on postdenervation decrease of membrane potential in rat diaphragm is directed towards furosemide-sensitive chloride transport

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The membrane potentials of denervated muscle fibers of the rat diaphragm kept in a tissue culture medium are depolarized by about 8–10 mV (10–12%) within 3 h after denervation (1). This early post-denervation depolarization (EPD) is augmented when inward-directed chloride transport is enhanced by high osmolarity (500 mosmol/L) produced by addition of sucrose or NaCl. The EPD and the effect of hyperosmolarity are effectively prevented by the Cl^- transport inhibitor furosemide (1 ± 10^{-4} M) or by a chloride-free bathing medium. The EPD is also substantially reduced (2–3 mV) when muscle strips are bathed with 1 mM L-glutamate (GLU) which is found in motor nerve endings, or with 5 ± 10^{-8} M carbachol (CCh), which mimics the effect of non-quantally released acetylcholine (ACh) (2,3). The hyperpolarizing effects of GLU and CCh on EPD are not influenced by ouabain, an active sodium transport inhibitor, but are absent when NO-synthase is inhibited by L-nitroarginine methylester (NAME) (4) or when Cl^- transport is augmented by increased osmolarity. It is suggested (4, 5) that the post-denervation cessation of non-quantal ACh release (6) and probably also GLU release from nerve endings leads to the activation of the furosemide-sensitive Cl^- transport in the sarcolemma which is responsible for the early post-denervation depolarization.

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112 Acetylcholine induces a greater production of prostacyclin in human pulmonary arteries than in veins

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Acetylcholine (ACh) is a more potent relaxant agonist in human pulmonary arteries (HPA) than in veins (HPV) (Walch et al., 1997). Vasodilatation is mediated by the release of endothelium-derived relaxing factor (EDRF; Furchgott and Zawadzki, 1980), identified as nitric oxide (NO; Palmer et al., 1987) and prostacyclin (PGI_2; Bunting et al., 1976). The aim of this study was to determine the involvement of PGI_2 in the different effects of ACh in HPA and HPV. To this end, the vasodilatation induced by PGI_2 or ACh and the production of PGI_2 were determined in human pulmonary vascular preparations.

Human lung tissue was obtained from patients who had undergone surgery for lung carcinoma. Arteries and veins were dissected from those macroscopically normal regions of the diseased lung. The vascular preparations cut as rings were set up in organ baths (10 mL) containing Tyrode's solution.

To determine the vasodilatations induced by PGI2, the vascular preparations were incubated (30 min) with Bay u3405 (1 μM), a TP-receptor antagonist, to avoid any contractile effect of the prostanoids. After a precontraction with noradrenaline (NA, 10 μM), PGI_2 was applied in a cumulative fashion. The sensitivity of HPA and HPV to PGI_2 was similar (pD_2 value, 6.81 ± 0.20 and 6.72 ± 0.19 for HPA and HPV, respectively; $n = 5$).

The effect of indomethacin (INDO), a cyclooxygenase inhibitor, on the ACh-induced relaxations was investigated in human pulmonary vessels. After an incubation (30 min) in absence or presence of INDO (1.7 μM), the tissues were contracted with noradrenaline (NA, 10 μM) and relaxed by addition of ACh in a cumulative fashion. In HPA treated with INDO, a significant decrease of sensitivity to ACh was observed (pD_2 value, 7.42 ± 0.12 and 6.90 ± 0.21 for control and treated preparations, respectively; $n = 5$; Norel et al., 1996). In contrast, in HPV, INDO did not reduce ACh relaxant potency (pD_2 value, 6.07 ± 0.15 and 5.83 ± 0.24 for control and treated preparations, respectively; $n = 5$).

PGI_2 production was determined after ACh stimulation in human pulmonary vessels by measuring the formation of 6-keto-$PGF_{1\alpha}$, the stable degradation product of PGI_2, using an enzyme immuno-essay. The vascular preparation were set up in the organ baths, contracted with NA (10 μM) and subsequentely relaxed with ACh (1 or 10 μM). The 6-keto-$PG_{1\alpha}$ production was measured in aliquots collected from the bath fluid at the end of 5 min prior to NAchallenge (basal) and 5 min following the ACh stimulation. Results are presented in *table I.* However, no difference between the different vessels was observed in the 6-keto-$PGF_{1\alpha}$ production for 10 min period after NA stimulation. The

Table I.

	Basal ($n = 17$	*ACh (1 μM)* ($n = 8$)	*ACh (10 μM)* ($n = 9$)
HPA	8 ± 1	126 ± 19	166 ± 33
HPV	9 ± 1	34 ± 11*	70 ± 17*

Release of 6-keto-$PGF_{1\alpha}$ (pg/mg of tissue) by HPA and HPV after stimulation with ACh. Values are means ± S.E.mean and (n) indicates the number of lung samples used. Results obtained after ACh stimulation were corrected for the 6-keto-$PGF_{1\alpha}$ production due to NA challenge *$P < 0.05$ relative to similar data obtained in HPA.

level of 6-keto-$PGF_{1\alpha}$ production was increased by 2.5-fold when compared with the basal production during the 10 min period before NA challenge (HPA, 16 ± 2 and HPV, 17 ± 2 pg/mg of tissue; $n = 17$).

6-keto-$PGF_{1\alpha}$ production was also evaluated in HPA and HPV before (basal) and after a stimulation with arachidonic acid (AA; 100 μM). The vascular preparations were set up in the organ baths and the 6-keto-$PGF_{1\alpha}$ production in the bath fluid was measured for the 15 min period under basal and stimulated conditions. In HPV, the 6-keto-$PGF_{1\alpha}$ levels measured were 28 ± 9 and 145 ± 19 pg/mg of tissue for basal and after AA challenge, respectively ($n = 7$) while those obtained in HPA were 28 ± 3 and 229 ± 43 pg/mg of tissue for basal and AA stimulated conditions, respectively ($n = 7$).

These results suggest that the greater sensitivity of HPA versus HPV to the relaxant effect of ACh is not due to a greater sensibility to the relaxant effect of PGI_2. However, this difference could be partly explain by a greater production of PGI_2 in HPA than in HPV. The greater production of this prostaglandin in HPA than in HPV may be in part due to a greater cyclooxygenase and/or PGI_2 synthase activities.

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113 The acetylcholinesterase (AChE) of the cobra *Naja naja oxiana*. Identification of residues involved in insensitivity towards fasciculin

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AChEs from the venoms of Elapid snakes have been recently characterised in detail. They present several specific properties when compared to other vertebrate enzymes: Venom AChEs are monomers (1) and their sensitivity towards fasciculin, a peptidic toxin purified from the venom of the mamba *Dendroaspis*, varies greatly depending on the species (2). In particular, AChE from the venom of the cobra *Naja* is almost insensitive to this peptidic inhibitor (with an IC_{50} value >> 10^{-5} M).

In an earlier paper (2), it had been shown that the low sensitivity to fasciculin of AChE from the venom of the krait *Bungarus* fasciatus (bfAChE) can be attributed to the presence of a methionine residue in position 70 and to a lysine residue in position 285, in place of a tyrosine and an acidic residue, respectively. To investigate the mechanism that explains the extremely high resistance of *Naja* AChE (nnAChE) to fasciculin, we have cloned a cDNA coding for AChE from venom glands of the Central Asian cobra *Naja naja oxiana* (nnAChE). The deduced peptidic sequence is 93% identical to bfAChE. Both enzymes share the features explaining the lower sensitivity of bfAChE to fasciculin (M70, K285), and in addition nnAChE carries two mutations in the peripheral site region (A276, K277), conferring a more positive charge to that region of the molecule.

The modification by site-directed mutagenesis of these residues to their counterparts in *Torpedo* or *Bungarus* AChE greatly increases the sensitivity of nnAChE to fasciculin. We describe a mutant which, at low ionic strength, is about four orders of magnitude more sensitive to fasciculin than wild type nnAChE. This shows that point mutations affecting charges in the peripheral site region have large effect on the sensitivity of AChE to fasciculin.

For this mutant, the inhibition by gallamine is strongly enhanced, whereas the inhibition by propidium and bis-quaternary compounds is almost not affected. This shows that the area around the mutated residues 276 and 277 forms a subsite essential for the binding of gallamine.

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114 Comparison of the effects of soman on recovery of brain functional acetylcholinesterase and signs of poisoning in the guinea-pig

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Organophosphate compounds such as soman produce irreversible inhibition of peripheral and central acetylcholinesterase (AChE), resulting in accumulation of acetylcholine (ACh) and a progression of cholinergic signs of poisoning including tremor, salivation, hypothermia and disturbance of motor activity. Studies in the rat have shown little correlation between the inhibition of total brain AChE and the signs of poisoning at 2–48 h (Jovic, 1974).

AChE exists as multiple molecular forms. In mammalian brain the G4 form is the functionally important form. AChE inhibitors have been shown to differentially inhibit the molecular forms of AChE in rat brain (Ogane et al., 1992). Therefore, it is possible that, following AChE inhibitors, a better correlation might exist between the signs of poisoning and the levels of the functional AChE.

Soman (27 μg/kg) produced the expected progression of signs of poisoning. In the brain areas the G1 activity recovered before that of G4. The maximum inhibition measured in the brain did not always reflect the rate of recovery *(figure 1)*. In the striatum the G4 form was inhibited by 78% at 4 h and by 44% at 24 h with recovery to normal levels by 7 days. However, in the cerebellum, the G4 form was inhibited by 73% at 4 h and by 67% at 24 h with little recovery by 7 days.

It seems likely that inhibited functional AChE may be down-regulated and then replaced via increased assembly of the G1 monomer (Chiappa et al., 1995). It has been proposed that the synthesis of precursor G1 may be controlled by the levels of ACh in the cleft. A previous study with a similar dose of soman, showed that ACh levels were significantly elevated in the striatum but not in the cerebellum (Fosbraey et al., 1990). In the present study, in the striatum, where the levels of ACh are high, AChE recovered relatively quickly, whereas, in the cerebellum where the ACh levels are low, the enzyme recovered at a slow rate even though the levels of AChE are relatively high.

The reason for the differential recovery rate of AChE following soman poisoning is unclear. However, the activity of the functionally important enzyme was still reduced 24 h after soman administration when the overt signs of poisoning had disappeared. It seems likely that there is a mechanism for the early recovery of AChE activity in brain areas known to utilise cholinergic transmission, which facilitates survival following soman poisoning. (c) British Crown Copyright 1998/DERA. Reproduced with the permission of the Controller of Her Britannic Majesty's Stationery Office.

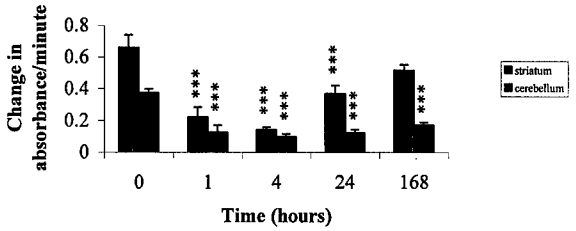


Figure 1. Effect of soman (27 μg/kg) on functional acetylcholinesterase activity in the striatum and cerebellum of the guinea-pig at different time points. The values are the means ± S.E.M. for four animals in each case. ***$P < 0.001$ compared to 0 h.

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115 Alzheimer's disease: Changes in the cerebrocortical expression of nicotinic receptors on the protein level

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In cognitive dysfunction syndromes like Alzheimer's disease (AD) impairment of cholinergic transmission and decreased numbers of nicotinic binding sites are well-known features (for review see Schröder and Wevers, 1998). The basic causes of the latter are not well understood and may be due to alterations in the expression of the nicotinic acetylcholine receptors (nAChR). Previous investigations on the mRNA level have shown that there are no overall alterations in the distribution pattern and in the densities of α4 and α7 transcript expressing neurons in the superior frontal gyrus of AD patients (Wevers et al., 1996). Therefore we now focused

our attention on the level of protein expression of both these subunits. Immunohistochemistry was performed using the subunit-specific antibodies mAb 299 (α4; Peng et al., 1994a) and mAb 306 (α7; Peng et al., 1994b; McLane et al., 1992) followed by the biotin-streptavidin technique. For quantitative analysis camera lucida-drawings were taken to assess the number of nAChR subunit-expressing neurons and the total number of neurons (Nissl-staining). Evaluation was carried out using a two-tailed *t*-test for independent random samples.

In AD there was a marked decrease of labeling intensity for both proteins as compared to controls. Furthermore, in AD the distribution pattern of immunoreactive neurons showed less labeled profiles, especially in the upper cortical layers, than the pattern observed for the neurons expressing the corresponding mRNA, whereas in the control group the patterns of transcript- and protein-bearing neurons looked similar. The quantitative evaluation revealed that the number of α4 and α7 protein expressing neurons was significantly reduced in AD, whereas the total number of neurons was not diminished. These results point to an impaired synthesis of nAChR subunits on the protein level as a possible cause for the cholinoceptive deficit in AD. Ongoing studies have to elucidate whether the maturation and assembly of receptor protein, the transport and or membrane insertion of the nAChR are affected. Supported by the Deutsche Forschungsgemeinschaft (Schr 283/18-1, 18-2) and the Stiftung Frau Maria Pesch.

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116 Immune-mediated slow-channel syndrome: A new form of myasthenia

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Autoimmune myasthenia gravis (MG) is caused by antibody mediated loss of functional acetylcholine receptors (AChRs). Here we describe a patient in whom the number of AChRs in the endplate was normal. However, the function of the AChR ion channel appeared to be altered.

We studied the clinical features of the patient, auto-antibodies, electrophysiological features and we did a passive transfer experiment to mice. A 32-year-old female had a 2-year history of diplopia and ptosis, dysphagia and generalised weakness. After rest, the force of the biceps brachii would improve strikingly after 2–3 maximal contractions, after which it decreased quickly. No or borderline level antibodies against AChRs were demonstrated with an immunoprecipitation assay, using denervated human muscle, but an assay using TE 671 cells expressing mainly adult type (AChR-ε) was positive (3.5 nmol/L). Pyridostigmine and plasmapheresis had no effect. Electromyography revealed decremental response to low frequency (1–5 Hz) but increment at high frequency (10–50 Hz) stimulation. There also were repetitive CMAPs, which decreased with 10 Hz stimulation. Thymectomy revealed a hyperplastic thymus. Electrophysiological study of an intercostal muscle revealed small MEPPs (0.22 ± 0.02 mV, vs. 0.56 ± 0.05 mV in controls) although the number of ^{125}I-α-bungarotoxin binding sites was normal. The decay time constants of EPPs were prolonged: 23 ± 3.6 ms (controls 5.3 ± 0.6 ms). Ultrastructurally, there was no destruction of the endplate. Injection of the patient's plasma into mice produced similar physiological changes in their diaphragms. We conclude that the patient has an antibody-mediated disorder in which the ion channel properties of the adult form (AChR-ε) of the ion channel is changed.

117 Muscarinic receptors in human melanomas

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In primary and metastatic human melanomas muscarinic cholinergic receptors are present. Muscarinic receptors are expressed, together with embryonic cholinesterase activity, in morphogenetically active embryonic cells. Therefore, the possibility exists, that in melanomas an embryonic trait is re-expressed after transformation. Therefore, we studied the presence of muscarinic receptors in the human melanoma cell line SK-mel-28 by immunofluorescence with the monoclonal antibody M 35 and characterized the receptors further by measuring calcium mobilization after muscarinic stimulation. Cell suspensions were stained with fura-2 and in a fluorimeter cuvette fluorescence was followed at 380 nm excitation. After addition of acetylcholine or carbachol a steep decrease in fluorescence intensity indicated calcium mobilization from intracellular stores (peak reaction), which was followed by constantly lowered fluorescence indicating a steady influx of extracellular calcium in the presence of agonist. By quantitative evaluation dose response curves were obtained, from which a ED of 4.3×10^{-6} M was calculated for acetylcholine and a ED of 2.2×10^{-5} M for carbachol. After pre-incubation with antagonists the dose response curve of acetylcholine was shifted to the right. The inhibition constant of pirenzepine was calculated as 3.9×10^{-7} M, of methoctramine as 6.8×10^{-7} M, and of 4-DAMP-mustard mustard as 1.9×10^{-8} M. Comparison with the data from the literature and those obtained in the chick embryo indicates that the muscarinic receptor in SK-mel-28 melanoma cells pharmacologically behaves as the M3 type and corresponds to the embryonic muscarinic receptor characterized by us in earlier studies.In a second experiment, studied the effect of acetylcholine and carbachol on cellular movements in the muscarinic receptor positive human melanoma cell line SK-mel 28 in a perfusion chamber. On perfusion with carbachol and acetylcholine the melanoma cells reacted within 2 to 10 min by concerted cell body contraction and retraction of cell processes. Addition of the muscarinic antagonist atropine to the perfusion medium abrogated the effects of the muscarinic agonists. The experiments demonstrate a direct link between the muscarinic acetylcholine receptor and the contractile apparatus of the melanoma cells. Since melanocytes are derived from neural crest cells which express cholinesterase activity and muscarinic receptors during their migratory phase in the embryo, the re-expression of the muscarinic cholinergic system in melanoma cells may be involved in invasive growth.

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