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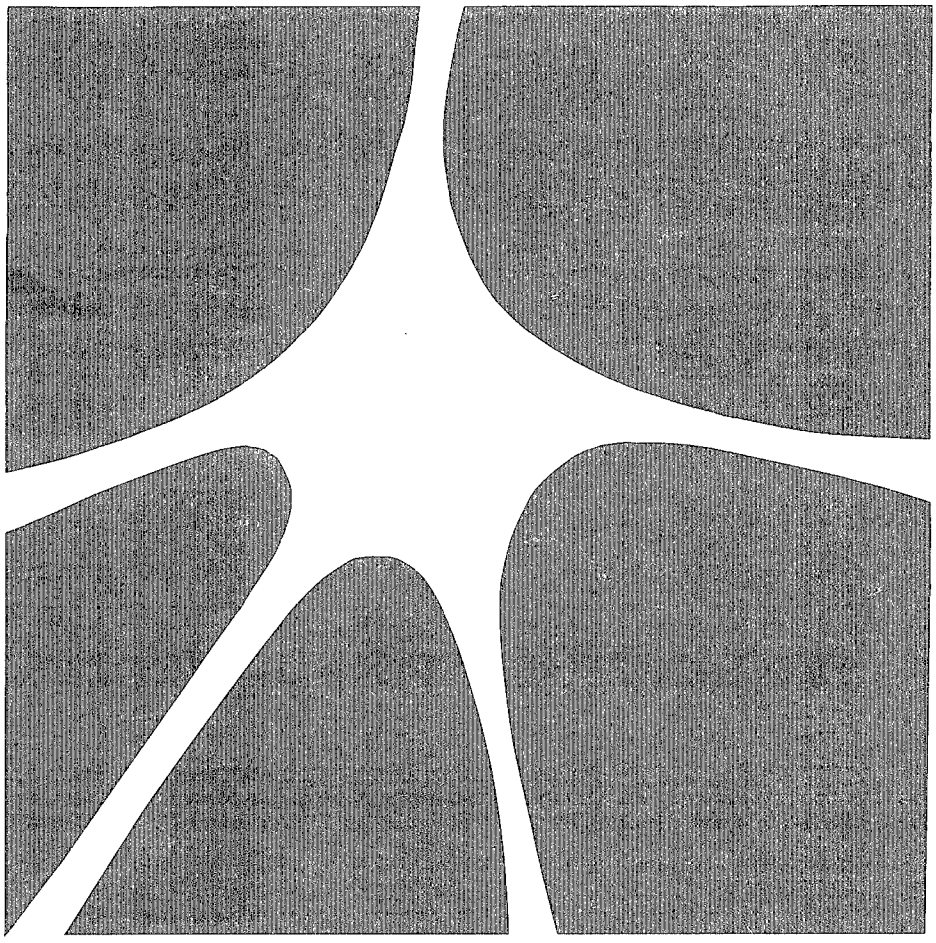

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Additional Abstracts

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CHARACTERIZATION AND DISTRIBUTION OF A MELATONIN BINDING PROTEIN

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We have recently identified in the rat and Syrian hamster brain, three proteins with apparent molecular weight values of 92, 55 and 45 kilodaltons (P92, P55 and P45) which become covalently labeled with the melatonin derivative bromoacetyl-2-[^{125}I]-iodo-5-methoxytryptamine (^{125}I-BIM). In the present report, the biochemical properties and tissue distribution of one of these proteins-P45 in the rat and hamster were investigated. Two dimensional polyacrylamide gel electrophoresis (isoelectric focusing followed by sodium-dodecyl sulfate polyacrylamide gel electrophoresis) indicated that P45 has an apparent pI value of 4.9. The labeling of P45 by ^{125}I-BIM was inhibited by the melatonin antagonist ML-23> 2-iodomelatonin>melatonin.

Specific polyclonal antisera to the Syrian Hamster P45 were raised in a rabbit using protein separated on 2D gels as the antigen. The antisera recognized a 45 kDa protein in the rat and hamster synaptosomes, shown to be P45 in Western blot experiments. P45 could be detached from the synaptosomal membranes by mild sonication. The ^{125}I-BIM labeled protein in this fraction was immunoprecipitated by the antiserum to P45. Melatonin binding in the sonicate, assessed using ^{125}I-melatonin as a probe, indicated that all of the specific binding in this fraction was inhibited by the antisera against P45 but unaffected by preimmune antiserum.

Tissue distribution studies in the rat and hamster indicated that P45 (identified by ^{125}I-BIM labeling and immunoblots) was present in the brain, retina, pituitary but not adrenal gland, pancreas, muscle, liver, heart, or lung tissue homogenate. Immunohistochemical studies in the rat indicated specific staining in the brain (median eminence arcuate nucleus, area postrema, and some pyramidal neurons in the neocortex) and in the pineal gland, with very little staining elsewhere. The staining was predominantly located in neuronal cell membranes; some glial staining was observed too. These results suggest that P45 is a melatonin target protein.

G proteins and the mechanism of action of mood stabilizers.

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G proteins play a pivotal role in post-receptor information transduction. An important characteristic of G proteins is their increased guanine nucleotide binding following agonist stimulation, which in turn leads to their activation. Using this characteristic of G proteins, we have developed a method which enables the measurement of early events in signal transduction beyond receptors, through activated receptor-coupled guanine nucleotide exchange on G proteins. By this method, lithium and other antibipolar treatments were demonstrated to inhibit the coupling of both muscarinic cholinergic and ß-adrenergic receptors to pertussis toxin-sensitive and cholera toxin-sensitive G proteins, respectively, thus suggesting alteration of the function of G protein by antibipolar treatments, as the single site for both the antimanic and antidepressant effects of these treatments. Differences between antibipolar (lithium, carbamazepine, ECT) and antidepressant (imipramine, clomipramine, clorgyline) patterns of interaction with G proteins were recently found. While antibipolar treatments attenuate both Gs and non Gs (Gi, Go)protein functions, only the first function was inhibited by antidepressant drugs. These results suggest that attenuation of b-adrenergic-coupled G_S protein function, which is common to both antidepressant and antibipolar treatments, may be the mechanism underlying their antidepressant therapeutic efficacy. Purely antidepressant drugs do not affect muscarinic receptor-coupled G protein function. The inhibition of these proteins, which was found to be a common characteristic of all antibipolar treatments, indicates that pertussis toxin-sensitive G proteins (Gi, Go) may be a molecular site for the antimanic therapeutic effect of these drugs. The ability to measure early events in signal transduction beyond receptors using the technique of receptor-coupled guanine nucleotide exchange on G proteins enabled us to extend our studies to human lymphocytes and measure functional alterations in members of the G protein family in patients with mood disorders. Moreover, this method suggests a mean to measure antidepressant and antibipolar drug effects in lymphocytes of affective patients. Manic patients lymphocytes show distinct hyperfunctional G proteins: both b-adrenergic receptor-coupled Gs and muscarinic receptor-coupled non-Gs (Gi, Go) protein functions are 2 to 3-times higher than in lymphocytes of normal subjects. In lymphocytes obtained from unipolar depressed patients Gs function is sharply reduced, while non-Gs function is almost unchanged. These changes in G proteins functions are normalized by specific treatments: lithium-treated bipolar patients, and antidepressant-treated euthymic affective patients show G proteins functions which are similar to the control subjects. The changes in G proteins functions in the affective patients are not due to motor hyper- or hypoactivity, as normal subjects after an intensive physical exercise show G proteins functions no different from the control subjects. The significance of these studies lies in two aspects: (a) better understanding of the mechanism of action of antimanic and antidepressant treatments which will enable an optimalization in their therapeutic use, and help in establishing biochemical correlates between drug reactivity and non-reactivity; (b) the identification of biochemical state or trait markers for the diagnosis of affective and other psychiatric disorders and their possible subclassification. Such biochemical markers may point to the involvement of G proteins in the pathogenesis of these disorders.

ROLE OF NITRIC OXIDE IN THE MECHANISM OF NEURONAL CELL DEATH

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It has been suggested that nitric oxide (NO) may play a role as neuronal messenger in the central nervous system (Garthwaite, TINS, 14: 60, 1991). *In vitro* electrophysiological experiments have involved NO in neuronal plasticity because N^W-nitro-L-arginine methyl ester (L-NAME), an inhibitor of NO synthase (NOS), prevents the formation of long-term potentiation (Schuman & Madison, Science, 254: 1504, 1991). Recently, we have reported that L-NAME protects rats from neuronal brain damage produced by the anticholinesterase agent, tacrine (THA) in lithium chloride (LiCl)-pretreated animals (Bagetta et al., Eur. J. Pharmacol., 213: 301, 1992) thus suggesting that NO may be involved. We now report further evidence which support this hypothesis.

Northern blot analysis of total RNA extracted from the hippocampus of rats (n=6) receiving LiCl (12 mEq/kg i.p.) 24 h before THA (5 mg/kg i.p.) showed a 6-fold increase in the expression of NOS mRNA and this was prevented by corticosterone (35 mg/kg i.p. given 30 min before THA; n=6) that *per se* did not affect the expression of NOS mRNA in control (untreated) animals. Preliminary *in situ* hybridization studies have revealed that NOS mRNA expression is significantly enhanced in the dentate gyrus granule cell layer of the hippocampal formation. The increase in NOS mRNA expression induced by LiCl and THA was accompanied by an approximately 80% increase in Ca^{2+}- calmodulin-dependent NOS activity in rat hippocampal homogenates and by a 3-fold raise in hippocampal cGMP levels. The increase in NOS activity was prevented by atropine (5 mg/kg i.p., given 15 min before THA; n=12), L-NAME (300 ug, given icv 10 min before THA; n=12) and by corticosterone (35 mg/kg i.p., given 30 min before THA).

FACILITATION AND INHIBITION OF PROTEIN SECRETION BY MELATONIN VIA A CHOLERA TOXIN SENSITIVE MECHANISM

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The constitutive secretion of proteins from animal cells involves several G-proteins but there is very little evidence for hormonal modulation of this process. We have studied the effect of melatonin on the constitutive secretion of newly synthesized proteins from cultured melanoma cells. At low cell density, melatonin (0.5-10 nM) inhibited whereas at high density it facilitated secretion, without affecting protein synthesis. The melatonin-mediated effects on protein secretion were prevented by pretreatment of the cells with cholera (CTX) but not pertussis (PTX) toxin.

These results indicate a see-saw type regulation by a hormone (melatonin) of protein secretion, mediated by at least one heterotrimeric GTP binding protein of the Gs class. The prevailing state of activation of the Gs protein involved may determine the direction and magnitude of melatonin's effects.

SEE-SAW EFFECTS OF MELATONIN AND GTPγS ON PROTEIN SECRETION OF MELANOMA CELLS

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The rate of secretion of newly synthesized, ^{35}S-methionine-labeled, proteins from melanoma M2R cells was assessed and found to increase transiently after plating, and then decay. Melatonin (0.5-10 nM) inhibited the constitutive secretion early after plating but facilitated it later on, generating a see-saw type response. Similar melatonin-mediated inhibition and facilitation of secretion was observed when the cells were synchronized and plated at low cell and high cell densities, respectively. Guanosine 5'-O-(3-thiotriphospate) (GTPγS; which was introduced into the cells during the process of permeabilization and resealing with ATP) affected protein secretion in a similar manner. However, in the presence of GTPγS and melatonin combination, protein secretion was enhanced at low as well as at high cell density. These results indicate that: a) Cell density alters the steady-state activity of secretory GTP binding proteins (G proteins) and subsequently the rate of constitutive protein secretion. b) The effects of melatonin on the secretory pathway negate those of cell density. c) The effects of melatonin are bi-phasic and involve G protein-mediated processes, with inhibitory and stimulatory consequences.

REGULATION OF ACETYLCHOLINE RECEPTOR GENE EXPRESSION IN MUSCLE AND BRAIN

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At the adult motor endplate, the AChR protein [α2βγ/εδ] as its subunits mRNAs are localized exclusively under the motor nerve ending. Denervation of the adult muscle causes a reappearance of unspliced and mature mRNA in extra-junctional areas. On the other hand, in the non innervated embryonic myotube, the α, β, γ and δ subunit mRNA are distributed all over the cell. A compartmentalization of gene expression at the level of subneural "fundamental" nuclei therefore takes place during development and is analyzed by the methods of cell biology and recombinant DNA technology (transfection, transgenic mice, DNA injection, adenoviral vectors) with both cultured and developing muscles *in situ*.

The data are interpreted in terms of a model which assumes that : 1) in the adult muscle fiber, nuclei may exist in different states of gene expression in subneural and extrajunctional areas ; 2) different second messengers elicited by neural factors such as CGRP (cAMP) or ARIA (tyrosine kinase) (under the nerve endings) or electrical activity (Ca^{++}, protein kinase C) (outside the endplate) regulate the state of transcription of these nuclei via trans-acting allosteric proteins binding to distinct cis-acting DNA regulatory elements ; 3) in the chicken α-subunit enhancer, consensus E Boxes (CANNTG) play a differential role in the regulation by electrical activity while in mouse ε-subunit promoter a different 83 nucleotides sequence confers preferential synaptic expression ; 4) a regulation of the expression of myogenic protein genes takes place during endplate formation ; 5) multiple post-transcriptional processes involving, in particular, the *Golgi apparatus*, proteins from the basal lamina and from the cytoskeleton (e.g. the 43 KD protein) contribute to the clustering, and stabilization of the AChR in the postsynaptic membrane.

A similar "promoter approach" is extended to the analysis of the expression of brain nicotinic receptor genes α2, α3, α4, β2, β4 by *in situ* hybridization, transfection of cultured nerve cells (β2-subunit promoter) and transgenesis in mice (chick α2 subunit gene and mouse β2-subunit promoter).

MOLECULAR BIOLOGY of DOPAMINE RECEPTORS

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Until 1990, the dopaminergic system was thought to rely on the interaction of dopamine with two receptors, the D1 and D2 receptors. Since, molecular biological analyses have revealed that there exist three more genes encoding other dopamine receptors, the D3, D4 and D5 receptors. On the basis of their primary sequences, pharmacological profiles and biological activities, these five receptors can be classifed into two subfamilies, the D1 receptor subfamily comprising the D1 and D5 receptors which induction stimulates adenylyl cyclase function and the D2 receptor subfamily, comprising the D2, D3 and D4 receptors, which bind with high affinities classical neuroleptics. The D2-like receptors however differ in their abilities to recognize particular neuroleptics, for instance the D3 and D4 receptors have higher affinities for particular atypical neuroleptics. Furthermore, the brain localization of the D3 and D4 receptors is similar but significantly different than that of the D2 receptor. Altogether the unexpected diversity in dopamine receptors leads to a reevaluation of our understanding of the dopaminergic system and of its implications in brain function.

Applications of Magnetic Resonance Spectroscopic Imaging and Magnetic Resonance Imaging (MRI) to Brain Pathophysiology.

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Magnetic resonance imaging and spectroscopy (MRI and MRS) have become important techniques for studying CNS pathophysiology due to the inherent non-invasiveness of these techniques. One main advantage of MR is the combination of MRS and MRI which enable to relate morphological changes with biochemical process *in vivo*. In addition, the contrast in MR images may be based on many different parameters. However, the main disadvantage of these techniques is their low sensitivity.

In this lecture we will present data concerning the spatial and the temporal evolution of lactic acid following trauma as obtained by ^{1}H MR spectroscopic imaging technique and conventional T_2-weighted MRI. The total acquisition time of the spectroscopic imaging spectra was 8 min for 16 slices of 1.1 mm width of a rat cortex (voxel size of less than 150μl). The early detection of stroke using diffusion-weighted MRI will be demonstrated in a cat middle cerebral artery occlusion (MCAO) model. This technique allow delineation of the ischemic zone within minutes after the ischemic event. The correlation between diffusion weighted hyperintensity and the metabolic disruption as obtained by ^{1}H and ^{31}P MR spectroscopy will be presented. The high sensitivity of diffusion weighted MRI for early detection of stroke is thought to be related to the accumulation of water in the intracellular space following the ischemic event. This conclusion is supported by a recent study in which changes in the apparent diffusion coefficient (ADC) of brain water in some pathophysiological states were obtained with a temporal resolution of 10 s.The potential of anisotropic diffusion-weighted MRI will demonstrated in cat CNS.

Characterization of the CAG Repeat, Structure and Expression of the Huntington's Disease Gene

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Huntington's disease (HD) is an autosomal dominant disorder characterized by progressive choreic movements and mental deterioration due to a loss of neurons, particularly in the striatum. An expansion of a trinucleotide (CAG) repeat in a novel gene called IT15 located in 4p16.3 has been identified as the mutation causing HD. In the expanded state the repeat is unstable, with more than 80% of meiotic transmissions involving a change in the number of CAGs. The greatest size changes are seen in paternal transmissions. The length instability appears to occur during gametogenesis, without leading to appreciable somatic mosaicism. Age of onset of the disease is inversely correlated with the length of the CAG repeat, with the juvenile onset cases, which most often result from paternal transmission, displaying the longest repeats. To begin to understand the mechanism by which the triplet repeat expansion in IT15 leads to the pathology of HD, we determined the structure of the disease gene and examined its expression. The IT15 gene consists of 67 exons ranging in size from 48bp to 341bp. It is expressed as two alternatively polyadenylated forms in a wide variety of fetal and adult tissues including the brain where the effects of the expansion are most prominent. Both normal and HD alleles are represented in the mRNA population, indicating that expanded triplet repeat does not prevent transcription. Morever, an individual carrying a balanced translocation with a breakpoint between exons 40 and 41 is phenotypically normal indicating that neuronal cell loss in HD is not likely to be a result of direct gene inactivation by the triplet repeat expansion.

GLYCOLIPID ANTIGENS-CSF MARKERS FOR DEGENERATIVE PROCESSES IN THE NERVOUS SYSTEM

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Glycolipids, mainly located to the cell surface and particularly abundant in the nervous system, show a cell type associated and developmentally regulated pattern. Normal cell membrane turn over results in shedding of these molecules to the intracellular space and metabolic and/or degenerative processes might be reflected by altered glycolipid concentration in CSF. By identifying the altered glycolipids it one might also be able to show which cell or cells that are involved in the process. CSF from patients with Alzheimer's disease show a significant increase of ganglioside GM1. As GM1 is enriched in synaptic membranes this finding is likely to reflect the loss of synapses in this disease. Patients with autism have a significant increase of nerve cell associated gangliosides. Patients with GM2 gangliosidose show increases of this ganglioside in CSF. Sulfatide, a myelin associated glycolipid, is found in increased concentration in CSF from patients with demyelination. Human meningiomas is associated with an increase of meningioma associated glycolipids in CSF. Being cell surface components, glycolipids are also suitable targets for antibodies and of great interest in autoimmune diseases. Glycolipid antibodies have so far mainly been investigated in serum from patients with neuropathies but have also been detected in CSF, in multiple sclerosis and Guillain Barré syndrome. Glycolipid and glycolipid antibody analyses in CSF may thus be useful in clinical neurochemistry.

HEMOGLOBIN: A POSSIBLE MELATONIN BINDING PROTEIN

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Previous studies indicated the presence of cytosolic-melatonin binding sites. We have attempted to purify these proteins from cytosolic fraction of crude bovine cortex, using ^{125}I-melatonin as a probe. Purification was pursued by 60% ammonium sulfate precipitation, followed by gel filtration chromatography on Biogel P200, anion-exchange chromatography on DE52 and hydrophobic chromatography on Fractogel. The purified protein had an apparent molecular weight of 16 Kilodalton on SDS-polyacrylamide gel. Sequence analysis revealed homology to the α-hemoglobin subunit. Thus, the binding of ^{125}I-melatonin to bovine hemoglobin (Sigma, 0.4μg/1μl) was studied. Specific binding of ^{125}I-melatonin binding to hemoglobin was inhibited by Serotonin> 5'-Methoxytriptamine> iodo-Melatonin>Melatonin in a dose dependent manner. Unlike the ^{125}I-melatonin binding site in the pars tuberalis and suprachiasmatic nucleus, GTPγS, GTPβS or Gpp(NH)p had no effect on ^{125}I-melatonin binding to hemoglobin. Sodium cyanide, 1nM-10μM had no effect on ^{125}I-melatonin binding, indicating that ^{125}I-melatonin does not bind to the Heme group. On the other hand, at physiological concentrations (4mM) 2,3-Diphosphoglycerate (DPG) inhibited the binding of ^{125}I-melatonin to hemoglobin by 50% . These data suggest that ^{125}I-melatonin binds to a specific hemoglobin conformation. The physiological significant of these observations are not clear. Hemoglobin may serve as a carrier protein for melatonin, and subsequently increase the efficacy of the antioxidant properties of melatonin at target organs.

ROLE OF THE 5-HT$_2$ RECEPTOR IN LEARNING AS REVEALED BY NEGATIVE INTRINSIC ACTIVITY OF RITANSERIN.

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Drugs classified as partial agonists at 5-HT$_2$ receptors (LSD, DOM, MDA, MDMA) have been demonstrated to enhance associative learning with a rank order of potency that corresponds to their ability to increase PI hydrolysis. Drugs active at the 5-HT$_{1A}$ receptor (lisuride and 8-OHDPAT) have no significant effect on learning. We have now examined the effects of the 5-HT$_2$ antagonists, ritanserin, BOL, MDL-11,939 and LY-53,857 on learning as measured by classical (Pavlovian) conditioning of the rabbit's nictitating membrane response. Ritanserin and MDL-11,939 retarded learning in a significant and dose-dependent manner, but BOL and LY-53,857 had no effect. However, all four drugs blocked the enhanced learning produced by LSD (0.013 mg/kg) confirming their antagonist action at the 5-HT$_2$ receptor. Thus BOL and LY-53,857 are neutral antagonists while ritanserin and MDL-11,939 are inverse agonists (antagonists with negative intrinsic activity). The inverse agonist actions of ritanserin and MDL-11,939 suggest the 5-HT$_2$ receptor has an endogenous level of activity that is critical for normal learning to occur. This, hypothesis is compatible with the reported decrease in 5-HT$_2$ receptors that occurs in dementia of the Alzheimer's type. This research was supported by a MERIT AWARD, MH 16841.

BIDIRECTIONAL MODULATION OF THE BRAIN REWARD SYSTEM BY OPIOIDS

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Opioids exert marked effects on motivation; μ- and δ-receptor agonists function as positive reinforcers, while κ-agonists induce aversion. Place conditioning was used to examine the neuro-anatomical substrates mediating motivational effects and in-vivo microdialysis was applied to assess the neurochemical effects of specific opioid ligands. Conditioned place preference was obtained after microinjection of the μ-receptor agonist DAGO into the ventral tegmental area (VTA), whereas conditioned place aversion was induced by microinjection of κ-receptor agonists into the VTA, the nucleus accumbens (NAC) and other mesolimbic structures. While DAGO microinjections into the VTA increased dopamine (DA) release, blockade of μ-receptors in this structure by CTOP decreased DA release in the NAC. The κ-receptor agonist U 69853 and antagonist nor-BNI were ineffective when injected into the VTA. In contrast, U-69853 decreased, and nor-BNI increased DA release when applied into the NAC; the μ-receptor agonist (DAGO) and antagonist (CTOP) did not affect DA release. These data show that the same mesolimbic pathway is modulated by two opposing, tonically active endogenous opioid systems. The concerted action of both (possibly mediated by β-endorphin and/or enkephalin in the VTA, and presynaptically-located dynorphin in the NAC) allows maintenance of basal mesolimbic DA release and thereby, an equilibrated emotional state. This last view is supported by the changes in DA release observed during withdrawal.

DEVELOPMENT OF LIGANDS FOR DEFINING NEW SUBTYPES OF PURINOCEPTORS.

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The structure-activity relationships for a variety of adenine nucleotide analogues at P_{2X}- and P_{2Y}-purinoceptors were investigated. A functionalized congener approach was applied to design analogues of 2-MeSATP as P_{2Y} receptor probes. ATP analogues having long chain 2-thioether groups (such as 6-cyanohexyl) had enhanced potency (particularly at P_{2Y}-receptors) or selectivity (particularly within the P_{2X}-class), and tended to be stable to nucleotidases. There was a significant correlation for the 2-thioether triphosphates between the $pK_{0.5}$ values for inositol phosphate production in turkey erythrocyte membranes and the pD_2 values for relaxation mediated via the P_{2Y}-purinoceptors in the guinea-pig taenia coli, but not for the vascular P_{2Y} receptors or for the P_{2X} receptors. At P_{2X} receptors, no activity was observed in the rabbit saphenous artery, but variable degrees of activity were observed in the guinea pig vas deferens and bladder depending on distal substituents of the thioether moiety. 2-Thioethers of ADP were equipotent to the ATP analogues, and the corresponding monophosphates were weak, but full agonists at P_{2Y}-purinoceptors. N^6-Methyl-2-(5-hexenylthio)-ATP was potent and selective for P_{2Y} receptors.

Certain analogues displayed selectivity or specificity within the P_{2X}- or P_{2Y}-purinoceptor superfamilies, suggesting possible subclasses. For example, 8-(6-aminohexylamino)ATP and 2',3'-isopropylidene-AMP were selective for endothelial P_{2Y}-purinoceptors over P_{2Y}-purinoceptors in the guinea pig taenia coli, rabbit aorta, and turkey erythrocytes. These compounds were both inactive at P_{2X}-purinoceptors. The potent agonist N^6-methyl ATP and the somewhat less potent agonist 2'-deoxy-ATP were selective for P_{2Y}-purinoceptors in the guinea-pig taenia coli, but were inactive at P_{2X}-purinoceptors and the vascular P_{2Y}-purinoceptors. Modifications at the ribose 3'-position were highly variable in receptor selectivity. For example, 3'-deoxy-ATP was a weak, but selective P_{2X} agonist, while 3'-acetamido-3'-deoxyATP was active at both P_{2X}-receptors and mesenteric artery P_{2Y}-receptors. 3'-Benzylamino-3'-deoxyATP was very potent at the P_{2X}-purinoceptors in the guinea-pig vas deferens and bladder, but not in the rabbit saphenous artery and inactive at P_{2Y} receptors.

NEW MEMBERS OF THE SAP90 (SYNAPTIC ASSOCIATED PROTEIN90) FAMILY OF PROTEINS RELATED TO THE DROSPHILA TUMOR SUPPRESSOR GENE PRODUCT DLG-A

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Synapses are highly specialized sites of cell-cell contact involved in signal transfer. We report a new family of Synaptic Associated Proteins (SAPs) related to PSD95/SAP90 (Cho et al., Neuron 1992, 9:929-942, Kistner et al., JBC 1993, 268:4580-4583) which has been previously described as a presynaptic protein. SAP90/PSD95 and the new family members, SAP97 and SAP102, are mosaic proteins containing three 90 amino acid residue repeats, an SH3 domain and a region homologous to guanylate kinases. The proteins share domain specific homology to a superfamily of proteins involved in the assembly and possibly the stability of sites of cell-cell contact. These include the product of the *lethal (1) discs-large-1* (*dlg*-A) tumor suppressor gene and the zonula occludentes proteins ZO-1 and ZO-2 (Anderson et al., Curr. Op. Cell. Biol. 1993, 5:772-778). We compared the immuno-staining of a general antibody detecting a variety of SAP90 family members with antibodies specific for single proteins identified up to now. Whereas SAP90 is localized in the presynaptic termini of inhibitory synapses in the rat cerebellum, SAP97 is found in the presynaptic termini of excitatory synapses and in unmyelinated axons througout the rat brain. Myelinated axons where never seen stained. The third protein SAP102 is the only postsynaptic member identified up to now. Preliminar data show the localization in dendritic spines in the hippocampus. These data implicate a similar function of the SAPs as suggested for *dlg*-Ap, ZO-1 and ZO-2 that is in the assembly of different neuronal compartments, for example specific subtypes of synapses. Further shown are data about the ability of Glutathione-S-transferase (GST)-SAP90 fusionproteins to bind specifically ATP and GMP with different affinities. This implicates that the proteins perform their function by guanylate kinase activity or by nucleotide binding itself.

TITLE : THE TYROSINE HYDROXYLASE LOCUS AND MANIC-DEPRESSIVE ILLNESS

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Abstract

Tyrosine hydroxylase (TH), the key enzyme in catecholamine synthesis, is of significant interest as a candidate gene in studies of manic-depressive illness (MDI). We have reported a positive association between a TH RFLP and this affection. In order to confirm and extend to new cases the association study, we have typed with a polymorphic (penta-allelic) tetranucleatide repeat located in the TH gene 110 patients diagnosed having MDI based on DMS-III criteria and 110 unaffected controls. Our results show a significant difference in the distribution of the allele frequencies between the MDI affected population and the control population ($X^2 = 10.8$ $P < 0.05$) strengthening the case for a vulnerability region to MDI on the TH locus.

In addition, we will discuss results obtained with classical linkage analyses and other non parametric methods which also indicate that a factor lying in the TH region may determine susceptibility to MDI in a large number of cases.

INVOLVEMENT OF NITRIC OXIDE IN CBF REGULATION UNDER PATHOPHYSIOLOGICAL STATES

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Nitric Oxide (NO) was suggested to be an important factor in CBF regulation as well as having direct effects on neuronal elements. Recent studies have shown some similarities between Carbon Monoxide (CO) and NO effects in biological systems (for review see Maines, M.D. Molecular and Cell NeuroSci. 4: 389-397, 1993). Exposure to low concentration of CO leads to a significant increase in CBF without a significant effect of intramitochondrial redox state. The interrelation between CO and NO in CBF regulation was an initial subject of the present study. Cortical spreading depression (SD) involves changes in ionic, metabolic, hemodynamic and electrical activities of the brain. The involvement of NO in the regulation of CBF during SD was documented (Goadsby *et al*., Brain Res. 595: 167-170, 1992; Duckrow, Brain Res. 618: 190-195, 1993) but the exact mechanism behind it is unclear. In order to clarify the role of NO in this multifactorial event (SD) as well as during CO exposure we adopted the multiparametric monitoring system (MPA) by which the hemodynamic, metabolic, ionic and electrical activities were measured from the surface of the brain (Mayevsky *et al*. J. of Basic and Clinical Physiol. and Pharmacol. 3: 323-342, 1992). Energy state and metabolism were evaluated by monitoring the cerebral blood flow and volume (Laser Doppler flowmetry) as well as intramitochondrial redox state (surface NADH fluorometry reflectometry). Ionic homeostasis was evaluated by the extracellular K^+, Ca^{2+} and H^+ activities (surface mini-electrodes). Electrical activities were assessed by the ECoG activity as well as by DC steady potential. Male Wistar rats (200-240 gr.) were anesthetized and the brain was exposed by a 6 mm diameter hole made in the parietal bone. The MPA was placed on the exposed brain using micromanipulator. In order to induce SD a push-pull canula was placed 3-4 mm anterior to the MPA. Dental acrylic cement was used to glue the MPA in place for the entire day. Two hours of recovery from the operation was given and then the rat was exposed to SD by epidural application of KCl solution (0.1-0.5 M). IP injection of Nω-nitro-L-arginine methyl ester (L-NAME) was given (50 mg/Kg) 90 minutes later. The second SD wave was induced 30-60 minutes after the injection of the drug. The main effects of NO synthesis inhibition before CO exposure or under spreading depression will be presented. We concluded that the inhibition of NO synthesis led to changes in CBF regulation during CO exposure. Also it decreases the ability of the brain to autoregulate CBF and O_2 supply during the initial phase of SD without significant detectable effects on the SD wave itself.

Supported by the Committee for Research and Prevention in Occupational Safety and Health, Ministry of Labour and Social Affairs, Israel and by the Research Committee of Bar-Ilan University.

CIRCADIAN RHYTHMS IN THE CULTURED SUPRACHIASMATIC NUCLEUS: INFLUENCE OF GLUTAMATE AND GABA

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An overwhelming body of evidence indicates that the suprachiasmatic nucleus (SCN) is the biological clock of the brain. In order to study electrophysiological mechanisms underlying the generation and entrainment of circadian rhythms in the SCN, we developed cultured organotypic SCN explants taken from 12-15-day-old rat pups grown for several weeks in a chemically defined medium. Recording continuously over a period of 1-3 days we were able to show circadian rhythms in the frequency of neuronal discharges in our SCN explants. Microiontophoretically applied glutamate increased spontaneous activity of the majority of the regular/irregular neurons and evoked activity in the spontaneously silent ones. GABA inhibited both the spontaneous and the glutamate-evoked activity. The magnitude of glutamate responses varied inversely as a function of the spontaneous firing frequency of regular SCN neurons. The latter suggests response efficacy changes as a function of the circadian time. MK-801 partially blocked glutamate responses but rarely influenced spontaneous activity of the cells. N-acetyl-aspartyl-glutamate (NAAG), a dipeptide found in the retinohypothalamic fibers (in addition to glutamate), directly increased or potentiated glutamate responses.

Bos, N.P.A. and Mirmiran, M. Brain Res. 511 (1990) 158-162.
Bos, N.P.A. and Mirmiran, M. Brain Res. Bull. 31 (1993) 67-72.

CALCIUM DEFICIENCY AFFECTS S100 IMMUNOREACTIVITY IN THE DEVELOPING RAT BRAIN

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S100 proteins are a group of closely related dimeric acidic proteins that belong to the EF-hand calcium-binding protein family. In the nervous system they are specifically concentrated in glial cells and a neurotrophic activity for the S100β subunit has been sugg ested. In the present study we investigated the effects of a calcium-deficient diet given to pregnant and nursing rats on the development of brain S100 immunoreactivity in the offspring. When forebrain and mid-hindbrain extracts were tested by ELISA method, lower levels of S100 were found in the treated animals, particularly in the mid-hindbrains of 7-day old rats ($p<0.01$). Immunocytochemical examination showed that S100 staining was restricted to glial cell, as expected, and that the number of S100-labelled cells was not appreciably different in treated and untreated offspring. Glial Fibrillary Acidic Protein immunoreactivity appeared to be essentially the same in treated and control offspring, suggesting that the lower level of S100 did not necessarily indicate a more general glial defect. The possible implications of this phenomenon in relation to suggestions of a neurotrophic role for S100β protein are currently being examined.

COMBINATION OF CHOLINERGIC AND ADRENERGIC DRUGS IN ALZHEIMER'S DISEASE

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Alzheimer's disease (AD) is a syndrome which includes the degeneration of number of neurochemical transmitter systems. The prevention of the degeneration of the basal forebrain cholinergic system has been a major target in development of drug therapies in AD. However, recent studies have demonstrated that serotonergic and noradrenergic systems, regulating information processing in a number of brain areas, also degenerate in AD. Our own studies have focused on a combined role of cholinergic and noradrenergic system in the symptomatic treatment of AD. The following basic and clinical research data will be presented: 1) Individuals with a decrease in noradrenergic markers, dopamine-β-hydroxylase or norepinephrine the brain in autopsy and who also show damage of cholinergic nerves, have more severe clinical symptoms than individuals with only basal forebrain damage and cholinergic depletion. 2) In experimental animal models of aging, subcortical regulation of spindling and information processing is better restored with a combination of the α_2-receptor antagonist, atipamezole and muscarinic receptor agonist, pilocarpine or cholinesterase inhibitor THA, than with cholinomimetics alone. 3) Norepinephrine depletion induced by DSP-4 aggravates the spatial learning defect produced by a cholinergic lesion or receptor blockade. 4) Combination treatment improves hippocampal theta rhythm and facilitates LTP in hippocampus better than either of the treatments alone. 5) In humans with age-associated memory impairment, treatment with an α_2-receptor antagonist improves vigilance, concentration and problem solving. In summary, our animal and human data emphasize that combination of treatments should be developed for clinical studies in order to treat different symptom complexes and the combination of α_2-receptor antagonist and cholinomimetic may prove to be an optimal treatment for the alleviation of the symptoms of AD.

DIFFUSIBLE SIGNALLING MOLECULES AND DISTRIBUTED SYNAPTIC ENHANCEMENT IN THE HIPPOCAMPUS. Erin M. Schuman, Division of Biology, 216-76, California Institute of Technology, Pasadena, CA 91125.

Long-term potentiation (LTP) is the activity-dependent long-lasting enhancement of synaptic transmission observed in many brain areas, including the hippocampus. Several different diffusible messengers such as arachidonic acid, carbon monoxide, nitric oxide, and platelet-activating factor have been proposed to act as intercellular signals in the production of LTP. Although LTP has generally been regarded as "synapse specific", the postsynaptic generation of any one or combination of these molecules leads to the prediction that other synapses in the vicinity will also be influenced. We have tested this prediction directly by recording simultaneously from two postsynaptic CA1 neurons in the hippocampus and recording synaptic strength in response to Schaffer collateral stimulation before and after LTP induction (by pairing 1 Hz stimulation with postsynaptic depolarization) in one neuron (see also Bonhoeffer, 1989). In experiments where we impaled two pyramidal neurons in close proximity (~ 140μm, soma-to-soma), the paired cell exhibited robust LTP (~ 190% of baseline) and the nearby neighboring neurons also exhibited a significant increase in synaptic strength (~ 135% of baseline; n = 20). Although it differed in magnitude, the potentiation observed at the neighboring neuron synapses had the same time course and duration as the potentiation observed in the paired neurons. This spreading of potentiation was spatially restricted as it was not observed when the neighboring cell was >500 μm away. Postsynaptic injection of an NO synthase inhibitor (L-Me-Arg) into the paired cell blocked potentiation at the synapses of both neurons. The combination of postsynaptic dialysis, $Ca2^+$ chelation, and hyperpolarization in the nearby neighboring cell also blocked the spreading of enhancement usually observed. This result suggests that the neighboring cell may play an active role in the distributed enhancement, either as a target site for a diffusible signal, or perhaps as a generator of additional messengers. We are currently examining the potential role of additional signaling molecules, such as neurotrophic factors, in the enhancement of hippocampal synaptic transmission.

ICER SHEDS LIGHT ON GENE REGULATION IN THE PINEAL GLAND

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The development and adaptive plasticity of biological systems depends on the regulated expression of specific genes. Within such regulative processes the activity modulation of transcription factors is pivotal. The transcription factor CREM plays an important role in the dynamics of (neuro)-endocrine processes[1-3]. Recently, a novel CREM isoform, ICER (inducible cAMP early regulator) was shown to have a nocturnally elevated expression in the rat pineal gland [3]. ICER encodes a potent repressor of cAMP-induced transcription[3]. Using in situ hybridization, Northern blotting and RNase protection analyses we now investigated the developmental appearance and temporal regulation of ICER in the rat central nervous system. In the course of ontogenetic development ICER mRNA was not detectable in the central nervous system prenatally. The nocturnally elevated expression of ICER previously described in the pineal gland of adult rodents[3] was first observed in 8-day old animals. Expression gradually increases and reaches adult levels at postnatal day 15. The only other structure in the rat central nervous system expressing ICER beside the pineal gland was shown to be the lamina intercalaris (deep pineal). The developmental appearance of a day/night difference in pineal ICER expression coincides with maturation of the sympathetic innervation of the gland and thus, with the onset of melatonin synthesis. The release of noradrenaline from sympathetic nerve endings was shown earlier to cause ICER induction in rat pineal[3]. The same mechanisms of regulation apply for the regulation of ICER expression in the lamina intercalaris. Our data provide a conclusive range of evidence for a fundamental role of the transcription factor ICER within cAMP-induced transcriptional events in neuroendocrine tissues.

[1]Foulkes et al., Nature 355:80 (1992); [2]Foulkes et al., Nature 362:264 (1993); [3]Stehle et al., Nature 365:314 (1993)

STRUCTURE, FUNCTION AND REGULATION OF THE THREE β-ADRENERGIC RECEPTORS

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Three β-adrenergic receptors are now known to be functionally expressed in mammals. All three belong to the superfamily of receptors coupled to GTP binding proteins. Accordingly, they display striking structural similarity : a glycosylated extracellular N-terminal region, seven presumed transmembrane domains, an internal C-terminal region. All three bind the same natural catecholamines, adrenaline and noradrenaline, couple to what appears to be the same trimeric Gs protein, and stimulate apparently the same adenylyl cyclase. Nevertheless, these three receptors display striking differences in terms of pharmacology and regulation. A number of β1/β2 antagonists (the well known β blockers) thus appear to be β3 agonists. The β1 and β2 in contrast to β3 receptors become phosphorylated upon short-term desensitization induced by agonist. Prevalence of expression vary widely in a given species (eg : β1 in heart, β2 in lung or uterus, β3 in fat and gut) or from one species to another. The relationship between the structural and functional properties of these three receptors has now been analyzed using site-directed mutagenesis and chimeric β2/β3 receptors. The results confirm that subtle differences may explain important differences between seemingly similar proteins.

Strosberg, A.D. "Structure, function and regulation of adrenergic receptors" Protein Sci. 12, 1198-1209 (1993).

INTERACTION OF ACETYLCHOLINESTERASE WITH GLYCOSAMINOGLYCANS.

Tarrab-Hazdai, R., Croitoru, S., Rossi, M., Schechtman, D. and Arnon. R.
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Schistosoma is a trematode that causes a debilitating disease called *Bilharzia*.The enzyme acetylcholinesterase (AChE) is present in all stages of the life cycle of the schistosome and is located on the muscle and on the surface of the parasite. Many of the drugs used for curing the disease are anti-cholinergic indicating the physiological importance of this enzyme. In this work we are studying the interaction of immobilized heparin with the AChE. Solubilized extract of cercaria were bound to sepharose-heparin, binding efficiency of the total AChE activity was ~50%, indicating the presence of two different AChE species. One that has a site for the heparin molecule and the other species without the binding site for heparin. All the bound AChE was progressively displaced from the heparin-agarose column by increasing salt concentration with a maximum release of about 0.5M NaCl concentration. The molecular forms that have binding properties for heparin correspond to G_2, and G_4, forms, with a sedimentation coefficient of 6.5-7S and 8S. The AChE released from the surface of the schistosomula by PILPC does not bind the immobilized heparin and corresponds to the 8S form. Monoclonal antibodies raised against the parasite AChE recognize in a selective manner the heparin bound AChE and the non bound. MoAb SA57 recognizes only the enzyme eluted from the immobilized heparin. Meanwhile, MoAb SA7 recognized the non bound enzyme. The functional implication of these findings will be discussed.

ALTERNATE SOMATIC PROMOTER AND/OR TRANSCRIPTIONAL ATTENUATION AND ANTISENSE TRANSCRIPTS ARE INVOLVED IN PREPROENKEPHALIN RNA CONTROL Gary Weisinger[1,2], Oren Zinder[1], Joseph D. DeCristofaro[2] & Edmund F. LaGamma[2], Technion, Israel[1] and SUNY at Stony Brook, NY, USA[2]

Preproenkephalin (ppEnk) RNA expression results from the interplay of a multitude of signal transduction pathways which we already know effects the gene's promoter at the level of RNA initiation and start site usage. In whole animal cholinergic agonist treatments, striatal ppEnk steady state RNA initiation levels are 10-15 fold greater than in untreated animals. In similar experiments no differences in the level of induced ppEnk mRNA levels (1.4kb) were noted when compared to untreated animals. This observation was verified by experiments probing the same northern blots with different oligomeric fragments which hybridize to ppEnk exons 1 and 3. These data suggested a down regulating transcriptional or posttranscriptional event occuring between the end of ppEnk exon 1 and the beginning of exon 3. To address this issue run-on transcription assays were initiated using single-stranded DNA (M13) probes derived from different regions of the rat ppEnk gene. These data would directly measure the rate of ppEnk RNA elongation at different places along the gene at a particular time. Our resulting data is consistent with the presence of a new species of somatic ppEnk RNA initiated within the first intron of the gene, which is active in basal rat striata, while the proximal promoter is induced following cholinergic agonist treatments. It seems transcriptional attenuation may also be occuring between exon 1 and the new somatic start site. Whether this new somatic start site is related to the germ cell promoter will be determined. Additionally, our data indicates the presence of small antisense ppEnk RNA transcripts that may play a role in the regulation of this gene. This new data will go along way in refining our mechanistic understanding of the regulation of this gene.

SORTING OF GPI-ANCHORED PROTEINS IN EPITHELIAL CELLS.

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In this study we analyzed the targeting of GPI-anchored proteins in epithelial cells in view of a sorting model that proposes the aggregation of apical and GPI-anchored proteins together with glycosphngolipid (GSL) rafts in the TGN. We have used GD1-DAF, a GPI-anchored protein, which is vectorially targeted to the apical membrane of MDCK cells. In FRT cells this protein is directly delivered to the basolateral domain while in MDCK-ConA^r mutant cells it is not sorted in a polarized fashion. Although GPI proteins are not sorted in the ConA^r mutant, the glycolipid, glucosylceramide (GlcCer) was found enriched in the apical membrane, as previously shown in MDCK cells. In contrast, GlcCer was basolaterally targeted in FRT cells. The analysis of the GSL composition in FRT and MDCK cells showed some major differences, nonetheless we demonstrated that, in both cell lines, they form complexes resistant to TX100 extraction. TX100 extractability studies demonstrated that GD1-DAF becomes insoluble during transport to the surface in both MDCK and ConA^r mutant cells and comigrates with GSLs on sucrose density gradients. Surprisingly, in FRT cells, gD1-DAF did not form clusters with GSLs and, therefore, remained completely soluble. This clustering defect in FRT cells correlated with the lack of expression of VIP21-caveolin, a protein localized to both the plasma membrane caveolae and the TGN. This suggests that VIP21/caveolin may have an important role in recruiting GPI-anchored proteins into GSL complexes, necessary for their apical sorting. However, since MDCK-ConA^r cells expressed caveolin and clustered GPI-anchored proteins normally, yet missorted them, our data also indicate that clustering and caveolin are not sufficient for apical targeting and that additional factors are required for the accurate apical sorting of GPI-anchored proteins to the plasma membrane of epithelial cells.

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Abstracts

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Classification of Abstracts by Session

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Thursday

Plenary Session

Symposia, Colloquia, and Workshops

Poster Session

A

THE MECHANISM AND REGULATION OF GLUTAMATE EXOCYTOSIS FROM CEREBELLAR GRANULE CELLS

D.G. Nicholls, J.M. Pocock, H. Hurst and M. Cousin, Department of Biochemistry, University of Dundee, Dundee, Scotland, U.K.

Cerebellar granule cells are the most abundant neurons in the CNS. They release glutamate and possess both ionotropic and metabotropic glutamate receptors as well as both L-type and non L-type voltage-activated Ca^{2+} channels. Glutamate exocytosis is evoked by a very high threshold Ca^{2+} channel whose properties do not correlate well with N-, P- or Q-type classifications. The channel is apparent in neurites and is blocked by the novel toxin from *Agelenopsis aperta*, Aga-GI, although there is an apparent switch to an L-type Ca^{2+} channel (the nature of which will be discussed) when cells are exposed to high KCl in the initial absence of external Ca^{2+}. Exocytosis from these cells can also be monitored in the imager at the single varicosity level by monitoring the release from synaptic vesicles of the fluorescent styryl dye FM1-43. In addition we present evidence that exogenous D-aspartate has access to the vesicular pool of transmitter in granule cells and can apparently be released by an exocytotic mechanism in contrast to its cytoplasmic location in isolated terminals (synaptosomes).

Na^+/K^+-ATPase inhibition of granule cells by ouabain initiates a complex chain of events including Na^+ entry, plasma membrane depolarization, Ca^{2+} channel activation and an extensive release of glutamate from the cells which is largely exocytotic as judged by its Ca^{2+}-dependency and sensitivity to tetanus toxin. Release is much more extensive than following a clamped KCl depolarization which may reflect the ability of synaptic vesicles to undergo several cycles of release in the presence of ouabain.

B

MODULATION OF GLUTAMATE EXOCYTOSIS BY PRESYNAPTIC METABOTROPIC GLUTAMATE RECEPTORS.

J. Sánchez-Prieto, I. Herrero and E. Vázquez. Departamento de Bioquímica, Facultad de Veterinaria, Universidad Complutense de Madrid, Madrid-28040, Spain.

The exocytotic release of glutamate is triggered by a localized increase in the $[Ca^{2+}]c$ in the active zone after depolarization. Isolated nerve terminals (synaptosomes) from the cortex of adult (2-3 months old) rats contain metabotropic glutamate receptors (mGluRs) that upon activation potentiate the increase in the $[Ca^{2+}]c$ evoked by depolarization with the result of a facilitation of glutamate release. The facilitatory effect is observed only with the transient depolarizations induced by 4-aminopiridine, 4AP, but not with KCl, and requires the presence of arachidonic acid because the protein kinase C that control the facilitatory pathway respond to the coincident activation by the diacylglycerol generated by the mGluR and the unsaturated fatty acid. In contrast, cerebrocortical synaptosomes from young (2-3 weeks old) rats contain mGluRs that reduce the elevation in $[Ca^{2+}]c$ induced by depolarization with the subsequent inhibition of glutamate release. The inhibitory effect is observed both with the transient and the permanent depolarizations induced by 4AP and KCl, respectively, indicating that the target of the inhibition is the Ca^{2+} channel coupled to glutamate exocytosis. Developmental studies indicate the presence of the inhibitory mGluR effects in 1, 2 and 3 weeks old rats, while the effects of the facilitatory mGluR are observed only in 3,4, weeks and adult rats. These results show changes of presynaptic control of glutamate release by mGluRs with development

C

REGULATION OF EXCITATORY AMINO ACID RECEPTORS (EAARS) IN CEREBELLAR GRANULE CELLS

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Granule cells develop survival requirements which in vitro can be met by treatment with high K^+ or EAAs, the effect of which is mediated through Ca^{2+} influx triggered activation of a calmodulin kinase. These growth conditions seem to mediate a trophic influence, mimicking the in vivo effect of afferent stimulation on granule cells. High K^+/EAA treatments also modulate the expression EAARs in the cultured cells, including inducing an increase in the activity and the density of NMDA receptors (NR) (which constitute an important route of Ca^{2+} entry), thus providing a positive feedback for strengthening the trophic influences. Although growth conditions do not modify the overall developmental increase in the NR mRNA pool, they exert an influence on NR subunit expression. In addition, high K^+/EAA treatments modulate the expression of AMPAR subunit mRNA and protein. In cultured granule cells, metabotropic Glu receptors (mGluR1) attain maximal activity relatively early and thus may be crucial for providing the Ca^{2+} signal at a time when other relevant activities are immature. Mechanisms underlying developmental changes affecting mGluR1 include maturation-dependent alterations in receptor desensitization. In addition, interactive effects involving activated NMDARs and mGluRs are also evident. In particular in high K^+-treated cells, exposure to NMDA results in the downregulation of NMDARs and mGluR1. In general, EAARs are subject to regulation by epigenetic factors and depending on the receptor class the control involves critically, in addition to gene transcription, translation and posttranslational events.

D

THE GUANINE NUCLEOTIDE BINDING SITE OF THE CHICK CEREBELLAR GLIAL KAINATE BINDING PROTEIN AND ITS RELEVANCE TO THE LIGAND BINDING SITE OF GLUTAMATE RECEPTORS

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The ligand binding subunit of a chick cerebellar kainate (KA) binding protein (KBP) is present exclusively in Bergmann glia and displays the typical pharmacological profile of low affinity KA receptors. It shares amino acid sequence homologies with the C-termini of KA/AMPA receptors and shows the hallmarks of ligand-gated channels. Yet, upon injection of KBP cDNA or KBP gene to *Xenopus* oocytes no KA-induced ionic currents are detected. Since KBP harbors a glycine-rich motif known to be involved in the binding of ATP and GTP by kinases and G proteins respectively, we explored the possibility that its function is regulated by nucleotides. Indeed, guanine but not adenine nucleotides interact with KBP by inhibiting KA binding in an apparent competitive manner. With the aid of photoaffinity labeling techniques, anti-peptide antibodies and KBP mutants we have demonstrated that the glycine-rich motif along with the N-terminal region of KBP form the guanine nucleotide binding site. Following this observation, we established, by ligand binding studies on rat cortical membranes and electrophysiological recording from cDNA injected oocytes, that guanine nucleotides behave as antagonists of all glutamate ionotropic receptors. Accordingly, we propose that the glycine-motif contributes to a common regulating site for glutamate and guanine nucleotides. The physiological significance of the interaction of ionotropic glutamate receptors with guanine nucleotides remains elusive since the latter are not known to be present in the extracellular milieu. Supported by the Pasteur-Weizmann Association , the Reich Foundation and the Yosef Cohen Center for Biomembranes.

A

ENDOTHELIN RECEPTORS: HETEROGENEITY AND SIGNAL TRANSDUCTION

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Binding data point to coexistence of several endothelin receptors (ET-R) in rat heart and cerebellum. Induction of phosphoinositide hydrolysis in these preparations by endothelins (ET-1 and ET-3) and sarafotoxins (SRTX-b and SRTX-c) was demonstrated by measurement of labeled inositol phosphate generation. Pertussis toxin (PT) enhanced the induction of phosphoinositide hydrolysis by all four peptides. The process is mediated by at least two heterotrimeric G-proteins, the one sensitive and the other insensitive to PT. Measurement of the GTPase activity induced by the peptides in rat heart and cerebellum preparations indicates for the first time the direct functional coupling between ET-R and a G-protein. These GTPase activity experiments provide evidence that phosphoinositide hydrolysis is stimulated via functional coupling between the endothelin receptor of the ET_A-R and ET_B-R subtypes and a PT-insensitive G-protein, Gq/11. The involvement of PT-sensitive G- proteins, i.e Gi-like and/or Go-like proteins, in the signal transduction pathways of ETs and SRTXs will be discussed.

B

MAPPING THE SUPERFAMILY OF G-PROTEIN COUPLED RECEPTORS IN OLFACTION.

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A gene superfamily of olfactory receptors (ORs) has recently been identified in a number of species (cf. Curr. Biol. 3:668-674, 1993). The human genome contains an estimated 130 OR genes clustered on several chromosomes. We have identified and mapped a 0.35Mb OR gene cluster on human chromosome 17p13.3, containing 16 intronless OR coding regions, two of which are pseudogenes (Hum. Mol. Genet. 3:229-235, 1994). The human OR genes in the cluster displays as much sequence variability as any randomly selected group of ORs. This suggests that the cluster may be one of several copies of an ancestral OR gene repertoire predating the divergence of mammals. In order to gain more insight on the evolution of OR clusters, we have recently also screened a chromosome 11-specific cosmid library. 18 different OR coding regions from this chromosome, all found to be pseudogenes, were assigned to several gene subfamilies, one of which is new. Three chromosome-11 pseudogenes are very similar to the two pseudogenes on chromosome 17, and appear to be their paralogues. A cluster comprised exclusively of OR pseudogenes, may be a result of a loss of a transcription control mechanism shared by the entire gene cluster, followed by a rapid accumulation of mutations. An OR was expressed both *in vitro* and *in vivo* and its product was studied employing specific antibodies. Immunohistochemical studies have shown the protein to be expressed in the plasma membrane of the host cells. In addition we were able to partially purify the 29 kDa OR protein. The mapping of OR gene clusters throughout the human genome, combined with their sequence analysis may help achieving a better understanding of human olfaction at the genetic level, as well as the ontogeny and phylogeny of the OR gene superfamily. In parallel, expression of the OR proteins, revealing their odorant ligands and mutation studies are expected to shed light on the structure-function relationship.

C

MOLECULAR BIOLOGY AND PHARMACOLOGY OF HISTAMINE RECEPTOR SUBTYPES

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Histamine, an ubiquituous cell-to-cell messenger, exerts its action through activation of three pharmacologically defined receptor subtypes termed H_1, H_2 and H_3. All three were shown after cloning (H_1, H_2) or suggested on pharmacological grounds (H_3) to belong to the superfamily of G protein-coupled receptors.

The histamine H_1 receptor was recently shown to be encoded in several animal species by an intronless gene and, when expressed in transfected CHO cells, to be coupled to a variety of signalling systems. It activates phospholipases C and A_2, generates calcium transients and potentiates cyclic AMP-generating stimulants.

The histamine H_2 receptor is also encoded by an intronless gene but displays limited sequence homology with that of the H_1 receptor. When stably expressed in CHO cells it activates adenylyl cyclase and inhibits arachidonate release.

The histamine H_3 receptor is essentially characterized by its pharmacological profile, functions and localisations distinct from those of H_1 and H_2 receptors. Initially discovered in the brain as an autoreceptor modulating histamine synthesis, it appears now to inhibit the release of a variety of aminergic and peptidergic neurotransmitters in the CNS and peripheral tissues.

D

D_2 AND D_3 DOPAMINE RECEPTORS IN MURINE EMBRYONAL DEVELOPMENT.

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Dopamine receptors play a central role in CNS neurotransmission, and receptors of the D2 subclass in particular have been associated with a number of neuropathological conditions, such as Parkinson's disease, schizophrenia and Huntington's chorea. The D2 subclass contains the cloned D_2R, D_3R and D_4R subtypes, including the short and long alternatively spliced isoforms of the D_2R and D_3R. In order to determine whether the D_2R or D_3R subtypes may have a role in embryonic development, we have studied the mRNA distribution of these receptors at different stages of murine embryonic development. PCR on cDNA from embryonal RNA at progressive stages of development shows that the long and short isoforms of the D_2R are not detectable at early stages, but appear together at day 13.5 post conception (p.c.), and continue to be expressed at slowly increasing levels during the following stages. The D_3R appears earlier than the D_2R, being detectable even at day 9.5 p.c. D_3R mRNA levels increase until day 12.5 p.c., and then show a slight decrease, levelling off at approximately day 14.5 p.c. The long and short isoforms of the D_3R show some slight variation in the pattern of their appearance, in contrast to spliced isoforms of the D_2R which show an identical pattern. Whole mount in situ hybridisation using specifc cRNA probes shows that at 9.5 days p.c. the D_2R cannot be detected, whereas D_3R mRNA is present in the optic pit. At 10.5 days p.c., D_2R mRNA signals can be detected in the otic vesicle, and D_3R mRNA is present in the otic vesicle and to some degree in the forebrain. At 11.5 days p.c. the distribution of both D_2R and D_3R transcripts is more widespread, being detectable in neural tissue. Thus the D_3R may be the dominant D2-type receptor in early embryos, in contrast to the adult where D_3R mRNA levels are significantly lower than D_2R mRNA levels. The importance of the presence of D_3R mRNA in the optic pit, which develops into the eye, and the otic vesicle, which forms the inner ear, is presently under investigation.

A

ENDOGENOUS LIGANDS FOR THE CENTRAL AND PERIPHERAL CANNABINOID RECEPTORS.

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Two cannabinoid receptors have been identified so far: a receptor in the brain (CBR-B) and a peripheral receptor in the spleen (CBR-P). Ligands for CBR-B, named anandamides have been isolated from the brain. Endogenous ligands for the peripheral receptor, (CBP-P), have not been isolated so far from peripheral tissues. We shall describe the isolation of such a ligand from canine intestines. It is an arachidonic acid derivative which differs from the anandamides.

The effects produced by the new endogenous ligand do not differ much from those caused by anandamide. Both compounds bind to CBR-B and CBR-P, reduce the formation of cAMP, and produce a tetrad of behavioral effects in mice. In most but not all of these assays the peripheral ligand is less potent than anandamide. However it is almost as potent as Δ^9-THC in inhibiting cAMP formation in the spleen. It binds to membranes of cells transfected with the CBR-P gene with K_D values close to those recorded in transfected cells with the CBR-B gene.

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B

ROLE OF GLUTAMATERGIC SYSTEM AND NITRIC OXIDE IN THE DEVELOPMENT OF SENSITIZATION TO COCAINE

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Repeated exposure to cocaine results in sensitization ("reverse tolerance") to many actions of the drug, a phenomenon common to the effects of other psychostimulants. The development of "pharmacological kindling", typified by increased sensitivity to the convulsive response of cocaine over time, is thought as the major psychopathological consequence that occurs in humans abusing cocaine. Recent studies have focused on the role of excitatory amino acids in the action of psychostimulants, and studies from our laboratory indicate the involvement of the N-methyl-D-aspartate (NMDA) type of glutamate receptors and the putative neurotransmitter nitric oxide (NO) in the process of sensitization to the toxic effects of cocaine in mice. Repeated administration of cocaine (45 mg/kg/day; 7 days) to Swiss-Webster mice results in a progressive increase in the convulsive response to cocaine and augmentation in lethality rate. Pretreatment of animals with the noncompetitive NMDA receptor antagonist, MK-801, completely abolishes the sensitization to cocaine-induced toxicities. Similarly, pretreatment with NO synthase inhibitors (L-NAME and N-nitro-L-arginine) provides a full protection against the development of cocaine-induced seizures and death. On the other hand, exacerbation in cocaine-induced sensitization is observed in animals that are treated with L-arginine, a substrate of NO synthase, prior to cocaine administration. Ligand receptor binding assays indicate that repeated exposure to cocaine causes up-regulation of cortical NMDA receptors, a phenomenon that is attenuated in animals that are pretreated with either MK-801 or NO synthase inhibitors. Taken together, both *in-vivo* and *in-vitro* studies support the involvement of NMDA/NO systems in the process of sensitization to cocaine. Although the mechanism underlying cocaine-induced NMDA receptor up-regulation remains to be determined, it is conceivable that supersensitivity of the NMDA receptor stimulates the production of NO. Therefore, blockade of NMDA/NO pathway may provide protection against cocaine-induced toxicities.

C

THE SYNAPTIC VESICLE PROTEIN, SYNAPSIN: ITS ROLE IN OPIATE DRUG ADDICTION

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Long-term use of opiates leads to development of tolerance and addiction. The classical biochemical model for opiate dependence is based on opiate-induced changes in adenylate cyclase activity and in the cellular levels of cAMP. We now propose that the changes in cAMP (as well as alterations in Ca^{2+} entry into the cells) are accompanied by a cascade of events that involve other cellular elements. Using spinal cord cells in culture as a model system, we have shown that opiate agonists inhibit the phosphorylation of synapsin I, a synaptic vesicle-associated protein whose phosphorylation is known to be regulated by cAMP and Ca^{2+} concentrations. Phosphorylation of synapsin I plays an important role in neurotransmitter release. Therefore, opiate inhibition of synapsin phosphorylation is a possible mechanism by which opiates inhibit neurotransmission. In addition, we have shown that chronic opiate treatment increases the immunoreactive levels of synapsin I in the treated cells. This accumulation of synapsin may have an important role in the development of tolarance to opiates. Moreover, it explains the enhanced synaptic release observed during opiate withdrawal. *This work was supported by grants from the Anti-drug Authority of Israel, and from the National Institute of Drug Abuse USA.*

D

Title: LIS1, a gene deleted in lissencephaly, a human neuronal migration disorder: gene isolation and expression.

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We have recently reported isolation of a gene *LIS1* located on chromosome 17p13.3 (Reiner et al.,1993). *LIS1* was found to be involved in Miller-Dieker lissencephaly syndrome (MDS). Lissencephaly, or "smooth brain," refers to the absence of normal convolutions or gyri (agyria/pachygyria), and produces severe mental retardation, seizures, and other neurological problems in all affected patients. The lissencephaly is due to a neuronal migration abnormality. In contrast to the normal six-layered brain, the neocortex in classical lissencephaly patients consists of four layers.

In order to conduct expression studies of Lis1 in mice, we have cloned and mapped the corresponding murine homolog. Lis1 expression was studied in developing mouse brain using in situ hybridization. At embryonic day 15 Lis1 expression is localized and is enhanced in sensory organs, the neural layer of the retina, the hippocampal formation, dorsal root ganglia, cortical plate and the thalamus. Most interestingly, Lis1 is expressed in a high levels in the purkinjie cell layer of the cerebellum while the granular cells are migrating inwards. The expression in purkinje cells of the adult is markedly reduced. The expression pattern may explain neuronal migration defects in the patients.

A

ATAXIA-TELANGIECTASIA: POSITIONAL AND COMPLEMENTATION CLONING CONVERGE

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Ataxia-telangiectasia (A-T) is an autosomal recessive disorder involving cerebellar degeneration, immunodeficiency, cancer predisposition, chromosomal instability and radiosensitivity. A-T is heterogeneous, with four complementation groups designated A, C, D and E. The group A and group C mutations, together accounting for 85% of patients, have been localized to chromosome 11q22-23. We have set up a combined system of positional and complementation cloning to identify and isolate the genes harboring the A-T(A) and A-T(C) mutations. Highly polymorphic markers (microsatellites) were generated at the A-T locus and used for recombination mapping and linkage disequilibrium analysis. A consortium of 3 laboratories containing 250 A-T families used these markers to narrow the A-T interval to 1.5 Mb of DNA. Linkage disequilibrium analysis among Moroccan Jewish patients points, however, to a possible A-T(C) locus slightly distal to this interval. The entire region was cloned in yeast artificial chromosomes (YACs), and a cosmid contig is being completed. Identification of transcribed sequences is based on direct selection and exon trapping. Concomitantly, cDNA libraries in episomal vectors are being transfected into A-T cell lines in search of cDNAs that complement the radiomimetic sensitivity of these cells. The two different cloning methods interact with each other, and converge at various points into one integrative system.

B

ON THE STRUCTURE ACTIVITY RELATIONSHIPS OF SCORPION TOXIN BLOCKERS OF POTASSIUM CHANNELS

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In recent years more and more peptidic toxins have been purified from scorpion venoms as they have proven to be useful tools for studying ionic channels. The first toxins which have been characterized are those active on sodium channels and responsible for the high toxicity of scorpion venoms. More recently, shorter toxins (31 to 39 residues, 3 disulfide bridges) have been isolated according to their blocking activity on potassium selective channels. These toxins are generally present in very small amounts in scorpion venoms but may be chemically synthesized. Using natural and synthetic toxins, synthetic analogs of the toxins and shorter peptides derived from them, a structure activity relationship study was made (1,2,3,4,5) that leads to a better understanding of the structural features that may be involved in recognition by Leiurotoxin I-like toxins of the apamin-sensitive Ca^{+}-activated K^{+} channel and by kalotoxin of the high conductance BK-type neuronal channel.

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C

THE INSECT SODIUM CHANNEL AS THE TARGET FOR INSECT SELECTIVE NEUROTOXINS.

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The combined employment of protein chemistry, electrophysiology and neurochemistry enabled the chemical and pharmacological characterization of two classes of neurotoxin polypeptides, the excitatory and the depressant, derived from the venom of Buthinae scorpions which selectively paralyze and kill insects (1). These insect selective neurotoxins:

a) Affect insect neuronal sodium conductance (2).
b) Serve as unique and exclusive probes of the insect voltage gated sodium channels.
c) Bind to these channels through multiple attachment sites which include segments of external loops in domains I, III and IV of the insect sodium channel (3).
d) Distinguish among sodium channels of different groups of insects (4).
e) Are employed as pharmacological tools for the study of insect excitability and the design of future selective insecticides.

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D

ON THE SITES BY WHICH SNAKE CURAREMIMETIC TOXINS AND ACETYLCHOLINE RECEPTORS INTERACT

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To delineate the site by which erabutoxin a (Ea) from *Laticauda semifasciata* interacts with the nicotinic acetylcholine receptor from *Torpedo marmorata* (AcChoR) we generated by site-directed mutagenesis a series of toxin mutants and investigated their affinities for AcChoR on the basis of competition experiments. We found that most residues functionally conserved within the family of curaremimetic toxins are important though not essential for binding of the toxin to AcChoR. These residues all belong to the second and third loops of the toxin. We also found that some variant residues located on the first and second toxin loops are also important for the toxin function. Our findings were supported by data based on the use of a monoclonal antibody which partially mimics the binding properties of AcChoR. Together, available data led us to propose the first experimentally-based delineation of the site by which a short-chain toxin recognizes AcChoR.

To identify the binding site of curaremimetic toxins on AcChoR we investigated, using a solid-phase assay, the capacity of some toxin derivatives to inhibit the binding of labeled toxins on the disulfide-containing fragments 128-142 (Tα128-142) and 185-199 (Tα185-199) of the α-subunit of AcChoR. The data led us to propose a tentative and preliminary model depicting the region to region interactions between curaremimetic toxins and AcChoR.

A

A SELECTIVE CHOLINOTOXIN

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An agent which selectively attenuates cholinergic function in vivo would be an extremely useful tool for: 1) studying factors controlling cholinergic function in vivo; and 2) providing an animal model which is designed to mimic clinical states in which a cholinergic deficiency plays a significant role. AF64A (ethylcholine aziridinium) is a close structural analog of choline with an aziridinium ring inherent in its structure. AF64A competes with choline: a) for its high, as well as low affinity uptake systems; b) at other cellular systems which utilize choline for their function; and c) in enzymatic processes which utilize choline as a substrate. At low concentrations, AF64A induces its neurotoxicity selectively at cholinergic substrate sites. At higher concentrations AF64A also interacts with the low affinity choline uptake system, which is ubiquitous at the membrane level; hence its biological effect no longer remains cholinospecific. AF64A binds covalently with reactive substrate sites, and causes damage as a result of their alkylation. Studies of the molecular basis of action of AF64A have shown that in the N-myc gene, it selectively alkylates DNA at N7 guanine sites. This affinity of AF64A for the guanine site also is reflected in its preferential inhibition of transcription of the G,C-rich acetylcholinesterase (AChE) gene, compared to that of the A,T-rich butyrylcholinesterase (BuChE) gene. This inhibition of AChE and BuChE transcription in vitro implies that a similar effect may occur in vivo as well. In vivo studies following intracerebroventricular administration of AF64A have demonstrated that reduction of cholinergic enzyme levels can be induced in a dose-dependent manner in the hippocampus, and recovery from this cholinergic hypofunction is dose-and time-dependent. In vivo levels of AChEmRNA in hippocampus, on the other hand, are increased following AF64A administration. This, and related information will be reviewed, with the aim of assessing the possible mechanism(s) of action of AF64A to induce its cholinotoxicity in vivo.

B

TETANUS TOXIN BLOCK THE SODIUM-DEPENDENT, HIGH-AFFINITY [^{3}H]SEROTONIN UPTAKE IN RAT CNS SYNAPTOSOMES.

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Tetanus toxin (TeTx) is a powerful neurotoxin that inhibits Ca^{2+}-dependent release of neurotransmitters from all presynaptic nerve endings. The last progress in protein composition of synapsis with the finding that Clostridium neurotoxins are zinc-endopeptidases indicate that TeTx could block neurotransmitter release by cleaving proteins that play an important part in release fenomena. Tetanus disease has been ascribed to an inhibition of both GABA and Glycine release in CNS, however some symptoms such as insomnia or sensorial hyperactivity could not be explained with this single alteration. We therefore are studying other mechanisms that could help in the understanding of the disease. We find that TeTx produces time- and dose-dependent inhibition of Na^+-dependent [^{3}H]serotonin (5-hydroxytryptamine, 5-HT) uptake in rat CNS synaptosomes. The uptake inhibition is found in all serotonergic areas, being the maximum uptake reduction in hippocampus and occipital cortex, but also significant in other CNS areas except in pons-medulla and cerebellum. Maximal reduction of [^{3}H]5-HT uptake arrives in hippocampal synaptosomes to 60% with TeTx treatment (10^{-9} M, 37°C, 60 min) but started to be significant at lower doses (10^{-12} M) or shorter incubation time (10 min). Of the two papain splits TeTx fragments, L-H_N (which is responsible for the toxic action) is consistently more effective than H_C (which is responsible for the binding to the nerve tissue), as inhibitor of [^{3}H]5-HT uptake in synaptosomal preparations. Also, simultaneous treatment of synaptosomes with the two TeTx fragments results in the same inhibition as with the whole neurotoxin. Treatment of synaptosomes with protein kinase C (PKC) inhibitors H-7 or sphyngosine, or with PKC activator phorbol 12-myristate 13-acetate (PMA), shows no clear implication of this regulatory kinase. In conclusion, the reduction of serotonin uptake represents a direct action of TeTx on the CNS, and could be responsible for tetanic symptoms that have been related to the serotonergic system.

C

GPI ANCHOR-HYDROLYZING PHOSPHOLIPASES

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GPI anchor-hydrolyzing phospholipases (GPI-PL) include C- and D-type enzymes which have been described in a number of organisms including bacteria, protozoan parasites, plants, and mammals. Although, these phospholipases efficiently cleave GPI structures *in vitro*, the physiological role of GPI hydrolysis by GPI-PL is still unclear. PI-PLC has been studied extensively over the past decades, mainly due to its involvement in signal transduction events. Interestingly, a particular subgroup, the bacterial PI-PLCs, was found not only to hydrolyze phosphatidylinositol (PI) but also protein GPI anchors. In fact, it was this unique property of bacterial PI-PLCs which initially lead to the discovery of GPI-anchored proteins. Today, bacterial PI-PLCs are widely used in the detection and characterization of GPI-anchored proteins and lipids, and several of these enzymes have recently been characterized in terms of their ability to hydrolyze GPI structures and PI. In mammals, the only GPI anchor-hydrolyzing enzyme known today is GPI-PLD which occurs in large amounts in serum. Although, it is not known whether the relative abundance has any biological significance, GPI-PLD was suggested to play a role in releasing GPI-anchored proteins from cell surfaces *in vivo*. In serum, GPI-PLD occurs in association with apolipoprotein A-1, a constituent of the high density lipoprotein fraction which *in vitro* stimulates GPI-PLD activity. GPI-PLD is activated by limited proteolysis, and the proteolyzed form displays a high resistance towards further degradation by proteases. This suggests that *in vivo*, the enzyme might undergo intracellular processing leading to a membrane active form of the enzyme.

D

FUNCTION AND CELLULAR DISTRIBUTION OF THE F3 GPI-ANCHORED NEURONAL MOLECULE.

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Using the neuronal glycoprotein of the immunoglobulin superfamily F3 as a model we will discuss how its GPI anchor might confer on molecules specific properties which may be particularly well suited to a role in modulating neuronal interactions.

1) When anchored to plasma membrane, F3 fulfills the operational criteria of an adhesion molecule; this activity had been mapped to its fibronectin type III repeats. Under soluble form F3 promotes neurite outgrowth of cultured neurons and the activity is displayed by the immunoglobulin-like domains. 2) To establish the physiological importance of these two forms we undertook a comprehensive description of the cellular localization of GPI -membrane anchored and soluble forms in the hypothalamo-neurohypophysial system. This system offers the opportunity to dissect neuronal cell bodies independently of their terminals. F3 is more abundant in the neurohypophysis where the axons terminate than in the hypothalamic nuclei where the molecule is synthetized. In addition, soluble forms are prevalent in the neurohypophysis suggesting that there is conversion of the GPI-bearing form to the soluble form during axonal transport and that GPI could be implicated in sorting to plasma membrane or to the extracellular space. 3) In the cerebellum, F3 is polarized to the tips of the axons of granule cells, the major neuronal population of this system, as an indication that indeed GPI might be a signal for targeting molecules to axons. However, there are exceptions such as Golgi neurons or hypothalamic neurons that express F3 all over their entire surface.

A

SITES OF MEMBRANE INSERTION AND INTERNALIZATION OF GPI-ANCHORED PROTEINS IN GROWING AXONS

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Neuronal growth proceeds by insertion of new membrane material in the growing axon or dendrite. Newly synthesized material (comprised of proteins and lipids) is assumed to be synthesized in the cell body and transported in vesicles by axonal transport to the growing processes. We have recently shown that inhibition of sphingolipid (SL) synthesis affects axonal outgrowth in cultured hippocampal neurons [*J. Biol. Chem.* (1993) **268**, 14476]. However, inhibition of SL synthesis had no apparent effect on the delivery (analyzed by immunofluorescence) of two GPI-anchored proteins, Thy-1 and Tag-1, to either axons or dendrites, even though considerable evidence suggests that, at least in polarized epithelial cells, glyco-SLs are involved in the targeting and transport of GPI-anchored proteins to one or other plasma membrane domain.

At short times after removal of Tag-1 from the cell surface by phosphatidylinositol-specific phospholipase C (PIPLC), Tag-1 immunofluorescence reappeared along the entire length of the axon surface, and not just at the growth cone, both in cells treated with or without inhibitors of SL synthesis. The reappearance of Tag-1 at the cell surface was completely blocked by incubation with Brefeldin A, which inhibits transport of vesicles out of the Golgi apparatus, confirming that Tag-1 is delivered to the cell surface by vesicular transport. Finally, labeling of live cells with an anti-Tag 1 antibody demonstrated that Tag-1 is rapidly internalized from axons and dendrites. These data suggest that there is no preferential addition of Tag-1 at the growth cone of growing axons, and indicate that at least one class of vesicles (i.e. those containing GPI-anchored proteins) is able to be inserted into the growing axon along its entire length.

B

DEVELOPMENTAL BRAIN DAMAGE, PKC AND COGNITIVE DYSFUNCTION

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A great body of evidence suggest that cognitive processes are confined to specific brain areas and that learning and memory are accompanied by changes in synaptic efficacy. The molecular mechanisms responsible for these changes are under extensive investigation and the central role of phosphorylation processes has clearly emerged. Recent in vitro studies have established a link between electrophysiological forms of synaptic plasticity and protein kinase C (PKC)-dependent processes at hippocampal synapses: however, this has been much more difficult to prove in vivo.

We have addressed this point by inducing selective and non-invasive ablations of cortical and hippocampal neurons by administering methylazoxymethanol acetate (MAM) to rats at day 15 of gestation. Since MAM has a selective antiproliferative effect on neuroepithelial cells, the offspring show a considerable reduction in the number of neurons in both brain areas and are characterized by severe defects in learning and memory. Biochemical investigation of the synapses of MAM-treated rats shows that a long lasting increase in the translocation of presynaptic PKC is paralleled by an increased basal phosporylation state of its specific protein substrate B-50/GAP-43 and an increased glutamate release. On the other hand, long-term potentiation (LTP) at these synapses is selectively impaired, in the absence of any change in the low frequency transmission. However, LTP could be restored by pharmacological manipulation of the glycine modulatory site at the NMDA receptor complex, indicating that the chronic increase in the transmitter release machinery brings about an adaptive change at post-synaptic glutamatergic receptors.

All these data point to the necessity of having appropriate animal models to study in vivo molecular mechanisms of synaptic plasticity in order to understand the importance of different synaptic elements in the complex biochemical pathways leading to cognitive deficits under situations mimicking pathological conditions.

C

RELATIONSHIP BETWEEN NEUROFIBRILLARY DEGENERATION, NEUROTRANSMITTER DEFICIENCY AND SEVERITY OF COGNITIVE DYSFUNCTION IN ALZHEIMER'S DISEASE

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The degree of cholinergic cortical deafferentation arising in the basal forebrain (BF) as well as the extent of neurofibrillary degeneration within the cerebral cortex found post mortem have repeatedly been described to correlate with the degree of cognitive dysfunction in AD. In the present study, we have comparatively analysed the process of neurofibrillary degeneration in the BF, in the cerebral cortex and in 12 further subcortical brain structures including those giving rise to the aminergic innervation of the cerebral cortex. The distribution of neurofibrillary degeneration was mapped both biochemically (ELISA) and immunohistochemically using a monoclonal antibody specific to abnormally phosphorylated tau proteins. Multiple regression analyses were performed to characterize the relationship between the extent of neurofibrillary degeneration, regional changes in the activity of choline acetyltransferase (ChAT), and in the content of aminergic neurotransmitters and the severity of cognitive dysfunction. The highest degree of correlation between cognitive dysfunction and post mortem measures was obtained for the extent of neurofibrillary degeneration and cell loss in the BF. Degeneration in other subcortical areas, although sometimes being quite severe, showed either no or only a weak relationship. Degeneration within the BF which rather selectively affected cholinergic neurones while sparing other cell types, showed a heterogeneous involvement of cell clusters throughout its rostro-caudal extension. This pattern of degeneration is reflected at the cortical level by an increase in the amount of abnormally phosphorylated tau protein as well as decrease of ChAT activity in the respective target area. The strongest relationship with cognitive dysfunction was observed for the density of neuropil threads present in those cortical areas which were affected by cholinergic deafferentation. The results indicate that the formation of abnormally phosphorylated tau proteins in cortical cholinergic axon terminals which might be related to a loss of cholinergic synapses and to cholinergic disconnection of the cortex, provides the most reliable correlate of cognitive dysfunction in AD. (Supported by the BMFT: 01 ZZ 9103-2.7)

D

FASTER INHIBITION RATES OF NORMAL BuChE AS COMPARED WITH AChE AND THE D70G "ATYPICAL" BuChE MUTANT PREDICT INDIVIDUAL VARIABILITIES IN RESPONSE TO ANTICHOLINESTERASE THERAPY

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Application of anticholinesterase drugs is one of the leading experimental approaches for treating patients suffering from Alzheimer's disease (AD) in attempt to balance their cholinergic system. These drugs are targeted at the acetylcholine hydrolyzing enzyme acetylcholinesterase (AChE) but also inhibit butyrylcholinesterase (BuChE), known for its numerous genetic variants. The most common variant involves replacement of Asp70 by Gly (D70G) through a point mutation and is termed the "atypical" enzyme which displays a considerably reduced sensitivity to various inhibitors. The relatively high frequency of "atypical" BuChE (11% heterozygotes in certain populations) implies that in each group of AD patients treated with anticholinesterase drugs, carriers of one or two alleles with this mutation should be expected. To predict their responsiveness to this treatment, we examined the susceptibility of AChE as compared to that of BuChE and the "atypical" BuChE variant towards several such anticholinesterase drugs. Rate constants reflecting inhibitor susceptibilities were calculated for various recombinant human cholinesterases produced in *Xenopus* oocytes and immobilized onto microtiter plates through selective monoclonal antibodies. BuChE's pseudo first order inactivation rate constants for the carbametes heptyl-physostigmine ($0.139min^{-1}$, 10nM inhibitor), physostigmine ($0.3min^{-1}$, 1μM inhib.) and SDZ-ENA713 ($0.139min^{-1}$, 10μM inhib.) were found to be higher than or equal to that of AChE, suggesting that BuChE serves as a second primary target for this drug. Moreover, the common D70G "atypical" variant of BuChE displayed considerably slower inactivation rates to these drugs ($0.01min^{-1}$, $0.025min^{-1}$, and $0.01min^{-1}$ respectively). The reversible amino acridinium compound Tacrine (1mM BtCh as substrate), also examined for AD therapy, diplayed 300-fold higher IC50 values for the "atypical" enzyme than for BuChE. These findings predict that carriers of the D70G BuChE mutation should vary from other patients in their susceptibility for the above drugs, which potentially contributes to the wide variability of responses observed in clinical trials.

A

GENE EXPRESSION IN THE AXONAL DOMAIN

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At variance with the opinion that the axon domain is unable to synthesize proteins, the axoplasm of the squid giant axon was found to contain all the components of the eukaryotic system of protein synthesis, including soluble translation factors, tRNA, rRNA, mRNA and active polysomes. The complex family of axoplasmic mRNAs was cloned in a lambda ZAP vector, and the clones encoding β-actin, β-tubulin and kinesin were characterized and sequenced. The axoplasmic localization of these mRNAs was confirmed by in situ hybridization analyses. The cell origin of axoplasmic RNAs was examined in isolated or perfused giant axons incubated with [^{3}H]uridine. Under these conditions, all the main RNA classes were recovered in the axon compartment, presumably derived from glial cells. In a separate set of experiments, the protein synthetic activity of a synaptosomal fraction from squid brain was found to be highly sensitive to cycloheximide and fully inhibited by hypo-osmotic shock. The large differences observed in the patterns of newly-synthesized synaptosomal proteins (and of immunoabsorbed NF proteins) in comparison with those of cell body proteins suggested that the activity of the synaptosomal fraction was significantly due to nerve endings. Similar results were obtained in cell-free translation experiments using purified polysomes prepared from the synaptosomal and microsomal fractions of squid brain.

B

DIADENOSINE POLYPHOSPHATES IN THE CENTRAL NERVOUS SYSTEM: THEIR ROLE AS NEW NEUROTRANSMITTERS

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Diadenosine polyphosphates, Ap_4A (diadenosine tetraphosphate) and Ap_5A (diadenosine pentaphosphate), are active substances stored in secretory vesicles of several neural models such as *Torpedo* electric organ and chromaffin cells, together with diadenosine hexaphosphate (Ap_6A). Diadenosine polyphosphates present in synaptic terminals are release by depolarizing agents in the presence of Ca^{2+}. In "*in vivo*", push-pull experiments the release of these compounds can be induced by amphetamine admininstration. The half life of the dinucleotides was very high in contrast with ATP and ADP which dissappear quickly from the perfusion media. This facts suggest a transmitter role for these substances at the synaptic cleft. Furthermore, the presence of P_2-purinergic receptors was investigated with the help of the radioligands [^{3}H]Ap_4A and [^{35}S]ADP-ß-S. The presence of two P_2-purinoceptors with Kd values of 10^{-10} M and 10^{-6} M was reported. The pharmacology of these receptors was rather different from the previously described in the literature for P_2-purinoceptors. Inhibition of synaptic transmission in the hippocampus by diadenosine polyphosphates has been reported. Studies carried out in synaptosomes, demonstrated the presence of two distinct purinoceptors. One is a P_2-purinoceptor and the other is a new one termed P_3-purinoceptor. The P_2-purinoceptor is only sensible to ATP and its synthetic analogs. This receptor seems to be coupled to the opening of voltage-sensible calcium channels as showed the studies performed with Ca^{2+} channel blockers.
The P_3-purinoceptor is sensible to diadenosine polyphosphates but not to ATP and its synthetic analogs. This receptor is a receptor-operated channel, not sensible to voltage-dependent channel blockers. Both receptors induce an increase in the cytosolic Ca^{2+}. In summary, diadenosine polyphosphates exert many of the typical features of neurotransmitters and they enlarge the possibilities of purinergic transmission in the central nervous system.

C

PUTATIVE ROLE OF ASTROCYTES IN GLUTAMATERGIC NEUROTRANSMISSION

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Using cultured striatal astrocytes from mouse embryos, we have shown that the activation of phospholipase C (PLC) mediated by α_1–adrenoceptors was potentiated by adenosine, somatostatin or α_2-adrenergic agonists. In fact, the resulting protein kinase C activation allowed A_1 adenosine, somatostatin or α_2-adrenergic receptors to be coupled to a phospholipase A2 (PLA2), leading to the release of arachidonic acid. Arachidonic acid inhibited the uptake of glutamate which is spontaneously released from astrocytes, resulting in the accumulation of this amino-acid in the extracellular medium. Glutamate, in turn, stimulated metabotropic receptors coupled to PLC, accounting for the apparent potentiation of the α_1-adrenergic PLC activation by adenosine, somatostatin or α_2-adrenergic agonists (1-3).
Additional experiments indicated that ATP alone (which is the cotransmitter of acetylcholine in cholinergic interneurons) or glutamate itself, also stimulated arachidonic acid release from astrocytes (4). Moreover, both mediators acted synergistically on these cells (4). In addition, in striatal neurons, the NMDA-induced arachidonic acid release is strongly potentiated by acetylcholine. This suggests that cholinergic interneurons, through a mechanism involving, in part, astrocytes, contribute to the modulation of glutamatergic neurotransmission. More generally, it might be proposed that in response to various stimuli, astrocytes, by regulating glutamate uptake, could participate to the regulation of glutamatergic neurotransmission, as recently confirmed by an electrophysiological study (5).

References: (1) El-Etr et al., J. Neurosci. 12:1363 (1992); (2) Marin et al. Proc. Natl. Acad. Sci. 88:9016 (1991); (3) Marin et al. Mol. Pharmacol. 44:1176 (1993); (4) Stella et al. J. Neurosci. 14:568 (1994) (5) Mennerick and Zorumski Nature 368:59 (1994).

D

TRANSGENIC MODELS FOR DOWN'S SYNDROME RELATED GENE-DOSAGE EFFECTS.

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Down's syndrome (DS) is one of the most common genetic abnormalities occurring once every 600-800 live births. The syndrome which results from the presence of an extra copy of chromosome 21 (Chr. 21), is unique in that it is caused by an overexpression of otherwise normal genes; the excess of gene products encoded by the extra Chr. 21 creates an imbalance in various biochemical pathways, giving rise to the clinical picture of DS. To dissect the complex phenotypic effects induced by altered expression of unknown number of Chr. 21 encoded genes, we have developed cellular and animal models which enabled the study of biochemical and physiological changes imposed by overexpression of isolated candidate genes. The cellular system consists of transfected cells overexpressing a single Chr. 21 gene while the animal model employs transgenic mice harboring extra copies of the gene and expressing elevated levels of the gene product. Using this approach it has been shown by us that overexpression of the Cu/Zn-superoxide dismutase (CuZnSOD) gene and the liver phosphofructokinase (PFKL) gene lead to specific phenotypic effects, similar to those observed in trisomy 21 cells and DS patients, thus providing insights into the genesis of these symptoms. For example, it was found that transfected PC12 cells overexpressing CuZnSOD had impaired uptake of neurotransmitters due to diminished pH gradient across their chromaffin granule membrane. This observation indicated that an imbalance in just one gene can produce alterations in neuronal cells which would impair the transduction of signals and mimic the deficiencies apparent in DS. In the animal model, transgenic-CuZnSOD mice overexpressing the gene, we found that the blood platelets have reduced uptake of serotonin due to a defect in the dense granule transport system. It is intriguing that this very same lesion appears both in the cellular and in the animal model system and that the consequent defect is a well known deficiency diagnosed in DS. These findings are the first example where a direct link between a clinical symptom of DS and a gene dosage effect of an individual gene has been established. Transfected cells and transgenic-PFKL mice overexpressing PFKL were also analyzed. Transfected PC12-PFKL cells with elevated activity of PFK exhibit altered glycolysis and the tetrameric phosphofructokinase purified from these cells had modified properties indicating that increased gene dosage can exert its influence not merely by enhancing the amounts of gene products but also by altering their biochemical nature.

A

Expression of mouse Pax genes in the developing nervous system.
Peter Gruss, Anastassia Stoykova, Ed Stuart and Ahmed Mansouri

Pax genes have been cloned on the basis of their homology to the Drosophila segmentation gene "paired". To so far, nine members of this family have been characterized and are expressed in a highly specific spatial and temporal pattern. In order to study the role of these genes, we have examined loss-of-function mutations. Pax3 was correlated with a pre-existing mutant named "splotch" (Sp). This mutant shows exencephaly, spina bifida and partial lack of spinal ganglia. A human homologue was identified to be mutated in individuals with "Waardenburg syndrome I". Pax6 has been correlated with a pre-existing mutant small-eye (Sey). *Sey* homozygous lack eyes and nose indicating a requirement of Pax6 in inductive processes. We have recently discovered a highly distinct Pax6 expression pattern in defined parts of the mouse brain. In particular, in the developing telencephalon and in thalamic structures. In Sey mutants we discovered a delayed development of thalamic structures indicating a role of Pax6 in differentiation of these brain tissues. In order to study the role of Pax7 we have performed a "knock-out" analysis. In Pax7 null mutants we observed a pointed snout which corresponds with Pax7 expression in nasal cartilage and/or nasal bones. Pax7 null mutants in general die within the first 3 weeks after birth. Since Pax7 and Pax3 belong to the same paralogous groups it is quite possible that regulatory complementation between these two genes occurs which predicts a strong phenotype in double homozygous mice. A misexpression of Pax genes revealed that murine Pax genes are proto-oncogenes. These in vitro results have been impressivly supported by Galli et al. 1993 and Shapiro et al. 1993 who discovered that in rhabdomyosarcoma Pax3 is fused with a novel fork head gene. These data indicate that Pax genes can play a role in tumor formation in vivo and in vitro. The molecular mechanisms of Pax gene function in development and oncogenesis will be discussed.

B

CLONING AND EXPRESSION OF RAT UDP-GAL:CERAMIDE GALACTOSYLTRANSFERASE (CEREBROSIDE SYNTHASE)

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Galactosylceramide (galactocerebroside) is highly enriched in the myelin sheath. Its expression on the cell surface is generally taken as the hallmark of differentiated myelinating cells, the oligodendrocyte in the CNS and the Schwann cells in the PNS. The last step of its synthesis, galactosylation of ceramide, is catalyzed by UDP-galactose-ceramide galactosyltransferase (CGT). We serendipitously isolated cDNAs coding for the rat CGT while attempting to clone adhesion molecules involved in myelination in the sciatic nerve. The cDNA consisted of 4188 bases, including an open reading frame of 1623 bp, which predicts a protein of M_r=61,126 Da with an N-terminal signal sequence and three potential glycosylation sites. The sequence was the same as that reported recently by Schulte and Stoffel (Proc. Natl. Acad. Sci., USA 90:10265, 1993) who cloned it on the basis of the partial amino acid sequence of purified enzyme. We expressed the catalytic activity in stably transfected CHO cells, providing the proof for its authenticity. Expression of its mRNA closely paralleled myelination in rat brain, while the developmental pattern was broader in the sciatic nerve. The absolute amounts of CGT mRNA in the brain and sciatic nerve were comparable to those which encode structural proteins of myelin. Following nerve injury, its expression in the sciatic nerve dropped significantly and then recovered more slowly compared to the expression of mRNAs coding for myelin-specific proteins. Non-neural tissues examined did not contain the CGT mRNA detectable by simple Northern blotting, except for the kidney which is known to contain small amounts of galactosylceramide. Southern blotting indicated that the rat CGT is likely to be encoded by a single, relatively large gene. Availability of the rat CGT cDNA should facilitate investigation of cell differentiation, myelin synthesis and maintenance.

C

BCL-2 IS IMPORTANT IN CONTROLLING CELL DEATH DURING THE DEVELOPMENT OF THE NERVOUS SYSTEM.

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Apoptosis, also known as programmed cell death, is an active process which is thought to be involved in controling cell numbers in various tissues of different species during embryonal development and throughout adult life. It is estimated that half of all neurons produced during embryogenesis die before adulthood. Although the mechanisms governing apoptosis are as yet unknown, it is suggested that in neurons cell death takes place when the number of neurons reaching their targets exceed the optimal number. In this case the concentration of target-derived neurotrophic factors available to the neuronal population is not sufficient to support their survival and therefore results in their death. The *bcl*-2 proto-oncogene product protects different cell types from apoptotic cell death and is expressed in the developing and adult nervous system. To study the role of *bcl*-2 in regulation of neuronal cell death we generated transgenic mice expressing *bcl*-2 in neurons under the control of the neuron specific enolase promoter. These transgenic mice express high levels of the *bcl*-2 transgene uniquely in neurons. Cultured sensory neurons isolated from dorsal root ganglia of newborn mice depend for their survival on nerve growth factor, however when these cells were isolated from the *bcl*-2 transgenic mice they survived in culture in the absence of neurotrophic factors. The number of different types of neurons in the central and peripheral nervous system was found to be at least 50% higher in the *bcl*-2 transgenic mice than in normal mice indicating the importance of *bcl*-2 in controlling neuronal survival during development.

D

BRAIN TUMORS IN BIGENIC MICE: MOLECULAR CHARACTERIZATION OF THE ONCOGENIC PROCESS

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A genetic model for brain tumors was developed in a binary system of transgenic mice. Mice that carry either the *c-myc* oncogene driven by a weak enhancer or the transactivator of that weak enhancer were created. The bigenic mice, carrying both the oncogene and the transactivator, exhibit dramatic and rapid tumor formation manifested by central nervous system tumors and abdominal lymphomas. The CNS tumors are composed of small round cells that can be grown in monolayer culture and express neuronal markers. To characterize the molecular events that are leading to the transformation, we are searching for genetic targets for c-*myc* oncogene in this system. We have constructed a cDNA library from mRNA of the brain tumor cells and used a subtraction/co-expression strategy to identify genes which are putative targets for c-Myc regulation. Of several genes that we have isolated, one, named *ECA39*, was studied in detail. The *ECA39* gene is expressed in several c-*myc* based tumors and bears a functional c-Myc binding sequence located 3' to its transcription start site. This sequence is required for expression of the gene and alterations in expression of Myc oncogenes affect expression of the *ECA39* gene. To further characterize *ECA39* gene, we have isolated the human homologue, and shown conservation of the peptide between nematode, mouse and human. Moreover, the recognition site for Myc binding, located 3' to the start site of transcription in the mouse gene, is also conserved in the human homologue. This suggests that in human, as in mouse, *ECA39* gene serves as a genetic target for Myc regulation.

A

ROLE OF MICROGLIA CELLS IN THE COURSE OF EAE

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Microglia cells are the most important resident immunocompetent cells in the brain. Their putative function in inflammatory brain lesions includes phagocytosis, antigen presentation as well as the secretion of immunoregulatory molecules. We have studied the role of resident microglia and hematogenous macrophages in acute EAE, induced in radiation bone marrow chimeras by passive transfer of encephalitogenic T-cells. The majority of activated effector cells in acute inflammatory lesions were found to be macrophages, entering the CNS from the circulation. In addition, however, the pool of resident microglia expressed macrophage activation antigens and MHC-molecules and was actively engaged in the removal of tissue debris. During recovery from the disease hematogenous macrophages were nearly completely removed from the lesions. Some activation markers, however, persisted to be expressed on resident microglia a considerable time after clearance of the inflammatory process.

B

ENERGY METABOLISM AND NEUROTRANSMITTER AMINOACIDS DURING EAE ATTACKS.

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The aim of this study was to detect and quantitate changes in spinal cord and brain during an EAE attack. EAE was induced in Lewis rats by subcutaneous inoculation with guinea pig spinal cord and complete Freund's adjuvant. All animals were weighted daily and examined for symptoms of EAE and accorded a score using a graded scale. Spinal cord and brain from the rats were extirpated under strict controled circumstances. Extracts from homogenates in D_2O and perchloric acid were prepared at low temperatures and used for ^1H NMR spectroscopy. Other extracts were made from sulphosalicylic acid homogenates and after neutralization used for biochemical analyses. Aminoacids were measured by HPLC, other biochemical components spectrophotometrically.

During attacks, aminoacids i.e. asparagine, glutamine, glycine and taurine were increased, while the levels of aspartate, glutamate and GABA were reduced. Energy metabolism indicators lactate and uric acid were increased. These results can be explained by a chronic energy stress perhaps caused by the local inflammations. Energy depletion impairs re-uptake of neurotransmitters into neurons and increases their extracellular concentrations causing neurotoxicity. The increase in aminoacids in our experiments can perhaps explain neuronal cell damage in chronic EAE and possibly the clinical symptoms during an attack.

C

THE ROLE OF SEX HORMONES IN EAE.*

An immunological approach.

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In the present study we examined the influence of sex hormones on the course of EAE, an animal model for multiple sclerosis. We induced EAE in male and female Lewis rats. All male rats developed a severe chronic (relapsing) course of EAE. By contrast, only 50% of the female rats developed a chronic course of EAE. The remaining 50% of the female rats showed complete and permanent recovery of the initial acute attack. Pathology in the central nervous system (CNS; infiltration of leukocytes and demyelination) was more severe in male rats than in female rats. In the immune system of male rats the frequency of leukocytes belonging to resting memory T-cell subset 2 (RTM2, Th_2-like cells) was lower and the frequency of antigen-activated $CD8^+$ T-cells was higher than in female rats. Upon gonadectomy, not only all male rats but also all female rats developed a chronic (relapsing) course of EAE and showed severe pathology in the CNS. Substitution therapy with estradiol restored recovery after the initial acute attack. Depletion of cells of the RTM2 subset in intact female rats postponed recovery of the initial acute attack. From these data we conclude that the ovarian production of estradiol (partially) protects female rats against relapses of EAE and pathology in the CNS. Probably estradiol (pre-) determines the course of EAE through an increase of the frequency of cells of the RTM2 subset. Epidemiological findings indicate that sex hormones modulate the course of MS as well. Therefore sex hormones may offer a new strategy to ameliorate the course of this human neurological disease of putative autoimmune etiology.

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D

IMMUNOMODULATION OF EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS WITH LINOMIDE, IN THE ABSENCE OF SYSTEMIC IMMUNOSUPPRESSION

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We investigated the effects and the mechanisms of action of linomide on experimental autoimmunity. Linomide, is a synthetic immunomodulator, that increases the natural killer (NK) activity and induces a stimulation of several other lymphocytic subpopulations. We have previously shown that linomide was extremely effective in inhibition of clinical and pathological signs of experimental autoimmune encephalomyelitis (EAE and CR-EAE), experimental myasthenia gravis, SLE-like disease and autoimmune diabetes, in experimental animals. EAE and CR-EAE were induced in SJL/J mice with s.c. injections over the flanks, of mouse spinal cord homogenate (MSCH) in adjuvant or by adoptive transfer of in vitro (with MBP or PLP) sensitized lymphocytes. Linomide was administered in the drinking water, in an estimated dose of 20-100 mg/Kg/day, starting either from the day of EAE/CR-EAE induction, or 8-20 days after the clinical onset of paralysis (22-37 days post immunization).Treatment with linomide totally protected all of the immunized mice from the development of EAE and CR-EAE. Linomide was equally effective in outbred animals. When given after the onset of CR-EAE, it inhibited the development of the disease; 22 spontaneous relapses of CR-EAE were observed in the untreated control mice (mean relapses/mouse:1.83±0.3), as compared to only 3 relapses in the linomide treated group (mean relapses/mouse:0.3±0.2, $p<0.001$). Treated mice were also resistant to an incipient attack, induced by a late rechallenge with MSCH. Almost all of the untreated controls (7/8) relapsed after the rechallenge, compared to only 2/8 of the linomide treated mice ($p<0.01$). Lymphocytes from treated mice showed a reduced in vitro proliferating response to MBP, PLP, PPD and to PMA+ionophore, but revealed a normal proliferation following anti-CD3 stimulation. These lymphocytes expressed the adhesion molecules LFA-1 and CD-18 at lower levels and produced significantly less IL-2 and IFNg (in vitro) compared to EAE-controls. Macrophages from linomide-treated mice showed a decreased abillity for antigen-presentation to MBP/PLP-specific T-cell lines. A significant reduction in the $Mac1^+$ cells was noted in the spleens of linomide treated animals. The enhanced NK activity that was found in mice treated with linomide could be associated with this reduction of the antigen-presenting cells. Linomide is very effective in regulation of experimental autoimmunity. It seems that it interferes with antigen-presentation and inhibits the T-cell activation process at very early stages, without inducing generalized immunosuppression.

A

PRENATAL ADMINISTRATION OF VIP ANTAGONIST PRODUCES SEVERE MICROCEPHALY

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Vasoactive intestinal peptide (VIP) produces dramatic growth of post-implantation mouse embryos that is characterized by large increases in mitosis (Nature 362:155, 1993). To assess the role of VIP on early nervous system growth *in vivo*, pregnant mice were treated with a specific antagonist to VIP (JPET 257:959, 1991). Prenatal administration of the antagonist early in development (E8-E11) produces a severe microcephaly, characterized by decreases in embryonic brain weight with reduced DNA and protein content. The retardation of growth was disproportionally manifested in the brain compared to the body and was prevented by co-treatment with the VIP, but not pituitary adenylate cyclase activating peptide. Identical treatment with the antagonist later in gestation had no detectable effect on embryonic growth. VIP binding sites were increased in the neuroepithelium of antagonist-treated embryos. The number of cells in S phase, as determined by bromodeoxyuridine immunocytochemistry, was significantly decreased, affecting neural (82% of control) tissue rather than non-neural tissue (96% of control). These data suggest that VIP is a regulator of brain growth and inhibition of its action provides new insight into a molecular mechanism for microcephaly.

B

PHARMACOLOGICAL DISCRIMINATION OF MULTIPLE VIP RECEPTORS WITH SUPERACTIVE LIPOPHILIC PEPTIDES

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To distinguish vasoactive intestinal peptide (VIP)[J. Molecular Neurosci. **4**, 1 (1993)] receptors in the brain that mediate neurotransmission from those involved in neuro-trophism, potent VIP analogues were designed. Using a single amino acid substitution and the addition of a fatty acyl moiety, an analogue was devised that exhibited both a 100-fold greater potency than VIP and specificity for a VIP receptor associated with neuronal survival. This VIP agonist increased neuronal survival via a cAMP-independent mechanism. Identical chemical modification of a prototype hybrid VIP antagonist ($neurotensin_{1-6}VIP_{7-28}$) also resulted in a 100-fold greater potency in blocking VIP-mediated increases in neuronal survival. Blockade of circadian activity rhythms was limited to VIP antagonists that could inhibit VIP-mediated increases in cAMP. The practical significance of VIP analogues is only beginning to be recognized with the advent of non-invasive treatment of human impotence with the superactive lipophilic VIP agonists [J. Clinical Investigation **90**, 810 (1992); Endocrinology in press) and the potential for blocking the autocrine growth properties of VIP in human lung carcinoma [Proc. Natl. Acad. Sci. USA, **90**, 4345 (1993)] and neuroblastoma [Brain Res. **624**, 339 (1993)]. The plausibility of pharmacologic intervention in human neurodegenerative disease approaches reality with the potential use of lipophilic peptides having potent neuroprotective effects.

C

GALANIN AGONISTS, ANTAGONISTS AND GALANIN RECEPTORS SUBTYPES

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Galanin actions in the CNS including the inhibition of hippocampal acetylcholine release, inhibition of neuronal firing in the locus coeruleus (LC) and stimulation of feeding and of growth hormone release can be elicited by N-terminal fragments of galanin such as galanin(1-15 or 1-16). The same N-terminal fragments acting at G_i/G_o protein coupled receptors inhibit the glucose induced insulin release from mouse pancreatic islets. Activation of smooth muscle contractile response in rat jejunum, however, requires both the N- and C-termini of galanin.

The high affinity, chimeric galanin receptor antagonists: M15 (galanin (1-13)-SP(5-11)), M35 (galanin(1-13)-bradykinin(2-9) and M40 (galanin(1-13)-(AlaPro)2Ala amide) inhibit the hippocampal; LC and hypothalamic responses to galanin, while the pituitary effect of galanin is not blocked by M15 and the pancreatic effect of galanin is not blocked by M40. At the smooth muscle galanin receptors both M15 and M35 are acting as agonists.

Thus *in vivo* and *in vitro* studies using high affinity agonists and antagonists of galanin receptors suggest that subtypes of galanin receptors may exist.

D

RECEPTOR SELECTIVE SUBSTANCE P ANALOGS

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The substance P related tachykinin peptides all share the C-terminal sequence Phe Xaa Gly Leu Met NH. This peptide sequence binds to the receptor and it has been strictly conserved through evolution. We have argued that in preparation of receptor-selective analogs, one should not use any of the modifications open to evolution or recombinant DNA methodology, as these modifications have all been tried. We have rather directed our modifications toward the peptide backbone. Of the many modifications that we have tried systematic N-methylation of each peptide bond in the substance P C-terminal hexapeptide sequence proved to yield receptor selective analogs. Furthermore, N-methylation of different peptide bonds gave rise to analogs with different receptor selectivity. We will discuss: 1. Application of the method for preparation of receptor selective analogs. 2. The molecular basis for receptor selectivity. 3. Potential use of the receptor selective analogs for dissection of complex biological responses and for rational drug design.

A

MEDIATOPHORE : A PROTEIN OF COMMUNICATION.

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The only known nerve terminal membrane protein that translocates acetylcholine and glutamate upon calcium action when reconstituted in proteoliposomes is mediatophore. This fully sequenced protein is a homooligomer of 15Kd subunits which has been localized at the presynaptic membrane active zone. Activated in high calcium but desensitized in low calcium, mediatophore has properties generally described for allosteric proteins which explain many characteristics of the release machinery. The 15Kd subunits are also found in the membrane sector of the complex V-ATPase associated to other components. It therefore controls through the proton gradient the vesicular storage of the transmitter. This 15Kd subunit also exists in several gap junctions. Hence, the 15Kd gene product seems to be an essential element of the cellular communication network. Perturbations of the 15Kd expression may lead to tumorigenic effects suggesting a link with cell communication.

B

INVOLVEMENT OF VACUOLAR H^+-ATPase IN THE NEURONAL SECRETORY PATHWAY

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Vacuolar H^+-ATPases are members of a larger family of proton pumps that includes the mitochondrial, chloroplast and bacterial ATP-synthases (F-ATPases). The catalytic sectors of V-ATPases are readily dissociated from the membrane sectors. Incubation of membranes containing V-ATPase on ice in the presence of MgATP causes the dissociation of the catalytic sectors from the membranes. This cold inactivation is general for all organelles tested so far. The free membrane sector may have other functions and the absence of the catalytic sector may serve a specific purpose. In contrast with the constitutive secretory pathway the regulated pathway requires ΔpH for its assembly, loading and timely secretion. It is assumed that this low pH is provided by V-ATPases starting in the Golgi and following up to the fusion of the vesicles with the plasma membrane. In the case of synaptic vesicles the V-ATPase is required not only for their proper assembly but primarily to provide the energy required for loading them with the proper neurotransmitters. It is likely that the new synaptic vesicles are equipped with sufficient amounts of membrane sectors but before leaving the Golgi apparatus they may have limited amounts of catalytic sectors. The mature synaptic vesicle may now get the rest of the catalytic sectors from preassembled complexes in the cytoplasm. The physical presence of large amounts of catalytic sectors may prevent the vesicle from fusion with the plasma membrane. We propose that some catalytic sectors should be dissociated from the synaptic vesicle to facilitate its fusion with the membrane. This temporal fusion is sufficient for releasing part of the stored neurotransmitters but should retain the bulk of the proteins in secretory granules.

C

FUNCTIONAL STUDIES ON THE MEDIATOPHORE PROTEIN

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The mediatophore is a protein of the presynaptic membranes. Reconstituted in proteoliposomes, it is able to translocate acetylcholine (ACh) in response to Ca^{2+}. The mediatophore has been isolated and cloned by the group of M. Israël (Gif-sur-Yvette) with which a part of the present data have been obtained. *In situ* physiological characteristics of ACh release in a cholinergic synapse (*Torpedo* nerve-electroplaque junction) are 1) a precise Ca^{2+}-dependency, 2) extremely rapid kinetics with emission of quantal amounts of ACh, each quantum being itself composed of *ca.*10'000 ACh molecules and presenting a fine substructure with discrete subunits, 3) the abrupt occurrence for 2-3 ms of a new population of intramembrane particles in the presynaptic membrane, 4) sensitivity to a variety of drugs and toxins. The release mechanism was reconstituded in *Xenopus* oocytes injected with mRNAs from cholinergic neurons. Expression of the mediatophore was correlated in oocytes with their ability to release ACh. The Ca^{2+}-dependency of the process and its pharmacology were like in the synapses *in situ*. Antisense oligos directed against the mediatophore mRNA reduced or suppressed in oocytes to the same extent both mediatophore expression and ACh release. The quantal and pulsatile features of release are being investigated with a variety of cell lines filled with ACh. Quantal release from these cells is assessed electrophysiologically with the *Xenopus* myoball technology (see Falk-Vairant *et al.* this meeting). Our aim in these experiments is to determine whether the mediatophore is directly involved in the production of quanta and to elucidate by which mechanism it operates.

D

STABILITY AND LABILITY OF PROTEIN INTERACTIONS IN SYNAPSES FROM TORPEDO ELECTRIC ORGAN

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Spatial and temporal precision is required for neurotransmitter (NT) release in all synapses. This precision is guaranteed through dynamic assembly of key components in the active zone. The notion that protein-protein interactions are central to NT release is supported by observations concerning *in-vitro* fusion systems, yeast genetics, and neurotoxin studies. We present a study on *Torpedo* synapses where interactions among key proteins is analyzed biochemically and morphologically.

Structural properties of major synaptic components in the electric organ synapses are elucidated. VAT-1 is a major 41 kDa protein from *Torpedo* cholinergic synaptic vesicles (SVs) which forms a high molecular weight complex within the SV membrane. The hydrodynamic characteristics of VAT-1 protein complex suggest that it is composed of four VAT-1 subunits. Synaptophysin, syntaxin, synaptotagmin and VAMP are not part of the VAT-1 protein complex. Interactions between the subunits within the protein complex do not depend on disulfide bonds or on ionic strength. However, partial dissociation of VAT-1 subunits from the complex occurs by chelating Ca^{2+} ions. A quantitative analysis on VAT-1 expressed in bacterial system reveals a 1:1 molar stoichiometry for binding of Ca^{2+} to VAT-1, with a dissociation constant of 0.13 mM. The lability of protein-protein interactions was further extended in studying syntaxin which was previously implicated in docking and priming of SVs. An alteration in the size distribution of syntaxin protein complex was detected upon extreme changes in the Ca^{2+} concentration. This is due to Ca^{2+}-dependent modification of the protein. As Ca^{2+} concentration changes dramatically during nerve cell activation, we suggest that interactions among key components in the *Torpedo* synapse are modified in response to environmental conditions, thus providing a vehicle for affecting NT release properties.

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A

DUCTIN- A MULTIPURPOSE MEMBRANE CHANNEL AND VIRAL ONCOPROTEIN TARGET.

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Many papillomaviruses encode a small hydrophobic polypeptide and in some instances this polypeptide has transforming activity: e.g. the E5 oncoprotein of bovine papillomavirus type I. The E5 oncoprotein binds tightly to a highly conserved channel forming polypeptide recently called ductin. Ductin is now thought to be the main component of the connexon channel of gap junctions and has been identified as the subunit c proteolipid of the vacuolar H^+-ATPase. It also could form a pathway for neurotransmitter release from the synaptic vesicle acting as a *fusion pore*, or directly through synaptic membrane as a component of the *mediatophore*. The E5 oncoprotein may therefore be disturbing a number of cellular activities such as cell-cell communication, receptor cycling and vesicle trafficking. The structure and function of ductin will be presented together with the nature of the binding activity of the E5 oncoprotein and effects on cellular activities.

B

A TORPEDO ELECTRIC LOBE cDNA ENCODES A VESAMICOL BINDING PROTEIN WHICH IS HOMOLOGOUS TO THE VESICULAR MONOAMINE TRANSPORTER.

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The highly cholinergic electric lobe of marine ray Torpedo abundantly expresses a messenger RNA of ~3kb encoding a 56KD protein which is homologous to the putative vesicular transporter UNC-17 identified by Alfonso et al (1993) in Caenorhabditis elegans. Both Torpedo and UNC-17 proteins have a high degree of identity with the vesicular monoamine transporters VMAT1 and VMAT2. CV1 fibroblasts transfected with unc-17 or Torpedo full length cDNA express high affinity binding sites for vesamicol, a drug which specifically binds to and inhibits acetylcholine transport into Torpedo cholinergic synaptic vesicles. This study clearly demonstrates that the vesamicol binding protein belongs to the family of the vesicular neurotransmitter transporters which use proton antiport to sustain neurotransmitter uptake. On the basis of these results and of preliminary experiments showing that the Torpedo cDNA transfected cells express an acetylcholine transport sensitive to vesamicol, we conclude that this protein is the vesicular acetylcholine transporter which we propose to call vAChT.

C

WHAT HAVE NON-PEPTIDE ANTAGONISTS TOLD US ABOUT NEUROPEPTIDE FUNCTION?

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Naloxone and naltrexone were until recently almost the only non-peptide antagonists of neuropeptide receptors. In the past few years there have been rapid developments, with the discovery of non-peptide antagonist drugs acting on cholecystokinin, substance P, angiotensin II, oxytocin, vasopressin and neurotensin receptors.

Devazepide (MK-329) is a potent and selective antagonist of cholecystokinin CCK-A receptors, developed in our laboratories from the natural product asperlicin., while L-365,260 and more recently described water soluble analogues are potent and selective antagonists at CCK-B/gastrin receptors. The latter compounds potentiate opiate-induced analgesia, but their effects in animal models of anxiety/panic are equivocal. Orally administered L-365,260 was able to block CCK-4 or pentagastrin-induced panic in normal volunteers or in patients with panic disorders, but clinical trials have so far failed to reveal any significant effects on endogenous panic or anxiety.

Tachykinin antagonists are now available with selectivity for NK-1 or NK-2 receptor subtypes. These potent and selective CNS penetrant compounds offer powerful new research tools. Neither NK-1 nor NK-2 antagonists have obvious effects on normal animal behaviour, although they are able to block behaviours elicited by centrally administered tachykinin agonist peptides. NK-1 antagonists are potent and efficacious in a broad range of animal models of emesis and in suppressing neurogenic extravasation of plasma proteins. They also prevent the augmentation of spinal reflexes evoked by noxious stimuli. These compounds may thus be effective as novel anti-emetic, anti-inflammatory and analgesic agents.

D

SODIUM–COUPLED NEUROTRANSMITTER TRANSPORT: STRUCTURE, FUNCTION AND REGULATION.

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The removal of neurotransmitters by their transporters — located in the plasma membranes of nerve terminals and glial cells — plays an important role in the termination of synaptic transmission. In the last three years many neurotransmitter transporters have been cloned. Structurally and functionally they can be divided into two groups: glutamate transporters, of which to date three have been cloned, couple the flow of glutamate to that of sodium and potassium. The second group of transporters includes those for GABA, glycine, taurine, norepinephrine, dopamine and serotonin. They are sodium and chloride dependent, but do not require potassium for function. One of these, the $GABA_A$ transporter, encoded by GAT-1, is perhaps the best characterized. It has been purified and reconstituted and has a molecular mass of around 80 kDa, of which 10—15 kDa is sugar. Amino- and carboxyl-terminii (around 50 amino acids each) are not required for function. The transporter is protected against proteolysis at multiple sites by GABA, provided that the two cosubstrates — sodium and chloride — are present. Several amino acid residues, which are critical for function, have been identified in the GABA transporter. These include arginine–69 and tryptophan–222 located in the first and fourth putative transmembrane helices, respectively. The first is possibly involved in the binding of chloride. The tryptophan appears to serve as a binding site for the amino group of GABA.

A

CONDUCTING STATES OF A MAMMALIAN SEROTONIN TRANSPORTER

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We have studied permeation at a cloned rat 5-HT transporter expressed in Xenopus oocytes. [^{3}H]5-HT uptake and [^{125}I]RTI-55 binding yield a turnover rate of ~1/sec that does not depend on membrane potential. In voltage-clamp experiments, three distinct currents result from 5-HT transporter expression. (1) A steady-state, voltage dependent transport-associated current is induced by 5-HT application. (2) A transient inward current is activated by voltage jumps to high negative potentials in the absence of 5-HT and is blocked by 5-HT itself. (3) A small leakage current is observed in the absence of 5-HT. All the observed currents are blocked by inhibitors of 5-HT uptake but are differentially affected by Na^+, Li^+, K^+, Ba^{2+}, Cs^+, Cl^-, and amiloride. The conducting states of the 5-HT transporter may reflect the existence of a permeation pathway similar to that of ionic channels.

B

VESICULAR NEUROTRANSMITTER TRANSPORTERS: FROM BACTERIA TO MAN.

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Vesicular transport has been observed for several classical transmitters, including acetylcholine, glutamate, GABA, glycine and the monoamines. The vesicular monoamine transporter (VMAT) has been the one most intensively studied and is the one for which most molecular information has been obtained. VMAT's are 12-membrane spanners with a large glycosilation loop facing the lumen. The recent cloning of cDNA's coding for VMAT's from various species and organs will be discusssed. Studies in which histidyl residues have been mutagenised to Arg or Cys will be presented. These studies suggest an important role of HIS in H^+ translocation or conformational changs induced thereby. Finally, the evolving concept that VMAT's may function as multidrug transporters will be addressed[1]. VMAT's are the first mammalian members of the TEXANs, a superfamily of transporters found in bacteria and higher organisms: they can all remove toxic cations away from the cytoplasm by a mechanism of H^+-substrate antiporter. They also have a very broad substrate and inhibitor specificity and they even share some of them with the P-glycoprotein MDR.

1. S. Schuldiner, *J. Neurochemistry* in press, (1994)

C

CHARACTERIZATION OF SEROTONIN UPTAKE SITES ON THE BLOOD-BRAIN BARRIER

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The blood-brain barrier (BBB) represents a complex epithelial interface in vertebrates that separates the blood compartment from the extracellular fluid compartment of the brain. Isolated microvessels are a tool to study the function of this interface in vitro. Here we report on attemps to characterize serotonin uptake sites on microvessels from the pig hippocampus. For comparison membrane preparations of hippocampal tissue were used. The enrichment of the microvessel fraction determined by measurement of alkaline phosphatase activity was 28-fold. Microvessels and hippocampal membranes were incubated at 4°C with [^{3}H]imipramin. The binding assays were terminated by rapid filtration through GF/B glass fiber filters. Equilibrium of specific binding was reached at about 30 minutes. Therefore, an incubation time of 60 min was choosen for the inhibition studies. Different concentrations of unlabelled imipramin were used to inhibit the binding of ^{3}H-imipramin. In the hippocampus a two-site model gives a better fit to the data than a one-side model (K_{d1}=1.4±0.6 nM, B_{max1}=1.2±0.5 pmol/mg; K_{d2}= 112±25 nM, B_{max2}=112±19 pmol/mg). In the microvessels only a one-side model fits to the data (K_d=100±18 nM, B_{max}=140±23 pmol/mg). However in saturation experiments also high affinity binding of [^{3}H]imipramin could be demonstrated (K_d=0.18±0.16 nM, B_{max}= 95±22 fmol/mg). Imipramin binding could be inhibited by potent nontricyclic inhibitors of the serotonin transporter such as paroxetin (K_i=29 nM) and fluoxetin (K_i=35 nM) but also by the tricyclic antidepressant drug chlorimipramin (K_i=27 nM) and serotonin (K_i=1230 nM). Therefore, we conclude that ^{3}H-imipramin labels serotonin uptake sites localized on porcine brain microvessels.

D

IDENTIFICATION OF THE MAMMALIAN VESICULAR ACETYLCHOLINE TRANSPORTER AND ITS EXPRESSION FROM A 'CHOLINERGIC' GENE LOCUS

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The rat vesicular acetylcholine transporter (VAChT) has been identified based on the acquisition of high-affinity vesamicol binding and proton-dependent, vesamicol-sensitive acetylcholine accumulation by a fibroblast cell line transfected with the cDNA encoding this protein. The distribution of VAChT mRNA is identical to that reported for choline acetyltransferase (ChAT), the enzyme required for acetylcholine biosynthesis, in the peripheral and central cholinergic nervous systems. A human VAChT cDNA was used to localize the VAChT gene to chromosome 10 at position q11.2, also the location of the ChAT gene. The entire sequence of the human VAChT cDNA is contained within the first intron of the ChAT gene locus. The transcription of VAChT and ChAT mRNAs from a single gene locus provides a mechanism for regulated expression of the cholinergic neuronal phenotype.

A

EFFECT OF NCAM EXPRESSION ON CELL MORPHOLOGY, MOTILITY AND INVASIVE CAPACITY

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In order to evaluate the effect of NCAM expression on cell morphology, motility and invasive capacity different cell-lines were permanently transfected with cDNA vectors encoding various NCAM isoforms or with vector alone. The transfected cell-lines were characterized by demonstration of expression of NCAM polypeptides of correct sizes, demonstration of surface localization of the molecule and determination of the amount of NCAM per cell. The function of the molecule was demonstrated by a simple aggregation assay. Cell morphology was assessed by an image analysis system allowing quantification of cell area, perimeter and form factor. Furthermore, the localization of NCAM was investigated by means of confocal laser microscopy. Motility *in vitro* was estimated by image analysis of motility of cell centroids and d-shape analysis. Invasive capacity was evaluated by *in vitro* assays for penetration of collagen I or artificial basal membrane. Invasive capacity was also investigated *in vivo* by subcutaneous and intracranial injections of transfected cells. Finally, the effect of NCAM expression on secretion of metalloproteinases was investigated by zymography. It was found that NCAM expression changed cell morphology, decreased cell motility, inhibited cell capacity for penetration of extracellular matrix, changed invasive capacity towards a more benign behaviour and decreased cell secretion of a matrix metalloproteinases of 92 kDa.

B

STRUCTURE FUNCTION RELATIONSHIP AT THE BINDING SITE OF THE ACETYLCHOLINE RECEPTOR: ANALYSIS BY POINT MUTATIONS IN THE MONGOOSE RECEPTOR.

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The ligand binding site of the nicotinic acetylcholine receptor (AChR) is located at the α-subunit, within a small fragment containing the tandem cysteins at positions 192 and 193. Earlier work from our laboratory on the ligand binding site of the snake and mongoose AChR, both of which do not bind α-bungarotoxin (α-BTX), indicated that several amino acid substitutions occur in the putative binding site close to cysteines 192 and 193. Sequence comparison suggests that amino acid residues at positions 187, 189, 194 and 197 of the AChR α-subunit are most important in determining resistance to α-BTX. To further identify the amino acids participating in ligand binding a series of point mutations were performed, changing amino acid residues at these four positions, each alone or in combination, from the mongoose to the mouse sequence. Fragments corresponding to amino acid residues 122-205 of the mongoose AChR α-subunit, and the respective mutated fragments were amplified by reverse transcription and PCR and expressed in E. coli. Analysis of the binding of the various mutated fragments to α-BTX indicated that only fragments in which all four positions were mutated to the mouse amino acid residues, exhibited α-BTX activity, similar to that of the mouse fragment. Interestingly, this mutated fragment bound very weakly to a monoclonal antibody (mAb5.5) directed against the ligand binding site of AChR, suggesting that it has acquired a different conformation of the binding site. Mutations of one, two or even three of these residues to the ones present in the mouse AChR resulted in just a small increase in toxin binding, and almost no change in mAb5.5 binding. Thus, our results indicate that changes in amino acid residues in the binding site domain, are important in creating the appropriate structure that determines α-BTX resistance.

C

ANTISENSE OLIGONUCLEOTIDE INHIBITION OF ACETYLCHOLINESTERASE GENE EXPRESSION PROMOTES STEM CELL EXPANSION AND SUPPRESSES HEMATOPOIETIC APOPTOSIS EX VIVO. D. Patinkin[1], E. Lev-Lehman[1], M. Grifman [1], D. Ginzberg[1], F. Eckstein[2], H. Zakut[3]and H. Soreq[1]. Dept. of Biol. Chem.[1], Hebrew Univ., Jerusalem,Israel; Max Planck Inst. for Exper. Med.[2], Gottingen,Germany; Dept. of Obstet. and Gyn.[3], Tel Aviv Univ.,Tel Aviv, Israel. Increased occurrence of myeloid leukemia in farmers exposed to organophosphorous insecticides inhibiting acetylcholinesterase (ACHE) suggests an involvement of ACHE in hematopoiesis. To analyze this possible link we introduced phosphorothioate antisense oligodeoxynucleotides (AS-oligos) targeted to CHEmRNAs into murine bone marrow serum-free cultures. AS-ACHE addition to cultures (designated CFU-GEMM) grown with erythropoietin and interleukin-3 (IL-3) caused 5-and 2-fold increases in colony and cell numbers at 12 uM, suggesting increased stem cell expansion and survival. Fewer colonies were formed in cultures grown only in IL-3 (CFU-MK) and AS-ACHE as compared with controls but cell numbers were increased 2-fold, reflecting enhanced proliferation. PCR analysis of ACHEmRNA levels revealed a 10-fold drop in ACHEmRNA levels 2 hours after AS-ACHE addition to CFU-MK cultures. A striking increase in macrophages was noted in AS-ACHE-treated CFU-GEMM while 50% of CFU-MK cells appeared to be primitive blasts, staining positively with both gpIIb/IIIa and glycophorin antibodies, megakaryocyte (MK) and erythroid markers, respectively. An RT-PCR of CFU-MK exhibited a 10-fold increase in GATA-1mRNA (an MK and erythroid transcription factor), as compared to S-ACHE cultures. Finally, DNA extracted from AS-ACHE-treated cultures was 5-fold higher in yield and revealed suppressed apoptosis-related fragmentation compared to non-treated or S-ACHE DNA. These results suggest that the ACHE function is to reduce expansion and proliferation of multipotent hematopoietic stem cells and to promote apoptosis of their progeny.

D

ANTISENSE INTERVENTION OF GENE EXPRESSION

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Antisense regulation of cellular function is first demonstrated in osmoragulation of OMP gene in E. coli. This technique has now been introduced for intervention of cellular function. I will introduce two examples. One is myelin deficient (mld) mice and the other is antisense knock out of inositol 1,4,5-trisphosphate receptor (IP3-R).

mld mutation: Myelin deficiency results in abnormal behavior such as intensional tremor and tonic convulsion. Myelin basic protein (MBP) is one of the major proteins consisting of about 30% of total myelin proteins. Shiverer mutant mice deletes part of the gene (Kimura et al.(1), Roach et al (2)) resulting in the absence of MBP. mld mice also showed symptoms. In mld mutant, MBP gene was duplicated. Downstream gene was intact, however, the deleted part of the MBP gene in shiverer was inverted in the upper gene (Okano et al (3), Popko et al (4)). Antisense nucleotide corresponding to the inverted portion (Okano et al. (3), Popko et al. (4), Tosic et al (5)) was detected. Finally, RNA-RNA duplex was detected in the mld (Okano et al. (6)), which is probably the reason for the suppression of the downstream intact MBP gene expression.

IP3-R knock out: We cloned IP3-R cDNA from Xenopus oocyte. Antisense nucleotide introduced into the oocyte suppressed the expression of IP3-R. Egg activation using cortical contraction as an indicator was also suppressed in the oocyte (Kume et al. (7)). This result clearly demonstrated the important roles of IP3-R upon fertilization.

Ref. 1. PNAS 86, 5661 (1989), 2. Cell 34, 799 (1983), 3. EMBO J. 7, 77 (1988), 4. Cell 48, 713 (1987), 5. EMBO J. 9, 401 (1990), 6. J. Neurochem. 56, 560 (1991), 7. Cell 73, 555 (1993)

A

ANTISENSE OLIGONUCLEOTIDES MAY AFFECT PROCESSES INVOLVED IN LEARNING AND MEMORY IN A SEQUENCE-SPECIFIC MANNER

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Long-term plastic changes of the brain are assumed to depend on a sequential induction of regulatory and functional target proteins, resp. As one of the earliest responses after training of rats on a foot-shock motivated brightness discrimination a differential induction of immediate early genes like *c-fos, c-jun, jun-B* and *zif/268* in hippocampal and cortical structures of rat brain was evident. Introducing the antisense technology as a tool to study *in vivo* the functional importance of gene expression in rat brain during processes of neuronal plasticity, experiments assaying the effects of inhibition of c-*jun* and *jun*-B expression by intrahippocampally applied antisense phosphorothioate oligodeoxynucleotides (S-ODN) on acquisition and retention of the brightness discrimination were performed. Ten hours following an injection of FITC- and ^{35}S-labelled randomized S-ODN, parts of the pyramidal tract of hippocampal fields CA1/CA2 and dentate granule cells are labelled in addition to fibres of corpus callosum and to cortical areas close to the site of injection. After intrahippocampal injection of anti c-jun S-ODN, performance of brightness discrimination was considerably impaired on three consecutive days of training compared with saline treated control rats, whereas activity in an open field test was not affected. Inhibition of *jun-B* expression did not impair the brightness discrimination score. Thus, suppression of immediate early gene expression in rat brain by topical application of antisense S-ODN affects processes involved in learning and memory formation in a sequence-specific manner.

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B

ANTISENSE OLIGODEOXYRIBONUCLEOTIDES: STABILITY AND DISTRIBUTION AFTER INTRA-STRUCTURE INJECTION INTO RAT BRAIN

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As a prerequisite for blocking specific gene expression in the brain, pharmacokinetics of radiolabelled two analogs of antisense oligodeoxyribonucleotides: unmodified O-ODN and nuclease resistant phosphorothioate S-ODN, inoculated into baso-lateral nucleus of amygdala of the rat brain, were studied. Both ODN analogs penetrated at restricted distance into the brain tissue. Rapidly after injection O-ODN were almost completely degraded. On the contrary, S-ODN remained intact up to 24 h following administration. The tissue clearance of the radioactivity delivered in a form of O-ODN and S-ODN were also different, the former characterized by much better tissue retention. Microscopic studies revealed that S-ODN penetrate across cell membrane and accumulate in the cytoplasm as well as in the cell nuclei. The in situ hybridization experiment (antisense probe to injected sense strand of ODN) proved that injected S-ODN were in a form available for annealing with the complementary strand. Our results provide basic description on distribution, retention and stability of anti-sense oligonucleotides injected into brain structure.

C

REGULATION OF CELL NUMBERS BY NEUROTROPHINS

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During the development of the vertebrate nervous system, many (apparently superfluous) cells are eliminated when long lasting relationships begin to be established, for example when the axons of embryonic neurons first contact their target cells, or oligodendrocytes their axons. It appears that in part, this regulation of cell numbers makes use of a neurotrophin-suppressible cell death program that is operative in many cells when they establish contact with other cells. Indeed, there is a slowly growing list of examples indicating that cell numbers in the developing nervous system can be regulated by the limited availability of factors belonging to a gene family -the neurotrophins. Thus for example, nerve growth factor (NGF) prevents the death of neural crest-derived neurons, and brain-derived neurotrophic factor (BDNF) saves motoneurons both during development and after axotomy. In addition, recent results indicate that neurotrophin-3 (NT-3) regulates neuronal numbers in vivo long before normally occurring cell death is observed in NT-3-responsive ganglia. Already during gangliogenesis, the limited availability of NT-3 regulates neuronal proliferation and/or the differentiation of neuronal progenitor cells. Also, a direct mitogenic effect of NT-3 (when used together with PDGF) has been demonstrated in vitro on oligodendrocyte precursor cells. It thus appears that the neurotrophins control cell numbers in the developing nervous system of more than one cell type, and that this control involves mechanisms that operate before (and probably in addition to) those long described for NGF, i.e. the prevention of the death of neurons that have already sent their axons to their target cells.

D

MOLECULAR MECHANISMS OF ACTION BY NGF RECEPTORS.

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Cell differentiation and survival are mediated by many trophic factors, including NGF, BDNF, NT-3 and NT-4/5. All of these factors interact with two different Type I transmembrane receptor subunits, the *trk* tyrosine kinase receptor family and the p75 neurotrophin receptor, a member of a family that includes the TNF receptors, the Fas antigen, CD30, OX40, and CD40. The *trk* receptors transduce neurotrophin signaling through autophosphorylation and increased tyrosine phosphorylation of key cellular substrates, whereas p75 is believed to participate in high affinity NGF binding with $p140^{trk}$, and to increase responsiveness to NGF.

Both p75 and $p140^{trk}$ are co-expressed in the majority of NGF-responsive neurons. Comparative measurements indicate that p75 is found in abundance relative to $p140^{trk}$ in target cells. An essential question is why neurotrophins interact with these two distinctive receptor molecules. We have approached this problem by altering the levels of the two receptors in PC12 and other transfected cells. We have found that the ratio of *trk* and p75 gives striking differences in high affinity binding and responsiveness toward neurotrophic factors. This two-receptor system respresents a unique growth factor mechanism for mediating neuronal cell survival and differentiation.

A

Trk receptors and neurotrophins *in vivo* models and functional assays
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The development of the vertebrate nervous system is characterized by over production of neurons which exhibit differential requirements for neurotrophins. The limited availability of neurotrophins at axonal target sites serves as a mechanism to selectively reinforce survival of appropriate connections while permitting death of wayward neurons which do not gain access to neurotrophins. Hence, survival of primary neurons in culture has served as a long standing assay for neurotrophin activity. The protooncogene receptor tyrosine kinase (RTK) and its related gene family members function as receptors for the neurotrophins, NGF, BDNF, NT-3, and NT4/5. These findings now permit more detailed investigation into the molecular mechanisms that mediate neuronal response. Through the manipulation of these genes as well as other pertinent protooncogenes, current efforts are aimed at understanding the ligand specificity and signal transduction pathway of these ligands within the context of *in vivo* models.
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B

THE ROLE OF TRK-FAMILY OF NERVE GROWTH FACTOR RECEPTORS IN THE DEVELOPMENT OF THE MAMMALIAN NERVOUS SYSTEM.
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The Trk family of tyrosine kinases have been shown to be the functional receptors for the NGF family of neurotrophins. The product of the *trk* protooncogene serves as the high affinity receptor for NGF, *trk*B for BDNF and NT-4, and *trk*C for NT-3. Using homologous recombination techniques we have generated mice lacking either the *trk*, *trk*B or *trk*C kinase receptor genes. Mice lacking the Trk receptors have severe sensory and sympathetic neuropathies and usually die by 1 month. In the P0 dorsal root and trigeminal ganglia there is a loss of >70% of the neurons. In the P10 SCG, approximately 99% of the neurons are absent. In the CNS, lack of the Trk receptor results in the loss of cholinergic projection fibers from the basal forebrain to the hippocampus and cerebral cortex. Mice lacking the TrkB receptor do not appear to feed and usually die by P4. Examination of the neural pathways involved in feeding showed significant deficits in the trigeminal ganglion and facial motor nucleus at P0. Deficits were also present in the P0 dorsal root ganglion and motor neurons of the spinal cord. Mice lacking a functional TrkC receptor appear normal at birth. By P4, however, these mice exhibit motor incoordination resembling pseudoathetosis. Analysis of the sensorimotor pathways in the TrkC-null mice revealed a complete absence of the Ia muscle afferents that convey proprioceptive information. The higher order proprioceptive pathways traveling through the fasciculus cuneatus and gracilus also have been demonstrated to be deficient in these mice. Further analysis of these Trk-family of knockout mice should help to resolve the role of growth factors in the development and maintenance of the nervous system.

C

FGF in the Repair of Dopaminergic Neurons

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Dopaminergic neurons of the nigrostriatal system degenerate in Parkinson 's disease. The etiology of this disease has not been clarified as yet. Novel therapeutic concepts include putative neuroprotective effects of trophic factors. FGF-1 and -2 (acidic and basic FGF) promote survival and differentiation of embryonic and lesioned adult neurons. We have previously reported that FGF-2 applied unilaterally in a piece of gelfoam to the striatum of MPTP-treated mice causes a marked protection of the dopaminergic innervation and non-dopaminergic transmitters of the striatum. In vitro studies on fetal mesencephalic dopaminergic neurons have suggested that the neurotrophic effects of FGF-2 are mainly indirect and mediated by glial cells. Likewise, FGF-mediated neuroprotection in MPTP-treated mice seems to be largely indirect, since FGF-2 fails to penetrate into the striatum. Moreover, a prominent neurotrophic activity that can be eluted from the striatal gelfoam differs from FGF, shares biological properties with CNTF, and may represent a neurotrophic factor for the MPTP-lesioned nigrostriatal system. CNTF administered instead of FGF-2 is not neuroprotective. Studies are in progress to explore (i)whether astroglial and/or microglial cells are potential mediators of the neuroprotective effect of FGF-2, (ii) what cytokines are induced by FGF-2, and (iii) what the molecular properties of the striatal CNTF-like activity are.

Supported by Deutsche Forschungsgemeinschaft (Forschergruppe "Plasticity and Gene Expression in the Nervous System under Pathologic Conditions")

D

TROPHIC FACTOR THERAPY OF DEGENERATING NEURONS OF THE NUCLEUS BASALIS MAGNOCELLULARIS OF RAT AND PRIMATE.
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A number of neurotrophic factors have been seen to display a protective effect on the maintenance of phenotypic characteristics of injured CNS forebrain cholinergic neurons in adult animals. In the basalo-cortical lesion model, NGF remains more effective than other neurotrophins or acidic-FGF in attaining these effects. Much of the experience gathered in rodents can possibly be applied to primates. Thus, we have observed in ***C. aethiops*** that human-recombinant NGF (Genentech) is capable of long term protection (6 months) of cholinergic neurons of the nucleus basalis of Meynert.

Beyond the maintenance of neuronal phenotypic characteristics, NGF is capable of altering the pattern of the cortical terminal network of the cholinergic projections. Experimental evidence accumulated in our laboratory (high resolution immunocytochemistry and image analysis) would indicate that the application of NGF to rats bearing partial, unilateral cortical infarcts results in a marked synaptic remodelling of cholinergic synapses in the remaining neocortex. These compensatory synaptic changes in the cerebral cortex observed in adult animals might explain the improved behaviour of these CNS-lesioned rats in the Morris water maze after the administration of NGF.

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A

G-PROTEINS - A POTENTIAL THERAPEUTIC TARGET?

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The heterotrimeric guanine nucleotide-binding proteins (G-proteins) act as switches that regulate the flow of information from cell surface receptors to a variety of intracellular effector systems. Multiple isoforms of the α, β and γ subunits exist giving rise to multiple G-proteins. Evidence is emerging that receptors may prefer specific G-protein combinations but that they are capable of multifunctional signalling in that they can couple with other G-proteins, giving rise to so-called promiscuous coupling. In addition receptors are not the only components of the G-protein linked cascades that can be regulated. Although the βγ -dimer may also have a role in signal transduction and regulation (1) this symposium concentrates on the regulation of the α subunit under physiological and pathological conditions. This includes the regulation of G-protein expression both when cells are challenged by the continued presence of an agonist (G. Milligan), and by antidepressant drugs (P. Lesch). The role of G-proteins in the mechanism of action of mood stabilizers (S. Avissar) and in the development of tolerance to and dependence on opioids (R. Schulz) will also be discussed. In conclusion, G-proteins have an important role in the regulation of cellular signalling sensitivity both in physiological and pathological conditions. G-Proteins are not simple transfer systems but are integral components of a regulatory system which determines the final cellular response. As such, G-proteins may represent a potential therapeutic target.

1. D.E. Clapham & E.J. Neer (1993) Nature 365: 403-406

B

REGULATION OF CELLULAR Gsα LEVELS AND BASAL ADENYLYL CYCLASE ACTIVITY BY EXPRESSION OF THE β2-ADRENOCEPTOR IN NEUROBLASTOMA CELL LINES.

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Mouse neuroblastoma x rat glioma hybrid NG108-15 and mouse neuroblastoma x embryonic hamster brain NCB20 cells were transfected with a construct containing a human β2 adrenoceptor cDNA under the control of the β actin promoter. Clones were selected on the basis of resistance to geneticin sulphate and those expressing a range of levels of the receptor expanded for further study. Membranes from a clone of NG108-15 cells expressing high levels of the receptor (βN22) but not one expressing only low levels of the receptor (βN17) exhibited a markedly elevated adenylyl cyclase activity when measured in the presence of Mg^{2+} compared to wild type cells. This was not due to elevated levels of the adenylyl cyclase catalytic moiety however as there was no difference in these membranes when the adenylyl cyclase activity was measured in the presence of Mn^{2+} or to altered levels of the guanine nucleotide binding protein G_s. Furthermore, the elevated basal activity was partially reversed by addition of the β-adrenoceptor antagonist propranolol. Agonist activation of βN22 but not βN17 cells led to a large selective down-regulation of cellular $G_s\alpha$ levels which was independent of the generation of cyclic AMP. Isoprenaline stimulation of adenylyl cyclase activity was achieved with substantially greater potency (some 30 fold) in βN22 cell membranes than in βN17. By contrast agonist activation of the endogenously expressed IP prostanoid receptor caused stimulation of adenylyl cyclase which was equipotent in each of βN22, βN17 and wild type NG108-15 cells. Agonist activation of the IP prostanoid receptor caused an equivalent degree of $G_s\alpha$ downregulation in each cell type. Isolation of clones of NCB20 cells expressing high levels of the β2 adrenoceptor also resulted in a specific agonist induced downregulation of $G_s\alpha$. Agonist stimulation of high affinity [^{3}H]forskolin binding in cells βN22 and βN17 cells (which is a measure of the formation of the complex of $G_s\alpha$ and adenylyl cyclase) followed a similar pattern to that of adenylyl cyclase stimulation as the dose effect curve for isoprenaline in βN22 cells was some 30 fold to the left of that in βN17 cells. However, at maximally effective concentrations of the agonist essentially all of the cellular adenylyl cyclase was activated. Analysis of levels of $G_s\alpha$ 1.25 x 10^6 copies per cells and adenylyl cyclase (1.7 x 10^4 copies per cell) in NG108-15 cells demonstrated that the G-protein was present in some 70 fold molar excess of its effector molecule.

C

EFFECTS OF ANTIBIPOLAR/ANTIDEPRESSANT DRUGS ON G PROTEIN EXPRESSION IN RAT BRAIN AND A HUMAN NEUROBLASTOMA CELL LINE

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Although G proteins control multiple cell functions, they are especially important in the CNS, where they serve the critical roles of first amplifying extracellularly generated neuronal signals and then transmitting these integrated signals to effectors, thereby forming the groundwork for complex information processing. It is therefore not surprising that recent research has begun to focus upon the elucidation of the role of G proteins in the etiopathogenesis of various psychiatric disorders and in their treatment. Growing evidence that subsensitive functional responses to receptor activation during chronic antidepressant and antibipolar drug treatment are not invariably a consequence of desensitization and downregulation of the receptor itself, and that psychotropic agents may also modulate postreceptor signal amplification, directed the focus on the role of G protein subunits in drug-induced neural plasticity. We examined the effects of long-term treatment with various antidepressant/antibipolar drugs on steady-state concentrations of Gα subunit mRNA and protein in various rat brain regions. The results indicate that chronic treatment with antidepressant and antibipolar drugs exerts differential effects on Gα mRNA and protein expression thus modifying signal transduction as an integral part of complex neuroadaptive mechanisms on which their therapeutic efficacy is potentially based. Currently, we are investigating the direct effect of antidepressant and antibipolar drugs on G protein α and β subunit expression and G protein-effector interaction in cultured SK-N-SH human neuroblastoma cells. This overview provides a survey of the molecular biology, physiology and pharmacology of G proteins of relevance to psychiatric disorders and their treatment. It emphasizes those G protein subtypes which are potential targets for the development of site-selective therapeutic strategies and drug development. Finally, novel findings regarding the role of G proteins in the etiology and pathophysiology of mental illness and their impact on the understanding of the molecular mechanisms of action of various psychotropic agents are discussed.

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D

G PROTEINS IN THE MECHANISM OF OPIOID TOLERANCE AND DEPENDENCE

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Chronic opioid treatment has been reported to affect the abundance of inhibitory acting G proteins in distinct tissues. Though these findings may contribute to the understanding of tolerance, they fail to explain dependence. As predicted on theoretical grounds, dependence would result from adaptations in excitatory signal pathways. In fact, opioid tolerant /dependent systems are well known to exhibit supersensitivity when challenged with excitatory drugs, a phenomenon specifically well documented for adenylate cyclase (AC) in neuroblastoma x glioma hybrid cells (NG 108-15 cells, δ-opioid receptors). In those cells, however, we found no change in the concentration of Gsα, the G protein mediating stimulatory signal transduction, no altered intrinsic activity of AC, and no change of coupling between Gαs and AC. Examining in NG cells the functional state of substituted, exogenous sα-subunits, the increase of PGE1 (PG)-triggered cAMP concentration was found consistently higher during tolerance/dependence as compared to controls. These results, together with the above mentioned, suggest an increased coupling efficiency between PG-receptors and Gsα, explaining the observed AC-supersensitivity. To verify this notion in chronically opioid exposed NG-cells, we employed PG-receptor stimulated, cholera toxin catalyzed ADP-ribosylation of Gsα which accounts as direct parameter for the functional interaction of PG-receptors with Gs *in situ*. This method revealed that several fold less PG is required to activate (ribosylate) Gsα in morphine dependent cells as compared to controls. Surprisingly, these tolerant/dependent cells also display a reduced number of functional PG-receptors. However, the enhanced coupling of PG-receptors to Gsα fully compensates the receptor down-regulation, and may even account for the sensitization of the AC-system. We conclude that chronic activation of inhibitory δ-opioid receptor in NG-cells increases the coupling efficiency between excitatory PGE1-receptors and Gsα. The increased rate of Gsα-subunits activated upon prolonged morphine exposure contributes to AC-supersensitivity, and, thus, to opioid dependence. Supported by SFB 220

A

GENE STRUCTURE AND THE MOLECULAR ESTABLISHMENT OF THE NEURONAL PHENOTYPE

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We have been studying the regulation of phenotypic diversity of the mammalian central nervous system using the enkephalin (ENK) as a model gene. The peptide neurotransmitter, enkephalin mediates several important neuronal functions such as analgesia, reward, motivation, as well as regulates cellular proliferation and organogenesis, indicating a very complex and precisely regulated spatial and temporal expression of the ENK gene during development in a tissue and cell-specific manner. Establishment of the enkephalin phenotype, i.e. expression of the ENK gene during neurodevelopment, is influenced by epigenetic factors (J. Neurosci. Res. 28:140,1991; Dev. Brain Res., 73:185,1993). Our pharmacological experiments using primary developing spinal cord neurons indicate that various first and second messengers affect the expression of the ENK gene in a selective, developmental-specific manner and the embryonic expression of the ENK gene is dependent on intact de novo protein biosynthesis. Based on these findings, we employed gelshift assays and DNA-footprinting and identified several developmental- and tissue-specific DNA binding proteins and their corresponding cis-elements. Using a novel detection system for studying far distant DNA-DNA interactions, we have identified di- and trinucleotide repeats with specific and restricted interactions and ion-induced structural changes exclusively during the early phase of phenotypic differentiation. We have been testing the physiology of these repeats by molecular decoy using primary embryonic neurons as well as generating transgenic animals.

B

CELLULAR MECHANISMS UNDERLYING NEURONAL REMODELING INVOLVES THE ACTIVATION OF LOW AFFINITY CALCIUM DEPENDENT PROTEASES : EXPERIMENTAL RESULTS AND HYPOTHESIS.

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Peripheral and central adult neurons undergo significant cytoarchitectural alterations during development as well as throughout adulthood. In adults, functional and structural plasticity of neurons have been shown to occur after nerve injury or in association with learning processes.

In this presentation we will describe experimental results indicating that the activation of low affinity calcium dependent proteinases underlies the initiation of growth cone formation, cytoarchitectural remodeling and neuritogenesis.

Following axonal transection of cultured aplysia neurons , the free intracellular calcium concentration ($[Ca^{2+}]_i$) is elevated from a resting level of 60-150nM to above 500μM (as revealed by fura-2 and mag fura-2 digital video imaging). This elevation is associated with reorganization of the membrane skeleton and cytoskeleton, microtubules and endoplasmic reticulum (as revealed by EM and video enhanced contrast differential interference microscopy) and with massive membrane recycling(as indicated by changes in membrane capacitance of the neuron). These processes lead to the formation of a growth cone and to vigorous extension of neurites.

A similar cascade of events can be triggered by a transient elevation of $[Ca^{2+}]_i$ to the milimolar level .

Vigorous neuritogenesis preceded by similar reorganization of the cytoskeleton and an increase in membrane recycling, but without changes in the $[Ca^{2+}]_i$ can be induced by intracellular micro injections of proteinases (trypsin, chymotrypsin and papain).

These results are consistent with the hypothesis that long-term neuronal remodeling is the consequence of the activation of Ca^{2+} dependent proteinases.

C

OLIGODENDROCYTE-ASSOCIATED NEURITE GROWTH INHIBITORS RESTRICT REGENERATION AND PLASTICITY IN THE MAMMALIAN CNS

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CNS myelin and oligodendrocyte plasma membranes contain specific membrane proteins with powerful neurite growth inhibitory and growth cone collapsing activity. The effect is probably mediated by neuronal receptors, and the second messenger cascade includes a massive elevation of intracellular Ca in the growth cone. Neutralization of this inhibitory activity by a mAB (IN-1) allows long-distance regeneration of lesioned corticospinal (CST), septo-hippocampal and optic nerve fibers in young adult rats. CST fibers react to lesions by spontaneous sprouting, the extent of which can be greatly and specifically increased by neurotrophin-3 (NT-3). However, elongation of these regenerative sprouts over distances exceeding 0.5 - 1 mm is only possible in the presence of mAB IN-1. Behavioral studies recently showed significant functional improvement in IN-1 treated spinal cord lesioned rats. - In the normal adult CNS the local occurrence of the plasticity marker GAP-43 shows an exact inverse relationship to the presence of oligodendrocytes and myelin. Preventing oligodendrocyte development in spinal cord and optic nerve resulted in very high local GAP-43 levels in this tissue. In optic nerves, significant sprouting of optic axons could be shown in these animals. Application of antibodies IN-1 to developing rats also resulted in aberrant growth and increased sprouting of corticospinal and retinal axons. These results suggest that oligodendrocyte-associated neurite growth inhibitors control and restrict sprouting and plasticity of the developing and adult CNS.

D

FORMATION OF FUNCTIONAL CONNECTIONS BY RETINAL GRAFTS

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We are particularly interested in how functional circuits are formed in the brain and whether ectopic neural tissue can replace circuits lost as a result of developmental disorder or damage. Embryonic retinae implanted in the brains of neonatal rats emit axons which project to many of the regions normally innervated by host optic axons. The patterns of outgrowth suggest that two mechanisms direct the process - one, a substrate-dependent event and the other, dependent on target-derived diffusible molecules. Grafts implanted into adult brains also show specific outgrowth to visual centres, but only when placed in close proximity. The grafts are capable of mediating a variety of visual behaviours, including pupilloconstriction, conditioned suppression, alerting, and photophobic responses. These observations show that damaged circuitry can be replaced using neural transplants and that these transplants are capable of encoding input signals and relaying information to circumscribed brain regions, sufficient to implement at least relatively simple sensorimotor responses.

A

CELLULAR AND MOLECULAR MECHANISMS OF PURKINJE CELL REPLACEMENT IN ADULT CEREBELLUM BY NEURAL GRAFTING.

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Embryonic Purkinje cells (PCs) grafted into adult cerebellum can replace missing neurons and become functionally integrated into the synaptic circuit of the cerebellar cortex of the host. The integration takes place in all tested cerebellar mutant mouse strains devoid of PCs (pcd, nr, Lc). The use as donor tissue of a transgenic mouse line (ß 2nZ3'), in which the reporter gene lacZ is expressed in cerebellar interneurons but not in PCs, has allowed us to demonstrate that only PCs are attracted for long distances by the deficient host molecular layer. The implanted PCs leave the graft and penetrate the host, first by tangential migration at the surface of the folia and later on by radial migration into the molecular layer along Bergmann glial fibers. During this radial migration, the embryonic PCs induce in the host Bergmann glial axes re-expression of nestin, an intermediary filament normally present only during the active phase of granule cell migration. Thus, the expression of glial cell components involved in neuronal cell migration may be inducible by adjacent immature cells. These results emphasize the importance of neural grafting in the search for cellular and molecular mechanisms operative during adult CNS restoration, that could recapitulate those transiently employed during normal cerebellar ontogeny.

B

Mapping the binding sites, the gate, and the channel of the acetylcholine receptor.

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In muscle-type ACh receptors, the two ACh binding sites are formed in the interfaces between α and γ (or ϵ) and between α and δ. By chemical crosslinking and site-directed mutagenesis, we have identified negatively charged residues in the δ and γ subunits that contribute to the binding of ACh. As in other proteins that bind the choline moiety, both negatively charged residues and aromatic residues contribute to ACh binding. The location of the binding sites between subunits suggests that the induced sliding of one subunit relative to another might transduce the free energy of binding into gating. In order to identify the residues lining the channel and to locate the gate, we have mutated to cysteine each of the residues of the mouse-muscle-α M2 segment and have expressed these mutant αs, together with wild-type β, γ, and δ, in Xenopus oocytes. We have determined the ability of small, charged, sulfhydryl-specific reagents to react with these cysteine residues and thereby irreversibly alter channel conductance or gating kinetics. The following residues in the α subunit are exposed in the channel lumen: Glu241, Thr244, Leu245, Ser248, Leu250, Leu251, Ser252, Val255, Leu258, and Glu262. These results are consistent with an α-helical conformation from Glu241 to Val249, an extended conformation from Leu250 to Ser252, and an α-helical conformation from Leu253 to Glu262. The accessibilties of some of these residues change with channel opening. Cysteine substituted for Glu241, however, is accessible to reagent added to the extracellular end of the closed channel; the gate, therefore, is more cytoplasmic than Glu241.

C

The secondary structure of the nicotinic acetylcholine receptor

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Transmembrane folding models of ligand-gated ion channels have been developed largely using hydropathy plots of primary structures deduced from cDNAs. Based on these plots, it was predicted that the nicotinic acetylcholine receptor spans the post-synaptic membrane by means of four alpha-helical segments. Recent high resolution EM-spectroscopy [1] provided evidence for only one intramembrane alpha-helix per subunit. We removed the extra membrane portion of the receptor protein by proteinase treatment and subjected the remaining intramembrane domain to FTIR spectroscopy. In this preparation we found about equal amounts of alpha-helical and beta structures [2]. Measurement of the linear dichroism of an oriented membrane sample showed that the alpha-helical structure is oriented roughly perpendicular to the membrane plane. The beta structure seems to not be uni-directionally oriented. These data are incorporated into a new secondary structure model of the receptor's transmembrane domain.

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D

APPROACHES TO THE ELUCIDATION OF THE SUBUNIT COMPOSITIONS OF GABA$_A$ RECEPTOR SUBTYPES

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Inhibitory signal transduction in the vertebrate brain is mediated predominantly by γ-aminobutyric acid (GABA) type A (GABA$_A$) receptors, subtypes of which are thought to be assembled from pentameric combinations of different subunits (named α1—α6, β1—β4, γ1—γ3 and δ). Polypeptide variants (e.g. two forms each of the β2 and γ2 subunits) also arise by the alternative splicing of primary gene transcripts, thus providing an additional level of receptor complexity. However, the exact polypeptide compositions of many *in vivo* GABA$_A$ receptor subtypes remain uncertain. We have taken several approaches, including the detailed *in situ* hybridization localization of receptor mRNAs in brain and retina, and a molecular analysis of cell-lines that express functional channels, to try to elucidate the subunit compositions of native GABA$_A$ receptors. These studies have revealed, for example, that the distributions of the chicken β2S- and β2L-subunit transcripts mirror those of the γ2S- and γ2L-subunit mRNAs, respectively. Furthermore, the α1-subunit mRNA is found in all of the brain regions that contain both β2- and γ2-subunit transcripts. In addition, we have recently isolated a complementary DNA that encodes a fourth γ subunit of the vertebrate brain GABA$_A$ receptor. The mRNA for this polypeptide is found, in the chick brain, in the hyperstriatum ventrale, an area in which biochemical changes are known to occur during the learning process known as imprinting, and the optic tectum, nucleus rotundus and ectostriatum, regions which are part of one of the pathways (the tectofugal pathway) that process visual information.

A

FACTORS DETERMINING AGONIST SELECTIVITY IN MUSCARINIC ACETYLCHOLINE RECEPTOR SUBTYPES.

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We investigated the molecular basis of agonist selectivity among muscarinic acetylcholine receptors (mAChR) subtypes. Carbachol (CCh) and oxotremorine significantly increased phosphoinositides (PI) hydrolysis in CHO cells transfected with the human m1AChR (Hm1) and m3AChR (Hm3). However, in both Hm1 and Hm3 cells adenylate cyclase (AC) was strongly activated by CCh and weakly by oxotremorine. Prolonged incubation of Hm1 cells with CCh induced marked receptor down-regulation and a loss of the AC response, whereas the PI response remained pronounced. Atropine inhibited muscarinic-induced PI hydrolysis and AC activation. However, the Ki of atropine was two orders of magnitude lower for AC than for PI in both Hm1 and Hm3 cells. These findings suggest that the preferential activation of the PI versus AC is due to existence of spare receptors for PI hydrolysis. investigating agonist selectivity among receptor subtypes, we found that the prototype M1 agonist, McN-A-343, activated more potently PI hydrolysis in Hm1 than in Hm3 cells. However, binding studies showed that McN-A-343 had similar affinities to m1AChR and m3AChR, indicating that the selectivity demonstrated by McN-A-343 is not at the recognition level. Since spare receptors for this transduction pathway are similar in Hm1 and Hm3 cells, we conclude that differences in the ability of the receptor to transduce the signal are responsible for the observed functional selectivity. Presently we test this hypothesis utilizing various chimeras of mAChR subtypes. Preliminary studies indicate that the 7th transmembrane helix is an important constituent for antagonist binding and may play a role in transducing the signal.

B

POLYGLUTAMYLATION OF TUBULIN: GENERAL FEATURES AND POSSIBLE ROLE AS REGULATOR OF MAP BINDING

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Tubulin polymorphism is due to the differential expression of several tubulin isogenes and, predominantly, to various posttranslational modifications (PTM), some of which being specific to this protein. Among the diverse PTM described so far, the recently-discovered polyglutamylation appears as the most complex one. It consists of the sequential addition of up to six glutamyl (glu) units, linked together by α or γ amide bonds, onto the free γ-carboxylic group of a glutamate residue located close to the C-terminus of α- (E # 445 for mα1) as well as β-tubulin (E # 435 and # 438 for class II and class III, respectively). Using polyclonal and monoclonal antibodies raised against glutamylated synthetic octopeptides and specific metabolic labeling, we showed that polyglutamylated tubulin accumulates throughout brain development and differentiation of primary neurons, with a regular increase in the length of the polyglutamyl chain carried by β-tubulin. We also showed that polyglutamylation is a reversible PTM: while the addition reaction utilizes microtubule polymers as preferential substrates, the reverse deglutamylation reaction is independent of the polymerization state of tubulin. Moreover, we found that the deglutamylase activity operates following biphasic kinetics: distal glu units (6th > 4th) are removed rapidly whereas the proximal ones (3rd > 1st) are excised much more slowly. Altogether, these results explain the asymmetrical distribution of polyglutamylated tubulin isoforms observed between microtubules and soluble tubulin. By ligand blotting (blot overlay) experiments, we found that polyglutamylation regulates the binding of the microtubule-associated protein Tau as a function of its chain length. Unmodified tubulin isotypes display a very weak, if any, affinity for Tau because of the constitutive inhibition exerted by the unmodified C-termini. As the polyglutamyl chain grows, this inhibition is progressively released and tubulin isoforms carrying around 3 glu units have an optimal affinity for Tau. Then, as the chain lengthens further, the binding inhibition is progressively restored and fully-glutamylated α- and β-tubulin isoforms do not bind Tau anymore. This effect is thought to occur indirectly via progressive conformational changes of the C-terminal domain driven by the growing polyglutamyl chain. Potential consequences of this "potentiometric" regulation on microtubule dynamics as well as the possible extension of the regulatory role of polyglutamylation to the other structural or motor MAPs will be discussed.

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C

THE ROLE OF MAP2 IN NEURITE FORMATION AND STABILISATION Andrew Matus*, Kevin Ashbridge, Beat Ludin, Jacqueline Ferralli and Thierry Doll, Friedrich Miescher Institute, P.O.Box 2543, 4002, Basel, Switzerland.

Microtubule-associated proteins (MAPs) are particularly abundant in neurons where they are present in both axons and dendrites. The two most abundant forms, MAP2 and tau, are the products of related genes and bind to microtubules via a domain containing 3 or 4 repeats of an 18 amino acid sequence motif. When either of these proteins is expressed in cultured non-neuronal cells the microtubules undergo a characteristic rearrangement in which they are stabilized independently of the centrosomal microtubule-organizing centre and form bundles. They also become capable of supporting process outgrowth from transfected cells, apparently because of additional rigidity which they acquire when MAPs bind to them.

To investigate the molecular mechanism underlying these changes we have transfected cells with mutated versions of MAP2 containing with deletions in the protein sequence. These results identify one region of the molecule as responsible for all the effects of MAP2, binding to microtubules, stabilization, polymer elongation and stiffening. The part of MAP2 required for these properties contains the 18 amino acids repeats together with neighbouring sequences. The repeats themselves are spaced along the length of the MAP2 and tau molecules so that they are able to bind to neighbouring tubulin subunits in the wall of the microtubule. Since both molecules are abundant in neuronal processes, this multimeric binding domain can coat the microtubules, tethering the tubulin subunits to one another and restricting their freedom of movement. We hypothesise that this is the source of the stiffening effect of MAPs on microtubules which makes them capable of supporting process outgrowth, either in transfected non-neuronal cells or in neuronal processes where they are normally found. The loss of these properties when the interaction of the MAPs with microtubules is disturbed, for example through abnormal phosphorylation which inhibits binding, could therefore have profound consequences for the maintenance of neuronal morphology and function.

D

DIFFERENTIAL DISTRIBUTION OF CYTOSKELETAL ELEMENTS DURING DIFFERENTIATION OF HUMAN NEUROBLASTOMA CELLS

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During differentiation of cultured human neuroblastoma LA-N1 cells extensive changes in the spatial organization and distribution of cytoskeletal elements take place. Thus, upon neurite extension, microtubule bundles are formed in which MAP2 is found in the proximal part, while MAP5 is distributed along the entire length of the neurite. Peripherin and vimentin were found to relocate to the growth cones.The differentiation of neuroblastoma cells is also accompanied by increased adhesion to laminin. The major binding site in laminin mediating cell attachment was identified as the YIGSR sequence on the B1 chain.The surface receptors of these cells were labeled by biotinylation and the solubilized particulate fraction was subjected to $C(YIGSR)_3NH_2$-AffiGel 10 affinity chromatography. Bound proteins were then eluted with 0.1 mg/ml of free $C(YIGSR)_3NH_2$, analyzed by SDS-PAGE, transferred onto a nitrocellulose membrane and incubated with streptavidin conjugated to horse radish peroxidase. This procedure revealed one major YIGSR-binding protein of apparent molecular weight of 67 kDa. This surface membrane protein was not eluted from the YIGSR column with an unrelated peptide, nor could it be eluted from an unrelated peptide column with the YIGSR peptide. Comparison of Bt_2cAMP differentiated neuroblastoma cells with undifferentiated cells did not show significant differences in the level of the 67 kDa binding protein. Thus, the enhanced binding of differentiated cells to the YIGSR sequence of laminin is probably due to changes in spatial organization, or modification of the binding protein or to expression of an additional binding protein that interacts with a different site on the laminin molecule. We have recently found, however, that during the differentiation process there are considerable changes in the pattern of tyrosine-phosphorylated proteins. These findings may shed some light on the regulation of neuroblastoma differentiation and the mechanisms of laminin signal transduction.

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A

NEURONAL POLARITY: MOLECULAR MECHANISMS LEADING TO AXONAL LOCALIZATION OF TAU-MICROTUBULE ASSOCIATED PROTEINS

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Tau microtubule-associated protein is a neuron-specific protein found primarily in axons and is developmentally regulated. The function of tau is in stabilization of microtubules, which is important in establishing and maintaining neuronal morphology. Axonal localization of tau involves a multistep process which is studied in differentiating primary neuronal culture. The initial step involves sorting and subcellular localization of its encoding mRNA into the proximal portion of the axon. Using the transfection assay into neuronal cells, we have demonstrated that sequences located in the 3'-untranslated region include a *cis*-acting sequence, which is involved in tau mRNA targeting. In addition, using ultraviolet cross-linking assay, two RNA-binding proteins of 43 and 38 kDa were identified, that exhibit specific binding to a minimal sequence of 91 nucleotides located within the same region, which is involved in targeting. These proteins are present in cytoplasmic extracts, prepared from neuronal cells, and in isolated microtubule preparations. Treatment of neuronal cell cultures with cytoskeletal inhibitors demonstrates that tau mRNA is associated with the microtubule system and not with actin filaments. These results support the hypothesis that mRNA localization pathway in neurons involves recognition between *cis*-acting signals and RNA-binding proteins that associate with microtubules, which ultimately will lead to axonal localization of tau protein.

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B

Microtubule stability in neurons: markers and mechanisms

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Neuronal cells contain an abundant subset of highly stabilized microtubules, which resist cold temperature induced disassembly. Such microtubule stabilization is developmentally regulated and seems to be important to neuronal morphogenesis and maintenance. We have recently described a major tubulin variant (Delta 2-tubulin) which cannot be tyrosinated because it lacks the two last carboxyterminal amino acids on the tubulin α-subunit. In most cell types, the Delta 2-tubulin isotype is restricted to very stable microtubule structures such as centrosomes and axonemes and behaves as a marker for extreme microtubule stability. Delta 2-tubulin is highly abundant in neurons where it represents at least 40% of total neurotubulin. The appearance of Delta 2-tubulin is upregulated during the course of brain development. The abundance of such a tubulin isotype in neurons shows that neuronal microtubules are much more stable than other cytoplasmic microtubules. We show that a single protein, STOP, seems to be responsible for the occurrence of highly stabilized microtubules in neurons. The primary structure and major functional properties of STOP will be presented.

C

THE ASTROGLIAL MARKER GFAP EXPRESSED IN CULTURED LIVER CELL POPULATIONS CONTAINING STELLATE CELLS

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Astroglial cells in culture express metabolic features of liver such as storage of glycogen, gluconeogenesis and generation of ketone bodies. Therefore, it was tempting to assume the presence in liver cells also of structural features of astrocytes. We confirmed previous reports of others that an astrocyte marker, the intermediate filament protein (IFP) GFAP, as well as another IFP occurring in astroglial cells, vimentin, can be immunocytochemically detected in liver slices. Consequently we investigated by double-labelling immunofluorescence techniques the presence of GFAP and vimentin in cultures of isolated hepatocytes, of isolated stellate cells (Ito cells; SC), and of co-cultures of isolated hepatocytes and isolated SC, as well as the appearance of a third IFP, desmin, in hepatocyte cultures. Hepatocytes lacked any of these IFPs. In contrast, in the hepatocyte culture a population of (phase-contrast invisible) star-shaped cells, reminiscent of astrocytes, were strongly expressing vimentin or GFAP and vimentin immunoreactivity. The expression of vimentin increased from 4 h to 4 d, while GFAP expression had a tendency to dercrease. These star-shaped cells were identified as SC by their numerical preponderance in the SC cultures and their expression of desmin. The morphological resemblance and the occurrence of GFAP suggest some common cell physiological traits in SC and astrocytes.

D

NEUROTROPHINS-SIGNALS BETWEEN THE NERVOUS AND IMMUNE SYSTEMS

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Neurotrophins, including nerve growth factor (NGF), are clearly multifunctional. Increasing evidence indicates that NGF, in addition to its neurotrophic functions, acts as an immunomodulator mediating 'cross-talk' between neurons and immune cells, including T lymphocytes.

We have analyzed murine CD 4^+ T-cell clones for their ability to express transcripts encoding NGF, low-affinity NGF receptor, and trk protooncogene, the signal-transducing receptor subunit for NGF. We found that two $CD4^+$ T-helper (Th) clones, Th 0-type clone 8/37 and Th 2-type clone D10.G4.1, express NGF and Trk mRNA after stimulation with mitogen or with antigen in the presence of antigen-presenting cells. NGF and trk induction occurred to a similar extent and over a similar time course in activated 8/37 T cells, raising the possibility that NGF and trk genes are under coordinate control. NGF and NGF receptor expression does not seem to be a universal property of all activated $CD4^+$ T cells, since the Th 1-type clone 9/9, a nonspecific killer clone expressing natural killer surface markers, failed to express detectable NGF or trk transcripts after either mode of stimulation The absence of detectable low-affinity NGF receptor mRNA in resting and activated T cells implies that it is not involved in NGF signal transduction in $CD4^+$ T cells. Our finding that activated $CD4^+$ T-cell clones not only express Trk but also synthesize and release biologically active NGF implicates NGF as an autocrine and/or paracrine factor in the development and regulation of immune responses.

In addition, a possible pathophysiological role of NGF in autoimmune disorders is suggested by our findings that NGF levels are makedly raised in cerebrospinal fluid of patients with multiple sclerosis, in serum of patients with systemic lupus erythematosus and in joints and serum of patients affected by rheumatoid arthritis.

A

CYTOKINES AND CYTOKINE-RELATED SUBSTANCES REGULATING GLIAL CELL RESPONSE TO INJURY NEEDED FOR CENTRAL NERVOUS SYSTEM (CNS) REGENERATION

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Axons of the mammalian CNS, unlike those of fish and amphibian CNS, have a negligible ability to regenerate after injury. Nevertheless, these axons are apparently able to elongate if the milieu through which they grow is permissive to growth. Among the elements making up the milieu are astrocytes, oligodendrocytes, resident microglia and invading blood-borne cells (macrophages and lymphocytes). Astrocytes, the cellular elements known to be supportive of growth, form a scar tissue that has been considered as a barrier to growth. Oligodendrocytes were shown to be inhibitory to axonal growth. In the injured fish optic nerve we found a factor that selectively kills mature oligodendrocytes *in vitro* and identified it as an interleukin-2 (IL-2)-like substance of twice the molecular weight of lymphocytic IL-2. We have also identified in the fish a nerve-derived transglutaminase enzyme capable of dimerizing IL-2. The dimeric IL-2 kills oligodendrocytes in an apoptotic manner. We also have preliminary results pointing to the possibility that the enzyme has the potential to facilitate nerve growth *in vivo*. The cellular source of the enzyme and of IL-2 is currently under investigation. Our preliminary findings suggest that their production may be related to the inflammatory reaction, i.e., to invasion by macrophages and the availability of their products. Our studies of the astrocytic response to injury indicate also that posttraumatic migration of astrocytes may be an essential precondition for axonal regeneration across the injury site. Astrocytes of fish optic nerve migrate whereas those of mammals do not, but the migration is apparently amenable for treatment with inflammation-associated products – e.g., IL-1β, tumor necrosis factor-α. We therefore propose that CNS, in many of its aspects and mainly with respect to nonneuronal elements, is not exempt from the body's normal healing mechanism in response to trauma; besides, that postinjury inflammation provides factors which may regulate the response of astrocytes and oligodendrocytes to injury, making them permissive to and/or supportive of growth, provided that this response is in synchrony with axonal growth.

B

THE HYPOTHALAMIC POOL OF IMMUNOMODULATORS

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The nervous and immune systems appear to communicate with each other by virtue of signal molecules - immunomodulators. In the course of the study of molecular mechanisms of the interrelationship between the two systems we have isolated from the soluble fraction of bovine hypothalamus a number of polypeptides related to the immune system. The purification procedure was based on reverse phase HPLC. The isolated polypeptides have been tentatively identified by mass spectrometry, Edman microsequence analysis and subsequent protein database searching as thymosin b4 (1-39), thymosin b1 (1-74) and b1 (1-76) - ubiquitin. To our knowledge, this is the first demonstration of endogenously derived thymosin b4 containing 39 amino acid residues. We have also isolated simultaneously with the thymosins and identified a receptor for a novel macrolide antibiotic - FK 506 which has received much attention due to its immunosuppressive activity in the areas of transplant rejection, autoimmune diseases, etc. This FK 506 - binding protein possesses peptidyl-prolyl cis-trans isomerase activity which catalyzes the interconversion of the cis- and trans-rotamers of a prolyl amide bond in peptide and protein substrates and facilitates protein folding. Mechanisms of biochemical actions of immunomodulators in the neuroendocrine system are discussed.

C

REGULATION OF PLATELET-ACTIVATING FACTOR (PAF) METABOLISM IN NERVOUS TISSUE AND IN CULTURED NEURAL CELLS.

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PAF is a lipid mediator which exerts its functions in cell-to-cell communication but has also intracellular targets. The roles of PAF in the nervous tissue are not completely known but must be of physiological relevance because it is involved in the neurotransmission and in pathological events since its concentration in brain increases during ischemia and convulsions *(Kumar et al.,1988)*. Neural cells are able to synthesize PAF *ex novo* or from endogenous 1-alkyl-2-acyl-*sn*-glycero-3-phosphocholine present in their membranes. The *de novo* synthesis needs 1-alkyl-2-acetyl-sn-glycerol and CDP-choline *(Francescangeli and Goracci, 1989)* whose availabilities may represent the limiting factors for their conversion to PAF by a specific enzyme (PAF-cholinephosphotransferase; PAF-CTP). This path is inhibited by an elevation of intracellular Ca^{2+} which is required for the activation of phospholipase A_2 (PLA_2), the enzyme mainly responsible for the degradation of membrane glycerolipids and for the liberation of fatty acids at the onset of ischemia or other cerebral metabolic disturbances. The activation of PLA_2 leads to a parallel production of lysophospholipids among them 1-alkyl-2-lyso-*sn*-glycero-3-phosphocholine (lyso-PAF) which is acetylated to PAF by an acetyltransferase (AcT) in the presence ofG acetyl-CoA *(Goracci and Francescangeli, 1991)*. Thus, this path may contribute to the production of PAF better than the *de novo* synthesis during brain metabolic disorders. Brain cytosol has an acetylhydrolase (AH) which inactivate PAF and contribute to the control of the levels of PAF in the nervous tissue. Differences in the activities of the enzymes involved in PAF metabolism have been found in various rat brain areas and this may be of relevance in connection to the localization of the cellular damage following an ischemic insult. The specific activities of PAF-synthesizing enzymes increase when neuronal cells from chick embryo in culture form synaptic-like junctions *(Francescangeli et al., 1993)* and this may be related to one of the proposed physiological functions of this lipid mediator which implies its action in gene expression by its binding to intracellular receptors *(Marcheselli et al., 1990)*. Furthermore, our recent data indicate that during differentiation of neuroblastoma cells (LA-N-1) induced by retinoic acid the activity of PAF-PCT is greatly inhibited whereas that of AT and AH are unaffected by the treatment.

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D

MODULATION OF LIPID SECOND MESSENGERS IN PROLIFERATING AND DIFFERENTIATED CELL LINES

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Many cellular processes are regulated by the binding of agonists to specific receptors leading to the liberation of lipid second messengers through the activation of phospholipases. Most of these second messengers modulate the activity of various protein kinases (PKC) which catalyse protein phosphorylation and thus induce short and long term cellular responses. The mechanism involved in the induction of specific cellular responses are unknown. This prompted us to investigate the production of lipid second messengers in LA-N-1 neuroblastoma cells, including the nuclei under various physiological conditions. The stimulation of LA-N-1 cells with phorbol esters (TPA) elicited a biphasic increase in diglycerides (DAG). In proliferating cells the TPA stimulation produced mainly saturated/monounsaturated and disaturated DAG species, whereras in cells differentiated due to retinoic acid the liberation of saturated/polyunsaturated and diunsaturated molecular species was observed. No increase in DAG was observed when isolated nuclei were treated with TPA. However, the nuclei contained endogenous PLD and PKC which were activated by TPA. Moreover, PA is able to stimulate nuclear PKC by a mechanism which differ from the activation by DAG. The data indicate that the stimulation of LA-N-1 cells by TPA elicites the production of specific DAG molecular species in the plasma membrane depending on the physiological status of the cells. These lipid messengers may stimulate the translocation of cytosolic PKC to the nucleus or activate endogenous nuclear PKC, which may stimulate PLD by phosphorylation of either the enzyme or a regulatory protein inducing a long term cellular response

A

GANGLIOSIDES AND SECOND MESSENGERS AS THE MODULATORS OF FREE RADICAL REACTIONS IN BRAIN

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The role of signal transduction systems and their modulators in the regulation of free radical reactions has not been practically studied yet, for the exception of phagocytic cells, though unbalanced activation of these reactions is one of the main causes of cell death and dysfunctions at various pathologies. Preincubation of rat brain synaptosomes with nanomolar concentrations of exogenous gangliosides (GM1, GD1a or GT1b) was shown to inhibit induced lipid peroxidation (LPO), to protect lipid polyenioic fatty acids, enzymes and receptors from oxidative destruction. In liposomes, made from synaptosomal membrane lipids, gangliosides in nanomolar concentrations had no effect on LPO process. Phorbol esters (PMA) were found to have dose-dependent inhibitory effect on LPO induced in synaptosomes. Polymyxin B (inhibitor of lipid-dependent protein kinases) abolished LPO inhibition by PMA or gangliosides, while dibutyryl c-AMP significantly diminished the degree of inhibition. But the data obtained seem to exclude the possibility that inositol polyphosphates or DAG mediate the inhibitory effect of gangliosides. Other signalling systems appear to be involved in the realization of their effect on LPO. The inhibitory effect of gangliosides on free radical reactions was confirmed using ischemic heart as a model and studying erythrocyte and synaptosome lipid composition in ischemic rats in vivo.

B

REGULATION OF PHOSPHATIDYLSERINE SYNTHESIS IN RAT CEREBRAL CORTEX

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Phosphatidylserine (PS), as other membrane phospholipids, is involved in signal transduction mechanisms. The role played by PS as a cofactor for protein kinase C (PKC) activity is well established and it has been proposed that its concentration in the membrane might modulate PKC specificity (1). In mammalian tissues serine synthesis occurs by base-exchange. This mechanism contributes also to the synthesis of ethanolamine and choline glycerophospholipids. In rat cerebral cortex experimental hypoxia increases the rate of PS synthesis (2). The possibility that such an increase is, at least in part, responsible for the development of brain damage by interfering with PKC activity has been suggested. In rat cerebral cortex serine incorporation into PS decreases during development but the increase in the synthesis of PS due to hypoxia is greater in adult than in younger animals (3). This observation might be related to the better resistance of young animals to hypoxia. Moreover, the effects of hypocapnia and calcium ions on PS synthesis seem to play a role in the hypoxia-induced synthesis of this phospholipid (3). Although the role played by base-exchange enzymes is not completely clear, the mechanism of regulation is of great interest. A phosphorylation-dephosphorylation mechanism, acting especially on choline and ethanolamine base-exchange, has been reported (4) and choline base-exchange seems to be regulated by a Gs protein in liver plasma membranes (5). In rat brain cerebral cortex a Gi protein might be involved in the regulation of PS synthesis by base-exchange (6). The possibility exists that different mechanisms can be involved in the regulation of base-exchange enzymes, depending on the specificity for the free base and the source of the enzyme. The regulation of PS synthesis by a Gi protein regulating the serine base-exchange enzyme will be discussed on the basis of results obtained by assaying the enzyme activity in cerebrocortical plasma membranes in the presence of G protein activators. The activation of the enzyme activity exerted by heparin and the effect of γ-D-glutamylglycine on PS synthesis in cerebrocortical homogenates will be also discussed in order to suggest a possible role for the regulation of base-exchange enzymes.

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C

PLATELET ACTIVATING FACTOR (PAF) AND RELATED LIPID DERIVATIVES IN THE FETAL BRAIN DURING ISCHEMIA

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PAF is a biological lipid autacoid which contributes to the regulation of normal cellular functions and participates as a potent extracellular or intracellular mediator in diverse pathological stress conditions. Generation of PAF, its putative targets and its clearance in the developing fetal brain under normal and ischemic conditions has not been studied previously.

Tracer amounts of [^{3}H]PAF administered through the uterine wall into the embryonic brain of 20-days old pregnant rats, was taken up and subsequently removed by a biphasic two-component ($t_{1/2}$ =10 and 41 min) system. The disappearance of [^{3}H]PAF was accompanied by an increase in the radioactivity associated with lyso-PAF which attained a plateau after 2.5 min, possibly indicating the degradation of the fast component. In fetal brains subjected to a complete obstruction of the maternal-fetal blood circulation for 20 min, a delayed elimination of [^{3}H]PAF via the slow component was noticed.

Intracranial administration of PAF enhanced release of arachidonic acid, caused a decrease in fetal brain poly-phosphoinositide (poly-PI) and stimulated production of thromboxane. The calcium ionophore A23187 stimulated *in vitro* formation of PAF and lyso-PAF from [^{3}H]choline-labeled fetal brain phospholipids suggesting that intracellular [Ca^{2+}] is involved in PAF production.

During intrauterine life the developing brain is capable of synthesizing its own PAF from endogenous precursors as determined by [^{3}H]serotonin release from platelets. Repetitive administration of the PAF antagonist BN52021 *in vivo* prevented poly-PI degradation irrespective of maternal-fetal blood flow occlusion. The data suggest that endogenous PAF generated either by decapitatitive ischemia or after blood flow occlusion is associated with a phospholipase C activation.

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D

XENOPUS OOCTYES AS A TOOL TO STUDY EXOGENOUS PROTEINS

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Xenopus oocytes are now widely used for study of different receptors and ionic channels after injection of the mRNA coding for these exogenous proteins. When using oocytes for expression of G-protein coupled receptors, it is important to be aware that the evoked responses relies on the activation of the endogenous transductional mechanisms of the oocyte. One of the most important second messengers in the oocyte, as in many other cells, is the inositol 1,4,5 trisphosphate ($InsP_3$). For that reason, the knowledge of its function is of special interest to understand the responses elicited by the expressed receptors. When $InsP_3$ reaches theshold levels, with a short latency, it releases calcium from intracellular stores in a localized all-or-none manner. Small increases of intracellular calcium levels facilitate release of more calcium by $InsP_3$ but when calcium rises to higher concentrations, it inhibits further release by $InsP_3$. This mechanism is underlying the oscillatory changes in calcium levels and, subsequently, the calcium dependent chloride current evoked when receptors coupled to the inositol phosphate cascade are activated.

Another important aspect to consider when studying membrane proteins coded for heterologous mRNA in oocytes is that all postranslational processing is done by the endogenous oocyte machinery and perhaps it may be not exactly the same as in the donor cell. That is why we have introduced a new technique by which purified and reconstituted proteins in a defined lipid matrix can be functionally incorporated into the oocyte membrane.

A

XENOPUS OOCYTES AS REPORTERS FOR CALCIUM INFLUX

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The popular oocyte expression system has an inherent cellular calcium reporting mechanism arising through the native calcium activated chloride conductance. This process has a large intrinsic amplification factor, so that small increases in intracellular Ca^{2+} can be detected. This useful property is demonstrated below by three examples. 1. By sequence comparison between Na^+-channels and Ca^{2+}- channels, two point mutations were localised, which were able to confer specific permeation and divalent-block characteristics of Ca^{2+}-channels on to Na^+-channels. The detection of these specific changes was helped by the fact that the Ca^{2+} permeating through mutated Na^+-channels activated the Cl^--current. 2. Permeation of Ca^{2+} through "classical" K^+-channel was detected by the presence of the Ca^{2+}-activated Cl^- current. This Ca^{2+} permeation is able to explain specific characteristics observed in Drosophila mutants where this channel type is deficient. 3. Depletion of intracellular Ca^{2+} stores, in this particular case through the expression of cloned $5HT_{1C}$ receptors that couple to IP3, causes a secondary small Ca^{2+} influx response that is significant for refilling internal stores. The primary response to 5HT is IP3-induced Ca^{2+} release, depleting the intracellular stores. A novel diffusible messenger seems to provide the retrograde signal to the plasma membrane for increasing a Ca^{2+}-permeability (capacitive calcium entry), which causes an elevation of intracellular Ca^{2+}. This Ca^{2+} then activates the native Ca^{2+}-dependent Cl^--currents.

B

Expression cloning and molecular studies of G protein-activated potassium channels in *Xenopus* oocytes.

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The inwardly rectifying atrial K^+ channel is activated by acetylcholine via a direct coupling with a G protein and plays and important role in regulation of heartbeat. Until recently, no molecular information on this channel has been available. To clone the cDNA of this channel, that we termed KGA, we have expressed it in *Xenopus* oocytes injected with cardiac mRNA. The expressed KGA channel showed the distinct pharmacological and electrophysiological features such as block by Ba^{2+}, inward resctification, and high selectivity to K^+ over Na^+. A directional library was made on the template of atrial mRNA, and RNA was syntheiszed *in vitro* from cDNA extracted from pools of plasmid-containing *E. coli*. Ooocytes were coinjected with the cRNA of these pools and cRNA of the 5HT1A receptor known to couple to the KGA channel. After a sib-selection process, a single cDNA was cloned corresponding to a subunit of the channel. The channel belongs to a new family of inward rectifier potassium channels with two putative transmembrane segments and a pore homologous to that of voltage-dependent channels. Deletion mutations at the C-terminus of KGA suggested that this part of the molecule participate in interaction with G-proteins. Additional studies on the nature of G protein-KGA interaction are in progress.

C

SUBUNIT ASSEMBLY AND FUNCTIONAL MATURATION OF Na,K-ATPase STUDIED IN XENOPUS OOCYTES.

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Na, K-ATPase is an ubiquitous ion transporter responsible for the maintenance of cellular potassium and sodium homeostasis. In its active form, the enzyme is composed of an α-subunit which carries all catalytic functions and a glycosylated ß-subunit. We have studied the role of the ß-subunit in the functional expression and the transport activity of the enzyme by using the Xenopus oocyte as an expression system. Assembly of the ß-subunit with the α-subunit turns out to be essential for the α-subunit to escape cellular degradation at the level of the ER, as well as to acquire the ability to hydrolyze ATP. Initial assembly competent folding of the two subunits appears to be mediated by a temporary interaction with the chaperone Bip. Besides its role in the structural and functional maturation of the α-subunit, the ß-subunit also modulates the transport activity, namely the apparent K^+ affinity of the functional Na, K-pumps expressed at the cell surface. Structure-function studies have revealed that at least one assembly site is formed by a hydrophobic domain within a ß-strand like structure of the extracytoplasmic C-terminus of the ß-subunit. On the other hand, the short N-terminal cytoplasmic tail is not involved in assembly with α-subunit but contributes to the functional interaction between the two subunits. A short motif of 8 amino acids in the cytoplasmic tail of the ß-subunit appears to be involved in the modulation of the properties of the external K^+ binding site in the α-subunit.

D

ALTERNATIVE EXON E6 DIRECTS SYNAPTIC LOCALIZATION OF RECOMBINANT HUMAN ACETYLCHOLINESTERASE IN NEUROMUSCULAR JUNCTIONS OF *XENOPUS LAEVIS* EMBRYOS

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We have expressed and characterized catalytically active recombinant human acetylcholinesterase (rHAChE) produced in microinjected oocytes of *Xenopus laevis*. However, the highly specialized, single-cell nature of the oocyte limits its usefulness in addressing questions regarding tissue-specific processing and the biological roles of cloned nervous system proteins. Therefore, to study the role of 3' alternative splicing in regulating tissue-specific expression of the human ACHE gene encoding AChE, we established an *in vivo* model in transiently transgenic *Xenopus* embryos. Following injection into *in vitro* fertilized *Xenopus* eggs, whole-mount cytochemistry and electron microscopy revealed that ACHE DNA bearing the alternative 3' exon E6 (ACHE-E6) induced prominent overexpression of catalytically active AChE in myotomes of 2- and 3-day-old embryos, including neuromuscular junctions (NMJs). NMJs from ACHE-E6-injected embryos displayed, on average, 4-fold greater AChE-stained areas (SA) and 80% increased post-synaptic lengths (PSL) compared to age-matched uninjected controls. Perhaps more significantly, ACHE-E6 overexpression stimulated the appearance of a class of large NMJs (PSL>4 μm) rarely observed in control embryos, apparently at the expense of small (PSL<3 μm) NMJs. Homogenates prepared from these embryos demonstrated increased binding of biotinylated α-bungarotoxin, indicating enhanced expression of the endogenous *Xenopus* acetylcholine receptor and suggesting coordinated regulation of cholinergic proteins in the developing NMJ. When exon E6 was replaced by ACHE DNA encoding the pseudo-intron I4 and 3' alternative exon E5, overexpressed rHAChE accumulated in epidermal cells, but not in muscle or NMJs. These findings, therefore, attribute an evolutionarily conserved NMJ-targeting role for the C-terminal peptide encoded by exon E6 and provide *in vivo* evidence for tissue-specific management of alternative AChEs.

A

$G_{\alpha q}$ DISCRIMINATES BETWEEN TWO BOMBESIN RECEPTOR SUBTYPES EXPRESSED IN XENOPUS OOCYTES.

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$G_{\alpha q}$ and $G_{\alpha 11}$, two members of the G_q class of G protein α-subunits, have been implicated as transducers of the pertussis toxin-insensitive activation of phosphoinositide-specific phospholipase C. This signal transduction pathway has been shown to be activated by bombesin receptor subtypes, gastrin releasing peptide receptor (GRP-R) and neuromedin B receptor (NMB-R). To determine which G-proteins couple bombesin receptor responses, we cloned *Xenopus* $G_{\alpha q}$ and $G_{\alpha 11}$ and specifically disrupted their synthesis with selective antisense oligonucleotides. $G_{\alpha q}$ antisense inhibited responses mediated by NMB-R by 74%, but had no effect on responses mediated by GRP-R. $G_{\alpha 11}$ antisense had little effect on either GRP-R- or NMB-R-mediated responses. These findings suggest that NMB-R exerts its effect by activating $G_{\alpha q}$, and that GRP-R and NMB-R show distinct G-protein coupling preferences *in vivo*.

B

RAPID DESENSITIZATION OF THE RESPONSE TO TRH IN OOCYTES IS AN AMPLIFIED PROCESS THAT PRECEDES CALCIUM MOBILIZATION.

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The Ca-mediated chloride current response to thyrotropin-releasing hormone (TRH) in Xenopus oocytes injected with RNA encoding the TRH receptor (TRH-R) was strongly desensitized. Sub-optimal concentrations of TRH caused a marked desesitization of the subsequent response to a higher concentration of the agonist, even though the second challenge was given before the onset of the response to the first challenge (within 3-15 sec). [Ca]i did not increase within this interval, suggesting it was not involved in desensitization. The latency of the second response, was often shortened, implying additive effects of processes initiated by the first challenge. Longer interval (30 sec) between the two challenges brought about a more pronounced desensitization and a prolongation of response latency. The Ca mobilization profile to a second challenge with high concentration of the agonist exhibited longer latency, lower maximal rise rate and lower amplitude. Minimal stimulation of co-expressed cholinergic-muscarinic m1 receptors resulted in heterologous desensitization of the TRH response. Activation of protein kinase C by β-phorbol 12-myristate,13-acetate resulted in a pronounced inhibition of the response to TRH. Chelerythrine, a specific inhibitor of protein kinase C abolished a large part of rapid desensitization. A mutant of the TRH-R that does not exhibit prolonged desensitization in mammalian cells and lacks protein kinase C concensus phosphorylation sites in the C-terminal region, exhibited similar rapid desensitization properties, suggesting that rapid desensitization is not targeted at this part of the receptor molecule. These results suggested that very low receptor occupancy activates an amplification step that results in heterologous desensitization. This process is mediated, at least partly, by the activation of protein kinase C, acting on a target proximal to Ca mobilization.

C

METALLOENDOPEPTIDASE FAMILIES IN NEURONAL FUNCTION

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Several distinct metalloendopeptidases have been implicated in peptide and protein processing in the brain. These include endopeptidase-24.11 (neprilysin) and angiotensin converting enzyme (ACE) as well as activities that apparently exist in membrane-associated and soluble forms such as thimet oligopeptidase (EC 3.4.24.15) and neurotensin degrading enzyme (neurolysin; EC 3.4.24.16). Molecular cloning of some of these enzymes have revealed the common HEXXH zinc-binding motif which is also present in botulinum and tetanus neurotoxins. These toxins also act as endopeptidases cleaving specifically proteins involved in neurotransmitter release. Endopeptidase-24.11 is a type II integral membrane protein topologically organised as an ectoenzyme. The enzyme is widely distributed and, in the nervous system, is particularly abundant in a striatonigral pathway. Its substrate specificity allows it to hydrolyse a range of neuropeptides including the tachykinins. In addition to its localisation at opioid synapses, e.m. studies reveal co-localisation of E-24.11 with substance P. ACE, a type I integral membrane protein, also regulates neuropeptide concentrations and exists in distinct glycosylated forms in brain. Release of ACE from the cell-surface by a membrane-bound secretase may reflect a more general mechanism for regulating the levels of certain neuronal cell-surface proteins, e.g. the amyloid precursor protein. Neuropeptidases provide accessible targets for therapeutic agents designed to modulate neuropeptide activity.

D

Streptomyces griseus Aminopeptidase (SGAP): Molecular Tool in the Study of Neutral Endopeptidase(NEP/CALLA)

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Streptomyces griseus aminopeptidase (SGAP) is a calcium-activated zinc metalloenzyme, characterized by high enzymic reactivity, high thermal stability and low molecular mass. The enzyme contains two zinc atoms per molecule, m ~ 30kDa, and it can bind Ca^{2+}, concomitant with an increase in activity. Procedures developed by us for high purification of the enzyme readily yield 200mg quantities, suitable for biochemical and structural investigations and with potential for a variety of biochemical and biomedical applications. Due to the unique physico-chemical properties and high enzymic reacitvity of SGAP it has been used as an additive in a two-stage assay developed by us to investigate the distribution, specificity and biological roles of the mammalian zinc ectoenzyme neutral endopeptidase 24.11 (NEP/CALLA). These studies allow screening of different cell types and body fluids that highly express NEP/CALLA and help to characterize novel putative substrate(s) of the enzyme/antigen. The studies assist in investigating the specificity of the ectoenzyme and its preference for small size peptides.

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A

THE MOLECULAR MECHANISM OF ACTION OF TETANUS AND BOTULINUM NEUROTOXINS

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Tetanus (TeNT) and botulinum neurotoxins (BoNT, seven different serotypes A-G) are proteic neurotoxins responsible of tetanus and botulism respectively. In their active form, they are composed of two fragments: the heavy chain is involved in the neurospecific binding and the cell penetration of the light chain, which is accountable for the blockade of neurotransmitter release. Comparison of the aminoacid sequences of clostridial neurotoxins reveals a highly conserved segment in the central part of light chain, containing the HExxH zinc binding motif of zinc-endopeptidases. In the native toxins, this segment coordinates one zinc atom, which is essential for the toxin-mediated inhibition of neurotransmitter release in Aplysia-injected neurones. Specific inhibitors of zinc-endopeptidases, prevent completely the intracellular activity of the toxins, strongly suggesting that they act via a zinc-dependent protease activity. We have demonstrated that TeNT and BoNT serotypes B, D, F and G cleave specifically VAMP-synaptobrevin, a 13 kDa integral membrane protein of synaptic vesicles present in two isoforms (-1 and -2) in the nervous system. TeNT and BoNT/B cleave only rat brain VAMP-2, while BoNT/D, BoNT/F and BoNT/G cleave both VAMP isoforms. Both TeNT and BoNT/B act at the same single site, while BoNT/D, /F and /G cleave VAMPs at different positions. BoNT/A and BoNT/E show a different target specificity by cleaving a short peptide at the carboxy-terminal of SNAP-25 (synaptosomal-associated protein of 25 kDa), a protein localized on the presynaptic membrane. BoNT/C cleaves both isoforms of HPC1/syntaxin, a membrane protein localized at the synaptic terminal. VAMP, SNAP-25 and syntaxin were demonstrated to be a component of the synaptic docking and fusion complex, together with NSF (N-ethylmaleimide-sensitive factor) and two SNAPs (soluble NSF attachment proteins). These and other results, that will be presented, provide compelling evidence that these neurotoxins inhibit the neurotransmitter release via the specific proteolysis of proteic components of the synaptic vesicles docking and fusion apparatus.

Hutmer, W.B. (1993) Nature 365, 104-5.
Montecucco, C. and Schiavo, G. (1994) Mol. Microbiol. in the press.

B

RECENT ADVANCES ON ENDOPEPTIDASE 3.4.24.16

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Endopeptidase 24.16 was first detected and purified on the basis of its ability to inactivate the tridecapeptide neurotensin. We have recently developed specific inhibitors of this peptidase. We show that these agents lead to: 1) a strong protection of neurotensin after intestinal infusion in dog; 2) a potentiation of the neurotensin-induced hypothermia after icv administration. By means of specific polyclonal antibodies, we demonstrate the dual neuronal and astrocytic distribution of endopeptidase 24.16. However, the subcellular localization appears different according to the cellular type (cytosoluble in astrocytes, cytosoluble and membrane-bound in neurons). Furthermore, the enzyme appears developmentally regulated in neurons but not in astrocytes in culture. The membrane-bound form of endopeptidase 24.16 is further documented by light and electron-microscopic approach in rat mesencephalon. The occurence of such form appears also in kidney as deduced from biochemical analysis using the triton X114 partition phase technique.

C

PURIFICATION, CHARACTERIZATION AND CLONING OF THE HORMONALLY REGULATED TRH-DEGRADING ECTOENZYME

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Thyrotropin-releasing hormone (TRH) is an important extracellular signal substance which acts as a hypothalamic releasing factor stimulating the release of the adenohypophyseal hormones and functions as a neurotransmitter/neuromodulator in the central and peripheral nervous system. The inactivation of TRH after its release is catalyzed by an ectoenzyme localized preferentially on neuronal cells in the brain and on lactotrophic pituitary cells. This enzyme exhibits a very high degree of substrate specificity as well as other unusual properties. The activity of the adenohypophyseal enzyme is stringently controlled by estradiol and thyroid hormones, indicating that this enzyme itself may serve regulatory functions.

After solubilization by limited trypsin digestion we succeeded in purifying this enzyme (200 000 fold) from rat and pig brain. Peptide fragments were then generated by enzymatic digestion or cyanogen bromide cleavage, purified by reverse-phase HPLC and sequenced. PCR-amplification and screening of cDNA libraries from rat brain and pituitary led to the identification and isolation of a cDNA that encodes a protein of 1025 amino acids. The analysis of the deduced amino acid sequences was consistent with the identification of the enzyme as a glycosylated, membrane-anchored Zn-metallopeptidase and transient transfection of COS-7 cells with this cDNA led to the expression of an active ectopeptidase that displayed the characteristics of the TRH-degrading ectoenzyme. Furthermore, Northern blot analysis demonstrated that the mRNA levels paralleled the tissue distribution of the enzyme and that in pituitary tissue the transcript levels rapidly increased when the animals were treated with triiodothyronine and decreased after injection of estradiol.

D

THE INDUCTION OF A TRANSFORMATION-ASSOCIATED METALLOPROTEASE TRANSIN mRNA IN THE RAT HIPPOCAMPUS FOLLOWING KAINIC ACID INDUCED SEIZURES

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Transin is a neutral calcium-dependent metalloprotease (EC.3.4.24) initially isolated from malignantly transformed rat fibroblasts and subsequently shown to be homologous to human stromelysin. We show the presence of transin mRNA in brain by demonstrating that kainic acid, but not metrazol induced seizures lead to the induction of transin mRNA in the rat hippocampus. Northern analysis and *in situ* hybridization were used to study the site and timing of the expression of transin mRNA in the rat brain. The maximum of induction by kainate in the hippocampus was 8h after the drug injection. The process was dependent on protein synthesis as cycloheximide injected before kainate abolished the effect and transin can be classified as late gene. One of the possible functions of this transin induction could be release of protease in the axon endings promoting later occuring mossy fiber sprouting induced by kainate. Thus, transin represents a new member of the rapidly increasing family of candidate plasticity related genes.

A

METABOLISM OF GALANIN AND GALANIN(1-16) IN ISOLATED CEREBROSPINAL FLUID AND SPINAL CORD MEMBRANES FROM RAT.

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The occurrence of galanin (GAL(1-29)) in the central nervous system and the possibility that it acts as an endogenous inhibitory spinal modulator in sensory/noxious processing and transmission, have focused interest on its metabolism. Studies on the structural requirements of GAL(1-29) for biological activity have shown that the N-terminal fragment GAL(1-16) is an equipotent agonist at the spinal GAL receptor, with a K_D value of 3 nM, but with slightly superior pharmaco-kinetic properties e.g. penetration and diffusion constants compared to GAL(1-29)[1]. The half-lives and degradation patterns of GAL(1-29) and GAL(1-16), were studied in isolated cerebrospinal fluid (CSF) from rats, and analysed by HPLC. The half-lives for GAL(1-29) and GAL(1-16) in isolated CSF were 112 min and 50 min, respectively. Those degradation products which appeared first were collected and further characterised by PTC amino acid analysis. The first independently appearing fragments for GAL(1-16) were GAL(3-16) and GAL(3-12) and for GAL(1-29) were GAL(1-5) and GAL(1-4), all biologically inactive peptides. Degradation studies in a lumbar dorsal spinal cord P_2-membrane preparation, in absence or presence of different class specific protease inhibitors were performed, to determine which protease class is the one responsible for the degradation of GAL(1-29) and GAL(1-16). These studies indicated that a zinc-metallo protease is mainly responsible for the degradation of GAL(1-29) and GAL(1-16) in spinal cord membranes, since *o*-phenanthroline (0.3 mM) is able to substantially prolong their half-lives from 109 min to 415 min (n=3) and from 25 min to 270 min, respectively.

1) Xu X. J., Wiesenfeld-Hallin Z., Fisone G., Bartfai T. and Hökfelt T. (1990) The N-terminal 1-16, but not C-terminal 17-29, galanin fragment affects the flexor reflex in rats. Eur J Pharmacol, 182, 137-141.

B

IMAGING OF CALCIUM AND SODIUM IN DENDRITES AND SPINES OF CULTURED HIPPOCAMPAL NEURONS.

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Dissociated rat hippocampal neurons were grown on glass in tissue culture plates for 1-6 weeks. Cells were loaded with the calcium indicator Fura-2 through sharp-tip microelectrodes or with Fura-2, Fluo-3 or calcium-green through a patch pipette. Segments of dendrites containing dendritic spines were visualized with 100x oil objective in an inverted microscope, equipped with a cooled CCD camera. [Ca]i variations in spines ([Ca]s) and dendrites ([Ca]d) resulting from exposure to NMDA, synaptic stimulation and drugs which regulate [Ca]i were measured. A brief exposure to NMDA caused a rapid rise of [Ca]s and a parallel, but smaller (by up to 40%) rise of [Ca]d. This rise of [Ca]i was seen both in current and voltage-clamped cells, indicating that the NMDA-evoked rise involves minimal, if any, activation of voltage-gated calcium channels. The recovery of [Ca]i to pre-NMDA occurred in the spine and the parent dendrite simultaneously, and was delayed in sodium-free medium.

Synaptic potentials were activated using alpha latrotoxin. In these cases [Ca]s was elevated more than the adjacent [Ca]d. Conversely, release of Calcium from mitochondria, using proton uncouplers, elevated [Ca]d more than [Ca]s. The presence of voltage-gated calcium channels in the spines was indicated by the higher rise of calcium in the spines upon depolarization of the soma by current injection. In fact, a calcium spike-associated rise in [Ca]i is faster and larger in the spine than in the parent dendrite. It is suggested that regulation of [Ca]s and [Ca]d are independent, but closely coupled. [Na]i concentration was measured with the sodium -sensitive dye SBFI. [Na]i went up to about 40-50mM following exposure to NMDA. The rise was higher in current-clamped than voltage clamped cells, and was slower than the rise of [Ca]i. As with [Ca]i, [Na]i rise appeared to be higher in spines than dendrites The link between the morphological and functional changes is currently under investigation.

C

LIGAND DEVELOPMENT FOR *IN VIVO* IMAGING OF MONOAMINE TRANSPORTERS

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Transporters for norepinephrine, serotonin and dopamine play a major role in terminating transmitter action. Inhibitors of serotonin and norepinephrine reuptake are effective antidepressants while dopamine transporter blockade appears to be related to the addicitive properties of drugs such as cocaine. A large numebr of reasonable ligands have become available over the years for labeling these transporters in vitro, in membrane preparations (binding) as well as in brain sections (autoradiography). However, attempts to use such ligands in vivo have failed repeatedly. The in vivo distribution pattern of radioactivity in the brain did not match the in vitro results or the known distribution of the relevant transporters provided by other techniques. In addition, most of the binding could not be displaced in vivo with the expected phamacological profile. Thus it appears that in vivo, most transporter ligands lose their specificity and bind to an addiotional , large and mostly intracellular population of sites possibly representing nonspecific vesicular, rather than specific membranal, transporters. A recently synthesized moclecule; 5-iodo-6-nitroquipazine (INQUIP), seems to be the first serotonin transporter ligand free of this problem. INQUIP has an extremely high (Kd=30pM) affinity to the serotonin transporter. Six or more hours after in vivo administration, the ligand distribution in the brain coresponds very well with the transporter neuroanatomy and the in vivo binding is completely and selectively displaceable by other serotonin uptake blockers. This opens the way for in vivo imaging of serotonin transporters in the human brain under various physiological and pathological conditions.

D

METHODOLOGICAL ASPECTS OF NEURORECEPTOR IMAGING

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More than one hundred radioactive ligands for in vivo imaging of neuroreceptors by PET or SPECT have been described. There are, however, major difficulties involved in quantitating such images in terms of the concentration of the receptor in the brain (B_{max}) and its affinity to the ligand (K_d). The best understood receptor is the Central Benzodiazepine Receptor, that is part of the GABA-ergic postsynaptic receptor system. This is so because the radioactive tracers C-11-flumazenil (for PET) and 1-123-iomazenil (for SPECT) have very suitable properties: (1) low non-specific binding; (2) no metabolism in brain; (3) hydrophilic metabolites do not enter the brain; and (4) a K_d in the range allowing to record both uptake and release of the ligand. Moreover, the brain cortex has a very high concentration of Bz receptors. With these tracers B_{max} and K_d can be indeed measured, most suitably by using a steady state approach and taking the plasma water phase as the reference fluid, and performing two tracer studies per subject, one at zero occupancy, and one at about 50% occupancy with non-radioactive ligand we used flumazenil. The results obtained show a variation of B_{max} in grey matter structures with highest values of about 120 mM in the occipital cortex and with a K_d of about 12 nM for flumazenil in all regions. With several of the other receptor/ligand systems there are major problems. In some cases the binding is so firm, that is essentially irreversible within the time available for recording. In this situation only the uptake constant or "clearance" from the blood K_1, can be derived. It reflects the blood flow times the extraction fraction at the blood:brain:barrier. Thus, even though a highly specific receptor ligand is used (such as C-11-methyl-spiperone) and a delayed image is recorded, the information gained is dominated by blood flow information if the tracer is binding too well to the region of interest. This also pertains to some ligands, binding to presynaptic neurotransmitter uptake sites. In other cases, when on uses less firmly bound tracers, as one must in order to calculate the equilibrium constant, K_d and B_{max}, the receptor uptake is impractically low. It can be concluded, that the methodological aspects of neuroreceptor studies are far from solved.

A

IMAGING OF DOPAMINE D2 RECEPTORS USING ^{123}I-IBZM AND SPECT IN PATIENTS WITH PARKINSONISM, WILSON'S DISEASE AND SCHIZOPHRENIA

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Single Photon Emission Computed Tomography (SPECT) with ^{123}I-Iodobenzamide (IBZM) allows semiquantitative measurements of striatal dopamine D2 receptors by calculating basal ganglia to frontal cortex ratios (BG/FC). We applied this procedure in 83 patients with de novo parkinsonism (29 of these received a second scan 3 to 6 months after initiation of dopaminergic therapy), in 19 patients with Wilson's disease (2 de novo and 17 under stable therapy), 27 patients with schizophrenia under neuroleptic treatment, and 14 controls. In patients with parkinsonism IBZM-SPECT predicted a positive or negative response to apomorphine in 69 of 76 patients (91%; the apomorphine test was equivocal in 7 patients). The response to long term oral dopamimetic therapy was correctly predicted in 54 of 62 patients (87%). In 29 of these patients the effect of treatment with L-DOPA, a dopamine agonist, or both was intraindividually assessed. In 10 patients treated with L-DOPA alone specific IBZM binding was unchanged. Treatment with a dopamine agonist ($n=11$, $p<0.05$) or a combination of L-DOPA and dopamine ($n=8$, $p=0.08$) agonist resulted in a reduction of specific IBZM binding. In 17 patients with Wilson's disease under stable therapy with D-penicillamine or zinc, we observed a highly significant correlation of reduction of specific IBZM binding and the severity of neurological signs ($r=-0.84$, $p<0.01$). In two additional de novo patients who were investigated before and 4 months after initiation of treatment with D-penicillamine, we found 16% and 24% increase of specific IBZM binding. In 27 patients with Schizophrenia treated with haloperidol ($n=18$) or clozapine ($n=9$) we observed a threshold of reduction of specific IBZM binding for the occurrence of extrapyramidal side effects (Chi square=23.1, $p<0.000$). In addition, we found an exponential dose response relation between striatal dopamine D2 receptor blockade and total daily dosage of both, haloperidol and clozapine, although there was a highly significant difference in dopamine D2 receptor occupancy between the two drugs ($p<0.000$). IBZM-SPECT may aid in the differential diagnosis of parkinsonism and in monitoring therapy in patients with Wilson's disease or schizophrenia.

B

THREE-DIMENSIONAL NEURORECEPTOR IMAGING IN THE HUMAN BRAIN BY PET

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We have previously developed several selective high affinity [^{11}C]labeled ligands to examine neuroreceptor distributions and ligand binding functions in the living human brain using positron emission tomography (PET). These studies have supplied quantitative data for neuroreceptor densities and affinities in several brain regions for dopamine receptor subtypes and benzodiazepine receptors. These methods have been used to compare receptor characteristics in patients with neuropsychiatric disorders as Huntington's chorea and schizophrenia. They have also been used to visualize and quantify the effect of clinical drug treatment on the neuroreceptors (receptor occupancy). So far, the display of ligand distribution has been limited to a series of 2D-planes showing the receptor populations in the brain. The development of high resolution PET technology (HRPET) has significantly increased the number of planes, presently about fifty. New approaches are called for when extracting all the important information from such a set of 2D-images. The rapid development of fast processors, high speed large capacity memories and the declining cost of data storage has made it practical to use 3D-graphical technics to display the results. This computer technology and data from HRPET scan experiments with three high affinity neuroreceptor ligands: [^{11}C]FLB457 (dopamine D_2), [^{11}C]NNCO112 (dopamine D_1) and [^{11}C]flumazenil (BZ) have now been used to obtain spatial displays of the radioligand binding structures in the brain of healthy volunteers. It is obvious from such displays that receptor populations can be correctly visualized with regard to volume of distribution and anatomical localization. Structures with high receptor content are observed as objects that can be rotated and examined from different angles within a brief period of time giving a strong visual perception of their 3D extension. Discrimination levels based on certain intensities can be used to define volumes. This methodology can be efficiently used to make quantitative comparisons between neuropsychiatric patient groups and in relation to drug treatment.

C

HISTOCHEMICAL TRACING OF IMPLANTED NEURONS IN THE HIPPOCAMPUS (HOECHST 33342 AND GOLGI IMPREGNATION)

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Dorsal blade granule cells of the dentate gyrus were eliminated by means of fluid injection into the infragranular cleavage plane of adult rats. Neuronal suspension, made of the dorsal hippocampi of 17-days-old embryos, was used both to produce the cleavage and to replace the lost cells. One group of animals received a suspension with nerve cells stained with the fluorescent dye bisbenzimide (Hoechst 33342). Survival and organization of the grafted cells was investigated 30-60 days later. In Nissl stained sections an extensive accumulation of neuronal tissue was found in the location of the grafts. In well positioned grafts a new, irregular granule cell layer reappeared. Using an epi-fluorescent illumination we could confirm, that only the cells within the graft were labelled. In Golgi impregnated material various forms of neurons with very variable dendritic tree were observed. Some of the neurons in the newly formed granule cell layer resembled to the usual forms of granule cells. It is possible to conclude that the cells found in the grafted area came from the donor tissue. They could partly restore the morphological picture of the dentate gyrus and they could probably reestablish some of the intrahippocampal connections.

D

PHOSPHOLIPASES AND SIGNAL TRANSDUCTION, AN INTRODUCTION

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G protein-activated phospholipases are present in many neuronal and non-neuronal cells. They take part in cell signaling by neurotransmitters, hormones and factors controlling growth, immune response and inflammation. Membrane phospholipids, such as phosphatidylcholine (PC), phosphoinositides (PIs) and sphingomyeline (SM) are substrates of the phospholipases (PLs) PLA_2, PI-PLC, PC-PLC, PLD and sphingomyelinase. Some of the produced second messengers are common to different PL-mediated pathways..

Substrate	PL	Product/second messenger
PC	PLA_2	Arachidonate → prostaglandins & leukotrienes Lyso-phospholipid → platelet activating factor
	.PLD	Phosphatidate → diglyceride Choline
	PC-PLC	Diglyceride → phosphatidate
PI	PI-PLC	Inositol phosphates Diglyceride → phosphatidate Diglyceride → arachidonate
SM	SMase	Ceramide → sphingosine (phosphoryl) choline

Receptor agonists may recruit, in the same cell, multiple PL-mediated pathways which synergize to finally establish a certain cellular response. Also, crosstalk among these pathways at the level of receptors, G proteins and protein kinases contributes to the complexity of the PL-mediated signal transduction. - Specific inhibitors of the PLs which are still lacking, and the elucidation of receptor subtypes and isoforms of G proteins and protein kinases would help to assign the relative importance of a certain PL for a particular cell response.

A

PHOSPHATIDYLCHOLINE-DERIVED PUTATIVE SECOND MESSENGERS IN MITOGENIC SIGNALING.

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Phosphatidic acid (PA) is rapidly produced in agonist-stimulated cells through activation of phospholipase D (PLD) and/or the concerted action of phospholipase C (PLC) and diacylglycerol (DG) kinase, but its physiological function is unknown. We have examined the effects of PA on distinct isoforms of protein kinase C (PKC) using a cell-free assay system. Addition of PA to cytosols from COS cells overexpressing PKC-α, -ϵ or -ζ causes activation of all three isotypes, as evidenced by PKC autophosphorylation, and prominent phosphorylation of multiple endogenous substrates. The diacylglycerol-insensitive ζ-isotype of PKC appears to be a preferential target for both PA and bisPA, a newly identified product of activated phospholipase D (EMBO J. 1993; 12: 2655-2662), with each lipid inducing its own characteristic profile of protein phosphorylation. PA activation of PKC-ζ is unique in that it causes an upward shift in electrophoretic mobility of PKC-ζ, which is not observed with PKC-α or -ϵ, or with other acidic lipids. We therefore suggest that PA is a candidate physiological activator of PKC-ζ. We have also investigated whether a phosphatidylcholine (PC)-specific PLC or PLD may be involved in PDGF-induced mitogenic signaling, which is known to proceed via Ras, Raf-1 and MAP kinase. We found that, in Rat-1 fibroblasts, PDGF stimulates a PC-PLC and PLD. Treatment of these cells with exogenous PC-PLC or PLD activates MAP kinase, independent of phorbol ester-sensitive PKCs. The PDGF-induced MAP kinase activation can be mimicked by the exogenous PLC, but not exogenous PLD, in that it is fully inhibited by Ro 31-8220, a highly selective PKC inhibitor, as well as by elevation of intracellular cAMP. Treatment with exogenous PC-PLC results in Raf-1 autophosphorylation but not in GTP loading of Ras. These results support the hypothesis that a PC-PLC is acting between Ras and Raf-1 in PDGF mitogenic signaling.

B

The regulation of G protein-coupled phospholipases C in membranes and monolayers

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Many cellular stimuli evoke the production of the polyphosphoinositide-derived second messengers, inositol 1,4,5-trisphosphate and 1,2 *sn*-diacylglycerol by activating intracellular phospholipases C. Three major subfamilies of phospho-lipase C have been described, termed β, γ and δ whose regulation have been the subject of much investigation. It is now well-established that activation of the β-isoforms of phospholipase C is mediated by G proteins of the G_q family, and phospholipase C-γ is regulated by alterations in its phosphotyrosine content. However, the interaction of the phospholipases with their lipid substrates and the regulatory influences exerted by substrates are only poorly understood. We have investigated the catalytic activity of the β-isoform of phospholipase C of turkey erythrocytes using both *in situ* and *in vitro* assays to study the effects of various characteristics of lipid interfaces on enzyme activity. The two dimensions of catalysis, penetration of the lipid interface and hydrolysis of substrate, were studied using both vesicular and monolayer assays, and results show that although both phosphatidylinositol 4-phosphate (PtdInsP) and phosphatidylinositol 4,5-bisphosphate (PtdInsP$_2$) are effective substrates in *in vitro* assays, turkey erythrocyte phospholipase C binds with faster kinetics and greater affinity to PtdInsP$_2$ than PtdInsP. In addition, enzyme catalysis of both lipids was greatly dependent on surface pressure and at pressures equivalent to that in natural membranes, PtdInsP$_2$ is favoured as a substrate over PtdInsP. Furthermore, stimulation of turkey erythrocytes with receptor agonists and guanine nucleotides shows that PtdInsP is hydrolysed at approximately 15% of the rate of PtdInsP$_2$. These data argue therefore that several characteristics of lipid interfaces regulate phospholipase catalytic activity, including lipid penetration, catalytic rate and substrate specificity. The discussion of the data will be extended to show that the regulation of lipase activity by substrate-containing lipid interfaces is not specific for the phospholipases C and can be extended to other enzymes.

C

THE REGULATION OF PHOSPHOLIPASE D BY ARF's.

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Phospholipase D (PLD) hydrolyses phosphatidylcholine to produce choline and phosphatidic acid, a phospholipid that may have important intracellular functions. PLD activity appears to be very widely distributed in eukaryotes and is thought to be regulated by a large number of cell surface receptors by mechanisms dependant on GTP-binding proteins and/or protein kinases. By using streptolysin-O permeabilisation we can control the conditions within HL60 cells and demonstrate that PLD activity is membrane bound, activated by GTP-binding proteins and modulated by calcium. Depletion of cytosolic proteins by extension of the permeabilisation period indicated that at least one component of this system was a soluble protein. This factor could be replaced by cytosols from various mammalian tissues and was later identified as ARF1 and ARF3, two members of the ubiquitous ARF family of small GTP-binding proteins. Membranes and enriched preparations can be used to examine the properties of this PLD. In conjunction with cellular fractionation techniques this provides some insight into the possible role of PLD as ARF's effector in the described physiological functions of this protein.

D

REGULATION OF PHOSPHOLIPASE D IN THE BRAIN

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Phospholipase D (PLD) is the key enzyme of a signal transduction pathway involving the hydrolysis of phosphatidylcholine yielding free choline and phosphatidic acid (PtdOH); PtdOH can be dephosphorylated to diacylglycerol, the endogenous activator of protein kinase C (PKC). In hippocampal slices from young rats (7d), PLD activity was found to be activated by glutamate (Holler et al., J. Neurochem. 61, 1569-1572, 1993) via metabotropic receptors. The EC_{50} was 0.2 mM, and maximal stimulation was three-fold. We now report that PLD activity has a distinct ontogenetic expression; in the rat, basal activity increases steadily during the first six weeks of life whereas the stimulation of PLD by glutamate decreases with age and is no longer significant in slices from adult animals. - In slices from both young and adult rats, an increase of PLD activity was observed in the presence of 0.1 - 1 μM of 4ß-phorbol-12ß,13 - dibutyrate (PDB), an activator of PKC. In young rats, the stimulation of PLD by glutamate and by PDB was found to be additive. Moreover, the presence of the PKC antagonist H-7 (50 μM) did not influence the glutamate-mediated PLD stimulation. We conclude that PLD is involved in a glutamatergic signal transduction pathway which is regulated independently of phosphoinositide hydrolysis.

A

NOVEL FUNCTION OF PHOSPHATIDYLINOSITOL-4,5-BISPHOSPHATE AS A COFACTOR FOR PHOSPHOLIPASE D

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Phosphatidylinositol-4,5-bisphosphate (PIP_2) is a quantitatively minor, highly acidic phospholipid that serves as a substrate for phosphoinositide-phospholipase C and phosphoinositide 3-kinase. The activation of these enzymes by cell surface receptor stimulation is often associated with activation of another signal transduction pathway, involving phospholipase D (PLD). We have previously suggested that acidic phospholipids may act as essential cofactors for PLD activity. We now report that PIP_2 is required for PLD activity in brain membranes and in permeabilized cells. Furthermore, evidence is provided that PIP_2 is a potent and specific activator of partially purified PLD activity in vitro. Neomycin, a high affinity ligand of PIP_2, inhibits PLD activity in brain membranes. Detergent-solubilized membranes exhibit reduced PLD activity which is insensitive to neomycin. Addition of PIP_2 to solubilized or partially purified enzyme greatly potentiates PLD activity and restores its sensitivity to neomycin. PIP_2 stimulates ca. 10-fold the maximal velocity of PLD with an EC_{50} of 0.5 mol%. Other acidic phospholipids are completely or nearly ineffective, indicating that the action of PIP_2 on PLD is specific. Additional studies in permeabilized U937 cells show that GTPγS-induced activation of PLD is stimulated by MgATP and inhibited by monoclonal antibodies to phosphatidylinositol 4-kinase, indicating that PIP_2 biosynthesis is required for optimal PLD activity. These results define a novel function of PIP_2 as a cofactor for membrane PLD and suggest that PIP_2 hydrolysis and re-synthesis could be important determinants in regulating signaling via PLD.

B

NEUROTRANSMITTER RECEPTOR-SPECIFIC PROFILES IN RAT BRAIN CORTEX: RELATION TO THE MODE OF RECEPTOR ACTIVATION OF PHOSPHOINOSITIDE PHOSPHOLIPASE C.

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Phosphoinositide breakdown as stimulated by six different neurotransmitter receptor agonists (carbachol, serotonin, noradrenaline, *trans*-ACPD, endothelin-1, and histamine) has been studied in rat brain cortical slices. We have monitored the accumulation of total ^{3}H-inositol phosphates (InsPs) and [^{3}H]CDP-diacylglycerol (CDP-DAG) in [^{3}H]inositol or [^{3}H]cytidine-prelabeled tissue, respectively, as well as the profile of the major InsPs, separated by HPLC, and quantified as the index log [(Ins4P+Ins1,4P_2)/Ins1P]. The efficacy of the six agonists to stimulate the accumulation of CDP-DAG, relative to that of InsPs, was not constant, revealing varying degrees of defective recycling of diacylglycerol to CDP-DAG. The value of the index for the profile of InsPs was not constant either, but characteristic of each agonist. Both parameters (ratio of efficacies CDP-DAG/InsPs and InsPs profile) were not independent, and defined two groups of agonists: (a) carbachol and serotonin, with balanced CDP-DAG and InsPs responses, and Ins1P prevailing against Ins4P+Ins1,4P_2, and (b) noradrenaline, *trans*-ACPD, endothelin-1, and histamine, with weak CDP-DAG responses, and high accumulation of Ins4P+Ins1,4P_2 over that of Ins1P. In a membrane preparation from brain cortex, only agonists of group (a) stimulated phospholipase C in the presence of guanosine 5'-O-(3-thiotriphosphate) and in a receptor antagonist-sensitive fashion, indicating that brain cortical α_1-, H_1-, endothelin-, and glutamate metabotropic-receptors stimulate phospholipase C indirectly. These results show that both the efficacy of the CDP-DAG response and the profile of InsPs are characteristics inherent to the proper mode of receptor activation of phospholipase C, direct (via receptor-G protein-phospholipase C interaction), or indirect, probably secondary to calcium entry.

C

THE ADRENAL MEDULLA RESPONSE TO STRESS

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The adrenal medulla (AM) is one of the major components in the response cascade to stress, The human (AM)contains both adrenaline (A) and noradrenaline (NA) cells in a ratio of approx. 75:25 respectively. The cholinergic stimulus invoked following stress, activates the (AM) to secrete vesicle contents, containing bioactive peptides and catecholamines (CA), and affects peripheral tissues. The response of (AM) cells to stress, is preferential secretion of (NA)after nicotinic stimulation, while muscarinic activation causes predominant secretion of (A). The amino acid neurotransmitter glycine can also cause secretion from the (AM) which is blocked by strychnine. Basal secretion is primarily of (A) so that it can "fine-tune" metabolic homeostasis by control of blood glucose levels. The (AM) also contains very high levels of enkephalins and other bioactive peptides. Cholinergic agonist treatment of the rat induces a 100-fold increase in pre-pro-enkephalin (ppEnk) mRNA levels in the rat (AM). This involves new RNA synthesis from E3 and E4 start sites, via ppEnk regulatory DNA elements, indicating a stress mediated regulation of peptide gene expression in the adrenal medulla.

D

TRANSCRIPTIONAL MECHANISMS IN REGULATION OF CATECHOLAMINE BIOSYNTHETIC ENZYMES AND NEUROPEPTIDE Y GENE EXPRESSION BY IMMOBILIZATION STRESS

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Immobilization stress was found to transcritionally activate expression of adrenomedullary catecholamine biosynthetic enzymes, tyrosine hydroxylase (TH), dopamine ß-hydroxylase (DBH), phenylethanolamine N-methyltransferase (PNMT) and neuropeptide Y, with different kinetics. To characterize the molecular events involved in this activation, changes in immediate early genes were examined at various times of a single and repeated stress were determined by Western blots. Electrophoretic gel shift assays characterized binding to regulatory regions of the TH and DBH promoters.

As little as 10 min immobilization increased phosphorylation of CREB which remained elevated for at least one hr. c-fos protein was induced maximally by about 30 min of stress. With repeated stress, members of the jun family were also elevated.

Increased binding of c-fos and c-jun to the AP-1 region of the TH gene was observed after a single immobilization and was not further elevated by repeated stress. Repeated immobilization increased binding of a complex, one partner of which is c-fos or fos related proteins to a regulatory region of the DBH promoter

The results indicate that several transcriptional regulatory pathways are activated by different extents of immobilization stress.

A

NEURONAL AND HORMONAL REGULATION OF GENE EXPRESSION OF CATECHOLAMINE BIOSYNTHETIC ENZYMES IN STRESS.

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Tyrosine hydroxylase (TH) and phenylethanolamine N-methyl transferase (PNMT) are enzymes involved in the biosynthesis of catecholamines (CA). Immobilization (IMO) stress has been shown to elevate TH and PNMT activities as well as mRNA levels in rat adrenals. The aim of this study was to examine the mechanism of IMO-induced changes in mRNA levels. The innervation of the adrenal medulla is cholinergic and is mediated predominantly via the splanchnic nerve. The IMO-induced rise in TH and PNMT mRNA levels was not reduced by antagonists of cholinergic receptors. Denervation of the adrenals by splanchnicotomy did also not block the IMO-induced rise in TH and PNMT mRNA found in innervated adrenals. Hypophysectomy did not significantly affect TH mRNA levels but completely abolished the IMO-induced rise in PNMT mRNA levels. Administration of ACTH (4 IU., sc., 7 days) to hypophysectomized rats fully restored the stress-induced increase in PNMT mRNA levels. Nitric oxide (NO), a newly discovered second messenger, might affect TH and PNMT gene expression. Inhibition of NO synthesis by L-nitro-arginine (40 mg/kg, sc.) reduced the IMO-induced rise in TH mRNA but did not significantly affect PNMT mRNA levels. These data suggest that during a single immobilization stress, neuronal and pituitary factors do not play a significant role in regulation of TH gene expression in the rat adrenal medulla. Nitric oxide appears to be involved in this regulation. Immobilization-induced PNMT gene expression depends primarily on intact pituitary-adrenocortical axis and splanchnic innervation or nitric oxide do not seem to play any significant role.

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B

TRANS-SYNAPTIC MODULATION OF ADRENAL TH AND PNMT GENE EXPRESSION DURING COLD STRESS.

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It is well established that cold stress induces catecholamine (CA) synthesis and release from adrenomedullary chromaffin cells. Our previous findings have demonstrated that cold exposure also results in a robust induction of adrenal TH mRNA, immunoreactivity and enzyme activity. Modest elevations in PNMT mRNA and protein levels also occur. Regulation of TH expression during cold stress is complex and may involve both transcriptional and post-translational control mechanisms. At the transcriptional level, cold exposure causes rapid and selective alterations in the binding of adrenomedullary nuclear proteins to the promoter region of the TH gene as judged by gel mobility shift assay. The most striking alteration occurs in a region which contains an AP1 binding site. Pretreatment of adrenomedullary nuclear extracts with antibodies to either c-Fos or c-Jun completely inhibits this cold-induced alteration in DNA/nucleoprotein binding. Results of adrenal denervation experiments demonstrate that cold-induced alterations in TH mRNA levels and nucleoprotein binding are neurally mediated, requiring intact sympathetic innervation of the gland. In contrast, splanchnectomy only partially inhibits the PNMT adaptive response. Surprisingly, the trans-synaptic modulation of TH and PNMT expression could not be attenuated by peripheral administration of chlorisondamine, although this drug completely inhibited Ach-mediated sympathetic responses. Similar results were obtained with hexamethonium and atropine. Additionally, peripheral administration of the cholinergic agonists, carbachol or nicotine failed to elevate adrenal TH mRNA levels. Taken together, these findings indicate that: (1) adrenal TH and PNMT expression are mediated differentially during cold stress, (2) cold-induced increases in adrenomedullary TH and PNMT expression are mediated, at least in part, through the interaction of a phorbol ester-responsive element and proto-oncogene transcription factors, and (3) trans-synaptic modulation of TH expression involves a non-cholinergic mechanism and is not coupled to Ach-mediated CA release.

C

THE EFFECT OF THE ADRENO-CORTICAL AXIS ON THE CLINICAL OUTCOME. BRAIN EDEMA AND PROSTAGLANDINS FOLLOWING CLOSED HEAD INJURY IN RATS.

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Glucocorticoids (GC) were reported to enhance neuronal damage and to potentiate behavioral deficits after brain ischemia or trauma. We investigated the effect of adrenalectomy (ADEX) or corticosteroid receptor antagonists on motor functions, brain edema and prostaglandin production after closed head injury (CHI). The following groups were studied: 1. control 2. ADEX (6 d) 3. rats treated for 5 d with GC antagonist (RU-38486) or mineralocorticoid (MR) antagonist (RU-28318). Rats were subjected under ether anesthesia to CHI (by a weight-drop device) or sham. 24 h later, the clinical status was assessed and brain edema and PGE_2 content were measured. CHI caused motor deficits, a 2.5-fold increase in serum corticosterone levels at 2-8 h; at 24 h significant elevations in water content and in PGE_2 levels (6-fold) in the cortex of the contused hemisphere were found. In ADEX rats, the CHI-induced impairment of motor function and the extent of edema were markedly reduced ($p=0.008$ and $p=0.026$, respectively). The increase in PGE_2 was abolished. In rats treated with GC or MR antagonists, the effects of CHI were similar to control. These results suggest that: 1. Removal of GC by ADEX, have a beneficial effect on the outcome of CHI. 2. This effect may be mediated by PGE_2. 3. The lack of effect following blockage of the GC or MR receptors may indicate that other factors associated with ADEX (e.g. ACTH hypersecretion) may be involved in the protective effect of ADEX following CHI.

D

Determinants of the Catalytic Machinery and of Allosteric Regulation of Cholinesterases.

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Development of efficient expression systems[1,2] of recombinant human acetylcholinesterase (HuAChE) were utilized to generate over 60 site directed mutants of HuAChE. A combination of immunological and binding assays, molecular modelling and kinetic studies of these mutants with various substrates and inhibitors revealed that: a. Ser203, His447 and Glu334 constitute the catalytic triad[3] as predicted by the 3D-structure of *Torpedo californica* AChE. b. Trp86 is the "anionic subsite" involved in π-cation interactions with the quaternary ammonium of ACh[4,5]. c. Phe295 and Phe297 constitute the acyl pocket and determine specificity towards alkyl moiety of different substrates and organophosphorus inhibitors. d. Glu202 and Glu450 at the bottom of the "gorge" modulates the catalytic activity towards charged and noncharged substrates and reactivity towards organophosphorus inhibitors[4,6]. e. The aromatic amino acids Tyr72, Tyr124, Trp286, and Tyr341 located close to the rim of the active site gorge together with Asp74 constitute degenerate peripheral anionic sites which allosterically modulate the catalytic activity via a cross talk with residues (Trp86 and Tyr337) at the active center of HuAChE[3-5,7].

[1]Velan *et. al.* 1991. J.Biol. Chem **266**, 23977. [2]Kronman *et. al.* 1992. Gene **121**, 295. [3]Shafferman *et al.* 1992. J.Biol.Chem **267**, 17640. [4]Shafferman *et. al.* 1992. EMBO J., **11**, 3561; [5]Ordentlich *et al* 1993 J.Biol.Chem **268**, 17083. [6]Ordentlich *et al*..1993. FEBS Lett. **334**, 215. [7]Barak *et al*. 1994 J.Biol. Chem **269**, 6296.

A

Transgenic Overexpression of Human Acetylcholinesterase in Mouse Brain

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To experimentally modulate cholinergic neurotransmission in the mammalian brain, we established stably transgenic mice overexpressing human acetylcholinesterase (AChE) in their brain. Directed by the human ACHE promoter, the transgene was found by RT-PCR to faithfully yield hACHEmRNA transcripts. These further produced correctly assembled monomers, dimers and tetramers, presenting sucrose gradient sedimentation profiles identical to those of the mouse brain enzyme. The transgenic human enzyme displayed with selective monoclonal antibodies, identical immunochemical interactions to those of the corresponding protein from human brain. In homogenates from basal brain nuclei, it caused a 2-fold increase in total AChE activity. Cytochemical staining by the Karnovsky technique revealed intense accumulation, high above control levels, of its reaction products in all of the brain regions normally expressing this enzyme. Moreover, in situ hybridization with the hACHEcRNA probe and subsequent immuno-decoration with alkaline phosphatase demonstrated normal, intensified patterns of ACHEmRNA transcripts in brain sections from the transgenic mice as compared with controls. Gross development, general features and behavior of these mice were apparently normal, suggesting that the excess human AChE activities produced by AChE expressing cells in these transgenic mice were tolerated through adjustment mechanism(s) maintaining normal cholinergic neurotransmission.

B

LOCALIZATION OF ACETYLCHOLINESTERASE AND ITS mRNA DURING EARLY DEVELOPMENT AND INNERVATION OF HUMAN MUSCLE COCULTURED WITH EMBRYONIC RAT SPINAL CORD.

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In situ hybridization and modified thiocholine procedure were used respectively for the localization of acetylcholinesterase mRNA (AChE mRNA) and the active enzyme during differentiation and synaptogenesis of the human muscle cocultured with embryonic rat spinal cord. AChE as well as its message were detected already at the mononuclear stage; they both increased during fusion. After the onset of nerve-induced contractions AChE mRNA became restricted to only few nuclei located in the synaptic region of the innervated muscle fibres. In the non-innervated myotubes it remained localized along the entire length. AChE patches, never observed in the noninnervated control cultures appeared at the nerve contacts at this stage. Throughout the differentiation process, except during fusion, a substantial differences in AChE mRNA expression could be observed among the individual myonuclei, which supports the hypothesis (Jasmin et al., Neuron 11; 467, 1993) that AChE transcription proceeds intermittently, rather than constitutively. Our results suggest that intermittent expression of AChE mRNA proceeds asynchronously at the level of individual nuclei. During fusion it seems to become generally upregulated as high message was observed around apparently all nuclei in the newly formed myotubes. At the onset of contractions it became downregulated so that all extrasynaptic nuclei became apparently silent. It seems that some factor(s), probably nerve-derived, prevent(s) contraction-mediated downregulation of AChE mRNA expression in the synaptic region.

C

Signal Transduction Therapy: A Novel Approach to Disease Management

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In the past 15 years it has become apparent that many proliferative diseases such as cancers, leukemias, psoriasis and atherosclerosis as well as inflammatory conditions such as rheumatoid arthritis and sepsis are diseases of cellular signaling.
Proto-oncoproteins and oncoproteins have been identified as the signaling molecules driving cells to proliferate. Their abnormally high activity, which results from a mutation or overexpression, signal the cell to divide faster then normal cells. The disruption of normal signaling leads to further mutations such as the loss of tumor suppressers as well as other molecular changes leading to aggressive tumors. Many of the oncoproteins and the proto-oncoproteins are receptor protein tyrosine kinases (RTKs) and cellular protein tyrosine kinases(CTKs). The enhanced tyrosine kinase activities is the hallmark of all cancers and of many other proliferative diseases. Therefore, these signaling molecules present themselves as targets for drugs. We have developed selective tyrosine kinase blockers which we named "Tyrphostins": EGF receptor kinase selective tyrphostins(e.g. AG213,AG 555,AG974,AG1478) block effectively EGF driven tumor growth as well as TGFα dependent growth of psoriatic keratinocytes. PDGF receptor directed tyrphostins like AG 1295 reverse the transformed phenotype of *sis* transformed cells and block the growth of glioblastomas, which are PDGF driven tumors, in nude mice. Similarly other families of tyrphostins were shown to induce apoptosis in K562 cells derived from **CML** patients, inhibit **Her-2/neu** overexpressor cells derived from breast and ovary tumors and eliminate recurrent human B-cell leukemia and therefore save the lives of SCID mice implanted with the leukemic cells. These results as well as the success of **Ras** blockers ("**Rastatins**") and of the **antiestrogen ICI 172,280** demonstrate the validity of the **signal transduction therapy** approach. Another aspect of this approach is the demonstration that certain tyrphostins are extremely potent in blocking inflammatory responses mediated by CTKs, such as Lipopolysaccharide (LPS, endotoxin) induced lethality in mice. This protective effect of tyrphostins is due to their blockade of the production of TNFα and NO which mediate LPS toxicity. Thus, tyrphostins may be used as drugs to block sepsis and septic shock.

D

MODEL SYSTEMS TO STUDY EXPRESSION, SPLICING AND PROCESSING OF THE ALZHEIMER'S AMYLOID PROTEIN PRECURSOR

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It is now widely accepted that the deposition of βA4 amyloid is a necessary and sufficient cause for the synaptic dysfunction and loss in Alzheimer's disease (AD). The biogenesis of the amyloid protein precursor (APP), βA4 protein and βA4 amyloid has been studied by us both *in vitro* and *in vivo*. Synthetic βA4 protein readily forms β sheets, filaments and amyloid at micromolar concentrations. The principle to inhibit this process has been worked out by our groups with βA4 variants. We also showed that at physiological concentrations (nanomolar) βA4 aggregation requires amino acid oxidation and protein cross-linking by radical generation systems. The addition of radical scavangers such as ascorbic acid, tocopherol derivatives, and free amino acids prevent radical induced βA4 aggregation. From necropsy examinations we estimate that a thirty-year period of βA4 amyloid accumulation follows this initiation before clinical recognition. In cells that exhibit cell interaction, APP exists in eight different isoform generated by alternative splicing of exons 7, 8 and 15. L-APP's, lacking exon 15, are abundant isoforms except in muscle and neurons. Neurons, the primarily affected cells in AD, are the only cells in brain that constitutively express APP. Splicing in neurons generates the four APP isoforms containing exon 15 but not L-APP's. In contrast, only activated microglia and astrocytes highly express all eight APP isoforms. APP metabolism in microglia is regulated by cytokines and molecules of the ECM. Because APP is a synaptic protein, we propose βA4 amyloid formation occurs at synapses and leads to neuronal deafferentation by a mechanism resembling "synaptic stripping" mediated by microglia.

A

MOLECULAR DISSECTION OF THE NEUROFIBRILLARY LESIONS OF ALZHEIMER'S DISEASE

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The paired helical filament (PHF) is the major fibrous component of the neurofibrillary lesions (neurofibrillary tangles, neuropil threads and senile plaque neurites) of Alzheimer's disease. It consists predominantly and probably entirely of microtubule-associated protein tau. PHF-tau contains all six adult human brain tau isoforms in proportion to their natural abundance, but in a hyperphosphorylated state. Hyperphosphorylation is believed to lead to the self-association of tau through its microtubule-binding domain, resulting in PHF formation. Phosphorylation-dependent anti-tau antibodies were used to identify some of the phosphorylated sites in PHF-tau; the sites identified by these antibodies were serine/threonine-prolines. Consequently, recombinant tau could be phosphorylated at some or all of these sites by mitogen-activated protein (MAP) kinase, glycogen synthase kinase-3 (GSK3) or cyclin-dependent kinase 5 (cdk5). The trimeric form of protein phosphatase 2A was the major brain phosphatase activity towards recombinant tau phosphorylated by MAP kinase or cdk5.

The identification of candidate protein kinases and protein phosphatases for the phosphorylation state of tau in developing and adult brain and in Alzheimer's disease brain represents a first step towards a true understanding of the molecular mechanisms that lead to PHF formation. This knowledge can now be used in attempts to produce an animal model for the neurofibrillay lesions of Alzheimer's disease.

B

THE BETA-A4 AMYLOID PROTEIN AND ITS PRECURSOR IN THE CAUSATION OF ALZHEIMER'S DISEASE

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The amyloidogenic processing of the βA4 amyloid precursor protein (APP) may be the final common pathway for the patho-genesis of Alzheimer's disease (AD). The factors which alter the balance between non-amyloidogenic (α-secretase) and amyloidogenic (β-, γ-secretases) processing of APP are not yet defined. Equally important may be conditions which favour the polymerisation of βA4 itself.
We are approaching the molecular basis of AD directly through two strategies: first, the study of circulating levels of APP in plasma as a reflection of the general metabolism of APP in the body; second, the quantitative assessment of APP in the post mortem human brain. Circulating plasma levels of APP, derived in large part from platelets, show a relative increase in the 130 kD band in AD. This species of APP contains immunoreactivity to the Kunitz protease inhibitor (KPI) domain. Factors which affect circulating levels include metal ions (oral administration of zinc sulfate, 100mg/day for three days causes a sharp rise in plasma APP at 48 hours, with a return to base-line by 7 days) and glucose load (50gms of oral glucose causes a decline of plasma APP within 2 hours). The metabolic basis of these changes in plasma APP are not yet identified.
APP has been purified from post mortem AD brains (n=10), normal controls (n=7) and other neurologic disease (n=4). The total yield of APP from AD brain tissue is decreased. Three separate assay techniques (Western blot; trypsin inhibition; tryptic digestion of APP) show a selective increase in the KPI-containing APP species in the soluble fractions of the AD brain. These data are consistent with a number of other studies at the mRNA level showing an increase of APP-KPI in AD. It is not yet certain that the origin of these APP-KPI species is neuronal rather than astrocytic.
We conclude that increased plasma and brain levels of KPI-containing APP species may reflect a fundamental disturbance in the βA4 amyloidogenic pathway in AD.

C

MODEL STUDIES OF IMMUNE MEDIATED NEURODEGENERATION IN ALZHEIMER'S DISEASE

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Different neurons contain distinctly phosphorylated isoforms of the heavy neurofilament protein NF-H. Alzheimer's disease (AD) and Down's syndrome are associated with serum Abs directed against phosphorylated epitopes highly enriched in NF-H of cholinergic neurons. Production of such Abs in the rat results in memory impairments and in derangements of the spatio-temporal organization of their behavior. This animal model is termed experimental autoimmune dementia (EAD). Immunohistochemical studies revealed that IgG accumulate in the septum, hippocampus and in the entorhinal cortex of the EAD rats. This is accompanied by a marked reduction in the density of septal cholinergic neurons. Both of these effects, as are the behavioral deficits, are specific to EAD rats and were not observed in rats immunized with other NF-H isoforms. An inverse correlation was observed between the level of IgG in the septum of individual EAD rats and the density of their septal cholinergic neurons. The decrease in the density of cholinergic neurons in the septum of EAD rats and the accumulation of IgG in this brain area have a similar time course and are both significant by three to four months following the initiation of immunization with NF-H, whereas the cognitive deficits of the EAD rats evolve more slowly. These animal model studies support a role for specific anti NF-H Abs in neurodegeneration in AD.

D

PEPTIDES IN THE SUPRACHIASMATIC NUCLEUS: MESSENGERS OF THE CLOCK

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The suprachiasmatic nucleus (SCN) located in the basal part of the hypothalamus imposes its circadian rhythm upon the rest of the brain by its projection to hypothalamic and thalamic target structures. We investigated the participation of several peptides in the transfer of information leading to the release of corticosterone. It appeared that vasopressin (VP) and not other peptides of the SCN are able to suppress the release of corticosterone from the adrenal. In an attempt to establish the morphological basis of this phenomenon we also investigated the anatomical connections between the SCN and hypothalamic targets. In addition, the colocalization with the inhibiting amino acid GABA was investigated. It appeared that there is no direct projection between the SCN and corticotrophin-releasing hormone neurons. Instead there are indirect connections (most probably via neurons in the dorsomedial nucleus of the hypothalamus). Furthermore, the presence of GABA was demonstrated in the intra- and extranuclear projections of the SCN. Colocalization of GABA was demonstrated with vasopressin, somatostatin and vasoactive intestine polypeptide. At most 30% of these peptidergic terminals exhibited GABA colocalization. It is concluded that the SCN conveys its circadian message by an intricate network of fibers and a delicate balance between peptidergic and amino acid neurotransmitters.

A

CAN FOS EXPRESSION TELL US ABOUT SIGNAL TRANSDUCTION IN THE SUPRACHIASMATIC NUCLEUS (SCN)?

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Expression of Fos in the rodent SCN depends on light stimuli via the retina (1). During the dark phase, exposure to a light pulse causes Fos induction. Stimuli initiated by light pulses are conveyed, via glutamatergic retinal ganglion cells, to post-synaptic elements within the SCN. Nuclear Fos-immunoreactivity (Fos-ir) denotes activated cells. Fos-ir in the SCN has been demonstrated at light- and electron-microscopic levels. Our data and that of others (2), indicates that various populations of SCN cells may respond to Fos induction differently. After brief light exposure (±15 mins), there is Fos-ir in the ventral SCN; some of the neurons involved contain vasoactive intestinal peptide (VIP) and/or gastrin-releasing-peptide (GRP). Light exposure of ±30 mins leads to Fos-ir also in the dorsal SCN, within vasopressinergic neurons. After 1-2 hrs light exposure Fos-ir occurs throughout the SCN, within neurons and within putative glial cells. There are recent precedents implicating glia in the clock mechanism (3,4). One possible working hypothesis is that a light-initiated cascade, commencing within retinal ganglion cells, stimulates VIP- and/or GRP-containing neurons monosynaptically, which in turn innervate vasopressinergic neurons, after which glial cells are recruited. Hence, Fos-induction for increasing periods of time may be a valid approach for delineating signal-transduction pathways within the SCN.

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B

G-PROTEIN MEDIATED MODULATION BY MELATONIN OF CONSTITUTIVE SECRETION

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The effect of melatonin on secretion of ^{35}S-methionine labeled proteins from cultured melanoma cells was investigated. At physiological concentrations (0.5-10 nM), melatonin inhibited the release early after plating or at low cell density, but facilitated the release later on, or at high cell density. To elucidate the involvement of G-proteins in these responses, the effects of melatonin on protein secretion from the cells were assessed in the absence and presence of guanosine 5'-O-(3-thiotriphospate) (GTP S; which was introduced into the cells during the process of permeabilization and resealing with ATP), pertussis (PTX) and cholera (CTX) toxins. At low cell density, melatonin inhibited the release, but paradoxically enhanced it when GTP hydrolysis was blocked (by GTP S or CTX treatment). At high cell density, melatonin facilitated the release and so did GTP S. CTX treatment prevented the melatonin-mediated facilitation. Similar treatment of the cells with PTX, did not affect the melatonin-mediated inhibition or facilitation. The ability of melatonin to affect binding of GTP ^{35}S to melanoma proteins was examined. Melatonin significantly enhanced GTP ^{35}S binding to the melanoma cells proteins; this effects was more pronounced at the inhibition than at the facilitation phase. Pretreatment of the cells with CTX also increased GTP ^{32}S binding; the binding was further enhanced in the presence of melatonin. Photoaffinity labeling of GTP binding proteins in the cells in the absence and presence of melatonin indicated that melatonin enhanced the incorporation of the label into a protein with an apparent molecular weight of 45 kDa and decreased incorporation into a 40 kDa protein. These results indicate that the effects of melatonin on protein secretion are mediated by at least two G proteins, one of which belongs to the Gs class. The direction and magnitude of melatonin's effects are dynamic and determined by the prevailing state of activation of the G proteins involved, thus generating a see-saw type response.

C

EXPRESSION OF INDUCIBLE NITRIC OXIDE SYNTHASE IN CEREBELLAR NEURONS BOTH IN VITRO AND IN VIVO.

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Nitric oxide (NO) has been identified as an important neuromodulator, as well as implicated in mediating neurotoxicity. Nitric oxide synthase (NOS) catalyzes the formation of NO from arginine. Two major isoforms of NOS have been described, a constitutive form (cNOS) which generates NO tonically, and an inducible enzyme (iNOS) which mediates primarily toxic effects, and requires de novo gene transcription. In the brain, NOS enzyme is a constitutive one. Since in the CNS NO mediates neurotoxicity and on the other hand, because of the potential role of iNOS in neurotoxicity, the present study was designed to examine whether cerebellar neurons can be stimulated by cytokine and endotoxin to transcribe the gene encoding iNOS. Cerebellar granule cells were prepared from postnatal day 8 rat pups and RNA was purified from cultures after 10 days in vitro. Transcription of the gene encoding iNOS was examined by RNA-specific reverse transcription followed by polymerase chain reaction (RS-PCR) and by fluorescent in situ hybridization, using specific oligonucleotide primers to the macrophage inducible NOS gene. We show that a primary culture of cerebellar granule cells expresses iNOS following the treatment of the neuronal culture with interferon-gamma (IFN-γ) and lipopolysaccharide (LPS) for 2-6 hours. The iNOS transcripts were not detected in untreated cultures. Furthermore, immunohistochemical analyses confirmed the identity of the neuronal culture and the expression of the cNOS protein. In order to detrmine whether neurons in the whole animal also have the capacity to be triggered to transcribe iNOS after induction, we injected IFN-γ and LPS to adult rat cerebellum. Fluorescent in situ hybridization revealed that cerebellar neurons in vivo can be triggered to transcribe the gene encoding iNOS after induction with LPS and IFN-γ. Results obtained by in situ hybridization and immunohistochemical analysis show that the same cerebellar neurons that express cNOS start to transcribe also iNOS, both in vitro and in vivo. These results raise the possibility that iNOS plays a role in neuronal damage following endogenous activation of cytokines in various brain insults.

D

INDUCTION OF NO-SYNTHASE TRANSCRIPTION IN CULTURED ASTROCYTES AND MICROGLIA

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The potential role of nitric oxide and the regulation of NO-synthesizing enzymes (NOS) in brain has become a major focus of research efforts recently. Data about NOS gene expression, however, are sparse. We have used RT-PCR to amplify a 382 bp piece of iNOS from enriched astrocyte and microglial RNA, have cloned the cDNA into an SP6/T7-containing vector and sequenced the insert. Changes between murine and rat sequences will be shown. A 148 bp restriction fragment was excised from the insert and shortened cRNA transcripts were used as internal standards for quantitation of PCR product. Lipopolysaccharide (LPS) (100 ng/ml) maximally induced iNOS mRNA in astrocyte cultures within 1h. This effect was observed with LPS concentrations as low as 10 ng/ml. Maximal induction in purified microglia was achieved within 30 min. Investigations with interferon-γ, IL-1 and other cytokines are underway and results will be presented. Pretreatment of cultures with 1mM LiCl resulted in an induction of iNOS transcription. More data on effects of lithium on iNOS expression will be shown. Better insights into gene regulation of iNOS in glial cells will further our understanding on long-term effects of NO both in long term potentiation and in chronic diseases of the brain.

A

NITRIC OXIDE IS INVOLVED IN THE REGULATION OF STRIATAL TRANSMITTER RELEASE

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Nitric oxide (NO) is a recently discovered messenger molecule that mediates a wide variety of physiological and pathological effects in the cardiovascular, endocrinological, peripheral nervous and central nervous systems. NO is produced from the terminal guadine group of L-arginine by nitric oxide synthetase (NOS). In the brain NOS occurs in vascular endothelium, nerve fibres of cerebral arteries, central neurons and astrocytes. Current evidence indicates that in central neurons NO is produced in postsynaptic structures in response to activation of exitatory amino acid (EAA) receptors. A major action of NO is to activate guanylate cyclase and thus elevate cGMP level in the target cells.

The striatum receives a massive excitatory glutamatergic input from the cerebral cortex, and EAA receptor have been shown on striatal dopaminergic and cholinergic neurons. Furthermore the NOS activity is high in the striatum. These facts rise the question wheather NO is involved in the modulation of striatal acetylcholine (ACh) and dopamine (DA) release.

The experiment were carried out on isolated superfused striatal slices of rats, loaded with either [^{3}H]-choline or [^{3}H]-dopamine. The superfusate was collected in 3 min samples. During the collection of the 3rd and 12th samples the tissue was stimulated electrically (S_1 and S_2). Drugs were applied 15 min prior the second stimulation and left in the solution throughout the experiments. The effect of drugs was studied on the S_2/S_1 ratio.

We found that NOS antagonist N-nitro-L-arginin methylester (L-NAME) reduced the release of DA, while its enantiomer, N-nitro-D-arginin (D-NAME) was ineffective. In the presence of N-methyl-D-aspartate (NMDA) receptor antagonist MK-801 L-NAME failed to decrease the release of DA. In contrast, L-NAME had no modulatory effect on the release of ACh.

These findings indicate that NO is involved in the effect of NMDA receptor activation on the release of dopamine in the striatum.

B

IRON DELOCALIZATION AND THE RELEVANCE OF $O^{\cdot}_2$ PRODUCTION BY NITRIC OXIDE TO NIGRAL CELL DEATH.

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The etiology of progressive nigral dopamine neuron death in Parkinson's disease (PD) remains elusive. Our previous observations on the chemical pathology of Parkinsonian substantia nigra (SN) support the concept of oxidant stress. These include increases of iron and ferritin and hydrogen peroxide producing monoamine oxidase B activity, decrease of reduced glutathione (GSH) and inhibition of mitochondrial respiratory chain enzyme, Complex I. The dopaminergic neurotoxin 6-hydroxydopamine can bring about similar changes in the animal model of PD. If oxidant stress is an initial trigger for neurodegeneration an endogenous or exogenous produced neurotoxin may initiate it. We have shown that nitric oxide may fulfil this role since it is able to delocalized ferritin bound iron, inhibit Complex I activity, act as signal transducer of superoxide, $O^{\cdot}_2$, production in activated microglia-macrophages and induced membrane lipid peroxidation. The dramatic proliferation of reactive macrophage-microglia in SN of PD and innervation of SN by cortico-striatal glutaminergic neurons suggests close attention needs to be paid to NO produced by these cells in PD.

C

THE EFFECT OF NITRIC OXIDE ON BRAIN ISCHEMIA

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The interaction between nitric oxide (NO) and ischemic brain tissue has been the focus of extensive research, as well as the center of much controversy. In a previous study we demonstrated a detrimental effect of a single high dose of nitroarginine, an irreversible inhibitor of NO-synthase (NOS), on behavior and brain histopathology following a mild forebrain ischemia. The present study was designed to explore the effect of gradual NOS inhibition on the same parameters. Gerbils were treated with N^G-nitro-L-arginine (NARG) 4 hours before they were subjected to a 5 min forebrain ischemia. Two control groups received either ischemia without NARG pretreatment (control ischemia group), or the highest dose of NARG without ischemia (control NARG group). A dose-dependent spontaneous hyperactivity was observed at 24 and 48 hours following ischemia, with a tendency towards recovery during the 6 days' follow-up period. On the 6th post ischemia day the gerbils were sacrificed and their brain removed and processed for histologic and morphometric analysis. Control NARG animals displayed a normal histology. A highly significant increase in the number of damaged cells and decrease in the number of normal cells was observed in the hippocampal CA1 cells of control ischemia group, and a dose dependent aggravation of these morphometric indices was observed across the higher NARG doses: 10, 25 and 50 mg/kg. The lowest, 5 mg/kg NARG dose was not statistically different from control NARG (histologically normal) group, and was better than control ischemia group, and better than each of the other (higher) NARG dose groups. Thus, while moderate NOS inhibition in vivo is deleterious to ischemic brain tissue in a dose-dependent manner, mild NOS inhibition is beneficial to the ischemic tissue. The effect of NO on cerebral blood flow might be a crucial factor underlying these changes, but other putative cellular mechanism(s) cannot be ruled out.

D

MOLECULAR CHARACTERIZATION OF OPIOID RECEPTORS.

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Opioid receptors (mu, delta and kappa) have long been described as membrane receptors of the nervous system which mediate the analgesic effect as well as the addictive properties of opium-derived alkaloid drugs. We (Kieffer et al., 1992, PNAS, **89**, 12048) and others (Evans et al., 1992, Science, **258**, 1952) used an expression cloning approach to achieve the first molecular characterization of a mouse delta opioid receptor. The cloned receptor presents all the structural characteristics of G-protein-coupled receptors. Heterologous expression indicates that it also exhibits typical pharmacological as well as functional properties of a receptor of delta subtype. Structurally related murine receptors have since been cloned by cross-hybridization and identified as mu and kappa receptors. The overall homology between the three receptor subtypes is 65% with alternating highly divergent and almost identical domains. The further molecular characterization of human receptors will contribute to our general understanding of nociceptive and reward pathways and might lead to improved treatment of pain and addiction.

A

CHARACTERIZATION OF NEW OPIOID LIGANDS

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Recently a great interest has been focused on delta opioid receptors following the discovery of the heterogeneity of this receptor type. We have developed a number of ligands in normal and in tritiated form with delta receptor specificity. The new compounds were tested by the use of *in vitro* binding assays in rat brain membranes. Tritiated Deltorphin II was found to be specific for the delta$_2$ sites(1). More recently Ile was incorporated into position 5 and 6. These hydrophobic residues resulted in an increased affinity and selectivity(2), moreover this ligand exhibits high specific radioactivity (49 Ci/mmol). The tritiated form of the tetra-peptide, H-Tyr-Tic Phe-Phe (TIPP) was found to be highly delta specific with antagonistic properties(3). The modified form of this ligand, containing a reduced peptide bond [TIPPΨ] showed a high stability against enzymatic degradation and unlike other delta antagonist, it was free of mu and kappa antagonist properties(4). A non-peptide delta receptor specific antagonist has also been prepared in radiolabelled form. Tritiated naltrindole displays very high affinity towards the delta sites; the binding of it to rat brain membrane was found to be partially wash-resistant(5).

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B

THE ROLE OF THE NITRIC OXIDE PATHWAY IN THE CENTRAL MECHANISMS OF MORPHINE ACTION.

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Inhibition of nitric oxide (NO) synthase by N^G-nitroarginine methyl ester (L-NAME) profoundly potentiated the antinociceptive effect of morphine administered intraventrically or intrathecally to rats. On the other hand, the antinociceptive effect of morphine was potentiated by the NO scavenger hemoglobin, but it was abolished by intrathecal administration SIN-1, which decomposes to NO. The effect of L-NAME and N^G-nitroarginine on the development of morphine tolerance and dependence was further examined in mice. Administration of NO synthase inhibitors diminished in a dose-dependent manner the tolerance to the analgesic effect of morphine. Inhibition of NO synthase also attenuated some signs of morphine dependence. In a consecutive study, the effect of chronic morphine administration on expression of NO synthase was studied in brain structures, by an in situ hybridization. The preliminary study has demonstrated that chronic morphine has little, if any, effect on the levels of NO syntase mRNA.

These results indicate that NO may play an important role in neurochemical mechanisms of the opiate-mediated antinociception, which suggests that NO takes part in the processes triggered by acute activation of opioid receptors, as well as in the development of morphine tolerance and dependence though the neurochemical mechanisms involved remain to be clarified.

C

RECENT PROGRESS IN THE DEVELOPMENT OF μ-SELECTIVE OPIOID ANTAGONISTS

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Cyprodime ((-)-N-Cyclopropylmethyl-4,14-dimethoxymorphinan-6-one) is a pure opioid antagonist with high selectivity for μ receptors (H. Schmidhammer et al., *J. Med. Chem.* 1989, *32*, 418). Since it has the highest selectivity of non-peptide, competitive μ opioid antagonists reported, this ligand is being used as biological and pharmacological tool in opioid research. For instance in bioassays (H. Schmidhammer et al., *J. Med. Chem.* 1990, *33*, 1200) and to characterize cloned and expressed opioid receptors (Y. Chen et al., *Mol. Pharmacol.* 1993, *44*, 8). Cyprodime has also been tritium labelled (F. Ötvös et al., *Helv. Chim. Acta* 1992, *75*, 1718). In an attempt to enhance μ receptor affinity and/or selectivity, a 3-hydroxy group was introduced and additional changes in position 4 (e. g. n-butoxy) and position 14 (e. g. ethoxy, allyloxy) were made. The novel compounds were tested for opioid receptor affinity and selectivity by ligand binding assays in guinea-pig homogenates and by bioassay in the mouse vas deferens preparation. It was found that a 3-hydroxy group was able to increase μ affinity approximately ten-fold. In some derivatives κ affinity was also increased. Small fluctuations in affinity and selectivity were afforded by substitutions in positions 4 and 14. In all cases the compounds had weakest affinity for δ opioid receptors.

D

MOLECULAR BIOLOGICAL STUDIES OF MODULATION OF EXPRESSION OF GENES OF THE ENDOGENOUS OPIOID SYSTEM

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With the recent cloning of the genes encoding all three types of the specific opioid receptors, it is now possible to determine normal levels of mRNA in specific regions of the brain of both the endogenous opioid receptors as well as endogenous opioid ligands using a very sensitive and specific modified technique of solution hybridization protection assay. In on-going studies in our laboratory we have initially mapped and determined the levels of the proenkephalin (ppENK) gene messenger RNA in rat brain regions and then determined the effects of a binge pattern of cocaine administration (Branch et al, Mol. Brain. Res., 14:231-238, 1992). Similarly, we have mapped the distribution and levels of the message for the prodynorphin gene (ppDYN) and determined the effects of "binge" pattern cocaine administration (Spangler et al, Mol. Brain Res. 19:323-327, 1993). Whereas the binge pattern cocaine administration only transiently effects elevations and mRNA of ppENK, this pattern of cocaine administration causes significant and persistent increase in ppDYN in the caudate putamen, one of the rat brain regions abundant in dopaminergic terminals. In related work we have found that this binge pattern of cocaine administration significantly increases the density of both mu (Unterwald et al., Brain Res., 584:314-318, 1992) and also kappa opioid receptors as determined by autoradiography in the nucleus accumbens, caudate putamen, and related regions with abundant dopaminergic terminals. Work is currently in progess to map mu opioid receptor mRNA distribution and levels in rat brain regions and determine the effects of drugs of abuse and treatment agents on gene expression of this receptor. Similar work has also been initiated to study the distribution and levels of mRNA of the kappa opioid receptor gene, and the effects of drugs of abuse and treatment agents on this gene's expression. All of these studies are currently being carried out in the rat model, with initial work performed to conduct similar studies in two other rodent species as well as in human tissue. These studies should give new insights into the effects of both drugs of abuse, cocaine, opiates, and alcohol, and also treatment agents.

A

INHIBITTORS OF ENKEPHALIN DEGRADING ENZYMES : AN ALTERNATIVE APPROACH ON OPIATE DEPENDENCE TREATMENT.

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Central administration of enkephalin catabolism inhibitors decrease the severity of naloxone precipitated morphine withdrawal[1]. RB 101, a new mixed inhibitor of enkephalin catabolism able to cross the blood-brain barrier have been recently synthesized[2]. The antinociceptive responses induced by RB 101 are potentiated by the co-administration of antagonists of the cholecystokinin-B (CCK-B) receptors[3]. In this study, the effects induced on naloxone-precipitated morphine withdrawal syndrome by RB 101, given alone or associated with the selective CCK-B antagonist PD-134,308, have been investigated. Morphine dependence was induced by administration of progressively increased doses of morphine (from 8 to 30 mg/kg, i.p.), twice daily, during five days. Withdrawal syndrome was precipitated by s.c. injection of naloxone (1 mg/kg), and behavioral and vegetative signs of withdrawal were observed during 25 min. The systemic administration of RB 101 (5, 10 and 20 mg/kg, i.v.) elicited a significant decrease in 8 of the 14 withdrawal signs evaluated. PD-134,308 (3 mg/kg, i.p.) did not modify the expression of morphine abstinence when given alone, but induced a strong facilitation on RB 101 responses (12 of 14 withdrawal signs were decreased). This facilitatory effect was particularly intense on peripherally mediated withdrawal signs, such as salivation, lacrimation, rhinorrhea and diarrhea. In a second experiment, the effects induced by RB 101 given alone were investigated on spontaneous morphine withdrawal. In this case, dependence was induced by administring higher doses of morphine (from 8 to 50 mg/kg, i.p.), twice daily, during seven days. Withdrawal syndrome was observed 24 h after the last morphine injection during a period of 30 min. RB 101 administration (40 mg/kg, i.p.) also decreased the presence of spontaneous morphine withdrawal. The signs more affected were teeth chattering, mastication and rearing. These results suggest that RB 101 could represent a new approach in the management of opiate withdrawal syndrome. CCK-B antagonists, as PD-134,308, may be useful in potentiating this anti-withdrawal effect.

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B

GLUTAMATE, Na-NITROPRUSSIDE AND cGMP ENHANCE DOPAMINE UPTAKE; IMPLICATIONS TO DEGENERATION OF DOPAMINERGIC NEURONS

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Rat primary mesencephalon neuronal cultures have been used to investigate, at the cellular and second messenger levels, the interactions between glutamate and dopaminergic neurons. Glutamate enhances ^{3}H-dopamine uptake in a dose and time-dependent way. This effect of the excitatory amino-acid precedes neurotoxicity. Similar enhancement in ^{3}H-dopamine uptake has been observed upon treatment with Na-nitroprusside or dibutyryl-cGMP, suggesting that NO might mediate glutamate effect on dopamine uptake. A human neuronal cell line that incorporates ^{3}H-dopamine is being used to further analyze the interrelationship between glutamate and dopaminergic cell degeneration. Preliminary results indicate that partial inhibition of dopamine uptake with a pcDAT vector expressing the rat dopamine transporter cDNA in the antisense orientation, or with a synthetic antisense thiol-oligonucleotide, partially block neurotoxicity. The implications of these results to the mechanisms underlying dopaminergic neuron degeneration will be discussed.

C

THE ROLE OF C-JUN IN DEVELOPMENT, REGENERATION AND NEURONAL SURVIVAL.

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The protooncogene transcription factor c-jun has characteristically rapid and transient transcriptional activation in many neural and other cell types. However it is now clear that c-jun can be expressed over long periods of time in neurons and glial cells following a variety of treatments including axon damage and during regeneration. This suggested that c-jun expression may facilitate the normal processes of differentiation and repair and perhaps be involved in cell survival. During development c-jun mRNA expression and protein is found in neurons from E11 and is seen up to the adult stage. Quinolinic acid injection into the striatun results in a delayed expression of c-jun (at 24h) in neurons that are destined to die from the excitotoxic insult (Purkiss et al., 1993). These results suggest that c-jun expression under these conditions will not prevent cell death. Following crush or section of the sciatic nerve in rats (or block of axoplasmic transport), there is a long term expression of c-jun in motor neurons which is maintained until the nerve has regenerated (Brown et al., 1994). This relationship to regenerative capacity is also found in central motor pathways where damage to the rubrospinal pathway or nigrostriatal pathway is accompanied by the attempt, but ultimate failure of regeneration. There is a correspondingly transient expression of c-jun and to a minor extent, c-fos (Jenkins et al., 1993). Overexpressing c-jun in neuronal cell lines results in a greater propensity to differentiate and is consistent with the recently reported (Johnson et al., 1993) poor response to growth factors of fibroblasts taken from c-jun null embryos. We suggest that the expression of c-jun facilitates differentiation but is not essential for neuronal survival.

D

INDUCTION OF VIP EXPRESSION IN SENSORY NEURONS

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Adult rat dorsal root ganglion (DRG) neurons exhibit increased expression of both VIP (vasoactive intestinal peptide) and the c-*jun* immediate-early gene following peripheral axotomy in *vivo* or when grown in culture. Since Jun protein can regulate transcription *in vitro* by binding to the VIP cAMP responsive element (CRE), we sought to determine whether it could act as a transcription factor responsible for VIP induction in DRG neurons. We blocked Jun expression in newly cultured neurons by microinjection of an antisense oligonucleotide and 2 days later found significant reductions in the proportion of injected neurons immunostained for Jun (39.4% (292/741) vs. 78.8% (1065/1351) in controls, $p<0.0001$) and VIP (17.3% (194/1123) vs. 30.1% (362/1203) in controls, $p<0.0001$) while staining for substance P and CGRP remained unaffected. The corresponding sense oligonucleotide had no significant effect on either Jun or VIP immunostaining. To test the ability of the rat VIP CRE to regulate transcription, we transfected DRG neurons in culture by microinjection of reporter plasmids containing either,the CRE as a synthetic oligonucleotide sequence driving expression through a c-*fos* promoter or 1.5 kb of 5'-flanking DNA (including the CRE and promoter) from rat VIP. Although the VIP sequences did not direct spontaneous expression, the CRE did mediate enhanced reporter gene expression in newborn rat DRG neurons exposed to 40 mM potassium and 10 µM forskolin. This is consistent with the ability of these stimuli to induce endogenous VIP expression in newborn rat DRG cultures which express little VIP spontaneously and in which neuronal Jun was less abundant (16.2% of neurons) than in adult rat DRG cultures. Thus, we have found that spontaneous VIP induction in cultured neurons is dependent upon the presence of Jun and that the rat VIP CRE is a functional enhancer in DRG neurons. Our results suggest that spontaneous induction of VIP in culture or after axotomy *in vivo* could be regulated by a Jun-containing transcription factor complex binding to the CRE and that transcriptional regulation of the VIP gene through other regulatory sites is probably also involved.

A

SEIZURES- AND APOPTOSIS-RELATED ACTIVATION OF GENE EXPRESSION IN THE RAT BRAIN.

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Kainate (KA) is a potent neuroexcitatory and neurotoxic analog of glutamate. Long term kainate effects are apparently dependent on its influence on gene expression mediated by inducible transcription factors like AP-1 (activator protein 1) made of proteins belonging to Fos and Jun families. In our studies we have analyzed the AP-1 DNA binding activity in nuclear protein extracts collected from rat brain at different times after systemic injection of kainate. Two phases of elevated AP-1 DNA binding activity were observed in the hippocampus and entorhinal cortex, correlating with period of seizures (2-6 hours following KA injection) and neuronal cell death (72 h). At 72 h after kainate treatment the DNA fragmentation, believed to be diagnostic for programmed cell death, was also noted. Contrary to AP-1, no effect of KA on the DNA-binding activities of two other transcription factors: CREB/ATF and Octamer was detected. These data point to selective effect of KA on AP-1 in brain structures known to be particularly vulnerable to KA. We have found that at 2-6 hours after the treatment, AP-1 consisted predominantly of Fos B and Jun B, while at 72 h Jun D comprised major AP-1 component in place of Jun B. Our findings implicate the AP-1 transcription factor and selectively Jun D protein in KA-driven neuronal cell death.

B

PRESYNAPTIC GLUTAMATE RECEPTORS ARE VOLTAGE DEPENDENT.

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In the crayfish neuromuscular junction the excitatory transmitter is glutamate. We show here that at as low a concentration as $5 \cdot 10^{-7}$M glutamate affected the release of neurotransmitter. Furthermore, for the first time, to our knowledge, we report a voltage dependent effect of glutamate on release, presumably indicating the existence of voltage dependent glutamate receptors in nerve terminals.

The deep extensor abdominal muscle and the opener muscle of the crayfish *Procambarus clarkii* were used. Release was monitored by recording single quantum events with a macropatch electrode. The nerve terminals were exposed to $5 \cdot 10^{-7}$M tetrodotoxin and were depolarized to different levels by the macropatch electrode. Glutamate ($5 \cdot 10^{-7}$-10^{-5}M) had a dual effect on release depending on the amplitude of the depolarizing pulse. At low depolarizing pulses it reduced release while at high depolarizing pulses it enhanced release. In preparations not treated with tetrodotoxin, action potential induced release was increased by 10^{-6}M glutamate but was inhibited by 10^{-4}M glutamate.

N-methyl-D-aspartate was also found to affect release in a voltage dependent manner. N-methyl-D-aspartate (10^{-7}-10^{-5}M) reduced release at low depolarizing pulses but it had no effect at higher depolarizations. Thus NMDA mimicked only one of the effects of glutamate. DL-2-amino-5 phosphor-valerianic acid blocked the effect of NMDA.

C

GABA-A RECEPTOR SUBTYPES: FUNCTIONAL AND MOLECULAR PROPERTIES

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The $GABA_A$ receptors are fast-acting ligand-gated chloride ion channels. Multiple $GABA_A$ receptor genes have been identified which encode highly homologous subunits i.e. the α1-6, ß1-3, γ1-3, δ and ρ1-2 polypeptides. Different pentameric combinations of these are thought to coassemble to yield receptors with distinct pharmacological and biophysical properties. Particularly, the characterisation of defined (αßγ) cloned receptors has shown differences in agonist sensitivity and benzodiazepine pharmacology. In adult brain, in situ hybridisation, immunoaffinity purification of subpopulations and immunocytochemical mapping of $GABA_A$ receptor subunit isoforms yields limited major forms of the receptor. This is in contrast to a more widespread pattern of expression of different subunit combinations during development. These observations will be discussed with respect to the putative distinct functional roles played by $GABA_A$ receptor subtypes. Recent work on the developmental expression of α1 and α6 subunit-containing receptors in cerebellar granule cells in primary culture will be described.

D

MOLECULAR PROPERTIES OF GABA TRANSPORTERS

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The removal of neurotransmitters by their transporters - located in the plasma membranes of nerve terminals and glial cells - plays an important role in the termination of synaptic transmission. In the last three years many neurotransmitter transporters have been cloned. Structurally and functionally they can be divided into two groups: glutamate transporters, of which to date three have been cloned, couple the flow of glutamate to that of sodium and potassium. The second group of transporters includes those for GABA, glycine, taurine, norepinephrine, dopamine and serotonin. They are sodium and chloride dependent, but do not require potassium for function. One of these, the $GABA_A$ transporter, encoded by GAT-1, is perhaps the best characterized. It has been purified and reconstituted and has a molecular mass of around 80 kDa, of which 10-15 kDa is sugar. Amino- and carboxyl-terminii (around 50 amino acids each) are not required for function. The transporter is protected against proteolysis at multiple sites by GABA, provided that the two cosubstrates - sodium and chloride - are present. Several amino acid residues, which are critical for function, have been identified in the GABA transporter. These include arginine-69 and tryptophan-222 located in the first and fourth putative transmembrane helices, respectively. The first is possibly involved in the binding of chloride. The tryptophan appears to serve as a binding site for the amino group of GABA.

A

Functional consequences of forward and reversed operation of neurotransmitter uptake carriers.

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The postsynaptic action of GABA and glutamate is partly terminated by uptake into glial cells and neurons. The GABA and glutamate uptake carriers belong to different gene families and have different ionic stoichiometries. GABA uptake is driven by the co-transport of 2 sodium ions and a chloride ion, while glutamate uptake is driven by the co-transport of 2 sodium ions and the counter-transport of a potassium and a hydroxide or bicarbonate ion. Both carriers can reduce the external transmitter concentration to close to the bottom end of the dose-response curves for the receptors they act on.

Inhibiting GABA uptake prolongs the synaptic current evoked by GABA acting on $GABA_A$ channels, and allows GABA to diffuse to other nearby neurons where it acts on $GABA_B$ receptors and reduces transmitter release. Inhibiting glutamate uptake has different effects at different synapses: it prolongs the synaptic current at cerebellar synapses from parallel and climbing fibres to Purkinje cells, but has little effect at cerebellar mossy fibre to granule cell synapses.

In certain conditions the uptake carriers can run backwards, releasing transmitter. For GABA this happens normally in the retina, where transmission from horizontal cells to bipolar cells appears to be by reversed uptake. The rundown of ion gradients during brain anoxia leads to reversed uptake raising the external [glutamate] to neurotoxic levels.

B

Compartmentalised distribution of immunoreactivity for $GABA_A$ receptors on the surface of neurons in relation to single or multiple sources of GABAergic synapses

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Quantitative, electron microscopic immunogold localisation was used to establish the relative density of the α1 and β 2/3 subunits of the $GABA_A$ receptor on the extrasynaptic somatic and dendritic membrane, or in the synaptic junctions of cerebellar granule cells receiving GABA only from Golgi cells. In addition the precise subcellular localisation of these subunits was established on granule cells of the dentate gyrus innervated by several distinct types of GABAergic interneuron.

$GABA_A$ receptors were highly concentrated in the synaptic junctions between the GABAergic Golgi cell terminals and granule cell dendrites. Immunoparticle density in the synaptic junction for the α1 and β2/3 subunits was approximately 100 and 50 times higher respectively, than on the extrasynaptic dendritic membrane. The immunoparticle densities on the somatic membrane were significantly lower than on the extrasynaptic dendritic membrane. The synaptic junctions between granule cell dendrites and the glutamatergic mossy fibre terminals were always immunonegative.

We compared the above distribution of immunoreactivity to the distribution on granule cells of the dentate gyrus receiving a segregated input from at least 6 types of GABAergic neuron. Preembedding immunogold localisation of immunoreactivity for the α1 and β2/3 subunits consistently showed extrasynaptic localisation of the $GABA_A$ receptor on the somatic, dendritic and axon initial segment membrane. A highly increased level of immunoreactivity for both subunits has been found in the synaptic junctions between basket cell terminals and somata, and between axo-axonic cell terminals and axon initial segments of granule cells. The results demonstrate that there is no qualitative difference in the α1 and β2/3 subunit content of basket versus axo-axonic cell synapses on hippocampal granule cells. Receptor immunopositive synapses were found at all depths in the molecular layer providing evidence that GABAergic cells that terminate only on the dendrites of granule cells also act through $GABA_A$ receptors.

The results demonstrate that both cerebellar and hippocampal granule cells exhibit a compartmentalised distribution of the $GABA_A$ receptor on their surface, the postjunctional synaptic membrane having the highest concentration of receptor. Hippocampal granule cells show a similar distribution of the same subunits in synapses receiving GABA form several discrete types of cell.

C

An ATP-Activated Na^+ Channel is Involved in Fertilization.

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A rapid positive shift in fertilized egg's membrane potential resulting from an increased Na^+ permeability, and a high ATP concentration in sperm cells, initiated the exploration of the ionic events induced by application of extracellular ATP in ***Henopus laevis*** oocytes. A saturating inward current with maximal amplitude E_{max}=2426±240 nanoampers and an apparent Km of 197.6μM was observed in defolliculated ***Henopus*** oocytes by extracellular ATP application. Current voltage analysis showed a reversal potential of 1.6±2.8mV and predominance of sodium ions. The ATP response is effectively antagonized by GTP (Kd=41.9μM), and its non-hydrolyzable analogues GppNHp and GTPγS (Kd=3.9 and 3.5μM respectively). GTP alone induces a smaller magnitude depolarization response in oocytes with higher EC_{50} values, compared to ATP, suggesting that GTP acts as a partial agonist at the ATP site. A potent inhibitory action of the ATP evoked inward current was observed by amiloride, similar to inhibition of amiloride-sensitive epithelial Na^+ channel (IC_{50}=2.12±0.96μM).

Likewise, in vitro fertilization, using mature eggs and *Xenopus* sperm, was inhibited by amiloride, GTP, and GppNHp. Hence, an ATP receptor on egg-membrane may be the recipient target for ATP originating in sperm. Suggesting that ATP induced increase in Na^+ permeability mediates the initial sperm to egg signal in the process of fertilization.

D

PURINOCEPTORS: HISTORICAL OVERVIEW AND NEW DIRECTIONS

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The potent actions of purine nucleotides and nucleosides on the heart and vasculature was first recognized in 1929 by Drury and Szent Györgyi. The 'purinergic nerve' hypothesis was proposed in 1972 (Burnstock, 1972), with evidence to suggest that ATP was the neurotransmitter in non-adrenergic, non-cholinergic nerves supplying the intestine, urinary bladder and parts of the vascular system. Later it became evident that ATP was also a co-transmitter with classical transmitters and peptides in many autonomic nerves (Burnstock, 1990). Receptors to purines were shown to belong to two main subtypes (Burnstock, 1978), namely P_1-purinoceptors, that are selective to adenosine, are activated via adenylate cyclase and are competitively blocked by methylxanthines; and P_2-purinoceptors that are selective for ATP and ADP. Further division of both P_1-purinoceptor into A_1, A_2 and A_3 subtypes and P_2-purinoceptors into P_{2X}, P_{2Y}, P_{2T}, P_{2U}, P_{2D} and P_{2Z} subtypes have been described (see Burnstock 1991). Using data obtained from new diagnostic agonists and the cloning of some P_2-purinoceptor genes (Barnard, Burnstock & Webb, 1994), a new basis for subclassification of purinoceptors into P_{2X1-3} subtypes of a ligand-gated cation acting family and P_{2Y1-7} subtypes of a G-protein mediated family has been proposed (Abbracchio & Burnstock, 1994). New developments include investigations of the therapeutic potential of selective agonists and antagonists of the different P_2-purinoceptor subtypes and of the long-term (trophic) actions of purines in development and regeneration.

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A

TROPHIC ACTIONS OF PURINES

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Several lines of evidence suggest that purines modulate proliferation in a variety of cell types. Both stimulation and inhibition of DNA synthesis have been demonstrated depending upon the purine derivative and the cell system; moreover, purines and pyrimidines have been also suggested to affect differentiation parameters, such as neurite outgrowth in neuronal cells. The available data on the trophic effects of purines will be reviewed, with particular attention to the mechanism(s) at the bases of these activities and to the possible involvement of the different P1 and P2-purinoceptors. Particular emphasis will be given to the possible key role in patho-physiological events, such as wound healing and tumor development, hypoxia-induced angiogenesis, injury-associated astrogliosis and nerve regeneration. In previous studies, we have characterized the effects of ATP and adenosine analogues on astroglial cell proliferation in primary cultures of rat striatum (Abbracchio et al., Neurosci., **59**: 67-76, 1994). More recent data obtained in the same experimental model suggest that purines might also affect astroglial cell differentiation. We have studied the effect of the ATP analogue αβmeATP on an astroglial differentiation parameter (the expression of glial fibrillary acidic protein (GFAP)-positive processes) in comparison with a known astroglial cell trophic factor, basic fibroblast growth factor (bFGF). Our data suggest that, in serum-free medium, αβmeATP promotes glial cell differentiation by producing dose-dependent elongation of astrocytic processes, with effects quantitatively comparable to those induced by bFGF. These data indicate that, besides cell proliferation, purines can also modulate differentiation parameters in target cells, which might play an important role in their trophic activities.

B

VESICULAR AND CYTOPLASMIC RELEASE OF TRANSMITTERS

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Over the past years only little progress has been made in characterization of mechanism of neurotransmitter release. It is generally accepted that the influx of calcium through voltage-sensitive channels serves as a link between depolarization of the nerve terminal and the activation of transmitter release of vesicular origin. There are types of release: (i) Ca_o-dependent and associated with axonal activity and (ii) Ca_o-independent (e.g. due to inhibition of sodium pump, reversed uptake etc.). While the former one is vesicular in origin and subject to presynaptic modulation through stimulation of presynaptic hetero- and autoreceptors (α_2, μ, A_1, D_2 etc.), the latter one is of cytoplasmic origin and cannot be modulated. Whether calcium entry without depolarization (Stanley, 1993) is sufficient to trigger exocytosis is still a problem remained to be solved.

When the effect of NaF + $AlCl_3$, a direct activator of G proteins, on the release of [^{3}H]dopamine ([^{3}H]DA), [^{3}H]gamma-aminobutyric acid ([^{3}H]GABA), [^{3}H]acetylcholine ([^{3}H]ACh) from slices of rat striatum, and on $[Ca^{2+}]_i$ in striatal synaptosomes was investigated evidence was obtained that under Ca^{2+}-free conditions the release of [^{3}H]DA and [^{3}H]GABA was even more enhanced. Since reserpine-pretreatment prevented the effect of the complex on [^{3}H]DA release, it is concluded that a non-receptor-mediated activation by NaF + $AlCl_3$ of α-subunit of G protein coupled to phospholipase C (PLC) results in a $[Ca^{2+}]_o$-independent release of transmitters stored in vesicles. It seems that an increase in $[Ca]_i$ without Ca-influx is able to produce exocytosis.

C

THE REGULATION OF THE BIOSYNTHESIS OF LARGE DENSE CORE VESICLES

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Most of the peptide constituents (soluble and membrane bound) of large dense core vesicles (LDV) have been cloned. We have analyzed their biosynthesis by measuring peptide and mRNA levels. Reserpine treatment of rats induced in the adrenal medulla high levels of the mRNAs of secretory peptides (especially chromogranin B, VGF and NPY) whereas those of partly membrane-bound ones and that of an intrinsic membrane protein, cytochrome b-561 were much less or not upregulated. For brain analogous results were obtained when defined neurons were stimulated by salt loading or kainic acid treatment. Thus mRNA levels of secretogranin II (the precursor of the new neuropeptide secretoneurin) and chromogranin B, but not of carboxypeptidase H and synaptophysin were elevated. We conclude that a strong stimulation of chromaffin cells or neurons leads to a biosynthesis of LDV with increased quanta of secretory peptides. In PC12 tissue culture the second messengers involved in this regulation were analysed. The mRNA levels of chromogranin B and NPY were upregulated by phorbolester, forskolin and calcium, respectively. A combination of these agents had a cumulative effect.

D

Structure and dynamics of cholinergic vesicle proteins

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Synapic vesicles are key organelles in synaptic transmission. On induced transmitter release they undergo cycles of exo- and endocytosis. The integral membrane synaptic vesicle protein SV2 remains associated with the organelle not only during induced transmitter release but during its entire life cycle. It has been proposed that rab3A - a small GTP-binding protein belonging to a protein family involved in regulating cellular membrane traffic - dissociates from the vesicular compartment during exocytosis and reassociates after a period of rest. Recently we sequenced a cDNA clone encoding the electric ray homologue o-rab3. O-rab3 is specifically associated with synaptic vesicles of the electric ray electric organ and with subpopulations of nerve terminals in *Torpedo* brain. We further investigated the question of a dynamic association of o-rab3 with synaptic vesicles during cycles of exo- and endocytosis and compared it to that of the membrane integral protein SV2. Sustained low frequency stimulation (0.1 Hz) of the nerves innervating the *Torpedo* electric organ results in recycling of vesicles without a decrease in vesicle number. Analysis of the subcellular localization of proteins using immunoelectron microscopical and biochemical techniques demonstrate that recycled synaptic vesicles retained their complement of both SV2 and o-rab3. In contrast, multivesicular bodies and vacuoles occasionally observed in nerve terminals contained SV2 but little if any o-rab3. It is concluded that o-rab3 remains associated with the synaptic vesicle membrane compartment during stimulation induced cycles of exo- and endocytosis. But it may be lost once the vesicle enters the prelysosomal pathway.

A

Regulation of Synapsin I Gene Expression

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The synapsins are a family of neuron-specific phosphoproteins that selectively bind to small synaptic vesicles in the presynaptic nerve terminal. To identify control elements directing the neuronal expression of synapsin I, we functionally analyzed the promoter region of the human and the mouse synapsin I gene. Using transient transfections of cell lines of neuronal and nonneuronal origin, we showed that the 5´-upstream region of the synapsin I gene containes elements responsible for the neuron-specific expression of synapsin I. An analysis of the proximal region of synapsin I promoter by electrophoretic mobility shift assay and south-western blotting revealed that there are DNA-binding proteins restricted to brain nuclear extract that bind to the synapsin I promoter and therefore indicate the likelihood of their participation in neuron-specific gene expression of synapsin I. In addition, we showed that the zinc-finger transcription factor zif268/egr-1, an immediate-early response gene involved in the regulation of growth and differentiation, bound *in vitro* to two sites in the proximal regulatory region of the human synapsin I gene. The zif268/egr-1 transcription factor also stimulated transcription from this control region in transactivation assays indicating that the synapsin I gene might be a downstream target gene for zif268/egr-1. Finally, we compared the "cyclic AMP-responsive element" (CRE) present in the upstream regions of the synapsin I and the chromogranin B gene. While the CRE in the chromogranin B promoter functioned as a cAMP-inducible enhancer, we demonstrate that synapsin I gene expression is not regulated by cAMP.

B

PRESYNAPTIC MODULATION OF TRANSMITTER RELEASE IN HUMAN BRAIN

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Understanding synaptic transmission in the human brain is of the uppermost importance due to the involvement of neurotransmitters in several neurological and psychiatric disorders. Studies of animal pharmacology and of molecular biology are revealing that transmitter receptors are highly heterogeneous. It is therefore essential, also in view of using animal models in the development of therapeutically useful drugs, to establish if functionally corresponding receptors in men and animals also display identical pharmacological profiles. Using human brain tissue samples removed during neurosurgery and monitoring transmitter release as a functional response, a number of neurotransmitter receptors have been identified, localized and pharmacologically characterized as types and subtypes.
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C

ROLE OF CALPAIN IN DEGENERATION OF AXON AND MYELIN IN CNS

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Calpain, a calcium-activated neutral proteinase, is associated with myelin, degrades cytoskeletal and myelin proteins and has been implicated in degenerative brain diseases. The protein degradation indicated calpain's pivotal role in tissue destruction in experimental spinal cord injury (SCI) and demyelinating diseases [e.g., multiple sclerosis (MS)]. To demonstrate calpain's role in nervous system degeneration, we examined (1) activity and immunocytochemical localization of mcalpain in rat SCI lesion after injury; (2) calpain activity in experimental allergic encephalomyelitis (EAE), an animal model close to MS; and (3) efficacy of cell permeable calpain inhibitors as neuroprotective agents. Our results indicate increased mcalpain activity and immunoreactivity in glial cells in SCI as compared to control. The increased NFP, MAP_2 and MAG degradation in SCI is prevented by calpeptin and methylprednisolone (MP). There is degradation of MAG with formation of dMAG in EAE as compared to control. The extent of dMAG formation depends on EAE severity. These results indicate calpain's involvement in axon and myelin breakdown in SCI and demyelinating diseases and that the inhibition of calpain activity by calpeptin and MP may provide neuroprotection. Partly supported by NIH-NINDS NS11066 and NS-31622, NMS Society RG-2130 and Paralyzed Veterans of America SCRF-1238.

D

CHARACTERISATION AND CLONING OF A METALLOPROTEASE CAPABLE OF DEGRADING MYELIN BASIC PROTEIN.

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When brain myelin membrane preparations are incubated at physiological ion concentrations and neutral pH, myelin basic protein (MBP) is rapidly degraded. The protease responsible for this activity has been purified from bovine brain myelin preparations. Biochemical analysis has shown that the enzyme is a zinc metalloprotease of molecular weight 58 kDa which cleaves MBP at the Pro73-Gln74 bond. Within neural tissue, immunohistochemistry has shown that this protease is concentrated in myelin forming cells *i.e.* oligodendrocytes and Schwann cells in the CNS and PNS respectively. However the protein is not confined to neural tissue, but is also present in the particulate fraction of a number of organs including heart, spleen, kidney, muscle and lung. Protein sequence derived from a tryptic peptide was used to design an oligonucleotide probe which was used to isolate a cDNA clone encoding this enzyme from a bovine brain cDNA library. Sequence analysis has shown that this is a novel protease composed of four domains; a long pre-pro sequence, a metalloprotease, a potential integrin binding (disintegrin) and a cysteine rich domain. We have named this protein MADM (*M*embrane *A*ssociated *D*isintegrin-containing *M*etalloprotease). MADM shows homology with certain snake venom metalloproteases and a number of recently described mammalian metalloproteases which contain integrin binding domains. Our *in vitro* studies using bovine kidney distal tubule epithelial cells (MDBK cells) have shown that MADM is cell-associated rather than secreted, despite the presence of a signal sequence and the apparent absence of a transmembrane domain. Thus MADM may bind to integrins present on the cell surface and thereby may modulate integrin function. We suggest that MBP, a cytoplasmic protein, is unlikely to be the physiological substrate for MADM, and that cell surface glycoproteins or proteins of the extracellular matrix are more likely candidates. We thank SmithKline Beecham for funding for L.H.

A

EXTRINSIC AND INTRINSIC CATHEPSIN D ACTIVITY OF MYELIN: A PARADIGM

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Cathepsin D activity of isolated myelin membranes is used as a paradigm to illustrate salient aspects of the limited proteolysis of myelin proteins, in particular myelin basic protein (MBP), by endogenous proteinases of the myelin sheath in early non-inflammatory demyelination. It is also used to highlight conceptual and methodological pitfalls. Latent-bound cathepsin D activity of myelin may be elicited upon solubilization of extrinsic membrane components of myelin by high ionic strength. However, as they were found to comprise several proteinases in addition to cathepsin D, as well as other lysosomal hydrolases they strongly appear to be artifactual in that they become only adsorbed during the isolation of myelin membranes. Yet there is distinct residual detergent-soluble cathepsin D activity of salt-extracted myelin membranes that appears to be truly intrinsic in origin. Results of the degradation of exogenous and endogenous MBP by intrinsic cathepsin D activity of myelin membranes will be presented as well as results of its current isolation and characterization.

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B

ANTIBODY-MEDIATED DEMYELINATING PATHWAY : STIMULATION OF THE MYELIN PROTEASE IS ASSOCIATED WITH SPECIFIC RECOGNITION OF EXTERNALLY LOCATED ANTIGENS OF MYELIN

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The origin of proteases involved in demyelination includes myelin-associated proteases. We have shown the presence in isolated human myelin of a calcium-activated protease acting on myelin basic protein (MBP). There was no difference in the activity of the protease itself in human myelin isolated from control or multiple sclerotic (MS) brains, and we suggested that the myelin protease could be activated in vivo by an increased availability of free calcium released upon destabilization of the myelin sheath. In MS, disruption of myelin could possibly be mediated by binding of intrathecally synthesized immunoglobulins (Igs). We derived strong evidence for this postulate by quantifying the extent of MBP degradation in human myelin incubated in the presence of Igs isolated from control or MS brains; the latter incubation resulted in significantly increased MBP degradation. To investigate the specificity of autoantibody(s) involved, we have assayed the ability of experimentally raised antibodies directed against specific myelin antigens, to mediate the stimulation of the myelin protease. Relevant Igs tested included monoclonal antibodies directed against myelin oligodendrocyte glycoprotein (MOG), MBP, myelin-associated glycoprotein, myelin proteolipid protein, the HNK-1 carbohydrate epitope and galactocerebroside (GalC). Highly significantly increased MBP degradation resulted from incubation of human myelin with anti-MOG and with anti-GalC antibodies, suggesting that specific recognition of externally located, readily accessible antigens, is a potential trigger for the myelin protease acting on MBP.

C

The cytokine-protease axis in autoimmune demyelination

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It has been recognized that demyelination is an enzymatic process, in which the initial phase is caused by the resident or infiltrating macrophages and lymphocytes. We advocate that the cytokine-regulated production of proteases leads to demyelination and initiates the autoimmune process in multiple sclerosis. The balances between disease-promoting and disease-limiting cytokines of the cytokine network and between proteases and protease inhibitors of the enzyme cascade determine the generation of remnant epitopes from intact myelin substrates. We proposed the REGA (Remnant Epitope Generates Autoimmunity)-model as an early step in the MS pathology (1). Experimental results on the regulatory role of primary and secondary cytokines, e.g. chemokines, and on the enzymatic attack of myelin by the matrix metalloproteinase gelatinase B will be presented and discussed.

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D

CSF PROTEOLYTIC ACTIVITY AND MRI-DETECTABLE DEMYELINATION IN HIV-1-INFECTED PATIENTS WITH NEUROLOGICAL DISEASES

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HIV-1 infection may indirectly promote demyelination in the central nervous system (CNS), but the pathogenetic mechanisms responsible for myelin breakdown are as yet unknown.

We have previously shown that the myelin basic protein (MBP), a major component of the myelin sheath, is released from the membrane and can be detected in the cerebrospinal fluid (CSF) of HIV-infected patients with active demyelination, often in the presence of an antibody response.

Since MBP is very susceptible to proteolysis and has been found in the CSF in the form of small fragments, proteases have been implicated in myelin degradation. In fact, an increase in proteolytic activity has been observed in experimental allergic encephalomyelitis and in acute phases of multiple sclerosis. However, no information is as yet available on the HIV-related demyelination.

In the present study, proteolytic activity towards purified MBP was detected by SDS gel electrophoresis in CSF samples from HIV-1-infected patients with neurological complications and MRI detectable demyelination.

MBP degradation was observed in 24/32 (75%) of the patients with AIDS dementia complex, or in 7/9 (78%) patients with progressive multifocalleukoencephalopathy (PML) and 1/2 patients with cerebral toxoplasmosis. In 39/43 (91%) of these patients MBP degradation and/or high MBP levels (> 2.2 ng/ml) were found.

To identify the proteases involved, the CSF samples were subjected to zymography on gels copolymerized with either gelatin or MBP. A number of bands between 63 and 132 kDa were observed on gelatin. In particular, a 72 kDa band was present in all samples; a 95 kDa and a 89 kDa band bands were found in many samples independently from the pathology. A less frequent 63 kDa band seemed to be more specifically related to demyelination. In MBP-containing gels, four bands with MW of 87, 83, 79 and 72 kDa were observed. Again, the 72 kDa was present in all samples. The 79 and the 83 kDa bands were present frequently, but the 79 kDa band appeared to be more specific.

Identification of type and origin of proteases involved is beeing studied.

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A

APPEARANCE AND ACCUMULATION PATTERNS OF MYELIN OLIGODENDROCYTE GLYCOPROTEIN DURING DEVELOPMENT

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The formation of functional myelin requires the expression of several major myelin specific proteins (MBP, PLP, MAG and CNP) as well as other quantitatively minor glycoproteins. Myelin/oligodendrocyte glycoprotein (MOG), one such glycoprotein, has been the focus of recent attention in view of the postulate that this protein plays a key role in the completion and/or maintenance of the myelin sheath. We, and others, have recently reported that this CNS specific glycoprotein, belonging to the immunoglobulin supergene family, has an approximate MW of 28 kD with a further band reacting with the monoclonal anti-MOG antibody (8-18C5) at approximately 55 kD. In order to further characterise MOG and elucidate its putative role in the synthesis of myelin we have purified MOG from several different species, studied its developmental regulation and begun to investigate by immunoblotting the possibility that MOG, like other myelin proteins, may occur as isoforms. The results show that purified MOG from different species is highly homologous and appears as a number of bands on SDS-PAGE analysis and immunoblotting. The appearance and accumulation of MOG appears to be developmentally regulated and, as reported for MBP and PLP, shows a caudo-rostral gradient of expression through development.

B

NEW AMPEROMETRIC MICROBIOSENSORS FOR THE DETERMINATION OF EXTRACELLULAR NEUROTRANSMITTERS

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Recently, we have reported a novel immobilized enzyme technology which provides sensitive and fast responsive amperometric biosensors. This biosensor construction is based on the electropolymerization of a amphiphilic pyrrole enzyme mixture previously adsorbed on an electrode surface. Following this strategy, we have prepared two amperometric biosensors based on polyphenol oxidase or glutamate oxidase. These biosensors exhibit fast response times towards dopamine and L-glutamate with attractive detection limits of 5×10^{-8} M and 5×10^{-7} M respectively. The miniaturization of these biosensors has been accomplished by modifying microelectrodes of platinum (diameter 50 and 200 µm) and carbon (diameter 8 µm). In collaboration with a team of neurosurgeons working on the Parkinson's disease, the microbiosensor based on glutamate oxidase has been used to localize in rat brains a subthalamic nucleus implicated in this disease. In parkinsonian animal models, this nucleus presents an increased glutamatergic activity and thus can be localized through the detection of extracellular glutamate. On the other hand, the glutamate microbiosensor has been applied to the detection of extracellular glutamate released during electrical stimulations of isolated neurons.

C

MICRODIALYSIS AND BIOSENSORS FOR MONITORING BRAIN METABOLISM

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In the last decade several technologies have been developed to monitor analytes *in vivo*. Among these, microdialysis has been applied to monitor neuro-transmitters, metabolites, drugs and hormones. Examples for application include glucose metabolism in the rat brain during drug treatment and behavior, and will be reveiwed by Korf. Clinical application of microdialysis include peripheral metabolism during excersice, in the intensive treatment units or during drug treatment. Examples on such clinical application will be shown by Hamberger and Korf.

Microdialysis may be combined with biosensor technology for *in vivo* monitoring, thereby combining the advantages of both techniques, e.g. biocompatibility and clean sampling, as revealed with microdialysis and on-line and rapid detection with biosensors. Developments in biosensor research include the combination of proteins to determine selectivity and silicon based technology to give rapid and transmittable signalling. Recent technical advances will be summarized by Bergveld. All three speakers will deal with future technical developments and biomedical applications.

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D

RECORDING OF EXTRACELLULAR GLUTAMATE BY ENZYME AMPEROMETRY

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An enzyme amperometric cell (biosensor) was developed for flow analysis of glutamate (GLUT) in the dialysate as it emerges from an implanted microdialysis probe. We have studied the effects of cerebral ischaemia and spreading depression (SD). GLUT-biosensors were of the thin-layer type with optimized geometry and electrode configuration to accommodate the slow flow used in microdialysis. Specific features included: Ag/AgCl reference and two platinum electrodes facing each other along their whole length; narrow input and output tubing inserted on either side of the electrodes; and thin gasket to reduce the internal volume of the cell. GLUT-oxidase was immobilized into a mixture of albumin and gelatin. O-1,2-diaminobenzene electropolymerized onto the working electrode eliminated interferences from endogenous electroactive compounds. *In vitro*, GLUT-biosensors showed a time resolution of 30 sec, minimal oxygen-dependency, and no influence of pH or ionic changes within the range encountered *in vivo*. GLUT-responses were linear up to 200 µM, with a detection limit of 0.5 µM at 1 µL/min. GLUT changes evoked by SD in the rat striatum were better resolved by the biosensor than enzyme-fluorescence. Comparison with the latter showed an excellent specificity of the biosensor for GLUT. SD-evoked changes in GLUT and DC potential were remarkably synchronous; there was no sign of an increase in extracellular GLUT preceding SD. A biphasic increase in GLUT was found during ischaemia. Only the first phase, very small relatively to the overall change, was Ca-dependent. This strongly suggests that most the of GLUT release in ischaemia is of metabolic origin.

A

BIOSENSOR AND MICRODIALYSIS FOR MONITORING GLUCOSE

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We have shown previously, using microdialysis in the rat, that mild behavioural stimulation can be used to stimulate the release of a number of neurochemicals. In order to investigate how the energy requirements of this release are met we have developed an enzyme-based assay system for glucose Glucose oxidase is immobilised together with peroxidase on 10μm silica beads and packed into a 2x20 mm column. Flow injection of dialysate into a buffer stream,which contains ferrocene as a mediator, pumped through the column gives a single current peak at a downstream electrode without interference from other species present in the dialysate. Used on-line glucose may be assayed at 1-2.5 min intervals. Experiments were performed at a flow rate of 2 μL/min in freely moving rats 24h after surgery. We used the variation of concentration method of Lönnroth to determine the extracellular level of glucose to be 0.47±0.18 mM (n=5), much lower than that of plasma glucose. Furthermore, it was possible to alter this level by both local pharmacological and physiological stimulation suggesting that supply and utilisation of glucose were not always completely matched. Thus local TTX (1μM) increased and local veratridine (50 μM) decreased dialysate glucose levels. Mild tail-pinch caused a biphasic increase in glucose. The first increase was accompanied by an increase in regional blood flow. A possible second source of glucose is ß-adrenoceptor mediated breakdown of astrocytic glycogen (Sorg & Magistretti 1991). We found local propranolol (50 μM) to abolish the tail pinch glucose increase, and a 5 min local pulse of isoprenaline (50 μM) to increase dialysis glucose content by 1.42±0.46 nmol (n=7) compared to an increase of 0.34±0.07 nmol (n=4) found with a 5min tail pinch. This suggests that ß-adrenoceptor play a role in physiologically induced increases in extracellular brain glucose.

Sorg, O. & Magistretti, P. J. (1991) *Brain Res.*, **563**, 227-233

B

QUANTITATIVE MICRODIALYSIS - THEORETICAL MODELS AND PRACTICAL IMPLICATIONS

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The technique of in vivo microdialysis has be proven as an efficient tool for monitoring local chemistry in a living body. One of the points still remaining in the debate on the usefulness of microdialysis is the question of recalculating absolute concentrations of a given substance sampled from the extracellular fluid. Microdialysis in most cases operates under quasi steady-state conditions, which means that only a certain part of a substance can be recovered.

A number of methods for quantitative microdialysis was reported in the literature. The methods can be divided into eight groups, depending on the mathematical models and theoretical principles used to describe convective diffusion in the extracellular space. Each method will be reviewed from a historical perspective, showing its main contribution to recent knowledge, as well as its limitations and drawbacks. It can be concluded that those methods based on explicitly derived equations for in vivo recovery are still too approximative and not suitable for routine applications. Therefore, empirical models based on varying perfusion flow rates or concentrations of substances in the perfusion solution, found several practical implications. Methods using a reference substance as a marker of in vivo recovery will be also discussed.

The review is aimed to emphasise the increasing importance of methods allowing the quantitative evaluation of microdialysis data whenever measuring neurotransmitter release, drug concentrations or pharmacokinetic variables.

C

NEUROPROTECTIVE EFFECT OF (-)-DEPRENYL AGAINST DSP-4 TOXICITY.

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Oral pretreatment with (-)-deprenyl prevents the DSP-4 induced noradrenaline (NA) depletion in the hippocampus of rats and domestic pigs. DSP-4 was injected i.p. to rats (50 mg/kg) and i.v, to pigs (4 mg/kg). The NA concentration of the hippocampus was determined by HPLC 7 days after DSP-4 administration. DSP-4 reduced the NA content more than 80% compared to the control level of both animal species. Pigs as experimental animals were chosen because their platelet MAO-B activity can easily be determined by radiometric method. (-)-deprenyl in a dose of 1 mg/kg p.o. evoked a remarkable prevention, when it was administered 1 to 4 h before DSP-4 treatment on both animal species. The preventive effect of (-)-deprenyl went parallel with the blood level of (-)-deprenyl metabolites (methylamphetamine and amphetamine) and did not show any relation to the inhibition of MAO-B activity. This finding was substantiated in experiments with SKF-525A. When SKF-525A was administered i.p. (50 mg/kg) to rats 1 h before (-)-deprenyl pretreatment, MAO-B inhibition was enhanced, while the protective effect of (-)-deprenyl was markedly reduced. We suggest that (-)-deprenyl as a MAO-B blocker is inactivated, while, as an uptake inhibitor, is activated by a metabolic process. Since the metabolites are more potent neuroprotectors than the parent compound, their actual concentration are critical in preventing the toxic effect of DSP-4.

D

(-)-DEPRENYL REDUCES PROGRAMMED CELL DEATH BY INDUCING NEW PROTEIN SYNTHESIS

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(-)-Deprenyl has actions that are independent of MAO-B inhibition including the reduction of neuronal death after toxic, hypoxic or axonal damage and alterations in protein synthesis and process growth in astrocytic or PC12 cells. We examined the capacity of (-)-deprenyl (-D), (+)-deprenyl (+D), (-)-deprenyl metabolites, and other MAO-B or -A inhibitors to reduce the programmed death (PD) of PC12 cells caused by trophic withdrawal. PC12 cells, cultured in serum with NGF for 6 days, were washed to eliminate trophic proteins before placement in serum with NGF, minimum essential media (MEM) or MEM with -D, +D, (-)-methamphetamine (-meth.),(-)-amphetamine (-amp.) or MAO-A or -B inhibitors in concentrations of 10^{-3} to 10^{-13} M. The PC12 cells died gradually over five days in the MEM solution with about 50% dying in the first 24 hours. In situ marking of DNA 3' ends and DNA electrophoresis revealed that the internucleosomal DNA fragmentation characteristic of PD began in most cells by 9 to 12 hours after trophic withdrawal. -D markedly reduced the PC12 cell death and internucleosomal DNA fragmentation at concentrations of 10^{-5} to 10^{-11} M according to a relationship with a maximum at 10^{-9} M. (+)-D did not alter PC12 cell survival at concentrations of 10^{-5} to 10^{-13} M showing that -D interacted with a stereospecific site to increase survival. Translational or transcriptional blockade with cycloheximide, actinomycin or camptothecin showed that the increased PC12 cell survival depended on new protein synthesis induction by -D which reached sufficient levels to reduce PD at 6 hours after -D addition. Most MAO-A and MAO-B inhibitors (iproniazid, phenelzine, semicarbazide, tranylcypramine, nialamide, MDL 72974A, RO-16-6491, clorgyline, and brofaromine) failed to increase PC12 cell survival. Pargyline increased the survival with an IC_{50} 1000 fold greater than -D. The major metabolites of -D, -meth. and -amp., increased the PC12 cell PD at 10^{-3} to 10^{-5} M. Furthermore, -amp. and -meth. at 10^{-7} to 10^{-11} M antagonized the increased PC12 cell survival induced by -D. We propose that a non-MAO binding site for -D mediates the reduction of PD and that -D actions in both neurons and astrocytic cells involve the induction of new protein synthesis.

A

THE ENIGMA OF MONOAMINE OXIDASE B

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The presence of monoamine oxidase A (MAO) within the aminergic neurotransmitter (noradrenergic, dopaminergic and serotonergic) neurons in the CNS is understandable. This enzyme operates to maintain the cytoplasmic concentration of the neurotransmitters low within the neurons. Thus, functionally MAO-A operates to regulate neurotransmitter release and metabolism. However, the function of a large extraneuronal pool of MAO-B, mainly present in glia is not understood. Unlike MAO-A, it oxidizes non-hydroxylated amines and its endogenous substrates are limited to phenylethylanine and probably dopamine. The many features of brain MAO-B that distinguish it from MAO-A include its (a) relatively slow rate of postnatal development, (b) lack of regulation in response to steroid hormones, (c) sole presence in adrenal chromaffin cell and platelets containing the highest concentration of catecholamines and serotonin respectively in the body. Thus, the wrong enzyme is present for the right reason in cell types to eliminate indirectly acting sympathomimetic amines capable of releasing catecholamines and serotonin.

B

RECENT DEVELOPMENTS WITH REVERSIBLE MAO-A INHIBITORS

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The new generation of reversible MAO-A inhibitors has been discovered in the late '70s. Two compounds, toloxatone and moclobemide, were shown to produce both in vitro and in vivo a selective "short-acting" inhibition of MAO - A. Since then, several other compounds from different chemical series has been developped. Among these the most advanced are brofaromine (phase III), befloxatone (phase II), BW-1370U87 (phase I), Esuprone (phase I) and RS-8359 (phase I).

The reversible interaction between the inhibitor and the enzyme represents the rationale to avoid the side effects associated with irreversible inhibition of both isoforms of MAO (e.g. the potentiation of the pressor effect of tyramine and the other pressive amines which are inactivated by deamination), or with a lack of specificity vs the enzyme, (e.g. drug interactions following inhibition of some cytochrome P-450 isoenzymes or other FAD dependent enzymes). Furthermore, some irreversible phenyl hydrazine derivatives have shown hepatic toxicity. In the chemical series of oxazolidinone (befloxatone, toloxatone) SAR studies indicate that the interaction between the inhibitor and the enzyme, occurs by multiple recognition with the cofactor FAD and the protein.

The higher affinity of befloxatone for MAO-A (nM) compared to that of toloxatone (μM) is the result of more favourable interactions of the two side chains of the oxazolidinone ring with the protein of the enzyme. This also determines the very high specificity of befloxatone for MAO-A.

Several preclinical and clinical pharmacological studies have shown that reversible MAO-A inhibitors do not present the feared interactions observed with the first generation of irreversible MAOI's and allow a safer treatment.

The present development of new reversible MAO-A inhibitors is expected to confirm the potential of these drugs as valuable tools for the treatment of depression as well as some forms of anxiety.

C

SULFATIDE -A POTENTIAL ANTIGEN INVOLVED IN THE DEVELOPMENT OF RETINOPATHY AND NEUROPATHY IN DIABETES TYPE 1

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It has been suggested that there is a common antigen between endocrine pancreas and nerve tissue involved in the pathogenesis of insulin dependent diabetes mellitus(IDDM).. We are presenting data suggesting that the glycolipid sulfatide might be such a shared antigen. Increased titers of sulfatide antibodies have, by us and others, been found in serum from patients with neuropathy. Anti-sulfatide antibodies was found in serum from 88% of 57 patients with newly diagnosed IDDM and 76 % of these patients were positive 6 months later (titers >1:400). All 136 healthy controls, age matched blood donors, were negative (titers <1:400) (Lancet 342, 840, 1993). The sulfatide reactivity was recovered in the IgG fraction using protein A column chromatography. Immunohistochemical analyses of the tissue distribution of sulfatide was performed with a monoclonal antibody specific for sulfatide, sulfated lactosylceramide and seminolipid. This antibody recognise the lipid part of the antigen and thus do not react with sulfated glycoproteins. The staining correlated to organs affected in this disease. In the human, pig, rat and mouse eye we found in the choroid layer a distinct spotted staining, by electronmicroscopy shown to be pericytes, cells known to disappear during development of retinopathy. In the nerve tissue myelin associated structures were most intensively stained. Biochemical analyses or retina, nerve tissue and pancreas revealed only one antigen, sulfatide. Thus, sulfatide antibodies sulfatide may play a role in development of retinopathy and neuroapthy.

D

BRAIN SPECIFIC PROTEINS IN CSF AS DIAGNOSTIC TOOLS

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In the clinical setting the main effort in CSF diagnostics is devoted to the measurement of blood-brain-barrier dysfunction, IgG concentrations and oligoclonal IgG bands, as well as cell count and cytology. However, achievements in the field of neuroscience opens new perspectives and we have chosen to use "brain specific" proteins as markers of various cell populations in the CNS. A sensitive ELISA for determination of glial fibrillary acidic protein (GFAp) has been developed. Reference levels in the CSF range from very low in children (15-100 pg/ml) to 500-1500 pg/ml at the age of 80 years. Levels were intermediately to substantially increased among patients with cerebral infarctions, encephalitis and asphyxia. In chronic disorders levels were moderately to intermediately increased (multiple sclerosis, infantile autism, Alzheimer's disease, vascular dementia, low pressure hydrocephalus and neuroborreliosis).

GFAp is the structural protein of the astroglial filament. The increased CSF levels observed in the different patient groups either reflect astrogliosis and/or destruction of astrocytes. To further characterise the astroglial response in some of these diseases, the CSF S-100 protein concentrations were determined. Acute disorders such as brain infarctions caused a transient increase of both proteins in CSF whereas chronic disorders with astrogliosis were associated with a predominant GFAp increase.

We will also report preliminary results of the neuronal marker proteins neuron specific enolase and neurofilament protein.

A

MEMBRANE LIPIDS, SIGNAL TRANSDUCTION AND AGING: IMPLICATIONS FOR NEUROBIOLOGY.

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We have demonstrated an age-related decline in the ability of lymphocytes and neutrophils to respond to agonist-induced stimulation. This is reflected in a diminished proliferative response in lymphocytes and an impaired ability to generate superoxide in neutrophils. These declines are associated with highly significant impairments in the ability of these cells to generate the second messengers inositol tris phosphate (IP_3) and diacylglycerol (DAG). Mobilization of internal calcium stores and influx of extracellular calcium is also compromised. Impaired autophosphorylation of critical tyrosine kinases is also noted. We have compelling evidence that alterations in membrane lipid composition plays a critical role in these declines in cellular function. A reduction in the concentration of polyphosphoinositride bisphosphate accounts for the impaired ability to generate IP_3 and DAG. A decreased concentration of cellular myristate results in impaired function of critical tyrosine kinases involved in cell activation. Of particular importance is the in vivo and in vitro observations that age-related declines in lymphocyte function can be corrected by feeding a diet high in saturated fats or by exposing lymphocytes to myristate rich medium in vitro. Work by others has demonstrated age-related alterations in the membrane lipids of cells of the neurologic system and improved function by dietary manipulation. A detailed understanding of the mechanism whereby age-related alterations in membrane lipids should provide important insights into the fundamental mechanisms resulting in reduced cellular function with aging and age-dependent diseases. This in turn could lead to interventional strategies aimed at maintaining function and minimizing the impact of disease.

B

IgG-ANTIBODIES IN PATIENT CSF AS DIAGNOSTIC TOOLS

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The brain was earlier considered to be immunologically priviledged. Investigations during the last decade have shown, however, that brain is patrolled by immunocompetent cells. Cells normally residing in the CNS have capacity to antigen presentation, e.g. astro- and microglia.

High levels of autoantibodies have been observed in brain disorders. Using rat brain sections as a "fishing net" for detecting neuron specific antibodies we found that CSF of Parkinson's disease (PD) patients contain antibodies which recognize DA-ergic structures in rat brain sections, and these antibodies kill DA neurons in culture. Alzheimer disease (AD) CSF also contain IgG antibodies, recognizing cholinergic neurons. Later studies involved embryonic brain and neuronal cultures, and a large number of patient CSF have been screened. Thus, antibodies in the CSF may mirror the degenerating neuron species.

About 50 % of AD CSF recognize amoeboid microglia in embryonic brain and in tissue cultures. Cell cultures were used to screen CSF antibodies. Correlations with the staining and the diagnosis of 4 different dementia populations revealed that AD CSF displayed remarkable selectivity towards microglial cells, even in familial AD before clinical diagnosis. The results add support to the concept that inflammation and immune mechanisms may contribute to AD pathogenesis and may play a role also in other neurodegenerative diseases. It also provides a diagnostic tool for detecting early AD, important for perventive measures to be taken before too many neurons have died.

C

Regulation of Phenotype Expression in the Mesostriatal Pathway

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We have previously reported an increase in the expression of the gene coding for tyrosine hydroxylase (TH), the rate-limiting enzyme in the biosynthetic pathway for catecholamines, in subsets of dopamine (DA) neurons, related to the establishment of synaptic connections with their target cells in the striatum. We have also observed that certain gene products in the striatal neurons are expressed at the same time that TH mRNA is up-regulated. This suggested that DA projections from the SN are simultaneously conducting regulatory messages in both anterograde and retrograde directions. In order to test this, we conducted an experiment in which the catecholamine neurotoxin 6-hydroxydopamine was unilaterally injected into the SN of newborn rats. Forty-eight hours later this treatment had effectively eliminated the DA innervation to the striatum on the injected side, but failed to reduce the expression of NT or the calcium binding protein, calbindin, leading us to formulate an alternate hypothesis: Induction or regulation of these striatal phenotypes is a function of afferent innervation from non-DA areas. Postmortem injections of the carbocyanine dye DiO into the striatum of postnatal rats has subsequently demonstrated that at birth and through the first day, virtually all striatal afferents originated from nigral DA neurons. At two days, when activation and peptide expression are first observed in the striatum, a robust afferent innervation was observed to arrive from both cerebral cortex and ventral thalamus, in addition to the midbrain DA group, raising the possibility that input from these areas might influence expression of specific striatal phenotypes in the perinatal rat. Supported by the Israel Institute for Psychobiology (ZS) and the Clore Foundation (YS).

D

CHANGES IN PRESYNAPTIC Ca++ RESPONSIVENESS AFTER THE INDUCTION OF LONG-TERM POTENTIATION IN HIPPOCAMPAL NEURONS.

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Long-term potentiation (LTP) is an activity-dependent change in synaptic efficacy that may be essential to learning and memory in the mammalian brain. At hippocampal CA3-CA1 synapses, LTP is initiated by postsynaptic events that are relatively well-understood. In contrast, the mechanism(s) of expression of increased synaptic strength remain controversial. To address the site of action of the LTP process we have developed a culture preparation where mature CA3-CA1 hippocampal pyramidal neurons can be kept in vitro for prolonged periods of time after which they display robust LTP when synapses are challenged with glutamate, hypertonic solution or high [KCl]o. In this system the long lasting phase of LTP appears to involve alteration(s) in presynaptic neurotransmitter release as it is accompanied by an increased frequency of miniature synaptic events (minis). This synaptic enhancement might result from increased efficiency in some neurosecretory steps, such as those controlling vesicle availability or vesicle fusion and exocytosis. To further address possible changes occurring during LTP of mini frequency we developed a simple approach to manipulate intracellular Ca++ in the vast majority of synaptic terminals impinging upon a given postsynaptic cell, while monitoring quantal vesicular release. For these experiments we chose two different, but complementary, approaches: i) manipulation of presynaptic Ca++ entry via rapid variations in the [Ca++] of the bathing medium and ii) rapid changes in the bathing medium osmolarity, which presumably does increase internal Ca++ concentration. As graded stimulations induced graded quantal release of glutamate we were able to construct input output curves where release rate are expressed as a function of tonicity or external Ca++ concentration. In particular, at higher level of stimulation individual responses become biphasic as the initial high rate of release is not mantained and quickly decays back to a lower plateau level. The area of the initial peak probably reflects the number of readily available vesicles, while the plateau phase gives an indication of the ability of the system to refill empty sites. In these output curves we then express the peak - plateau charge tranfer and the τ of accomodation as a function of stimulation strength in various conditions. Using this approach, we have compared LTP of mini frequency to elevated mini frequency triggered by α-latrotoxin. In unpotentiated synapses, hypertonic solutions does greatly enhance mini frequency above that observed at basal, normotonic (200 mosm) levels. When the basal rate of release is augmented by LTP or α-latrotoxin, then the steady-state degree of response to high hypertonicity level is comparably reduced. Evidently, both LTP and α-latrotoxin increase basal release without elevating a ceiling attained by strong, sustained stimulation. Hypotonic challenges (250 mosm) presumably leading to a reduction in the $[Ca^{++}]_i$ did reveal interesting differences: after α-latrotoxin (as in the unpotentiated case), hypotonicity was largely ineffective in suppressing basal mini frequency; on the contrary during LTP hypotonicity decreased mini frequency several-fold. The α-latrotoxin results suggest that some release sites lost an inhibitory constraint and support a $[Ca^{++}]_i$-independent exocytosis (Ca^{++} independent minis): the resulting pedestal of mini frequency is then insensitive to hypotonicity. In contrast, mini LTP appears associated with a leftward displacement of the intermediate part of the stimulus-response curve at steady-state , toward lower values of tonicity. This findings appear not compatible with an increase in Ca^{++} independent exocytosis as the mechanism behind LTP. Furthermore, the mini frequency LTP cannot be explained by an increase in vesicle availability because this would lead to an increase in the release ceiling attained with strong hypertonic solution. This is also confirmed by a conservation of the maximal peak area . These data seem partially consistent with an enhanced $[Ca^{++}]_i$-sensitivity of the secretory machinery. At the moment we are also testing possible alternative explanations by computer modelling: among the various currently tested hypothesis we are also considering a reduction in the undocking reaction, that could manifest itself only for low level of stimulation.

A

SUBCELLULAR ANALYSIS OF Ca^{2+} HOMEOSTASIS IN NEURONS AND MYOCYTES

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Ca^{2+} is a ubiquitous second messenger, which transfers the information carried by of a variety of extracellular agonists (hormones, neurotransmitters, growth factors) into a large number of cell responses, such as secretion, motility, proliferation, etc. The Ca^{2+} signal exhibits a high degree of spatio-temporal organization, the significance of which is only partially understood. In this context, organelles play a key role, not only because they participate in cytosolic Ca^{2+} homeostasis, but also because the $[Ca^{2+}]$ in the lumen has been shown to control organelle functions (such as gene expression in the nucleus, protein sorting in the endoplasmic reticulum, ER, dehydrogenases activity in the mitochondria). We have developed a new methodology for the measurement of $[Ca^{2+}]$ within defined compartments of living cells, which is based on the modification of the cDNA for the Ca^{2+}-sensitive photoprotein aequorin, in order to include organelle specific targeting sequences, and on the recombinant expression of the chimeric photoprotein in cultured cells. Three chimeras were constructed, which allow, upon transfection of cultured cells, the monitoring of Ca^{2+} concentrations in the mitochondria ($[Ca^{2+}]_m$), nucleus ($[Ca^{2+}]_n$) and ER of living cells ($[Ca^{2+}]_{er}$). In the first and second case, a mitochondrial presequence and a typical nuclear localization signal, respectively, were added to aequorin. In the case of the ER probe, the photoprotein was fused to an immunoglobulin domain, which binds specifically to a resident ER protein. In this contribution, we describe the use of these probes for the study of Ca^{2+} homeostasis in neurons and myocytes. Cell stimulations coupled to rises in cytosolic Ca^{2+} concentration invariably result in increases of $[Ca^{2+}]_m$ and $[Ca^{2+}]_n$, with different kinetics and amplitudes. In neurons, large changes of $[Ca^{2+}]_m$ are evoked by the activation of plasma membrane channels, while in myotubes large $[Ca^{2+}]_m$ changes are associated to Ca^{2+} release from sarcoplasmic reticulum. $[Ca^{2+}]_n$ closely follows cytosolic Ca^{2+} both in kinetics and amplitude. Finally, a large drop in $[Ca^{2+}]_{er}$ occurs upon depletion of intracellular Ca^{2+} stores, though the kinetics of this decrease are complex.

B

In vivo phosphorylation of the Na,K-ATPase α-subunit in rat sciatic nerves: role of calcium and protein kinases

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In view of the critical importance of the Na,K-ATPase in the ionic homeostasis in excitable tissues including neurons, it is important to elucidate the regulatory mechanisms that govern its activity. In particular, the question arises whether Na,K-ATPase is itself a direct target for protein kinase C (PKC) or the cAMP-dependent protein kinase (PKA). In the present study, the phosphorylation state of the Na,K-ATPase α-subunit and its sensitivity to modulators of protein kinases has been examined in endoneurial preparations of rat sciatic nerves. Nerves metabolically labeled (60 min) with ^{32}P-orthophosphate were challenged (15 min) with protein kinase modulators in the presence or absence of extracellular calcium, and α-subunit ^{32}P-labeling was analyzed by SDS-PAGE and fluorography after immunoprecipitation. Treatment of the nerves with the PKC activator phorbol-myristate-acetate (PMA) barely changed the α-subunit ^{32}P-labeling. In contrast, application of the PKC inhibitor staurosporine decreased the ^{32}P-labelling of the enzyme. Interestingly, extracellular calcium omission also led to a decrease in the ^{32}P-labeling and PMA application under these calcium-free conditions restored labeling to basal level. These observations suggest that in the intact rat sciatic nerve, the Na,K-ATPase α-subunit is phosphorylated in a tonic manner by a Ca^{2+}-dependent process involving PKC. Treatment of the nerves with the cAMP-raising agent forskolin reduced the α-subunit ^{32}P-labeling, suggesting that PKA participates in the negative modulation of the phosphorylation process. Since forskolin was shown to stimulate $^{45}Ca^{2+}$ efflux and to decrease $[Ca^{2+}]_i$ in peripheral nerve, we tested whether these effects could explain the results of Na,K-ATPase phosphorylation. Indeed, when forskolin was added in Ca^{2+}-free buffer its inhibitory effect was not additive to calcium removal alone. Finally, the rapid reduction of the ^{32}P-labeling caused by both staurosporine and forskolin is suggestive of the presence of a strong protein phosphatase activity in the vicinity of the pump. Therefore, the effect of the protein phosphatase inhibitor okadaic acid was tested on the basal labeling of the α-subunit. Okadaic acid treatment enhanced the ^{32}P-incorporation in the α-subunit by 3 fold. In conclusion, Na,K-ATPase phosphorylation in the rat sciatic nerve appears to be tonically controlled via the regulation of PKC activity. The latter is proposed to depend on the rate of diacylglycerol production by the nerve phospholipase C under the influence of both $[Ca^{2+}]_i$ and PKA activity. The dynamic phosphorylation state of the α-subunit would thus result mainly from the balance between a strong phosphatase activity and the degree of PKC activation.

C

ZINC APPEARS IN METABOLISING CORTICAL SLICES INDUCED BY GLUTAMATE AND NMDA : AN NMR STUDY.

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Our previous studies using the ^{19}F-magnetic resonance indicator for calcium (5FBAPTA) showed an increase in free intracellular calcium ($[Ca^{2+}]_i$) in actively metabolising whole tissue preparations of the guinea pig cerebral cortex resulting from depolarization, combined hypoxia and hypoglycaemia, and after exposure to NMDA. We have now studied the effects of glutamate and more prolonged exposure to NMDA. Glutamate (0.5 or 1 mM) caused a 75-100% increase in $[Ca^{2+}]_i$, and a new resonance was attributed to zinc, which was suggested by its specific chemical shift in the ^{19}F-MR spectrum and confirmed from its disappearance in the presence of a high affinity chelator of heavy cations, TPEN. The appearance of zinc occurred with or just after the rise in $[Ca^{2+}]_i$, and was independent of Mg^{2+}. NMDA, NMDLA and NMLA (10-200 μM) all caused a slower increase in $[Ca^{2+}]_i$, and zinc was observed in some but not all experiments. When present, zinc appeared later than the increase in $[Ca^{2+}]_i$. These changes were also independent of Mg^{2+}. Zinc was not observed when $[Ca^{2+}]_i$ was increased by depolarization or by metabolic deprivation. Decreases in both PCr and ATP occurred in all of these studies. The results are discussed in terms of the proposed role of zinc as a modulator of excitotoxicity. Observations of zinc after exposure to glutamate or to NMDA, but not after depolarization or deprivation of glucose and O_2 (where increases also occur in $[Ca^{2+}]_i$), suggest that the cellular damage caused by the latter insults (depolarization and fuel deprivation as in ischaemia) involves mechanisms not solely attributable to release of excitotoxins.

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D

LONG LASTING CHANGES IN THE FREQUENCY OF INTRACELLULAR CALCIUM OSCILLATIONS IN ASTROCYTES

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Mixed primary cultures of astrocytes and neurons from the rat visual cortex were loaded with the Ca^{2+} indicator fura-2 and analyzed by the fluorescence video imaging technique. Perfusion of the cell monolayer with the neurotransmitter L-glutamate caused in astrocytes sustained, regular, oscillations of cytosolic Ca^{2+} concentration, $[Ca^{2+}]_i$. These oscillations were insensitive to NMDA and AMPA receptor antagonists and were induced also by the selective metabotropic agonist 1-aminocyclopentane-1,3-dicarboxylic acid (t-ACPD). Removal of the stimulus for a period ranging from 1 to 60 minutes and restimulation with the same glutamate concentration induced another oscillatory response, but with an increased frequency. Repetitive (up to 4) stimulations with this protocol resulted in a continuous increase in the oscillation frequency. Local brief (100 ms) pulses of glutamate applied at a frequency of 1 Hz to individual astrocytes elicited $[Ca^{2+}]_i$ oscillations whose frequency increased following a second series of pulses. A similar stimulation protocol, but with a pulse frequency of 0.1 Hz, did not induce $[Ca^{2+}]_i$ oscillations, nor modifications of the response pattern following repetitive stimulations. The present findings represent the first example of a long-term modification induced by a neurotransmitter in a non neuronal cell.

A

TRANSLOCATION OF PROTEIN KINASE C_γ AFTER INDUCTION OF LTP *IN VIVO* DEPENDS ON THE ACTIVATION OF METABOTROPIC GLUTAMATE RECEPTORS

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Protein kinase C (PKC) has been frequently cited as a candidate for playing a crucial role in cellular mechanisms underlying long-term potentiation (LTP), a long-lasting activity-dependent increase in synaptic transmission. In this study the possible involvement of the Ca^{2+}-dependent PKC isoenzymes α/β and γ was investigated 5 and 15 minutes after tetanic stimulation of the perforant path *in vivo*. In respect to the different physiological significance of the EGTA-extractable membrane-associated PKC and the detergent-extractable membrane-inserted PKC, our biochemical experiments focussed on a possible redistribution of PKC immunoreactivity between cytosol and the two membrane fractions.
Fifteen minutes after induction of LTP the γ PKC isoenzyme was redistributed to the cytosolic fraction relative to unstimulated paired controls in the dendate gyrus as well as in the combined CA1-CA3 hippocampal regions. This increase in cytosolic γ PKC immunoreactivity was prevented in animals injected with (R,S)-α–methyl-4-carboxyphenylglycine, a competitive antagonist of G protein-coupled metabotropic glutamate receptors (mGluR's) which blocks the potentiation at perforant path/dendate gyrus synapses. We have never observed a translocation of α/β PKC to cytosolic or membrane fractions when analyzed in the same samples. These results strongly implicate a role of postsynaptic mGluR's in LTP and support the idea of distinct physiological roles of different PKC isoenzymes in synaptic plasticity.

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B

METABOLISM OF U-[^{13}C] GLUTAMATE TO GLUTAMINE AND LACTATE IN CORTICAL SLICES : AN NMR STUDY.

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Guinea pig cerebral cortical slices were incubated with 0.2 mM U-[^{13}C] glutamate in the presence of 10 mM glucose for 3 hr. Samples of the incubation media were taken at 1 hr intervals, and PCA extracts of the tissue were prepared at 3 hr. ^{13}C-MR spectroscopy revealed clear signals from glutamine and lactate in the media, and from glutamate, glutamine, aspartate and GABA in the tissue extracts. The lactate released to the medium showed a doublet in C-3 (i.e. label also on adjacent C-2) and a multiplet in C-2 (label also on adjacent C-1 and C-3) confirming that the lactate must have arisen from the exogenous glutamate, since if it were due to naturally abundant ^{13}C in the lactate, only singlets would have appeared. The isotopomer patterns of the glutamine and lactate were qualitatively similar to those observed by Sonnewald et al, 1993 (*J. Neurochem.* **61**, 1179-1182) in cultured astrocytes. However the doublet of the glutamine C-3 (i.e. C-3 with C-4 *or* C-2) was higher relative to the triplet (i.e. C-3, C-2 *and* C-4) in the intact tissue than in the astrocytes. This doublet of the C-3 which indicates the relative amount of incorporation of ^{12}C (mainly from unlabelled glucose first into C-4, then C-2) of the glutamine released to the medium over 3 hr accounted for 30 % of its total label. The % ^{13}C enrichments confirm the conclusions of Sonnewald et al that there is higher incorporation of ^{13}C from exogenous glutamate into lactate than into glutamine.
We are grateful to Ursula Sonnewald for the gift of U-[^{13}C] glutamate and to the M.R.C. for research support.

C

SEROTONIN COUNTERACTS THE NMDA-EVOKED ARACHIDONIC ACID RELEASE IN THE HIPPOCAMPUS

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Serotonin receptor systems whose activation leads to a hyperpolarization of the membrane and to a decrease of the cell's input resistance can be considered as functional antagonists to the activation of NMDA receptors. It is known from the literature that serotonin $5HT_{1A}$ receptor agonists reduce the input resistance of neurons in the hippocampus. In this study we have investigated the effect of blockade of serotonergic receptor $5HT_2$ and activation of $5HT_{1A}$ on glutamatergic receptor(s) evoked arachidonic acid (AA) release in the hippocampus. It was observed that stimulation of glutamatergic receptor in adult brain hippocampal synaptoneurosomes induces statistically significant liberation of AA from prelabeled lipid. The effect of glutamatergic receptor agonists, as 1 mM glutamic acid added together with glycine in the absence of $MgCl_2$ and the action of 100 uM NMDA was abolished by inhibitor of receptor–operated calcium channels, MK–801, and by inhibitor of phospholipase A_2, quinacrine. Serotonin at 10 uM added together with 10 uM pargyline decreases significantly activated by glutamic acid and NMDA AA release. The effect of serotonin was not altered by ketanserin, antagonist of $5HT_2$ receptor. More effective action as serotonin itself exerts agonist of $5HT_{1A}$ receptor, which potently eliminates the action of excitatory amino acids and in this way may protect the brain against AA accumulation.

D

DIFFERENTIAL EXCITOTOXIC EFFECTS OF GLUTAMATE AND ITS ANALOGUES ON RETINAL CELL TYPES IN VITRO

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Tissue culture models of retinal neurons (RN) (primary cultures from newbom rats) or Müller glia (RMG) (early passaged cultures from young rats) were established using published methods. Addition of 250μM glutamate (Glu) reduced the total neuronal count by 40%, affecting immunocytochemically identified bipolar and amacrine cells whereas photoreceptors appeared unharmed. Measurement of lactate dehydrogenase activity in treated cultures confirmed the loss of cells. Addition of 250μM kainic acid (KA) only slightly reduced total neuronal numbers, and appeared selective for amacrine cells (amacrine cells represent 13% total neurons in controls, 2.3% in KA tests, or 90% reduction). Ganglioside analysis of KA-treated neuronal cultures revealed a simpler profile compared to controls, containing large amounts of GM3 and lesser quantities of complex GG. Addition of KA up to 10mM had no visible effect on RMG growth in vitro. However, 1mM KA was toxic for RMG in whole retina. Glu showed a dose-dependent effect between 1 and 10mM on RMG proliferation as measured by ^{3}H-thymidine uptake: 1mM Glu = control (100%); 2.5mM Glu, 74%; 5mM Glu, 49%; 10mM Glu, 26%. These data reflect the presence of different Glu receptors on distinct retinal cell types. Different neuronal populations appear to retain similar spectra of actions to their in vivo counterparts, while RMG seem to be less sensitive in vitro than in vivo. These culture models should be useful in testing the effects of other Glu analogues, such as NMDA and AMPA, as well as possible beneficial effects of different trophic factors and pharmacological agents on retinal excitotoxicity.

A

INHIBITORS OF AMINO ACID UPTAKE INTO VESICLES

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We have examined a series of potential inhibitors of amino acid uptake into vesicles. Evans Blue and Chicago Blue was by far the best competitor inhibitors of glutamate uptake and their Ki was in the nanomolar range. It seems that 2 sulphuric acid groups separated by 2-carbon atoms and aromatic rings are essential for inhibition. Amino groups are essential for inhibition, but their spatial position seems less important. The compounds donot affect the membrane potential or ΔpH at this concentration.

Evans Blue and Chicago Blue will also inhibit uptake of GABA, but at a concentration which is 1000 fold higher than for glutamate.

Several GABA analogues were inhibitor of GABA and glycine uptake. So fas no inhibitors are of the same efficacy as the glutamate inhibitors. The resiprocal inhibition of GABA and glycine was also found in fish and avian species, confirming our previous finding in rat. The vesicles from fish and avian also took up β-alanine. Thus GABA and glycine may be taken up by the same transporter into vesicles.

B

INTERACTION OF S-ALKYL GLUTATHIONES WITH THE NMDA RECEPTOR-IONOPHORE

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The mechanism of neuroprotection of glutathione, the most abundant thiol peptide in the brain, is still unknown. The antagonists acting at the NMDA receptor-ionophores are potentially neuroprotective. We have earlier shown that both reduced and oxidized forms of glutathione inhibit the NMDA-induced influx of Ca^{2+} into neurons either by displacing glutamate from the NMDA recognition site or by stabilizing the redox state of this receptor-ionophore. We now studied the effects of some alkylated analogues of glutathione on the binding of glutamate, glycine and MK-801 (ligands for the agonist-, coactivator- and within-channel sites, respectively) to brain synaptosomal fractions and on the glutamate-activated influx of Ca^{2+} into neurons. S-Methyl-, S-ethyl-, S-propyl-, S-butyl- and S-pentylglutathione strongly inhibited the Na^+-independent binding of glutamate, being more effective than reduced or oxidized glutathione. The peptides failed to affect the binding of glycine but slightly activated that of MK-801. Their activatory effects were additive to that of glycine. All peptides significantly inhibited the activation of MK-801 binding by glutamate or by glutamate together with glycine. Except for S-methyl-glutathione, the peptides inhibited the Ca^{2+} influx into neurons evoked by glutamate. We conclude that S-alkyl analogues of glutathione are promising NMDA antagonists, acting mainly at the agonist recognition site.

C

SIDE-CHAIN MODIFICATION OF AMINO ACIDS IN THE NMDA RECEPTOR-IONOPHORE ALTERS THE BINDING OF LIGANDS

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Functional groups of amino acids are important for the selectivity of ion channels and for the ligand recognition in the glutamate receptors. We tested now how the modification of cysteine and histidine side chains by N-ethylmaleimide (NEM) and diethylpyrocarbamate (DEP), respectively, affects the binding of glutamate (GLU) and MK-801 to pig brain membranes. A pretreatment of the membranes with NEM dose-dependently increased the binding of [^{3}H]glutamate. The binding to the NMDA-sensitive site was enhanced more than that to the kainate- or AMPA-sensitive site. The basal binding of MK-801 was inhibited by 70 %. However, the activation of binding by glycine (GLY) and GLU was even enhanced. DEP increased the binding of GLU and decreased that of MK-801. The activation of MK-801 binding by GLU and GLY was attenuated. The pretreatment with NEM followed by DEP decreased both GLU and MK-801 binding. The enhancement of GLU binding by the modification of cysteine and histidine side chains indicates that these amino acids are not present in the GLU binding sites but probably located near to the ion channel. They may regulate the opening and closing of the NMDA receptor-governed Ca^{2+} channel labelled by MK-801.

D

EFFECTS OF GLUTAMATE ON CULTURED CEREBRAL ENDOTHELIAL CELLS

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Jorgensen and Diemer proposed in 1982 (Acta Neurol. Scand. 66:536-546) that glutamate is involved in the production of ischemic damage in the hippocampus. Direct measurements with intracerebral microdialysis showed (Benveniste et al., J. Neurochem. 43:1369-1374, 1984) the elevation of extracellular concentrations of glutamate and aspartate during transient cerebral ischemia.

The aim of our studies was to establish if glutamate could influence the state of phosphorylation of Calcium/calmodulin-stimulated protein kinase II [CaM-PK II], a multifunctional kinase with broad substrate specificity, which was shown (Deli et al., J. Neurochem. 60:1960-1963, 1993) to be present in cerebral endothelial cells. The experiments were carried out at day in vitro 7 on confluent rat brain endothelial cultures grown on petri dishes coated with collagen. Cultures were exposed to 2 mM glutamate for 30, then possible changes in phosphorylation pattern were checked at 0, 10 60 minutes after exposure. Significant increase in the phosphorylation of CaM-PK II was observed 0 and 10 minutes after 30 minutes exposure with glutamate, which could be prevented by pretreatment with MK-801 (100 μM). These results confirm the finding of Koenig et al. (Brain Res., 558:297-303, 1992) on the occurrence of NMDA-receptors on cerebral endothelial cells, and suggest that these receptors may take part in the reactions of cerebral endothelial cells in ischemic brain oedema.

A

NMDA AND AMPA RECEPTORS IN THE DEVELOPMENT AND PLASTICITY OF THE BARREL CORTEX OF THE MOUSE

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It is believed that NMDA and AMPA receptors are involved in mechanisms of cortical plasticity. The ontogeny and distribution of the NMDA and AMPA receptor sites in the barrel cortex, the cortical representation of mystacial vibrissae, was examined in the mouse by quantitative autoradiography and membrane binding. Initially low binding of [^{3}H]MK 801 sharply increased during the second week of postnatal life reaching the adult level by the end of the fourth week. Scatchard analysis indicated that this rise of [^{3}H]MK 801 labeling was due to an increase in the number of binding sites. In adult the highest binding was found in supragranular layers. In layer IV of the SI cortex, the pattern of labeling resemble pattern of morphologically identified barrels field. AMPA receptor binding sites after initial low levels rose reaching the peak values at p.d. 14. At 70 p.d. a significant decline of binding was noted. The laminar differences were poorly pronounced. Adult unilateral removal of all vibrissae or denervation of selected rows of vibrissae did not alter the pattern and intensity of [^{3}H]MK 801 labeling. Time course of developmental changes of NMDA and AMPA receptor sites suggested that NMDA and AMPA receptors are not directly involved in early morphogenetic plasticity of the barrel cortex.

B

NMDA RECEPTOR-MEDIATED INTRACELLULAR $^{45}Ca^{2+}$ MOBILIZATION IN RAT BRAIN IN VIVO

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Receptors for N-methyl-D-aspartate (NMDA) mediate massive calcium influx to brain neurons that has been implicated in neuronal plasticity and also in excitotoxic cell damage in several neurodegenerative disorders. Recent in vitro data indicate that apart from calcium influx from the extracellular space, NMDA receptors may mediate Ca^{2+} mobilization from intracellular pools. This subject was not studied in vivo. Here we report the results of microdialysis experiments on the adult rats, aimed at the detection and characterization of intracellular Ca^{2+} mobilization in different brain regions. Microdialysis probes (CMA/11, Microdialysis AB, Stockholm) were implanted stereotaxically to the hippocampus, striatum and thalamus of urethane-anesthetized rats under post hoc morphological control. NMDA was given in the dialysis medium. Changes in extracellular Ca^{2+} concentrations were detected by direct measurements of ionized Ca^{2+} in initially calcium-free dialysates. Moreover ^{45}Ca efflux from the prelabeled endogenous Ca^{2+} pools was measured. [^{14}C]sucrose was used as an indicator of changes in the extracellular space volume. The application of 5 mM NMDA for 20 min to the striatum did not change significantly ^{45}Ca and Ca^{2+} concentrations, whereas in the hippocampus, particularly in CA4/DG, and in the thalamus, an approx. 50% decrease in the Ca^{2+} concentration was accompanied by a huge increase in ^{45}Ca efflux, exceeding the basal level in the hippocampus by 1500%. This effect was dependent on NMDA concentration and sensitive to NMDA receptor antagonists. In all the studied brain regions NMDA application resulted in only approx. 15% increase in the [^{14}C]sucrose efflux reflecting cellular swelling and corresponding reduction of the extracellular space volume, thus indicating that this effect can not explain a huge increase in ^{45}Ca efflux. Our results suggest that in vivo in several rat brain regions NMDA induces the release of ^{45}Ca from the neurons, originating from different intracellular Ca^{2+} pools. The participation of calcium bound to the calcium-binding proteins, and of IP3 and ryanodine-sensitive ER pools will be discussed.

C

ROLE OF GLUTAMATERGIC INPUT IN THE NICOTINIC MODULATION OF STRIATAL ACETYLCHOLINE RELEASE

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Previous studies of our laboratory have shown that nicotine failed to increase ACh release from striatal slices when the dopaminergic input was not impaired but it enhanced the release from slices dissected from 6-hydroxydopamine pretreated rats or in the presence of sulpiride. We concluded that somatodendritic nicotinic receptors exist on striatal cholinergic neurons. The question arises whether nicotine acts directly on the striatal cholinergic interneurons or glutamate mediates the nicotinic effect on the ACh release through nicotinic receptors located on the terminals of the corticostriatal tract.

Therefore we investigated the effect of nicotine on acetylcholine release from striatal slices of the rat in the presence of *N*-methyl-D-aspartate (NMDA) receptor antagonist, MK-801. Experiments were carried out on isolated superfused striatal slices of male Wistar rats, loaded with [^{3}H]choline. Drugs were applied 90 minutes after the start of the perfusion. Transmitter release was measured by radioassay. We found that NMDA receptor inactivation by MK-801 led to a further enhancement of striatal tritium outflow indicating that the effect of nicotine on the release of acetylcholine occurs on the cholinergic neuron directly not through the activation of the release of glutamate. Our results corroborate the earlier reports of somatodendritic localization of nicotic receptors on the cholinergic neurons and the involvement of the dopaminergic and glutamatergic inputs in the action of nicotine on the striatal transmitter relase.

D

CHANGE IN THE CONCENTRATIONS OF AMINO ACIDS IN CSF AND SERUM OF PATIENTS WITH ESSENTIAL TREMOR

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The concentrations of amino acids in cerebrospinal fluid (CSF) (n=19) and serum (n=10) taken from patients with essential tremor were measured using HPLC and compared with those of controls (n=15). Reduced concentrations of some inhibitory amino acids (serine $p<0.05$, glycine $p<0.05$) were observed in both fluids taken from patients with tremor. Significant increase was detected in the concentrations of glutamate ($p<0.001$) and aspartate ($p<0.01$). The tendency of changes in CSF and serum was similar, nevertheless highest difference in amino acids concentrations in serum of patients with essential tremor was observed. This study arises the possibility that a genetically determined metabolic disorder may be involved in the etiology of essential tremor. The slight increase in the concentration of glutamate together with reduced levels of GABA, glycine and serine in CFS may form the neurochemical basis of the central oscillation observed in essential tremor.

A

IMMUNOCHEMICAL DETECTION OF GLUTAMATE RECEPTOR SUBTYPES IN RAT BRAIN

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Polyclonal antibodies were made against synthetic peptides corresponding to specific sequences of the large extracellular amino terminal part of the GluR1, overlapping regions of GluR5/6/7 and KA-1/2 and the carboxy terminus of GluR1, GluR5, GluR7, KA-1, KA-2 and NMDAR1 glutamate receptor subunits. Peptides were used directly for immunisation (multiple antigenic peptides) or after coupling to ovalbumin or thyroglobulin with glutaraldehyde (monomeric peptides) and either injected subcutaneously or into the rear marginal ear vein of rabbits following co-adsorption with an adjuvant peptide to colloidal gold particles, which causes rapid and massive immune responses.

Antibodies to GluR1, GluR5, GluR7 and KA-1/2 were able to recognize the solubilized receptor subunit in a native conformation, as demonstrated by selective immunoprecipitation of [^{3}H]AMPA or [^{3}H]kainate binding activity. After purification the antibodies selectively recognized the peptides to which they were raised and stained bands on immunoblots of rat brain membrane fraction corresponding to the molecular weights of the native subunit. These antibodies could be used for immunoprecipitation of the detergent solubilized hetero-oligomeric complexes of the glutamate receptor, and for the analysis of their subunit composition.

B

EFFECTORS OF NEUROTRANSMITTER GLUTAMATE RELEASE FROM RAT CEREBELLAR SLICES.

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The effect of aminooxyacetate (AOAA, 5mM), phenylsuccinate (PhS, 5 mM), NH_4^+ (4mM), ketone bodies (3mM), glutamine (1mM) on the K^+ stimulated Ca^{2+} dependent release of D-[^{3}H]- aspartate from rat cerebellar slices was studied by superfusion. AOAA, PhS or NH_4^+ increase the release of D-[^{3}H]-aspartate by 30% ($p<0.05$), 188% ($p<0.01$) and 68% ($p<0.05$) respectively. Ketone bodies or glutamine have no effect. Increase in the K^+ stimulated Ca^{2+} dependent release of D-[^{3}H]- aspartate corresponds to a decrease of glutamate release (1). Therefore AOAA, PhS or NH_4^+ inhibit glutamate Ca^{2+} dependent release. Because NH_4^+ is a strong inhibitor of phosphate activated glutaminase (PAG) and ketone bodies are inhibitors of glycolysis these results show that in the cerebellum glutamine is a major precursor of neurotransmitter glutamate and glucose is not. That glutamine has no effect is probably due to the replenishment of the glutamine pool through the uptake of glutamate by glia and the glutamine synthetase reaction. The inhibitory actions of AOAA and PhS are in line with previous experiments (1) with cerebellar granule cells, supporting the suggestion that glutamate from glutamine has to enter the mitochondrion before supplying the neurotransmitter pool.

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C

GLUTAMATE- AND ISCHEMIA-INDUCED INHIBITION OF PROTEIN SYNTHESIS: NO EVIDENCE FOR A COMMON MECHANISM

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The neurotoxic effect of glutamate is considered to play a major role in cell damage in different pathological states of the brain, cerebral ischemia being one. The effects on protein synthesis of tissue exposure to glutamate or ischemia were tested in hippocampal slices in order to determine whether glutamate produces the same effects as glucose/oxygen deprivation (*in vitro* ischemia). 400-µm hippocampal slices were exposed to glutamate (1-10 mM) or ischemia for 15 min and protein synthesis estimated by measuring incorporation of ^{14}C-leucine into proteins immediately after exposure, 2 and 24 hours later. In addition, adenine nucleotides were measured in order to establish the energy status of slices. 1-mM glutamate did not have an effect on protein synthesis or energy status in hippocampal slices, neither immediately nor later on; blockade of glutamate uptake did not alter the situation, suggesting that extracellular glutamate concentration was not the limiting factor. 10-mM glutamate did inhibit protein synthesis for about 50% and reduced ATP levels as well, but these effects were reversed within the next two hours; in addition glutamate antagonists (MK801, NBQX, and 5-AP) did not counteract effects of glutamate, indicating that these effects were not receptor-mediated. In contrast, ischemia produced lasting inhibition of protein synthesis, and this inhibition persisted over 24 hours (30%, 50%, 70% of controls immediately after ischemia, 2 hours and 24 hours after ischemia, respectively); this inhibition could not be related to the energy deprivation, as adenine nucleotides essentially recovered within the first 30 min of reintroduction of oxygen and glucose. Ischemic inhibition of protein synthesis could not be reversed by glutamate antagonists. It is concluded that ischemia caused an inhibition of protein synthesis by mechanism which is not glutamate-receptor mediated.

D

RNA EDITING OF GLUTAMATE RECEPTOR SUBUNITS

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Editing of mRNA of glutamate receptor subunits GluR2, GluR5 and GluR6 is a reaction important for the control of calcium fluxes through ion channels of the non-NMDA receptor type: The calcium conductance of the channel is markedly reduced when the respective mRNA is completely edited. We developed a new technique to quantify the extent of mRNA editing in different developmental stages of the brain.

RNA was isolated from various brain regions of adult animals and animals at day 19 of gestation. RNA was reverse transcribed into cDNA which was taken as template for PCR using subunit-specific primers. PCR products were incubated with Bbv 1 which recognizes the base sequence of the non-edited receptor subunit around the edited base A (GCAGC) but not that of the edited subunit (GCGGC, A edited to G). Quantification of the extent of RNA editing was done by gel electrophoresis of the restriction digest and image analysis of the electrophoresis bands.

The GluR2 subunit was completely edited in the embryonic and adult state. Editing of the GluR6 subunit was above 90% in all brain structures studied consisting predominantly of gray matter, but it was only about 55% in white matter. In contrast, only 33 to 52% of GluR5 mRNA was edited in the cortex, striatum and hippocampus of adult animals but 70 to 80% in the thalamus and cerebellum. Interestingly, the extent of GluR5 mRNA editing was considerably less at day 19 of gestation as compared to the adult state; these differences were most pronounced in the cerebellum where the extent of GluR5 mRNA editing was 78% in adult brains but only 27% in the embryonic state. Knowledge of the extent of mRNA eding will add to our understanding of the function of non-NMDA glutamate receptors in different physiological and pathological states of the brain.

A

EFFECTS OF GM1 ON NMDA-INDUCED NEURONAL INJURY IN RABBIT HIPPOCAMPUS: IN VIVO MICRODIALYSIS STUDY

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Microdialysis was used to apply N-methyl-D-aspartate (NMDA) to the hippocampus of rabbits, control and pretreated with GM1 ganglioside (i.m. injections of 30 mg/kg for 3 days, twice a day). Calcium transients were followed by measurements of ^{45}Ca efflux from the prelabeled hippocampus and by direct detection of ionized Ca^{2+} in dialysates. Thromboxane B_2 and 6-keto-PG-$F_{1\alpha}$ were measured in dialysates. [^{14}C]sucrose was used to monitor changes in the extracellular space volume. Morphologic changes in the hippocampal neurons were examined after 24 h. 20-min, 1 mM NMDA application resulted in 1-h, max. 40% decrease ^{45}Ca efflux, in a brief, <20% increase in the [^{14}C]sucrose efflux and in a huge release of both eicosanoids to dialysis medium. A degeneration of pyramidal neurons of the hippocampus CA1 in the vicinity of a microdialysis probe was observed. In GM1-treated rabbits NMDA application induced a 70% increase in ^{45}Ca efflux, whereas [^{14}C]sucrose efflux did not differ compared to control. The NMDA-induced release of 6-keto-PG-$F_{1\alpha}$ to dialysate was reduced in 38%. Experiments with nominally calcium free dialysis medium showed that NMDA-induced a decrease in Ca^{2+} concentrations in the dialysates was reduced in GM1-treated rabbits. These results indicate that in the rabbit hippocampus in vivo GM1 activates the release of calcium from the NMDA-stimulated neurons, that partially counterbalances Ca^{2+} influx and reduces net calcium redistribution to neurons. Inhibition of 6-keto-PG-$F_{1\alpha}$ release suggests that GM1 stabilizes intracellular calcium homeostasis and reduces intracellular Ca^{2+} concentration. These mechanisms may participate in GM1-evoked protection of the hippocampal neurons from NMDA excitotoxicity.

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B

MECHANISM OF NMDA RECEPTOR-EVOKED REDUCTION OF ARACHIDONIC ACID INCORPORATION INTO PHOSPHATIDYL-INOSITOL IN RAT BRAIN CORTEX SYNAPTONEUROSOMES

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Recently, we have shown that glutamate induces inhibition of arachidonic acid (AA) incorporation into brain membrane lipids. In this study, we have investigated the effect of stimulation of several subtypes of glutamatergic receptors (AMPA, NMDA, kainate and quisqualate) on AA acylation of phosphatidylinositol (PI) in the absence and presence of Ca^{2+} in synaptoneurosomes. It was observed that mainly NMDA receptor activation leads to reduction of AA uptake into PI, whereas quisqualate receptor is not involved in this process. Due to determination of the mechanism of NMDA-dependent inhibition of PI acylation we have found higher effect of NMDA in the absence than in presence of Ca^{2+}. MK-801 increases inhibitory action of NMDA on Ca^{2+}-dependent AA uptake into PI. High extracellular K^+ or veratridine also reduces AA incorporation into PI and NMDA has no further effect. The Na^+ channel antagonist, tetrodotoxin, reverses action of K^+ and NMDA almost completely in the absence and partially in the presence of Ca^{2+}. The inhibitor of voltage-gated Ca^{2+} channels, conotoxin, exclusively in the presence of Ca^{2+} eliminates K^+ and NMDA mediated decrease of AA acylation of PI. Moreover, intracellular Ca^{2+} antagonist, TMB-8, abolishes NMDA effect on AA uptake into PI in the absence and presence of Ca^{2+}. Our results show for the first time, that NMDA by stimulation of voltage-operated Na^+ and Ca^{2+} channels and connected biochemical processes induces blockade of AA acylation of PI. This mechanism may be involved in mantaining of excessive level of free AA in the brain, that may have important implication in NMDA receptor-mediated neurotoxicity under pathological conditions.

C

EXCITOTOXINS AND ENERGY DEPRIVATION IN HIPPOCAMPAL SLICES

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The excitotoxic hypothesis attributes a major role to excitatory amino acids (EAAs) and their receptors in the neuronal damage that follows brain disorders where energy deprivation is involved (cerebral ischemia, hypoxia, hypoglycemia). A major premise of this hypothesis is that blockade of EAA receptors could protect neuronal tissue against the consequences of energy deprivation.

Energy-deprived hippocampal slices (Sprague-Dawley rats) were used to evaluate EAA receptor agonists and antagonists as enhancers or blockers of neuronal damage, respectively. Transverse slices (400 μm) were prepared and placed in a dual incubation/recording chamber. They were perfused with artificial cerebrospinal fluid (ACSF), supplied with a gas mixture of 95% O_2/5% CO_2 and temperature of 34°C. Hypoxia was produced by replacing O_2 with N_2 in the gas mixture. Glucose deprivation (GD) was produced by reducing the glucose concentration in the ACSF from 10 mM to 0.7 mM. EAA receptor agonists and antagonists were supplied via the ACSF. Electrically-evoked population spikes (neuronal function) were recorded before, during, and after an energy-depriving insult. A slice in which a response could not be evoked was considered to be neuronally damaged.

After exposure of slices to 10-min hypoxia or 75-min GD, 85% and 81% of the slices recovered their neuronal function, respectively. Ten μM NMDA had no effect on normoxic slices, but was toxic to hypoxic or glucose-deprived slices. The hypersensitivity of energy-deprived slices to excitotoxic compounds such as NMDA and glutamate, makes this *in vitro* preparation an excellent model system for the study and screening of EAA receptor ligands and their derivatives. Our studies suggest a role for the kainate/AMPA receptor in ischemic neuronal damage. We evaluated the potential of several aromatic and heterocyclic derivatives of EAAs as either agonists or antagonists of EAA receptors. In addition, quinolinate, an endogenous tryptophan metabolite, was found to be not only an excitotoxin but also a potentiator of the toxicity of other EAA receptor agonists. Finally, we found cysteate, cysteine, kainate and AMPA to differentially affect hypoxic and hypoglycemic slices. This prompted us to postulate the existence of mechanistic differences between these two conditions.

D

CHARACTERIZATION OF (^{3}H)GLUTAMATE BINDING IN MEMBRANES OF ENTORHINOHIPPOCAMPAL COMPLEX OF DEVELOPING RAT BRAIN INJURED BY I.C.V. INJECTION OF QUINOLINATE

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Quinolinic acid (QUIN), an endogenous excitotoxic metabolite of tryptophan with binding affinity to NMDA-type of glutamate (GLU) receptor, causes slowly progressing degeneration of vulnerable neurons. Excitotoxic injury was quantified by means of changes in (^{3}H)GLU binding to membranes isolated from the entorhinohippocampal complex 4 days after the injection of QUIN. Binding was significantly decreased only in the hippocampus of 30- and 50-day-old rats (by 25 and 32%, resp.). In contrast, binding to cortical membranes was significantly elevated (by 29 and 56%, resp.). Observed changes in the binding of GLU were caused by modifications in the equilibrium binding constants rather than by the density of the receptors. Further analysis revealed that both ionotropic (NMDA-type) and metabotropic (quisqualate-type) GLU receptors were involved in the changed GLU binding. The alteration of NMDA-type of the receptor was only evident when glycine was present. In the case of metabotropic GLU receptor, the sensitivity to L-serine-O-phosphate and L-AP3 was found. The results suggest that in 30- and 50-day-old rats QUIN is implicated in neurodegeneration of rat entorhinohippocampal complex in young adult rats and that both, ionotropic and metabotropic, receptors are involved.

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Key words: quinolinate - (^{3}H)glutamate binding - neurodegeneration - entorhinohippocampal complex - brain development

A

NITRIC OXIDE IS INVOLVED IN NMDA-EVOKED INHIBITION OF ARACHIDONIC ACID INCORPORATION INTO LIPID OF BRAIN CORTEX SYNAPTONEUROSOMES

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Our recent studies indicate that stimulation of glutamatergic receptor(s) induces reduction of arachidonic acid (AA) incorporation into brain cortex membrane lipid. In this study, we have investigated the role of nitric oxide (NO) in the NMDA receptor–mediated inhibition of AA uptake. The effect of NMDA receptor activation on AA incorporation was determined in the absence and presence of Ca^{2+} ions. Moreover, the effect of NO synthase (NOS) inhibitor, N^G–Nitro–L–arginine (NNLA), was studied in two different conditions: NMDA was added simultaneously with NNLA or after preincubation of synaptoneurosomes with this inhibitor for 15 min at 37°C. It was found that NMDA evokes significant reduction of AA incorporation in the absence and presence of Ca^{2+}. NNLA applied simultaneously with NMDA diminishes this inhibition. Receptor–operated Ca^{2+} channel blocker (MK–801) has no effect on the NMDA–evoked AA uptake inhibition into membrane lipid. When synaptoneurosomes were preincubated with NNLA (15 min before addition of agonist) NMDA induced more pronounce inhibitory effect and suppressed AA acylation by about 40–50% and NNLA has no further effect. Our results indicate that NO is involved in NMDA–dependent inhibition of AA incorporation into brain membrane lipid. NNLA protects the neurons against NMDA–evoked events exclusively if it acts simultaneously with glutamatergic agonist. Inhibition of NO synthase before stimulation of NMDA receptor potentiates the action of NMDA.

B

GLUTATHIONE IS AN ENDOGENOUS LIGAND OF NMDA RECEPTORS

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We have recently shown that a number of endogenous γ-glutamyl peptides interact with glutamatergic neurotransmission. The effect of glutathione, the most abundant γ-glutamyl peptide in the brain, was now subjected to study. Both reduced (GSH) and oxidized (GSSG) forms of glutathione inhibited the Na^+-independent, NMDA-sensitive binding of glutamate (GLU) to synaptic membranes, GSH being more effective of them. The strychnine-insensitive binding of glycine (GLY) was not affected. Both peptides dose-dependently enhanced the binding of MK-801. The effect of GSH was biphasic, tending to diminish at concentrations above 0.05 mM. The enhancement of MK-801 binding by GLY was potentiated by both peptides but they inhibited the effect of GLU and that of GLU together with GLY. The antagonist 7-Cl-kynurenate, acting at the GLY recognition site in the NMDA receptor complex, inhibited both the basal binding of MK-801 and the binding activated by GLY and enhanced by GSH. The binding of MK-801 was activated fastest by GLU followed by GSH, GSSG and GLY. The results indicate that GSH and GSSG bind to the recognition sites in the NMDA receptor but have no significant effects at the GLY sites. The actions are probably due to the γ-glutamyl moiety in these peptides.

C

LIPOXYGENASE AND CYCLOOXYGENASE INHIBITORS MODULATE THE EFFECTS OF FATTY ACIDS IN RAT BRAIN.

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Dietary manipulation studies in domestic cats have sown that post-delta-6-desaturation fatty acids (PDFA's) are required for normal brain structure and dopaminergic function. The effects of individual fatty acids have been studied using rat striatal slices. Three of the PDFA are the direct precursors of the three series of eicosanoids, and these were inhibitory of dopamine outflow at low, but stimulatory at high, concentrations. All other fatty acids were either stimulatory or had no effect. The eicosanoid synthesis blockers, nordihydroguaiaretic acid (NDGA) and indomethacin (INDO) were used to assess eicosanoid involvement. INDO did not alter the effects, whilst NDGA eliminated the inhibition of dopamine release. The effects of the three PDFA possibly related to their lipoxygenase products, but whether involving leukotrienes, HPETEs or HETEs is still unclear. Structural similarities between NDGA and dopamine also permit the effects to relate to competition for receptors to be a mechanism of action.

D

MODULATION OF DOPAMINERGIC NEUROTRANSMISSION BY FATTY ACIDS IN CAT AND RAT BRAIN.

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Long-chain polyenoic fatty acids are present in large amounts in neural tissues. Most mammal species are capable of the hepatic production of such fatty acids from dietary essential fatty acids (EFA's). This process utilises the desaturase enzyme cascade and produces both n6 and n3 post-delta-6-desaturation fatty acids (PDFA's). The Felinae however, lack the first enzyme, delta-6-desaturase and are thus dependant on a dietary supply of the PDFA. Long-term dietary deprivation of PDFA in domestic cats has induced modulation of both structure and dopaminergic neurotranmission in cat brains and has indicated requirements for both n6 and n3 PDFA, but for different functions. Subsequent incubation of rat brain slices in vitro with PDFA has shown different effects depending on whether the PDFA were direct eicosanoid precursors or not. Low eicosanoid precursor PDFA concentrations were inhibitory of dopamine release, whilst high concentrations of all PDFA were stimulatory. This indicated probable involvement of eicosanoids in the modulatory effects of the PDFA.

A

PROPERTIES OF ENDOGENOUS FACTOR ACTIVATING Na,K-ATPase INDUCED BY BLOCKADE OF ADRENOCEPTORS

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Previously a peripheric mechanism was found for the reverse transsynaptic regulation of catecholamine uptake and synthesis in adrenergic neurons, in which the initial links are postsynaptic adrenoceptors of the effector cell. Blocking these receptors leads to the formation and release from the effector cell of chemical regulatory factors. The latter reach the presynaptic membrane through the synaptic gap and produce an increase in the rate of transport into the adrenergic neuron of noradrenaline and its major precursor amino acid tyrosine. It was established that the regulatory factor released from the effector cells under the blockade of postsynaptic alpha- and beta-adrenoceptors by phentolamine and propranolol (0.5-1.0 μM) and activating noradrenergic uptake and synthesis increased the activity of Na,K-ATPase of the isolated rat organs and partially purified Na,K-ATPase isolated from the rat brain.The regulatory factor is a protein with MW 25 - 100 kDa, adsorbed on phenylsepharose CL-4B and eluated with 30 mM KCl. It consists of two poorly active components with pI 5.1 and 5.9. Their mixture (1:1) activates Na,K-ATPase by 60%.

B

CLONING AND FUNCTIONAL CHARACTERISATION OF THE HUMAN 5-HT_{2B} SEROTONIN RECEPTOR

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We have isolated a human 5-HT_{2B} receptor clone from a cDNA library derived from SH-SY5Y cells. Although the receptor sequence was only 80% homologous to the rat sequence (Foguet et al., 1992, EMBO J. 11, 3481-3487) the exon-intron distribution was conserved between the two species. After expression in HEK 293 cells, activation of the receptor stimulated the production of phosphatidylinositol. The pharmacology of this functional response correlated well with that of the rodent receptor. 5-HT_{2B} receptors are closely related to 5-HT_{2C} receptors. It has been suggested that activation of 5-HT_{2C} receptors is involved in the initiation of a migraine attack. Antagonist pA_2 values from the rat stomach fundus and pK_D values from a 5-HT_{2C} receptor binding assey both correlate highly significantly ($p<0.005$) with the daily dose of eight migraine prophylactic compounds. This implies that the 5-HT_{2B} receptor is an additional candidate for serotonin receptors involved in the onset of migraine attacks.

C

THE ROLE OF SEROTONIN RECEPTOR SUBTYPES IN SMOOTH MUSCLE CONTRACTION

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Serotonin is an important modulator of smooth muscle relaxation and contraction. This role of serotonin has been well documented in various blood vessels, including the pulmonary artery, cerebral artery, renal artery, coronary artery, jugular vein, and saphenous vein. Furthermore, it regulates the contraction of the gut and the rodent stomach fundus. Given the large number of cloned serotonin receptors (more than a dozen), it became increasingly difficult to discriminate their role in the function of serotonin using pharmacological procedures. We have designed specific PCR primers which distinguish between all cloned subtypes of serotonin receptors coupled to G-proteins. They were used to analyze the distribution of serotonin receptor subtype expression in different smooth muscle tissues. A specific set of receptor subtypes is apparently responsible for smooth muscle contraction, another for relaxation. The distribution of the subtypes in these tissues will be discussed in correlation with serotonin action.

D

EFFECTS OF INTRASTRIATAL DOPAMINE INFUSION ON D2 DOPAMINE RECEPTORS IN THE 6-OHDA LESIONED RAT: NEUROCHEMICAL AND BEHAVIORAL STUDIES

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The present study examined the efficiency of intrastriatal dopamine (DA) infusion in the 6-Hydroxydopamine (6-OHDA) lesioned rat model as a potential approach for the treatment of Parkinson's disease. The completeness of the (6-OHDA)-induced nigrostriatal injury was confirmed using [^{3}H]-mazindol autoradiography and apomorphine-induced rotation. Lesioned rats were treated with one of 3 different DA solutions (50 mM, 100 mM, or 150 mM) or vehicle solution (Na-metabisulfite). Intrastriatal DA infusion significantly reduced the apomorphine-induced rotation 7 days after initiation of treatment. [^{3}H]-spiperone autoradiography performed one day after the termination of DA infusion into the striatum, revealed a clearly defined area with a dramatic reduction of D2-DA receptors. This area was 1.0 - 1.8 mm in radius and enclosed 21.8 - 52.5 % of the cross-section of the striatum. The percent reduction of D2 receptors in this area was 40.5 ± 4.0 % for the 50 mM DA-group, 54.0 ± 8.4 % for the 100 mM-group and 54.4 ± 27.0 % for the 150.0 mM-group when compared to the same area on the unlesioned side. However, the specific binding of [^{3}H]-spiperone in the entire lesioned striatum following DA treatment was still higher on the lesioned side compared with the unlesioned side. The present results suggests that intrastriatal DA infusion may be an useful therapeutic approach for the treatment of Parkinson's disease but a more uniform delivery of DA into the striatum may be required.

A

A NOVEL LIGAND FOR CANNABINOID RECEPTORS, ARACHIDONYL GLYCEROL, BINDS TO BOTH THE NEURONAL AND PERIPHERAL RECEPTOR FORMS

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Recently, a peripherally expressed human cannabinoid receptor has been cloned. This receptor is similiar to the central nervous system cannabinoid receptor at a level of 44% homology that increases up to 68% homology within the binding domain (Monro, et al. Nature 365:61, 1993) We have transiently expressed the human peripheral and rat neuronal cannabinoid receptors in COS 7 cells and assessed the binding of various cannabinoid ligands. We found that the high affinity ligand for the brain cannabinoid receptor, ^{3}H-HU243, binds to the neuronal and peripheral receptors with Kd values of 40.3 ± 20.3 and 81.8 ± 7.7 pM, respectively. In addition, heterologous competition binding assays with the endogenous ligand, ananamide, shows slight preferential binding to the neuronal over the peripheral form, displaying Ki values of 252 ± 47 and 581 ± 111 nM, respectively. Another endogenous ligand, arachidonyl glycerol was recently identified in dog small intestines (Mechoulam et al., unpublished). We have found that both forms of the ligand, ANE1 and ANE2 (arachidonic acid group on positions 1 or 2 of the glycerol, respectively) bind to both cannabinoid receptors. The neuronal receptor displayed Ki values of 580 ± 62 and 610 ± 75 nM while the peripheral receptor binds with Ki values of 570 ± 51 and 1400 ± 172 nM. Glycerol or arachidonic acid did not compete with the binding of ^{3}H-HU243 to either receptors. These findings indicate that the endogenous ligand arachidonyl glycerol interacts with both cannabinoid receptors with similar affinities.

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B

DALARGIN BINDING PROTEINS - THE COMPONENTS OF THE BRAIN OPIOID RECEPTORS

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One of the priority trends in modern narcology is the study of molecular mechanisms of opioid CNS receptors alteration under the influence of morphine group substances. The main goal of our investigation is to reveal the molecular brain structures which are responsible for binding morphine group substances.

The specific membrane protein - dalargin binding protein (DBP) was isolated and purified 4000 times. The DBP specific were bound with ^{3}H-naloxone and ^{3}H-DADLE. The DBP molecular mass and Stok's radius were determined, electrofocusing of protein revealed a single band with pI 5,4.

Polyclonal antibodies to DBP inhibited their binding with ^{3}H-naloxone. Immunoblotting of the rat brain membrane proteins revealed specific components of opioid receptors with molecular mass 45 and 65 kD. The immunohistochemical staining of the rat brain slices by polyclonal antibodies showed the differences in localization of opioid receptors.

The DBP functions as a component of mu- and delta-receptors are discussed.

C

EFFECT OF DSIP ON THE CONTENT OF BIOGENIC AMINES AND ITS METABOLITS IN THE BRAIN AFTER THE AMPHETAMINE ADMINISTRATION

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Metabolic changes in the brain under the effect of some regulatory peptides are significantly stronger in the conditions of pathological or experimental injury. The biochemical investigation of neurotransmitter systems were performed on the subcellular level, using the model of amphetamine pharmacological action on metabolic parameters and the further correction by means of Delta-sleep inducing peptide (DSIP). Amphetamine was injected to the rats during 20 days at the dose 2,5 mg/w.v and DSIP once time later (60 mcg/w.v). The effect neuropeptide were studied on the biogenic amines contents (DA, NA, 5-HT), its metabolits (HVA and 5-HJAA) and the activity of MAO (A and B forms) in the subcellular fractions of brain rats structures (cortex and striatum). It was shown, that in the experimental conditions effect of DSIP on MAO A activity and 5-HYAA content was inereased without the significant changes of DA and NA content; on the contrary HVA content was decreased. The activation serotoninergic system and the maintaining of the balance level neurotransmitters seems to be a basis of DSIP antistressor action and may play an important role in the adaptive processes in CNS by restorating of the nerveus activity and animals behavior to the normal level.

D

INHIBITION AND SENSITIZATION OF THE CANNABINOID RECEPTOR BY VERY LOW DOSES OF ANANDAMIDES

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Recently a novel family of endogenous compounds, named anandamides, was isolated from brain tissue (Devane et al. 1992; Hanus et al. 1993). They are ethanol amides of long chain unsaturated fatty acids. The anandamides bind to, and activate cannabinoid receptors.

Since in some tests, the anandamides exhibit a partial agonist profile, they may cause antagonism under certain conditions. Hence we investigated whether the anandamides inhibit cannabinoid-induced effects (I). In addition, we investigated whether chronic treatment with anandamides will induce tolerance and/or sensitization to a challenge dose of anandamides (II). (I) Various doses of two anandamides (20:4, n-6 or 22:6, n-3) were injected i.p. in mice in the presence of Δ^9-tetrahydrocannabinol (THC). Effects were measured by a standard test battery (Martin et al. 1991). The results indicated that very low doses of anandamides (about 500x lower than their agonistic dose) inhibited Δ^9-THC-induced effects.

(II) After repeated administration (10x) of a high (20 mg/kg) or low (0.001mg/kg) dose of anandamides (20:4, n-6 or 22:6, n-6), animals were challenged with anandamide (20:4, n-6, 20 mg/kg) 24h after the last injection. After chronic treatment with the high dose of anandamide, full (catalepsy, analgesia, hypothermia) or partial (motor activity) tolerance to the challenge was observed. In contrast, after chronic treatment with the low dose of anandamide, sensitization to the effects of the challenge dose was recorded.

Based on these data and previous results from IN VITRO experiments (Fride et al. 1994), we suggest that a cannabinoid receptor subtype, or Gs-protein, activated by acute low doses of anandamides and desensitized by chronic low doses, may explain both the inhibition and sensitization of cannabinoid- or anandamide-induced effects.

A

ALCOHOL OF ABUSE, DESYMPATHIZATION AND BRAIN EDEMA: ENZYMES OF CHROMATIN - PHOSPHOLIPID INTERRELATION AS MOLECULAR AND PHYSIOLOGICAL TAPGETS.

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It was demonstrated by us the existence of similarities in pathogenesis of brain morpho - functional and metabolic abnormalities under the condition of alcohol abuse,unilateral remuve of the upper cervical sympathetic ganglion(desympathization) experimental brain edema (intraperitoneal administration of tetraethyltin). The pathologies studied are accompanied by significant changes in composition of histonic, nonhistonic, cytoplasmatic proteines, nucleic acids levels, and in proteinkinase activities.These changes are characterised also by structural, functional and physico - chemical properties disorders of chromatin. According fluorimetric, infrared and ultraviolet spectroscopy observations more pronounced changes were noticed in the activity of phosphatidatphosphohydrolase, phosphatidylinositol-specific phospholipase C activity, chromatin - bound phospholipids and their fatty acid composition. The chromatin matrix and RNA - polymerises (I and II) activities disorders and their correlation with abnormalities in transcription, translation processes, as well as, with depression of phospholipides biosynthetic and demyelinization reactions have been demonstrated. The role of sympathetic nervous system and the cell membrane lipids in pathogenetic mechanisms of diseases studied is discussed.

B

EFFECT OF MORPHINE AND CH-38083, A SELECTIVE α-2 ADRENOCEPTOR ANTAGONIST ON THE PREOVULATORY GONADOTROPINE AND PROLACTIN SECRETION IN RATS.

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It is well documented that exogenous and endogenous opioids inhibit the secretion of gonadotropine, TSH and ACTH and stimulate the release of PRL and GH. However, there are growing evidence that in rats, during both the preovulatory LH surge or steroid-induced LH hypersecretion the opiates' effects on pituitary hormone secretion, pain perception, as well as on thermic and locomotor activity are reduced. To test the proestral influence of opioids on FSH, LH and PRL secretion we injected morphine hidrochloride (10 mg/kg) s.c.) alone or combined with CH-38083 (5 or 10 mg/kg s.c.), a novel selective α_2 adrenoceptor antagonist, to young female rats in proestrus. Injections were performed between 10.30-12.00 hours a.m., the animals were sacrificed between 3.00-4.00 hours p.m. Blood gonadotropine and PRL contents were measured by RIA. Morphine alone or combined with CH-38083 did not change significantly the FSH and LH plasma levels indicating that the responiveness to opiates was altered during the proestral LH surge. Furthermore, morphine slightly reduced the PRL level and potentiated the PRL lowering effect of CH-38083. The inhibition of PRL secretion by the latter suggests a stimulatory role for α_2 adrenoceptors on the PRL surge. Similar effects were described in the literature for idazoxan, another α_2 adrenoceptor blocker.

C

REGULATION OF PRODYNORPHIN GENE EXPRESSION BY CHRONIC OPIATE AGONISTS AND ANTAGONISTS.

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The effects of chronic exposure to opiates have been long investigated. Neurochemical alterations occur during the development of opiate tolerance. We investigated the possibility that opiate agonists and antagonists affect the gene expression of one of the opioid precursor, prodynorphin, when chronically administered to the rat.
The opiate receptor μ agonist morphine (20 μg/die), the δ agonist deltorphin (2 μg/die), the k agonist U50-488H (100 μg/die) and the specific antagonists naloxone (72 μg/die), naltrindole (20 μg/die) and norBNI (20 μg/die) were icv administered to the rat for 7 days. At the end of the treatment the hypothalamus, hippocampus and striatum were dissected, total RNAs were extracted, subjected to electrophoresis and blotted onto membranes for Northern Analysis with a cDNA probe, pBgBa.
The prodynorphin mRNA levels resulted significantly diminished in all tissues investigated after chronic treatment with morphine (55±7 % of control), deltorphin (63±8 % of control) and U50-488H (33±5 % of control). The chronic exposure to the antagonists naloxone, naltrindole and norBNI elicited a significant increase in prodynorphin mRNA levels (from 150±12 to 220±22 % of control) in all tissues).
Taken together these results indicate that opiate agonists, acting on any type of opioid receptor, induce a down-regulation of opioid gene expression, evaluated as prodynorphin mRNA levels, whereas all antagonists elicit a marked up-regulation of prodynorphin gene expression. The comprehension of these effects on the biosynthesis of the opioid neuronal system could contribute to the elucidation of the mechanisms underlying the development of opiate tolerance.

D

NEUROCHEMICAL CHARACTERISTICS OF THE BRAIN OF WISTAR RATS DIFFERING IN LOCOMOTOR ACTIVITY DURING DOPAMINE SYSTEM DYSFUNCTION

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Investigation of morphochemical criteria of compensatory-accommodative processes in central nervous system on experimental models of dopamine system hypo- and hyperactivity has been performed using Wistar rat strains with different locomotor activity in "open field", and the developed dysfunction was further corrected by means of neuropeptide (tuftsin or DSIP) injections. It has been shown, that in control conditions the indices of protein metabolism (aminopeptidase, content and concentration of structurated proteins) as well as the activity of some oxidative enzymes were significantly higher in rats with low motor activity in "open field" as compared to those with high activity. Under the experimental conditions the differences in the response to the injury and to neuropeptide correction between these two groups of animals have been revealed on systemic, cellular and molecular levels. The development of the investigations in this field will allow to solve the question of the role and the degree of involvement of cortical and subcortical brain structures in the development of pathologic symptoms caused by dopamine system dysfunction (Parkinsons disease, schizophrenia).

A

Evaluation of gelatinases and IL-6 in the cerebrospinal fluid of patients with optic neuritis, multiple sclerosis and other inflammatory neurological diseases

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The activities of the metalloproteinase, gelatinase B, and the presence of IL-6, an inducer of metalloproteinase inhibitors, were investigated in CSF samples of 190 patients with MS, ON, OIND or control patients. IL-6, measured as hybridoma growth factor was found in only 4 samples. Elevated CSF gelatinase B levels were found in 40%, 35%, and 54% of MS, ON and OIND patients respectively while all control CSFs were devoid of gelatinase B activity. Clinical and laboratory data were compared with gelatinase B levels. No correlation was found between the CSF cytosis and gelatinase B suggesting that this enzyme in the CSF originates from CNS lesions rather than from passive leakage from CSF cells. However, the occurrence of gelatinase B correlated with the IgG index in the MS patient group. This study stimulates further investigation into the possible usage of protease inhibition in demyelating diseases.

B

URINARY δ-ALA CORRELATES WITH EVOKED POTENTIAL PARAMETERS: AN APPROACH TO INDICATE NEUROTRANSMITTERS INVOLVED IN ASPECTS OF NERVOUS SYSTEM FUNCTION

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Lead is one of the oldest neurotoxic substances known to man. The present study was designed to evaluate a spectrum of evoked potential and biochemical parameters whose functional sensitivity could supplement the present blood lead measurement used to monitor individuals occupationally-exposed to lead at permitted levels. A second objective was to relate the chosen biochemical parameters measured in blood and urine to nervous system function. This is a report of the results obtained from the simultaneous measurement of blood lead, urinary delta-aminolevulinic acid (δ-ALA), visual evoked potentials (VEP) and event-related potentials, utilizing a target detection task in lead-exposed and control human subjects. The VEP P100 latency and target detection P300 latency (related to stimulus evaluation) were significantly lengthened in lead-exposed subjects, and these parameters correlated with blood lead indicating that lead affects the generators of these components. They also correlated with urinary δ-ALA, with the VEP N75 latency also correlating with urinary δ-ALA. It is well established that lead-exposure results in elevated levels of δ-ALA; but, at least at "safe" levels of exposure, no effects have been related to this elevation. There is evidence in the literature that δ-ALA can compete with GABA at receptors for this neurotransmitter. and a mechanism of lead neurotoxicity related to this was proposed by Silbergeld and Lamon (1980). Subjects treated with benzodiazepines have also demonstrated significantly longer target detection P300 latencies, implying GABA receptor neuron involvement in the generation of this latency. There is extensive evidence in the literature of GABA receptor neuron involvement along the entire length of the visual pathway. This is consistent with the interpretation that the observed effects on the target detection P300 and VEP N75 and P100 latencies were due to δ-ALA competing at GABA receptors. The results of this study suggest that simultaneous measurement of biochemical parameters in ethically-accessible body fluids and evoked potentials could provide *in vivo* data on involvement of specific neurotransmitters in generating evoked potential components.

Reference: Silbergeld, E. K. and Lamon, J. M. Role of Altered Heme Synthesis in Lead Neurotoxicity. J. Occup. Med. 22, 680-684, 1980.

C

DOPAMINE -B- MONOOXYGENASE AS A TARGET FOR MYCOTOXIN ZEARALENON ACTION

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The nature of Zearalenon (mycotoxin of fusarium) toxic action on the activity electrophoretically homogenous preparations of dopamine-B-monooxygenas (the enzyme catalysing the reaction of transformation of dopamine into noradrenaline) have been investigated. The data obtained have shown th Zearalenon has the dose-depending effect on the process mentioned. Th small concentrations of toxin intensify the noradrenaline formation process, while the high quantities of Zearalenon lead to the development of the contrar effect. The experiments have been carried out using two independen spectrophotometric assays:thyramine and ascorbate were used as substrate and electrons donor correspondingly. An additional we have examined also the kinetic parameters (K_m and V_{max}) of dopamine - B - monooxygenase catalysing reaction under the conditions of Zearalenon toxic action. The role of mycotoxins, particularly of Zearalenon, in the disturbances of catecholamines biasynthesis and release in discussed.

D

EFFECT OF 6-HYDROXYDOPAMINE ON MITOCHONDRIAL NADH OXIDASE (COMPLEX I)

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6-Hydroxydopamine (6-OHDA) is thought to owe its dopaminergic neurotoxicity to formation of oxygen free radicals and lipid peroxidation. However the biochemical mechanism of its neurotoxicity is still unknown. We found that the first enzyme of mitochondrial respiratory chain, NADH oxidase (complex I), was completely inhibited by 6-OHDA (EC50=10.5 μM). The enzyme inhibition was insensitive to the change of NADH or ADP concentrations. EDTA did not prevent the inhibition, thus excluding a possible substantial role of metal cations or peroxidation products. The extent of the inhibition decreased as a result of preincubation of 6-OHDA with mitochondria suspension. Both non-enzymatic oxidation and monoamine oxidase-catalyzed oxidation of 6-OHDA are responsible for this loss of the enzyme inhibition. Non-enzymatic oxidation in the absence of mitochondrial suspension brought about the most rapid and almost complete decay of the inhibitor. During the preincubation of 6-OHDA with mitochondria the extent of inhibition rapidly decreased to the constant level, which was significantly higher than that of non-enzymatic oxidation. Thus evidence is provided, that 6-OHDA uses intramitochondrial sources of reducing agents (probably GSH) and presumably can deplete them. Oxidation of 6-OHDA by monoamine oxidase was significant within the first 10-15 min of preincubation of 6-OHDA with mitochondria. Inhibition of both monoamine oxidase A and B increased the extent of the inhibition during this period, thus increasing the 6-OHDA toxicity. Since oxidized 6-OHDA looses its ability to inhibit NADH oxidase, 6-OHDA itself and not its oxidative products are responsible for the inhibition of the complex I.

A

INHIBITION OF MITOCHONDRIAL RESPIRATION BY 6-HYDROXYDOPAMINE

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Energy depletion is regarded as a cause of cell death. Consequently, inhibition of mitochondrial respiration is thought to be toxic, since the respiration is a main source of cell energy supply. We found already that 6-hydroxydopamine (6-OHDA) inhibits the first enzyme of the brain mitochondrial respiratory chain NADH oxidase (complex I) with EC50=10.5 μM. The terminal respiratory enzyme cytochrome c oxidase (complex IV) is less susceptible to the inhibition by 6-OHDA: its EC50=34 μM. Low substrate (reduced cytochrome c) concentrations increased the enzyme susceptibility to the inhibition because of the substrate participation in non-enzymatic oxidative-reductive recycling with oxidized inhibitor, which decreases the effective substrate concentration. This recycling was markedly prevented by EDTA addition, which decreased the extent of inhibition. EDTA action reveals a role of a metal cation, presumably iron, in this recycling. However, with higher substrate concentrations the enzyme susceptibility to the inhibition did not depend on EDTA addition. Similar to case of NADH oxidase, neither high substrate concentrations, nor ADP were able to prevent cytochrome c oxidase inhibition by 6-OHDA. The blocker of electron flow from cytochrome c oxidase to O2, cyanide, did not change the susceptibility of the enzyme to 6-OHDA and vice versa. Consequently, 6-OHDA binding does not strongly influence the heme a3 environment. The inhibition of both the first and the terminal enzymes of the mitochondrial respiratory chain may thus account for the 6-OHDA neurotoxicity and cell death.

B

DIFFERENTIAL DISPLAY OF HIPPOCAMPAL, STRIATAL AND SEPTAL RNAs FROM AF64A TREATED RATS REVEALS ALTERATIONS IN THE EXPRESSION OF G,C RICH TRANSCRIPTS

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The differential PCR display approach was adapted to study transcription patterns in specific brain regions following intracerebroventricular (ICV) administration to rats of ethylcholine mustard aziridinium (AF64A). The cholinotoxin AF64A is actively uptaken by cholinergic cell bodies via the choline transport system. Once there, it can interact with G residues and affect gene expression. Genes with higher G,C contents will hence be more sensitive to AF64A. Total RNAs were extracted from hippocampus, septum and striatum of 3 rats per sample at 7 and 60 days post administration. First and second strand cDNA synthesis from these RNAs, under low stringency annealing conditions, was followed by PCR amplification, using arbitrary 17-mer primers designed to include high G,C content to increase the probability of amplifying G,C rich genes. At least 50 conspicuous DNA products were detected in each region. A large part of the observed procucts were common to all regions, however some of the products were region-specific. The general pattern of displayed products did not change in brain regions of AF64A-treated rats as compared to control rats. While no AF64A-dependent changes could be observed in striatal RNAs, we were able to detect several quantitative changes in PCR products displayed from hippocampus and septum . In the septum levels of 3 transcripts were decreased on day 7 and remained low through day 60. At least 3 other PCR fragment appeared to be higher in level in the treated hippocampus than in controls at both 7 and 60 days post AF64A administration. Thus, using the powerful approach of differential PCR display, we were able to identify yet uncharacterized transcripts subject to modulation in brain regions undergoing cholinotoxic damage. Future identification and cloning of these transcripts will deepen our understanding of the transcriptional changes that take place in cholinodegenerative diseases such as Alzheimer's and Huntington's.

C

THE ABILITY OF LATROTOXIN-LIKE BRAIN PROTEIN TO INDUCE FUSION OF NEGATIVELY CHARGED LIPOSOMES

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A soluble protein interacting with polyclonal antibodies to natural toxin from the black widow spider venom (α-latrotoxin) was identified in bovine brain previously. A protein fraction containing latrotoxin-like, or L-protein, was obtained using anionic and hydrophobic chromatography methods from the bovine brain cortex cytoplasm. The ability of this fraction to induce fusion of negatively charged liposomes consisting of phosphatidylcholine, phosphatidylethanolamine and cardiolipin in a molar ratio 2:3:5, respectively, was investigated. The extent of fusion was significantly increased at pH 6.0 and under denaturation condition (4M urea, 0.1% SDS). By the use of the fluorescent probe ANS the fusogenic activity is shown to enhance simultaneously with the hydrophobicity of the protein under investigation. The presence of the promoting fusion conformational changes in the protein molecule and the possible relationship of the L-protein to the neurosecretion processes are discussed.

D

DECREASE OF LOCOMOTION IN MICE C57Bl/6 AFTER ADOPTIVE TRANSFER OF LYMPHOCYTES FROM ANIMALS WITH MPTP-INDUCED PARKINSON'S SYNDROME

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Mechanisms of CNS degenerative damage on Parkinson's disease are not clear at present. Nowadays much attention is payed to studies on the role of antibodies but not the cellular factors of immune system in pathogenesis of Parkinson's disease. In present investigation oligokinezia as a main symptome of Parkinson's syndrome was assessed by changes of locomotion automatically in mice-recipients on days 7 and 14 after adoptive transfer of $2x10^7$ splenocytes or B-lymphocytes obtained by anti-Thy-1 monospecific serum. 10 months old mice with experimental Parkinson's syndrome induced by MPTP injections (20 mg/kg twice a day for 10 days) were used as donors. Decrease of horizontal activity was observed both after splenocytes and B-lymphocytes adoptive transfer as compared to control animals. Velocity of movement was also reduced. Splenocytes obtained from intact mice or treated by MPTP *in vitro* were unable to induce changes in locomotion. It is supposed that cellular factors of immune system (exactly B-lymphocytes) are involved in pathogenesis of MPTP-induced Parkinson's syndrome in mice.

A

TH-ACTIVITY AND THE CONCENTRATION OF DA AND ITS METABOLITES IN STRIATUM AND SUBSTANTIA NIGRA IN RATS

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The aim of the present investigation was to study the effects of severe neonatal asphyxia in a model of delayed cesarean section on the TH-(Tyrosine-3 mono-oxygenase, EC 1.14.16.2) activity in substantia nigra (SN) and nucleus caudatus (NC) immediately after asphyxia of the newborn animals and in the age of 4 weeks. Method used are: TH-activity of the P2-fraction by kinetic method (14C-tyrosine), DA and metabolites in the homogenate by HPLC with electrochemical detection. In controls the levels of DA and its metabolites increase 2-4 times in SN and about 10 times in NC. These developmental changes may reflect the very active synaptogenesis in postnatal period in NC. In the newborn rat asphyxia acutely reduced the TH-activity in SN by nearly 60 %. In the 4 weeks old rat no changes in TH-activity are seen. In the newborn rats exposed to asphyxia the DA and DOPAC levels were increased by about 30 % in SN, at the age of 4 weeks a decrease of DA and 3MT levels by about 30 % was found. In NC of newborn rats exposed to asphyxia the concentration of HVA increases nearly by 30 %, in the 4 week old rats a decrease of the DA level by about 50 % was seen. In 4 weeks old rats the utilization of DA decreases dramatically in NC but only slightly in SN. The results indicate that severe asphyxia in the perinatal period effects DA-metabolism and TH-activity differently in projecting axons and in the cell body.

B

INHIBITION OF SPHINGOLIPID SYNTHESIS IN THE RAT BRAIN: POSSIBLE EFFECTS ON LEARNING AND MEMORY.

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Sphingolipids (SL), particularly gangliosides (GM), are enriched in neuronal membranes where they have been implicated as mediators of various regulatory events. We recently provided evidence that SL synthesis is necessary to maintain neuronal growth by demonstrating that in hippocampal neurons cultured at low density, inhibition of ceramide synthesis by Fumonisin B1 (FB1) affected axonal outgrowth [Harel and Futerman (1993) JBC **268**, 14476]. The changes that take place during neuronal development are considered to be similar to the neuronal plasticity exhibited by neurons during learning [Pulvirenti (1992) Funct. Neurol. 7, 481]. In an attempt to understand the biochemical changes underlying the formation of new memory, we are studying the role of SLs in neuronal plasticity. We have microinjected specific inhibitors of SL synthesis or transport into the hippocampus and the insular cortex (containing the gustatory cortex) and examined their effect on biochemistry and behavior. Specifically, FB1 injected into the hippocampus or the insular cortex inhibits the synthesis of ceramide, the precursor of all SLs, in a dose dependent manner over a period of 6-12 hours. It also produces hyperactivity and epilepsy at high doses. We are currently testing the effects of FB1 and Brefeldin A (a drug which blocks the movement of vesicles from the Golgi apparatus to the plasma membrane thereby blocking the delivery of newly synthesized materials to their target sites) on taste learning. Preliminary results suggest that these drugs interfere with conditioned taste aversion when injected to the insular cortex before introduction of a novel taste.

C

ABNORMALLY PHOSPHORYLATED TAU PROTEINS IN AD: REGIONAL PATTERN AND RELATIONSHIP TO SEVERITY OF DEMENTIA

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The distribution of abnormally phosphorylated tau proteins which is a constant and early marker of Alzheimer's disease (AD) was investigated in a number of cortical areas and in the basal nucleus of Meynert of twelve patients with AD. Quantification was performed with a sensitive sandwich ELISA employing the monoclonal antibody (mab) B5-2 which immunoreacts with neurofibrillary tangles, dystrophic neurites surrounding amyloid cores of plaques and neuropil threads. On western blots, the mab B5-2 specifically labels the abnormally phosphorylated tau species A68 in a phosphorylation dependent manner but shows no crossreactivity to normal human adult or foetal tau proteins. The preferential involvement of cytoarchitectural defined cortical areas showed marked individual differences in AD, and no unique distribution pattern of abnormally phosphorylated tau proteins could be established. While regional heterogeneities along the rostro-caudal extension of the brain declined with the progression of the disease, lateral differences which were largely non-directional, appeared to be more stable over time. The mean content of abnormally phosphorylated tau proteins of individual cases was significantly related to the severity of the disease. On a regional scale, this correlation was highest for the basal nucleus. The formation of abnormally phosphorylated tau was associated with a loss of normal soluble tau proteins. Those cortical areas which in normal brain showed the highest amount of normal soluble tau proteins appeared to be particularly prone to deposition of abnormally phosphorylated tau proteins. The present results indicate that the formation of abnormally phosphorylated tau proteins can be initiated in the neuropil at more than one brain area. The spreading of the pathology appears to involve both intracortical and subcortical pathways but largely spares interhemispheric pathways. It is hypothesized that regional differences in the compartimentation and metabolism of tau proteins might reflect the molecular basis for the regional different vulnerability in AD.
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D

cGMP-DEPENDENT PROTEIN KINASE-LIKE IMMUNOREACTIVITY IN RAT BRAIN

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Although the physiological function(s) of cyclic GMP-dependent protein kinase have not yet been identified unequivocally, findings from different tissues and cellular systems suggest that the enzyme is one major factor which regulates cytosolic calcium levels. In the brain cGMP kinase has been previously localized almost entirely to Purkinje cells including its axons and presynaptic boutons in cerebellar and vestibular nuclei. In the present study cGMP kinase-like immunoreactivity (-ir) was demonstrated with affinity-purified rabbit antibodies raised against bovine cGMP kinase peptide sequences. In addition to intracellular staining of Purkinje cells and its axonal and dendritic processes cGMP kinase-ir was found in the microenvironment of numerous nonpyramidal cells in the cerebral cortex and around neurons of different subcortical regions, such as basal forebrain diagonal band nuclei, reticular thalamic nucleus and red nucleus. Intracellular staining was also observed tentatively in neurons of certain subcortical nuclei. The combined fluorescence microscopic detection of cGMP kinase-ir and binding sites for the N-acetylgalactosamine-specific plant lectin *Wisteria floribunda* agglutinin (WFA) showed dual labelling of perineuronal nets in the cerebral cortex and many subcortical nuclei. It can be concluded that those neurons ensheathed with lattice-like, glia-associated extracellular matrix, are covered by presynaptic boutons and/or glial structures which are functionally dependent on high levels of cGMP kinase activity.

A

ACTIVITIES OF ACETYLCHOLINESTERASE, ADENYLATE CYCLASE, 5'-NUCLEOTIDASE IN THE SENSOMOTOR AND LIMBIC SYSTEMS OF RAT BRAIN AFTER MANIPULATORY TRAINING

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The activities of acetylcholinesterase (AChE), adenylate cyclase (AC), 5'-nucleotidase (NT) were studied in P2 fractions from the amygdala and hippocampus (limbic system) and from the cortex and striatum (sensomotor system) after training of rats to perform reaching with tactile control by preferable forepaw. The rats were subdivided into three groups: control (without training, C); trained animals with low (L) and high (H) ability to learn the experimental task. After the intensive training the significant decrease of AC ($P<0.001$) and AChE ($P<0.01$) was found only in the sensomotor system of H, while in the limbic system we have revealed the decrease of AC and the increase of AChE in both groups of trained animals. In the limbic system of H the activity of NT wasn't changed while it was decreased in the group of rats with low ability to learn the instrumental task.

In the striatum of studied animals a very specific character of the enzyme activities with the lowest level of NT in C (0.93 ± 0.03 mcg Pi/mg protein/min) was found. The activity of NT was found to be increased up to 1.28 ± 0.07 in L and up to 2.00 ± 0.13 in H (what was close to the activity of this enzyme in the amygdala). The activity of AChE decreased only in the group of H, however it remained 3-5 times higher in the striatum than in other investigated structures.

Thus, the changes of the activities of key enzymes of acetylcholine and adenosine metabolism are different in the sensomotor and limbic structures of brain in animals with different ability to learn the experimental task. The biochemical peculiarities of the striatum can reflect its specific role in the regulation of manipulatory acts with sensory control.

B

CORRELATIONS BETWEEN THE RESULTS OF BIOCHEMICAL STUDY OF INTRACRANIAL CYSTS AND ULTRALOW-FIELD MRI DATA

Petraikin A.V., Arutunov N.V., Demchuk M.L.

The results of complex investigation of 12 patients with intracranial cysts of varius ethiology are presented in this study. Ultralow-field MRI examinations ("MAGNAVIEW", 0.04 T) and biochemical estimation of total protein, Fe ⁺ concentration in the contents of intracranial cysts were performed. On T2-weighted MR-images (SE 2800/150) the high intensity signal was marked in all cases. On T1-weighted images (SE250/40) the signal intensity was different in every cases and it increase correlated markedly with protein concentration ($r=0.98$ $n=6$) in the interval of protein content 0 - 10 %. Further elevation of protein concentration (up to 40 %) $n=4$ didn't result in the increase of MR signal. It's necessary to note, that in ultralow-field relaxation time T1 fall connected with the increase of protein content is marked more sharply, than in moderate and high magnetic fields. The presence in cyst paramagnetic ions of Fe 3+ (reduced form of haemoglobin) lead to stronge increase signal on T1-wighted images, that permited us to differentiate the cyst n=2 with old haemorrages.

Thus, the use of non-invasive method of MR-tomography gives us opportunity to determinate the protein concentration in the intracranial cysts and to separate the cases with old haemorrages into the ciyst.

C

THE TYROSINE PHOSPHORYLATION OF A 180kD SYNAPTOSOMAL PROTEIN IN THE INSULAR CORTEX OF THE RAT IS MODULATED BY TASTE LEARNING.

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Protein tyrosine phosphorylation (PTP) is a major signal transduction pathway involved in cellular metabolism, growth and differentiation. Recent data suggest that PTP also plays a role in neuronal plasticity. To further elucidate the role of PTP in behavioral plasticity, we are investigating its modulation by taste learning in the taste cortex of the rat. Taste learning was chosen because it can occur after a single trial training, hence permitting correlation of molecular phenomena with behavioral ones. A robust single trial taste learning paradigm is conditioned taste aversion (CTA), in which rats learn to associate a novel taste with delayed poisoning. We provided rats with water (a familiar taste) or saccharin (a novel taste), or subjected them to CTA training (pairing saccharin with malaise induced by i.p. injection of LiCl). We found that CTA training, and to a lesser extent exposure to saccharin, increased tyrosine phosphorylation of several proteins in the insular cortex, as detected by immunoblotting of the cortical homogenate with anti-phosphotyrosine antibodies. A major protein so modulated is a glycoprotein of MW 180kD, abundant in synaptosomal membranes. The protein was partially purified by subcellular fractionation combined with affinity chromatography on anti-phosphotyrosine antibody-coupled resin and on lectin-coupled resins. The 180kD protein was modulated by taste (but not by odor) only in the insular cortex but not in other brain areas. However a protein of similar properties is present in other cortici. We have detected a similar 180kD protein tyrosine phosphate protein also in calf brain.

D

192IgG-SAPORIN INDUCED IMMUNOLESION OF THE BASAL FOREBRAIN CHOLINERGIC SYSTEM DIFFERENTIALLY REGULATES CHOLINERGIC PARAMETERS AT THE PROTEIN AND mRNA LEVEL

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The cholinergic basal forebrain (CBF) system is known to play an important role in cortical activation and normal cognitive function. To mimic cholinergic cortical dysfunction a number of excitotoxins have been used to produce lesions in the CBF which are far from beeing selective to cholinergic cells. Recently, a monoclonal antibody to the p^{75}LNGFR (192 IgG) coupled to a cytotoxin (saporin) was introduced and described as an efficient and selective immunotoxin for NGF- receptor bearing CBF neurons (Wiley, 1992).

The aim of this study was to elucidate how reduced cortical cholinergic activity affects cholinergic markers in cholinoceptive target regions of the basal forebrain.

Seven days after an intracerebro-ventricular injection of 4 µg of 192 IgG-saporin conjugate in rats, consecutive serial coronal brain sections were assayed for cholinergic markers i.e. muscarinic and nicotinic acetylcholine receptors (mAChR and nAChR, respectively) aetylcholinesterase and high affinity choline uptake sites using both receptor autoradiography and in situ hybridization.

Evaluation by quantitative image analyses revealed a dramatic loss in acetylcholinesterase staining following immunolesion in all regions of the cerebral cortex, hippocampus and olfactory bulb by up to 90%, whereas corpus striatum, cerebellum and the brain stem were unaffected. Similary, hemicholinium-3 binding to high affinity choline uptake sites on cholinergic nerve terminals was significantly reduced in the fronto-parietal cortex and in the hippocampus but to a lower extent (reductions by up to 50% of control values). Receptor autoradiography demonstrated a considerable increase of the M1 mAChR subtype in the frontal, parietal temporal and occipital cortices (up to 30%), which was more pronounced in layer II/III and layer VI as compared to other cortical layers. In situ hybridization revealed an increase of m1 and m3 mRNA by about 10% each in all layers of the frontal, parietal, temporal and occipital cortices. The M2 mAChR subtype was slightly elevated after immunolesion in temporal and occipital cortices suggesting that a postsynaptic population of M2 receptors exists. This is supported by moderate elevated m2 and m4 mRNA levels by 10 to 15 % in these cortical regions.

Immunolesion did not affect mAChR binding in the CBF nuclei. In contrast the m2 mRNA level was differentially reduced in rostral-caudal direction of CBF nuclei (to -60 to -20% of the control value).

The data suggest that (i) the immunotoxin is a useful tool to mimic cholinergic dysfunction and (ii) reduced cholinergic activity differentially affects cholinergic markers in cortical target regions.

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A

EXPERIENCE-DEPENDENT CHANGES IN RECEPTOR GENE EXPRESSION IN RAT VISUAL CORTEX

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The developmental regulation of the expression of muscarinic acetylcholine (m1-m4) and AMPA-type glutamate receptor (GluR-A through GluR-D) mRNA subtypes were studied in the visual cortex of both normally raised and monocularly deprived rats (one eyelid sutured at the age of 11 days) using in situ hybridization histochemistry and computer-assisted image analysis. Both muscarinic m1 to m4 and AMPA receptor GluR-A through GluR-D transcripts exhibit a differential laminar expression pattern in the developing rat visual cortex. At birth the levels of GluR-A transcripts are lower by about 50% in each visual cortical layer as compared to the adult values. In contrast, GluR-B to GluR-D mRNAs are expressed in all cases at higher levels at birth than in the adult brain. The m2 receptor gene transcript was found to be homogeneously distributed until postnatal day 10. From day 10 onwards a bimodal laminar pattern gradually develops with increased mRNA levels in layer IV and upper layer VI. From postnatal day 21 onwards the hybridization peak in layer VI decreases as compared to the peak level in layer IV reaching the adult pattern already on day 25. Unilateral eyelid closure from postnatal day 11 resulted in transient changes in the laminar distribution of m2, m3, and m4 as well as GluR-A through GluR-C receptor mRNA between postnatal days 18 and 25, whereas the development of the laminar pattern of the m1 and GluR-D receptor mRNA was hardly affected regardless of the length of visual deprivation. The data suggest that the expression of muscarinic and AMPA receptor genes in rat visual cortex are developmentally regulated and differentially affected by visual experience.

B

INHIBITION OF MEMORY CONSOLIDATION AFTER ACTIVE SHOCK AVOIDANCE CONDITIONING BY INJECTION OF L2/HNK-1 ANTIBODIES INTO GOLDFISH BRAIN

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In Goldfish a CNS-specific glycoprotein, named ependymin, is synthesized in meningeal fibroblasts, secreted and incorporated into ependymal cells, radial glial cells and type I and XIV neurons. It is rapidly induced after learning and during optic nerve regeneration. Injected antisera and antisense probes interfere with the consolidation of such adaptations. Because its carbohydrate residues comprise a sulfated glucuronic acid epitope typical of several cell adhesion molecules, we investigated, whether the function of ependymin may be linked to cell adhesion properties: (1.) Ependymin provides a good substrate for growing axons in vitro. (2.) The monoclonal rat antibody L2, directed against the HNK-1 epitope, recognizes glycosylated ependymins on Western blots and (3.) labels the same cell populations as anti-ependymins. (4.) Goldfish trained on active shock avoidance in a shuttle-box were intracerebroventricularly injected with L2/HNK-1 antibody 15 min after learning. When tested 3 days later, they were unable to recall the task. However, this antibody did neither interfere with acquisition nor with performance of the task in overtrained animals. - We conclude that the function of ependymin in CNS plasticity may be mediated by the L2/HNK-1 carbohydrate epitope and cell adhesion phenomena which provide a basis for structural reorganizations. (Supported by DFG grant Schm 478/4-4)

C

G PROTEINS IN BIPOLAR AFFECTIVE DISORDER: THE EFFECTS OF LONG TERM LITHIUM TREATMENT

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Our objectives were to study recent suggestions from a number of investigators that signal transducing G proteins may be involved in the pathophysiology of Bipolar Affective Disorder, and may represent molecular targets for lithium's mood-stabilizing actions.

We utilized selective antibodies to quantitate the levels of the G protein α subunits which regulate adenylyl cyclase activity ($G\alpha_s$ and $G\alpha_{i2}$) and phosphoinositide turnover ($G\alpha_{q/11}$), and also carried out pertussis toxin (PT) catalyzed [^{32}P]ADP-ribosylation in platelet and leukocyte membranes from a group of 14 untreated (predominally manic) Bipoloar patients, 20 lithium-treated euthymic Bipolar patients and 11 healthy controls.

In both tissues, the immunolabeling of the 45 kDa form of α_s was higher in the Bipolar group considered as a whole (treated or untreated) compared to controls, effects which reached statisitcal significance in leukocytes. There were no significant differences in the immunolabeling of α_{i2}, α_{q11}, or PT-catalyzed [^{32}P]labeling in either tissue in the untreated Bipolar group compared to the control. In both tissues, lithium-treated subjects demonstrated lower levels of α_{q11} and higher PT catalyzed [^{32}P]ADP-ribosylation, which reached significance in platelets.

Our results are complementory to the findings of G α_s in postmortum brain tissue in BAD, and suggests that the previously reported elevation in leukocytes [^{3}H]Gpp(NH)p binding in Bipolars may be due, at least in part, to elevations in the 45 kDa form of $G\alpha_s$. The significantly higher PT catalyzed [^{32} P] ADP-ribosylation in the chronically lithium-treated subjects replicates our findings in rat cortex and adds to the growing body of evidence implicating G α_{i2} as a target of lithium's actions.

D

SYNTHETIC COPOLYMER 1 INHIBITS THE BINDING OF MBP, PLP AND MOG PEPTIDES TO CLASS II MAJOR HISTOCOMPATIBILITY COMPLEX MOLECULES ON ANTIGEN-PRESENTING CELLS.

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Copolymer 1 (Cop 1) is a synthetic basic random copolymer of amino acids that has been shown to be effective in suppression of experimental allergic encephalomyelitis (EAE) and has been proposed as a candidate drug for multiple sclerosis (MS). We have previously demonstrated that Cop 1 bound extensively and immediately to the MHC class II molecules on APC of both mouse and human origins. Moreover, Cop 1 inhibited the binding of biotinylated derivatives of myelin basic protein (MBP) and of p84-102 (an immunodominant epitope of MBP) to the MHC class II molecules, and even displaced these antigens when already bound. In the present report, we extended our studies to other myelin proteins that have been suggested as autoantigens in MS, e.g. proteolipid protein (PLP) and oligodendrocyte glycoprotein (MOG). We used seven MBP, three PLP and five MOG biotinylated peptides to demonstrate their direct binding to MHC molecules on living APC, which was monitored by flow cytometry using phycoerythrin (PE)-streptavidin. MBP, PLP and MOG peptides bound to APC of both mouse strains with different H-2 restrictions (SJL/J, H-2^s and PL/J, H-2^u), and EBV- transformed B cells from MS patients with various HLA haplotypes. The binding of peptides to the mouse APC was indiscriminate, although with different efficiencies, while the binding to the EBV-transformed B cells was restricted to several haplotypes. The specificity of the binding to the MHC class II molecules was confirmed by its inhibition with anti-I-A antibodies. In addition, the binding of all the peptides tested, was completely inhibited by the unlabeled Cop 1. These results suggest that Cop 1 indeed competes with MBP, PLP and MOG for MHC binding, indicating its potential as a broad-spectrum drug for MS.

A

METABOLISM OF BRANCHED-CHAIN AMINO ACIDS IN ASTROGLIA-RICH PRIMARY CULTURE

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Leucine, isoleucine and valine, the three branched-chain amino acids (BCAAs), are the only amino acids (AAs) the catabolism of which is initiated by transamination primarily in extrahepatic tissues. BCAAs are metabolized in the brain at high rate. However, the cell type which their degradation takes place in remains to be elucidated. Bearing in mind that glutamine synthetase, which is involved in the detoxification of NH_3, is exclusively localized in astrocytes we assume that BCAAs are preferentially degraded in this cell type. Therefore we used astroglia-rich primary cultures derived from rat brain for studying by an hplc technique the metabolism of BCAAs.
Astroglial cells in culture removed BCAAs rapidly from the culture medium. The disappearance of the AAs followed first order kinetics. This happened inspite of the presence of sufficient substrates for the generation of energy, such as glucose.
On the basis of the fact that the only way the ketogenic AA leucine can be degraded is to acetyl-CoA and acetoacetate and that astrocytes have the capacity to generate ketone bodies from fatty acids [Auestad et al. (1991) J. Neurochem. 56,1376-1386], [4,5-^{3}H]leucine was used in further metabolic studies. Radioactive metabolites released by astroglial cells into the culture medium were analysed using ion exchange chromatography. Surprisingly, α-ketoisocaproate accumulated during the incubation period. Two additional metabolites could be detected which, most likely, are acetoacetate and ß-hydroxybutyrate.
These results indicate that at least astroglial cells in culture degrade leucine to metabolites which are released. It is suggested that in the brain astrocytes may behave similarly and thus might meet the energy requirements of neighbouring cells such as neurons and oligodendrocytes. In view of these and other results, astrocytes may be considered as the brain´s fuel processing plants.

B

DISTRIBUTION OF CALPASTATIN IN RABBIT CNS AND SKELETAL MUSCLE

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Calcium-dependent neutral proteases (calpains) are distributed widely in cells and tissue. In skeletal muscle, a typical source of these enzymes, calpains are thought to be involved in the degradation of myofibrillar proteins, but other proteins are also considered their potential substrates (myosin light-chain kinase, calcium ATPase, protein kinase C etc.). In the nervous system calpains play an important role in the proteolysis of cytoskeletal proteins and myelin basic protein; furthermore they partecipate to the modulation of synaptic receptors as well as of many enzymes (protein kinase C, for example). In both tissues calpains are normally regulated by a specific proteic inhibitor, calpastatin, whose role and distribution, however, is still unclear. In the present experiments we have used a polyclonal antibody raised in sheep against the 34 KDa form of the inhibitor, purified from rabbit skeletal muscle. For the immunohistochemical studies adult rabbits were deeply anesthetized and perfused by 2% paraformaldehyde and 0.3% monomeric glutaraldehyde. Cryostat sections (5µm thick) were processed by an indirect immunoperoxidase method (ABC Elite, Vector) using DAB as a substrate. The inhibitor-related immunoreactivity in vastus lateralis muscle was distributed: 1) at endomysial level, 2) on sarcolemma and 3) inside the muscle fibres. A strong specific immunoreactivity was also detected in peripheral nerves (perinevrium, endonevrium) but, only vascular structures were stained in the CNS (vessels and meninges). Neurons and glial cells were completely devoid of labeling. Immunoblotting experiments are in progress to characterize further this vascular form of calpastatin. The present data indicate that in rabbit CNS *neural* calpastatin, if any, may differ completely (or in an important extent) from that of *muscular* or *vascular* origin.

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C

AN ENDOGENOUS INHIBITOR OF DYNORPHIN CONVERTING ENZYME IN HUMAN CEREBROSPINAL FLUID

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Neuropeptide peptidases deriving from the central nervous system are present in a large excess over the peptide pool and, in the absence of an efficient regulatory mechanism, they are capable of conversion or degradation of the particular peptide within minutes. An endogenous inhibitor has been partially purified from human cerebrospinal fluid and further characterized by means of physico-chemical parameters. This protein of the relative molecular mass of 50 kDa is a potent competitive inhibitor (Ki = 84 nM for DYN A and 94 nM for DYN B, resp.) of dynorphin converting enzyme derived from human CSF. It also efficiently inhibits trypsin, chymotrypsin and plasmin through rapid formation of complexes. Studies on the reactive site of the isolated protein indicate that an arginine residue is essential for the inhibitory activity against trypsin. Its activity is temperature- and pH-dependent. Antibody-based techniques show, that it is different from α_1-protease inhibitor, α_1-antichymotrypsin or protease nexin-II. Its inhibitory properties are, however, similar to those of α_1-antiplasmin but the estimated molecular mass is about 20 kDa lower.

The above findings suggest the presence of a substantial quantities (50 pmol/liter CSF) of a serpin-type inhibitor in the CNS. It may have an influence on neuropeptide peptidases, including dynorphin converting enzyme, derived from the central nervous system, thus, indicating the new modulatory mechanism for these enzymes.

D

STRUCTURE-FUNCTION RELATIONSHIP IN THE MELANOTROPIC AND PHEROMONOTROPIC INSECT NEUROPEPTIDE PBAN/MRCH

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Pheromone biosynthesis activating neuropeptide (PBAN, also termed melanization and reddish colouration hormone - MRCH) is a C-terminally amidated 33 amino acid cerebral neuropeptide known to regulate sex pheromone biosynthesis and cuticular melanization in insects. In the present study we have analyzed the structure-function relationship of PBAN/MRCH with respect to its pheromonotropic and melanotropic activities in order to identify the shortest sequence which evokes biological activity and to determine whether the same sequences are responsible for both functions. C-terminally free and amidated fragments derived from the amino acid sequence of the PBAN/MRCH were synthesized and their biological activities were examined. Pheromonotropic activity was tested by the ability of the PBAN derived fragments to induce sex pheromone biosynthesis in adult female moths. Melanotropic activity was examined by the ability of the same fragments to induce cuticular melanization in moth larvae. Sex pheromone content in the pheromone gland was determined by capillary gas chromatography and cuticular melanization intensity by a computerized optical density scanner. Structure-activity analysis revealed that the sequences responsible for each activity are different. The pheromonotropic activity is evoked by the C-terminal part of the neuropeptide, whereas the melanotropic activity requires the presence of the N- as well as the C-terminal regions. Both activities require a C-terminally amide. The identification of the sequences which regulate these activities may serve as a basis for the design of peptidomimetic agonists and antagonists.

A

THE CHIMERIC PEPTIDE M35 IS A PARTIAL AGONIST AT RAT INSULINOMA Rin m 5F GALANIN RECEPTORS.

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Galanin is a widely distributed neuroendocrine peptide with inhibitory actions on insulin release from the pancreas and on acetylcholine release from the ventral hippocampus. Galanin receptors have so far been studied using ^{125}I-labeled galanin as ligand in receptor autoradiography and in binding studies. We have studied the rat β-cell insulinoma Rin m 5F galanin receptors using a radioactively labeled chimeric galanin receptor ligand ^{125}I-M35: {[^{125}I]monoiodo-Tyr^{9}}galanin-(1-13)-bradykinin-(2-9) amide. This chimeric peptide is a high affinity galanin receptor ligand, which has earlier been shown to act as a galanin antagonist in the rat spinal cord, hippocampus and on isolated mouse pancreatic islets [1,2,3]. Equilibrium binding of ^{125}I-M35 to Rin m 5F cell membranes showed the presence of a single class of high affinity binding sites with a K_D of 0.5 ±0.2 nM. Displacement of ^{125}I-M35 or ^{125}I-galanin by unlabeled M35, galanin and galanin(1-13) gave equal affinities for the ligands independently of the tracer ligand used. M35 has a dual effect on the forskolin stimulated cAMP production in Rin m 5F cells; at low concentrations (<30pM) it acts as an antagonist, whereas at higher concentrations (>30pM) it acts as a galanin receptor agonist. It thus appears that Rin m 5F cells express a subtype of galanin receptor at which M35 acts as a partial agonist.

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B

BACKBONE CYCLIZATION OF PEPTIDES AND DRUG DEVELOPMENT

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Neuropeptides are involved in numerous important biological activities and in divers pathological states. However, very few of them are used as drugs. Three main reasons hamper the use of various native peptides as drugs: (i) they undergo fast enzymatic degradation (ii) they simultaneously activate multiple receptors thus causing unwanted side effects and (iii) natural peptides suffer from undesired bioavailability characteristics. These deficiencies stem by large from the conformational flexibility of peptides. Therefore, imposition of conformational constraint (e.g. cyclization) on peptides may achieve metabolic stability, receptor selectivity and controlled bioavailability.

Our approach for the conversion of native peptides into peptidomimetics with desired pharmacological features involve gradual rigidification of the structure and correlation with biological activity, as well as conformational analysis and conformationally based computerized drug design. This approach is based on a new concept for imposing conformational constraints on peptides that we have recently introduced. According to this method, called "backbone cyclization", constraints are achieved by joining N and/or C alpha atoms in the peptidic backbone through an appropriate linker. This technology affords the production of an enormous number of chemical entities. We will demonstrate our approach for the design and synthesize of bradykinin antagonists.

C

NON-IDENTITY BETWEEN BRAIN AND KIDNEY PHOSPHATE ACTIVATED GLUTAMINASE AS REVEALED BY AN ANTISERUM AGAINST THE C-TERMINAL SEQUENCE.

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Phosphate activated glutaminase (PAG) catalyzes the hydrolytic cleavage of glutamine to glutamate and ammonia. Kidney PAG is important for the acid-base balance, and the brain PAG is probably responsible for the formation of transmitter glutamate. In spite of their different physiological functions, no kinetic or immunological differences between the purified kidney and brain enzymes have previously been found. The cloned DNA sequence is known for the brain PAG, but not for that of the kidney. In the present study we have shown that consistent with previous reports regarding antibodies raised against kidney PAG, antibodies raised against the purified brain enzyme have comparable affinity to PAG from both tissues. However, the purification of glutaminase from brain is very time consuming, and in addition the protein has weak immunogenic properties, leaving us with antibodies of low avidity. In a trial to avoid these problems we raised rabbit antibodies to a synthetic decapeptide corresponding to the deduced C-terminal amino acid sequence of the brain enzyme. Using this method we obtained antibodies of higher avidity. However, these antibodies showed a much higher affinity for the brain than for the kidney enzyme as determined by immunoblotting and immunoprecipitation. These results suggest that the two enzymes either deviate in their C-terminal sequence, or that the C-terminal end of the kidney PAG is posttranslationally modified to be unrecognizable for the antibodies.

D

OVEREXPRESSION OF ACETYLCHOLINESTERASE VARIANTS INDUCES MORPHOGENIC CHANGES IN RAT GLIOMA CELLS

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In search for the biological role of Acetylcholinesterase (AChE) in malignant glioblastoma tumors and brain glia, rat C6 glioma cells were microinjected or transfected with a series of DNA constructs expressing 3'-terminally alternative forms of ACHEmRNA under the control of the native human ACHE promoter, or of the potent cytomegalovirus promoter. The glioma cells expressed cytochemically-stainable AChE activity following injection or transfection with any of these DNA constructs and exhibited intracellular accumulation of AChE. Within 4 hours and up to 48 hours post-injection, cytoplasmically-labeled cells displayed increase in cell body volume and extension of long processes. In stably transfected cells these morphogenic features were inheritable into progeny cells up to 1 month, were dependent on endogenous production of the enzyme and did not occur in neighbouring cells not expressing the enzyme. The brain ACHEcDNA form, carrying exon 6 at its 3'-end, encoded an AChE form that was detected in cytoplasm and medium. The enzyme in cells expressing ACHEDNA containing at the 3'-end of its coding sequence intron 4 and exon 5 was mainly detected in association with nuclei, was secreted to the medium 5 fold more effectively than the brain form of the enzyme and appeared to be more negatively charged as compared with the brain form in electrophoretic separation under non-denaturing conditions. These findings demonstrate that the C-terminus of AChE dictates the distribution of this enzyme within the cell and its export for secretion and provide direct experimental evidence for a morphogenic role for AChE in glioma cells.

A

THE Na:Ca EXCHANGE AS A UNIVERSAL SENSOR FOR LOW CONCENTRATIONS OF BIOACTIVE SUBSTANCES

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The effect of very low (less than 10^{-10}M) concentration of synaptic transmitters and cardiac glycosides on membrane chemosensitivity, Na-K pump, Na:Ca exchange, intracellular messengers level and membrane lipid composition in molluscan neurons were studied. The traditional electrophysiological, isotope and biochemical methods were used. ACh, GABA, specific inhibitor of ATPase - ouabain in very low concentrations, not inducing transmembrane currents, modulated membrane responses to higher concentration of transmitters. Such modulation was due to the change of probability of receptor-bound ionic channels' activation, and not the change of the amplitude and kinetics of single-channel currents. Low concentration of ACh, GABA and ouabain had various effect on Na-K pump activity(activation, inactivation and no effect, accordingly) while they had elevating effect on Na:Ca exchange, intracellular messengers level, and stimulatory effect on membrane lipid turnover. The Na:Ca exchange is suggested as a universal gate through which low concentrations of biologically active substances affect the intracellular metabolic pathways.

B

BIOCHEMISTRY AND MOLECULAR BIOLOGY OF THE BOVINE VESICULAR MONOAMINE TRANSPORTERS. B. Gasnier, C. Sagné, D. Botton, M.F. Isambert, E. Krejci, J. Massoulié and J.P. Henry. Institut de Biologie Physico-Chimique and Ecole Normale Supérieure, Paris, France.

In monoaminergic cells, the monoamines are concentrated in secretory vesicles by a transporter catalyzing a monoamine/H^+ antiport and utilizing the energy of the H^+-electrochemical gradient generated by a V-type H^+-pump. From the data obtained by several laboratories, in rats the transporter is encoded by two different genes, $VMAT_1$ and $VMAT_2$, the former one being restricted to adrenal medulla. In bovine, we have observed two related genes, $bVMAT_1$ and $bVMAT_2$, in adrenal medulla. This result is supported by *in situ* hybridization experiments. It is surprising in view of our previous pharmacological and biochemical results which did not detect such an heterogeneity of the bovine adrenal medulla transporters. The corresponding clones are now characterized. The possible biological significance of this heterogeneity of the vesicular monoamine transporters will be discussed. From the structural point of view, the sequence of bVMAT cDNA suggests the existence of 12 transmembrane segments and of an intravesicular loop bearing glycosylation sites. The binding site of the inhibitor tetrabenazine on $bVMAT_2$ has been localized on that sequence using several approaches including photoaffinity labelling, site-directed mutagenesis and use of proteases and antibodies against various sequences of the transporter.

C

TPA HAS A LONG-TERM EFFECT ON SEROTONIN TRANSPORT

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Recent studies have shown that short incubation of various cell types with phorbol-12-myristate-13-acetate (TPA) decreases ^{3}H-serotonin uptake. The question whether TPA has also a long-term effect on serotonin transporters has been addressed herein, using the human placental cell line JAR. Upon incubation of these cells with TPA for 24-72 hr we have observed a dose- and time- dependent increase in ^{3}H-serotonin uptake. Burk-Linweaver plot of the data shows that TPA increases the Vmax of ^{3}H-serotonin uptake by 2-3 fold, with no significant effect on the Km. Phorbol analogues that do not activate protein kinase C, 4-α-phorbol-12-myristate-13-acetate or phorbol, do not increase ^{3}H-serotonin uptake. Data about interactions between the effect of TPA and cholera toxin, and the possible role of protein kinases A and C in regulating serotonin transporters, will be presented. The role of TPA in controlling serotonin transport in cells transfected with cDNA coding for the rat serotonin transporter is being analyzed as well.

D

Role of Aspartate and Histidyl residues in transport activity of the rat chromaffin granule amine transporter

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Vesicular monoamine transporters (VMAT) accumulate a variety of neurotransmitters into synaptic vesicles using the pH gradient generated by the vesicular H^+ATPase, in a process which involves exchange of lumenal protons for cytoplasmic amines. Transporters from rat, bovine and human sources have been isolated and shown to belong to a large family of Toxins Extruding Antiporters (TEXANs)[1]. When expressed in cultured cells, these proteins demonstrate neurotransmitter transport activity and are inhibited by the plant alkaloid Reserpine, which binds to the transporter at the site of amine recognition. In structure-function relationship studies of the rat chromaffin granule amine transporter, we have used site-directed mutants that exhibit modified specificity of the transporter to its substrates. Since inhibition of transport activity, as well as reserpine binding was inhibited by the chemical modifier DCC (N,N'-dicyclohexylcarbodiimide, which react with carboxyl groups), mutagenesis studies of Asp residues, which are conserved among all members of the VMAT family was carried out. Two Asp residues have been mutagenized to cystein, and the mutant proteins were shown to have neither transport activity nor reserpine binding, suggesting that these residues are involved in substrate binding. A role for Histidyl residues in transport activity has been implicated, based on inhibition of proton transport by DEPC (diethyl pyrocarbonate). Therefore, the one and only conserved Histidine throughout the VMAT family was changed to either Cystein or Arginine. When expressed in CV-1 cells, these mutants showed no transport activity, and their resperpine binding becomes energy independent and drops to a similar level of the wild type energy independent reserpine binding. The results suggest that the conserved Histidine plays a crucial role in proton translocation occuring after substrate binding.

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A

VESICULAR MONOAMINE TRANSPORTERS DISPLAY SUBSTRATE SPECIFICITY SIMILAR TO MULTIDRUG TRANSPORTERS

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The Chromaffin Granule Amine Transporter from rat PC12 cells (r-CGAT or r-VMAT1) was cloned by taking advantage of selection in the neurotoxin N-methyl-4-phenylpyridinium (MPP^+) [1]. The sequence predicts a protein that shares a distinct homology to a class of bacterial drug resistance transporters. In our work we demonstrate that several classic cytotoxic compounds (substrates of the Bacterial Multidrug Resistance and the P-glycoprotein) are able to interact specifically with the bovine chromaffin granule amine transporter (b-VMAT2; which is 66% identical to r-VMAT1) [2]. Ethidium, Isometamidium, Verapamil, TPP^+ and Doxorubicin inhibit the transport of serotonin and binding of reserpine with half inhibiting concentrations in the order of few micromolars. In contrast Vincristine did not affect either activity even at relatively high concentrations. Ethidium inhibition of serotonin transport is 5 fold less potent for r-VMAT1 than b-VMAT2.

The results indicate that VMATs have a very broad specificity, the first requirement for being a multidrug transporter. We speculate that VMATs may confer resistance to cells because they sequestrate cytotoxic compounds into intracellular compartments and thereby lower the toxin level in cytoplasm, as in the case of MPP^+.

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B

BRAIN LECTINS AND THEIR POSSIBLE ROLE IN LONG-TERM MEMORY FORMATION

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The activity of neurolectins (NL) in the rat brain nuclear (P1) and crude mitochondrial (P2) fractions at postnatal development (PD) from 2 up to 15-16 days of age has been studied. It is shown that agglutination of rabbit trypsinized erythrocytes by P1 and P2 depends on the PD stages and is changing undulately. Unlike that of P1, the NL activity of P2 correlates with the synaptogenesis. The P1 and P2 display a peculiar sensitivity to the freezing-thawing. It predominantly stimulates the NL activity of P2 and is less effective for the P1. NL are completely inactivated after 5 min of exposition to a boiling water. In the brain cytosol fraction by means of biospecific chromatography 3 NL have been revealed whith the affinity to mannose, N-acetylglucosamine and galactose respectively. Simultaneously membrane bound NL were extracted with glycine buffer (pH3) and some separate NL were identified by a corresponding PAGE band disappearence after pH3-fraction pretreatment whith the trypsinized and glutaraldehyde fixed erythrocytes. All the pH3 NL are of a synaptosomal membrane origin and modulate the Ca-ATPase activity. The long-term memory consolidation model will be discussed, where NL have a signal significance in the recognition of neuronal contact centres.

C

NCAM DEPENDENT CELLULAR INTERACTIONS WITH COLLAGEN SUBSTRATUM

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Interactions between cells and extracellular matrix play a crucial role in the modulation of cell motility, growth and differentiation. To evaluate the functional significance of the association between the neural cell adhesion molecule (NCAM) and extracellular matrix constituents, we have used L cells transfected with cDNA constructs encoding for transmembrane human NCAM 140 (LBN) and GPI linked human NCAM120 (LCN), in a collagen type I gel invasion system. The penetration capacities of the cells were strongly dependent on cell density, being significantly higher for vector transfected (LVN) control cell lines than for LBN and LCN lines, both at low and high cell densities. LBN and LCN cells cultivated on a collagen substratum, were studied by confocal laser scanning microscopy. A patchy like distribution of NCAM on the cell surface was seen with abundant staining of the tips of processes and the filopodial spikes at leading edges. Double staining for NCAM and collagen I showed that NCAM transfected cells when grown on plastic were able to retain newly synthesized and secreted collagen on the surface. To estimate the behaviour of an individual motile cell on collagen, we used a computer assisted image analysis system with automated time lapse recording followed by calculation of a number of motility and morphology parameters (velocity, area, perimeter, formfactor, shape changes) as time series. Remarkable variations of these parameters dependent on cell line and substratum were observed. The data indicate a distinct effect of NCAM expression on cellular interactions with interstitial collagen.

D

TISSUE-ASSOCIATED SERUM PROTEINASE INHIBITORS IN MOUSE AND HUMAN NEUROMUSCULAR SYSTEM

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Numerous factors are important in the development and maintenance of skeletal muscle and its motor innervation. Among these, plasminogen activation plays a major role due to tPA, urokinase and their inhibitors (PAI 1,PAI 2 etc.).However, less is known about other proteinase inhibitors (some plasmatic inhibitors, for example) which also interfere with fibrinolysis by regulating plasmin activity in the extracellular space. In this context of potential interest are α-1-antitrypsin, α-1-antichimotrypsin and α-2-macroglobulin, three serum proteinase inhibitors which have been also detected in some organs and cells.In the present experiments we searched for the tissue-associated counterpart of these inhibitors, if any, in mouse skeletal muscle using anti-human inhibitor antibodies. C57 mice were perfused with PBS (containing heparin) under deep anesthesia (diazepam 12.5 mg plus Ketamine 125 mg pro Kg body weihgt i.p.) to remove any plasmatic contamination. Cryostat fresh sections (5μm thick) of soleus or extensor digitorum longus muscles were then cut, fixed (2 min) with a mixture of 2% paraformaldehyde and monomeric glutaraldehyde (0,5%) and finally processed for light microscope immunocytochemistry (ABC Elite, Vector). In both muscles the inhibitor-related immunoreactivity occurred at endomysial level as well as inside the muscle fibres. These specific immunoreactivities were tissue-associated, as indicated by release experiments done by extensive wash (up to 12 hr) of sections before fixation and immunohistochemistry. Preliminary experiments using normal biopsies confirmed the occurrence of tissue-associated plasmatic inhibitors also in human muscles. Western blotting experiments are in progress to characterize further the inhibitor-related immunoreactivities. The present results suggest that also plasmatic inhibitors may play a role in the adult neuromuscular system.

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A

(Ca^{2+}–Mg^{2+})-ATPase ACTIVITY OF THE NEURAL CELL ADHESION MOLECULE OF ADULT RAT BRAIN

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Increasing evidence indicates a structural and functional connection between various cell adhesion molecules and different membrane ATPases. In the present study a possible association between (Ca^{2+}–Mg^{2+})-ATPase activity and the neural cell adhesion molecule (NCAM) was investigated. The effects of various detergents on total and solubilized ATPase activities were compared. It was found, that 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) released a major fraction of the (Ca^{2+}–Mg^{2+})-ATPase activity together with NCAM, while Triton X-100 produced strong inactivation of the enzyme. Using different types of solid phase immunoadsorption polyclonal anti-NCAM antibodies selectively isolated ATPase activity from CHAPS-solubilized supernatant fractions. Neither antibodies nor Fab fragments did inhibit ATPase activity. This enabled detection of ATPase activity in NCAM immunoprecipitates after immunoelectrophoresis in agarose gel, using a procedure developed for ATPase staining in gels. Thus, both approaches demonstrated (Ca^{2+}–Mg^{2+})-ATPase activity associated with NCAM. The enzyme showed a broad nucleoside triphosphate specificity, capability for ADP but not AMP hydrolysis and it was insensitive to known inhibitors of P- or F_1F_0-type ATPases. The enzyme exhibited a higher rate of Ca^{2+}-stimulated ATP hydrolysis in relation to Mg^{2+}-stimulation without strict selectivity for divalent cations. These characteristics as well as an extracellular localization of a consensus ATP-binding motif in the NCAM sequence indicate that the enzyme probably is an ecto-ATPase.

B

DEVELOPMENT OF PROTEOGLYCAN-CONTAINING PERINEURONAL NETS IN POSTNATAL RAT BRAIN

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Glial cell processes together with substances of extracellular matrix form a specific microenvironment of neurons. One type of such glia-matrix-neuron-interfaces are perineuronal nets which are enriched in chondroitin sulfate proteoglycans containing N-acetylgalactosamine (GalNAc), hyaluronan as well as hyaluronectin. In the adult rat brain perineuronal nets enwrap several non-pyramidal neurons in the cerebral cortex and neurons in numerous subcortical regions. In the present study, different detection reagents for extracellular matrix such as the GalNAc-binding plant lectin *Wisteria floribunda* agglutinin (WFA) and markers for glia and neurons were applied to elucidate cell and tissue differentiation. Already during the first postnatal week a strong staining was observed in glomerular and plexiform layers of the olfactory bulb as well as in layer Ia of the piriform cortex. In the neocortex, WFA-labeling of neuropil was predominant in layers V / VI. In the somatosensory area including its barrel-field a significant labeling of layer IV was found. In the primary visual and auditory cortex this pattern appeared only at the end of the second week. Diffusely stained perineuronal nets occurred in low density in these neocortical layers at postnatal day 7. During the second week the density of nets increased in layers IV and V / VI and newly formed nets were found in other cortical layers. In subcortical regions, such as basal forebrain complex, red nucleus and substantia nigra, lattice-like perineuronal nets were sharply contoured at postnatal day 7. It is concluded that GalNAc-containing proteoglycans, distributed in the neuropil and concentrated in perineuronal nets, sequentially appear in patterns related to spatio-temporal gradients of brain maturation in cortical and subcortical regions.

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C

GLYCONEOGENESIS VIA A GLUCONEOGENIC PATHWAY IN ASTROGLIAL CELLS

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The incorporation was studied of the gluconeognic substrates lactate, alanine, glutamate and aspartate into glycogen of astroglial primary cultures derived from mouse brain. The incorporation was inhibited by 3-mercaptopicolinate, an inhibitor of phosphoenolpyruvate carboxykinase (PEPCK), one of the characterisitc gluconeogenic enzymes. In contrast to PEPCK in rodent hepatocytes, astroglial PEPCK activity was not affected by dibutyryl cAMP and dexamethasone, and only the mitochondrial isoenzyme of PEPCK was detectable in the astroglial cells. The contribution of 3-carbon compounds to total astroglial glycogen amounted to about 15 %, under the condition that cellular glycogen was replenished on refeeding of glucose-starved cells. A participation of gluconeogenic substrates in glycogen metabolism was also detectable under steady state conditions, when glycogen content was invariant with culture time. Immunohistochemical examinations of rat brain slices demonstrated a colocalization of fructose-1,6-bisphosphatase (FBPase), the other characteristic gluconeogenic enzyme, with the astrocyte marker glial fibrillary acidic protein, indicating a restriction of the gluconeogenic pathway in brain to astrocytes. From the gluconeogenic capacity and the localization of FBPase we hypothesize that the gluconeogenic pathway could serve as a mechanism by which astrocytes dispose of lactate generated by neighboring cells such as neurons.

D

DNA PRECURSOR INCORPORATION INTO THE HIPPOCAMPUS OF THE HYPO- AND HYPERTHYROIDIC ADULT RATS

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Thyroid hormone deficiency or excess early in life produces severe changes in the development of the central nervous system. Thyroid hormones primarily affect cell acquisition in these parts of the brain where neurogenesis is significant postnatally, like cerebellum and dentate gyrus (DG). Studies of experimental hypothyroidism in rats performed early during development suggest that in both structures cell migration and maturation are affected, but not cell proliferation. There is no direct evidence on the influence of thyroid hormone on the DG neurogenesis, which persists in adult rodents. In this study we aimed to evaluate the effect of hypo- and hyperthyroidism on the DG cell acquisition, estimated by incorporation of bromodeoxyuridine (BrdU), a precursor of DNA synthesis to the DG cells. Euthyroid (E), hyperthyroid (E+T3), hypothyroid (H) and hypothyroid given replacement treatment by T3 (H+T3) rats were studied. Offspring of naive Wistar rats born on the same day were pooled and randomly distributed into families of 6 pups per dam (E and E+T3 groups). Offspring of dams fed diet containing 0.4% propylthiouracyl beginning on embryonic day 19 in order to make pups hypothyroid were divided into H and H+T3 groups. Additionally, pups from H and H+T3 groups underwent partial thyroidectomy on postnatal day 30. At 2 months of age the animals were weaned and received 1 week treatment with BrdU (i.p., 30mg/kg body weight, daily). Three weeks later (3 months of age) rats were sacrificed by perfusion for immunocytochemistry and BrdU labeling of DG neuronal nuclei was determined with PAP technique. Counts of immunopositive cells revealed that BrdU incorporation was significantly reduced in hypothyroidic group, while in H+T3 group BrdU labeling was slightly below that one in control groups. Also the distribution of the labeled cells within DG among the groups was different. Data indicate that in the adult rats hypothyroidism affects the proliferation and possibly migration of DG neuronal precursors and that this effect can be abolished by T3 treatment. (Supported by MRC, Canada, and the Network of Centres of Excellence for Neural Repair and Functional Recovery).

A

ALTERATIONS OF NEURONAL DIFFERENTIATION *IN-VITRO* AND OF ANIMAL BEHAVIOUR *IN-VIVO* AFTER SELECTIVE GENE SUPPRESSION WITH ANTISENSE OLIGONUCLEOTIDES

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Selective inhibition of gene expression with antisense oligonucleotides has become a powerful tool for functional analysis. Phosphorothioate oligonucleotides (S-ODN) are extremely stable and the most successful antisense molecules to date. Injection of S-ODN into the brain allows selective suppression of the synthesis of single proteins for a limited time in a restricted brain region. We have previously shown that selective inhibition of c-Jun and JunB transcription factor synthesis strongly alters neuronal differentiation *in-vitro*.
Using FITC- or ^{35}S-labelled S-ODN we now analyzed cellular uptake and S-ODN distribution after *in-vivo* brain injections.
Two *in-vivo* experiments demonstrated the role of Jun transcription factors in neuronal plasticity:
1. In brightness discrimination experiments, retention of a learning task could be strongly inhibited by c-*jun* suppression.
2. Co-suppression of *jun*B and c-*fos* in the suprachiasmatic nucleus (SCN) prevented light induced resetting of the circadian rythm in rats housed under constant darkness.

In summary, the antisense technology allows functional analysis of the role of single proteins in complex physiological processes. Furthermore it holds great therapeutic potential.

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B

RAPID INDUCTION OF EPENDYMIN mRNA AFTER ACTIVE AVOIDANCE CONDITIONING AND ANTISENSE INTERVENTION WITH MEMORY CONSOLIDATION

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Goldfish were trained in a shuttle-box to avoid electric shocks administered after a conditioning light stimulus and killed 10 min to 16 h after acquisition of the avoidance behaviour. In situ hybridizations using a 30mer 35S-labeled anti-ependymin oligonucleotide revealed, that ependymin mRNA is exclusively expressed in the leptomeninx and rapidly induced after conditioning as compared with passive, active and yoked controls. Increased mRNA expression was folllowed by enhanced translation and secretion of this L2/HNK-1-bearing cell adhesion protein, that is redistributed after learning and incorporated into specific neurons of goldfish optic tectum. - In order to inhibit de novo synthesis of ependymin molecules without interference with pre-existing molecules, 18mer phosphorothioate oligodeoxynucleotides (S-ODN) were injected into the perimeningeal brain fluid before training. When tested 3 days later, these animals were amnestic. Fish treated with randomized S-ODN sequences or injected with antisense probes into brain ventricles (i.e., away from the site of ependymin synthesis) served as controls. Results support the conclusion that only newly synthesized ependymins, that have not yet undergone further modification, are involved in memory formation.

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C

NGF EFFECTS ON GENEREGULATION OF INTERLEUKIN 1α IN PC12 CELLS

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PC12 cells exposed to Nerve Growth Factor (NGF, 7S, from mouse sub maxillary gland) show a time and dose-dependent induction of Interleukin 1α (IL-1α) (Alheim et al., 1991)

To determine the transcriptionfactors involved in the NGF induction of IL-1α expression in PC12 cells, the 5' regulatory region of the IL-1α gene was studied, using chloramphenicol acetyltransferase (CAT) as a reporter protein. A 227 bases long fragment of the 5' region of the IL-1α gene (-163 - +64) was inserted into the promoterless plasmid pBLCAT3, immediately preceding the gene coding for CAT, and this fragment was tested as the promoter of CAT expression.

PC12 cells were transfected with these plasmids, using electroporation. The PC12 cells were exposed to NGF for two days, before harvesting. The expression of CAT protein was measured, using a CAT-ELISA. The expression of CAT was increased 4 to 10-fold in the cells exposed to NGF, compared to control. This shows that, at least part of, the NGF responsive element on the IL-1α gene is located within this 227 basepair region.

Trying to pinpoint the specific bases responsible for the upregulation of IL-1α expression, three different (9-15 bases) deletions were made. The deletion sites were choosen based upon comparison to other NGF-induced proteins (e.g. ERK2, galanin). However, so far, no significant reduction in CAT expression was noted, using these plasmids for electroporation, as compared to the non-deleted fragments.

There are two putative AP-1 binding sites within the IL-1α gene fragment used, which may mediate NGF induction. These AP-1 sites are separated by 50 bases and presented as inversed repeats. By deletions of these two AP-1 sites, we are examining whether or not the AP-1 site is responsible for the NGF induction of IL-1α .

Reference: Alheim, K., Andersson, C., Tingsborg, S., Ziolkowska, M., Schultzberg, M. and Bartfai, T. (1991) Interleukin-1 Expression Is Inducible by Nerve Growth Factor in PC12 Pheochromocytoma Cells, *Proc Natl Acad Sci USA.* **88**: 20; 9302-9306

D

EXPRESSION OF NGF AND BDNFmRNAs IN ASTROCYTES UNDER NORMAL *IN VIVO* CONDITIONS AND AFTER CHRONIC INJURY

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Nerve growth factor (NGF), the prototype of the 'target derived neurotrophic factors' is an important mediator for the regulation of neuronal survival during development as well as for neuronal maintenance and repair in the adult brain. The cellular source of endogenous NGF in the CNS is still somewhat controversial. In the normal adult brain, expression of NGF mRNA has so far been identified only in neurones. We here report on the expression of NGF and BDNF mRNAs detected by in situ hybridization in rat brain astrocytes under normal in vivo conditions. Coronal sections of the rat forebrain were first processed for in situ hybridization to NGF mRNA or BDNF mRNA. Mouse antisense RNA fragments (NGF: 0.64kb; BDNF: 1.2kb) containing digoxigenin-uridine and previously subjected to limited alkaline hydrolysis were used. Sections were subsequently reacted for the presence of GFAP immunoreactivity. Besides the neuronal expression of both neurotrophins which was detected in a number of brain areas, the combination of in situ hybridization and immunocytochemistry allowed to clearly localize the hybridization signal over astrocytes as well. Astrocytes exhibiting a hybridization signal were most numerous in the hippocampal formation. They were also present, although fewer, in all cortical areas, in the amygdala and in the basal forebrain nuclei such as the medial septal nucleus, diagonal band nucleus and basal nucleus. After chronic neurotoxic injury, expression of NGF mRNA quantified by Northern blot analysis was found to be elevated in the neocortex, hippocampus, thalamus and basal forebrain. This increase in NGF mRNA expression was related to an increase in number of activated astrocytes expressing NGF mRNA while neuronal expression was only marginally changed. The localization of neurotrophin expression in astrocytes within cholinergic forebrain nuclei which have previously been shown to be one of the major cellular targets of NGF and BDNF both under normal conditions and after chronic neurotoxic injury seriously questions a solely 'target derived' origin of neurotrophic factors. (Supported by the BMFT: 0316914A).

A

TUMOR NECROSIS FACTOR α INDUCES A DELAYED OPENING OF THE BLOOD-BRAIN BARRIER IN VITRO

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Tumor necrosis factor α (TNFα), a proinflammatory cytokine, is involved in the pathogenesis of CNS infections and several neurodegenerative diseases. The possible role of the blood-brain barrier (BBB), the active interface between the blood and brain, during these changes is controversial. It is disputed whether TNFα results in an opening of the BBB for different tracers or not in animal models. No data are available on the direct effect of TNFα on the permeability of cerebral endothelial cell monolayers cocultured with astrocytes, the *in vitro* reconstituted model of BBB. Incubation of bovine brain capillary endothelial cells (BBCEC) with 50, 250, and 500 U/ml rhTNFα up to 4 h did not change significantly the transendothelial flux of either sucrose (m.w. 342 Da), or inulin (m.w. 5 kDa), except for a decrease ($p<0.05$) in sucrose transport at 120 and 240 min for the highest dose of TNFα. One hour preincubation of BBCEC monolayers with TNFα induced a significant ($p<0.001$) 2- to 3-fold increase in the permeability for both markers 16 hours after the challange at all 3 concentrations. These observations were confirmed by immunohistochemistry, where reorganization of F-actin, but not of vimentin structure, correlated with the increased permeability of BBCEC. In conclusion, the direct effect of TNFα on our *in vitro* BBB model was biphasic: it resulted in no change and/or decrease of permeability in the first 4 hours, and a significant increase of transendothelial flux with concomitant cytoskeletal changes 16 hours after the challenge.

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B

SIMPLIFIED GANGLIOSIDE COMPOSITION OF PHOTORECEPTORS COMPARED TO OTHER RETINAL NEURONS

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We have examined ganglioside (GG) content and composition in different fractions of developing and adult rodent retina in vivo and in vitro, paying particular attention to the GG levels in photoreceptors. Compared to normal retina, mutant RCS rat and rd mouse retina (exhibiting total degeneration of photoreceptors), despite their large cellular losses contained relatively higher levels of GG/mg protein, with increases in all GG classes especially complex di- and tri-sialoGG. Pure sheets of photoreceptors were also isolated from adult normal rats by vibratome sectioning, and contained only 34% the amount of GG detected in the remaining retina (2.2nmol vs 6.4nmol GG), and had relatively higher levels of GM3 and GD3, but greatly reduced levels of complex GG (eg. GT1b represented 13% total GG in internal retina, but was undetectable in photoreceptor samples). Purified photoreceptor outer segment organelles largely paralleled these data for whole cells. Finally, cultured newborn rat retinal neurons expressed largely similar GG profiles to young in vivo normal rat retina, with a complex distribution of all GG classes. Treatment with neurotrophic factors such as basic fibroblast growth factor led to reductions in GM3 and increases in complex GG. Immunocytochemical labelling of retinal cell cultures using cell-type specific and anti-GG antibodies (anti-GM1 and anti-complex GG, A2B5) showed a relative reduction of staining of photoreceptors compared to other retinal neurons such as amacrine cells. Taken altogether, these findings indicate that photoreceptor cells possess less GG and of a simpler form than those found in other retinal or brain-derived neurons. This may represent an adaptation towards their specialized function of detecting and transducing light energy.

C

NERVE GROWTH FACTOR: A CANDIDATE PARACRINE/AUTOCRINE MEDIATOR IN ASTROGLIA-MICROGLIA-NEURON INTERACTIONS

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It has been long known that both microglia and astrocytes are activated by CNS injury. Microglia are the first to react and their reaction is transitory, while reactive astrocytes, once transformed, persist in their newly acquired state. It was recently proposed that astrocytes and microglia exert opposing actions on the survival of neurons by releasing neurotrophic and neurotoxic factors, respectively. However, evidence indicating that, like astrocytes, microglia can also be stimulated to produce nerve growth factor (NGF, a potent neurotrophic factor for several classes of neurons), was at odds with that proposal and prompted us to investigate the localization of NGF. The present study in primary CNS cultures, provides evidence not only for the acummulation of NGF selectively in microglia, rather than in astrocytes, but also for chemotaxis towards and sequestration of NGF by microglia. The findings suggest that a local increase in NGF release may serve to attract more microglia and perhaps peripheral macrophages. And, that by means of sequestration, microglia can regulate NGF availability in a site-specific manner at critical times during CNS development and aging, and after neurotrauma, when this neurotrophin is needed for nerve cell survival and axonal growth and regeneration.

D

NEURONAL GROWTH CONE AND SYNAPTIC MEMBRANE PROTEIN BASP1.

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Recently (J. Neurochem. 61 Suppl.: S34D, 1993) we have described a group of brain acid soluble proteins (BASPs). All four members of the group (BASP1, BASP2-1, BASP2-2 and BASP3) are very acidic phosphoproteins (pI 4.3-4.6) soluble in 5% perchloric acid. BASPs abnormally migrate during SDS-PAGE: in 12% gel, mobilities of these proteins correspond to Mr 45-55 kDa, while their real molecular masses are 20-25 kDa. Two of these proteins (BASP2-1 and BASP2-2) proved to be two forms of well-known neuronal protein GAP-43 (B-50, pp46, F1, neuromodulin etc.). These two forms of GAP-43 can be resolved by PAGE using 0.9 M acetic acid-2.5 M urea, but not by SDS-PAGE. Two other proteins (BASP1 and BASP3) have not been identified with the known brain proteins.

This study focuses on the properties of BASP1. We show that BASP1 is a major phosphoprotein of growth cones and of synaptic membranes: its amount 3-4 times exceeds that of GAP-43. BASP1 can be considered as membrane integrated protein: for its release nonionic detergent (1% Triton X-100) must be used. In contrast to other BASPs, BASP1 shows signs of hydrophobicity: its movement during PAGE using 0.9 M acetic acid-2.5 M urea system is strongly retarded in the presence of 0.5% Triton X-100. Treatments of BASP1 with 0.5 M NaOH, 1 M hydroxylamine, as well as extraction with chloroform/methanol (2:1) and ethanol/ethyl ether (2:3) mixtures have not revealed the presence of lipid constituents,in BASP1. Therefore, the hydrophobicity of BASP1 is an intrinsic property of this protein. Similariy to GAP-43, BASP1 is abundant in cortex and hippocampus, but in contrast with GAP-43, BASP1 is abundant in cerebellum too. BASP1 samples obtained from different species (rat, cow, man) proved to be markedly distinct immunologically. Their peptide maps are also different, showing high variability of BASP1 in evolution.

A

EXPRESSION AND PHARMACOLOGICAL MODULATION OF NERVE GROWTH FACTOR (NGF) IN INTACT AND DAMAGED RAT BRAIN

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Nerve growth factor (NGF) is a proposed therapeutic for the treatment of neurodegenerative disorders such as Alzheimer's disease based on its ability to rescue cholinergic neurons in animal models of neurodegeneration. Since NGF is unable to cross the blood-brain barrier, the direct adminstration into the brain is the only means of drug delivery. Recently a great deal of interest is directed towards the mechanisms involved in the regulation of endogenous NGF in the brain and its pharmacological manipulation, as an alternative strategy for the treatment of neurodegeneration. We have recently shown, that although in intact brain NGF is mainly localized in target neurons, after electrolytic lesion of septo-hippocampal connections the activated astroglia in the septum and hippocampus may produce and secrete NGF like molecules (Oderfeld-Nowak at al., Neurochem. Int., 21, 455, 1992). We have now extended this observation showing that the induction of NGF immunoreactivity (NGF-IR) in astroglia occurs also after various mechanical and neurotoxic (eg. by trimethyltin) injuries. These results may be interpreted in terms of some self-compensatory mechanisms to the damage. We have also found that administration of interleukin-1β (IL-1β) stimulates the appearance of NGF-IR in astroglia and enhances NGF-IR in pyramidal and granular hippocampal neurons. Experiments with combined lesion and IL-1β administration effect upon NGF content (estimation by ELISA) suggest similar or the same mechanism in stimulation of NGF expression after both treatments. Our recent preliminary data indicate that the administration of NMDA receptor antagonist MK-801, prior to IL-1β, is able to block the increase in NGF-IR in pyramidal and granular hippocampal neurons caused by IL-1β, pointing to an interplay between immune and neurotransmitter systems in regulation of brain NGF expression.

B

GROWTH CONE-COLLAPSING ACTIVITY OF BRAIN-DERIVED NEUROTROPHIC FACTOR AND NEUROTROPHIN-4

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We studied the effects of different neurotrophins (NTFs): nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin-4 (NT-4) on the growth cones of the rat and chick embryonic dorsal root ganglia (DRG) and embryonic trigeminal ganglia (TG) using the growth cone collapse assay. Addition of BDNF or NT-4 at concentrations up from $4x10^{-10}$ M induced the growth cone collapse of DRG and TG neurons, which were grown in the medium containing $0.4x10^{-10}$ M of NGF or NT-3. NGF and NT-3 had no growth cone collapsing activity on DRG and TG neurons. In order to study the involvement of different NTF receptors in NTF-induced growth cone collapse we used K252a, an alkaloid that is known to inhibit specifically the autophosphorylation of *trk* neurotrophin receptors. K252a caused growth cone collapse and partially inhibited the neurite outgrowth of DRG and TG neurons at concentrations 50-200 nM. These results suggest that NTFs can guide neurite growth by chemorepulsion. The growth cone collapsing effect of BDNF and NT-4 may be mediated by *trkB* receptor in NGF- and NT-3-dependent neurons.

C

DEPOLARIZATION-INDUCED MODULATION OF THE ADP-RIBOSYLATION OF NUCLEAR PROTEINS IN RAT BRAIN STEM: POSSIBLE ROLE IN SIGNAL TRANSDUCTION

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Activation of pertussis-toxin-sensitive G-proteins by membrane depolarization was recently reported in rat brain and heart tissues (Cohen-Armon, M. and Sokolovsky, M. (1991) J. Biol. Chem. 266, 2595-2605; (1993) J. Biol. Chem. 268, 9824-9838). In the present study we observed reversible induction of ADP-ribosylation of proteins in crude nuclei of rat brain-stem, induced by membrane depolarization. The depolarization-induced effect on nuclear proteins was prevented by pertussis-toxin induced ADP-ribosylation. Two nuclear protein species are involved; 39-40 kDa proteins located in the nuclear membrane, specifically labeled by [α^{32}P]azido-GTP, and recognized by antibodies raised against the α-subunit of Gi- and Go-proteins, and 100 kDa proteins that seem to be bound to the DNA. The possible role of ADP-ribosylation of the nuclear GTP-binding and PTX-sensitive proteins in the transduction of signals was further supported by evidence for endogenous ADP-ribosylation of these proteins on cystein residues. These results may indicate a 'nuclear link' in a novel intracellular signal transduction mechanism.

D

G-PROTEINS AND LONG-TERM POTENTIATION IN RAT OLFACTORY CORTEX SLICES.

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Some of the factors determining the long-term potentiation (LTP) development are the changes of neurochemical characteristics of membrane proteins, in particular G-proteins.

In the dynamics of LTP maintenance in rat olfactory cortex slices we investigated the G-proteins GTPase activity (enzyme kinetic method, using p-NPP, GTP, GTP-γ-S and GDP-β-S) and G-proteins content (HPLC technique: "Gold System" chromatography system, reverse phase colums).

We observed, that the G-proteins GTPase activity was increased after tetanization(100/s frequency; 30s), reaching the maximum to 20 min and remaining at the increased level during 1 hour. These changes were the result from the correrspondings increase of G-protein fractions in cytoplasm.

Thus,it was shown that the changes of membrane conformation propertis and G-protein mobility takes an important part in the dynamics of LTP supporting phase.

A

STRATEGY TOWARDS A LOCALIZATION OF ADENYLATE DEAMINASE IN RAT BRAIN

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As part of the purine nucleotide cycle (PNC) adenylate deaminase (AMP-DA) catalyzes the hydrolytical deamination of AMP to IMP thereby releasing the neurotoxic NH_4^+. The physiological function in brain of the PNC is unknown. We consider a role in association with amino acid degradation in the brain. Thus, we propose transaminations of the amino group via glutamate to oxaloacetate resulting in aspartate that delivers the amino group of amino acids into the PNC in an essentially irreversible reaction.

In an effort to cellularly allocate AMP-DA in the brain a new efficient method of purification has been developed for AMP-DA from sheep brain. The enzyme will serve as an antigen for the production of antibodies that are to be used in immunohistochemical studies. An allocation for AMP-DA mRNA has recently been achieved by in situ hybridization. Using antisense cDNA probes against putative consensus sequences of AMP-DA isoenzymes mRNAs, it was shown that throughout the brain only neurons appear to contain AMP-DA mRNA.

B

SEROTONIN STIMULATION OF GTP BINDING IN RAT HIPPOCAMPAL MEMBRANES AND THE ACTION OF ANTIDEPRESSANT DRUGS.

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Changes in agonist stimulation of GTP binding have been used to demonstrate involvement of G proteins in the mechanism of action of psychotropic drugs. We here demonstrate that addtion of serotonin to hippocampal membranes results in an increase in 32P-GTP binding, and that use of selective agonists and antagonists enables characterisation of the receptor subtype involved. Membranes prepared from rats which had received chronic electroconvulsive shock (ECS) showed reduced binding when compared with membranes from rats which had received sham treatment only. Stimulation of GTP binding by carbachol was also significantly reduced in these membranes, in confirmation of results obtained by other workers using cortical membranes. These results provide further evidence for an action of antidepressants at a site distal to the receptor, very likely the G protein.

C

G PROTEINS IN CHEMOTRANSDUCTION OF THE CAROTID BODY

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Hypoxia increases the phosphatidylinositol 4,5-bisphosphate (PIP_2)-specific phospholipase C (PLC) in the carotid body (CB) tissue. In this study we tested the hypothesis that G proteins could be involved with the regulation of PLC in response to hypoxia. We addressed this issue by comparing the effects on PLC activity of pertussis (PTX) and cholera (CTX) toxins in guanosine 5'-O-(β-thiotriphosphate) ($GTP_\gamma S$, 100μM)-treated normoxic and hypoxic carotid bodies excised from anesthetized cats. The CB homogenate was the source of the enzyme and [3]$HPIP_2$ was the exogenous substrate. PLC activity, assessed from the formation of radioactive inositol 1,4,5-trisphosphate, was assayed in the presence of 10 mM LiCl and endogenous Ca^{2+}. We found that $GTP_\gamma S$ augmented PLC activity in both normoxic and hypoxic CB. The hypoxic but not normoxic augmentation of PLC was inhibited by preincubation with PTX. By contrast, CTX had no effect on $GTP_\gamma S$-stimulated PLC activity in hypoxia. These results suggest the functional involvement of a G_i-like protein in the transduction of the hypoxic signal. We conclude that a G protein-PLC interaction seems germane for the chemotransduction of the carotid body.

D

TRANSMITTER RELEASE BY NON-RECEPTOR ACTIVATION OF THE α-SUBUNIT OF G PROTEIN IN RAT STRIATAL SLICES

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The effect of 5 mM NaF + 10 μM $AlCl_3$, a direct activator of G proteins, on the release of [3H]dopamine ([3H]DA), [3H]gamma-aminobutyric acid ([3H]GABA), and [3H]acethylcholine ([3H]ACh) from slices of rat striatum, and on $[Ca^{2+}]_i$ in striatal synaptosomes was investigated.

In tissues preloaded with [3H]DA resting release in 3 min was 0.98 ± 0.08 (n=18) % of the content of label of the tissue. When the tissue was exposed to NaF + $AlCl_3$ for 30 min the basal release of [3H]DA, [3H]GABA, and [3H]ACh was enhanced 2.22 ± 0.32- (n=7), 1.21 ± 0.05- (n=5), and 1.23 ± 0.06 times (n=7). In calcium-free solution the release of [3H]GABA and [3H]DA was increased by NaF + $AlCl_3$ much more than in the presence of $[Ca^{2+}]_o$. In slice preparations taken from reserpinized animals NaF + $AlCl_3$ had no effect on [3H]DA release, i.e., under condition when the vesicular storage of [3H]DA was prevented. Neomycin (0.1 mM), a phospholipase C (PLC) inhibitor, significantly decreased the effect of NaF + $AlCl_3$ on [3H]DA and [3H]GABA release, suggesting that α-subunit of G_o protein coupled to phospholipase C may be at least partly responsible for the Ca-independent release. $[Ca^{2+}]_i$ in synaptosomes was enhanced by NaF + $AlCl_3$ in normal solution. HPLC analysis of the radioactivity of the perfusate showed that in the presence of NaF + $AlCl_3$ the content of dihydroxyphenylacetic acid (DOPAC) in perfusate sample increased significantly while in pargyline-treated animals only DA content increased significantly.

It is concluded that a non-receptor-mediated activation by NaF + $AlCl_3$ of α-subunit of G protein coupled to phospholipase C results in a release of transmitters stored in vesicles in a $[Ca^{2+}]_o$-independent manner.

A

PHENOTYPIC PLASTICITY OF THE EXPRESSION OF TYROSINE HYDROXYLASE IN THE ADULT RAT BRAIN

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In the rat Locus Coeruleus (LC) phenotypic expression of tyrosine hydroxylase (TH) has been evaluated after RU 24722 (20mg/kg IP), reserpine (mg/kg IP) and PCPA (300mg/kg IP daily during 4 days) administration. Quantitative analysis of TH protein after direct transfer onto nitrocellulose and adjacent immunohistochemical staining allowed us to analyse two distinct areas: 1) one strictly corresponds to the clustered noradrenergic perikarya and contains 30% of the total TH; the other represents a surrounding neuropile which contains 70% of the total TH and mainly corresponds to the radiated dense dendritic trees. In each compartment the number of TH positive cells , the TH protein content and their topological respective limits were quantitatively defined. In the 3 examined models, activation of TH expression led to a spatially organized and significant dendritic filling. RU 24722 but not reserpine and PCPA administration was found capable to induce TH expression in so called "sleeping cells" which appeared clustered in specific and identified cell populations. This phenotypic plasticity revealed in adult rat and the possible role of modulation of TH content in dendritic compartment will be discussed.

B

NGF PROMOTES M1 MUSCARINIC RECEPTOR-STIMULATED AMYLOID PRECURSOR PROTEIN SECRETION IN PC12 CELLS.

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Rat pheochromocytoma cells stably transfected with the M1 muscarinic receptor (PC12M1 cells) extend neurites in response to muscarinic receptor stimulation, and this is synergistic with NGF-induced neurites (Pinkas-Kramarski *et. al.*, J. Neurochem. 59:2158, 1992). Neurotrophic-like effects were also mediated in these cells by AF102B, an M1-selective muscarinic agonist (Gurwitz *et. al.*, Soc. Neurosci. Abs. 19:1767, 1993). M1 receptor stimulation of PC12M1 cells was also shown to release to the culture medium non-amyloidogenic fragments of the amyloid precursor protein (APP), which probably involved protein kinase C activation (Buxbaum *et. al.*, PNAS 89:10075, 1992). Kinase C mediated secretion of soluble APP was reported to be more robust in differentiated PC12 cells (Loffler and Huber, BBRC 195:97, 1993). We therefore studied APP secretion in PC12M1 cells following combined stimulation with NGF and muscarinic agonists, by immunoblotting with the 22C11 mAb (which recognizes the N-terminus of APP) followed by computerized video-imaging densitometry. Increased M1 receptor-mediated secretion of soluble APP was detected in PC12M1 cells following exposure to NGF (50 ng/ml) for 3 days, relative to non-differentiated cells. This was also evident from the increased disappearance of membrane-bound APP following M1 receptor stimulation in NGF-differentiated cells: exposure to carbachol or AF102B (both at 0.1 mM; 24 h) resulted in membrane APP levels of 70±6% and 72±11% of control in non-differentiated, *vs.* 46±7% and 39±14% of control in NGF-differentiated cells, respectively, and this was blocked by co-incubation with atropine. Thus, NGF promotes the M1 receptor-mediated APP secretion in PC12M1 cells, which may reflect enhanced levels and/or availability of protein kinase C or the proposed "secretase" which cleaves membrane-associated APP. The data also indicate that the M1-selective agonist AF102B, which is a partial agonist for M1-mediated phosphoinositide hydrolysis and arachidonic acid release in PC12M1 cells, is nonetheless as powerful as the full agonist carbachol for decreasing the levels of membrane-associated APP, assayed in these cells following 24 h incubation periods. This may reflect less pronounced desinsitization of M1-mediated signaling by partial agonists. Neuronal cell-death in AD may involve increased production of the amyloidogenic βA4 peptide, secondary to deficient secretion of APP. Our observations imply that AF102B may compensate for such deficiencies and thus may constitute a novel treatment for AD.

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C

THE MONGOOSE ACETYLCHOLINE RECEPTOR α-SUBUNIT: CLONING AND EXPRESSION.

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To elucidate the fine structure of the acetylcholine receptor (AChR) ligand binding site we have been studying the putative binding site from animal species that are resistant to α-neurotoxins. Previous work from our laboratory on the mongoose AChR binding site domain (Barchan et al, Proc. Natl. Acad. Sci. 89: 7717, 1992) demonstrated that there are five amino acid differences between the mongoose (α–bungarotoxin (α-BTX) resistant) and the mouse (α-BTX sensitive), which cluster in the presumed ligand binding site, close to cysteines 192 & 193. Four of these differences are at positions 187, 189, 194 &197 which are conserved in animal species that are susceptible to α-BTX. It should be noted that Asp 187 in the mongoose α-subunit is an additional putative N-glycosilation site. In order to find out whether other regions in the α-subunit of the mongoose AChR may participate in determining its resistance to α-BTX we have cloned by RT-PCR the entire AChR α-subunit gene from the mongoose and compared it to the α-subunit gene of the mouse. Alignment of the mouse and mongoose α-subunit sequences shows 89% homology in the nucleotides and 93% homology in the deduced amino acids. There are 29 amino acid differences between the mouse and the mongoose α-subunits, only six of which are at positions that are conserved in all animal species that bind α-BTX. These six differences include the four amino acids substitutions in the binding site domain and two others at position 112 (Tyr to His) and 153 (Gly to Ser). It is possible that the latter two substitutions may also contribute in determining the resistance of the mongoose to α-BTX. The other 23 amino acid differences are at positions which are not conserved among animal species that are susceptible to α-BTX and most of them represent minor conservative differences. Therefore, amino acids residues at these positions are probably not involved in conferring toxin resistance. The α-subunit specific cDNA from the mouse and the mongoose AChR were translated in vitro in a nuclease treated reticulocyte lysate system and were immunoprecipitated with polyclonal antibodies to the AChR. In both cases, distinct polypeptides of 41kDa were expressed, suggesting that both cDNA templates properly translated to yield their respective α-subunit precursors.

D

NATURAL MUTATIONS IN THE LIGAND BINDING SITE OF THE ACETYLCHOLINE RECEPTOR DETERMINES RESISTANCE TO α–BUNGAROTOXIN

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Previous work from our laboratory demonstrated that a synthetic dodecopeptide from the α-subunit of the acetylcholine receptor (AChR), which includes the two tandem cysteines at positions 192 and 193, contains the essential elements of the ligand binding site. In an attempt to elucidate the molecular basis for ligand binding we investigated the binding site structure of AChR from several animal species which show various degrees of resistance to α–bungarotoxin (α-BTX). Fragments of the AChR α-subunit encoding residues 122-205 from snakes, mongoose, hedgehog, human, shrew, cat and mouse were amplified by reverse transcription and PCR and then sequenced and expressed in E. coli. All fragments are highly homologous and contain the four cysteines at residues 128, 142, 192 and 193. The snake, mongoose and hedgehog fragments did not bind α-BTX and they all have substitutions at the conserved positions 187 and 189. This indicates that changes at these positions from the aromatic residues Trp and Phe respectively, to non aromatic ones, may be sufficient for conferring toxin resistance. The snake and the mongoose AChR have additional substitutions at positions 194 (snake and mongoose) and 197 (mongoose). Nevertheless, it should be noted that the human AChR fragment which is a partial binder and binds α–BTX to a lesser extent than the mouse, cat and shrew fragments, also has substitutions at the positions 187 and 189 from aromatic to non aromatic residues. This suggests that the nature of the subsititutions at the binding site, and possibly changes in other positions which may affect the charge and/ or conformation of the binding site, also contribute to determining α-BTX resistance.

A

CLONING OF A PUTATIVE NEURO-TRANSMITTER RECEPTOR,CV1711, FROM THE CIRCUMVALLATE TASTE PAPILLAE OF THE RAT

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Our objective is to reveal taste specificity by cloning and characterizing receptors from the taste tissue. Existing data support the hypothesis that putative G-protein coupled receptors are involved in bitter and sweet taste sensations. Although much research has been done, no single receptor, that is specific to taste tissue has been characterized. CV1711, G-protein coupled receptor (GCR), was identified by comparing PCR products amplified from the cDNA of circumvallate taste papillae cDNA to those amplified in nearby epithelia sheets, which are devoid of taste cells. The PCR primers utilized were designed from conserved sequences in transmembrane domains two and seven of the GCR superfamily. The deduced amino acid sequence of CV1711 exhibits 24-31% homology to many members of the neuropeptide GCR subfamily. Northern blotting of rat tissue RNAs indicated that the 3 kb mRNA of CV1711 was present only in epithelia that containing taste cells and not in nearby non-sensory epithelia without taste cells. This CV1711 gene may encode for taste receptor, or neuropeptide hormone receptor that is specific to taste tissue, but whose ligand is presently unknown.

B

MUSCARINIC RECEPTOR SUBTYPES IN CHICK HEART AND THEIR POSITIVE ALLOSTERIC INTERACTION WITH ALCURONIUM.

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Cardiac muscarinic receptors are of the m2 subtype in mammals. It has been shown by mRNA analysis (Tietje et al. 1990, Tietje an Nathanson 1991) that two subtypes of muscarinic receptors are expressed in chick hearts, but their quantitative proportion has not been established. We have now found that two populations of muscarinic binding sites may be distinguished in cardiac membranes from 1-day-old chicks by investigating the binding of N-(^{3}H-methyl)scopolamine ((^{3}H)NMS) and of unlabelled subtype-specific muscarinic antagonists. One population, corresponding to about 80% of binding sites, has a high affinity for AF-DX116 and methoctramine and the rate of (^{3}H)NMS dissociation from it is fast. The other population has a low affinity for the two unlabelled antagonists and the rate of (^{3}H)NMS from it is slow. Both populations have a high affinity for pirenzepine, which is higher in the second population. The two populations could be separated by selective inactivation with benzilylcholine mustard. The larger population appears to be related to mammalian m2, while the smaller population to mammalian m4 receptors. Alcuronium allosterically increased the affinity of both receptor subtypes to (^{3}H)NMS.

C

NEUROPROTECTIVE ACTION OF LOW INTENSIVE He-Ne LASER UNDER THE CONDITION CEREBRAL ISCHEMIA / REPERFUSION

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Investigation have been carried out on 75 white rats of 180-200 g weight anesthezied by intraperitoneal nembutal administration (3 mg/100 g body mass). Transitory ischemia / reperfusion was modeled by bilateral occlusion of common carotid arteries. Laser irradiation have been carried through parietal cranial bone. He-Ne laser parameters were: length -632,8 nm; spot diameter -1,52 cm; power - 1,1mWt; density - 0,6 mWt/cm^2; irradiation period - 10 min; stabile regime.

It was estabilished that laser exposure in pre-occlusive period prevented the decrease of triphosphoinositide (TPI), diphosphoinositide (DPI) and sphingomyelin (SPM) contents,observed in brain tissue during ischemia,and at the same time it had not almost influenced upon phosphatidylserine (PS) and phosphatidylethanolamine (PE) levels and normalized phosphatidylcholine (PCH) quantity in reperfusion. Lasere exposure in post-occlusive period didn't show the significant changes of TPI and DPI levels in reperfusion, but simultaneously revealed the increase of IMP, SPM, PCH and PS contents. Laser irradiation markedly increases ATP quantity in brain tissue, decreased in reperfusion period. The increase of total content of adenylnucleotides (AN) occurring at that time is accompanied by simultaneous stability of ADP, adenosine quantities and by increase of AMP and cAMP contents. Protective action of He-Ne laser in respect of AN pool was obtained also during cerebral radiation in post-occlusive period in the form of complete normalization of AMP, cAMP and adenosine contents, in increase of ATP/ADP and ATP/AMP coefficients. The role of lasers in the molecular mechanisms of regulation of structural, metabolic, functional properties and pharmacological effects is discussed.

D

VILIP - Ca^{2+}-dependent interaction with cell membrane and cytoskeleton

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The 22 kDa EF-hand Ca^{2+}-binding protein VILIP isolated from chick brain (1) is a member of a recently identified family of highly homologous neuronal proteins.There is only very little information about the funtion of these proteins.
Immunocytochemical studies revealed a specific expression paytern of VILIP in a subset of neurons of the entire brain and of the retina. In the chick cerebellum, a comparison with *in situ* hybridisation data indicates a transport from granular cell bodies to the axonal structures of the molecular layer.
Cell fractionation studies under physiological conditions showed that the distribution of VILIP between cytoplasm and membrane fraction is about 3:1. Using 1mM Ca^{2+} in the homogenisation buffer one can observe a significant shift into the membrane fraction. Further fractionation of the membrane fraction revealed that this shift is at least partly due to a Ca^{2+}-dependent interaction of VILIP with the cortical cytoskeleton. One of the responsible cytokeletal components was identified as actin using recombinant VILIP in a binding assay on Western blots. Another binding partner of about 50 kDa was not yet identified.
Our data suggest that VILIP is implicated in the Ca^{2+}-dependent regulation of the dynamics of the cortical cytoskeleton in neurons.

(1) Lenz et al. (1992), Mol. Brain Res. 15, 133-140

A

SELECTIVE REGULATION OF PROTEIN PHOSPHATASE I DURING DEVELOPMENT IN CHICKEN FOREBRAIN AND CEREBELLUM

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We have reported that the majority of protein phosphatases type I (PP1) and 2A (PP2A) in rat forebrain is membrane bound and less active than the cytosolic forms of these enzymes (Sim et al., J.Neurochem. 1994, in press). We have taken advantage of the protracted developmental changes and temporal separation of the formation and maturation phases of synaptogenesis in chicken brain to investigate if the appearance of factors responsible for this membrane association and inactivation of PP1 and PP2A is linked with a particular stage of synapse development. As in rat the majority of PP1 (measured by immunoblots) was present in the membrane fraction of adult chick forebrain and cerebellum, but most enzyme activity (measured using glycogen phosphorylase as substrate) was recovered in the cytosol. During posthatch development there was a progressive decline in the activity of both cytosolic and membrane bound PP1 in forebrain and cerebellum. Concomitantly, there was a progressive increase in the amount of PP1 associated with membranes and no change in the cytosolic concentration of PP1. Similar analysis of PP2A showed that membrane PP2A with low specific activity was present in chicken forebrain and cerebellum at 2 days but there were no significant developmental changes in enzyme activity or concentration in cytosol fractions or, apart from a small decline in activity shortly after hatching, in membrane fractions. These results demonstrate a selective developmental regulation of PP1 suggesting that such mechanisms may be important in synaptic plasticity.

B

IDENTIFICATION OF NOVEL PROTEIN COMPONENTS OF THE PSD FRACTION

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The constituent and associated proteins of synaptic junctions are thought to play a crucial role in the function of chemical synapses. They represent a distinct subset of proteins highly specialized in neurotransmission and capable of mediating plastic changes in the brain. Previously we reported a combined immunological and molecular biological approach to discover new protein components present at the PSD fraction from rat brain (Garner, C.C. et al., in: Neuronal Cytoskeleton - Morphogenesis, Transport and Synaptic Transmission, Japan Scientific Societies Press pp.317-329). From about 500 cDNA clones being immunoreactive with polyclonal PSD antisera we characterized more than 260 clones by sequence analysis and 180 clones by Northern blotting. DNA sequence comparisons revealed 26% of the clones having no correlates in public databases. Concerning the tissue distribution of their transcripts only 25% of the "unknown" clones hybridize with mRNAs exclusively expressed in the brain. Among all investigated clones 22% show equal expression in all examined tissues, 25% are differentially expressed, but in most cases predominant in the brain, and 28% fail to give any hybridization signal, which may be due to the low abundance of corresponding transcripts. Some sets of the "unknown" clones display interesting sequence similarities to previously identified proteins. PSD clones 7k and 37c2, for example, encode a protein that contains structural motifs characteristic of extracellular matrix proteins or cell surface receptors.

C

CHARACTERIZATION OF THE ANTI-OXIDANT CAPACITY OF 9L GLIOMA CELLS

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The glutathione pathway is thought to be the main anti-oxidant mechanism in the CNS. This pathway is coupled to the pentose phosphate pathway (PPP) so that cellular response to oxidative stress is manifested by stimulation of the PPP. We have previously described a novel technique, which allows for measurements of PPP activity *in vivo* and in tissue cultures[1,2]. Addition of H_2O_2 to the incubation medium for 30 min stimulated PPP activity in a concentration-dependent manner from basal levels of 2.9±1.0% (n=3±SE) to a maximum of 23.4±0.1% in the presence of 400 µM H_2O_2. Estimated from fractional survival assays, H_2O_2 was toxic with an EC_{50} value of 65 µM. Similarly, diamide (5 mM, 30 min), which oxidizes GSH to GSSG, produced a concentration-dependent stimulation of PPP activity to a maximum of 75.7±0.1%. Incubation with 5 mM diamide and 100 µM buthionine sulfoximine for 24 h, an inhibitor of γ-glutamyl-cysteine sythetase causing depletion of GSH levels, resulted in a decreased maximal PPP activity to 65.0±0.1% and an increased sensitivity to H_2O_2 toxicity to an EC_{50} of 22 µM. Phenazine methosulphate (5 µM), an electron acceptor for NADPH, resulted in stimulation of PPP activity to 71.71±0.4% while in the presence of dehydroepiandrosterone (DHEA, 60 µM), an inhibitor of glucose 6-phosphate dehydrogenase, a decreased maximal PPP activity of 51.0±0.5% was observed. Assays of G6PDH in the presence of DHEA in tissue extracts revealed 87% inhibition. This discrepancy is attributed to limited permeability of DHEA through the plasma membrane. We therefore conclude that; 1) the relatively low maximal PPP activity obtained with H_2O_2 suggests that glutathione peroxidase, rather than glutathione reductase or G6PDH, may be rate limiting for removal of H_2O_2, 2) cellular susceptibility to H_2O_2-induced oxidative stress can be augmented by agents that inhibit the glutathione pathway, 3) cellular response to oxidative stress can be observed by monitoring PPP activity.

1) Ben-Yoseph O. *et al.* (1993). *J. Neurochem.* 61 (Suppl.), 230D.
2) Ross B. D. and Ben-Yoseph O. (1993). *J. Neurochem.* 61 (Suppl.), 231A.

D

NEURONAL AND GLIAL RESPONSE TO OXIDATIVE STRESS AS INDEXED BY THE PENTOSE PHOSPHATE PATHWAY

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Hydrogen peroxide can give rise to cytotoxic formation of hydroxyl radicals through metal-catalyzed Haber-Weiss reactions. Oxidative stress has been implicated in a host of neuropathological conditions so that it would be beneficial to monitor neuronal capacity for H_2O_2 detoxification. The glutathione pathway, required for H_2O_2 degradation, is coupled to the pentose phospate pathway (PPP), so that monitoring PPP activity provides an index of neuronal oxidative stress. We have previously reported measurement of PPP activity both *in vitro* and *in vivo*[1,2], using (1,6-$^{13}C_2$,6,6-$^{2}H_2$)glucose. Addition of H_2O_2 to the incubation media of primary neuronal cortical cultures for 30 min stimulated PPP activity in a concentration-dependent fashion from basal levels of 2.1±1.6% (n=3±SE) to a maximum of 23.2±2.1% in the presence of 100 µM H_2O_2. Estimated by LDH release, H_2O_2 was toxic with an EC_{50} value of 85 µM. In the presence of 200 nM selenium (9 days), an activator of glutathione peroxidase (GPx), maximal H_2O_2-induced PPP was 27±2.2%, EC_{50}=153 µM. In the presence of 2 mM deferoxamine (DFX), a chelator that forestalls $OH^{\cdot}$ formation, maximal PPP stimulation by H_2O_2 was 33.4±1.9% with an EC_{50} of 329 µM. DFX thus protects from H_2O_2 toxicity, probably because chelation of iron inhibits $OH^{\cdot}$ radical formation. However, when the PPP became saturated, H_2O_2 caused neuronal toxicity. Higher maximal PPP activity, observed in the presence of DFX, supports the notion that the chelator blocks $OH^{\cdot}$ formation thereby preserving H_2O_2 as a substrate for GPx. Selenium plus DFX produced maximal H_2O_2-induced PPP of 41.4%, EC_{50}=341 µM. In astrocytic cultures, H_2O_2 stimulated PPP to 69.6±1.1%, EC_{50}=597 µM. The higher vulnerability to oxidative damage of neurons versus glia may be due to greater reserve capacity of glial glutathione pathway. This data suggest that PPP flux is a viable indicator of oxidative stress.

1) Ross B. D. and Ben-Yoseph O. (1993). *J. Neurochem.* 61 (Suppl.), 231A.
2) Ben-Yoseph O. *et al.* (1993). *J. Neurochem.* 61 (Suppl.), 230D.

A

OXIDATION THERAPY: THE USE OF A H_2O_2-GENERATING ENZYME SYSTEM FOR TREATMENT OF EXPERIMENTAL GLIOMA

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Hydrogen peroxide is known to induce cytotoxicity *via* mechanisms including DNA damage, lipid peroxidation and protein oxidation. This genotoxicity has been proposed to be the result of metal-catalyzed Haber-Weiss reactions leading to the generation of harmful hydroxyl radicals. In this study, we explore the possibility of harnessing the cytotoxic properties of an H_2O_2-producing enzyme to treat experimental malignant gliomas in the rat. Glucose oxidase (GO) catalyzes the formation of glucono 1,5-lactone and H_2O_2 from glucose and oxygen, both of which are readily available in tissues. Polyethylene glycol (PEG) moieties were attached to GO in order to increase the enzyme's *in vivo* half life and reduce antigenicity. PEG-GO (200 U), administered by two intratumoral injections 3 h apart, produced a 6 day growth delay in subcutaneous rat 9L gliomas as compared to control animals receiving heat denatured PEG-GO. *In vivo* tumor metabolic changes, monitored using ^{31}P MRS 5-6 hours following PEG-GO treatment, revealed a dramatic impairment of the energy state with a complete loss of phosphocreatine, a 96±2% (n=3±S.E.) reduction in the ATP/P_i ratio, and a 0.72±0.10 unit decline in intracellular pH. PEG-SOD and PEG-catalase were also administered i.v. in order to protect the animals from systemic toxicity due to the potential leakage of H_2O_2 and/or PEG-GO from the tumor site into the vascular system. This study demonstrates that the use of intratumoral H_2O_2-generating enzyme systems is a promising therapeutic approach meriting further evaluation. We are currently studying D-amino acid oxidase, which requires substrates that are not endogenous to mammalian systems. This enzyme is appealing since it will provide the unique ability to regulate the formation of H_2O_2 *in vivo* by controlling the enzymic activity by substrate availability and through the use of specific inhibitors. Also, H_2O_2-induced cytotoxicity can be augmented by sensitizing the tumor to oxidative stress. This can be achieved by inhibition of the glutathione pathway, which is the main anti-oxidant mechanism in these gliomas (Ben-Yoseph *et al.*, this issue).

B

THE EFFECT OF UNILATERAL 6-HYDROXYDOPAMINE(6-OHDA) LESIONS OF THE SUBSTANTIA NIGRA ON DOPAMINE TURNOVER(DATO) AND DOPAMINERGIC MARKERS IN RAT STRIATUM.

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Rats with unilateral dopaminergic nigrostriatal lesions are widely used as a model for human Parkinson's disease (Ungerstedt, 1971 Acta physiol. Scand. Suppl. 367, 49). We report the effect of discrete lesions produced by injecting 6-OHDA unilaterally into the substantia nigra(sn) on DATO and dopaminergic biochemical markers in rat striatum.In groups of 6-9 rats dopamine(DA) and metabolites were measured ex-vivo using HPLC with amperometric detection and DA reuptake sites were labelled with [^{3}H]-GBR12935 (Andersen 1987 J. Neurochem. 48, 1887). Measurements from the lesioned and non-lesioned sides were obtained for each rat and used in a paired comparison.

Table 1. Effect of unilateral nigrostriatal lesions on the rat striatal dopaminergic system.

6-OHDA (µg)	DOPAC+HVA/DA ratio Non-les.	Lesion.	%ΔDA level	%ΔDA reuptake
0	0.32 ± 0.06	0.35 ± 0.10	-9.4 ± 11.3	-30.6 ± 5.9
2	0.25 ± 0.05	0.41 ± 0.09#	-60.7 ± 11.8*	-61.3 ± 14.8*
4	0.36 ± 0.11	0.71 ± 0.25	-80.6 ± 5.1**	-75.8 ± 7.1**
8	0.34 ± 0.11	1.12 ± 0.74	-82.6 ± 5.0**	-77.7 ± 7.6**

*=P<0.05,**=P,<0.01 values compared by one-way ANOVAR followed by Williams test; #=P<0.05 Students paired t test.

6-OHDA injected into the sn produced dose-dependent lesions in the striatum. The dopamine depletions and [^{3}H]-GBR12935 binding were significantly correlated (r=0.755, slope=0.68, P<0.001). Lesioning increased DATO but only after the 2µg 6-OHDA lesion did the difference reached significance. Hence, DA content and [^{3}H]-GBR12935 binding site number are useful indices of the severity of nigrostriatal lesions.

C

NEUROTOXICITY OF ß-AMYLOID ANALOGUE PEPTIDES ON RAT HIPPOCAMPAL NEURONAL CULTURES.

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ß-amyloid peptide (ßA4) accumulates in senile plaques of Alzheimer's disease (AD) in the form of fibrils with a ß-pleated sheet conformation. This amyloidogenesis may have a key role in the progressive neuronal degeneration associated with AD. The establishment of cell culture models for evaluating the biological effects of ßA4 is potentially useful in the study of the cellular mechanisms involved in the pathogenesis of AD. Furthermore, recent data suggest that conformational changes in ßA4 or other amyloidogenic proteins could be responsible for the degree of neurotoxicity. In our study, using dissociated hippocampal cell cultures derived from 18 day rat embryos and the MTT colorimetric assay, we have examined the neurotoxic effect of ß-amyloid related peptides. Firstly, the synthetic peptide corresponding to the Dutch-haemorrhagic mutation ß1-40(E_{22}→Q), had comparable neurotoxic effects to rat and human ß1-40 peptides, between 0:1 and 20µM (at 20µM, 53±3, 57±2 and 54±12 % of toxicity, respectively). On the other hand, no toxic effect was observed with the N terminal fragment ß1-15, the extracellular fragment ß1-28 or the ß17-28 fragment of ßA4. Two amyloidogenic proteins, human full length amylin(1-37) and salmon thyrocalcitonin(1-32), which take a ß-pleated sheet conformation in solution, induced an important neurotoxic effect on the cultures (at 10µM, 90 and 62 % of toxicity, respectively). We have also tested two analogues of ßA4 with substitution of hydrophobic amino acids, ß1-40(F_{19}-F_{20}→T-T) and ß1-40(F_{19}-F_{20}→G-T), described by Hilbich et al. (J.Mol.Biol., 1992, 228, 460-473) to have a greatly reduced amyloidogenicity. In our hands, ß1-40(F_{19}-F_{20}→T-T) was not toxic at concentrations up to 20µM while ß1-40(F_{19}-F_{20}→G-T) had a weak neurotoxic effect at 20µM (34% of toxicity). Our results, associated with those of other teams on the aggregation state of the described peptides, suggest that the neurotoxicity induced by ßA4 is probably conformation-dependent *in vitro* and that a similar mechanism may be implicated in the neurodegeneration induced in neuritic AD pathology.

D

A possible role of iron ions in pathogenesis of hereditary degeneration of the retina in rats.

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Object of study: rats of Campbell strain with hereditary degeneration of the retina and rats of Wistar strain as a control.

Methods. Separation of subcellular fractions, purification and identification of proteins(electrophoresis on PAAG, different kinds of chromatography), spectrophotometrical methods.

Results. It was shown for the first time, that in Campbell rats at IO-20days of postnatal life(before an appearance of visible signs of the disease):1-the content of nonheme blood plasma iron decreased 2-fold;2-the content of plasma blood transferrin decreased and its saturation by iron ions increased 4-fold and 2-fold respectively, the affinity of apotransferrin to iron did not change;3-the content of apoferritin decreased IO-fold in liver;4-the content of nonheme iron decreased by 40% in microsomal fraction of brain cortex. All this shifts stabilized at 40 day of age. In the contrast to the nonheme iron containing proteins the level of heme iron containing protein cytochrome P-450 increased markedly in microsomal fraction of brain only to 40 day and ran up to 5-fold at 90 day of age.

Conclusions. Taking into account the data obtained a possible role of early disturbances of nonheme iron containing proteins in pathogenesis of hereditary degeneration of the retina is discussed.

A

Cerebral Free Sterols in Experimental Severe Hypoxia

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Free sterols of cerebral myelin under severe hypoxia were studied. Rats of Wistar strain were placed in the chamber for 3 min in a gas mixture containing 2% oxygen. The animals were sacrificed after 4, 24 hours and 2 and 8 weeks after hypoxia. Total free sterols of the myelin fraction were extracted and then separated by GC-MS (gas chromatography and mass spectrometry). Results of studied have shown that the level of main, typical myelin sterol--cholesterol decreases 14 days and 2 months after hypoxia. The next steroid after cholesterol in the respect of quality was prabable 4,4,14α-trimethyl-5α-cholest-24-en-3-one. The level of this derivative decreases in 4 and 24 h after hypoxia and increases in 2 months after experiment. Sterols of molecular weight 412, 414 and 426 were observed in all experimental groups in smaller amount in comparison to control group and the remaining derivatives only in trace amount. Persisting of free sterols for very long time after hypoxia my be the result of some disturbances in the metabolism of these derivatives, which are responsible for their transformation into cholesterol. The second possibility is the creation of free radicals in the result of hypoxia.

B

ALTERATIONS IN ENERGY METABOLISM OF SYNAPTOSOMES CAUSED BY CHRONIC LEAD ADMINISTRATION

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The mechanisms of lead toxicity to the CNS are not clear. It was postulated that Pb^{++}/Ca^{++} interactions may play an important role in toxicity of Pb^{++} in neurotransmission process. Our investigations suggested the existence of several mechanisms of lead toxicity, related to the individual neurotransmitter. Effect of Pb^{++} on synaptosomal energy metabolism was studied using rodent model of chronic poisoning. 100 mg $Pb(CH_3COO)_2/l$ drinking water was given to 3-week-old rats for 3 months. Synaptosomes were isolated from hemispheres using a discontinuous Ficoll gradient according to Booth and Clark (1978).
Our data indicated: 1. The lead level in the synaptosomal fraction obtained from Pb^{++}-treated rats was about twice higher than in the control. 2. The oxygen consumption by toxic synaptosomes in the presence of 5 mM KCl, with glucose or pyruvate as a substrate was higher by about 25% than that in the control and about 20% when K^+ concentration increased to 30 mM. 3. ATP level of toxic synaptosomes decreased by about 35%, levels of ADP and AMP were not changed. 4. Na^+-K^+-ATPase activity decreased by about 25%; Mg^{++}-ATPase activity was unchanged. 5. Electron microscopies studies showed, that mitochondria of toxic synaptosomes were morphologically changed. In conclusion – synaptosomal energetic processes may be the targer of lead action, what can be the reason of disturbances in the neurotransmission.

C

ADP-RIBOSYLATION OF THE NUCLEI OF RAT BRAIN CELLS

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Of all subcellular fractions the highest ADP-ribosyl transferase activity and a preferential ADP-ribosylation of non-histone proteins, including those of nuclear matrix, were found in the nuclei of brain cells of both newborn and adult rats. The matrix of brain nuclei was found to be enriched by ADP-ribosyl transferase. ADP-ribosylation by an endogenous enzyme modulates DNA topoisomerase II activity of neuronal nuclear matrix. The data obtained indicate that ADP-ribosylation participates in various genetic processes; primarily it is involved in the excision repair of nuclear DNA, damaged by X-irradiation of rats or by intraperitoneal injection of various DNA-damaging chemical agents. By our experimental findings it is most likely that fragmentation of DNA stimulates ADP-ribosylation of chromatin in the damaged regions, thus weakening DNA-protein interaction and modulating the structure of chromatin, which facilitates the penetration of DNA polymerase into DNA and enhances the DNA reparative synthesis in the cells of nervous tissue.

D

ROLE OF PAF IN NEURONAL RESPONSE TO BRAIN ISCHEMIA

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Platelet activating factor (PAF), 1-0-alkyl-2(R)-acetyl-glycerol-3-phosphorylcholine, is known as one of the most important lipid mediators. PAF is undetectable under resting conditions and accumulates in brain during seizures or ischemia and in neuronal cells under neurotransmitter stimulation. Pretreatment with PAF antagonists, BN52021, reduced significantly ischemia-evoked protein kinase C (PKC) activation/translocation and ornithine decarboxylase (ODC) induction in the hippocampi of gerbils subjected to 5 min forebrain ischemia and subsequent 3h recovery. Locally applied 0.5uM PAF itself triggered calcium decompartmentation in microdialised rabbit hippocampus *in vivo* and markedly potentiated this effect induced by NMDA. *In vitro*, PAF in physiological concentration (10nM) triggered Ca^{2+} influx and activated PKC in the rat synaptoneurosomal fraction. As the enhanced glutamate release have been implicated by the mechanism of PAF action (Clark et al., Neuron 9, 1992, 1211-1216) and also could explain our results, we measured 14C-aspartate release from hippocampal slices after transient anoxia and PAF or PAF-antagonist treatment. These studies suggest that PAF plays a role as an intensifier of glutamatergic neurotransmission and Ca^{2+} destabilizator and that PAF antagonists may be useful in the treatment of ischemic injury.

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A

INCREASED SUSCEPTIBILITY TO CONVULSANT IN INTACT MICE AFTER ADOPTIVE TRANSFER OF LYMPHOCYTES FROM PENTYLENETETRAZOL KINDLED MICE

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Kindling was induced in mice F1 C57B16/CBA by repeated pentylenetetrazol (PTZ) injection in a subconvulsive dose (30 mg/kg) intraperitoneally for 30 days. Susceptibility to PTZ was estimated in mice by intravenous titration with 1% PTZ solution at a rate of 0,01 ml/s on days 1, 7 and 14 after adoptive transfer of $2x10^7$ splenocytes from PTZ kindled mice. On day 1 after the adoptive transfer of lymphocytes seizure susceptibility to PTZ in mice-recipients were shown to be unchanged. On day 7 PTZ doses neccessary for provoking clonic convulsions and tonic convulsion attacks with letal outcome were decreased by 18,5 and 21,9% respectively as compared to control mice after transfer of lymphocytes from mice with 30-days saline administration. On day 14 only PTZ dose nessasary for provoking clonic convulsions was reduced. Thus, increased susceptibility to PTZ was established in mice-recipients after adoptive transfer of lymphocytes from kindled mice. The data confirm the hypothesis that cellular factors of immune system are essential for modulating central nervous system phenomena.

B

EFFECTS OF SYMPATHETIC DENERVATION ON SECOND MESSENGER FORMATION IN THE IRIS OF THE EYE

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The smooth muscles of the iris of the eye present an interesting system in which to study biochemical and functional interactions between peripheral adrenergic and cholinergic receptors. To throw more light on these interactions we have investigated the effects of NE, carbachol (CCh), NaF and IBMX on IP_3 accumulation, cAMP formation and contraction in the dilator and sphincter smooth muscles of the sympathetically denervated as well as the normal rabbit eye. We found: (a) In the denervated dilator, NE-stimulated IP_3 production and contraction are enhanced. (b) In the denervated sphincter, CCh-stimulated IP_3 production and contraction are attenuated. (c) The increase in tension by NaF (20 mM) in the normal and denervated dilator was 12.5 and 18 mg/mg wet wt., respectively, and in the sphincter was 33.8 and 18 mg, respectively. (d) Basal and isoproterenol-induced cAMP formation increased in both tissues. (e) Binding studies employing [^{125}I]-(iodocyanopindolol) showed that the density of β-adrenergic receptors does not change in the denervated sphincter, however, the K_d values decreased in this tissue. These data indicate profound alterations by sympathetic denervation of the iris in the coupling of adrenergic and muscarinic receptors to second messenger formation and muscle response in this tissue.

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C

PKC ISOFORMS IN CEREBRAL ENDOTHELIAL CELLS

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The protein kinase C (PKC) family is composed by at least four conventional (α,βI,βII andγ) and several related novel isoforms (δ,ϵ,ζ,η,θ) with different distribution and sensitivity to Ca^{++} and phorbol esters. The enzyme is known to be present in cerebral endothelial cells. We have investigated by using RT PCR the distribution of seven isoforms (α,β,γ,δ,ϵ,ζ,η.) in rat brain, freshly isolated brain microvessel fraction, primary cultures of rat brain endothelial cells, an immortalized rat brain endothelial cell line and aortic endothelium.
Brain contained all the seven investigated isoforms. The same expression pattern was shown by freshly purified microvessels except for γ isoform. Primary cultures of endothelial cells expressed α,β,δ and ϵ isoenzymes, whereas the immortalized cell line expressed only PKCα. The rat aortic endothelium contained PKC α, δ and possibly ϵ, η. The variety of expression pattern of PKC family members in endothelial cells of different type may have consequences in the functional responsiveness to environmental stimuli. Since PKC has been shown to be involved in the regulation of the blood-brain barrier permeability, the presence of different isoforms may confer a sophisticated intracellular regulatory mechanism to the brain endothelial cells.

D

CYCLIC AMP ACCUMULATION IN CHICK BRAIN AFTER DIFFERENT SOCIAL EXPERIENCE

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The MNH (medio-rostral neostriatum/hyperstriatum ventrale) is a forebrain region which plays an important role in acoustic imprinting of domestic chicks. A dramatic loss of synaptic spines as well as an elongation of PSDs, which presumably reflects the stabilization of the remaining synapses has been demonstrated in the neostriatal part of this area. It is still unknown, which transmitter and second messenger systems play a role in this synaptic selection process. Since cAMP stimulated phosphorylation of PSD - proteins has been shown to be critically involved in the maturation of synapses of the chick forebrain, we speculate that similar cAMP regulated mechanisms could be involved in synaptic stabilisation during imprinting. To test this hypothesis we investigated the time course of cAMP accumulation in the MNH of four experimental groups of chicks: isolated, social (reared in groups), imprinted (successfully imprinted on rhythmic tone) and non-imprinted chicks (imprinting procedure was unsuccessful). The right and left MNH and LPO (lobus parolfactorius - another region, involved in imprinting) were dissected out, crude membrane fractions were prepared and the cAMP accumulation were analysed for both hemispheres separately. In social, imprinted and non-imprinted chicks the forskolin stimulated cAMP accumulation in the right MNH was significantly higher than in the left, in isolated vice versa. A dopamine stimulation of cAMP synthesis could be detected in MNH of imprinted, non-imprinted and social chicks, but not in isolated. Here, there was no difference between both hemispheres. Obviously, in chick MNH the stimulation of adenylate cyclase by dopamine occurs as a result of social experience, acquired either by social housing or during the behavioural tests of the imprinting procedure. Parallel autoradiographical studies of dopamine receptors revealed a very high density in LPO (a putative analogue of mammalian basal ganglia) and intermediate levels in MNH. Further investigations will clarify, whether the observed changes are due to alteration of dopamine receptors, e.g. increased density and/or affinity of D_1/D_2 receptors, or whether they have to be explained by changes in G - protein synthesis.

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A

Actin-based protrusion of lamellipodia of growth cones is induced by activation of protein kinase C

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In human SH-SY5Y neuroblastoma cell bodies and growth cones, 1-10 nM of the protein kinase C (PKC) activator 12-myristate-13-acetate (PMA) within minutes induces the protrusion of flat lamellipodia. Fluorescence-staining by phalloidin and reversible inhibition by cytochalasin B shows that the lamellipodial protrusions are based on a rapid reorganization of actin involving its polymerization at the outer border of the forming lamellipodium. Pretreatment of the cells with the PKC inhibitors staurosporine (0,5 µM for 24h) or bis-indolylmaleimide (0,5 µM for 2h) completely abolished the PMA effect.

These data strongly suggest an involvment of PKC in lamellipodia induction as a key step in signal transduction regulating the dynamic actin cytoskeleton in growth cones.

B

PARTICIPATION OF SECOND MESSENGERS IN THE ELECTRICAL RESPONSE OF NEURONS TO APPLICATION OF ANTIBODIES AGAINST S-100 PROTEIN

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The electrical responses of isolated neurons of snail Helix and pyramidal neurones of rat brain hippocampus slices to rabbit's antibodies against S-100 protein of a rat brain (AS-100) were studied. It was shown that AS-100 induced a transient membrane depolarization and an inactivation of action potentials in both types of neurones. The mechanisms of AS-100 effect was studied in snail neurones using voltage clamp method and iontophoretical injection of the drugs trough multibarreled microelectrode. Under such conditions AS--100 application led to an increase in steady-state inward current in a half of neurones tested and a decrease in amplitude of high threshold Ca-current in all neurones tested. Intracellular injection of Ca-chelator EGTA led to an abolition of AS-100-induced effects. Intracellular injection of cyclic AMP (but not cyclic GMP) or extracellular application of dibutyryl cyclic AMP restored Ca-current that had been inhibited by AS-100. It can be supposed that intracellular free Ca- ions level increased after AS-100 application due to cessation of Ca-ions binding by S-100 proteins. Ca-ions, in turn, decrease the level of cyclic AMP -dependent phosphorylation of channels proteins because of the activation of phosphodiesterase and proteinphosphatase.

C

RELEASE AND EXTRACELLULAR METABOLISM OF ATP IN THE RAT HABENULA: NEUROCHEMICAL AND ENZYMECYTOCHEMICAL STUDY

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The release and extracellular metabolism of ATP and ADP, the putative central neurotransmitters were studied in the habenula by the combined luciferin-luciferase and creatine phosphokinase assay and by high performance liquid chromatography with UV-detection (HPLC-UV). Endogenous ATP is released stimulation-dependently by low frequency electrical stimulation (2 Hz, 480 shock) and the release could be reduced by tetrodotoxin, the inhibitor of the voltage-dependent Na^+ influx. Endogenous ADP, the primary metabolite of ATP could be detected in the effluent both at rest and after stimulation, but the relative amount of it in the total ATP+ADP content decreased after stimulation, indicating that the majority of the released compound is ATP in response to stimulation. ATP is decomposed to ADP and AMP in the extracellular fluid by the ectoATPase enzyme as demonstrated by HPLC-UV technique ($K_m = 811 \pm 68.88$ µM, $v_{max} = 23.1 \pm 2.75$ nmol/min/prep). The presence of ectoATPase enzyme was also visualised by electron microscopic enzymecytochemistry: it was present on the membranes of dendrites and nerve terminals as well as in the synapses. Taking into account the ATP-receptor mediated synaptic current in the habenula, identified recently by Edwards et al. (Nature, 359:144, 1992) our data provide evidence for the storage, release and inactivation of ATP all needed for an endogenous substance qualified as a transmitter.

D

THE ROLE OF PROTEIN KINASE C SUBTYPES IN TPA-ENHANCED NORADRENALINE RELEASE FROM SH-SY5Y CELLS.

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Our previous studies using immunoblotting have shown that the human neuroblastoma SH-SY5Y expresses protein kinase C (PKC) subtypes α, ε and ζ. Exposure of cells to 100 nM TPA for 48h resulted in down-regulation of PKC-α and ε but not PKC-ζ. Under these conditions TPA-enhanced K^+- and carbachol-evoked noradrenaline (NA) release was fully inhibited. Here we report the effects of prolonged treatment with different concentrations of the phorbol esters TPA and PDBu on PKC subtype levels and TPA-enhanced NA release. Incubation of SH-SY5Y cells with 10 nM TPA for 48h resulted in a marked reduction of PKC-α which fell to undetectable levels following 48h exposure to 100nM and 1000nM TPA. In contrast, PKC-ε was only partially down-regulated following 48h TPA treatment (10-1000 nM). Cells exposed to 10nM, 100nM and 1000nM TPA for 48h showed inhibition of acute TPA-enhanced K^+-evoked release by 80%, 100% and 100% and inhibition of acute TPA-enhanced carbachol-evoked release by 70%, 100% and 100% respectively. In contrast, 48h exposure to 10nM and 100nM PDBu did not down-regulate PKC-α; however 48h treatment with 1000nM PDBu led to almost a full reduction in PKC-α levels. 10nM PDBu (48h) also had no effect on PKC-ε levels, however PKC-ε was partially down-regulated following exposure to 100nM and 1000nM PDBu for 48h. Cells exposed to 10nM or 100nM PDBu for 48h showed no inhibition of TPA-enhanced K^+-evoked NA release, however 1000nM PDBu inhibited this release by 50%. Prolonged exposure to 10nM PDBu did not inhibit TPA-enhanced carbachol-evoked release, however 100nM and 1000nM PDBu inhibited this release by 30% and 70% respectively. These data show that under conditions where PKC-α can no longer be detected and PKC-ε is partially down-regulated by TPA and PDBu acute TPA-enhanced K^+- and carbachol-evoked NA release is inhibited, therefore implicating PKC-α and/or ε in the regulation of TPA-enhanced K^+- and carbachol-evoked NA release from SH-SY5Y cells.

A

NEW DATA ABOUT BRAIN HIGH MOLECULAR WEIGHT ASPARTIC PROTEINASE

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Recently it has been found that brain high molecular weight (HMW) aspartic proteinase (E.C. 3.4.23.-) in contrast to cathepsin D (E.C.3.4.23.5) generates the fragment 31-40 of the ß-chain of bovine hemoglobin (calmodulin (CaM)-binding coronaro-constrictory peptide) by cleavage of Leu30-Leu31 and Phe40-Phe41 bonds.(1). The aspartic proteinase was isolated from bovine brain cortex by a procedure involving ammonium sulfate precipitation (30-70%), gel-filtration on Sephadex G-100 and affinity chromatography on pepstatin-agarose. On polyacrylamide gel electrophoresis (7.5%) the purified native enzyme preparation produced 2 bands approximately corresponding to Mr 90 kD and 20 kD. The protein with Mr about 20 kD was digested by endoproteinase Lys-C at 37°, at pH 8.5, at enzyme:substrate ratio 1:10 for 6h. The peptide mixture was separated by reversed-phase HPLC. Selected peptides were analysed by N-terminal sequence analyses. A computer search revealed that the protein with Mr about 20 kD is CaM. It was shown that Ca^{2+} (in concentration 10^{-3} M) increased the HMW aspartic proteinase activity about 36%. Data obtained give us reason to suppose that HMW aspartic proteinase is probably a target enzyme for CaM.

1.Barkhudaryan N. et al.FEBS Lett.329,p.215,1993.

B

NEUROPROTECTIVE ACTIVITY OF HU-211: DOSE RESPONSE AND THERAPEUTIC WINDOW IN A GLOBAL ISCHEMIA MODEL IN THE MONGOLIAN GERBIL

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HU-211, a synthetic non-psychotropic cannabinoid acting as a functional non-competitive NMDA antagonist, was found in the past to induce neuroprotection in global ischemia in the Mongolian gerbil. In the present work, we tested two pharmacological aspects of HU-211; dose-response and therapeutic window in the same model. Mongolian gerbils were subjected to 10 min global forebrain ischemia via bilateral common carotid arteries (CCA) occlusion. HU-211 at dose levels of 1.5, 2, 3.5, 4, 5.5 and 6 mg/kg was administered i.v. 30 min post-ischemic insult. A week later, animals were sacrificed. Brains were removed and sections stained with cresyl violet and hemotoxylin eosin. The number of viable pyramidal cells in the CA1 subsector of the hippocampus was then counted for each animal. HU-211 at 3.5 and 4 mg/kg demonstrated more live cells compared to all other treatment groups, including vehicle treatment group (ANOVA followed by Duncan's post-hoc test, $p < 0.05$). For instance, in the middle subsector of the hippocampus, mean ± SEM viable cells per 0.8 mm; HU-211 3.5 mg/kg: 60±24 n=10; HU-211 1.5 mg/kg : 28±10 n=10; HU-211 5.5 mg/kg 34±14 n=10; vehicle 30±10 n=10). The same procedure was used in the therapeutic window study. Drugs were administered at 4 mg/kg, 30, 60, 120 and 180 min post-ischemic insult. HU-211 administered 30 and 60 min post-insult induced a statistically significant neuroprotection (ANOVA followed by Duncan's post-hoc test, $p < 0.05$) compared to all other treatment groups (e.g. viable neuronal cells in middle subsector of CA1 subfield of the hippocampus, mean±SEM HU-211; 30 min: 48±19 n=10; 60 min: 40±18 n=10; vehicle: 17±8 n=10; 120 min: 14±8 n=10; 180 min: 19±12 n=10). These results demonstrate that HU-211 may be an efficacious treatment after ischemic brain damage.

C

Compartmental analysis of blood-brain transfer and protein incorporation of L-[^{75}Se]seleno-methionine in rat brain

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[^{73}Se]Selenium-labeled methionine ($T_{1/2}$ = 7.1 h) may be an appropriate tracer for studying long-lasting metabolic processes like protein synthesis with positron emission tomographie (PET). In order to check this possibility, we have studied in 42 male Wistar rats the blood-brain transfer, protein incorporation and metabolism of L-[^{75}Se]selenomethionine (SeMet) with relatively high specific activity. The animals were slightly anesthetized with ether. SeMet (500 KBq, > 400 GBq mmol^{-1}) was injected i.v. and the rats were decapitated at 14 different times (3 animals/time) up to 360 min p.i. and the distribution of [^{75}Se]activity in different organs including the brain was measured. In addition, plasma and brain samples at 1.5, 5, 20, 120, 240, and 360 min were analyzed for labeled fractions of free SeMet, metabolites, and SeMet bound to t-RNA and proteins. A three-compartment model (see Brust et al. 1992: J. Neurochem. 59, 1421) was applied to the data to calculate the blood-brain transfer constant, K_1 (0.151 ± 0.031 ml g^{-1} min^{-1}), as well as the rate constant of SeMet incorporation into proteins (k_3 = 0.020 ± 0.003 min^{-1}). The apparent incorporation of methionine into proteins is estimated to be about 0.6 nmol g^{-1} min^{-1}, assuming a calculated content of free endogenous methionine in the brain of 28 nmol g^{-1}. This is very close to values obtained with L-[^{35}S]methionine (0.68 nmol g^{-1} min^{-1}) in rats (Grange et al. 1991: J Cereb Blood Flow Metab 11 (Suppl. 2), S356). It is concluded from the studies that the use of L-[^{73}Se]-selenomethionine in PET may be appropriate to measure brain protein incorporation in humans.

D

STUDY OF CONFORMATION LABILITY OF Na,K-ATPase USING TIME-RESOLVED PHOSPHORESCENCE ANISOTROPY.

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Conformational lability of the membrane bound Na,K-ATPase labelled with eosin-5'-isothiocyanate was studied by time-resolved phosphorescence anisotropy method. Phosphorescence anisotropy decay curves are satisfactorily described by a sum of two components with a constant residual level. The former (with rotational correlation time of 10-15 usec) was ascribed to monomeric form of the enzyme, whereas the latter (with rotational correlation time varied from 100 to 400 usec depending on temperature or ligands presented) was indicative of oligomeric ensembles with changeable amount of partners at different steps of Na,K-ATPase operation cycle. Potassium favorable to E2 state of the enzyme stimulates association of functional units of Na,K-ATPase, and sodium induces their dissociation. ATP stimulates E2 ---> E1 transition by facilitating shift of E2 oligomers into E1 monomers. Na,K-ATPase prepared in an active state by solubilization with C12E9 is characterized by a mono exponential phosphorescence anisotropy decay curve. The data presented confirms hypothesis of short-lived oligomer formation which is a ligand sensitive step of E2 ---> E1 transition of Na,K-ATPase cycle [Boldyrev A., Fedosova N., Lopina O. Biomed. Sci. 1993, 2, 451-454].

A

HU-211, A NON-PSYCHOTROPIC CANNABINOID AS A NOVEL NEUROPROTECTANT AGENT

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Accumulation of evidence suggests that multiple proccesses lead to neuronal death following cerebral ischemia. These are associated with excessive activation of excitatory amino acid receptors and involve the generation of free radicals from different sources. Previous studies have demonstrated the ability of HU-211, a synthetic non-psychotropic cannabinoid, and a non competitive NMDA receptor antagonist to protect cortical neurons from damage mediated via the NMDA preferring glutamate receptors. The current study was designed to explore potential protective effects of HU-211 on neuronal degeneration following anoxia or exposure to the nitric oxide (NO) donor sodium nitroprusside (SNP) in culture. Primary cerebral cortical cell cultures were prepared from 18-20 day old fetal rats and consisted of neurons plated over a confluent glial-cell feeder layer. Toxicity experiments were performed with cells at 10 day in culture and cell viability was evaluated by morphological criteria following neuron specific enolase immunostaining and assessed quantitatively by measuring the extent of mitochondrial activity in the cells. Subjection of cultures to 1 hour deprivation of oxygen and glucose was associated with neuronal cell injury and was markedly reduced by 10 μM HU-211 or by 30 μM MK-801 present either during or following anoxia. By contrast, exposure of cells to SNP resulted in a massive glial and neuronal cell damage which was attenuated by the addition of 10 μM HU-211, while not affected by the presence of 30 μM MK-801. In conclusion, HU-211 can protect neurons from damage associated with multiple biochemical pathways, probably via more than one mechanism of action. The broad spectrum neuroprotective activity of HU-211 suggests the use of the compound for preventing ischemia-related neuronal damage in which excitatory amino acid receptors and NO have been shown to play major roles.

B

Quantitation of Cerebral Metabolic Compartmentation as Revealed from [U-^{13}C]Glucose Metabolism by ^{13}C NMR Spin-Spin Couplings

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A method is presented for determining the compartmentation of amino acid metabolism in the brain. ^{13}C NMR spectroscopy, and more specificially homonuclear ^{13}C–^{13}C spin coupling patterns of ^{13}C labeled amino acids, was used to measure the relative flux of label from D-[U-^{13}C]glucose through the anaplerotic pathway versus the oxidative pathway (1). The complete analysis of the individual carbons of glutamate, glutamine, γ-aminobutyric acid (GABA) and aspartate ^{13}C NMR spectra permits the quantitation of ^{13}C enrichments and pyruvate carboxylase activity in astrocytes. Glucose flux through the pyruvate carboxylase pathway was quantitated after constant infusion of D-[U-^{13}C]glucose to young rabbits. We demonstrate, for the first time, that multiplet spectra of three adjacent ^{13}C in glutamine and glutamate, which are derived from [1,2,3-^{13}C$_3$] pyruvate, reveal different isotopomer populations in glutamine in comparison to that in glutamate. This is due to two different metabolic compartments characterized by the presence or absence of glutamine synthase activity and two different TCA cycles, one preferentially mediated by pyruvate carboxylase and the other by pyruvate dehydrogenase. The labeling patterns and ^{13}C isotopomer populations of glutamate and GABA carbons indicate that these amino acids were preferentially synthesized in the compartment where glucose is metabolized to acetyl-CoA by pyruvate dehydrogenase. In contrast, the labeling pattern and ^{13}C isotopomer population of glutamine indicate that this amino acid is preferentially synthesized in the compartment where glucose is metabolized to pyruvate followed by carboxylation to oxaloacetate by pyruvate carboxylase. The anaplerotic pathway accounts for 35% of glutamine synthesis and only 16% of glutamate and GABA syntheses under metabolic and isotopic steady state conditions. These results quantitatively support the concept that glutamine is supplied by astrocytes to neurons to replenish the neurotransmitter pool of GABA and glutamate.

1. A. Lapidot and A. Gopher (1991) Soc. Magn. Reson. Med. (U.S.A.) Vol. 1, p.444.

C

EXOGENOUS GANGLIOSIDES MODIFY THE ACTIVITIES OF MEMBRANE BOUND ENZYMES

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Previously we have found that i.p. administration of total bovine brain gangliosides (30 mg/kg of body weight) influences the process of memorization of learned movements in rats (1,2). The analysis of the activities of some membrane bound enzymes in the structures of rat brain participating in the control of motor behaviour showed that ganglioside (G) injections affect the activity of acetylcholinesterase (AChE), 5'-nucleotidase (NT), as well as of adenylate cyclase (AC). The activity of AChE was found to be increased in sensomotor cortex (Cx), but unchanged in hippocampus (Hp), amygdala (Am) and neostriatum (S) of G injected rats as compared with untreated ones. On the contrary, the activity of NT was decreased in S, Hp and Am but unchanged in Cx. The level of AC activity in G treated rats was higher in all studied structures. The increase of AC activity after G treatment was more pronounced in the brain of rats with low ability to learn. It seems to be possible that G influence the process of learning and consolidation modifying the activities of the enzymes participating in nervous transmission and its regulation.

1. Neurochem. Int. 1992. V. 21, Suppl. P. 165.
2. J.Higher Nervous Activity (Rus.).1993.V.43.N6.

D

IS QUINOLINIC ACID A POTENT LIPID PEROXIDANT IN RAT BRAIN ?

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Quinolinic acid (QUIN) is assumed as a potent lipid peroxidant in rat brain under in vitro (Nerochem.Res., 16:1139,1991) and in vivo (Neurosci.Lett., 159:51,1993). Lipid peroxidation was measured by thiobarbituric acid test and by its end-product (malondialdehyde, MDA) estimated by means of HPLC. For this purpose we used homogenates of the cerebral hemispheres an cerebella of 50-day-old rats. Homogenates were incubated with various concentrations between 50 μM and 50 mM of QUIN. In contrast to the previous results of Rios and Santamaria (1991,1993), we found that increasing concentrations of QUIN decreased levels of free and total lipo-peroxidative products below the controls as determined by spectrophotometric measurment of thiobarbituric acid reactive products. These results were supported by the MDA-TBA complex determination using HPLC equipped with Sepharon SGX C18 4x250 mm fy TESSEK (mobile phase methanol-phosphate buffer, pH 6.3, flow rate 0.7 ml/min., detection at 532 nm). We also assayed a signiiicant inhibition of MDA formation in brain homogenates incubated with QUIN. Hypothetical explanation of our findings is based on the possibility of scavenger action of pyridine cycle of QUIN molecule. (Supported by grants of GA/ČR 309/93/0577 and of MH 099-3.)

A

INFLUENCE OF NITRO-L-ARGININE METHYL ESTER (L-NAME) ON THE CEREBRAL RENIN-ANGIOTENSIN SYSTEM.

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Presence of NO synthase (Knowles et al,1989) and angiotensin forming enzymes in the brain of normal and hypertensive rats (Ganten et al,1983) determined us to study the effect of intravenously injected L-NAME on the cerebral renin and angiotensin-converting enzime content of the adult rats.

The experiments were performed on 15 male adult rats (200-300 g) killed by cervical dislocation. Brain stem, hypothalamus and fronto-parietal cortex were rapidly removed and homogenized in ice-cold washing buffer. Renin was measured as angiotensin I by radioimunoassay and angiotensin-converting enzyme was determined spectrophotometrically using the Depièrre and Roth technique (1976). The inhibition of NO synthase with L-NAME (100-200 µM/kg) induced an evident increase of the renin in the brain stem and hypothalamus without significant variations of the angiotensin-converting enzyme. The renin content increased from the mean value 11,5+3,2 ng.Ang I/g/h to 19,3+3,8 ng.Ang I/g/h in the brainstem and from 20,5+4,2 ng.Ang I/g/h to 31+4,8 ng.AngI /g/h. in hypothalamus.The obtained results suggest a possible relationship between the cerebral renin-angiotensin system and L-arginine-NO way.

B

VOLUME-REGULATORY TAURINE EFFLUX FROM HYPOSMOTICALLY STRESSED CORTICAL CELLS OCCURS VIA DISTINCT ANIONIC AND NEUTRAL PATHWAYS

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Preferential efflux of cellular taurine plays a major role in protection against cerebral oedema in various pathophysiological states including hyponatraemia. The object of the present study has been to examine transport mechanisms of efflux from cells in cortical slices pre-loaded with ^{3}H-taurine, and whether this occurs via anionic or neutral pathways. Efflux is known to be enhanced by reduction of medium o.p. from 315 to 265 mOsm, with moderate cell swelling, and to be partly dependent on external Cl^-. The main findings were (1) in both iso- and hyposmotic media efflux was accelerated by reducing pH from 7.8 to 7, or increasing bicarbonate from 2.5 to 60mM; these effects were more marked in iso- than hyposmotic media. Slowing of efflux was associated with cell swelling (decreased slice non-inulin space). (2) Dependence of efflux on pH and bicarbonate was abolished by acetazolamide (1mM) with marked cell swelling. (3) Trifluoperazine (50µM) impaired efflux and caused cell swelling only in hyposmotic media, with accentuated dependence on pH and bicarbonate. (4) Niflumic acid (50µM) inhibited efflux, with cell swelling, and abolished the pH and bicarbonate effects, in both media. The findings are interpreted in terms of volume-regulatory efflux by 2 separate mechanisms - (1) parallel antiports (anionic taurine/Cl^- and Cl^-/external bicarbonate) operative in iso- as well as hyposmotic media, and (2) neutral, calmodulin-dependent, pH and bicarbonate-independent efflux in response to hyposmotic stress.

C

EFFECT OF PROPYLTHIOURACIL-INDUCED HYPOTHYROIDISM ON CEREBRAL CORTEX OF YOUNG AND AGED RATS: LIPID COMPOSITION OF SYNAPTOSOMES, MUSCARINIC RECEPTOR SITES AND ACETYLCHOLINESTERASE ACTIVITY.

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The effect of hypothyroidism on the lipid composition of synaptosomes, density and affinity of muscarinic receptor sites, and acetylcholinesterase activity in the cerebral cortex of young and aged rats was investigated. The animals were made hypothyroid by adding 0.05% propyl-2-thiouracil to their drinking water for four weeks. This pathological state induced an increase in the relative percentage of sphingomyelin in young rats. In aged rats hypothyroidism induced a decrease of sphingomyelin and glycerophosphocholine and an increase of cholesterol. The effect of hypothyroid state on cerebral cortex resulted in an increase of acethylcholinesterase activity both in young and aged rats and was also reflected in an increase of density of M1-AChRs but only in the former.

D

Dissection of succesive organophosphorus inhibition and oxime reactivation by human cholinesterase variants. Mikael Schwarz[1], Yael Loewenstein[1], David Glick[1], Jian Liao[1], Bent Norgaard-Pedersen[2], and Hermona Soreq[1]. [1]Dept. of Biological Chemistry, The Hebrew University of Jerusalem, Israel. [2]Statens Seruminstitut, 2300 Copenhagen, Denmark.

Acylation and deacylation in cholinesterase catalysis were dissected using successive organophosphate phosphorylation and oxime promoted reactivation as analogous reactions. Rate constants were calculated for natural and site-directed variants of Butyrylcholinesterase (BuChE) and for two naturally occurring variants of Acetylcholinesterase (AChE) arising from alternative splicing. The enzymes were produced in *Xenopus* oocytes and enriched by adsorption onto immobilised antibodies. This allowed to control experimental inactivation and reactivation times, eliminate possible interference by reagents of the previous stage of the sequence and determine the amount of recombinant protein by the use of ELISA. The gorge-lining contributed to enzyme acylation as was seen by chimeric substitution of active site gorge lining residues of human BuChE with the more anionic and aromatic residues of AChE. This reduced phosphorylation 60-fold, down to the level displayed by AChE, while reactivation was reduced only 4-fold. In contrast, introduction of a positive charge at the acyl-binding site decreased phosphorylation 8-fold but reactivation 30-fold, suggesting that additional positive charges at the active site hamper deacylation. Furthermore, the rate-limiting step for catalysis was deacylation in the chimera, but acylation in the mutants with a basic group introduced into their acyl-binding site, revealing modulation of different catalytic steps by these two regions of cholinesterases. Both alternative AChE forms displayed similar inactivation and reactivation rates demonstrating that differences in the acyl-binding site and gorge lining of cholin-esterases contribute to catalysis far more than changes in their C-termini.

A

NEURAL REGULATION OF MUSCLE ACETYLCHOLINESTERASE

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Molecular forms of acetylcholinesterase (AChE) in rat slow soleus and fast EDL or sternomastoideus muscles were analysed by velocity sedimentation, and AChE mRNA was determined by northern blot analysis. Muscles were studied either after axotomy (denervation) or crush (reinnervation) of their motor nerves, or after botulinum toxin induced paralysis. AChE activity in rat fast muscles, especially in the extrajunctional region, is much higher than that in slow muscles which is due to higher level of AChE mRNA in fast muscles. AChE activity in muscles drops immediately after denervation because the level of AChE mRNA in muscles is critically dependent upon innervation. Extrajunctionally, identical effect occurs after botulinum toxin induced muscle paralysis indicating that electromechanical activity in muscles maintains high level of AChE mRNA expression. During reinnervation, AChE activity in the slow soleus muscles transiently increases far above normal. This is due to a) great increase of the extrajunctional asymmetric AChE forms mimicking the immature postnatal state, and b) increase of the G_1 form. No such increase was observed in the reinnervated fast EDL muscle in which activity of all AChE forms slowly returned towards normal. The transient changes observed in the reinnervated soleus muscles are probaly not due to the tonic pattern of neural stimulation because they appeared also in disused muscles in which the pattern of stimulation becomes phasic.

B

EARLY PLACENTAL EXPRESSION OF BUTYRYL-CHOLINESTERASE AND LOW INCIDENCE OF D70G BCHE IN PATIENTS WITH PREGNANCIES AT RISK SUGGEST SELECTION ADVANTAGE FOR "ATYPICAL" BCHE.

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In search for potential scavengers protecting the developing human embryo against cholinergic poisons, we characterized the cholinesterases expressed in placenta. RT-PCR performed on placental mRNA revealed efficient expression of both butyrylcholinesterase (BuChE) and the three alternative forms of acetylcholinesterase (AChE) mRNA in 1st and 3rd trimester placentae. Electron microscopy performed on placental tissues subjected to selective AChE or BuChE activity staining revealed that cells in 7 weeks placenta only expressed BuChE activity, whereas soluble AChE activity was conspicuous within blood vessels. This finding indicated that BuChE is the primary placental scavenger against anti-cholinesterase poisons to which the early fetus may be exposed. Knowing that the "atypical" BuChE (encoded by the G70 BCHE allele) is resistant to certain natural and man-made cholinergic poisons, we initiated a peripheral blood DNA PCR-RFLP test to determine the incidence of the D70G BCHE allele. This test was employed on a group of 43 patients with pregnancies at risk as compared with 69 age-matched controls. The G70 BCHE allele frequency was found to be 0.01 in the patients at risk, as compared with 0.03 frequency in the control group. The lower incidence of the poison-resistant "atypical" BCHE allele in patients with pregnancies at risk as compared to controls, suggests that cholinergic poisons could be the cause for part of the early pregnancy failures in the examined group and that the G70 BCHE allele can protect against such risks. If confirmed in a larger scale, this finding may explain the selection advantage apparent for G70D BCHE in particular populations.

C

PHOSPHORYLATION AND DEPHOSPHORYLATION OF DISTINCT ISOFORMS OF THE HEAVY NEUROFILAMENT PROTEIN NF-H

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Sera of Alzheimer's disease (AD) patients contain IgG which bind more readily to the heavy neurofilament protein NF-H of purely cholinergic bovine ventral root neurons than to NF-H of chemically heterogeneous neurons. This finding and immunohistochemical studies utilizing specific anti neurofilament antibodies led to the suggestion that distinctly phosphorylated neurofilament isoforms exist in different types of neurons. We have recently examined this hypothesis by direct biochemical experiments which revealed that the NF-H protein of bovine ventral root cholinergic neurons is more acidic and is markedly more phosphorylated than that of bovine dorsal root neurons.

In the present study we employed this system to study the degree to which distinctly phosphorylated NF-H isoforms differ in the extents to which they can be phosphorylated and dephosphorylated in vitro. This was performed utilizing the protein kinase $PK40^{erc}$ which is specific to the serines of the KSP repeats of the neurofilament subunits and alkaline phosphatase. The results obtained reveal that the more heavily phosphorylated ventral root NF-H is dephosphorylated more rapidly than dorsal root NF-H. Both of these molecules are poor substrates of $PK40^{erc}$ in their native form. However, following dephosphorylation, they are phosphorylated extensively and differentially by this kinase. Under these conditions, $PK40^{erc}$ catalyzes the incorporation of about four phosphates per molecule of ventral root NF-H and the ratio of phosphates incorporated per molecule of ventral root NF-H to those incorporated into a molecule of dorsal root NF-H is 1.46±0.17.

D

CATHEPSINS AND CYSTATINS IN ALZHEIMER'S DISEASE IMMUNOHISTOCHEMICAL AND FIA STUDIES

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Alzheimer's disease (AD) is neuropathologically characterized by massive loss of neurons in discrete brain areas, intracellular neurofibrillary tangles and extracellular deposition of amyloid. The formation of amyloid is thought to involve an altered proteolytic processing of the amyloid precursor protein (APP).

Two cellular pathways have been identified to manage APP processing the secretory (trans-Golgi) and the endosomal/lysosomal ones. There is evidence that the secretory pathway delivers both normally (non-amyloidogenic) and aberrantly (potenially amyloidogenic) cleaved fragments, whereas the endosomal/lysosomal way is thought to generate in a first step transiently existing COOH-terminal fragments, which are in a second step completely degraded by lysosomal proteinases (cathepsins), thus preventing amyloid formation.

According to our "lysosomal" hypothesis of amyloid formation, this *beneficial* action of cathepsins may be disturbed by interaction with proteinase inhibitors. We herein show by conventional immunohistochemistry and laser confocal microscopy that cathepsins B, D, L, and S appear within neuritic plaques in close association with their natural inhibitors, the cystatins A and C (and sometimes B).The origin of these inhibitors seems to be both brain-and blood borne. In is further demonstrated from the reaction pattern of cathepsins in AD brains that these enzymes are also involved in the formation and/or persistence of neurofibrillary tangles.

A sensitive fluorescence immunoassay (FIA) has been developed to quantify cystatin A in human liquor of AD patients.

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A

INTERACTION OF BRAIN PHOSPHOFRUCTOKINASE WITH ßA4-AMYLOID

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One of the main functional metabolic disturbances in incipient demenz of Alzheimer type is the reduction of cerebral glucose utilization. Morphologically the brains of Alzheimer patients are characterized by multiple depositions of ßA4-amyloid which is the major component of extracellular senile plaques. ßA4-amyloid, however, is also found intracellularly and can bind to different cellular components and structures.
The interaction of ßA4-amyloid with phosphofructokinase, one of the key enzymes in glycolysis, was studied *in vitro* under various conditions. ßA4-amyloid was found to bind in nanomolar concentrations to phosphofructokinase and decrease its activity. Binding was demonstrated by enzyme linked immunoassays and by gelfiltration studies. Inactivation of phospho-fructokinase by ßA4-amyloid could only partially be prevented by fructose 6-phosphate. In control experiments no interaction was detectable between lactate dehydrogenase and ßA4-amyloid.

B

THE DRASTIC REDUCTION OF BRAIN ISOFORM OF CREATINE KINASE LEVEL IN ALZHEIMER'S DISEASE BRAIN

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It has been known, that the disturbance in energy metabolism occurs in the brain of patients with Alzheimer's disease (AD), characterized by multiple impairment of memory and cognitive functions. Creatine phosphokinase (CPK, EC 2.7.3.2) is one of the ATP-regulating enzymes which plays an important part in energy metabolism. We have previously demonstrated the decrease of brain isoform of CPK (CPK BB) activity and content in the cytosolic fraction of the frontal cortex of patients with Alzheimer's disease. In the present work the determination of the CPK BB level was carried out on cytosolic and membrane fractions, obtained from postmortal different brain areas of non-demented controls and patients with AD. It was detected by the immunoblotting the drastic reduction of immunoreactive CPK BB level in cytosolic fractions in most of the structures, included in the memory system as well as: hippocampus, gyrus cinguli, temporal lobe and frontal cortex. In membrane fractions of the same structures the level of CPK BB was invariant or decreased slightly. Thus the decrease of CPK BB level may be important in the formation of energy metabolism impairment in AD brain, which in turn may lead to memory deficit.

C

DIFFERENTIAL SPLICING OF ß-AMYLOID PRECURSOR PROTEIN IN AGGREGATING BRAIN CELL CULTURES

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The prevailing splice form of ß-APP in brain is ß-APP_{695}, which lacks a proteinase inhibitor domain coded for by exons 7 and 8. Since the precursor protein gives rise to both a large extracellular fragment clipped off near the plasma membrane by a secretase and a short peptide (ßA4), which is the major component of senile plaques, it is hypothesized that ß-APP_{695} is a more suitable substrate for ßA4-producing enzymes than other ß-APP splice variants. It is shown here by use of RT-PCR, that ß-APP_{695}-specific splicing typically occurs in primary neurons but not in rat or human neuroblastoma or in glial cells. Aggregating brain cells were treated with either cytosine arabinoside (ara-C) or cholera toxin to eliminate glial cells or neurons, respectively. Ara-C treatment clearly increased percentage of ß-APP_{695}, and high K^+ (30 mM) had an additional effect. Surprisingly, cholera toxin treatment, which reduced other neuronal parameters (ChAT, GAD) to almost undetectable levels, did not bring about the "glial pattern" where ß-$APP_{770/751}$ predominate. Aggregating brain cell cultures highly enriched in neurons apparently are a rich source of splicing factors specifically excising exons 7 and 8 from ß-APP precursor RNA and, therefore, can be used for both identification and therapeutic strategies to inhibit these factors.

D

CHARACTERIZATION OF A 43 kd ANTIGEN IN RAT BRAIN RECOGNIZED BY A MONOCLONAL ANTIBODY RAISED AGAINST A PHF PREPARATION FROM ALZHEIMER'S DISEASE BRAIN

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Neuritic plaques (NP), neurofibrillary tangles (NFT) and neuropil threads (NT), the major pathological hallmarks of Alzheimer's disease (AD), are related to the formation of paired helical filaments (PHF), the principal component of neurofibrillary degeneration. PHFs comprise the microtubule-associated protein tau in an abnormally phosphorylated state. Previously, we have described a monoclonal antibody B5-2 raised against a preparation of PHF, which selectively stains NFT, NP and NT in AD. On Western blot, the mab B5-2 labels three bands corresponding to A68, but shows no crossreactivity to normal adult or foetal tau species. The immunoreactivity can be abolished by alkaline phosphatase treatment on both, immunohistochemistry and on Western blot.We found that this antibody crossreacts immunohistochemically with postnatal rat brain. On Western blot, the mab B5-2 labels in a phosphorylation-indepentend manner a 43 kd protein which cosediments with microtubules, when tubulin was polymerized in rat brain extract. Purification of this protein, which is not heatstable, followed by N-terminal sequencing of the V8 endoprotease digest revealed sequence homology to actin. Subsequent isoform mapping showed that the antibody reacts specifically with the cytoplasmatic ß-actin isoform. This suggests that the epitop of the mab B5-2 is situated on the first 15 N-terminal amino acids of ß-actin. As the mab B5-2 does not recognizes foetaltyp tau, which has been demonstrated to possess a phosphorylation pattern comparable to PHF-tau, we conclude that the epitop recognized by the mab B5-2 which appears to be shared by ß-actin and tau might be crucial for developing PHF. (Supported by the BMFT: 01 ZZ 9103-2.7)

A

CHARACTERIZATION OF THE RAT SUPRACHIASMATIC NUCLEUS (SCN) IN LONG-TERM ORGANOTYPIC CULTURE

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Hypothalamic slice explants containing the SCN were derived from neonate rats and maintained in stationary organotypic culture for up to 5 weeks. Extra- and intra-cellular electrical activity were recorded, including intrinsic circadian activity over 24 hour periods. Injection of the tracer neurobiotin into recorded neurons, confirmed their location within the confines of the SCN. Immunocytochemical characterization of the culture, at both light- and electron-microscopical levels, identified vasoactive intestinal peptide (VIP)-containing neurons in the ventro-lateral SCN, vasopressin (VP)-containing neurons medio-dorsally, and GABAergic cells throughout the nucleus. The GABAergic component was particularly profuse, projecting extensively into the surrounding hypothalamic tissue. Fos-induction, exemplified by Fos-immunoreactivity within nuclei of SCN cells, was induced by exposure to glutamate via the culture medium. Glutamate-receptor inhibitor abolished the reaction. In conclusion, this long-term in vitro model of the brain's circadian clock is highly viable, and is able to sustain essential characteristics of the SCN in vivo.

B

ELECTROPHYSIOLOGICAL STUDY OF SCN NEURONS MAINTAINED IN LONG TERM ORGANOTYPIC CULTURE.

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The suprachiasmatic nucleus (SCN) is the locus of the main circadian oscillator in mammals. Electrophysiological properties of neurons in long-term SCN cultures were compared to those of SCNs in freshly prepared slices. The submerged slice technique was used throughout. Extracellular recordings monitored the rate of spontaneous activity and intracellular recordings (whole cell patch) measured passive and active properties of the cells. Neurons from both long-term cultures and fresh slices generate spontaneous all-or-none activity in the range of 0.1-13 Hz. The mean firing rate was 3.7±2.4 (n=248; range 0.1-11Hz) in cultures, versus 3.5±2.4 (n=86; range of 0.1-13) in fresh slices. In both systems, monitoring neuronal activity over 24 hour periods revealed circadian variations in spontaneous activity: periodic shifts in firing frequencies between low (average 1Hz) and high level (average 6 Hz) levels.

Input resistance, measured from the slope of the I-V curves, was 480±160MΩ (n=9) for cultures and 500±220MΩ (n=29) for fresh slices. Anomalous rectification was found in 22% of the former and in 37% of the latter. Na^+ dependent fast action potentials were readily recorded from both experimental models, as were also low and high threshold Ca^{2+} action potentials. The excitability of the different neurons was estimated by measuring the frequency-current relationship. The first instantaneous I-f curves were linear with a slope of 780±150 spikes/sec/nA (n=8) for cultured SCNs and 840±380 spikes/sec/nA (n=19) for fresh slices. In summary the electrophysiological properties of SCN neurons in the long-term and short-term models are similar, underscoring the value of the long-term culture for future studies in which long survival times are mandatory.

C

NITRIC OXIDE IN ISCHEMIA - REPERFUSION BRAIN INJURY. EFFECT OF N^G-NITRO-L-ARGININE

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Reperfusion of previously ischemic brain has been shown to be associated with exacerbation of cellular injury by potentiation of activation or inhibition of intracellular enzymes, influx of Ca^{2+} and disruption of cell membranes, which may result in ultimate cell death. Until now little informations have been published related to the role of nitric oxide (NO) in pathomechanism of ischemia–reperfusion brain injury. In this study, the activity of NO synthase (NOS) measured by NO-dependent cGMP formation during the different time of reperfusion was evaluated. Brain ischemia was induced in gerbils by ligation of both common carotid arteries for 5 min and then subjected for reperfusion for 5, 30, 60 min and 2, 4 h and 4 days after ischemia. Some groups of animals were treated with N^G-Nitro-L-arginine (NNLA) i.p. in a dose of 30 mg/kg b.w. 5 min before and in some cases 1, 3, 36, 48 h after ischemia. During reperfusion time biphasic increase of NO-mediated cGMP level in brain cortex was observed. The initial time of reperfusion 5–15 min enhances cGMP level by about 75–100%, respectively, compared to control value. This level at 15 min reperfusion is significantly higher by about 50% than observed in ischemic brain. The later phase of NO-dependent cGMP elevation is observed 2 h after ischemia and the level is 60% above the control value and similar to that in ischemic brain. Inhibitor of NOS protects the brain against NO-cGMP elevation during ischemia and reperfusion period. These results indicate for the first time the biphasic excessive elevation of NO and related to this molecule cGMP production during reperfusion. These changes and probably also other biochemical processes mediated by NO may at least in part potently participate in pathomechanism of ischemia–reperfusion brain injury.

D

ROLE OF FREE RADICAL PROCESSES IN ENERGETIC BRAIN METABOLISM AFTER BRAIN INJURY

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One of the main factors,determining the conditions of life and death of brain cells after brain injury is the disoder in energetic brain metabolism.Evidently, the mechanism of this disorder is the universal reaction of brain on pathologic action -activation of lipid peroxidation (LP) and decrease of brain antioxidant activity (AOA).The toxic products,generated in these chain free radical reactions,destroy mitochondrial ultrastructure,that leads to the suppressing of oxidative phosphorilation,ATP synthesis,actvity of succinate dehydrogenase and other membrane enzymes.Such desorganization of brain chemistry and structure results in the changes of brain functional activity /disorder in the processes of excitation and depression of the CNS/.As we found out,after the stimulation of the nervous activity of injured animals ,the content of LP products and AOA in brain came to control values.So by the modification of the CNS functional state we can influence positively on LP processes,the processes, which launch the whole chain of further energetic brain metabolism disorders,acidosis of the brain and cell death after brain injury.

A

NITRIC OXIDE SYNTHASE/NADPH-DIAPHORASE IN HIPPOCAMPAL SYNAPSES IN RELATION TO LONG-TERM POTENTIATION

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Nitric oxide (NO) seems to be an endogenous neuromodulatur. The synthesizing enzyme, nitric oxide synthase (NOS), is thought to be largely identical with NADPH-diaphorase (NADPH-d). Using electron microscopical histochemistry for demonstration of NADPH-d, the reaction product BSPT-formazan was localized in many neurons predominantly at membranes of the endoplasmic reticulum, including the nuclear envelope, and sometimes at membranes of the Golgi-apparatus or the outer membrane of mitochondria. Subcellular studies present a detailed picture of the neuropil with synapses which show labeled structures closely associated with vesicles on the presynaptic side. Only exceptionally, membrane elements of the postsynaptic side were marked with reaction product.
Due to our findings on the subcellular localization of NADPH-d we were attempted to look for an induction of NOS/NADPH-diaphorase by experimental stimulation. A good model for plastic changes is the long-term potentiation (LTP) in the hippocampus. Some groups have demonstrated a principal involvement of NO by LTP in the hippocampus slice by the blockade of NOS-inhibitors. Therefore, we speculated that the induction of LTP will modify the subcellular correlate (i.e. NADPH-positive membranes) in LTP-involved synapses on the pre- and/or postsynaptic side.
The induction of LTP in the dentate area was performed by tetanising the perforant path with 10 short bursts of 200 Hz. After tetanisation the rats were perfused, vibratome sections were cut and stained for NADPH-d. Finally, the sections were flat embedded in Durcupan and dendritic regions of the stratum moleculare adjacent to the stimulation electrode were dissected and ultrastructurally analysed.
Most of the NADPH-d reaction product was again found on the presynaptic side (is NO really a retrograde transmitter?). There was a low, but statistically significant increase in the number and extent of NADPH-d labelled membranes on the presynaptic side.

B

κ OPIOID AGONIST INHIBITS ENDOTHELIN-MEDIATED Ca^{2+} MOBILIZATION IN RAT CARDIOMYOCYTES

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Binding studies indicate that κ opioid receptors are present in adult rat cardiac sarcolema. It has also been suggested that endogenous opioids may reach the heart via the systemic circulation or directly when released from nerve endings distributed within the cardiac tissue. The nature of the function of opioid binding sites in heart is still an enigma. We found that cultured cardiomyocytes prepared from ventricles of post natal day one rats bound the κ selective opioid U69593 with a Kd value of 1.8±0.7 nM and Bmax value of 76.4±9.7 fmol/mg protein. In addition, the κ opioid agonist U50488 was shown to affect Ca^{2+} mobilization in the cells. For example, we studied the effect of U50488 on endothelin-induced Ca^{2+} mobilization in cultured rat cardiomyocytes utilizing Fura-2AM as a fluorescence Ca^{2+} indicator. Computerized image analysis of the fluorescence ratio (340/380 nm) showed that preincubation of the cells for 30 min in the presence of U50488 significantly inhibited the endothelin-mediated Ca^{2+} release from intracellular stores. In addition, blocking calcium channels with 30 nM nifedipine significantly reduced the effect of endothelin and potentiated the inhibition by U50488 of the levels of free Ca^{2+}. The results thus suggest that extracellular Ca^{2+} is involved in the process. We propose that κ opioid receptors have an important role in the homeostasis of Ca^{2+} levels in cardiomyocytes. *This work was supported by grants from the Anti-drug Authority of Israel, the United States-Israel Binational Science Foundation, and the Israel Cancer Research Fund.*

C

BRAIN GLYCOLYTIC ENZYMES: POSSIBLE MOLECULAR TARGETS FOR SEVERAL ANALGESICS

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New morphine-like potent analgesic fenaridine and morphine inhibit direct and reverse reactions catalyses by glycolytic enzymes hexokinase (HK), neuron specific enolase (NSE) and pyruvate kinase (PK) which were purified from human and rat brain. Inhibitor's concentrations were within the range of pharmacological effects of morphine and fenaridine. The enzymes activities were insensitive to the analgesics antagonists naloxone and fenetam in the large concentration diapason. Fenaridine was competitive inhibitor for direct reactions of HK and NSE with the same $K_i=5\cdot10^{-5}$ M. PK direct reaction was inhibit by fenaridine in the noncompetitive way with $K_i=3\cdot10^{-6}$ M, while apparent $K_i=3.3\cdot10^{-7}$ M for purified PK covalently attached to the erytrocyte membranes. Rat brain synaptosomal membrane-bound PK was the most sensitive for these compounds (app. $K_i=10^{-7}$ M). Mechanism of such inhibition and it biological meaning is under investigation, meanwhile synaptosomal membrane-bound pool of the PK as the most sensitive probably can serve as a simple suitable in vitro model for express testing of the compounds with potential analgesic effect.

D

COMPARATIVE ANALYSIS OF [^{3}H]MET-ENKEPHALIN-ARG^6-PHE^7 OPIOID RECEPTOR BINDING IN FROG AND RAT BRAIN MEMBRANES.

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In previous work we reported on a [^{3}H]Met-enkephalin-Arg^6-Phe^7 (MERF) opioid binding site of frog brain membrane preparations (M. Wollemann, J. Farkas, G. Tóth and S. Benyhe: J. Neurochem. 61, S213 A 1993.). Binding experiments were performed at 4 °C in TRIS buffer containing proteolytic inhibtors at pH 7.4. Incubation lasted 40 mins. Under these conditions the binding reached equilibrium within 20 mins and was stable for 60 mins. In the frog brain membrane preparation 84 % of the radioligand remained unchanged during the incubation. However, in the rat brain membrane preparation 60 % of MERF was decomposed under similar conditions. Nevertheless, the rank order potency of the investigated ligands did not change substantially i.e. bremazocine > Naltrindole > Met-enkephalin > DADLE > EKC > DSLET > DPDPE > U-69593. Introduction of norleucine instead of methionine, and D-Ala^2 in MERF decreased somewhat affinity, but did not increase the stability of the ligand. Further attempts are made in order to stabilize the ligand binding without decreasing affinity by the use of other peptidase inhibitors and substitutions or changes of amino acids in the heptapeptide.

A

PROTECTIVE EFFECT OF BIMU-1 AND BIMU-8 ON AMNESIA INDUCED BY HYPERCAPNIA

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BIMU-1 and BIMU-8 are endowed with antinociceptive activity due to an amplification of cholinergic neurotransmission (1). Since during cerebral hypoxia the loss of mnemic capacity is attributed also to a reduced functionality of cholinergic neurons (2), it seemed worthwhile to investigate the effect of BIMU-1 and BIMU-8 on a hypoxic condition. The two substances were tested in the pass-through passive avoidance test (punishing shock adopted was of 0.22 mA for 1 s.) in albino Swiss-Webster mice whose learning and memory processes had been impaired by hypercapnia. Hypercapnia was performed soon after the training test by placing each mouse into a box (1 l) in which the air had previously been replaced with pure CO_2 at a constant flux. Mice remained in the box for exactly 25 s, which was the minimum time necessary to cause a statistically significant amnesia. With this procedure control mice had 75 % retention, while mice exposed to CO_2 only 44 %. BIMU-1 and BIMU-8, starting at the doses of 5 mg/kg i.p., were able to reverse the amnesic effect. Retention was dose-dependent up to a maximum of 20 mg/kg i.p. (31.8% - 90.5%). The smaller antiamnestic effect shown by the 30 mg/kg dose as compared to the 20 mg/kg one is probably due to the antinociceptive action of both BIMU-1 and BIMU-8 that impair the effectiveness of averse stimuli in the passive avoidance test. BIMU-1 and BIMU-8 were not used at higher doses than 30 mg/kg i.p. since collateral side effects became visible. Piracetam (30 mg/kg i.p.) completely reversed the amnesia. All drugs were administered 20 min before the training session. The present results show that the two 5-HT_4 agonists prevent amnesia following exposure of mice to a hypercapnic environment.

1) Ghelardini et al. *XXIII Meeting of Soc. for Neurosci. vol. 19, abstr. 565.2, p. 1376, 1993*
2) Mishra et al. *Neurochem. Res. Bull. vol. 18, p. 111-115, 1993*

B

EFFECT OF CARNITINE AND ITS DERIVATIVES ON PROLIFERATION OF NEUROBLASTOMA NB-2A CELLS AND PROTEIN KINASE C ACTIVITY

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The major function of L-carnitine in the eukaryotic cell is to transfer acyl compounds from the cytosol to the mitochondrial matrix, although several other functions have been ascribed to carnitine derivatives in neural cells. In cultured neuroblastoma NB-2a cells carnitine accumulated in a concentration dependent way, the uptake of this compound was observed to consist of a saturated transport (Km=170 μM) and a diffusion component. The relative content of free carnitine and its acyl derivatives has been different than in brain, the long-chain acylcarnitines reached 65% of total accumulated carnitine. The level of intracellular palmitoylcarnitine was either elevated by administration of high concentrations of carnitine, or titrated down with a specific inhibitor of carnitine palmitoyltransferase I (etomoxir) what allowed to estimate the effect of palmitoylcarnitine on the activity of protein kinase C (PKC) *in vivo*. It was established that NB-2a cells contain an α-isoform of PKC which undergoes a very quick transfer to the membranes and down regulation even after a short (2 h) treatment with 8 nM phorbol-12-myristate acetate. NB-2a cells did not proliferate after inhibition of carnitine palmitoyltransferase I, therefore the PKC activity was measured in cells permeabilized with streptolysin O (6U/ml) after modulating the intracellular level of palmitoylcarnitine. Measurements of PKC activity expressed as the phosphorylation of RFARKGSLRQKN peptide point to palmitoylcarnitine as a natural modulator of the activity of this enzyme in NB-2a cells.

C

ENHANCEMENT OF cGMP LEVELS ELICITED BY $GABA_B$ ANTAGONISTS IN RODENT BRAIN: POSSIBLE INVOLVEMENT OF NMDA RECEPTORS AND OF NITRIC OXIDE (NO)

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$GABA_B$ receptor agonists such as R(-)-baclofen decrease cerebellar cGMP levels of rodents in a dose-dependent manner. $GABA_B$ antagonists with affinities in the low nanomolar range such as CGP 55556 and its diastereoisomer CGP 56999, antagonized the decrease produced by (-)baclofen and elicited a dose-dependent increase of cerebellar cGMP. To test whether NMDA receptors were involved in the increase of cGMP induced by $GABA_B$ antagonists, we injected antagonists of the different binding sites of the NMDA receptor complex either alone or in combination with $GABA_B$ antagonists. The effects of $GABA_B$ antagonists were abolished by the competitive NMDA antagonist CGP 40116, by R(+) HA 996, a competitive antagonist at the strychnine-insensitive glycine binding site and by spermidine. The two NO-synthase (NOS) inhibitors N-monomethyl-Larginine and nitro-L-arginine also suppressed the increase in cGMP produced by CGP 56999. These results suggest that blockade of $GABA_B$ receptors increases glutamate release, which activates NMDA receptors and raises postsynaptic Ca2+ levels. This stimulates NOS to produce NO, which in turn activates soluble guanylate cyclase. In agreement with these biochemical results, doses of CGP 56999 which increase cGMP levels also induce epileptiform discharges in the EEG. This epileptic activity was antagonized by baclofen and by NMDA antagonists.

D

CHANGES OF ^{3}H-DIAZEPAM BINDING AND INTRACELLULAR REGULATORS CONTENT IN RAT BRAIN AFTER PENTYLENETETRAZOL KINDLING

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Levels of cAMP and cGMP in the left and right rat sensorimotor cortex (SMC) were determined 7 days after pentylenetetrazol (PTZ) kindling (30 days) by radioimmunological method. Levels of cyclic nucleotides in rat SMC 7 days after acute injection of seizure dose of PTZ were shown to be unchanged. Levels of cAMP and cGMP in SMC after 30-days administration of physiological saline were increased by 2-3 times as compared to acute injection; cortical asymmetry of cAMP and cGMP was not observed. 7 days after kindling cGMP level was increased in right SMC and decreased in left SMC as compared to control animals; cAMP level was unaltered in left and increased in right SMC. 11 months after the end of kindling and one day after acute PTZ injection (30 mg/kg) ^{3}H-diazepam binding with synaptosomal membranes of cerebellum was decreased and increased after single injection of seizure dose of PTZ; alterations in cortex were not observed. 7 days after kindling an increase by 60% in the free Ca^{2+} concentration (using fluorescent probe Guin-2) in control rat brain synaptosomes was revealed; the treatment by verapamil and ryodipine prevented this increase. There was no difference between the levels of cyclic nucleotides in the rats treated by calcium antagonists or physiological saline (30-days administration).

A

THE EFFECTS OF CONVULSANTS ON THE DESENSITIZATION OF $GABA_A$- RECEPTOR COMPLEX

REBROV IGOR, GLEBOV RUDOLF

The effects of convulsants on the desensitization (DS) of $GABA_A$-receptor complex (RC) were studied by $^{36}Cl^-$ influx assay into synaptoneurosomes from rat cerebral cortex. The degree of DS was determined by decreasing of $^{36}Cl^-$ influx after preincubation of synaptoneurosomes with muscimol. The studied convulsants were two types of inhibitors of $GABA_A$-RC. The first type were blokers of Cl^--channel, picrotoxin and pentylenetetrazol. The second type was antagonist of GABA -receptor, bicuculline. Both types of convulsants inhibited of $^{36}Cl^-$ influx and significantly decreased the degree of DS (3-4 times less with concentration of the preparetions near IC_{50}). The DS delay effect increased with rising of the convulsants concentration. Correlation between the inhibition of $^{36}Cl^-$ influx and the DS delay was different for all three convulsants. Increasing of muscimol concentration from 5 to 50 μM removed the DS delay by bicuculline, but didn't change the effect of picrotoxin. So, muscimol influenced the DS delay effect in the conditions of $GABA_A$-receptors inhibiting. The main result of the present work is the fact that mechanism of the $GABA_A$-RC DS isn't connected with changes only on the receptors level, but consequences from the functional activity of all the receptor -channel complex.

B

EXORPHINS IN SCHIZOPHRENIA AND AUTISM.

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We have isolated peptides immunoreactive to bovine casomorphin1-8 antibodies from urine and dialysis fluid (1,2) One peptide cochromatographed with bovine casomorphin . Another had amino acid composition and mass number equal to gliadinomorphin.The opioids show bell shaped dose respons curves (1). This may explain the contrary data on opioids and the effects of antagonists like naltrexone .We also find an increased level of IgA antibodies i serum to casein,lacto-globulin ,and gliadin .(2,3) This affirms Dohans hypothesis (4) on a possible etiology .

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C

NMR SPECTROSCOPIC STUDIES OF [U-^{13}C]GLUTAMATE and [U-^{13}C]GLUTAMINE METABOLISM IN CULTURED CORTICAL ASTROCYTES AND GABAERGIC NEURONS

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Cultured cortical astrocytes and GABAergic neurons were incubated in media containing 0.25 or 0.5 mM [U-^{13}C]glutamate or [U-^{13}C]glutamine for 2-3 h. Perchloric acid (PCA) extracts of the cells and of redissolved lyophilized media were subjected to ^{13}C-NMR spectroscopy in order to identify metabolic pathways. ^{13}C-NMR spectra of PCA extracts of neurons incubated with uniformly labelled glutamate or glutamine showed multiplets for aspartate which can only be formed through the tricarboxylic acid (TCA) cycle. The labelling pattern in GABA indicated that at least 30 % of labelled GABA was derived from TCA cycle intermediates. In the presence of the GABA transaminase inhibitor γ-vinylGABA (100 μM), aspartate and GABA were still labelled from TCA cycle intermediates indicating that labelled glutamate enters the TCA cycle. Furthermore, scrambling of label in GABA and aspartate was more pronounced after incubation with glutamine than with glutamate, suggesting metabolic compartmentation of neuronal glutamate metabolism. PCA extracts from astrocytes incubated with uniformly labelled glutamate showed multiplets for glutamate, glutamine, aspartate and malate and the culture medium showed peaks for glutamine and lactate, citrate and alanine. On the basis of the labelling pattern in the different metabolites it was concluded that exogenously supplied glutamate is preferentially metabolized through the TCA cycle (70%) and to a lesser extent to glutamine (30%). Lyophilized media from neurons incubated with uniformly labelled glutamate or glutamine did not show any labelled peaks for lactate and alanine. This observation is in agreement with the localization of the malic enzyme in astrocytes. This pathway may be of importance to supply neurons with energy substrates when neuronal activity is high.

D

EFFECT OF α_2-ADRENORECEPTOR ANTAGONIST CH-38083 ON LPS-INDUCED TNF-α AND IL-6 PLASMA LEVELS

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We investigated in mice the role of α_2-adrenoreceptors in the regulation of LPS (lipopolysaccharide)-induced production of cytokines. Animals were injected intraperitoneally by a novel, highly selective α_2-adrenoreceptor antagonist CH-38083 30 minutes before LPS administration. Plasma levels of tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) were monitored by ELISA. The plasma level of TNF-α was significantly decreased in CH-38083-pretreated mice as compared to the controls. On the other hand, LPS-induced IL-6 levels were higher in CH-38083-pretreated mice. Since it is believed that increased level of TNF-α is involved in the pathogenesis of inflammation, and that IL-6 plays an anti-inflammatory role by activating hepatic acute-phase protein production, the. the administration of a highly selective α_2-adrenoreceptor antagonist may be beneficial. The inhibition and augmentation of LPS-induced TNF-α and IL-6 production, respectively, by the inhibition of alfa-2 adrenoceptors might have immunosupressive effect during inflammatory response.

A

CHARACTERIZATION OF THE SUBTYPE OF PRESYNAPTIC α_2-ADRENOCEPTORS MODULATING NORADRENALINE RELEASE IN RAT HIPPOCAMPUS

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In vivo brain microdialysis and high performance liquid chromatography with electrochemical detection were used to study the effect of different selective α_2-antagonists on the hippocampal noradrenaline (NA) release in freely-moving awake rat. Systemic administration (0.5 mg/kg i.p.) of either the α_{2AD} antagonist BRL 44408 or the α_{2BC} antagonist ARC 239 did not change significantly the basal release of NA. In a higher dose (5 mg/kg i.p.) ARC 239 remained ineffective, whereas BRL 44408 caused a significant increase of the extracellular level of NA. Similar results were obtained from in vitro perfusion experiments. Rat hippocampal slices were loaded with [^{3}H]-NA and the electrical stimulation-evoked release of [^{3}H]-NA was determined. The α_2-antagonists were applied in a concentration range of 10^{-8} to 10^{-6} M. ARC 239 was ineffective, whereas BRL 44408 increased significantly the electrically induced release of [^{3}H]-NA. Our data indicate that hippocampal NA release in rat is regulated by α_{2D}-adrenoceptor, a species variation of the human α_{2A} subtype.

B

ADP-RIBOSYLATION OF RAT BRAIN SYNAPTIC MEMBRANES PROTEINS

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The rate of mono-ADP-ribosylation of synaptic membranes (SM) proteins from rat cerebral cortex increases more than 15-fold if SM are stored for 20 h at room temperature in the presence of inhibitors of proteolysis (PMSF, N-EM, EDTA and pepstatin). This effect is not associated with non-enzymatic incorporation into SM of free ADP-ribose, which may be generated by NAD cleavage. ADP-ribosylation reaction doesn't change under the influence of specific inhibitor of NAD-ase NMN, but almost completely prevented by ATP and GTP, probably due to phosphorylation of SM proteins. The enhancement of ADP-ribosylation is accompanied by increase of ^{14}C-ADP-ribose incorporation predominantly into the fraction of high molecular weight proteins of SM. The incubation of SM as a source of endogeneous ADP-ribosyltransferases with double labelled NAD and some amino acids (Arg, Cys, His) leads to formation of their adducts with ^{14}C-ADP-ribose, which can be separated on Dowex 1x4 and PEI-cellulose. Thus, cysteine and histidine alongside with arginine may serve as a target amino acids of ADP-ribosylation in SM proteins. This conclusion is supported by results showing the selectivity of forming ADP-ribose-amino acids links to specific compounds (NH_2OH, $HgCl_2$ and NaOH).The data obtained allow to suppose that mono-ADP-ribosylation of SM proteins is one of the possible mechanisms of modulation of synaptic processes by NAD.

C

LACK OF T-CELL DOES NOT AFFECT PEPTIDES AND SYNAPTIC VESICLE MEMBRANE PROTEINS IN SCG AND IRIS OF THE RAT

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The aim of the investigation was to study the T-cell influence in the modulation of superior cervical ganglion (SCG) and its target organ, the iris. We used athymic, nude (LEW/Mol-rnu/rnu) and euthymic (Lewis) rats as an experimental model. Cryostat sections of SCG, and pieces of irides, which were strech fixed, were incubated with antibodies against: 1) sympathetic adrenergic markers, tyrosin hydroxylase (TH) and neuropeptide Y (NPY); 2) growth associated protein, GAP-43; 3) synaptic vesicle membrane proteins, synaptophysin (p38), synaptotagmin (p65) and SV2. The samples were examined using a confocal laser scanning microscope. All of the markers labelled structures in the SCG and irides of athymic as well as in euthymic rats. In the SCG, TH-like immunoreactivity (LI) was mainly located in cell bodies of principal cells and SIF cells. Some of the TH- positive principal cells and all of the SIF cells were also NPY-positive. All of the other markers induced labelling in the intraganglionic nerve terminals around the cell bodies. In the iris, GAP-43-LI, p38, p65, SV2 and TH-LI were mostly located in the network of terminals. NPY-positive nerve fibres were fewer than TH-positive fibres and mostly accompanied blood vessels. There were no clearcut differences between athymic and euthymic rats observed using the present antibodies. The results suggest that lack of T-cells does not significantly influence the development of adrenergic neurons in the SCG and irides.

D

PHOSPHORYLATION OF PURIFIED IP_3-RECEPTOR FROM RAT CEREBELLUM BY CYCLIC GMP-DEPENDENT PROTEIN KINASE

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The inositol (1,4,5)-trisphosphate receptor/Ca^{2+}-channel protein (IP_3-R) has previously been shown to be a substrate for cyclic AMP-dependent protein kinase (PKA), Ca^{2+}/phospholipid-dependent protein kinase (PKC) and Ca^{2+}/calmodulin-dependent protein kinase in vitro. In the present study we have shown that membrane bound and immunopurified IP_3-R from rat cerebellum serve as a substrate for cyclic GMP-dependent kinase (PKG) in vitro. Our findings show that PKG is capable of phosphorylating immunopurified IP_3-R to a stochiometry of 1,5 pmol P_i/μg receptor whereas PKA phosphorylated the IP_3-R to a stochiometry of 3 pmol P_i /μg receptor. Thermolytic phosphopeptide maps of immunopurified IP_3-R phosphorylated by PKA and PKG indicate that PKG phosphorylates the receptor on one site located on the same peptide as one of the two PKA-derived phosphopeptides. The observation that phosphorylation by PKA followed by phoshorylation by PKG is not additive, while phosphorylation by PKG followed by phosphorylation by PKA is additive, further suggests one common phosphorylation site.

A

HISTAMINE-SENSITIVE POTASSIUM TRANSPORT BY CULTURED ASTROGLIAL CELLS OF RAT BRAIN

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The K^+ uptake into astrocytes consisted of three components:/a/ ouabain-sensitive component/representing Na^+ + K^+ ATP-ase/;/b/ furosemide-sensitive component /representing Na^+ + K^+ + Cl^-cotransport/ and /c/ a component,insensitive to ouabain and furosemide /representing passive K^+-channels/.

In this study, histamine/H/ and histamine/H/--receptor agonists and antagonists were examined as possible modulators of K^+-transport in cultured astrocytes of rat brain. For K^+-transport studies ^{86}Ru was used as tracer in a Krebs medium /pH 7.4/ during 5,10 and 60 min incubation. The ouabain--sensitive uptake of ^{86}Ru was about 50 % of the total uptake in these cultures of astroglial cells. 0.01-0.10 uM H and H-agonists, R-alphamethylhistamine /H_3agonist/ and thyazolyle+hylamine H_1--agonist/ produced remarkable apparent increases in ^{86}Ru uptake during 1-10 min and 60 min . Dimaprit /H_2-agonist/ was ineffective on the ^{86}Ru transport. The above effects were much higher in type-2-astrocytes than in the type-1 cells and could be reversed by H_2 and H_3 antagonists or both.

The sensitivity of ^{86}-Ru transport to H and H--agonists might indicate H-gated K^+-channels in astrocytes, especially in type-2-astrocytes of rat brain.

B

SYNTAXIN IN TORPEDO ELECTRIC ORGAN HAS SEVERAL VARIANTS WHICH ARE AFFECTED BY CALCIUM LEVEL

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Syntaxin is a protein which was implicated in vesicular targeting as well as in docking and priming of synaptic vesicles in the nerve terminal. Syntaxin was shown to interact with other proteins such as VAMP/synaptobrevin and munc/n-sec which are expected to be central in regulating neurotransmitter release. Moreover, cleavage of syntaxin by butulinum neurotoxin blocks neurotransmitter release.

In this report we show that syntaxin is highly enriched in synaptosomes of *Torpedo* electric organ. The *Torpedo* syntaxin resembles that of rat brain in several aspects. Both proteins are similar in size (35 kDa), and furthermore, are recognized by various independent monoclonal antibodies. In *Torpedo*, several variants which are slightly different in size are resolved by gel electrophoresis. By immunoelectron microscopy syntaxin is shown to be localized at the presynaptic membrane.

Ca^{2+}, which is a trigger for neurotransmitter release affects *Torpedo* syntaxin. The ratio between syntaxin variants is changed by altering Ca^{2+} level. When Ca^{2+} is omitted from the synaptosomal preparation (with 5mM EGTA), an additional variant is formed. This may reflect a Ca^{2+} dependent modification at the protein level.

The syntaxin variants interact with VAMP in a Ca^{2+} independent manner. However, the interaction of one of the variants of syntaxin with other unknown proteins is Ca^{2+} dependent as was demonstrated by a sedimentation analysis. Thus, Ca^{2+} level in the nerve terminal may modulate and regulate neurotransmitter release via an effect on the stability of protein complexes.

This work was supported by the Israeli Academy of Science.

C

Na,K-ATPase ACTIVITY AND LONG-TERM POTENTIATION IN RAT OLFACTORY CORTEX SLICES.

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The brain cell membrane changes are important in the neurochemical conception of the high nervous system plasticity.

On the model of the long-term potentiation(LTP) in rat olfactory cortex slices by means of the quantitative cytophotometry and computer densitometry we investigated the neuronal and glial membranebound Na,K-ATPase and Mg-ATPase activity and also 14-C-desoxiglucose (14-DOG) incorporation.

In the dynamics of LTP maintenance phase the neuronal Na,K-ATPase activity significantly decreased in 5 min after tetanization, with its following increasing only to 30 min. Glial (mainly astrocytes) Na,K-ATPase activity changes were opposite to neuronal enzyme: its activity was increased in 5 min and returned to control value in 30 min. There were no significant changes of neuronal and glial Mg-ATPase activity in this phase of LTP.

The small increasing of 14-C-DOG incorporation was observed in 5 min and 30 min after tetanization.

We will discuss the correlation of the neuronal and glial Na,K-ATPase activity changes with the functional nervous system state and the role of this enzyme in the exitability of the tissue.

D

PROTEOLYTIC PROCESSING OF CHROMOGRANIN A IN VARIOUS TISSUES

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Chromogranin A is an acidic protein that belongs to a group of proteins (chromogranins) which also include chromogranin B, secrtogranin II and 7B2. It is stored in the large dense core vesicles of various endocrine tissues and in neurons. Processing of chromogranin A starts at the N- and C-terminus of the molecule and results in the generation of multiple peptides of various sizes. Here we charactarized the formation of GE-25 a novel chromogranin A-derived peptide. GE-25 is located in the C-terminal domain of the primary amino acid sequence of chromogranin A. After separation of tissue extracts by gel-filtration HPLC processing of chromogranin A to GE-25 was investigated in several endocrine organs. In the adrenal medulla and anterior pituitary GE-25-IR was found to occur mainly as chromogranin A, whereas in the posterior pituitary, the small intestine and in the pancreas chromogranin A was degraded to a high degree to the free peptide. An analysis of the hypothalamus and hippocampus revealed equal amounts of precursor proteins and free peptide. These studies provide the basis for further experiments on a possible biological function of this novel peptide.

A

The *Drosophila* Rop protein may interact with the products of the *syntaxin* and *Ras2* genes

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The *Drosophila Ras2 Opposite* (*Rop*) gene is highly expressed in the *Drosophila* nervous system, and in the garland cell - the *Drosophila* nephrocyte[1]. The *Rop* protein is homologous to the products of the *C. elegans* gene *unc-18*, and the rat and bovine *munc-18* genes, implicated in the final steps of neurotransmitter exocytosis in nerve terminals. Munc-18 is highly associated with Syntaxin, an integral protein of the pre-synaptic membrane. Using a GST-Syntaxin fusion (a gift from R. Scheller), we have shown that the Rop protein can bind to mammalian Syntaxin. Thus, the *Drosophila* Rop, like its mammalian homologs, is probably a component of the nerve terminal.
Rop is regulated by a bidirectional promoter, shared with *Ras2*, a member of the *ras* gene family. The two genes are co-expressed in the garland cells, a small group of nephrocytes which take up waste materials from the haemolymph by endocytosis. The garland cell is characterized by long channels deep extending from the cell membrane, and a rich population of a variety of vesicles. Immunohistochemistry and electron microscopy revealed that the products of the two genes are co-localized to the cortex of the garland cell. They could be detected on the surface of the channels, and on the surface of many vesicles as well, suggesting that the Ras2 and Rop proteins may be components of the vesicular trafficking system in these nephrocytes.

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B

EFFECT OF HALOPERIDOL ON NEUROTRANSMITTER GLUTAMATE IN RAT HIPPOCAMPUS.

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It has been recently reported that haloperidol, a typical antipsychotic agent, acts at various sites of glutamatergic neuronal activity (NMDA hippocampal receptors, glutamate release in striatum or amygdala,GDH inhibition). Therefore the in vitro effect of haloperidol on synthesis and release of neurotransmitter glutamate was studied in rat hippocampus. Haloperidol was applied in a concentration of 100μM. Neurotransmitter release was studied by superfusion of hippocampal slices, preloaded with D-[H^3]-aspartate, a non metabolizable analogue of glutamate that labels also the neurotransmitter pool. Biosynthetic studies were performed in hippocampal homogenates; the effect of haloperidol on phosphate activated glutaminase (PAG) and glutamate dehydrogenase (GDH) major candidate enzymes of neurotransmitter glutamate metabolism was tested. 1. Haloperidol significantly reduces the K^+evoked release of D-[H^3]-aspartate in the presense of Ca^{2+}. 2. There is no effect on the K^+ evoked Ca^{2+}independent release (Ca^{2+} replaced by Mg^{2+}) of D-[H^3]-aspartate. Thus inhibition refers to the Ca^{2+}dependent component of D-[H^3]-aspartate release. 3. Haloperidol inhibits non activated GDH in a concentration dependent manner. 4. There is no effect on GDH activated by physiological concentrations of ADP or leucine. 5. There is no effect on PAG under elevated or physiological concentrations of its main activator, phosphate. It is suggested that in rat hippocampus haloperidol affects glutamate release through an action on presynaptic mechanisms regulating exocytosis and not on the biosynthetic pool.

C

P19 EMBRYONAL CARCINOMA CELLS DIFFERENTIATE INTO FUNCTIONAL NEURONS

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Embryonal carcinoma (EC) cells, the stem cells of teratocarcinomas, represent good model systems for the study of cellular commitment, differentiation and development. P19 is a mouse-derived EC cell-line capable of differentiation toward ectodermal, mesodermal and endodermal lineages. Thus, P19 cells represent an early stage of development. Following treatment with retinoic acid these cells differentiate into neurons, astrocytes and fibroblast-like cells.

We induce P19 differentiation under conditions which provide a homogenous neuronal culture (>98% neurons). We show that P19 neurons express a battery of neuronal genes that are not expressed before differentiation. These genes include cytoskeletal proteins (MAP-2, NF-M and tau), synaptic vesicle proteins (synaptophysin, SV2 and VAMP/synaptobrevin) and terminal specific proteins (SNAP-25 and Rab3A). All genes are induced at approximately the same stages of differentiation, which is just before neurite outgrowth. However, maturation of P19 neurons occurs relatively late after induction of the neuronal genes. This maturation is indicated by GAP-43 down regulation, which is seen after 12 days in culture. At approximately this stage, the neurites show characteristics of dendrites which express MAP-2, and of axons which express neurofilaments. Another aspect of maturation is the ability of the cells to release ACh following depolarization with KCl. The release is Ca^{2+} dependent, and drops to baseline levels at 0.5 mM Ca^{2+}. The cells at this age also respond to α-latrotoxin (0.5 nM) by an intense release of ACh. Finally, we show that P19 cells are also able to form synapses with a muscle cell line - C2.

We suggest that this cell line can serve as a model system for the study of neuronal differentiation from a multipotent cell up to a mature neuron.

D

DYSTROPHIN AND SPECTRIN ISOFORMS ARE PRESENT IN A GLYCOPROTEIN COMPLEX FROM MAMMALIAN BRAIN SYNAPTOSOMES

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Dystrophin, a protein whose deficiency leads to Duchenne muscular dystrophy, is thought to play an important role in the membrane physiology of muscle cells. It is also present in brain. Although dystrophin's functional role is still unknown, its localization and homology of sequence with spectrin suggest that it is an important structural component of membrane skeleton. In this study we examined the expression of spectrin isoforms in control and dystrophic mdx mice during neuronal development and investigated the subcellular localization of dystrophin in rat brain. No differences were detected in the expression of spectrin isoforms, α- and β-fodrin (αIIΣ2* and βIIΣ1) and β-spectrin (βIΣ2), between control and mdx mice. However, proteolysis of spectrin was observed in 2- and 3-week-old mdx mice. Dystrophin and βIΣ2 spectrin were enriched in postsynaptic density (PSD) structures of rat brain. Spectrin and dystrophin isoforms were also associated with a glycoprotein complex obtained by fractionation of bovine brain synaptosomes on a laminin-Sepharose column. Our results suggest that unique isoforms of spectrin together with dystrophin and dystrophin-associated protein, may play a pivotal role in organizing topographically defined clusters of receptors or cytoplasmic protein complexes that are functionally important in nerve cells.

The financial support of Telethon-Italy to the project "Role of spectrin isoforms in the central nervous system of normal and dystrophic mdx mice" is gratefully acknowledged.

A

EFFECTS OF β-HYDROXYBUTYRATE (HB) AND DIABETES ON ACETYL-CoA AND ACETYLCHOLINE SYNTHESIS IN BRAIN.

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The increase of HB and decrease of insulin level taking place in diabetes could influence both acetyl-CoA and ACh metabolism in the brain. In rat brain synaptosomes from control rats HB inhibited pyruvate oxidation (12%), but increased level of acetyl-CoA (15%) in synaptoplasm and the rate of ACh synthesis (20%). HB did not change proportions between indirect, ATP-citrate lyase dependent and direct, Ca-activated pathway, that provide acetyl-CoA from mitochondrial to synaptoplasmic compartment. Streptozotocin diabetes lasting 10 days caused 50% increase of acetyl-CoA formation from HB in the nerve terminals. Also inhibitory effect of HB on pyruvate oxidation was increased to 25% under these conditions. In spite of that the level of acetyl-CoA and ACh synthesis in diabetic synaptosomes were about 30% higher than those in controls. Intraperitoneal injections of insulin, in single doses of 10U/kg/day for 5 days, did not correct diabetes and ketonemia but caused further increase in acetyl-CoA content and ACh synthesis by synaptosomes to the levels 50% above control values. Significant correlation (r=0.93) was found between acetyl-CoA level and ACh synthesis in synaptosomes of all experimental groups. On the other hand, there was no correlation between rates of pyruvate oxidation and ACh synthesis as well as between pyruvate oxidation and acetyl-CoA level in synaptosomes. These data indicate that both HB and exogenous insulin interact in activation of ACh synthesis in brains of diabetic rats, without relevant changes in pyruvate oxidation. It may be due to increased provision of acetyl-CoA from mitochondria to cytoplasmic compartment of nerve terminals.

B

QUANTAL ACETYLCHOLINE RELEASE IN RECONSTITUTED MODELS.

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The mediatophore isolated from the presynaptic membrane of torpedo electric organ is a protein able to release acetylcholine (ACh) in a Ca^{2+}-dependent manner. This release was so far measured by biochemical means, but we didn't know if it showed the pulsatile and quantal features observed at natural synapses. To answer this question we used the patch clamp technique in whole-cell recording configuration applied to a Xenopus myocyte. It is a sensitive ACh detector with a high time resolution. Different cell types artificially filled with ACh were studied for their ability to release ACh on a Ca^{2+}-dependent manner upon electrical stimulation. A good correlation was found between: 1) The biochemical measurement of ACh release. 2) The expression of the 15 kD subunit of the mediatophore within the cell membrane. 3) The ability to release quanta of ACh. We are currently transfecting cells (initially unable to release ACh) with the mediatophore cDNA and will test whether they will acquire an ACh release ability. We also are going to transfect the mediatophore antisense into cells presenting the release mechanism and analyse if it is sufficient to inhibit quantal transmitter release.

C

SYNAPSIN-LIKE IMMUNOREACTIVITY IN INVERTEBRATE NEURONS *IN VITRO*: REDISTRIBUTION FOLLOWING THE ESTABLISHMENT OF SYNAPTIC CONTACTS

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The characterization of proteins involved in the storage and release of neurotransmitters has been a major step in understanding the synaptic function. Among them, the synapsins, a family of evolutionary conserved, neuron-specific phosphoproteins associated with mammalian small synaptic vesicles, appear to play a pivotal role in both mature and developing nerve terminals. Neuronal cultures from invertebrate nervous system represent a model particularly suitable to analyze the synaptic functions; in fact, in these cultures presynaptic and target neurons form active contacts which can undergo synaptic plasticity.
We studied the redistribution of the synapsin-like immunoreactivity in identified *Aplysia* and *Helix* cultured neurons following the establishment of synaptic contacts. To this aim we cocultured a large serotoninergic cell (**GCN**) from the cerebral ganglion and its target neuron (**B2**) from the buccal ganglion; these cells form *in vitro* reliable synaptic contacts that can be electrophysiologically tested. After different periods in culture we localized the synapsin-like immunoreactivity by means of a battery of anti-synapsin polyclonal antibodies. In the presence of the target neuron the synapsin-like immunoreactivity in the presynaptic cell substantially overlapped the serotonin immunostaining (used as a marker of active synapses). On the contrary, in the absence of the physiological target a diffuse labeling pattern along the outgrowing neurites was observed. This is in good agreement with the data showing that in cultures of hippocampal neurons of mammals synaptic vesicle proteins cluster in presynaptic specializations following contacts with dendrites of other neurons. Our results suggest that in invertebrate cultured neurons the sorting of synaptic vesicle proteins to the presynaptic ending is strongly affected by the presence of the target cell.

D

PHENOTYPIC DIFFERENCES IN THE EXPRESSION OF THE *PT* TRAIT IN HYPOMYELINATED MUTANT RABBIT.

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Paralytic tremor (pt) is an X-linked recessive mutation resulting in a severe hypomyelination of rabbit central nervous system (CNS). The *pt* mutants, although strictly controlled for their *pt* trait, differ significantly from one another in their phenotype. An onset of neurological disorders takes place usually at 10th postnatal day. Typical first symptoms include: tremors, an exaggeration of tendon reflexes and a weakness of limbs. Mutants are smaller and lighter than agematched controls. As they age, the intensity of enumerated symptoms increases, additionally a spastic paresis of hindlimbs might be developed, followed occasionally also by a paresis of forelimbs. In most severe cases, mutants try to move on the bellies with their limbs shifted aside; a lifespan of so strongly affected animals is reduced to a few months. A typical i.e. mild course of the *pt* disease excludes the heavy disorders as paresis and the affected animals have an almost normal lifespan. Also a spontaneous recovery after a typical onset of neurological symptoms is often observed. The aim of our present studies is to investigate whether there is any difference in the major myelin specific proteins expression in mutants which differ in their phenotypes. For our studies we have chosen 5 groups of animals aged 22, 42, 65, 80 and 150 days; each group consists of mutants expressing predominant phenotypes (i.e. two mutants with most sever and two another with the mild course of the disease) and agematched control animals. We have stated that degree of both the CNS hypomyelination and the underexpression of investigated myelin markers (PLP, MBP, MAG, MOG and CNP) observed in the brain homogenates, correlates with the severity of the neurological symptoms and is highest in the most affected animals. Studies on purified myelin have revealed a certain difference in PLP expression between both examined phenotypes, whereas contents of others investigated myelin proteins seem to be less affected.
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A

POSTASPHYXIAL CSF HYPERINSULINISM AND BRAIN OEDEMA FORMATION IN NEWBORN PIGLETS WITH PNEUMOTHORAX

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Insulin plays a crucial role in the maturation of neonatal brain and it may also be involved in the pathogenesis of brain injuries. Clinical studies pointed out that (1) neonatal asphyxia resulted in hyperinsulinemia, and (2) the increased plasma insulin levels correlated with higher rate of mortality and neurological sequalae. We measured both the insulin concentrations by RIA, and glucose levels using the glucose oxidase method in plasma and CSF during 3 phases (baseline, critical, and recovery after resuscitation) of experimental bilateral pneumothorax (PTX) in newborn piglets. BBB permeability for sodium fluorescein (SF, 376 Da) and Evans'blue-albumin (EBA, 67 kDa) was also determined by spectrophotometry during 4 h of experiments. We observed significant hyperinsulinism ($p<0.001$) both in plasma and CSF, and a mild hypoglycemia ($p<0.05$) in the reperfusion period. $Insulin_{CSF/plasma}$ ratio (means $\pm$ SEM, $n=16$) was decreasing during critical phase of PTX (0.09 ± 0.02, NS) compared to the baseline value (0.12 ± 0.04), it was elevating at 60 min (0.14 ± 0.04, NS), and increased significantly at 180 min (0.40 ± 0.14, $p<0.05$) after resuscitation. There was a similar increase in $glucose_{CSF\ plasma}$ ratio at 180-min reperfusion (0.99 ± 0.14 *versus* 0.76 ± 0.05 at baseline, $p<0.05$) the end. A selective BBB opening for SF ($P<0.001$) was seen in the critical phase of PTX, but significant EBA extravasation ($p<0.001$) occurred only in the 180-min recovery. In conclusion, neonatal asphyxia resulted in increased blood to brain transport of insulin and glucose, and the hyperinsulinism observed might alter the development of vasogenic brain oedema.

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B

CO-LOCALIZATION OF PYRUVATE CARBOXYLASE WITH PYRUVATE DEHYDROGENASE IN GLIAL PRIMARY CULTURES AND WITH FRUCTOSE-1,6-BISPHOSPHATASE IN BRAIN SLICES

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Recently we demonstrated that astroglial cells in culture are capable of gluconeogenesis. Therefore, it was important to investigate the relative cellular localization of key gluconeogenetic enzymes. To this end we used monospecific antibodies, i.e., a mouse monoclonal antibody and a rabbit antiserum raised against bovine liver pyruvate carboxylase (PC) as well as a monoclonal antibody against bovine liver fructose-1,6-bisphosphatase (FBP). Immunocytochemical examination of astroglia-rich primary cultures derived from mouse brain revealed co-localisation of PC with the mitochondrial marker pyruvate dehydrogenase (PDH) in many cells. In contrast to the uniform staining of PDH in all cells, the staining of PC varied in intensity from one cell to another. However, with both antibodies the punctuate staining of the mitochondria is of uniform intensity within one cell. Data obtained with fluorescence microscopy and laser scanning microscopy of cultured cells made it likely that in a given cell PC is colocalized with PDH in all mitochondria. This result does not support the hypothesis of two kinds of mitochondria within one cell. Double-labelling experiments involving an antiserum against PC and a monoclonal antibody against FBP revealed a copresence of the enzymes in astrocytes of brain slices. These findings suggest that the gluconeogenetic enzymes PC and FBP are colocalized in astrocytes. This notion is confirmed by the colocalisation of glial fibrillary acidic protein with PC and FBP, respectively, in brain slices.

C

MODULATORY EFFECTS OF IONTOPHORETIC DRUG ADMINISTRATION ON LOCAL PROCESSING IN THE AUDITORY CORTEX

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A multi-electrode setup for combined microiontophoretic drug administration and multiple single-unit recordings was developed. It consisted of a "combined electrode" (CE) and three glass-coated regular tungsten electrodes (TEs). The CE, 12 cm long, is composed of a tungsten rod inside the central channel of a seven-barrel micropipette. The CE's tip was ground to expose 10-30 µm of the tungsten rod, the tip diameter of each of six glass barrels was 1-3 µm. The CE and three TEs were arranged within a 6 cm long stainless steel guide and were microdriven independently into the auditory cortex of anaesthetized guinea pigs. The distance between the tips of the CE and TEs varied from 350 to 900 µm. Up to 12 single-units could be isolated using four spike sorters. Auto- and cross-correlation histograms of the simultaneously recorded neurons were calculated and displayed on-line. Administration of glutamate caused potent responses in "nearby" cells, which were recorded by the CE, and weak responses in "distant" cells which were recorded by the TEs. Of the responding cells, 89% and 11% were excited and inhibited, respectively, by glutamate. Both spontaneous neuronal activity and responses to auditory stimuli were modulated by acetylcholine (ACh). ACh potentiated and depressed the auditory responses of 25 and 34%, respectively, of the cells. The remaining 40% of the responding cells were not affected by ACh. In the presence of ACh, a few of the apparently non-responding cells responded to auditory stimuli. ACh, and also norepinephrine, modulated the firing pattern of single neurons and the pattern of interactions between simultaneously recorded neurons.

D

EFFECTS OF ANTIDEPRESSANTS ON CYCLIC AMP RESPONSES IN THE LIVING RAT

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Microdialysis was used to determine the effects of chronic electroconvulsive shock (ECS) and chronic imipramine on cyclic AMP responses to noradrenaline (NA, 100 µM) and forskolin (F, 50 µM) in cortex of living, freely-moving rats. ECS was applied via ear-clip electrodes daily for 10 days and imipramine via daily i.p. injections at 10 mg/kg for 3 weeks. Recovery of cyclic AMP was $17.9 \pm 0.6\%$ with a 2 mm dialysis probe, and $28.3 \pm 2.7\%$ with a 3 mm probe. NA resulted in a 2- to 3-fold increase in cyclic AMP levels in both sham-treated and ECS-treated rats, with no difference in either the height or duration of the response. F also resulted in a 2- to 3-fold increase in cyclic AMP levels, but the peak of the response occurred significantly later in ECS-treated animals. In rats which had received chronic imipramine, responses to both NA and F were increased compared to those in saline-treated rats. These results indicate that the effects of antidepressants on cyclic AMP responses in rat cortex may differ according to the type of preparation used, and that the desensitization of the response observed in the ex vivo slice preparation does not occur in vivo.

Supported by the Stanley Foundation.

A

***In Vivo* Estimation of the Rate of Spillover of Dopamine into Extracellular Fluid of the Brain.**

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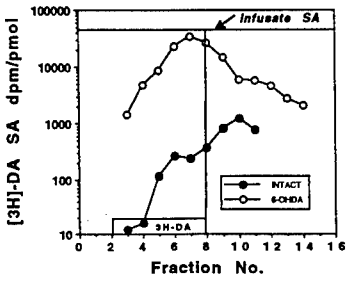
Microdialysis has proven to be an important tool for investigating changes in extracellular fluid levels of endogenous compounds *in vivo*. A simple modification of a commercially available microdialysis probe, by attaching a cannula 1 mm from the external surface of the probe, enables application of a tracer microinjection kinetic aproach to estimate the rate of entry (spillover) of transmitters into extracellular fluid in the brain *in vivo*. This study tested validity of this novel neurochemical approach.

^{3}H-dopamine (^{3}H-DA) was infused via the cannula into the striatum of anaesthetized rats and increments in microdialysate levels and specific activityies (SA's) of ^{3}H-DA were analyzed in 30 min intervals. The steady-state SA of ^{3}H-DA would be expected to be less than that in the perfusate, due to local dilution of the tracer by endogenous dopamine. This dilution would reflect spillover of dopamine into the local extracellular fluid. In intact animals, ^{3}H-DA SA was about 1% of that in the infusate (Figure).

Animals treated with 6-OHDA (8 µg/ 4 µl into medial forbrain bundle 3-4 weeks before the acute study) to destroy DA terminals, the SA of ^{3}H-DA approximated that in the infusate (Figure). The estimated spillover of DA were about 5 and 0.05 pmol/ min in intact and 6-OHDA treated rats.

Use of the microdialysis-tracer dilution technique should enable estimation of rates of release, reuptake, turnover, and metabolism of endogenous monoamines *in vivo*.



B

IN VITRO ANTIOXIDANT ACTIVITY OF NATURAL CARNOSINE- RELATED COMPOUNDS

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Previously the antioxidant activity of carnosine (b-alanyl-L-histidine) was demonstrated by a number of researchers [see 1 for review]. For further investigation this phenomenon we have used model of Fe^{2+}-induced peroxidation of human serum apo-B-lipoproteins in phosphate buffer [2]. Carnosin-related compounds (CRC) were added to the samples before addition of ferrous ions, then parameters of chemiluminescent curves were estimated.

It was found that carnosine as well as CRC at 10 mM concentration decrease intensity of lipid peroxidation: control - 100%, b-alanine - 84%, acetylcarnosine - 70%, acetylanserine - 60%, imidazole - 53%, homocarnosine - 34%, ophidine - 32%, carnosine - 22%, anserine - 6%. The last five compounds also inhibit the rate of the reaction. Difference in their efficiency may be due to different abilities of these compounds to act as reducing agents. This feature may be explained by formation of ring-chain tautomers of CRC [3]. Although mechanisms of antioxidant activity is not fully understandable CRC may be probably used in clinical practice as natural regulators of peroxidation processes in liquid tissues of the organism.

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C

THE EFFECT OF FREE RADICALS ON SYNAPTOSOMAL IONIC HOMEOSTASIS

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Formerly it has been shown that hydroperoxides are able to depolarize synaptosomal plasma membrane. The depolarization was slow and moderate, and was not associated with a significant release of glutamate and acetylcholine neurotransmitters.

In the present study the effect of different peroxidizing stimuli on synaptosomal ionic homeostasis was investigated. Iron-ascorbate and organic hydroperoxides were used to induce free radical production. Following cumene hydroperoxide administration slow increase of intrasynaptosomal [Na^+] could be observed by using SBFI fluorescent indicator. Similarly, synaptosomal [Ca^{2+}] has been increased by the peroxide. The increase of $[Ca^{2+}]_i$ can be prevented by previous potassium depolarization, which in itself enhanced $[Ca^{2+}]_i$. The mechanism of this "protective" effect is discussed in terms of Ca^{2+} channels and lipid peroxidation.

D

THE INFLUENCE OF STRUCTURE-FUNCTION RELATIONSHIP ON THE REGULATION OF BRAIN MITOCHONDRIAL ENZYMES

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One of the main properties of mitochondrial enzymes is the possibility to associate with the membrane. Hexokinase(HK) is the external mitochondrial membrane associated enzyme; malate dehydrogenase(MDH) can interacted with the inner mitochondrial membrane. This partial association can reflect a change of metabolic state modulated by various external factors, e.c. by regulatory neuropeptides. Neuropeptide regulation of the catalytic properties of HK and MDH have been investigated in rat brain mitochondria. Solubilization caused the loss of half of HK activity for intact animals. The intraperitoneal injection of Delta-Sleep-Inducing Peptide(DSIP) before hypoxic stress prevented the metabolic stress changes, reducing the enzyme solubilization and increasing the association with membrane. The intraperitoneal injection of DSIP before hypoxic stress as well as that to control animals increase V_{max} and does not effect on the another kinetic constants of MDH. Such diversions of enzyme characteristics depends on MDH distribution between membrane and mitochondrial matrix by DSIP action. Our results indicate that this regulatory peptide changes catalytic behavior of the both enzymes. The regulatory action of DSIP manifests itself by stabilizing enzyme catalytic properties in the higher level which prevents them from hypoxic stress changes.

A

GAP-43 RELATES TO THE AUTONOMIC AND SENSORY NERVES IN SCIATIC NERVE AND GASTROCNEMIUS MUSCLE IN THE RAT

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The distribution of growth associated protein-like immunoreactivity (GAP-43-LI) in the sciatic nerve and gastrocnemius muscle was investigated in Sprague-Dawley rats before and after lumbar sympathectomy, using confocal laser scanning microscopy (CLSM). In sham-operated controls, GAP-43-LI accumulated rapidly on both sides of the crush (6 hour crush; proximal and distal) and was mainly distributed in medium and thin sized axons. Using double immunolabelling, GAP-43-LI colocalized with TH- and NPY-LI, markers of sympathetic nerves, or with Substance P, a marker of sensory nerves. After lumbar sympathectomy (3 days), however, TH-LI or NPY-LI positive axons were reduced more than 90%, and GAP-43-LI positive axons were also reduced but not to the same extent. In gastrocnemic muscle, most of the GAP-43-LI positive perivascular nerve terminals were also TH- or NPY-positive, and some of them were SP-positive. Some GAP-43-LI positive terminals remained after sympathectomy, but very few TH- and NPY-positive nerve fibres were found around the blood vessels. On the other hand, SP-positive fibres, in the sciatic nerve or perivascularly, did not show a clear change after sympathectomy, and most of them were colocalized with GAP-43-LI. The results suggest that GAP-43-LI in the normal adult rat was mainly present in sympathetic adrenergic and sensory nerve fibers.

B

ASTROGLIAL RESPONSE TO BRAIN INJURY AND ITS MODULATION

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The diversity in the manifestation of gliotic response and its different susceptibility to various pharmacological manipulations has been described in several conditions [see Hatten et al., Glia(4), 1991; Norton et al., Neurochem. Res.(17), 1992]. The better understanding of the related mechanisms could prove useful in elucidating the beneficial or detrimental role of gliosis in brain repair. We have recently demonstrated an increase in GFAP content in the septum and hippocampus after the interruption of septohippocampal connections, and a differential reaction to pharmacological treatment with gangliosides: the inhibition of GFAP content increase in the hippocampus possibly was related to the diversity of astrocyte type and/or specific regional neuron-glia interaction [Oderfeld-Nowak et al., J.Neurochem 61(1), 1993]. We have found the increase in GFAP content after septohippocampal pathway lesion also in other structures, far from the lesion, such as thalamus, striatum and cortex. The increase in GFAP content was accompanied by an enhancement of GFAP-IR, but the magnitude of the latter and its relation to the enhancement of the former differed in various structures. We have found that the administration of a known modulator of inflammatory process - IL-1β causes a widespread enhancement of GFAP-IR in all structures investigated, which effect, however, was not paralleled by an increase in GFAP content, with an exception of the cortex. Combined effect of the septohippocampal pathways lesion and interleukin 1-beta administration resulted in diminishing the effect of lesion itself on the GFAP content in all investigated structures. An interesting observation has been made in our preliminary experiments pointing to the possible interplay between immune and neurotransmitter systems in regulating the astrogliosis. Non-competitive NMDA receptor antagonist- MK801 given prior to IL-1β administration blocked the IL-1β -evoked increase in GFAP-IR in the septum and hippocampus.

C

THE DIFFERENT MECHANISMS OF CHROMATIN PHOSPHORYLATION IN NEURONS AND GLIA DURING MEMORY MODULATION

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The study of regulatory mechanisms of gene expression in neurons and glia has significant value for understanding of the action of neuroactive drugs on brain function. We previously reported that the memory stimulant ethylnorantifein (nootropic drug −ethimizol) rises the transcription activity in the rat neuron nuclei, but is not in the glia. Therefore, the phosphorylation of nonhiston proteins in glia and neuron chromatin has been studied with the application of pharmacological tools from antifeine group. Nowadays, there are practically no experimental data on the properties of glial nuclear protein kinases (PC) and their protein substrates. We find that the level of cAMP−independent phosphorylation of chromatin and the amount and degree of nonhistone protein HMG (in particular HMG14 and HMG17) are much higher in neuronal nuclei than in glial. Ethimizol increases the chromatin phosphorylation in neurons. The memory deteriorating agent allylnorantifein decreases this phosphorylation and chromatin transcription. Both ethyl− and allylnorantifeine does not influence on phosphorylation of HMG proteins in glial chromatin. The activity of purified cAMP−independent casein−type PC N1 and N2 in neuronal chromatin are much higher than in glial chromatin. No influence of antifeines on activity of N1 PC was shown. At the same time the antifeines selectively modulate HMG14 proteins phosphorylation by N2 PC of neuronal and glial chromatins. Thus, the absence of antifeine effects on HMG phosphorylation of glial chromosomal proteins is not due to the specificities of protein kinases isolated from the two cell types. In general the data suggest that decreased amount of HMG proteins and low PC activity combined with dense chromatin compactness seem to be unfavorable for realization of these drug effects in glial cells. Control of gene expression in different brain cells and mnestic effects of neurotropic drugs is discussed.

D

ISOENZYME PATTERN OF PHOSPHOFRUKTOKINASE IN NEURONAL AND GLIAL CELLS AND CELL CULTURES FROM RAT BRAIN

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6-phosphofrukto-1-kinase (PFK) is a key enzyme in cerebral glucose metabolism. In the brain the enzyme exists as a complex mixture of all three isoenzyme types: muscle (M)-, liver (L)- and brain (C)-type which form heterotetramers. So far it is unknown whether specific brain cell types preferentially express one or more PFK isoenzymes. Since the different PFK isoenzymes have different allosteric properties their distribution and cellular localization with respect to different cell types in the brain might be critical for the regulation of cerebral glycolysis.

In this study poly- as well as monoclonal antibodies against the purified PFK isoenzymes were produced and the isoenzyme pattern in dissociated brain cell fractions, primary cell cultures as well as human gliomas were evaluated after separation by PAGE using Western blotting. In addition, total PFK activity in the different cell types was followed up. The cellular localization of the PFK isoenzymes *in situ* was studied by immunohistochemistry using biotinylated the corresponding secondary antibodies and ABC-complexes with Nickel-enhanced diaminobenzidine for visualization.

The results demonstrate that neuronal as well as glial cells express all three types of PFK isoenzymes, but with different intensity. As shown by the immunohistochemical data the distribution of PFK is also different between different types of neurons.

A

THE MOUSE BRAIN RAS ACTIVATOR CDC25Mm

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Ras genes participate in normal cell growth control and are also involved in terminal differentiation of several cell types. In brain ras genes, in particular H-ras, are higly expressed, but their function is still unknown. A critical step in the activation of ras proteins is the release of bound GDP, catalyzed by Guanine nucleotide Releasing Factors (GRFs), that leads to the active GTP-bound form. Using a cDNA mouse brain library we have cloned the catalytic domain of the mouse GRF CDC25Mm by complementation of the yeast cdc25-1 mutant of *S. cerevisiae* (1). The corresponding protein specifically increases up to 1000 fold in an "in vitro" system the GDP release from c-H-ras (2). Four full-lenght cDNAs, derived from the same gene, coding for protein of different sizes and having in common the last 661 aminoacids comprising the catalytic domain, have then been isolated from mouse brain (3). Using two polyclonal antibodies directed toward different regions in the C-terminal 472 aminoacids we have identified in mouse brain two proteins of 140 and 58 kDa: while the former is expressed in the adult mouse, the latter is present in the embryo and persists for few days after birth. In other tissues we have been unable to identify CDC25Mm products, in accordance with the fact that Northern blot analyses have revealed only in brain a messenger RNA corresponding to CDC25Mm. These findings indicate a specific expression in the brain of the CDC25Mm gene and suggest that differential expression of various forms of CDC25Mm may be involved in brain developement and maintainance or function. Experiments are in course to determine by immunohistochemistry and tissue fractionation the localization of the CDC25Mm product(s) in various brain areas and at a subcellular level.

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