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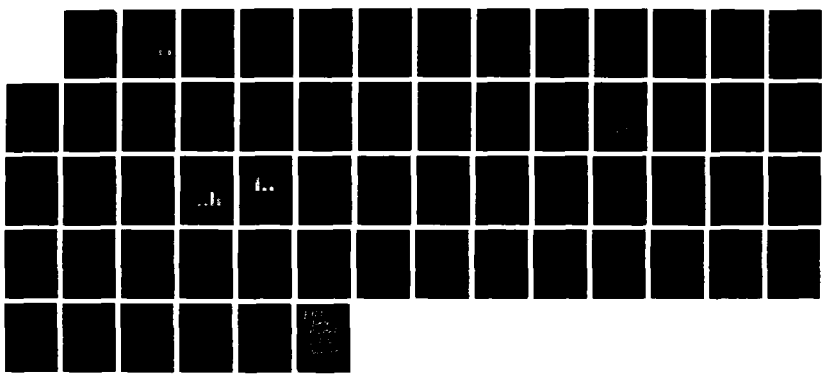

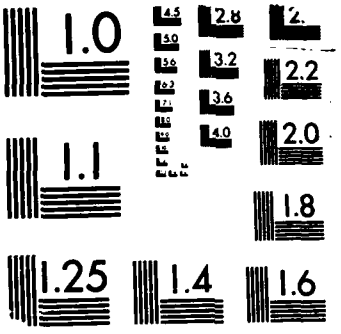

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NEUROLOGICAL CONSEQUENCES OF ACUTE AND CHRONIC CHOLINERGIC BLOCKADE

ANNUAL REPORT

EDWARD F. DOMINO

JUNE 2, 1986

Supported by

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21701-5012

Contract No. DAMD17-84-C-4157

University of Michigan
Ann Arbor, Michigan 48109-0010

A193 339

SECURITY CLASSIFICATION OF THIS PAGE

REPORT DOCUMENTATION PAGE

Form Approved OMB No. 0704-0188

1a. REPORT SECURITY CLASSIFICATION Unclassified	1b. RESTRICTIVE MARKINGS
2a. SECURITY CLASSIFICATION AUTHORITY	3. DISTRIBUTION/AVAILABILITY OF REPORT Approved for public release; distribution unlimited
2b. DECLASSIFICATION/DOWNGRADING SCHEDULE	
4. PERFORMING ORGANIZATION REPORT NUMBER(S)	5. MONITORING ORGANIZATION REPORT NUMBER(S)
6a. NAME OF PERFORMING ORGANIZATION University of Michigan 6b. OFFICE SYMBOL (If applicable)	7a. NAME OF MONITORING ORGANIZATION
6c. ADDRESS (City, State, and ZIP Code) Ann Arbor, Michigan 48109-0010	7b. ADDRESS (City, State, and ZIP Code)
8a. NAME OF FUNDING/SPONSORING ORGANIZATION US Army Medical Research & Development Command 8b. OFFICE SYMBOL (If applicable)	9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER DAMD17-84-C-4157
8c. ADDRESS (City, State, and ZIP Code) Fort Detrick Frederick, Maryland 21701-5012	10. SOURCE OF FUNDING NUMBERS PROGRAM ELEMENT NO. 62734A; PROJECT NO. 3M1 62734A875; TASK NO. AI; WORK UNIT ACCESSION NO. 440

11. TITLE (Include Security Classification)
Neurological Consequences of Acute and Chronic Cholinergic Blockade

12. PERSONAL AUTHOR(S)
Edward F. Domino

13a. TYPE OF REPORT	13b. TIME COVERED	14. DATE OF REPORT (Year, Month, Day)	15. PAGE COUNT
Annual	FROM 6/1/85 TO 5/31/86	1986 June 2	

16. SUPPLEMENTARY NOTATION

17. COSATI CODES		
FIELD	GROUP	SUB-GROUP
06	15	
06	16	

18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)
Aprophen, atropine, dexetimide, d-hyoscyamine, 3H-acetylcholine release, hippocampal slice, l-hyoscyamine, benactyzine, mecamylamine, methylatropine,, nicotine

19. ABSTRACT (Continue on reverse if necessary and identify by block number)
Various selective and nonselective M1 and M2 cholinergic antagonists have been studied for their presynaptic effects on 3H-acetylcholine release from the rat brain hippocampal slice preparation. Marked differences in potency and efficacy were observed that have direct implications with regard to the treatment of OP poisoning.
Organophosphate

20. DISTRIBUTION/AVAILABILITY OF ABSTRACT	21. ABSTRACT SECURITY CLASSIFICATION
☐ UNCLASSIFIED/UNLIMITED ☒ SAME AS RPT. ☐ DTIC USERS	Unclassified
22a. NAME OF RESPONSIBLE INDIVIDUAL Mary Frances Bostian	22b. TELEPHONE (Include Area Code) 301/663-7325; 22c. OFFICE SYMBOL SGRD-RMI-S

DD Form 1473, JUN 86 Previous editions are obsolete. SECURITY CLASSIFICATION OF THIS PAGE

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FOREWORD

In conducting the research described in this report, the investigator adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 86-23, Revised 1985).

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I. Introduction

A. Why study the effects of M1 and M2 cholinergic antagonists on presynaptic cholinergic receptors that modulate ACh release?

Since the early research of Langley (1901), the concept of neurohumoral transmission, both in the peripheral as well as central nervous system, has been well established. Rapid advancements in the neurosciences, including the development and refinement of methodological techniques, have resulted in a number of putative neurotransmitters being identified. Various pharmacological tools are available to intervene in their synthesis, release, their effects on postsynaptic membrane receptors, their degradation, etc. This has advanced immensely our understanding of the way communication in the nervous system occurs and its importance in various physio/pathological processes. Innumerable studies in recent years have been devoted to the mechanisms of release of neurotransmitters. Studies using various techniques have considerably enhanced our knowledge. The mechanisms of acetylcholine (ACh) release have received great attention since the early part of this century (see Magnus, 1930). The intestine of various animals was studied extensively in various species and the origin of ACh was the subject of considerable debate among researchers. In the classical work of Paton and his colleagues (1965, 1968, 1971) experiments with innervated longitudinal muscle strip, prepared from guinea pig intestine proved the neurogenic origin of ACh. This work also laid the foundation for understanding the factors affecting the release of ACh. Since the enteric nervous system resembles (1) morphologically, (2) ultrastructurally, and (3) pharmacologically the central nervous system, it is still used by many investigators and has been compared to brain slice preparations (e.g., hippocampus or cerebral cortex, see Kilbinger, 1982 for extensive review).

Of great interest is the description of a "negative feedback mechanism" for the control of ACh release that is triggered by activation of presynaptic muscarinic receptors. Stimulation of these receptors by ACh or other muscarinic cholinergic agonists leads to the inhibition of further ACh release. This inhibition is overcome by muscarinic antagonists like atropine (Szerb, 1979). This is an important mechanism of regulation of ACh release. Fig. 1 illustrates in diagrammatic form a summary of the major pre- and postsynaptic receptors and messengers involved in cholinergic transmission.

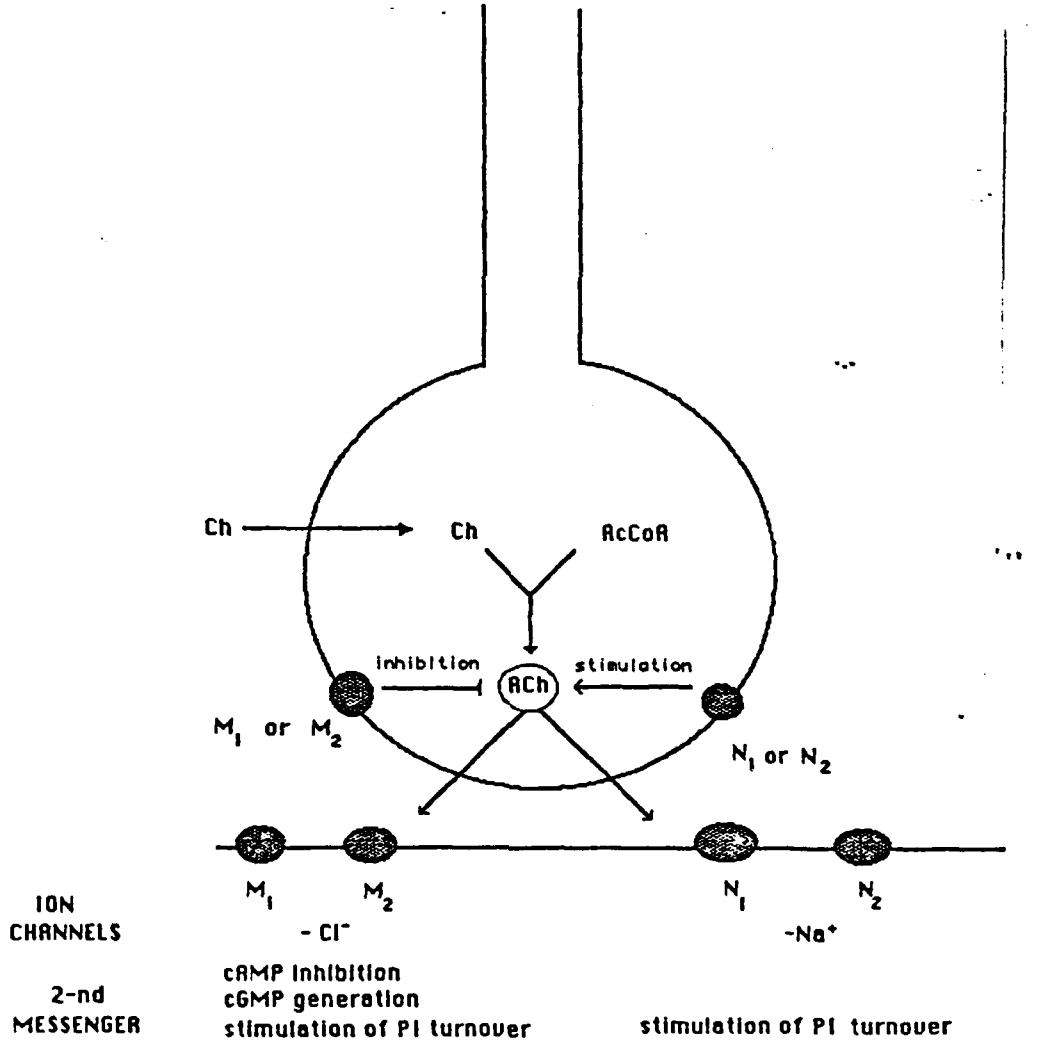

Fig. 1. Schematic diagram of multiple pre- and postsynaptic M and N receptors, ion channels, and messengers involved in cholinergic transmission.

This basic knowledge of cholinergic transmission has direct relevance to the problem of poisoning by organophosphate (OP) agents and subsequent treatment. Presently atropine is the drug of choice for treating OP poisoning because of its effective postsynaptic blockade of M1 and M2 (muscarinic) cholinergic receptors. Fig. 2 shows the effects of atropine on increasing ACh release from cholinergic nerve endings when atropine is used as an antidote to nerve agent poisoning. Benzodiazepines like diazepam (DZ) and midazolam (MZ) would enhance the effects of GABA and therefore tend to reduce ACh release in a beneficial manner.

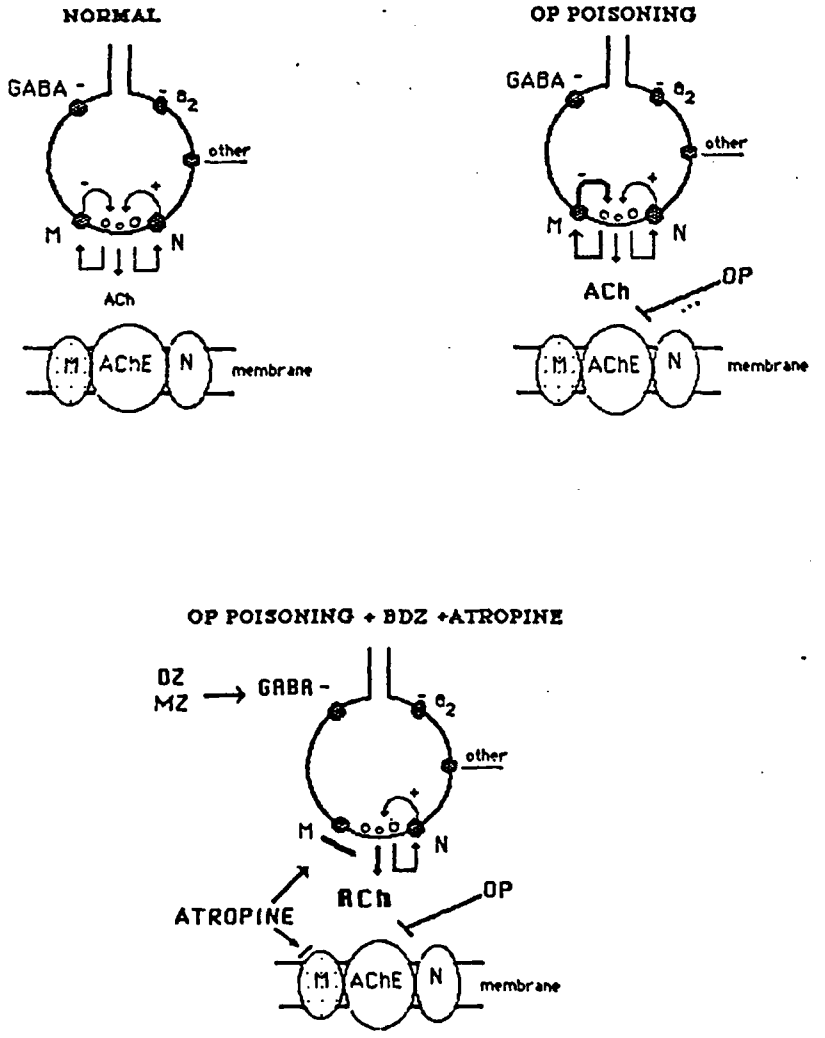

Fig. 2. Schematic diagram of the consequences of atropine on ACh release from cholinergic nerve endings, when atropine is used as an antidote to nerve agent poisoning.

For purposes of brevity and clarity, all sources of acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) are not illustrated. Under normal circumstances there is a very precise control of ACh release that is modulated by presynaptic M and N receptors. The M receptors exert a negative feedback on ACh release, while N receptors (at some sites only) exert a positive feedback on ACh release. In the presence of OP agents that inhibit AChE and BChE, a large excess of free ACh causes a predominant feedback inhibition of more ACh release through excitation of presynaptic M receptors. Since M receptors are more numerous than N receptors, no more ACh is released. However, in the presence of atropine as an antidote, this M mediated presynaptic inhibition of ACh release is blocked, causing further free ACh release to affect postsynaptic sites. Even though atropine is a competitive M1 and M2 antagonist, apparently enough atropine can be given so that postsynaptic M1 and M2 antagonism predominates in the presence of enhanced free levels of ACh in the biophase of M receptors. On the other hand, the N cholinergic receptors and other non-atropine sensitive receptor sites can still react to the enhanced levels of free ACh. Since OP agents also have direct membrane effects, it is obvious that a combination of antidotes is needed to protect against OP agents. Surprisingly, N-antagonists alone are not very effective OP antidotes. Instead it is becoming very clear that benzodiazepines such as diazepam and midazolam, in combination with atropine, do protect against paraoxon-induced OP intoxication (see Krutak-Krol and Domino, 1985).

The working hypothesis of the present research is that while atropine is clearly a suitable classical OP antidote, a more effective antidote would be a muscarinic cholinergic antagonist that would only act postsynaptically and not alter presynaptic ACh release.

The use of brain slices incubated with ^{3}H-choline offers a simple assay method for testing for the presynaptic activity of atropine like drugs. In the past 1½ years we have set up the rat hippocampal slice preparation for measuring ^{3}H-ACh release. Our accomplishments to date are described in the present annual report.

B. Why study the rat hippocampal slice preparation in vitro?

The rat in vitro hippocampal slice preparation was used for several reasons. These include: 1) It is a sensitive site of action of OP agents in general and nerve agents in particular. 2) The input-intrinsic-output relations and pharmacology of the hippocampus are becoming well known (see review by Dingledine, 1984). 3) The major cholinergic input into the hippocampus via the medial septum is cholinergic. 4) The slices can be cut so that most of the intrinsic hippocampal connections will remain intact. 5) Many similar slices can be obtained from the brain of one animal.

C. Functional neurotransmitter circuitry of the hippocampus

1. Neural circuitry of the hippocampus

The major contributions to our knowledge of the anatomy of the hippocampus were made in 1901 by Ramon y Cajal (see 1955) and later by Lorente de No (1934). The hippocampal formation represents a very regular laminated structure so that a description of its transverse section can be representative of the whole structure as shown in Fig. 3. The gross localization of some of the major neurotransmitters is illustrated in Fig. 4.

2. Perforant pathway and granular cells

Fibers originating in the ipsilateral entorhinal cortex enter the hippocampal formation and form the perforant pathway. A small portion of these fibers originate from the contralateral entorhinal region (Goldowitz et al., 1975). These fibers terminate on the granular cells. This input is organized in parallel lines as shown in Fig. 3. The number of fibers and

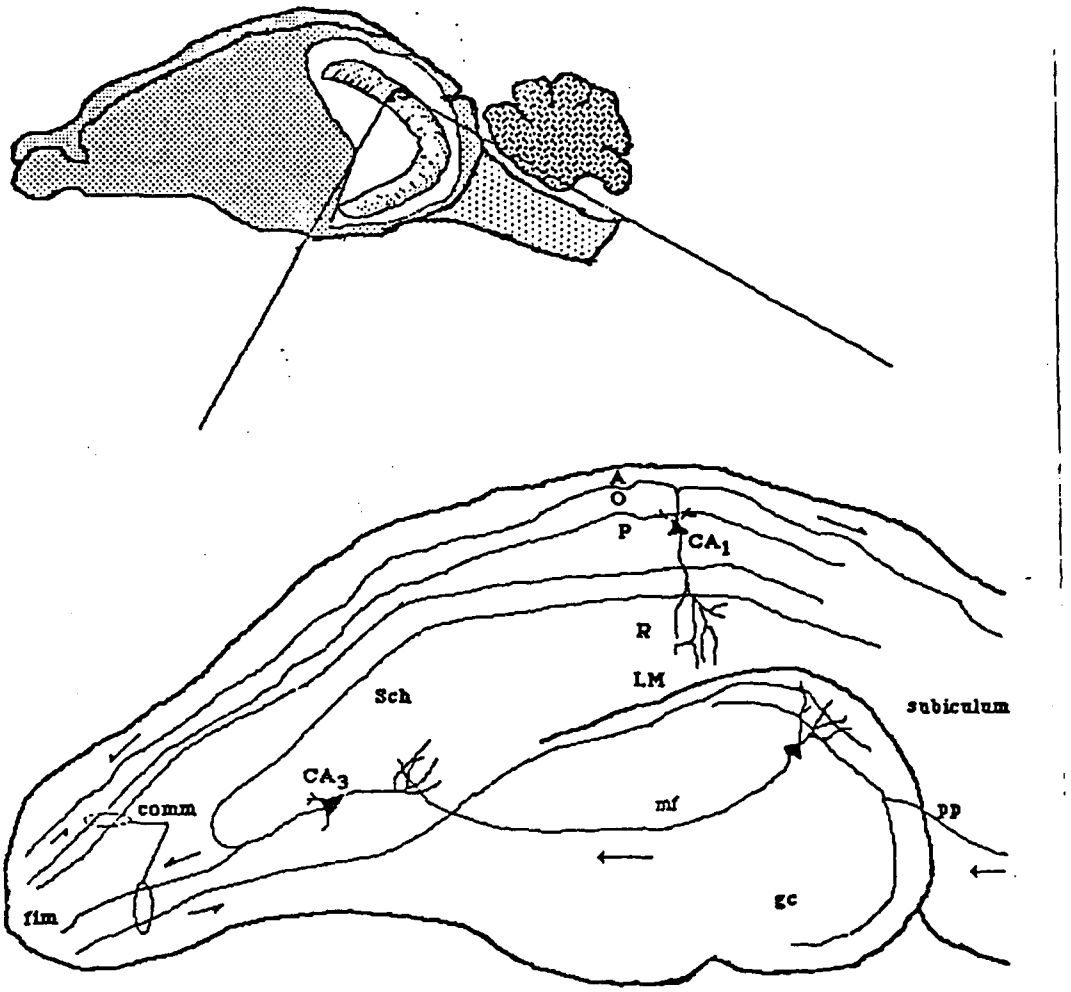

Fig. 3. Overview of the rat brain and anatomy of the hippocampal slice. The symbols are: A-alveus, O-stratum oriens, P-stratum pyramidale, R-stratum reticulare, LM-stratum lacunosomoleculare, CA_1-CA_1 region, Sch-Schaffer collaterals, CA_3-CA_3 region, comm-commissural fibers, fim-fimbria, mf-mossy fibers, gc-granular cells, pp-perforant path, subiculum-subiculum

granular cells per unit volume of tissue is constant (Lomo, 1971). The amino acid glutamate serves as an excitatory neurotransmitter in the perforant path fiber-granular cell synapse (Nadler et al., 1976).

3. Mossy fiber synapses in the CA_3 region

Axons of granular cells, known as mossy fibers form synapses with apical dendrites of CA_3 pyramidal neurons. These synapses are of the "en

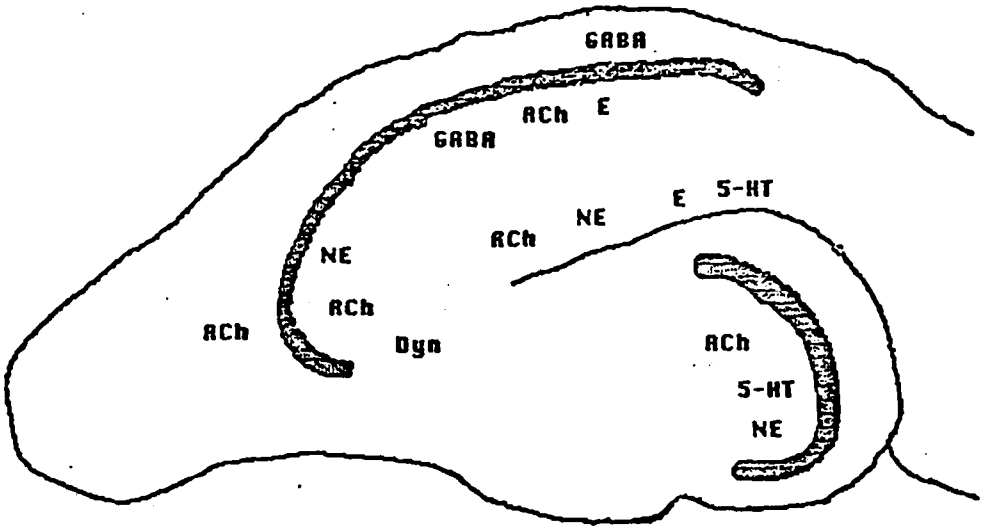

Fig. 4. Gross localization of some of the major neurotransmitters in the rat hippocampus.

The symbols are: ACh-acetylcholine, Dyn-dynorphin, E-enkephalins, NE-norepinephrine, GABA-γ-aminobutyric acid, 5-HT-serotonin

passage" type. The neurotransmitter utilized by these granular cells is unknown, but its effects are excitatory (White et al., 1979).

4. Commissural fibers and Shaffer collaterals

The commissural pathway arises from the CA_3 region in the contralateral hippocampus. It enters the hippocampus through the fimbria and provides afferents to apical and basal dendrites of pyramidal cells in the CA_3-CA_1 region and to the gyrus dendatus (Lynch and Cotman, 1975; Laurberg, 1979). The commissural fiber system may be employed in indirect inhibition. These fibers also connect to small, bipolar vasoactive intestinal peptide (VIP) immunoreactive interneurons (Leranth and Frotscher, 1983) as well as basket cells (Frotscher and Zimmer, 1983). Shaffer collaterals arise from pyramidal cells in the CA_3 region and terminate in the CA_1 region on basal dendrites of pyramidal neurons. The commissural pathway and Shaffer

collateral utilize glutaminic acid as their excitatory neurotransmitter (Wieraszko and Lynch, 1979; Wieraszko, 1981).

5. Intrinsic inhibition in the hippocampus

Axons of pyramidal and granular cells have recurrent collaterals which activate basket cells as described by Lorente de No (1934). Basket cells participate in classical recurrent inhibition (Anderson et al., 1964). Axons of basket cells in turn ramify among pyramidal cell bodies and dendrites, and in addition show lateral spread (Struble et al., 1978). As a consequence of the preparation of our hippocampal slices, some of the recurrent inhibition is lost because the recurrent collaterals are partially cut.

In addition to recurrent inhibition, there is also feed forward inhibition in the hippocampus (Alger and Nicoll, 1982a). The anatomical basis for this type of interaction has been shown recently and is consistent with earlier physiological findings. Frotscher and Zimmer (1983) described nonpyramidal neurons in striatum pyramidale of the CA_1 region. Their dendrites extended to stratum oriens and stratum lacunoso moleculare. Recently, Somogyi et al. (1985) described GABA immunoreactive axo-axonic cells in the CA_1 region. Their dendrite distribution corresponds to the distribution of pyramidal cell dendrites, suggesting the same input source. Additional anatomical evidence for feed forward inhibition is based on binding studies which reveal that a GABA antagonist, muscimol, binds to stratum oriens and stratum radiale areas (Chan-Palay, 1978). The transmitter utilized by basket cells is GABA (Curtis et al., 1971; Storm-Mathisen and Fonnun, 1972; Storm-Mathiesen, 1977). GABA has two independent actions on pyramidal cells. It hyperpolarizes their soma, and has biphasic depolarization/hyperpolarization actions when applied to the dendrites (Andersen et al., 1980; Thalmann et al., 1981; Alger and Nicoll, 1979; 1982b;

Blaxter and Carlen, 1985). The hyperpolarizing effect of GABA is thought to be due to an increase in chloride conductance. Reduction of the extracellular chloride concentration causes a shift of the reversal potential in a depolarizing direction (Andersen et al., 1980; Thalmann et al., 1981). The biphasic initial depolarization and the subsequent hyperpolarization are mediated by increased chloride and calcium activated potassium conductance, respectively. The phase of hyperpolarization of the biphasic response can be blocked by intracellular injection of EGTA or by washing the slice with a calcium free solution (Blaxter et al., 1984; Blaxter and Carlen, 1985). GABA analogues, such as 4,5,6,7-tetrahydroisoxazolo[5,5-C]pyridine 3-ol (THIP) and baclofen elicit hyperpolarization when applied to the dendritic layer (Alger and Nicoll, 1982b; Thalmann and Hershkowitz, 1985). Alger and Nicoll (1982b) have provided evidence for two kinds of GABA receptors on pyramidal cells in the hippocampus. They demonstrated that several GABA antagonists block primarily the depolarizing effect of GABA on dendrites. In the presence of a barbiturate in the medium, GABA and THIP applied to the dendrites had only depolarizing effects. Under these conditions, the hyperpolarizing response of the soma was unaffected. Diazepam, on the other hand, enhanced somatic hyperpolarization without changing the depolarizing response of the dendrites. Repetitive or prolonged exposure to GABA decreased both the hyperpolarizing and depolarizing response of these target cells to this neurotransmitter (Krnjevic and Phillis, 1963; Ben-Ari et al., 1979; Thalmann and Hershkowitz, 1985). This attenuation, often described as "fading" was thought to be due to GABA uptake by both glia and neurons (Baxter, 1976) and/or due to intracellular chlorine ion accumulation (Gold and Martin, 1982). Recently, Thalmann and Hershkowitz (1985) showed that this attenuation process is independent of GABA reuptake. These investigators used THIP and muscimol to

show that "fading" due to these agonists still occured. Both are known to be very poorly taken up by the GABA uptake system. The effect of THIP on pyramidal neurons did not reveal a marked and consistent decrement. The responses to THIP and muscimol were, however, attenuated after a long pulse of GABA. This latter observation makes it unlikely that attenuation depends on a GABA uptake process. The same investigators did not observe the change of THIP reversal potential after a long GABA pulse. The remaining possible mechanism of attenuation is a receptor desensitization process, similar to the desensitization of N cholinergic receptors.

6. Modulatory innervation of the hippocampus

The hippocampus receives many afferents which are denoted by the name "extrinsic modulatory pathways." In addition to the above described afferents from the entorhinal cortex and from the contralateral hippocampus, there are afferents arising from: a) septum, b) thalamus, c) hypothalamus, d) interpeduncular nucleus, e) medial raphe nuclei (nucleus raphe dorsalis, nucleus centralis), f) locus coeruleus, and g) dorsal tegmental nucleus. The last four originate in the mesencephalon and brainstem (Wyss et al., 1979; Riley and Moore, 1981). For clarity, we will discuss the influence of different neurotransmitters on pyramidal cells, specifying briefly the origin of fibers containing the given neurotransmitter substance.

7. Role of ACh

The cholinergic input to the hippocampal formation arises from nucleus septalis medialis and the vertical and horizontal limbs of the nucleus of the diagonal band. The cholinergic fibers enter the hippocampus through the fibria. The nerve endings are found in stratum orients, hilus fascia dendate, and stratum pyramidale (Lynch et al., 1978; Kimura et al., 1981; Woolf et al., 1984). Stimulation of the medial septum causes a marked increase of the

population spike in stratum pyramidale. Tetanic stimulation causes facilitation of the population spike followed by depression. The latter is followed by long lasting facilitation. These effects are blocked by atropine and scopolamine (Krnjevic and Ropert, 1982). The direct postsynaptic effects of acetylcholine are excitatory. It has a long time course comparable to that described in autonomic ganglia and is accompanied by a slow rise of input resistance (Dodd et al., 1981). Cole and Nicoll (1984) were able to demonstrate that repetitive stimulation of cholinergic fibers (stratum oriens) in vitro produced a fast e.p.s.p. followed by i.p.s.p. and then followed by slow e.p.s.p. The recordings were made in CA_1 region. Tetrodotoxin blocked the slow e.p.s.p. suggesting that the slow e.p.s.p. was dependent on propagated nerve action potentials. The slow e.p.s.p. was enhanced by physostigmine and blocked by atropine. Physostigmine also reduced the after hyperpolarization and this effect was also blocked by atropine. These investigators also studied the relationship between the stimulation of different layers of the hippocampal slice with the presence or absence of the above phenomena. Their finding confirmed earlier anatomical studies on the location of cholinergic nerve endings. The long lasting effect of ACh tends to facilitate the response of pyramidal cells to excitatory afferent impulses. A second action of ACh in the hippocampus is inhibitory. Valentino and Dingledine (1981) reported that the iontophoretic application of ACh to the pyramidal cell layer caused excitation (enhancement of evoked e.p.s.p. and attenuation of recurrent i.p.s.p.). However, the iontophoretic application of ACh to the dendritic zone resulted in a reduction of both the e.p.s.p. and recurrent i.p.s.p. The effect exerted on dendrites was blocked by atropine. The presynaptic inhibition involved both inhibitory and excitatory afferents. It was postulated by Valentino and Dingledine (1981) that ACh may decrease the

stimulus when applied to stratum radiale. They determined that this action is mediated through M receptors and occurs via the lateral septum. An interesting observation made by Finklestein et al. (1985) is that [^{3}H]-Ch uptake, [^{3}H]-ACh release, and [^{3}H]quinuclidinbenzilate (QNB) binding can be modified by stress. Choline uptake increased after 10 min of restraining a rat in the prone position. Increasing the duration of stress reduced choline uptake. Acetylcholine release and QNB binding increased proportionally to the duration of restraint induced stress.

8. Role of NE

Noradrenergic afferents to the hippocampus arise from the locus coeruleus and pass through the forebrain via the fasciculus medialis (Lindvall and Bjorklund, 1974). Noradrenergic nerve endings are found to be most dense in the hilus of the dentate gyrus, stratum laculosum-moleculare CA_1, and stratum radiatum CA_3. It has been demonstrated that both α and β adrenergic receptors are present in the hippocampus and that the distribution of norepinephrine correlates better with the density of α adrenergic receptors (Young and Kuhar, 1980). The selective β agonist, 2-fluoronorepinephrine (2 FNE) increases the population spike amplitude in vitro in hippocampal slices pretreated with the MAO inhibitor pargyline (Muller et al., 1982). This effect was blocked by the β-antagonist timolol, while the α-antagonist phentolamine did not have any effect. The α agonist, 6 fluoronorepinephrine (6FNE) produced a marked decrease in the population spike amplitude; this effect was blocked by phentolamine. The action of the indirect acting agonist tyramine was markedly potentiated by phentolamine. Metabolic studies showed that NE increased intramitochondrial fluorescence of NADH (Segal et al., 1980). The effect was blocked by propranolol and ouabain, and mimicked by cAMP. Surprisingly, the effect was still present after the pyramidal cells were destroyed by kainic acid. This suggests that the effect of NE on such cells is indirect.

Destruction of noradrenergic afferents to the hippocampus can occur with the selective action of N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine (DSP_4) which results in greater than 70% depletion of norepinephrine content. Such depletion of NE, however, does not change the population spike amplitude (Dunwiddie et al., 1983). Interestingly, it also does not change the number of β-adrenergic binding sites nor the response to the beta agonist isoproterenol (Dunwiddie et al., 1983; De Montigny et al., 1980). The inhibitory response of pyramidal cells to clonidine is also unchanged.

9. Role of 5-HT

Serotonergic afferents to the hippocampus arise from nucleus centralis superior and nucleus raphe dorsalis. Radiographic studies indicate that the serotonergic innervation is more abundant in stratum radiatum than in stratum pyramidole (Azmitia and Segal, 1978; Riley and Moore, 1981). Serotonin (5-HT) has two distinct actions. When applied to the CA_1 region it causes hyperpolarization and a decrease in resistance of about 30% (Segal, 1980). This action is blocked by methysergide. The action of 5-HT appears to be a direct effect since it is resistant to blockade by a medium containing low Ca^{+2} and high Mg^{+2}. When applied iontophoretically to the dentate region, serotonin causes depolarization with a decrease of input resistance similar to that obtained by applying GABA to the dendritic zone. Destruction of the serotonergic innervation by intraventricular injection of 5,7-dihydroxytryptamine does not affect the responsiveness of CA_3 neurons to iontophoretically applied serotonin (De Montigny et al., 1980). This suggests a lack of up regulation of serotonergic receptors after destruction of serotonergic afferents to the hippocampus.

10. Role of opioid peptides

There appears to be a general consensus that, with three exceptions,

the effect of opiates is to reduce spontaneous and evoked neuronal discharge (see Rosier and Bloom, 1982). The three exceptions are with hippocampal pyramidal neurons (Nicoll et al., 1977; Zieglgansberger et al., 1977), Renshaw cells in the spinal cord (Davies and Dray, 1976, 1978), and lateral reticular nucleus (Satoh et al., 1979).

Present data suggest that there may be three sources of opioid peptides in the rat hippocampus, a) granule cells-CA_3 mossy fibers system containing dynorphin, b) projection from lateral entorhinal cortex containing enkephalin, and c) intrinsic neurons (Gall et al., 1981). The first system is localized in the hilus area of the dendate. Axons of these cells terminate in stratum lucidum in the CA_3 region. The authors suggest that at least part of the granule cell and mossy fiber projection is "enkephalinergic." The second system arises from the lateral retrohippocampal cortex. The terminals are found in the outer molecular layer of gyrus dentatus and in stratum lacuhoso-moleculare in the CA_1 region. Additionally, immunoreactive cell bodies are found in stratum pyramidale of the CA_1 region, in stratum radiatum and stratum orients of the CA_1 region. These cells are described as interneurons. Stengard-Pedersen et al. (1983) offered evidence that besides opioid peptides, the mossy fibers contain cholocystokinine. McGinty et al. (1983) showed that the opioid staining of mossy fibers is probably exclusively due to the kappa agonist, dynorphin. Opioid peptides enhance the response of pyramidal neurons to excitatory synaptic activation. The site of action of opioid peptides is in stratum pyramidale, although they do not depolarize pyramidal cells, increase resistance, or lower the threshold of excitation (Nicoll et al., 1980; Dingledine, 1981). Dunwiddie et al. (1980) and Lee et al. (1980) demonstrated that the primary effect of iontophoretically applied opiates on pyrammidal cells is augmentation of their electrical activity due

to disinhibition. When D-Ala^{2}methionine- enkephalinoamide was applied iontophoretically to an identified putative inhibitory interneuron its firing rate decreased. This effect was antagonized by naloxone. Nicoll et al. (1980) used intracellular recordings to determine that feed forward inhibitory potentials to orthodromic stimulation are more attenuated than the responses to antidromic stimulation. The antagonism between GABA and opioids does not occur at the gabergic receptor level since the effects of exogenous GABA was not antagonized by D-Ala^{2}methionine-enkephalinamide. Dingledine (1981) reported that enkephalin exerts its action mainly on the feed forward inhibitory pathway. Mapping experiments suggest that the primary site of enkephalin action can be localized on the soma and axon of gabergic cells in the CA_1 region, which is most likely the cell described by Somogyi et al. (1985).

11. Role of other neurotransmitters and autocoids.

Immunoreactive staining methods applied to the hippocampal formation have revealed the presence of a number of neuroactive peptides. Vasoactive intestinal polypeptide (VIP) and cholecystokinin (CCK) staining have the same distribution, i.e., in stratum oriens and stratum radiatum of the CA_1 region, and near the dentate hilus (Greenwood et al., 1981; Leranth and Frotscher, 1983; Loren et al., 1983). However, CCK is much more abundant (Iverson, 1983). Both peptides have excitatory properties when applied iontophoretically to pyramidal cells. The octopeptide fragment, CCK-8, the principal form present in the brain, elicits potent excitatory responses as rapidly as L-glutamate (Dodd and Kelly, 1981). CCK at concentrations below an effect on the population spike blocks GABA-induced suppression of the activity of pyramidal neurons (Stittsworth and Giardina, 1985). Somatostatin containing neurons are present in the hilar region and extend to the molecular

layer of gyrusdentatus. When applied by iontophoresis or pressure injection to the CA_1 and CA_2 regions, it appears to excite pyramidal cells (Bakst et al., 1985). The excitation is fast in onset, resembling that evoked by glutamate (Dodd and Kelly, 1978).

Other substances present in the hippocampus include TRH prohormone located in the hilus of the dentate (Low et al., 1985). The TRH source is extrinsic. The nerve fibers containing TRH may enter the hippocampus via the fornix. In addition, corticotropin releasing factor (CRF) has been found in the hippocampus. Vasopressin increases the excitability of pyramidal neurons. Vasopressin causes constriction of some (not all) blood vessels in the hippocampus in vitro. Oxytocin excites inhibitory interneurons causing excitation of pyramidal cells (Chappell et al., 1985; Raggenbass et al., 1985; Smock and Topple, 1985).

The interactions of various neurotransmitters and autocoids are just now being studied so our knowledge in this area is sparse.

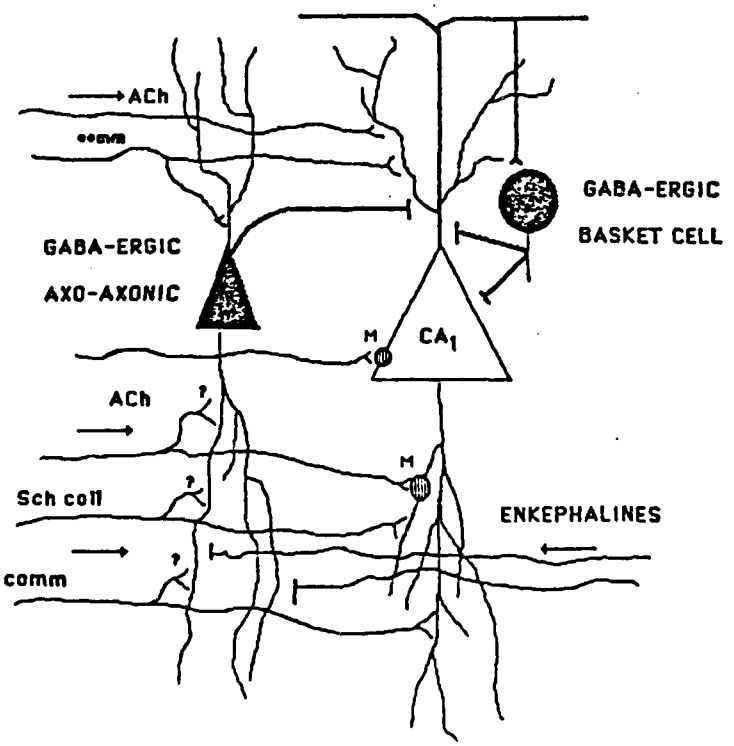

Fig. 5. Schematic diagram of the relationship between cholinergic inputs and intrinsic neurons of the rat hippocampus.

12. Summary diagram of the neurotransmitter anatomy of the hippocampus

A simplified diagram which summarizes the known neurochemical inputs, intrinsic connects and outputs of the hippocampus is illustrated in Fig. 5.

II. Materials and Methods

A. General

Tritiated choline (spec. activity 80 Ci/mmol) was purchased from New England Nuclear (Boston, MA 02118). ACS scintillation fluid and NCS tissue solubilizer were supplied by Amersham (Arlington Heights, IL 60005). Atropine sulfate, hemicholinium-3 (HC-3), carbamylcholine chloride (carbachol), scopolamine hydrochloride, l-hyoscyamine, methylatropine methylnitrate and benactyzine hydrochloride were purchased from Sigma Chemical Co. (St. Louis, MO 63178). Pirenzepine was obtained from Boehringer Ingelheim, Ridgefield, CN (06877). Mecamylamine was donated by Merck, Sharp and Dohme Research Lab (Rahway, NJ 07065). Dexetimide hydrochloride was donated by Janssen Pharmaceutics, Inc. (Piscataway, NJ 08854). d-Hyoscyamine-d-camphor sulfonate was obtained from the former Parke Davis and Co. when they were located in Detroit, MI 48207. Aprophen was the generous gift of J.F. Stubbins, Medical College of Virginia. All other chemicals were reagent grade. The preparation and perfusion buffer selected was Yamamoto solution (Wieraszko, 1982) containing 124 mM NaCl; 3.1 mM KCl; 25.6 mM $NaHCO_3$; 1.3 mM $CaCl_2$; 1.3 mM $MgSO_4$; 1.3 mM KH_2PO_4; and 10 mM glucose. Male HSD, Sprague Dawley outbred rats (150-250 g) from Harlan Sprague Dawley, Inc. (Indianpolis, IN 46229) were used for all experiments. Animals were housed in a temperature-controlled room on a 12 hr fixed light schedule (0700-1900 light and 1900-0700 dark). Food and water were available ad lib. Rats were killed by decapitation. Their brains were removed and placed in oxygenated ice cold (3-5^{o}C) Yamamoto solution. The hippocampi were dissected and transverse slices (400 μm) were

prepared using a McIlwain tissue chopper. The slices from the medial section of hippocampus were incubated for 30 min in 5 ml of artificial cerebrospinal fluid (Yamamoto solution) containing 20 µCi of ^{3}H-choline. The final concentration of choline was 1.05 µM. This low choline concentration has been shown to be optimal for high affinity uptake by cholinergic neurons (Weiler et al., 1979). After incubation the slices were washed, placed in the chambers and superfused for 40 min with a flow rate of 1 ml/min. It is important to stress that the flow rate for the individual chamber remained constant during the experiment. This constant flow rate assures that the changes in radioactivity collected in each aliquot reflect real changes in release. The perfusate solution contained HC-3 (10 µM) to prevent re-uptake of ^{3}H-Ch and was constantly equilibrated with 5% CO_2 and 95% O_2. By preventing the uptake, more accurate measures of released ACh could be made. All solutions and slices were held at 35°C. After 40 min of superfusion, the fluid was then collected at 5 min intervals for the subsequent 65 min. After 60 min and at 90 min the slices were stimulated for 2 min each with rectangular electrical pulses. At 65 min, after the first stimulation, all chambers except those designated to serve as controls, were perfused with solution containing the drug to be studied. The parameters of the stimuli were as follows: voltage = 25 V, pulse duration = 2 msec, frequency 1 Hz, current = 20-25 mA. In some experiments the frequency ranged from 0.25 Hz to 16 Hz. One ml of each collected perfusate (i.e., each 5 min interval) was removed and added to 15 ml of ACS scintillation fluid. At the termination of the experiment, the slices were dissolved in 100 µl NCS tissue solubilizer and counted in 15 ml of ACS. Total disintegrations per min (dpm) were determined using a programmed LS 9800 series liquid scintillation spectrometer (Beckman). Each evoked release was expressed as a percentage of the pre-existing pool of neurotransmitter before the stimulation. The ratio of the fraction released during the second

stimulation (S_2) over the fraction released during the first stimulation (S_1) represents a normalized value for the evoked release of ACh. The ratios from experiments with drug-treated slices were compared to the ratios of control slices. Another set of experiments was performed using a modified method. After placing the slices in the chambers and after 40 min of superfusion, fractions were collected every 5 min from 40 to 165 min usng an Ultrorac II fraction collector (LKB, Sweden). At 15 min intervals, slices were electrically stimulated for 2 min with rectangular pulses (1 Hz, 2 ms, 25 V, 25 mA). The first stimulation served as the control. The slices were perfused with the treatment drug at the lowest concentration prior to the second stimulation. The next higher concentration of the treatment drug was added to the perfusate after each succeeding stimulation period. One ml of each collected fraction was added to glass vials containing 15 ml ACS scintillation fluid. At the end of the experiment the slices were each dissolved in 100 µl of NCS tissue solubilizer and added to glass vials with scintillation fluid. Total disintegrations per min (dpms) were determined directly using a programmed LS9800 series liquid scintillation spectrometer (Beckman Instruments, Irvine, CA 92713).

B. Calculations and statistical analyses.

Each evoked ^{3}H-ACh release was calculated as a percentage of the total pre-existing radiolabeled ACh prior to that stimulation. The data were expressed as the percent difference between treated and control fractional release. Calculations were performed by computerized methods. The formula utilized for the calculations was as follows:

$$\text{Percent change from control} = \frac{\frac{Sn}{Cn} - \frac{S1}{C1}}{\frac{S1}{C1}}$$

Sn - Fraction released from a treated slice at a given stimulation period.
Cn - Corresponding fraction obtained from control slice.
S1 - Fraction released from a treated slice at the first stimulation period; no drug present
C1 - Fraction released from a control slice at the first stimulation period.

A critical calculation is the determination of the true basal release of ^{3}H-ACh prior to electrical stimulation of peak ^{3}H-ACh release. Hence, the true ^{3}H-ACh release is the difference between peak and basal release. We observed that a 5 min collection period was enough to "catch" all of the radioactivity released during one stimulation period. The baseline values for all the peaks obtained by electrical stimulation were calculated by obtaining the mean value of the baseline values before and after stimulation. This method allows one to take under consideration all of the peak radioactivity even if it was divided into two fractions. All of the calculations were performed by computerized methods and a programmable Hewlett Packard 29C calculator. The results are presented as percent change of control and in some instances as the S_2/S_1 ratio ± S.E. The latter method was used in many of the earlier Quarterly Progress Reports.

C. Tests of viability and criteria of inclusion/exclusion.

The major test of viability of the hippocampal slices was their ability to maintain ^{3}H-ACh release. In addition, before incubation the slices were inspected visually for anatomical integrity as per Teyler, 1980.

Ratios (i.e., S_2/S_1) of the two peak release values or % of total release expressed as % fractional release obtained during electrical stimulation of treated and untreated rat hippocampal slices were accepted or rejected based on the absence or occurrence of release during S_1 or S_2. If no appreciable release was observed, that slice was judged to be not stimulated and/or not viable. Those values where little to no release was evident during either one or both stimulation periods were rejected. The remaining values were utilized for the final data analysis.

D. What are the ^{3}H-products one is measuring using ^{3}H-Ch preincubation?

The major deficiency of the present research was that total ^{3}H was

measured without prior separation of ^{3}H-Ch, ^{3}H-ACh, etc. The literature on this issue indicates that 95-97% of total radioactivity is due to ^{3}H-ACh release (McGee et al., 1978; Richardson and Szerb, 1974; Weiler et al., 1979). Obviously, further research is needed to confirm these findings.

E. Pro and Con of biasing presynaptic M and N receptors with cholinergic agonists

Atropine is more potent in releasing ^{3}H-ACh from brain slices when the slice is electrically stimulated with higher frequencies (James and Cubeddu, 1984). Higher frequencies cause increasing rates of neurotransmitter release so at any given time a larger number of presynaptic receptors is occupied by ACh. Therefore, receptor blockers should be more effective at higher frequencies when the feedback system is more activated. Under these circumstances the action of atropine would be measured with a higher signal to noise ratio.

Exactly the same effect can be achieved when one uses muscarinic agonists in addition to lower frequencies of stimulation. The use of AChEI will also increase the concentration of free ACh in the synaptic cleft. This would make atropine more effective in antagonizing the inhibition of ACh release by the M autoreceptor. The present study was done under non-biased conditions and needs to be extended using various cholinergic agonists. For the purposes of screening nerve agent antidotes, one should extend the present research using OP substances like soman.

F. Equipment

In Fig. 6 is illustrated a schematic diagram of the system used for the in vitro measurement of ^{3}H-ACh release. It is important to stress that although the individual chambers may have different flow rates the flow rate for an individual chamber remains constant during the experiment.

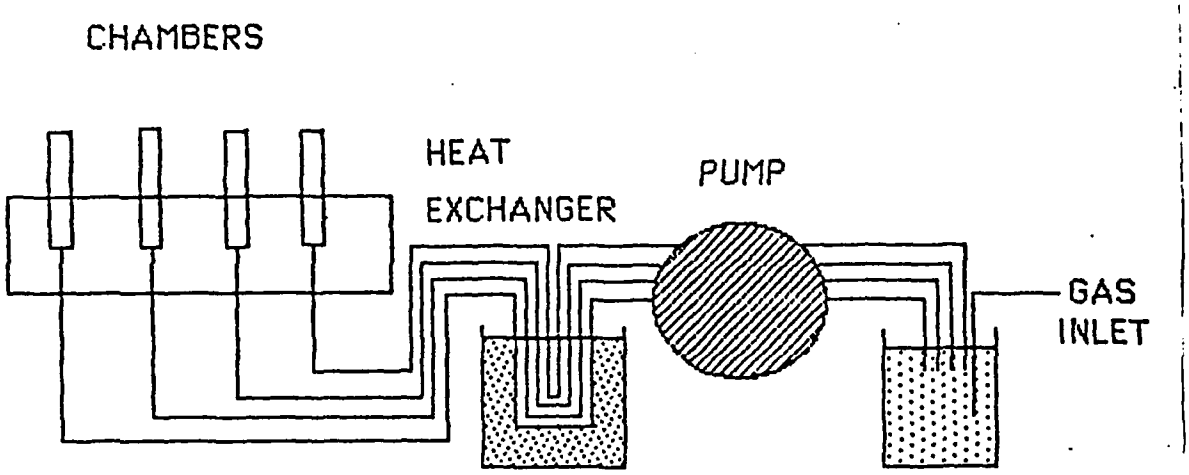

Fig. 6. System for in vitro assay of ^{3}H-ACh release from rat hippocampal slices

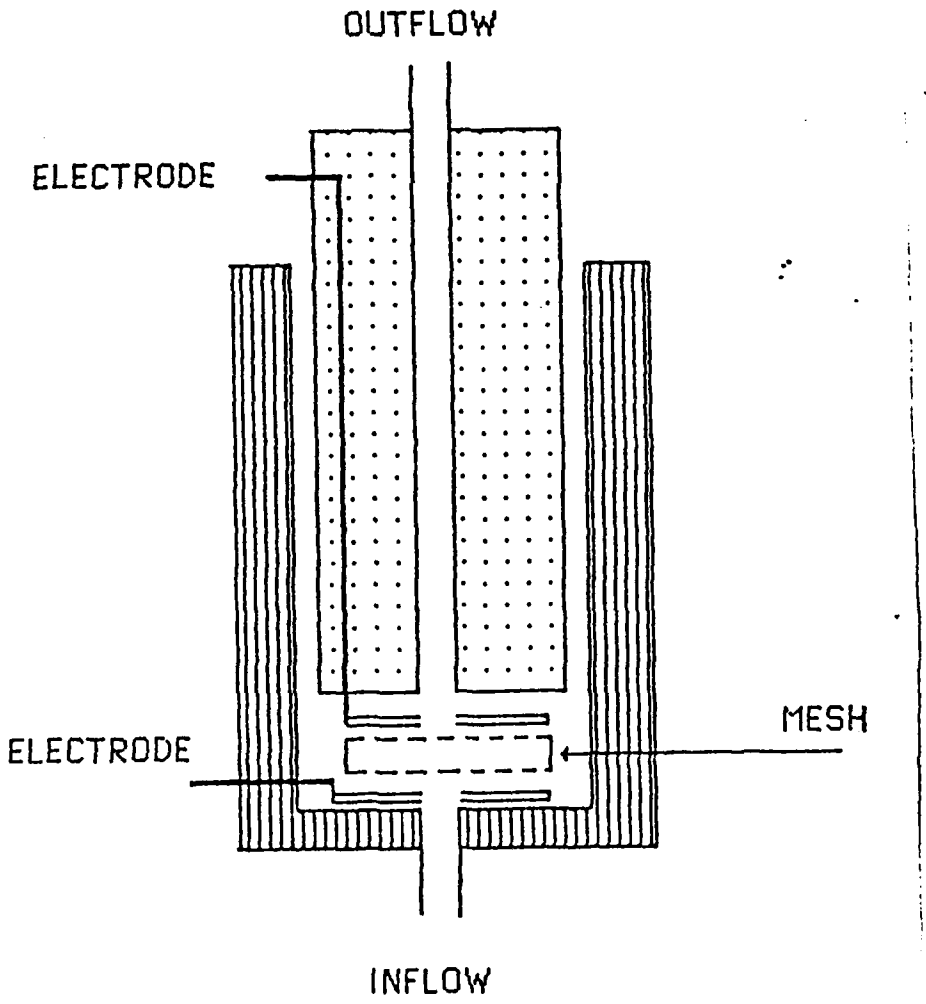

Fig. 7. Individual chamber used for the assay of ^{3}H-ACh release from rat hippocampal slices.

Fig. 7 is an expanded view of one of the individual chambers. The slice is placed between two rings of mesh and two platinum electrodes.

III. Results

A. Effects of frequency of electrical stimulation.

To establish the frequency of stimulation convenient for the required screening of muscarinic cholinergic agonists, we checked the relationship between frequency and ^{3}H-ACh release. The results are illustrated in Fig. 8. Increasing frequency of stimulation produced a correspondingly higher release of ^{3}H-ACh from the slices reaching an optimum of 4 Hz. A subsequent decline in ^{3}H-ACh release was evident at 16 Hz.

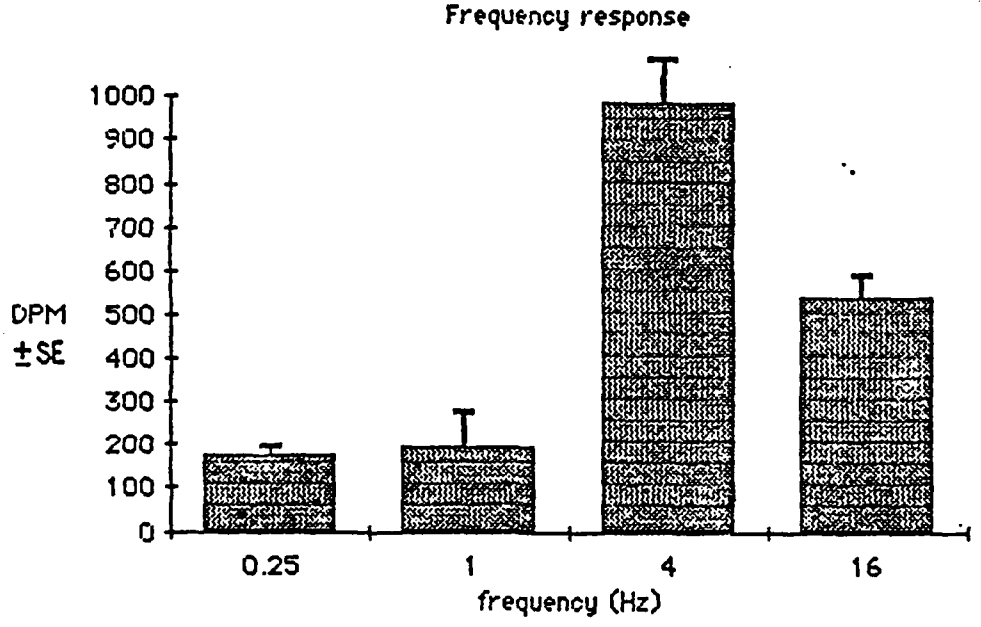

Fig. 8. Effects of frequency of stimulation on ^{3}H-ACh release from rat hippocampal slices.

When the data are expressed as the amount of ^{3}H-ACh released per one impulse (Fig. 9), a different relationship appears: the lower the frequency the higher the release of ^{3}H-ACh. We conclude from this set of experiments that one should use a frequency of 1 Hz as the standard for the subsequent experiments.

Additionally, the S_1 stimulated release of ^{3}H-ACh using the lower frequency will not deplete the ACh pool. Hence, the second stimulus (S_2) modified by the muscarinic cholinergic blocker (with an expected increase in the ^{3}H-ACh release) will not be limited by a depleted ACh pool.

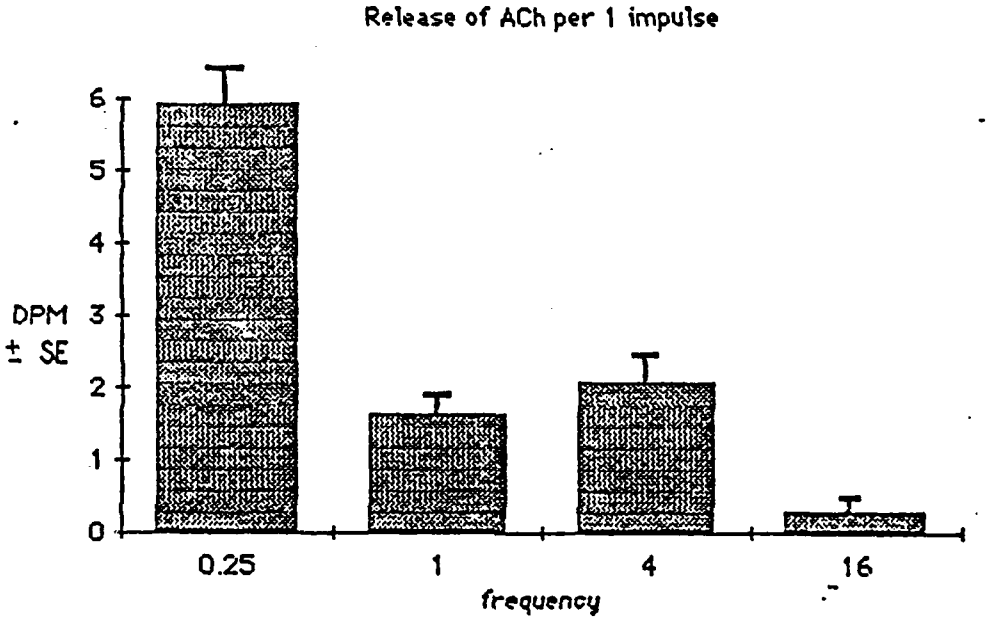

Fig. 9. Calculated release of ^{3}H-ACh per one electrical impulse.

Fig. 10 illustrates the results on ^{3}H-ACh release in a typical experiment using the hippocampal slice technique. The data are expressed as DMP of ^{3}H-ACh. Electrical stimuli were delivered at 60 and 90 min. The spontaneous release of ^{3}H-ACh over time decreased. This was another reason that the "fraction of release" method subsequently was utilized.

During the course of this research it became apparent that a more efficient method was needed to study ^{3}H-ACh release. Thus, a second accumulative method was developed. Rather than just study S_2/S_1 ^{3}H-ACh release, a series of increasing concentrations of drug were added in an

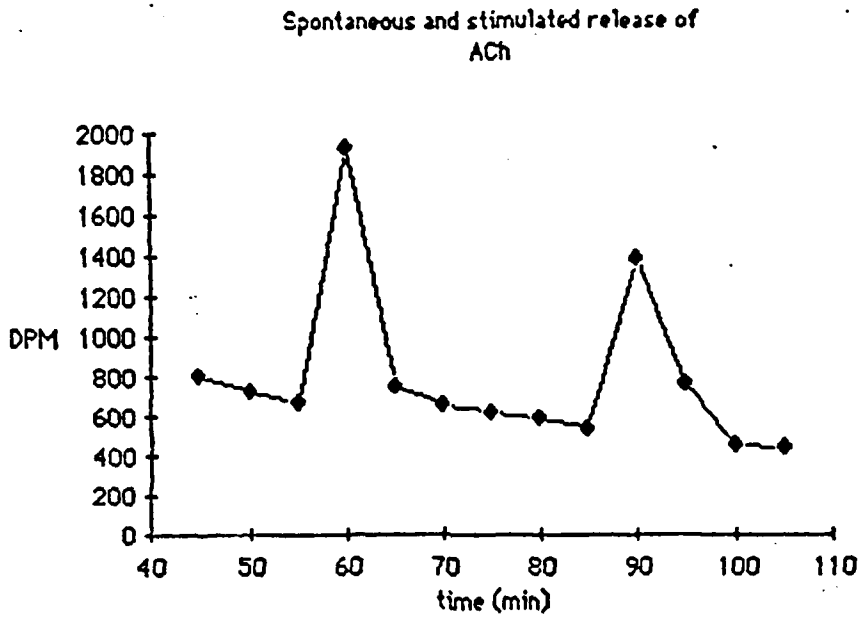

Fig. 10. Typical spontaneous and electrically stimulated release of ^{3}H-ACh *in vitro* from rat hippocampal slices using the S_1, S_2 method.

accumulative manner to obtain an S_1, $S_2 \ldots S_n$. Typical spontaneous and electrically stimulated release of ^{3}H-ACh *in vitro* from rat hippocampal slices is shown in Fig. 11.

B. Effects of HC-3 on S_1 release of ^{3}H-ACh products

HC-3 prevents the presynaptic re-uptake of ^{3}H-Ch, a ^{3}H-ACh breakdown product present in the presynaptic cleft. Use of HC-3 should result in more of the radioactive ACh products being left in the cleft and then transferred from the chamber to the fraction collection tubes than would occur in the absence of HC-3 (i.e., no blockade of Ch re-uptake). In fact, our data indicated that in the presence of HC-3 the total radioactivity collected for both the S_1 and the S_2 stimulation periods vs background were higher than when HC-3 was not used. To test whether the addition of HC-3 affects the proportional ACh release, as indicated by the S_2/S_1 ratios, atropine

experiments were conducted in the presence and in the absence of HC-3. All other experimental conditions were as detailed in the Methods section. Data from these experiments were analyzed first on an Apple MacIntosh computer using the software Statworks' two-factor analysis of variance (ANOVA) test to determine if any significant difference existed between the two groups (all of the concentrations plus control per group). Secondly, the Student's "t" test with the Welch approximation, programmed and run on a Hewlett-Packard 29C calculator, was used for estimating any significance of difference between each concentration with and without HC-3 (e.g., atropine 10^{-6} + HC-3) and between the two separate control sets. No significant differences between groups or sets of data were found with either test.

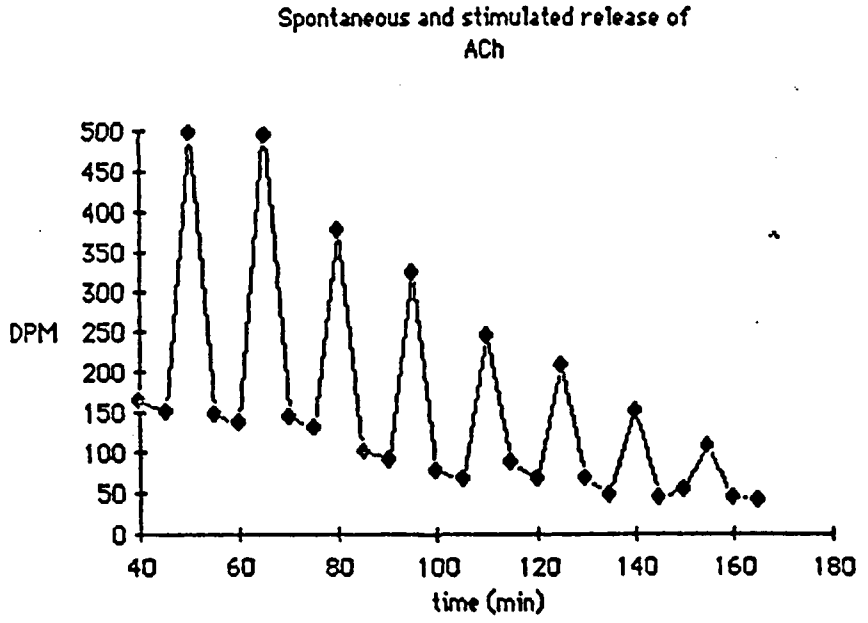

Fig. 11. Typical spontaneous and electrically stimulated ^{3}H-ACh release *in vitro* from rat hippocampal slices using an accumulative S_1, S_2,... S_n method

C. Effects of atropine

Atropine sulfate was chosen first because it is the classic nonselective muscarinic antagonist. The effect of increasing concentrations of atropine

alone on the evoked ^{3}H-ACh release were studied. Table 1 and Fig. 12 illustrate that the pattern of ^{3}H-ACh increase was dose-dependent. No appreciable differences were noted between data from experiments where HC-3 was or was not used. Therefore, all data were combined. In this and all subsequent tables, the number of experiments is given as N.

Table 1. Effects of atropine on ^{3}H-ACh release

Concentration	% of Control	S.E.	N
10^{-5}M	12.9	2.9	5
3 x 10^{-6}M	26.8	11.1	5
10^{-5}M	34.6	7.3	5
3 x 10^{-5}M	47.6	8.7	10
10^{-4}M	24.6	5.4	4

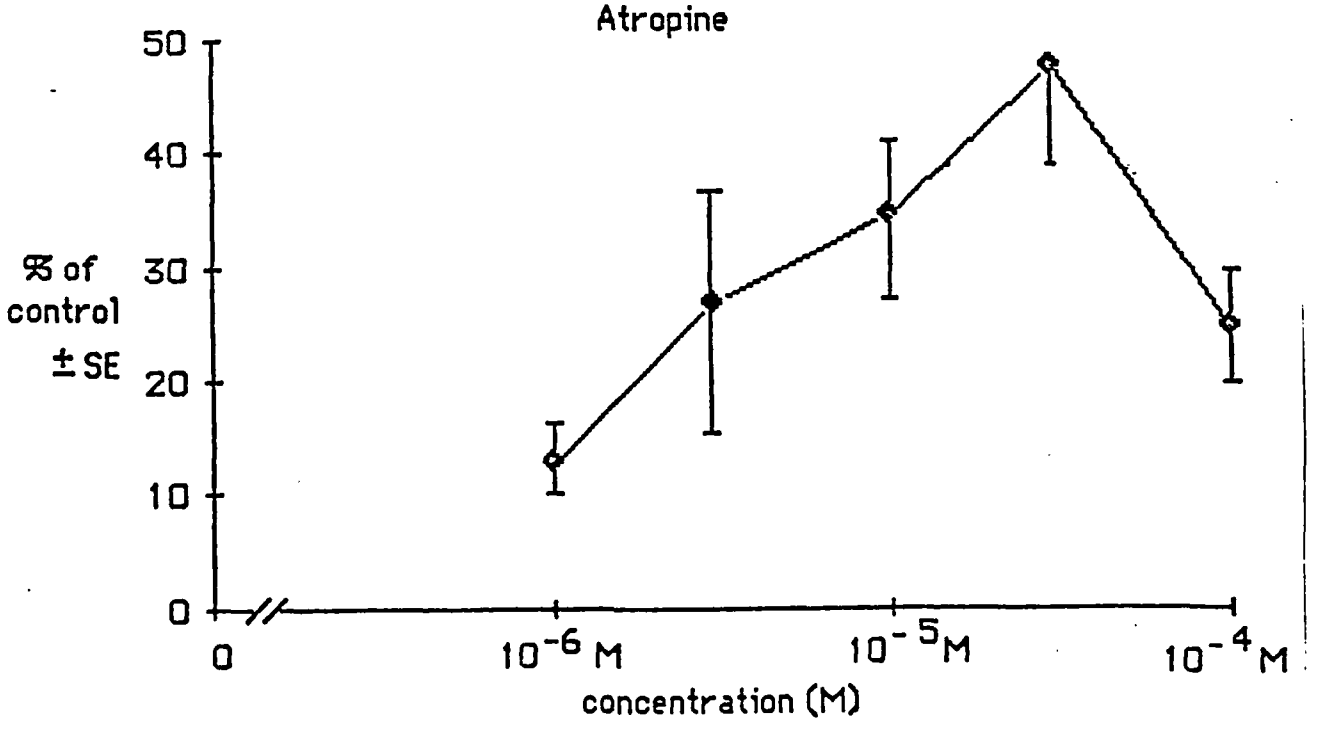

Fig. 12. Effects of atropine on ^{3}H-ACh release.

D. Effects of d-hyoscyamine

d-Hyoscyamine, one of two enantiomers of atropine, does not affect ^{3}H-ACh release when compared to control. This lack of effect is not unexpected since d-hyoscyamine has been shown to be inactive as a muscarinic antagonist *in vivo* when administered to experimental animals. The active form is its non-superimposable mirror image, l-hyoscyamine. The data obtained with d-hyoscyamine are given in Table 2 and Fig. 13 below.

Table 2. Negligible effects of d-hyoscyamine on ^{3}H-ACh release

Concentration	% of Control	SE	N
3×10^{-8} M	- 0.2	0.2	4
3×10^{-7} M	- 2.4	0.9	4
1×10^{-6} M	8.2	2.8	4
3×10^{-6} M	9.1	3.0	4
1×10^{-5} M	-16.9	2.5	4

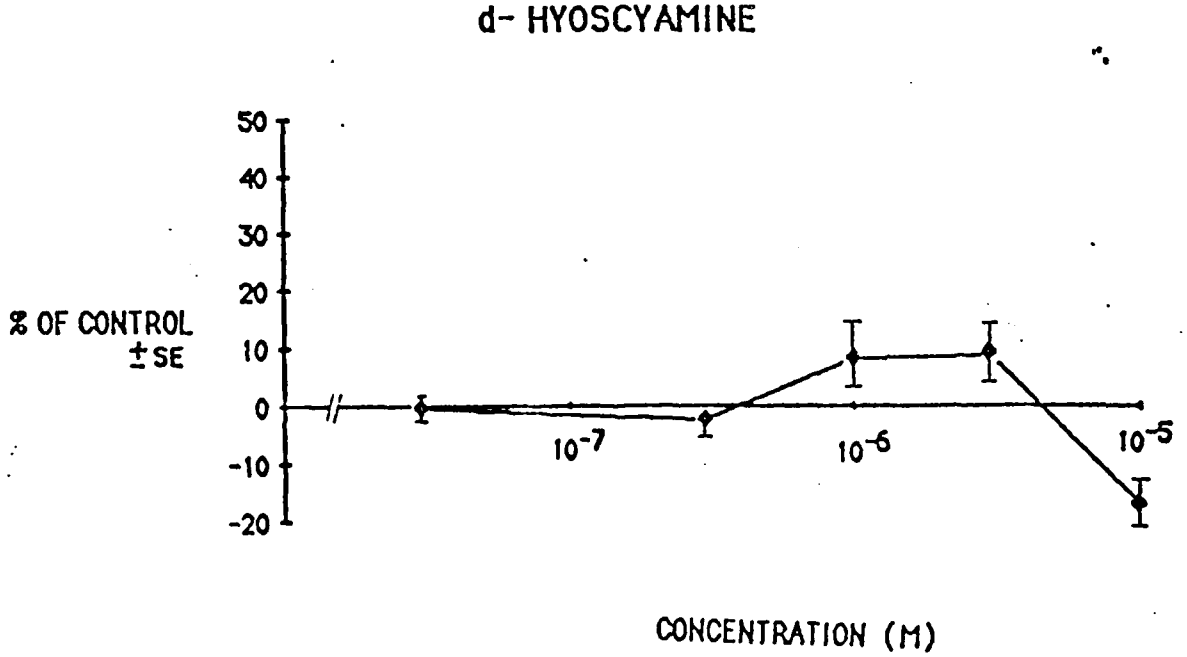

Fig. 13. Negligible effects of d-hyoscyamine on ^{3}H-ACh release.

E. Effects of l-hyoscyamine

Atropine is a mixture of two enantiomers, l-hyoscyamine and d-hyoscyamine. The substance, l-hyoscyamine, is the active form that binds cholinergic muscarinic receptors. Results showing the release of ACh versus atropine concentration are presented in Table 3. Fig. 14 displays the data in graphic form. As expected, l-hyoscyamine is more potent than atropine.

Table 3. Effects of l-Hyoscyamine on ^{3}H-ACh Release

Concentration	% of Control	S.E.	N
1×10^{-7}M	6.3	1.8	4
3×10^{-7}M	19.5	8.9	6
3×10^{-6}M	25.7	8.7	4
1×10^{-6}M	12.6	4.8	6

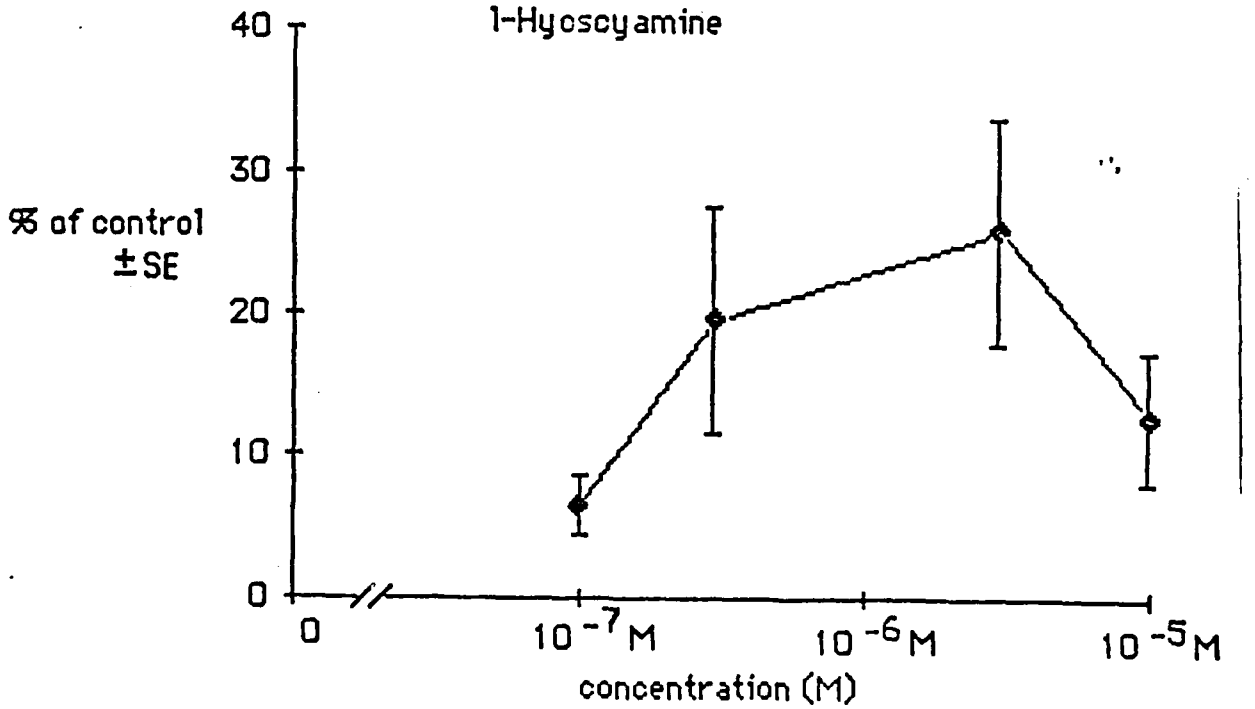

Fig. 14. Effects of l-hyoscyamine on ^{3}H-ACh release.

F. Effects of methylatropine

The response to methylatropine was not unlike that to atropine except that methylatropine was slightly more potent. In intact animals methylatropine exhibits its actions peripherally rather than centrally because it does not cross the blood-brain-barrier. This impermeability results from its hydrophilic properties due to its quaternary amine structure. However, with brain slices there is no blood-brain-barrier. When such slices were treated with methylatropine they showed an increase in stimulated acetylcholine (^{3}H-ACh) release as compared with the nontreated control slices. The molar concentrations used and resultant ratios are shown in Table 5 and Fig. 15.

Table 4. Effects of methylatropine on ^{3}H-ACh release

Concentration	% of Control	S.E.	N
1×10^{-7}M	10.9	3.5	4
3×10^{-7}M	21.7	16.5	3
1×10^{-6}M	26.8	2.3	4
3×10^{-6}M	40.0	24.0	4
1×10^{-5}M	17.8	5.7	5

Fig. 15 illustrates the data with the corresponding log concentrations in graphic form. The concentration-effect curve shows enhanced ^{3}H-ACh release with increasing concentrations of methylatropine to and including 3×10^{-6} M. At a higher concentration (1×10^{-5}M), ^{3}H-ACh release was decreased from peak release levels at 3×10^{-6}M.

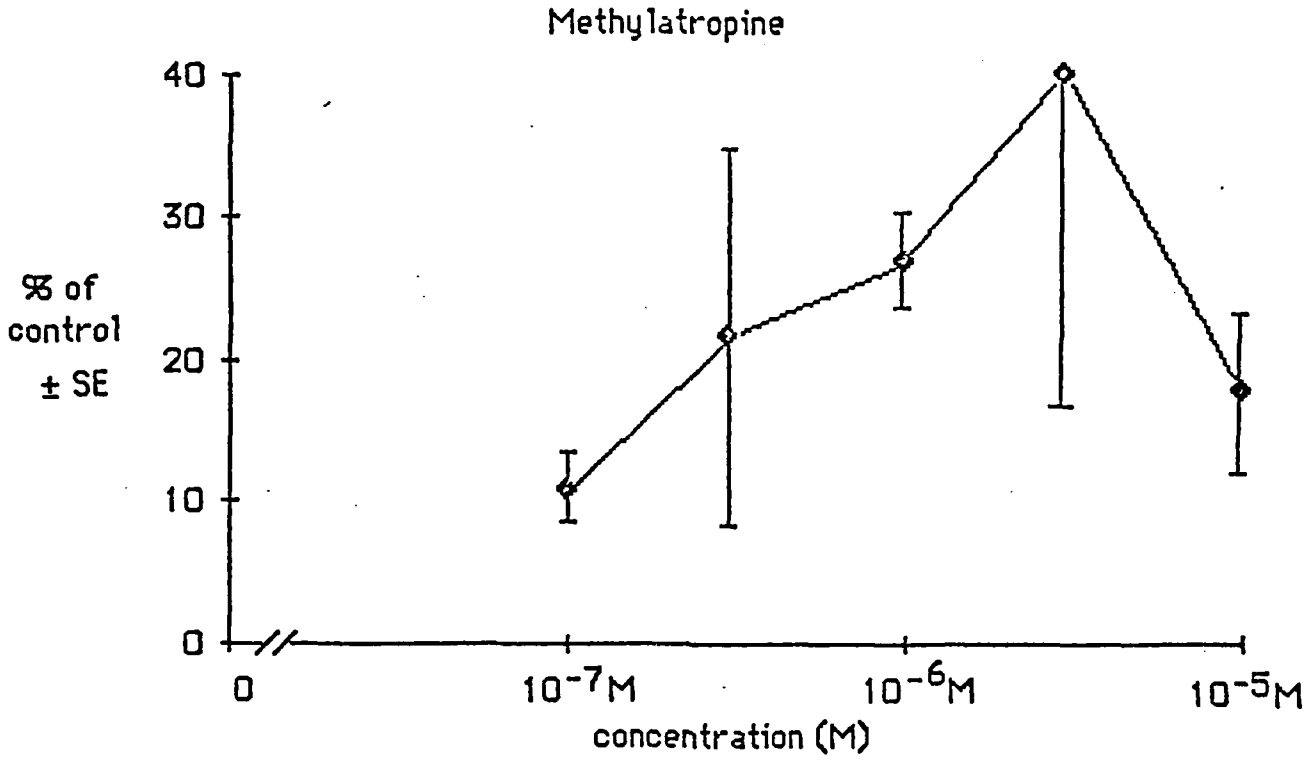

Fig. 15. Effects of methylatropine on ^{3}H-ACh release.

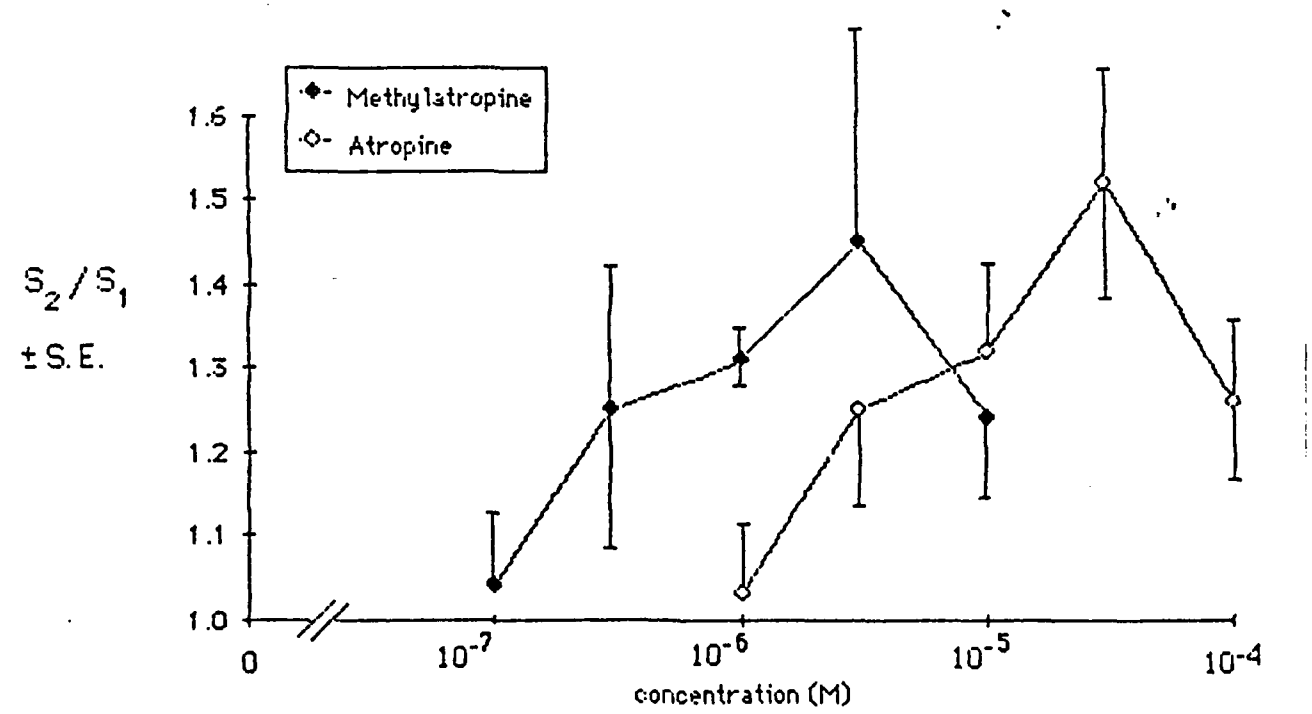

Fig. 16. Relative potency of atropine and methylatropine on ^{3}H-ACh release.

An interesting aspect of these data is that the concentrations of methylatropine vs those of atropine used to elicit the dose-effect curves in electrically stimulated slices differ by an order of magnitude. This shift is clearly shown in Fig. 16.

It has been reported that atropine obeys a classical one-site binding model while methylatropine binding can be analyzed to fit either a one-site or a two-site model depending upon the lipophilic nature of the ligand. These differences were proposed to be due to the lipophilic and and hydrophilic properties of atropine and methylatropine, respectively (Long-Hwa and El-Fakhany, 1985). The same authors reported that methylatropine displaces the hydrophilic ligand, ^{3}H-N-methylscopolamine (^{3}H-NMS) consistent with a two-site model. Atropine displaced both types of ligands, involving a one-site model in each case. Both sites are thought to be located on the same receptor. The possibility exists that binding of a hydrophilic ligand to the corresponding site on the presynaptic receptor is more efficient in inhibiting the action of the receptor. A second possibility is that presynaptic cholinergic receptors have more hydrophilic sites or more exposed hydrophilic sites than do the postsynaptic receptors. These findings may indicate a preferential binding of methylatropine to presynaptic versus postsynaptic receptors while atropine may bind to all receptors in an equivalent manner.

G. Effects of scopolamine

Scopolamine-treated slices exhibited stimulated ^{3}H-ACh release as compared with the nontreated control slices as summarized in Table 5 and Fig. 17. Five concentrations (ranging from 3×10^{-7}M to 10^{-5}M) with four slices per concentration were assayed. The results indicate that the responses were dose-dependent within that concentration range. These data are not unexpected because scopolamine is a muscarinic antagonist that has been shown to act on

the M1 and M2 receptors and affect ACh release in concentrations similar to those of atropine (Halim et al., 1982; Raiteri et al., 1984; Kellar et al., 1985).

Table 5. Effects of scopolamine on ^{3}H-ACh release

Concentration	% of Control	S.E.	N
3×10^{-7}M	14.2	3.2	4
1×10^{-6}M	9.3	5.6	4
2×10^{-6}M	34.0	11.7	4
1×10^{-5}M	32.5	5.5	4
3×10^{-5}M	8.7	3.5	4

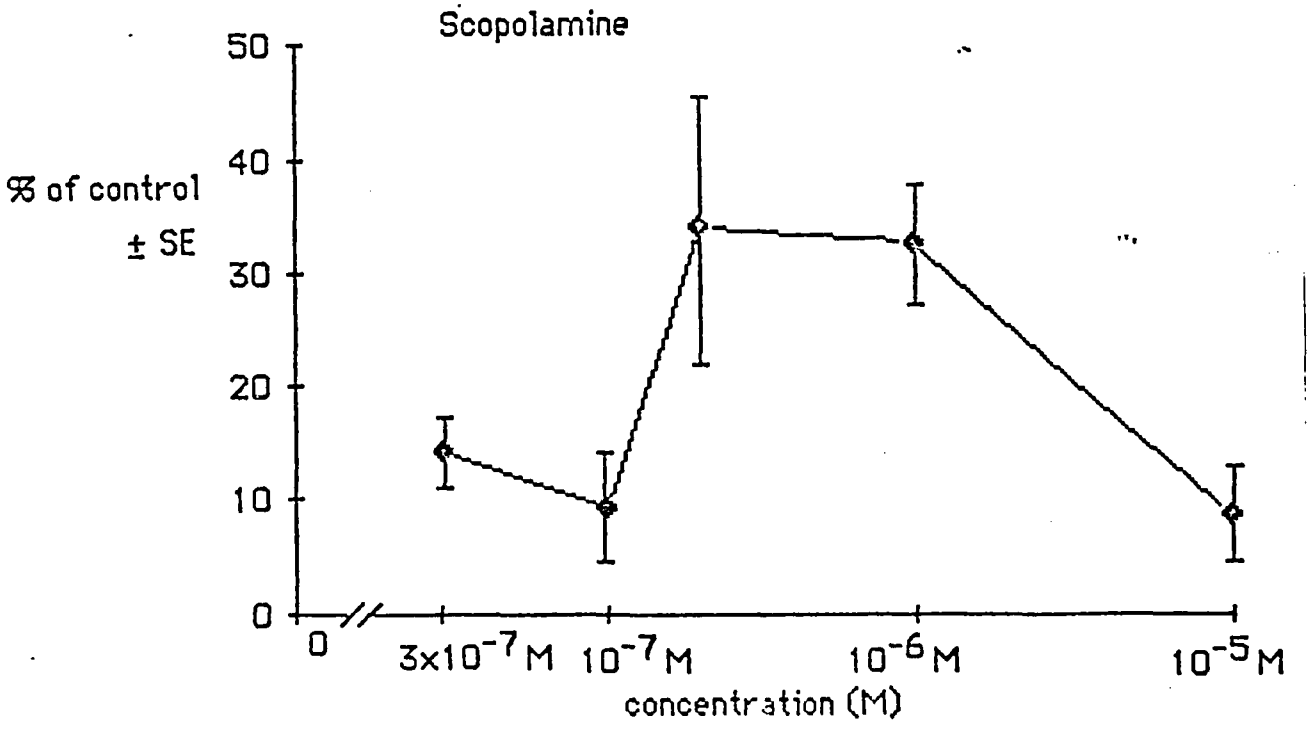

Fig. 17. Effects of scopolamine on ^{3}H-ACh release.

H. Effects of pirenzepine

As described earlier in this report, current research indicates that there are at least two types of muscarinic receptors. These receptors have been designated M1 and M2 and have been localized on the postsynaptic membranes. The differences between these receptors may be conformational rather than compositional (Sokolovsky et al., 1983). Pirenzepine binds with high affinity to the M1 receptor while atropine binds to both of them with equal affinity (Vickroy, 1984). Pirenzepine was a less effective promoter of the evoked ^{3}H- ACh release as shown in Table 6 and Fig. 18. Low concentrations of pirenzepine dihydrochloride (1×10^{-7}) seemed to cause a slight decrease in ^{3}H- ACh release, although the difference vs the control value is not statistically significant. However, this low dose pirenzepine experiment was repeated several times with similar results.

Table 6. Effects of pirenzepine on ^{3}H-ACh release

Concentration	% of Control	S.E.	N
1×10^{-7}M	-5.19	3.8	8
1×10^{-6}M	15.15	6.8	3
1×10^{-5}M	9.93	2.5	6

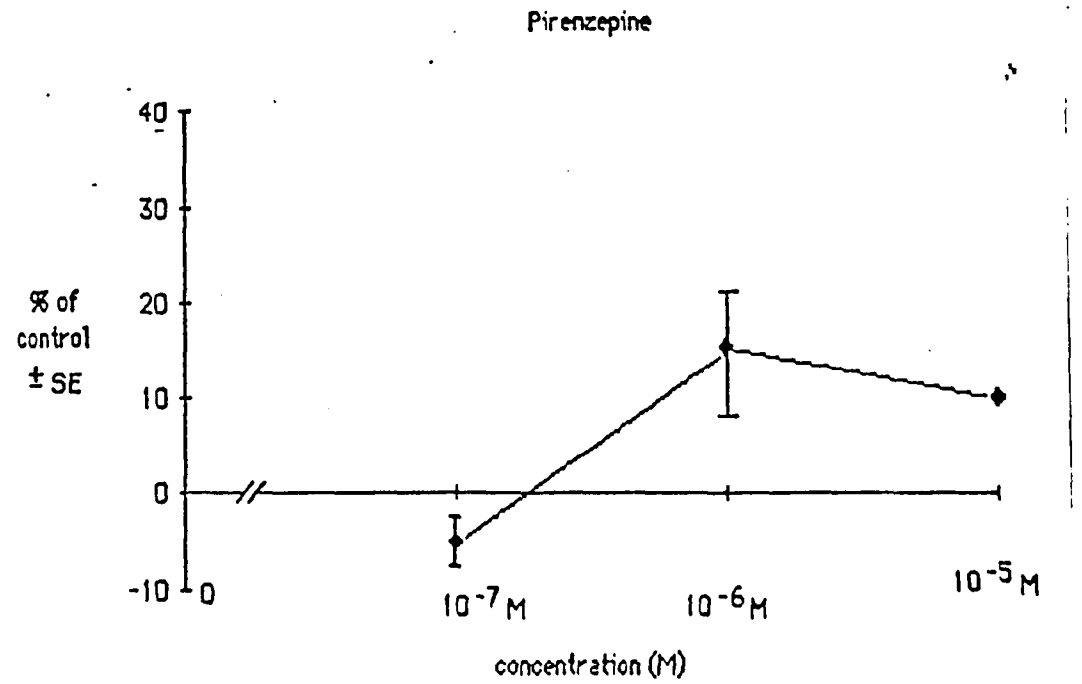

Fig. 18. Effects of pirenzepine on ^{3}H-ACh release.

I. Effects of mecamylamine

Mecamylamine is a well known nicotinic ganglionic cholinergic antagonist and was included in this series of compounds to confirm the inhibitory presynaptic control of ^{3}H-ACh release by muscarinic autoreceptors. The release experiments in which mecamylamine was used as the treatment drug yielded the data in Table 7 and plotted in Fig. 19. Three concentrations of 10^{-7} M, 10^{-6} M and 10^{-5} M were assayed. Clearly the results suggest that mecamlyamine does not enhance presynaptically-controlled release of ^{3}H-ACh.

Table 7. Effects of mecamylamine on ^{3}H-ACh release

Concentration	% of Control	S.E.	N
1×10^{-7} M	-11.5	3.5	3
1×10^{-6} M	- 8.6	2.5	3
1×10^{-5} M	-3.8	7.7	3

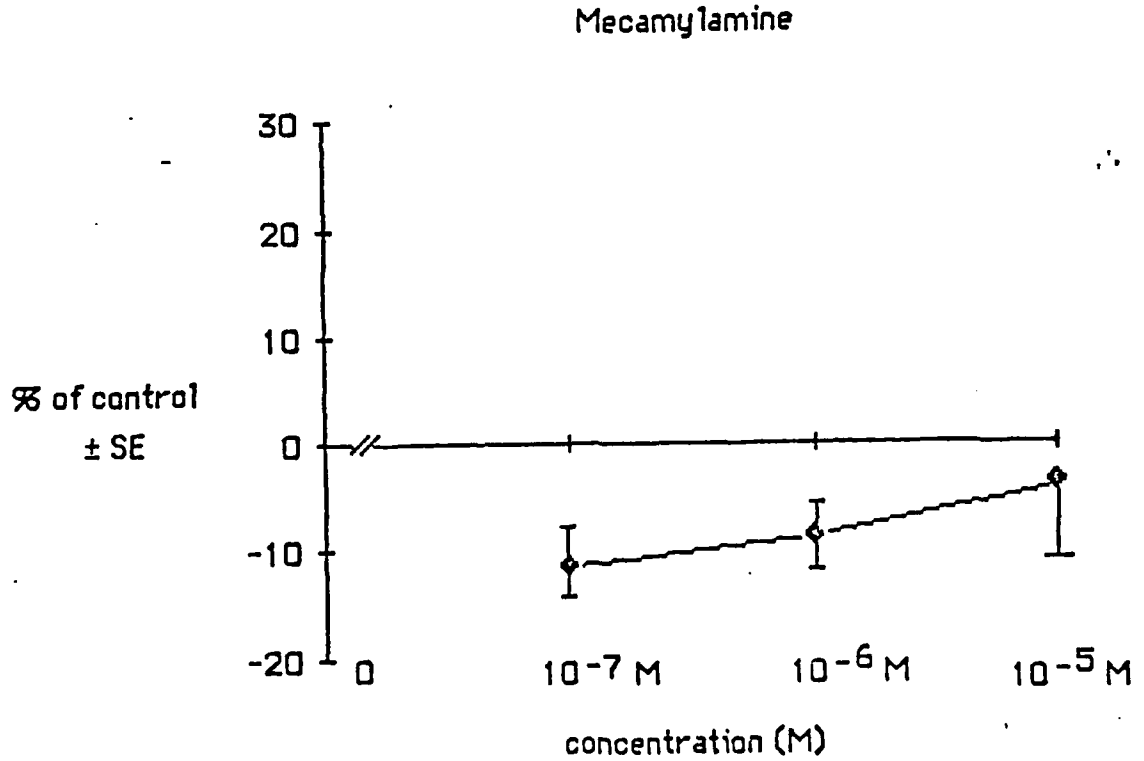

Fig. 19. Effects of mecamylamine on ^{3}H-ACh release.

J. Effects of aprophen

Data from the ^{3}H-ACh release experiments, using several concentrations of aprophen, are listed in Table 8 and are presented graphically in Fig. 20. Aprophen has the ability to block presynaptic muscarinic receptors, as exhibited by the concentration dependent increases of release of ^{3}H-ACh. At concentrations of 3×10^{-6}M and greater aprophen-treated slices showed reduced release as compared with control slices.

Table 8. Effects of aprophen on ^{3}H-ACh release

Concentration	% of Control	SE	N
3×10^{-9}M	8.1	3.0	4
1×10^{-8}M	13.2	4.2	4
3×10^{-8}M	20.3	4.0	4
1×10^{-7}M	23.1	2.3	4
3×10^{-7}M	32.5	1.1	4
1×10^{-6}M	36.0	3.0	4
3×10^{-6}M	21.0	4.1	4

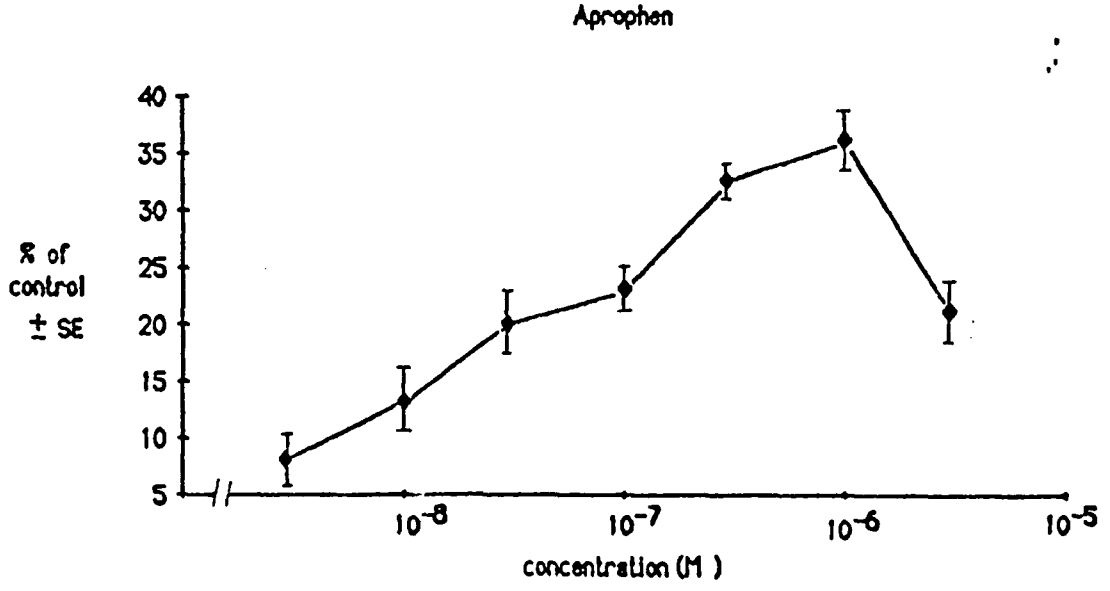

Fig. 20. Effects of aprophen on ^{3}H-ACh release.

K. Effects of benactyzine

Benactyzine also induces the release of ^{3}H-ACh from hippocampal slices as shown in Table 9 and Fig. 21. However, one observable difference is that the maximum effect of benactyzine is smaller than those elicited by the other muscarinic blockers, with the exception of pirenzepine. The maximum release mean value is only 22.9% above the control mean.

Table 9. Effects of benactyzine on ^{3}H-ACh release.

Concentration	% of Control	SE	N
1×10^{-8} M	6.0	3.1	6
3×10^{-8} M	16.9	2.6	6
1×10^{-7} M	22.9	3.5	6
3×10^{-7} M	17.8	2.8	6
1×10^{-6} M	19.2	2.5	6
3×10^{-6} M	8.7	3.3	6

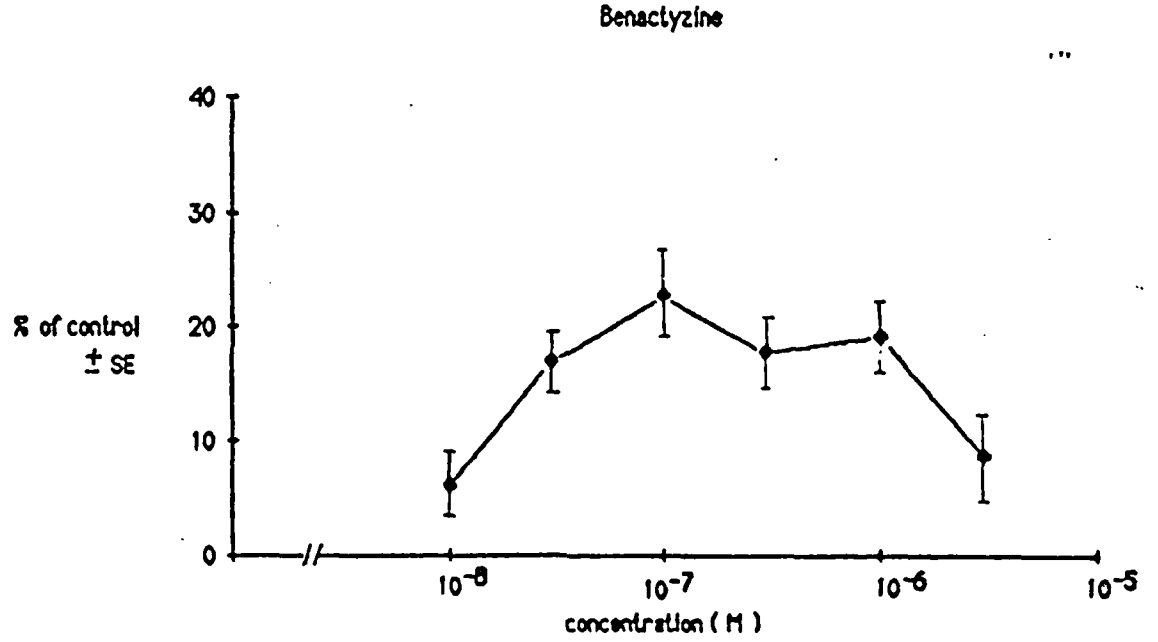

Fig. 21. Effects of benactyzine on ^{3}H-ACh release.

L. Effects of dexetimide

Dexetimide is a well known anticholinergic drug. It also is an effective releaser of ^{3}H-ACh as summarized in Table 10 and Fig. 22. A high concentration of 10^{-5}M dexetimide has less ^{3}H-ACh release effects. Its effects were very similar to atropine but it was more potent.

Table 10. Effects of dexetimide on ^{3}H-ACh release

Concentration	% of Control	S.E.	N
3×10^{-7}M	15.3	10.2	5
1×10^{-6}M	18.7	6.3	5
3×10^{-6}M	30.7	5.3	9
1×10^{-5}M	28.6	7.5	11
3×10^{-5}M	50.5	8.3	10
1×10^{-4}M	14.7	1.6	4

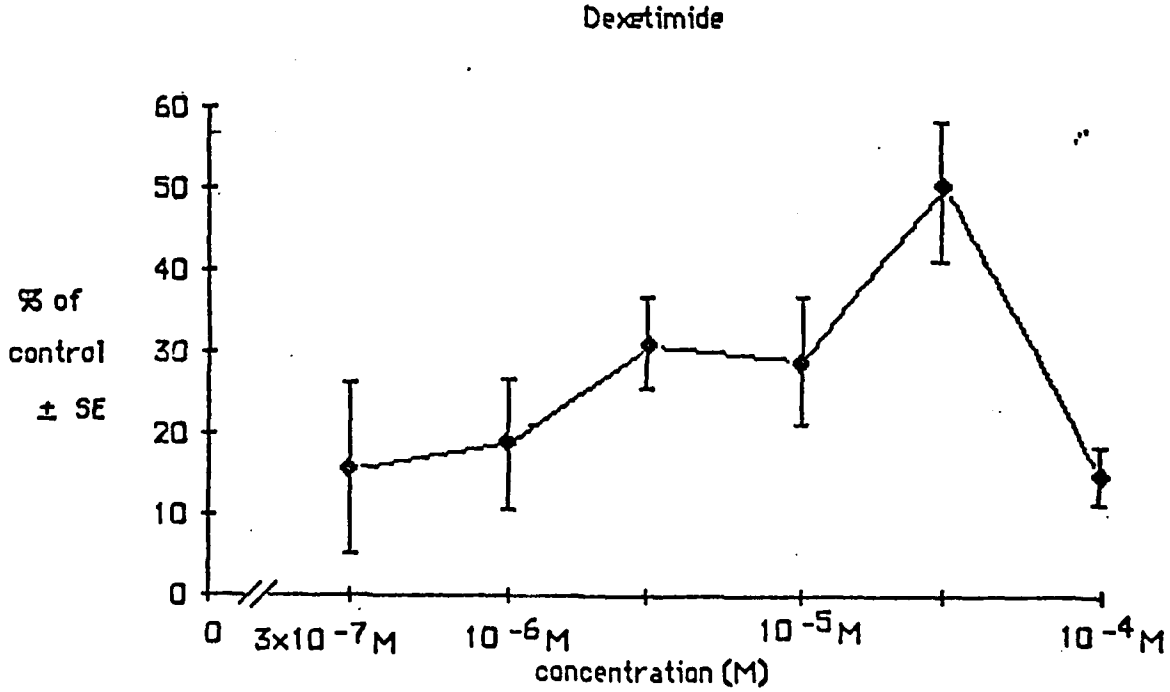

Fig. 22. Effects of dexetimide on ^{3}H-ACh release.

M. Effects of carbachol

As summarized in Table 11 and Fig. 23, carbachol in increasing concentrations reduced ^{3}H-ACh release. The EC50 was about 10 μM.

Table 11. Inhibitory effects of carbachol on ^{3}H-ACh release

Concentration	% of Control	S.E.	N
3×10^{-7}M	-23	10	4
1×10^{-6}M	-51	8	4
3×10^{-6}M	-86	1	4
1×10^{-5}M	-97	3	4
3×10^{-5}M	-104	1	4

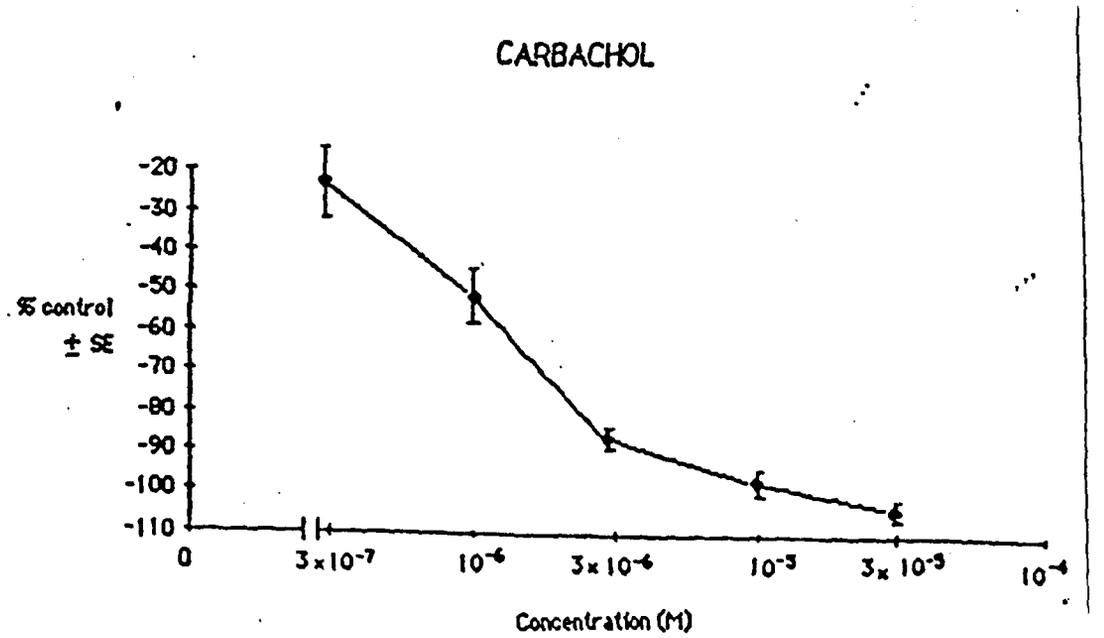

Fig. 23. Inhibitory effects of carbachol on ^{3}H-ACh release.

N. Effects of atropine on ^{3}H-ACh release in the presence of carbachol

In the presence of 10^{-6}M carbachol ^{3}H-ACh release was markedly suppressed. However, when this concentration of carbachol was used in combination with atropine a marked shift to the left in the concentration effect relationship for ^{3}H-ACh release was observed. These data are summarized in Table 12. In Fig. 24 are plotted the concentration effect relationships for atropine with and without carbachol. Note that in the presence of carbachol atropine increased markedly the release of ^{3}H-ACh, far more than without carbachol. The atropine concentration effect data presented on the right hand portion of Fig. 24 were obtained using the accumulative method and are similar to those of the S_2/S_1 method shown in Fig. 12. It also served as the control for the atropine plus carbachol data shown in the left hand portion of Fig. 24. Additional non-treated slices were also run as control.

Table 12. Effects of atropine on ^{3}H-ACh release in the presence of 1 μM of carbachol

Concentration	% of Control	S.E.	N
3×10^{-10}M	16.4	8	4
1×10^{-9}M	16.8	9	5
3×10^{-9}M	56.0	17	6
1×10^{-8}M	103.7	23	6
3×10^{-8}M	107.7	17	6
1×10^{-7}M	155.4	39	5

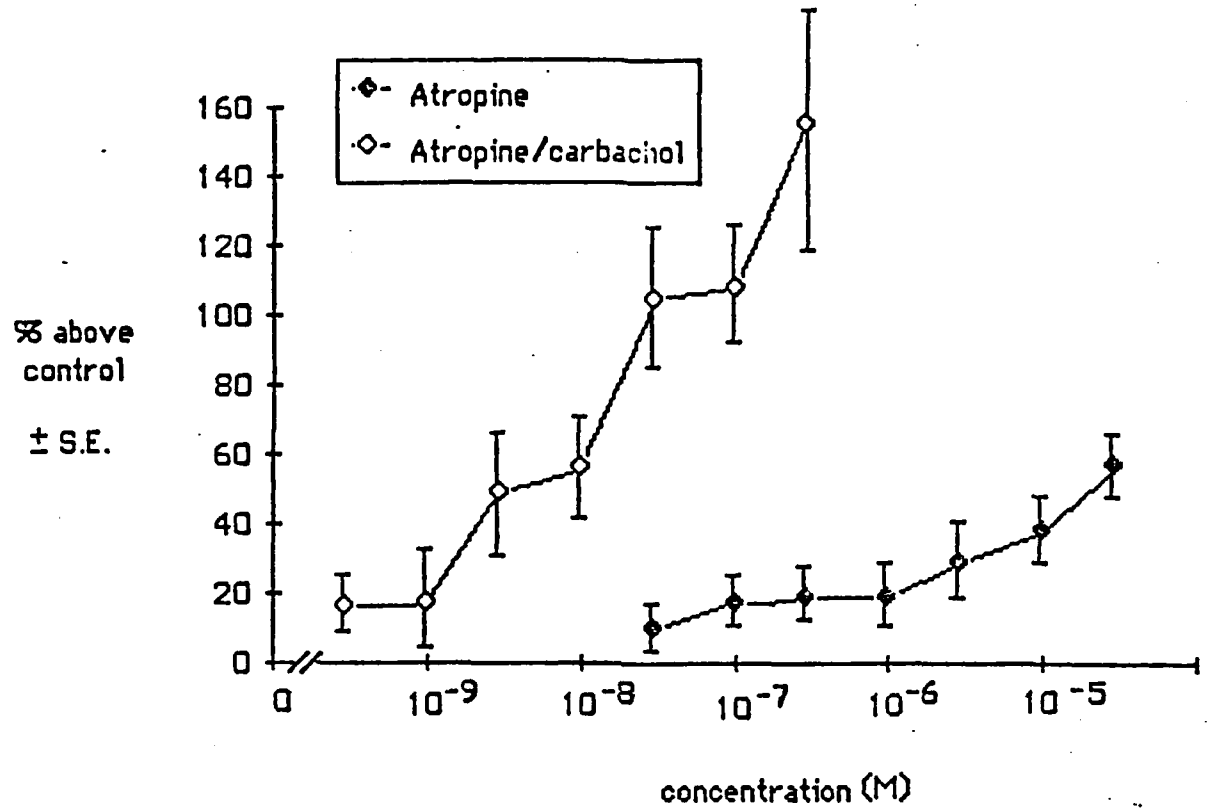

Fig. 24. Effects of atropine on ^{3}H-ACh release in the presence and absence of carbachol.

IV. Discussion and Conclusions

It is well known that the release of ACh is negatively controlled by presynaptic muscarinic receptors. The inhibition of ACh release can, in fact, be overcome by classical antagonists such as atropine (Szerb, 1979). Our task was to demonstrate first that atropine stimulates ^{3}H-ACh release in our model system of rat hippocampal slices. We then proceeded to test other atropine like analogs searching for a muscarinic antagonist with classical postsynaptic effects but without the presynaptic stimulatory effects of atropine.

In this second annual report we summarize the results found with atropine and related cholinergic antagonists on ^{3}H-ACh release. The present research

indicates that the methods used are useful for screening potential therapeutic agents for reduction of ^{3}H-ACh release. The use of the accumulative method is especially feasible and is recommended in future studies. To our knowledge, this latter technique has not been previously described in the literature and is a definite accomplishment for facilitating drug screening. Our data suggest that various M-cholinergic antagonists differ in their ability to release ^{3}H-ACh. There is considerable evidence that there are subclasses of muscarinic receptors (Birdsall et al., 1983; Hammer and Giachetti, 1984; Hirschowitz et al., 1984). Pirenzepine has been reported to bind with high affinity to a receptor designated M1 and with very low affinity to the receptor M2. Atropine binds with equal affinity to M1 and M2 (Vickroy, 1984). In the present studies pirenzepine showed very little effect, suggesting that the cholinergic autoreceptor is of the M2 type. This finding is in agreement with that of Mash and coworkers (1985) who have shown a selective loss of M2 muscarinic receptors in the cerebral cortex in patients with Alzheimer's disease and in animals with experimental cortical cholinergic denervation. The latter human disease and the animal experiments destroy cholinergic cell bodies and subsequently the cholinergic nerve endings which contain M2 presynaptic receptors.

Two major findings pertinent to the treatment of OP agents were made. The first was that benactyzine caused the least amount of ^{3}H-ACh release. The second was that the dose effect curve for atropine causing ^{3}H-ACh release was markedly shifted to the left and maximal release dramatically increased in the presence of carbachol, a mixed M and N agonist. It is esential that similar studies be done with nerve agents to bias the M2 presynaptic receptor and reexamine the effects of unusual muscarinic antagonists such as benactyzine.

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