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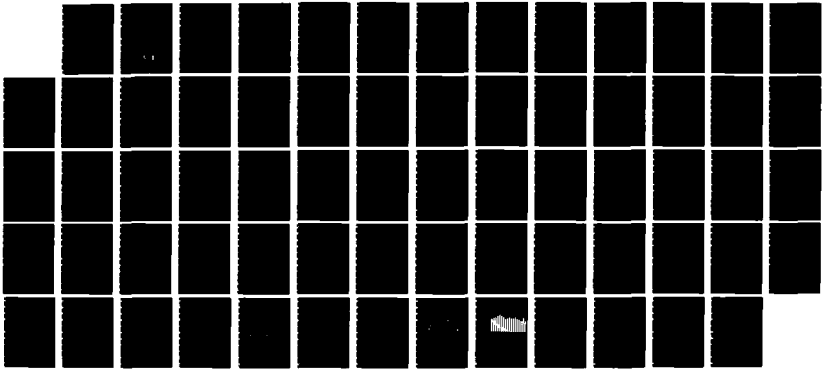

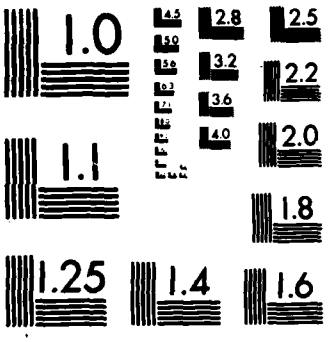

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The effects of acute and chronic treatments with the organophosphorous cholinesterase inhibitor DDVP (dichlorvos) (O,O-dimethyl O-(2,2-dichlorovinyl)phosphate), combined with drug treatments were studied on rat brain regional cholinergic parameters (acetylcholine content, choline content, acetylcholinesterase, choline acetyltransferase, sodium dependent high affinity choline uptake, muscarinic receptor subtypes). The results show that the increase in brain regional acetylcholine induced by DDVP can, to some extent, be dissociated from the cholinesterase inhibition of the drug. DDVP appears to stimulate a positive feedback mechanism to shut down cholinergic nerve terminals and this is held to be at least partly responsible for the increase in acetylcholine. This results in an intraneural accumulation of the cholinergic neurotransmitter. Drugs that possibly interfere with the feedback may cause a shift in the proportion of intraneural to extraneural acetylcholine, in favor of the

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ANTICHOLINESTERASE EFFECTS ON NUMBER AND FUNCTION OF BRAIN MUSCARINIC RECEPTORS AND CENTRAL CHOLINERGIC ACTIVITY.

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Milan, Italy
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18. brain;striatum;hippocampus;cortex;brainstem;rat;hydrophilic drugs;hydrophobic drugs;oxotremorine;physostigmine;choline chloride;pirenzepine;scopolamine;N-methyl-scopolamine; McN-A-343; quinpirole; putative neurotransmitters;dopamine;noradrenaline; glutamate;serotonin;dopamine receptors;cholinergic muscarinic receptors; muscarinic receptor subtypes; receptor accessibility; chronic treatment;acute treatment;tolerance; cross tolerance;down regulation;toxicity;mortality;routes of administration.

19. former, in cholinesterase poisoned rats.

In rats treated chronically with DDVP, AChE activity was reduced by more than 70% in the striatum,hippocampus and cortex. At the same time, ACh content in these regions was not altered. Marked tolerance to the ACh-accumulating action of DDVP and cross-tolerance to the increase produced by physostigmine were induced in the striatum but not in the hippocampus or cortex. Whereas in naive rats,DDVP, 10 mg/kg, p.o.,produced a 25% increase in striatal acetylcholine (please see Table 1, Interim Annual Scientific Report No.1, Grant No. AFOSR-82-0306 A, 30 September 1983), a 7-fold higher dose of DDVP (70 mg/kg) produced less than a half-maximal increase in ACh content which amounted to only a 10% increase. The effect of a 3.6-fold greater dose of physostigmine (1.8 mg/kg i.p., 20 min), which normally markedly increased striatal acetylcholine, was almost totally abolished. When chronic DDVP-treated rats were challenged with oxotremorine, marked cross-tolerance to the ACh-increasing action of the muscarinic receptor agonist was induced in both striatum and hippocampus: a 3-fold higher dose (3 mg/kg, i.p., 20 min) produced less than half-maximal increases. No cross-tolerance was produced to the marked decrease (-30%) in ACh content induced by the muscarinic receptor antagonist scopolamine (0.5 mg/kg, i.p., 20 min) in either striatum or hippocampus. In addition, no cross-tolerance to the ACh-increasing action of the dopaminergic agonists apomorphine or quinpirole was observed in the striatum. The results suggest that long-term treatment with DDVP produces tolerance and cross-tolerance specifically to muscarinic receptor activating drugs.

The chronic treatment with DDVP induced a decrease in muscarinic receptor density in striatum (128±3 pmoles/g) and hippocampus (104±3 pmoles/g) of 31% and 16%, respectively. In the striatum, there was also a two-fold increase in receptor affinity for the radioligand (from K_D 0.044 to 0.026 nM). In both regions, pirenzepine/(^{3}H)QNB competition experiments revealed the presence of two muscarinic receptor subtypes : M-1, the high affinity site,and M-2,corresponding to the low affinity site.In striatum, the ratio of the relative proportions of the M-1 and M-2 subtypes was 0.47 in the vehicle-treated group and rose to 1.44 in the chronic DDVP-treated rats and the nH increased from 0.7 to 0.8. It appears,therefore, that muscarinic receptor down regulation affects mainly the M-2 subtype. In hippocampus,the M-1/M-2 ratio of 1 and the nH of 0.7 were unchanged by DDVP treatment.

The predominant loss of the M-2 subclass in striatum may be responsible for the tolerance and the cross-tolerance towards the ACh-increasing action of muscarinic receptor activating agents.

Preface

Of pertinence to this study on possible feedback mechanisms activated by cholinesterase inhibitors as an acute adaptive response to their toxic actions is our ongoing work on elucidating the feedback neuronal loops in the striatum and hippocampus which regulate cholinergic activity. The identification of particular neurotransmitters in feedback loops would present novel means to mitigate the toxic effects of the cholinesterase inhibitors, perhaps by potentiating mechanisms that reduce acetylcholine release. We have recently proposed that cholinergic neurons intrinsic to the striatum appear to be under the dual control of inhibitory dopamine-containing afferents arising in the substantia nigra of the brainstem and excitatory, possibly glutamate-containing afferents from the frontal cortex (please see Fig. 1 and References 1 and 2 below). It was shown in Table 16 of Final Report, AFOSR-82-0306A, submitted 31 October 1984 by Herbert Ladinsky that the increase in striatal acetylcholine content induced by either DDVP (dichlorvos) or physostigmine was partially and significantly inhibited by undercutting the frontal cortex and destroying the excitatory input to the striatum. It is suggested that specific blockers of glutamatergic receptors in the brain could be possible effective antidotes because they might abolish the excitatory influence on the cholinergic neurons and perhaps diminish the release of acetylcholine. Several such compounds are currently available and have been useful in characterizing the subtypes of glutamate receptors in rat brain in in vitro studies but their use in vivo has thus far been limited. It is anticipated that many new, more potent and selective glutamate receptor antagonists will be developed in the near future. In addition, since the entire cerebral cortex projects to many nuclei throughout the brain through excitatory fibers it appears even more likely that such antagonists could mitigate central toxic effects of cholinesterase inhibitors.

It was noted in the Preface to Final Report, AFOSR-82-0306A (submitted 31 October 1984), that this laboratory has been carrying out, as part of of the EOARD/AFSC grant, a study on the effects of cholinergic muscarinic receptor agonists and antagonists specific for type I (M-1) or type II (M-2) muscarinic receptors, on brain regional acetylcholine content and acetylcholine turnover rate. Results from the study showed that the M-2 type of cholinergic muscarinic receptor appears to be predominantly involved in the action of the muscarinic drugs (please see Ref. 3). Indeed, the increase in striatal acetylcholine content induced by DDVP appears also to occur through activation of the M-2 type, e.g. pretreatment with pirenzepine, a selective and potent M-1 receptor antagonist failed to block the effect of DDVP whereas scopolamine, a potent "classical" antagonist which acts equally at all muscarinic receptor subtypes, strongly blocked the acetylcholine-increasing effect of DDVP (please see Results-Table 3).

At that time, specific M-2 antagonists were not available for testing but in the past year a patent was issued for compound AF-DX 116, a pirenzepine analog showing high selectivity for the M-2 receptor subtype. The compound was useful in revealing three muscarinic receptor subtypes in rat, guinea pig and rabbit peripheral and brain tissues (please see Ref. 4) and in human tissues. The receptors are now classified as cortical M-1, cardiac M-2 and glandular M-2 receptors. It is anticipated that in the near future more potent and selective compounds for each of these three subtypes will be available and can be tested for their ability to mitigate toxic symptoms of cholinesterase inhibitors.

We also demonstrated that these receptor sites are not readily accessible to hydrophilic receptor antagonists such as N-methyl-scopolamine, a classical, powerful muscarinic receptor antagonist which

does not discriminate between muscarinic receptor subtypes, and pirenzepine, a non-classical selective muscarinic receptor antagonist which shows selectivity for the M-1 subtype (please see Fig. 2). The findings suggest that tertiary amine muscarinic antagonists are able to label sites on the muscarinic receptor that are not easily accessible to the more hydrophilic quaternary ammonium compounds and that the interaction with these sites is indispensabile in the regulation of ACh release.

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TABLE OF CONTENTS

1. Statement of work

The ultimate objective of the proposed research is to clarify a basic, still unresolved problem connected with the mechanisms by which the anticholinesterases affect cholinergic nerves which should lead to a more thorough understanding of their most adverse reactions, i.e the generalized cholinergic stimulation, convulsions and neuromuscular paralysis. This may lead to safer management of the agents through new pharmacological means for preventing or counteracting the toxic effects.

The organophosphorous cholinesterase inhibitor DDVP (dichlorvos), will be employed. Dose-response effects and time course effects of DDVP on brain regional cholinergic biochemical markers, i.e. acetylcholine and choline contents, acetylcholine turnover, sodium-dependent high affinity uptake of choline, specific muscarinic receptor binding, acetylcholinesterase activity and choline acetyltransferase activity as well as brain monoamine levels in rats will be carried out. Highly sensitive and specific radioenzymatic methods methods and chromatographuc procedures will be utilized for the measurements. The curves obtained will be compared with one another to search for correlations.

Rats will be treated chronically with DDVP with the intention of looking for a change in specific muscarinic binding characteristics and then to correlate this with tolerance and cross-tolerance to muscarinic agonists on brain regional acetylcholine content.

Experiments will be designed to determine whether putative neurotransmitters play a role in mediating the action of DDVP. To this end

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various drug classes will be employed to interfere with, or facilitate, the neuropharmacological cholinergic effects of DDVP. Among the drugs to be used are: reserpine, alphamethylparatyrosine, parachlorophenylalanine, pimozide, atropine, phenoxybenzamine, prazosin and choline. As these powerful drugs affect known neuronal systems, their influence on DDVP will provide clues as to the mediator in a feedback loop. Lesions of known, central monoaminergic pathways will also be made to remove any suspected neuron before challenge with DDVP.

It is anticipated that the results will reveal: a) whether DDVP induces an intracellular as well as extracellular accumulation of acetylcholine and thus whether a negative feedback loop is operative; b) whether monoaminergic pathway(s) are activated by DDVP; c) whether the feedback loop can be manipulated pharmacologically; d) whether such manipulation can prevent or reduce the toxicity of cholinesterase inhibition; and e) whether DVP-induced changes in muscarinic receptor number leads to changes in the sensitivity of muscarinic receptors to stimulation.

2. Status of the research

The overall aims and scope of this project have been essentially met as originally outlined along the guidelines established in the Statement of Work of Proposal and Grant No. AFOSR-85-0025.

Results from this study and the previous one (Grant AFOSR-82-0306A) have yielded valuable information concerning basic problems connected with the mechanisms by which the anticholinesterase poisons affect cholinergic neurons to produce toxicity. We thus now have leads as to: 1, whether a cholinesterase inhibitor induces an intracellular as well as extracellular buildup of acetylcholine in brain - we believe that both processes occur but that the intrasynaptic buildup, although of smaller entity, likely produces greater toxicity; 2, whether certain neuronal pathways are activated by anticholinesterases - the corticostriatal glutamatergic pathway appears to play a major role in our conditions; and 3, whether these pathways can be manipulated to mitigate poisoning - a likely possibility but difficult to identify; glutamic acid blocking agents are suggested. In addition, we know now that chronic treatment with an anticholinesterae poison decreases muscarinic receptor number in the brain (known as down regulation), that both M-1 and M-2 muscarinic receptor subtypes are affected, that down regulation of the receptors (particularly of the M-2 subtype) appears to be mainly responsible for tolerance to the acetylcholine-increasing action of the anticholinesterase itself and to the cross tolerance towards other anticholinesterases or other non-anticholinesterase muscarinic receptor agonists. The M-1 muscarinic receptor subtype may be involved in toxic effects produced by cholinesterase inhibitors. Finally, the putative M-2 receptors involved in the feedback regulation of cholinergic neurotransmission in striatum do not appear to be located presynaptically on striatal cholinergic interneurons but the agonist-sensitive muscarinic receptor sites do appear to be located postsynaptically

A. Neuropharmacological characterization of DDVP

I. Acute treatments

I a. Drug Effects on Brain Acetylcholine Content

Table 1 shows the effect on striatal ACh (acetylcholine) content produced by the intracerebroventricular (i.c.v.) administration of 45 nmoles of the cholinesterase inhibitor DDVP (dichlorvos). The drug induced a 33% increase in ACh content 30 min after treatment without affecting the level of choline (data not shown). ACh content in the striatum of control, vehicle-treated rats was 65.1±3.6 nmoles/g wet weight and this was raised to 86.8±3.6 nmoles/g ($p<0.01$) in the DDVP-treated group. This i.c.v. dose of DDVP produced no noteworthy behavioral responses except for slight tremor.

Fig. 2 shows the dose-response curves of the muscarinic agonists oxotremorine and the butynyl base, McN-A-343, a selective M-1 muscarinic receptor agonist, as well as the antagonists, scopolamine, N-methyl-scopolamine (a quaternary hydrophilic agent) and pirenzepine, a selective M-1 muscarinic receptor antagonist, and nicotine on the content of ACh in striatum after i.c.v. administration of the drugs. The effects are expressed in percentage of the striatal ACh content of control (65.5 ± 3.2 pmoles/g wet wt). The powerful muscarinic receptor agonist oxotremorine had no

effect at the dose of 2.5 nmoles and significantly increased ACh content by 28% at the dose of 7.5 nmoles/rat. The maximal effect of about 40% was achieved at the concentrations of 250 and 500 nmoles/rat. Oxotremorine did not affect choline content at any dose (data not shown). For purposes of comparison, the DDVP data presented in Table1 is shown by the solid triangle. It can be noted that DDVP appears to be as potent as oxotremorine. Differently from DDVP, oxotremorine exerted more untoward behavioral responses. The drug produced tremor and diahrrea at 25 nmoles and sweating and chlordachylorrhea at 250 nmoles.

In vitro, DDVP (1 nM-1 mM) alone does not displace (^{3}H)QNB from its specific muscarinic binding sites in striatal membranes (please see Table 5) suggesting that DDVP does not act directly on the muscarinic receptor in striatum. This result supports the notion that DDVP produces an increase in ACh content via an indirect mechanism probably related to acetylcholinesterase inhibition. On the other hand, it is well recognized that oxotremorine both displaces (^{3}H)QNB from specific muscarinic receptors (please see Table 5) and is devoid of anticholinesterase activity. It thus increases ACh content by direct muscarinic receptor activation most likely acting at postsynaptic effector sites.

On the other hand McN-A-343, administered over the same dose range as oxotremorine (7.5-500 nmoles/rat) failed to produce any significant increase in ACh content. Nicotine, increased ACh content but required high doses of 250-2000 nmoles/rat to produce a modest increase of 20-25%. A time course of the increase induced by nicotine (308 nmoles) showed that the effect was terminated by 60 min at the highest dose used of 308 nmoles i.c.v. (Table 2).

The classical, non-selective but powerful muscarinic receptor antagonist scopolamine induced a dose-dependent decrease in ACh content in the range of 6.5-400 nmoles/rat. At the peak decrease, effected by 150 nmoles/rat, the striatal ACh content was depleted by around 50%. The potency of scopolamine determined in this experiment is corrected for the clearance of this hydrophobic agent from the brain. It was found using a radioreceptor assay, that approximately 30% of the drug was cleared from the brain during the course of the experiment.

N-methylscopolamine, a drug as potent as scopolamine as a muscarinic receptor antagonist of the classical type in vitro (please see Table 5), reacted much differently than scopolamine. Surprisingly, this hydrophilic quarternary compound produced only a small (15%) decrease in striatal ACh at low doses (5-7.5 nmoles) and then produced no further decrease even when the i.c.v. doses were raised to 150 nmoles.

By contrast, pirenzepine failed to affect the level of ACh over the entire dose range of 7.5-1000 nmoles/rat. Furthermore, at the dose of 500 nmoles, the same negative effect was seen over the time span of 15-60 min after i.c.v. injection (Table 2). Pirenzepine had no effect on striatal acetylcholinesterase activity in vitro when the drug was incubated with a striatal homogenate at a concentration up to 10 μM (data not shown). The activity of acetylcholinesterase in normal striatal homogenate was found to be 468.1±11.4 μmoles/h/g wet wt. Radioreceptor assay showed that pirenzepine, a highly hydrophilic compound, was not cleared at all by the brain up to 60 min after i.c.v. injection and was present in the striatum in a concentration of 783 nmoles/g striatal water (after i.c.v. injection of 2000 nmoles) which is equivalent to the calculated concentration of the drug of 736 nmoles or approximately 100 % of the dose injected.

Table 3 shows the increase in ACh content in striatum produced by the oral administration of DDVP (15 mg/kg, 30 min) and compares the ability of the selective M-1 muscarinic receptor antagonist pirenzepine, and the non-selective one scopolamine, to block DDVP. DDVP was found to produce a 41% increase in ACh content in striatum. The 15 min pre-treatment with pirenzepine at the i.c.v. dose of 500 nmoles failed to counteract the increase induced by DDVP but, as expected, the 15 min prior i.c.v. injection of scopolamine markedly, but not completely, prevented the increase at the much lower dose of 15 nmoles. Scopolamine, at the dose used, produced a 15% decrease of its own on striatal ACh content (Table 3 and Fig. 2).

Fig. 3 shows the dose-response effects of scopolamine, N-methyl-scopolamine and pirenzepine in antagonizing the increase in striatal ACh induced by the agonist, oxotremorine. The drugs were administered i.c.v. 10 min prior to the i.p. administration of 1 µmole/kg of oxotremorine, the minimum systemic dose producing the maximum effect (earlier data from this laboratory). Similar to the effects they showed on striatal ACh content by themselves, the three antagonists exhibited marked difference in their potencies against oxotremorine; scopolamine was clearly the most effective antagonist whereas N-methylscopolamine and pirenzepine showed virtually no inhibition except at the highest doses employed.

In hippocampus, pirenzepine and scopolamine showed different characteristics in their ability to block the DDVP, as compared with their actions in the striatum. Table 3 shows the increase in ACh content in hippocampus produced by the oral administration of DDVP (15 mg/kg, 30 min) and compares the the ability of the selective M-1 muscarinic receptor antagonist pirenzepine and the non-selective one scopolamine to block DDVP. DDVP produced a 26% increase in ACh content. Pretreatment with scopolamine completely blocked the action of DDVP in the hippocampus. At the dose used, 15 nmoles i.c.v., scopolamine did not produce an effect of its own on hippocampal ACh content.

Whereas the M-1 selective muscarinic receptor antagonist pirenzepine did not affect ACh content of its own up to the dose of 500 nmoles i.c.v. in striatum (Fig. 2), it did decrease ACh content in the hippocampus at a lower dose (Table 4). In a time-course experiment, it was found that pirenzepine decreased hippocampal ACh by 22% at 60 min and maximally decreased ACh by 27% at 240 min after the administration of 250 nmoles of the drug. Pirenzepine still maintained a strong and significant decrease in hippocampal ACh after 24 h again implying poor elimination from the brain. In line with the discriminating action of the drug, pirenzepine, although unable to prevent the action of DDVP in the striatum, prevented completely the increasing action of DDVP in the hippocampus. The results may suggest that pirenzepine, at the dose of 250 nmoles, may be acting at M-2 receptors to influence cholinergic activity (Table 3).

The data from competition experiments of (^{3}H)QNB binding by the agonists oxotremorine and the butynyl base, McN-A-343 to membranes of striatum (which contains almost equal proportions of both the M-1 and the M-2 subtypes) and the midbrain-hindbrain (which is rich in the M-2 subtype of muscarinic receptor) are shown in Table 5. The competition curves of OTMN/(^{3}H)QNB and McN-A-343/(^{3}H)QNB were shallow with Hill coefficients significantly different from unity and therefore the single IC_{50} shown reflects the average affinity of each drug for the heterogeneous agonist subtypes of the muscarinic receptors. Oxotremorine had nearly 13 times greater affinity for the muscarinic receptors of the midbrain-hindbrain than for those of the striatum (IC_{50}'s being 115 nM and 1460 nM, respectively). On the other hand, McN-A-343 showed slightly higher preference for the muscarinic receptors in striatum (IC_{50} = 5056 nM) than for those in the midbrain-hindbrain (IC_{50} = 8052). The ratio of the IC_{50} values of McN-A-343 to oxotremorine was 3.5 in striatum whereas in the midbrain-hindbrain it was 70.

In vitro, DDVP (1 nM-1 mM) alone was unable to displace (^{3}H)QNB from its specific muscarinic binding sites in striatal membranes (Table 5). IC_{50}'s of DDVP were greater than 1,000,000 nM in both tissues indicating no direct action of this cholinesterase inhibitor on either the M-1 or the M-2 subtypes of muscarinic receptors. This was further confirmed by an in vivo experiment (Table 6) in which pretreatment with DDVP, 15 mg/kg, p.o., 30 min, did not alter the binding characteristics of (^{3}H)QNB (B_{max} = 135±1.9 pmoles g; K_D = 0.038 nM; nH = <0.9) in the striatum.

Table 5 also presents the data from competition experiments of (^{3}H)QNB binding by the antagonists scopolamine, N-methylscopolamine and pirenzepine to membranes of striatum and midbrain-hindbrain.

The classical antagonist scopolamine competes with (^{3}H)QNB for the

receptor sites in a manner consistent with that expected for the competition of drugs for a single binding population with Hill coefficients close to unity. Among the antagonists, this drug showed the highest affinities for a single population of muscarinic receptors in both striatum and midbrain-hindbrain; the corrected IC_{50}'s were 0.44 and 1.5 nM for the two brain regions, respectively.

Unlike the "classical" antagonism curves, the pirenzepine/(^{3}H)QNB competition curve revealed a Hill coefficient significantly less than unity in striatum (nH=0.78). About 38% of muscarinic binding sites in striatum showed high affinity for PZ (IC_{50}-1=12.3 nM) and the remaining sites possessed lower affinity (IC_{50}-2=230.3 nM) for pirenzepine. The muscarinic receptors in midbrain-hindbrain had a uniformly low affinity (corrected IC_{50}=125 nM) for pirenzepine which was not much different from that of the lower affinity pirenzepine sites in striatum (230 nM).

N-methylscopolamine (NMS), although supposedly belonging to the class of classical, non-selective antagonists similar to scopolamine, demonstrated both potency for a high affinity site in both brain regions and the ability to discriminate between two receptor subtypes in both striatum and midbrain-hindbrain in competition experiments with (^{3}H)QNB. In striatum, unlike the "classical" antagonism curves, the NMS/(^{3}H)QNB competition curve revealed a Hill coefficient significantly less than unity (nH = 0.66) About 85% of muscarinic binding sites in striatum showed high affinity for NMS (IC_{50-1} = 0.67 nM)(slightly lower than scopolamine) and the remaining 15% of sites possessed lower affinity (IC_{50-2} = 58.5 nM) for NMS. About 79% of the muscarinic receptors in midbrain-hindbrain had a high affinity (IC_{50-1}=2.63 nM) (slightly lower than scopolamine) for NMS

which was 4-fold lower than that in striatum, whereas 21% of the muscarinic sites showed low affinity for NMS (IC_{50-2} = 165 nM).

It is not surprising that each muscarinic receptor antagonist would be able to exhibit some degree of selectivity in discriminating between the various subtypes of muscarinic receptors. On the other hand, we are dealing here with two compounds, scopolamine and N-methylscopolamine, with greatly different physico-chemical properties. The former is much more hydrophobic than the latter. This suggests that some antagonists do not have access to certain receptors which may be deeply embedded or surrounded by lipophilic material. Thus, at least in some brain regions, we have found that the highly hydrophilic compound N-methylscopolamine is weaker than scopolamine pharmacologically both in vivo - in decreasing ACh and in blocking DDVP) and in binding studies in vitro. The implications are that some muscarinic receptors may not be activated by drugs that are normally considered to be highly powerful. These receptors are thus located apart or compartmentalized.

I c. Toxicity

Toxic symptoms of some drugs including DDVP are described in individual chapters and more details on toxicity and mortality will be presented in chapter II. Chronic Treatments.

II. Chronic treatments with DDVP

II a. Drug Effects

Rats were treated with DDVP (10 mg/kg, p.o., twice daily for 11

consecutive days) and then treatment was suspended for 36 h to allow washout of the drug before the animals were challenged with other drugs. Acetylcholine content was unchanged by this treatment as will be seen in the following Tables. At the same time AChE activity most likely returned to normal values. Indeed, it was shown in a previous report (Tables 1 and 2, Final Report AFOSR-82-0306 A, 31 October 1984) that enzyme activity practically recovered within 24 h after a single dose of DDVP in both striatum and hippocampus. Complete tolerance to challenge by DDVP (15 mg/kg, p.o., 30 min), physostigmine (1 mg/kg, i.p., 20 min) and oxo-tremorine (0.54 mg/kg, i.p., 20 min) were obtained on the increase in ACh content in striatum but not in the hippocampus or cortex (these data were presented in Tables 17,18 and 19, Final Report AFOSR-82-0306A, 31 October 1984).

II b. Tolerance and Cross-Tolerance and Toxicity

The following data show that tolerance and cross-tolerance to challenge by these cholinergic drugs occurred in striatum even at several-fold higher doses in chronic DDVP-treated rats.

Table 7 shows the dose-response effect of DDVP on ACh content in chronicaly DDVP-treated rats. It is seen that an acute dose of DDVP, 15 mg/kg, produced the usual marked increase in ACh content in striatum of saline-treated rats. The dose of 20 mg/kg, p.o., produced a slightly greater increase of 41%. Higher doses (30 mg/kg or above) could not be tested for their effect on striatal ACh content because of the high mortality produced by the cholinesterase inhibitor. No significant effect of DDVP on striatal ACh was produced in the chronically DDVP-treated rats even when the dose of DDVP was raised almost 5-fold, up to 70 mg/kg.

Such measurements were not made in hippocampus of cortex because previous studies indicated that no tolerance was produced in these brain regions to chronic DDVP treatment.

The tolerance to the effect of DDVP on ACh content in striatum was accompanied by strong protection against the fatal action of high, acute challenging doses of DDVP in rats. The results are shown in Table 8. Whereas oral doses of DDVP of 30 mg/kg or higher produced mortality approaching or attaining 100% in naive rats, the same doses produced low (2/6) or zero (0/6) mortality in the chronic DDVP-treated animals.

Table 9 shows the dose-response effect of physostigmine (0.5, 1, 1.5 and 1.8 mg/kg, i.p., 20 min) on striatal ACh content in saline-treated and chronic DDVP-treated rats. It is seen that all the doses produced marked increases in striatal ACh content and that complete cross-tolerance to this effect was produced after chronic DDVP treatment.

On the other hand, no cross-tolerance was produced to the action of physostigmine on ACh content in the hippocampus (Table 10) even when a lower dose of physostigmine (0.5 mg/kg) was used.

Differently from the protection afforded by chronic DDVP treatment against DDVP mortality, the mortality produced by physostigmine was potentiated (Table 8).

When chronically treated rats were challenged with oxotremorine (1mg/kg, i.p., 20 min), there was a similar complete cross-tolerance reaction to the normally marked ACh increase produced by this potent drug in striatum (Table 11). The cross-tolerance persisted even as the dose of oxotremorine was raised to 3 mg/kg, a large dose which produces strong

cholinergic symptoms such as excessive sweating, diahrrea, chlordachylorrhea and tremors.

On the other hand, cross tolerance to the ACh increase by oxotremorine in the hippocampus of chronically DDVP-treated rats was not clearcut (Table 12). Cross tolerance was found to the marked increases in ACh content produced at the doses of 1.6 mg/kg and 3 mg/kg, but not at the doses of 1 mg/kg and 2.4 mg/kg. It is tentatively suggested from these difficult experiments that probably chronic treatment with DDVP did not lead to a cross-tolerance reaction to oxotremorine in the hippocampus. Oxotremorine produced no mortalities up to the dose of 3.2 mg/kg in saline-treated group and only 1 death in 7 rats in the DDVP-treated group (Table 8).

The i.p. administration of choline chloride, 250 mg/kg, elicited a 21% increase in striatal ACh content in saline-treated rats (Table 13). The mechanism by which this increase is produced is not clear. The compound is not an inhibitor of acetylcholinesterase but is the precursor of ACh. The drug is also recognized as being a weak muscarinic receptor agonist. Complete cross-tolerance to challenge by choline chloride was induced in chronically DDVP-treated rats, which would speak in favor of an agonistic mechanism for the increase.

As occurs with physostigmine, but not with DDVP or oxotremorine, chronic DDVP treatment potentiated strongly the toxic effects of choline chloride (Table 8). The compound produced 4 deaths in 11 rats in the chronically DDVP-treated group and no deaths in the saline-treated group.

In agreement with the lack of cross-tolerance found to acute challenge with apomorphine (Table 17, Final Report AFOSR-82-0306A, 31

October 1984) no cross-tolerance was obtained to challenge by quinpirole (LY 171555), a powerful D-2 dopaminergic receptor antagonist which produces a maximal increase in striatal ACh content at 150 ug/kg, i.p., 30 min (a supramaximal dose) (Table 14). However, cross-tolerance reaction was observed when a maximal dose of quinpirole, 90 ug/kg, was used. It is tentatively suggested from these difficult experiments that probably chronic treatment with DDVP did not lead to a cross-tolerance reaction to quinpirole in the striatum.

A summary Table of the tolerance and cross-tolerance results is given in Table 15. In conclusion of this phase of the work, the results show that long-term treatment with DDVP induces tolerance and cross-tolerance specifically to muscarinic receptor activating drugs acting through different mechanisms to increase ACh content. This is particularly clear in the striatum and less obvious in the hippocampus. Tolerance and cross-tolerance to the neuropharmacological cholinergic action of the drugs appear to be dissociated from the toxic actions of the drugs, when present. The overall results thus suggest that DDVP and physostigmine, which share the property of being powerful acetylcholinesterase inhibitors, exhibit some pharmacological effects which are not common to both drugs. Furthermore, oxotremorine and choline chloride share the property of being muscarinic receptor agonists but also have other and different pharmacological properties as well, as denoted by their opposite behaviors in causing fatalities.

As previously reported (Tables 17-19, Final Report AFOSR-82-0306A, 31 October 1984)), no cross-tolerance was found to the marked decrease in ACh content induced by an acute challenge with scopolamine (0.5 mg/kg, i.p., 20 min) in any of the three brain regions explored.

The phenomena of tolerance and cross-tolerance to acute treatment with such differently acting muscarinic receptor activating drugs on ACh content in rats treated subchronically with DDVP, as compared with the different reactions to the fatal actions of some of the drugs cannot be readily explained at the moment. The following section will show that the long-term treatment with DDVP results in loss (down regulation) of muscarinic receptors, both of the M-1 and the M-2 types and such results may lend some ideas concerning the tolerance and cross-tolerance phenomena.

II c. Binding Studies - Down Regulation

Both M-1 and M-2 muscarinic receptor subclasses were detected in rat striatum and hippocampus by means of pirenzepine-(^{3}H)QNB competition experiments (Fig. 4 and Table 22, Final Report AFOSR-82-0306A, 31 October 1984). The same Table is also shown in this Report as Table 16. In the striatum, the relative proportion of the receptor subtypes was 32% (R1 population) and 68% (R2 population) corresponding to the high affinity and low affinity sites, respectively. In the hippocampus, approximately equal proportions of the two affinity states was found to be present. The data in Table 16 were also reported previously in the Final Report AFOSR-82-0306A, 31 October 1984, Table 22.

The chronic treatment with DDVP caused a decrease in the muscarinic receptor densities in striatum and hippocampus of 31% and 16%, respectively as determined by Scatchard analysis of saturation isotherms

using (^{3}H)QNB as the ligand labelling the specific muscarinic receptors (Table 21, Final Report AFOSR-82-0306A, 31 October 1984). The same Table is also shown in this Report as Table 17. It was demonstrated earlier in this Report that an acute treatment with DDVP, 15 mg/kg, p.o., 30 min, did not alter the Bmax or the K_D of muscarinic receptors in striatum as determined by Scatchard analysis (Table 6) which provides further proof that the down regulation is due to the chronic treatment. In the striatum of the chronically DDVP-treated rats, there was also a steepening of the pirenzepine-(^{3}H)QNB competition curve (but not the oxotremorine/(^{3}H)QNB curve (Table 18), and an increase in the R1/R2 ratio to 59%/41%. In the hippocampus of chronically DDVP-treated rats, the R1/R2 ratio was not altered. The data in this Table were also reported previously in the Final Report AFOSR-82-0306A, 31 October 1984, Table 22.

In striatum, the increased proportion of high affinity M-1 sites (indicating a decreased number of M-2 sites), combined with the marked loss of total muscarinic receptor number in the chronically DDVP-treated rats may provide a feasible explanation for the tolerance and cross tolerance responses to muscarinic receptor activating drugs described earlier, i.e. our overall data suggest that these drugs increase striatal ACh content through M-2 receptor activation.

On the other hand, the relatively modest loss to total receptors and the unchanged R1/R2 ratio in the hippocampus provides the explanation for the lack of tolerance and cross-tolerance to muscarinic receptor activating drugs in this region. It should be pointed out here that these experiments do not indicate which muscarinic receptor subtype in the

hippocampus mediates the increase in ACh content produced by the muscarinic receptor activating agents.

In order to gain further information as to the muscarinic receptor subtype involved in the tolerance and cross-tolerance reactions, binding studies were performed using (^{3}H)pirenzepine (0.5 and 1 nM), the radioligand which at these concentrations specifically labels M-1 receptors. In Table 19 it is seen that chronic treatment with DDVP produced a marked 30-35% loss in the M-1 muscarinic receptor subtype in the striatum. A similar loss of 30% in M-1 receptors was found in the hippocampus, too (Table 20).

In the striatum of the chronically DDVP-treated rats, there was no change in the shape of the pirenzepine-(^{3}H)pirenzepine competition curve (Table 21) as denoted by the Hill coefficient around unity. The corrected IC_{50}'s were 16.6±1.7 (veh), 19.9±1.2 nM (chr. DDVP). Thus, a single population of high affinity sites (M-1 sites) for pirenzepine appears to be affected by the chronic DDVP treatment.

In the hippocampus, too, there was no change in the shape of the pirenzepine-(^{3}H)pirenzepine competition curve (Table 22) as denoted by the Hill coefficient around unity and similar corrected IC_{50} values of about 12-15 nM. Thus, a single population of high affinity sites (M-1 sites) for pirenzepine appears to be affected by the chronic DDVP treatment in the hippocampus as well.

In striatum, the increased proportion of high affinity M-1 sites (indicating a decreased number of M-2 sites), combined with the marked loss of total muscarinic receptor number in the chronically DDVP-treated rats may provide a feasible explanation for the tolerance and cross

tolerance responses to muscarinic receptor activating drugs described earlier, i.e. our overall data suggest that these drugs increase striatal ACh content through M-2 receptor activation.

It is intriguing to suggest that whereas the M-1 receptor subtype may not be involved in the effects of agonists on ACh content, this subtype may be involved in behavioral responses produced by agonists and toxic responses produced by cholinesterase inhibitors as well as the direct-acting agonists. This aspect is well worth following up (please see recommendation section).

II d. Acetylcholine release in vivo

The release of ACh in vivo was determined by the trans-striatal dialysis fiber method of Imperato and Di Chiara (1). This technique is based on stereotaxic insertion of a thin dialysis tube (0.2 mm outer diameter) through the brain which traverses both caudate nuclei (Fig. 4). One end is connected to a constant flow perfusion pump through polyethylene tubing, and through this end Kreb's solution is pumped into the dialysis tube. From the other end, the Kreb's solution is collected into 150 ul microtubes.

Experiments are performed 24-48 h after surgery in awake rats having free access to water and food. The dialysis tubing is perfused with Krebs solution at a speed of 2µl/min, and samples were collected every 10 or 20 min. The ACh collected was determined by the sensitive and specific radioenzymatic method of Consolo et al. (2) with some modifications The lower sensitivity of the method is approximately 1 pmole ACh.

Only minimally-detectable amounts of ACh outflow could be detected

by this trans-striatal dialysis technique when the rat striata were perfused with Kreb's solution in the absence of physostigmine. When the cholinesterase inhibitor (1 uM) was added to the Kreb's perfusing solution, then a large and constant release of ACh of about 7 pmoles/10 min/ 2 striata was detected over at least a 120 min period (Fig 5).

The necessity of employing a cholinesterase inhibitor provides strong evidence, in accord with our working hypothesis throughout the period of this research project with AFOSR, that normally released ACh is rapidly and completely destroyed by acetylcholinesterase. In the presence of a cholinesterase inhibitor, intrasynaptic, extracellular ACh is protected and is able to diffuse into surrounding regions. Some also diffuses into the dialysis fiber. The omission of Ca++ from the perfusion medium completely prevented the release of ACh even in the presence of physostigmine (data not shown). When rats were pretreated with scopolamine (0.8 mg/kg, s.c.), a marked release of ACh was provoked (data not shown).

II e. Materials and Methods

Female CD-COBS rats (Charles River,Italy), body weight 210-220g, were utilized. The animals were housed under constant temperature (22°C), humidity (60%) and 12 h dark-light cycles, and allowed free access to food and water.

Receptor Binding Experiments

The animals were killed by decapitation and the striatum and midbrain-hindbrain (Mb-Hb) regions were dissected out over ice and homogenized in distilled water in a final volume of 1:100 for striatum and 1: 50 for the Mb-Hb. The homogenates were then divided into several tubes

and stored at -80°C until used. In competition experiments, an aliquot of the homogenate containing 0.05 mg protein of striatum or 0.25 mg protein of Mb-Hb was incubated at 37°C with 0.05 nM or 0.75 nM (^{3}H)QNB, respectively, in 1 ml of 0.02 M HEPES buffer, pH 7.5, containing 100 mM NaCl and 10 mM $MgCl_2$. The incubation lasted for 1 h (in the case of antagonist/(^{3}H)QNB competition experiments) or 30 for min (in the case of agonist/(^{3}H)QNB competition experiments to avoid possible desensitization of receptors). The reaction was terminated by the addition of 1.5 ml of chilled buffer followed by rapid filtration over Whatman GF/B filters under vacuum and two subsequent rinses of the filters with 2.5 ml of cold buffer. The filters were placed in small "hang-in" polyethylene vials containing 5 ml Filter-Count scintillant (Packard) and counted in a Packard 300 ß-counter at the efficiency of 45%. Stereospecific binding was defined as that displaceable by 5 µM atropine and averaged 90% of total binding.

Data of the competition experiments were calculated by using a computerized log-logit equation to obtain the IC_{50}'s and Hill coefficients of the inhibition curves, with the exception that the data of the PZ/(^{3}H)QNB competition experiment were fitted to a computerized model for multiple independent binding sites.

Acetylcholine and Choline Assays

The rats were killed by fast focussed microwave irradiation to the head (1.3 kW at 2.45 GHz for about 4 sec) using an adapted commercial microwave system (General Medical Engineering Corp., Peabody, MA, U.S.A.). The striatum was removed from the brain and, after weighing, the tissue was homogenized in a mixture of 15% 1 N formic acid:85% acetone before proceeding to the measurement of ACh by the radioenzymatic method of Saelens et al. (3) with modifications. Choline-O-acetyltransferase enzyme,

used in the coctail for measuring ACh and choline, was obtained from bovine caudate nuclei and partially purified.

The drugs used were dissolved in saline and administered intracerebroventricularly in a volume of 15 µl through a Portex PP10 polyethylene guide cannula implanted in the third ventricle 24 h earlier, unless otherwise stated. The time interval selected for each drug after i.c.v. injection was determined in pilot studies to yield the peak effect on striatal ACh content (these data are not shown). In this condition, it is assumed that the peak drug concentration in the striatum has been reached, and since the drugs were injected for relatively short times (15-30 min) and directly into the brain, it is further assumed that the drug doses can be used to compare the in vivo potencies.

II e. References

1) Imperato, A. and Di Chiara, G., J. Neurosc. 5, 297-306, (1985).

2) Consolo, S., Romano, M., Scozzesi, C., Bonetti A.C. and Ladinsky, H. : in "Dynamics of the Cholinergic System". I. Hanin ed., Plenum Press, New York, (1986).

3) Saelens, J.K., Allen, M.P. and Simke, J.P., Arch. Int. Pharmacodyn., 186, 279-286, (1970).

B. Conclusions

DDVP, at the non-lethal but cholinomimetic dose of 15 mg/kg, strongly increased the content of ACh in rat brain hemispheric regions. It appears that at least part of the increase in brain ACh induced by DDVP is provoked by muscarinic receptor-stimulated positive feedback to shut down the cholinergic nerve terminals. This results in an intraneuronal accumulation of ACh. One possibility of overcoming the toxic effects of cholinesterase poisoning is to employ drugs that potentiate positive feedback mechanisms. Such drugs may possibly cause a shift in the proportion of intraneuronal to extraneuronal ACh in cholinesterase poisoned rats in favor of an intraneuronal buildup.

Our work has shown that both M-1 and M-2 subtypes of muscarinic receptors are present in rat hemispheric regions. The M-2 subtype appears to be the one most responsible for the effect of muscarinic receptor activatnig drugs in increasing ACh content and thus in decreasing ACh release and turnover. We have shown that it is possible to down regulate muscarinic receptors, both of the M-1 and M-2 subtypes by chronically treating rats with DDVP. The marked loss of the M-2 subclass of receptors may be responsible for the tolerance and cross-tolerance to

anticholinesterases and other muscarinic agonists in striatum. In hippocampus, although the down regulation of muscarinic receptors does occur, the decrease is modest and is also not relegated only to the M-2 receptor population. No tolerance or cross tolerance to any of the muscarinic agonists was observed.

The conventional antagonist scopolamine, which binds to a homogeneous population of muscarinic receptors in striatum, decreased ACh levels in striatum by about 40% in a concentration-dependent manner and antagonized the cholinergic action of DDVP and oxotremorine. Differently, the hydrophilic quaternary analog of scopolamine, N-methylscopolamine, exerted only a weak effect on ACh content in striatum and was not capable of antagonizing oxotremorine even at the highest dose used. These observations are consistent with recent reports indicating that a significant difference exists in the interaction of tertiary and quaternary muscarinic cholinergic antagonists with the receptor sites in rat brain homogenates, both in saturation and competition experiments. (^{3}H)N-methylscopolamine, used as the ligand, labeled only a fraction (65%) of the receptor population that is available to (^{3}H)QNB, suggesting that (^{3}H)N-methylscopolamine might label only a subpopulation of (^{3}H)QNB binding sites with high affinity. Our own results in this report support the results and the concept. Thus, although definitive conclusions are not warranted at this point, these recent findings and the lack of a full response of N-methylscopolamine in vivo suggest that tertiary amine muscarinic antagonists are able to label sites on the muscarinic receptor that are not easily accessible to the more hydrophilic quaternary ammonium compound and that the interaction with these sites is indispensabile in the regulation of ACh release.

On the other hand, pirenzepine, the novel muscarinic receptor antagonist which has been shown to interact with high and low-affinity subpopulations of muscarinic receptors in various brain areas, including the striatum, failed to elicit any change in striatal ACh content at i.c.v. doses up to 1000 nmoles/rat.

These results fit with the finding indicating that the agonist McN-A-343, which in in vitro binding studies, shows the same selectivity as pirenzepine for the M-1 sites, did not alter the striatal level of ACh. Taken together the data suggest that scopolamine drives the change in ACh level through an action at pirenzepine-insensitive sites, i.e. likely M-2 sites.

In summary, our data have provided in vivo pharmacological evidence that only one of the muscarinic receptor subtypes - likely the putative M-2 receptors - is involved in the feedback regulation of cholinergic neurotransmission in rat striatum. These receptors do not appear to be located presynaptically on striatal cholinergic interneurons since this area was shown to lack an autoreceptor mechanism. In accordance, evidence provided earlier from this laboratory indicates that oxotremorine-sensitive muscarinic receptor sites are located postsynaptically in the striatum. We also demonstrated that these receptors sites are not readily accessible to a particular hydrophilic receptor antagonist such as N-methylscopolamine. In addition, pirenzepine, although sharing the hydrophilicity of N-methylscopolamine, does not dislay any effect on the ACh level exhibiting selective action at pirenzepine-sensitive sites. These results taken together may provide clues as to the type of selective antimuscarinic agent would be most effective in overcoming DDVP toxicity.

C. Recommendations

1) It is recommended to look for selective glutamate-receptor antagonists as possible agents useful in the prevention and/or the mitigation of anticholinesterase poisoning. Such drugs may operate to reduce the excitatory action of glutamate and/or aspartate on cholinergic neurons and thus act to reduce acetylcholine release (please see Preface).

2) It is recommended to test selective antagonists of the cardiac M-2 type and glandular M-2 type as agents useful in the prevention and/or mitigation of anticholinesterase poisoning. We have recently shown in a paper submitted to Life Sciences, that the brain regions are rich in both muscarinic receptor subtypes, particularly of the glandular M-2 type. The advantage of using drugs showing selectivity for a certain muscarinic receptor subtype would be to markedly reduce the well known side effects of muscarinic receptor antagonists (please see Preface). Some drugs to be recommended are AF-DX 116, hexahydrosiladiphenidol, 4-DAMP, gallamine (a neuromuscular blocking agent but also an M-2 antagonist), and choline chloride and perhaps choline analogs (homocholine, betaine, false cholinergic transmitters and precursors, i.e. N-amino-N,N-dimethyl-aminoethanol, deanol.

3) It is of interest to suggest that whereas the M-1 receptor subtype may not be involved in the effects of agonists on ACh content, this subtype may be involved in behavioral responses produced by agonists and toxic responses produced by cholinesterase inhibitors as well as the direct-acting agonists. This aspect is well worth following up (please see Results section - chronic treatment)

3. Professional personnnnel

Silvana Consolo, Doctorate in Biological Sciences

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4. Interactions (Coupling Activities)

During a meeting on the Neurobiology of Acetylcholine, held in Maywood, IL, June 3-6, 1985, Dr. Ladinsky and I had the opportunity of exchanging ideas with Prof. Alex Karczmar, then retiring Chairman of the Department of Pharmacology, Univ. of Loyola School of Medicine, Maywood, IL and Consultant to the U.S. Army.

At a meeting on Muscarinic Receptor Subtypes held in Boston, Massachusetts, August 19-22, 1985 , Dr. Ladinsky met with Prof. Donald J. Jenden, Chairman, Department of Pharmacology, UCLA, Los Angeles, CA and with Prof. Henry I. Yamamura, Associate Professor, Department of Pharmacology, University of Arizona Life Sciences Center, Tucson, AZ, both of whom are well known for their expertise both in muscarinic receptor subtypes and cholinesterase inhibitors.

Dr. Ladinsky met Dr. Nigel Birdsall during a visit to Dr. Karl Thomae, GmbH, Biberach, Germany. Dr. Birdsall is a recognized expert on cholinergic mechanisms who is currently working on the purification and the chemical identification of muscarinic receptor subtypes in various mammalian organs. His intentions are to eventually clone these receptors. Dr. Ladinsky had the opportunity of exchanging views on accessibility of the various receptors to drugs.

We have met with Maj. Richard B. Drawbaugh, Ph.D., EOARD/AFSC, London in

Milan in Nov. 1985 to brief him on our results and to discuss the present proposal by Dr. Silvana Consolo for continuing work with AFOSR on projects of mutual interest.

Part of the work of this project will be presented to the meeting on the Cellular and Molecular basis of Cholinergic Function to be held in Buxton, Derbyshire, U.K., May 11-16, 1986. Copies of the abstracts are enclosed.

Table 1

Effect of the intracerebroventricular administration of DDVP on rat striatal acetylcholine content

Treatment	Striatal ACh (nmoles/g)
Saline	65.1±3.6
DDVP	86.8±3.6*

DDVP was administered i.c.v. to female rats at the dose of 45 nmoles and the rats were killed 30 min later.

*$p < 0.01$ vs the saline treated group ; Student's t-test

The data are the means ± S.E.M. (n = 6)

Table 2

Time course effects of nicotine and pirenzepine on acetylcholine content in rat striatum

Time (min)	Striatal acetylcholine (nmoles/g)	
	nicotine	pirenzepine
Saline	63.9±1.3	62.5±3.0
15	71.5±0.8*	58.5±2.9
30	75.0±3.0**	64.9±1.3
60	64.2±1.7	61.3±4.6

The drugs were administered intracerebroventricularly at the doses of 308 nmoles (nicotine) and 500 nmoles (pirenzepine).

* $p < 0.02$; ** $p < 0.01$ vs saline group; Dunnett's test

The data are the means ± S.E.M. (n=6)

Table 3

Effect of the classical muscarinic receptor antagonist scopolamine and the selective antagonist pirenzepine on the increases in acetylcholine content produced by DDVP in striatum and hippocampus of the rat

Drug	ACh content (nmoles/g)	
	Striatum	Hippocampus
Saline	64.3±1.8(8)	21.4±0.9 (6)
DDVP alone	90.0±2.3(9)*	27.0±0.8 (5)*
Pirenzepine + DDVP	93.5±3.0(8)*	23.4±0.7 (6)
Scopolamine + DDVP	63.0±2.3 (9)	22.0±1.0 (6)
Scopolamine alone	54.7±3.8 (8)*	21.0±0.8 (6)

Doses and times : DDVP 15 mg/kg,p.o., 30 min ; pirenzepine, 500 nmoles i.c.v. , 45 min ; scopolamine 15 nmoles i.c.v., 45 min.

The data were analyzed by ANOVA (2x2) factorial analysis and Tukey's test for unconfounded means

* $p<0.01$ vs saline treated group

Interactions : pirenzepine-DDVP in hippocampus $F_{1,22} = 5.7$; $p < 0.05$
" " in striatum N.S.
scopolamine-DDVP in hippocampus $F_{1,19} = 6.7$; $p < 0.02$
" " in striatum $F_{1,27} = 13,4$; $p < 0.01$

Table 4

Time course effect of pirenzepine on rat hippocampus acetylcholine content

Drug	Time (min)	Hippocampal ACh (nmole/g)
Saline	-	25.3±1.2
Pirenzepine	60	19.8±1.0**
	120	19.2±1.1**
	240	18.6±0.8**
	360	19.9±0.4**
	1440	20.9±2.0*

Pirenzepine was administered at the dose of 250 nmoles intracerebroventricularly and the rats were killed at the times shown for acetylcholine assay. At this dose, pirenzepine likely acts at M-2 muscarinic receptors.

** $p<0.01$; * $p<0.05$ vs saline

The data are means ± S.E.M. (n=6)

Table 5

Affinities of agonists and antagonists for the muscarinic receptors in rat striatum and midbrain-hindbrain regions

Inhibitor	Brain Area	R_1(%)	IC_{50_1} (nM)	R_2(%)	IC_{50_2} (nM)	nH
Pirenzepine	Striatum	38	12.3	62	230	0.78*
	Midbrain-hindbrain			95	125	0.94
Scopolamine	Striatum	98	0.44			0.88
	Midbrain-hindbrain	100	1.5			0.93
N-Methyl-scopolamine	Striatum	85	0.67	15	58.5	0.66*
	Midbrain-hindbrain	79	2.63	21	165	0.61*
Oxotremorine	Striatum	91	1460			0.67*
	Midbrain-hindbrain	94	115			0.72*
NcN-A-343	Striatum	95	5056			0.71*
	Midbrain-hindbrain	95	8052			0.67*
DDVP	Striatum		>1,000,000			
	Midbrain-hindbrain		>1,000,000			

The agonist /(^{3}H)QNB, antagonist/(^{3}H)QNB competition experiments were performed as described under 'Materials and Methods'. The data are the means of 3 experiments run in triplicate.

The IC_{50}'s of compounds showing Hill coefficients (nH's) different from unity were not corrected for the concentration of radioligand used

* Significantly less than Unity ($p<0.01$; Student's t-test)

Table 6

Saturation constants of the binding of (^{3}H)QNB to specific muscarinic receptors in striatal membranes of rats treated acutely with DDVP

	B_{max} (pmoles/g)	K_d (nM)	nH
Saline	135.64±1.9	0.038±0.0028	0.915±0.028
Chronic DDVP	136.33±5.12	0.044±0.0023	1.005±0.04

The data are the means ± S.E.M. of 3 saturation experiments run in triplicate.
Rats were given DDVP, 15 mg/kg,p.o. The animals were killed 30 min later for binding assay.

Table 7

Dose-response effect of acute DDVP administration on striatal acetylcholine content in rats treated chronically with DDVP : Tolerance Study

	Striatal ACh (nmoles/g)				
Acute dose DDVP (mg/kg,p.o.)	Saline	Saline + Chronic DDVP	Saline + Acute DDVP	Chronic DDVP + Acute DDVP	Interaction
15	66.4±2.4	69.6±1.4	89.5±1.0 **	73.4±2.4	p<0.01
20	66.6±2.3	62.1±3.3	94.1±5.4 **	67.4 ±2.2	p<0.01
50	65.0±3.4	64.8±1.3	high mortality	71.2 ±2.9	-
70	62.9±2.7	68.8±3.1	high mortality	75.2±2.8	-

The data were analyzed by ANOVA two-way factorial analysis, Tukey's test and Tukey's for unconfounded means.
The data represent the means ± S.E.M. (n=6-7)
Rats were treated with DDVP 10 mg/kg ,p.o.,two times daily for 11 days followed by 36 h withdrawal.

**p< 0.01 vs control group

Table 8

Mortality to acute challenge with high doses of cholinesterase inhibitors (DDVP and Physostigmine) or to other types of cholinergic activators (oxotremorine and choline chloride)

	Dose (mg/kg p.o. or i.p.)	Saline + acute drug challenge	Chronic DDVP + acute drug challenge
		No.of deaths/No.of animals treated	
Physostigmine	1.0 i.p.	0/7	2/6
	1.5 i.p.	0/6	5/15
	1.8 i.p.	2/7	7/7
Choline chloride	250 i.p.	0/7	4/11
Oxotremorine	1.0 i.p.	0/7	0/7
	1.6 i.p.	0/7	0/7
	3.0 i.p.	0/7	0/7
	3.2 i.p.	0/7	1/7
DDVP	15 p.o.	1/20	2/9
	20 p.o.	2/20	1/4
	30 p.o.	5/6	2/6
	40 p.o.	6/6	0/6
	50 p.o.	6/6	0/6
	60 p.o.	6/6	0/6

Rats were treated with DDVP 10 mg/kg p.o. twice daily for 11 consecutive days followed by 3 h withdrawal . The rats were acutely challenged with the drugs and doses shown in the Table. Death occurred in 30 min or the animals showed complete recovery.

Table 9

Dose-response effect of acute physostigmine administration on striatal acetylcholine content in rats treated chronically with DDVP : cross-tolerance phenomenon

Dose Physostigmine (mg/kg, i.p.)	STRIATAL ACh (nmoles/g)				Interactio
	Vehicle	Saline + chronic DDVP	Saline + acute physo-stigmine	Chronic DDVP + acute physo-stigmine	
0.5	69.1±4.2	68.0±4.4	96.7±0.2 *	73.8±2.9	p<0.01
1	69.1±4.2	68.01±4.4	95.8±4.6 *	68.2±1.2	p<0.01
1.5	67.8±3.1	66.7±2.6	110.5±2.0 *	71.5±2.6	p<0.01
1.8	-	-	high mortality	high mortality	-

* p<0.01 vs vehicle group.
The data were analyzed by ANOVA two-way factorial analysis ,Tukey's test and Tukey's test for unconfounded means.
The data are means ± S.E.M. (n=6)
The rats were treated with DDVP 10 mg/kg,p.o.,twice a day for 11 days 36 h withdrawal and were killed 20 min after physostigmine administration.

Table 10

Dose-response effect of acute treatment with physostigmine on hippocampal acetylcholine content in rats treated chronically with DDVP : Tolerance Study

	HIPPOCAMPAL ACh (nmoles/g)				
Dose Physost. (mg/kg,i.p.)	Vehicle	Chronic DDVP	Physostigmine	Chronic DDVP + Physostigmine	Interaction
0.5	22.8±0.8	23.6±2.1	33.3±1.0 *	31.0±1.8 *	N.S.
1.0	22.8±0.8	23.6±2.1	35.4±3.5 *	33.5±1.9 *	N.S.

See Legend Table 9 for details .

Table 11

Dose-Response effect of acute challenge with oxotremorine on striatal acetylcholine content in rats treated chronically with DDVP : Tolerance Study

STRIATAL ACh (nmoles/g)

Dose of Oxotremorine (mg/kg i.p.)	Vehicle	Chronic DDVP	OXO	Chronic DDVP + OXO	Interaction
1.0	62.0±2.1	65.4±2.1	81.8 ±1.8 *	60.1 ±0.3	$p < 0.01$
1.6	66.5 ±2.5	60.3 ±2.9	90.4 ±9.6 *	69.5 ±2.3	$p < 0.01$
2.4	66.0 ±1.4	60.3 ±1.1	93.9 ±2.1 *	62.2 ±3.5	$p < 0.01$
3.0	62.9 ±2.7	68.8 ±3.1	101.9 ±4.6*	78.8±2.7**	$p < 0.01$

The rats were treated with DDVP (10 mg/kg) two times daily for 11 days followed by 36 h withdrawal. The rats were killed 20 min after oxotremorine administration.
The data represent the means ± S.E.M. (n=6).
The data were analyzed by ANOVA (2x2) factorial analysis and Tukey's test for uncounfounded means.
* $p < 0.01$ vs vehicle
** $p < 0.01$ vs chronic DDVP

Table 12

Dose-response effect of acute challenge with oxotremorine on hippocampal acetylcholine content in rats treated chronically with DDVP : Tolerance Study

	HIPPOCAMPAL ACh (nmoles/g)				
Dose Oxotremorine (mg/kg i.p.)	Vehicle	Chronic DDVP	Oxo	Chronic + Oxo DDVP	Interaction
1.0	22.8±0.8	22.9±0.6	29.3±1.6*	26.9±0.8	N.S.
1.6	21.5±0.4	23.3±0.3	33.5±2.4*	26.1±0.8	$p < 0.01$
2.4	22.5±0.6	22.5±0.5	29.8±0.7*	26.4±1.2	N.S.
3.0	21.3±0.9	23.1±1.3	35.2±1.1*	27.5±1.1	$p < 0.01$

The rats were treated with DDVP 10 mg/kg p.o., two times daily for 11 days followed by 36 h withdrawal. The animals were killed 20 min after oxotremorine administration.
The data represent the means ± S.E.M. (n = 6)
The data were analyzed by ANOVA (2x2) factorial analyses and Tukey's test for unconfounded means.
* $p < 0.01$ vs vehicle.

Table 13

Effect of acute challenge with choline chloride on striatal acetylcholine content in rats treated chronically with DDVP : Tolerance Study

	Striatal ACh (nmoles/g)
Vehicle	65.9±2.0
Chronic DDVP	60.0±2.4
Choline chloride	79.8±2.6**
Chronic DDVP + Choline chloride	61.7±2.3

Choline chloride was administered i.p. at the dose of 250 mg/kg and the rats were killed after 20 min.
The data represent the means ± S.E.M. (n=6)
The data were analyzed by ANOVA (2x2) factorial analysis and Tukey's test for unconfounded means
$F_{int} = 6.7$ $p = < 0.01$
** $p < 0.01$ vs controls

Table 14

Dose-response effect of acute challenge with Quinpirole (LY 171555) on striatal acetylcholine content in rats treated chronically with DDVP : Tolerance Study

	STRIATAL ACh (nmoles/g)				
Dose Quinpirole (mg/kg i.p.)	Vehicle	Chronic DDVP	Quinpirole	Chronic DDVP + Quinpirole	Interaction
90	68.3±1.6	68.7±3.2	85.2±1.1*	73.9±3.4	$p < 0.02$
150	66.0±2.1	63.8±0.8	80.4±2.3*	77.6±3.5**	N.S.

The rats were treated with DDVP 10 mg/kg twice a day for 11 days, 36 h withdrawal and were killed 30 min after Quinpirole administration .
The data represent the means ± S.E.M. (n= 6)
The data were analyzed by ANOVA (2x2) factorial analysis and Tukey's test
* $p < 0.01$ vs vehicle
** $p < 0.01$ vs chronic DDVP

Table 15

Summary table on the tolerance and cross-tolerance to muscarinic receptor activating drugs and a D_2-dopaminergic agonist on brain regional acetylcholine content produced in rats treated chronically with DDVP

	BRAIN REGION		
Acute challenge with drugs	Striatum	Hippocampus	Cerebral cortex
DDVP	Tolerance	none	none
Physostigmine	Cross-tolerance	none	-
Oxotremorine	Cross-tolerance	none	-
Choline chloride	Cross-tolerance	-	-
Quinpirole	No cross tolerance	-	-
Scopolamine	No cross tolerance	none	none

Rats were treated with DDVP, 10 mg/kg, p.o., 2 x daily for 11 days followed by a 36 h washout period. The rats were then challenged with various doses of DDVP, physostigmine, oxotremorine, choline chloride and quinpirole for their increasing effects on brain regional acetylcholine content. The doses used are given in Tables 7, 9, 10,11, 12, 13, 14.

This Table should be compared with protection against or potentiation of mortality produced by the drugs in chronically DDVP-treated rats (Table 8).

Blank areas indicate brain regions in which the challenging drugs produce no or weak effects on acetylcholine content and so were not tested .

TABLE 16 - Effect of chronic treatment with DDVP on the heterogeneity of muscarinic receptors in rat striatum and hippocampus

	Chronic treatment	(n)	R_1 (%)	R_2 (%)	IC_{50_1} (M)	IC_{50_2} (M)	N_H
Striatum	Vehicle	(4)	31.92±3.79	68.07±3.79	$1.33 \pm 0.14 \times 10^{-8}$	$5.33 \pm 0.88 \times 10^{-7}$	0.71±0.02
	DDVP	(6)	58.71±4.54**	40.78±4.54*	$3.49 \pm 0.74 \times 10^{-8**}$	$5.59 \pm 1.22 \times 10^{-7}$	0.77±0.03
Hippocampus	Vehicle	(3)	51.01±10.49	48.84±10.32	$4.78 \pm 1.3 \times 10^{-8}$	$6.64 \pm 1.21 \times 10^{-7}$	0.78±0.05
	DDVP	(3)	54.41±8.57	45.29±7.85	$5.79 \pm 1.07 \times 10^{-8}$	$6.31 \pm 1.52 \times 10^{-7}$	0.74±0.06

* $p < 0.05$
** $p < 0.01$
vs vehicle ; Student's t test

DDVP, 10 mg/kg, p.o., 2 x daily for 11 days followed by 36 h withdrawal of drug.

Data were obtained by pirenzepine/(^{3}H)QNB competition experiments.

TABLE 17 - Effect of chronic treatment with DDVP on the muscarinic receptor binding in rat hippocampus and striatum

		B_{max} (pmoles/g wet wt)	K_D (nM)
Hippocampus	Vehicle	103.78±1.65 (4)	0.027±0.001 (4)
	DDVP	86.73±4.20 (4)*	0.029±0.003 (4)
Striatum	Vehicle	128.96±3.10 (4)	0.044±0.002 (4)
	DDVP	88.56±3.86 (4)*	0.026±0.003 (4)*

* $p < 0.01$ vs vehicle ; Student's t test

0.01-0.5 nM(^{3}H)QNB was used as radioligand .

Table 18

Binding constants of oxotremorine in striatum of rats treated chronically with saline or DDVP

Chronic treatment	IC_{50} (nM)	nH
Saline	5.05±0.78	0.80±0.41
Chronic DDVP	3.50±0.58	0.86±0.49

The data are the means ± S.E.M. of 3 competition experiments run in triplicate.
Rats were given DDVP, 10 mg/kg, p.o., two times daily for 11 days followed by 36 h withdrawal.
Oxotremorine /(^{3}H)QNB competition experiments were performed using 0.05 nM of the radioligand.
The IC_{50} values were obtained by non-linear least squares regression analysis on the basis of a one binding site model. Hill coefficients (nH) were calculated by linear regression analysis and assessed for statistically significant deviation from unity .

Table 19

Down regulation of the M-1 muscarinic receptor subtype in striatum of rats treated chronically with DDVP as determined by (^{3}H)pirenzepine binding.

Conc.of ligand (nM)	Specific binding of (^{3}H) PZ (pmole/g wet wt)		
	Saline	Chronic DDVP	% Control
0.5	4.00 (4.1;3.9)	2.85 (2.8;2.9)	68.25
1.0	8.10 (8.0;8.2)	5.25 (5.4;5.1)	64.81

The rats were treated chronically with DDVP for 11 days (10 mg/kg p.o.)x two times daily). The study was performed 36 h after last treatment.
The data represent the means of two experiments run in triplicate. The duplicate values are shown in brackets.

(^{3}H)PZ selectively labels M-1 receptors at the given concentrations.

Table 20

Down regulation of the M-1 receptor subtype in hippocampus of rats treated chronically with DDVP as determined by (^{3}H) pirenzepine binding

Conc.of ligand	Specific binding of (^{3}H)PZ (pmole/g wet wt)		
	Control (saline)	Chronic DDVP	% Control
0.5	2.07 (2.0 ; 2.15)	1.40 (1.3; 1.5)	69.24
1.0	3.89 (4.0 ; 3.8)	2.76 (2.7; 2.8)	70.86

The data represent the means of two experiments run in triplicate. The duplicate values are shown in brackets.

Please see Legend to Table 19

Table 21

Binding constants of pirenzepine in striatum of rats treated chronically with saline or DDVP

	IC_{50} (M)	nH
Saline	$1.66 \pm 0.0176 \times 10^{-8}$	1.0 ± 0.004
Chronic DDVP	$1.99 \pm 0.0119 \times 10^{-8}$	0.98 ± 0.05

The data are the means ± S.E.M. of 3 pirenzepine /(^{3}H)pirenzepine competition experiments run in triplicate.
Rats were treated with DDVP, 10 mg/kg,p.o., twice a day for 11 days followed by 36 h withdrawal.
(^{3}H) pirenzepine was used at the concentration of 0.5 nM which specifically labels M_1 receptor subtype.

Table 22

Binding constants of pirenzepine in hippocampus of rats treated chronically with DDVP

	IC_{50} (M)	nH
Saline	$1.22 \pm 0.08 \times 10^{-8}$	0.91 ± 0.04
Chronic DDVP	$1.53 \pm 0.23 \times 10^{-8}$	0.98 ± 0.09

The data are the means ± S.E.M. of 3 pirenzepine/(^{3}H)pirenzepine competition experiments run in triplicate.
Please see Legend of Table 21 for further details.

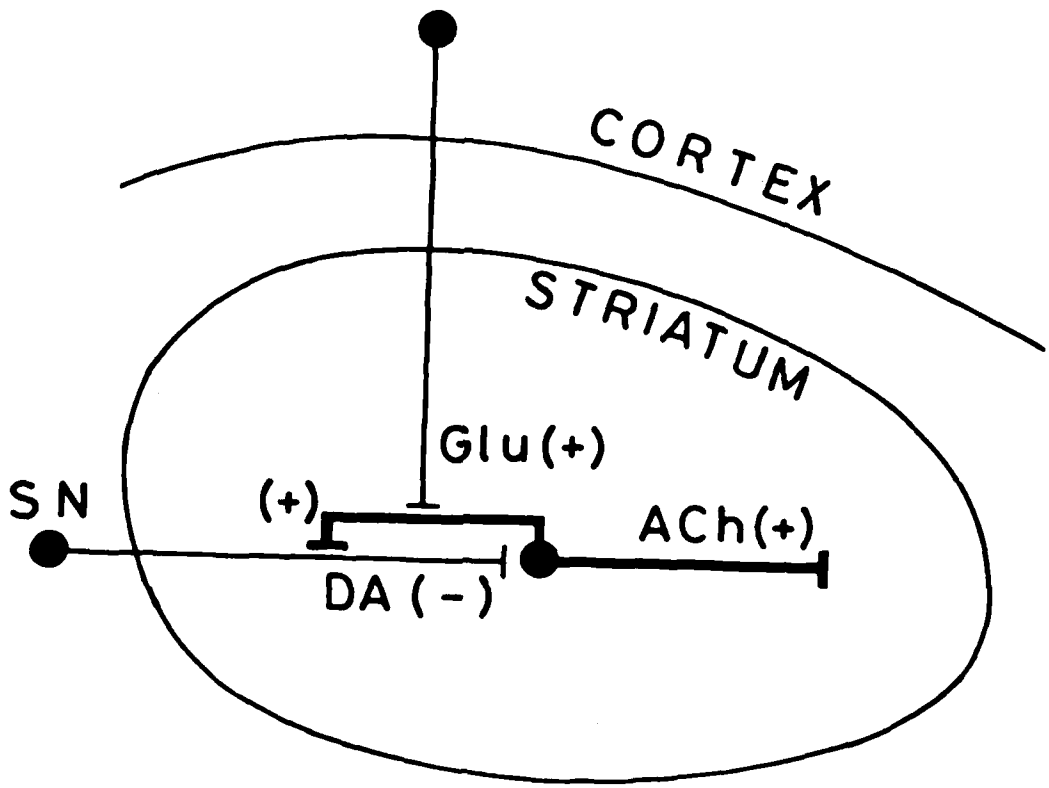


FIG. 1 -

Possible arrangement of the dopaminergic,cholinergic and putative glutamatergic neurons in the striatum. Cholinergic activity may be regulated by a balance between the excitatory and inhibitory neurotransmitter influences. Chronic interruption of the excitatory input alters the steady state and brings the cholinergic interneurons to a new,lowered functional equilibrium state. In this depressed condition,the cholinergic neurons are refractory to dopamine receptor agonists. Please see the Preface section for reference to this figure.

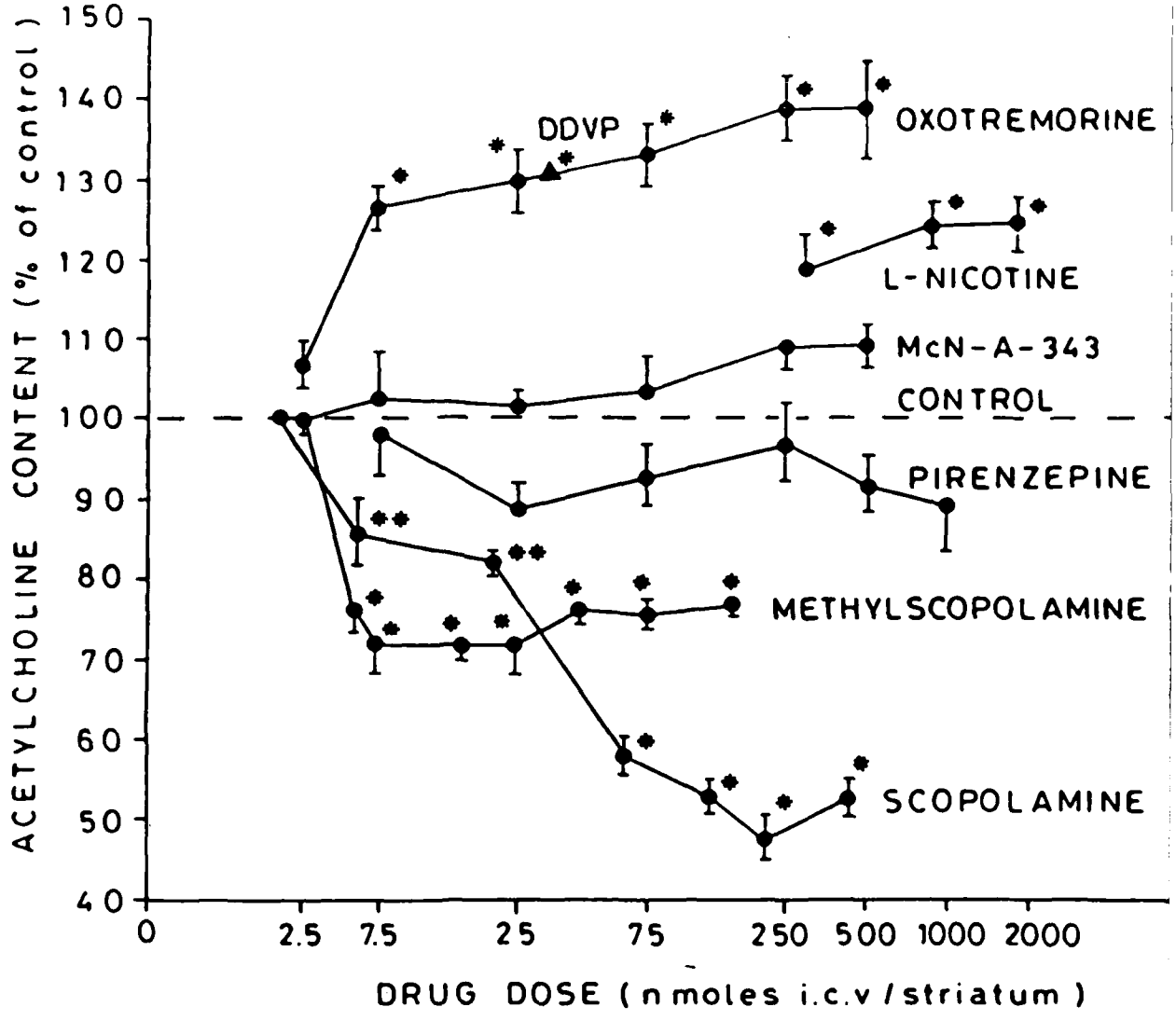


FIG. 2 -

Dose-response curves of the effect of cholinergic agents oxotremorine, DDVP, nicotine, McN-A-343, scopolamine, N-methylscopolamine and pirenzepine on rat striatal acetylcholine content. The drugs were given intracerebroventricularly to alert rats through a cannula implanted 24 h earlier and the animals were killed by focussed microwave irradiation to the head at the following times after drug administration: 20 min for oxotremorine; 15 min for McN-A-343; 30 min for the other drugs. Acetylcholine was determined by a radioenzymatic method. None of the drugs interfered with the assay when 500 nmoles of each were added to blanks and carried through the entire procedure. The abscissa shows the drug doses on a logarithmic scale and the ordinate gives the content of acetylcholine as a percentage of the control which was found to be 65.2±3.2 nmoles/g tissue. The concentrations of several of the drugs (scopolamine, N-methylscopolamine and pirenzepine) in striatum were determined by radioreceptor assay (please see Methods). The actual measured concentrations of scopolamine, a hydrophobic drug that rapidly leaves the brain, are given. Vertical bars represent the S.E.M. of 6-8 rats per point.

* = $p<0.01$; **=$p<0.05$ vs saline-treated group; Dunnett's test.

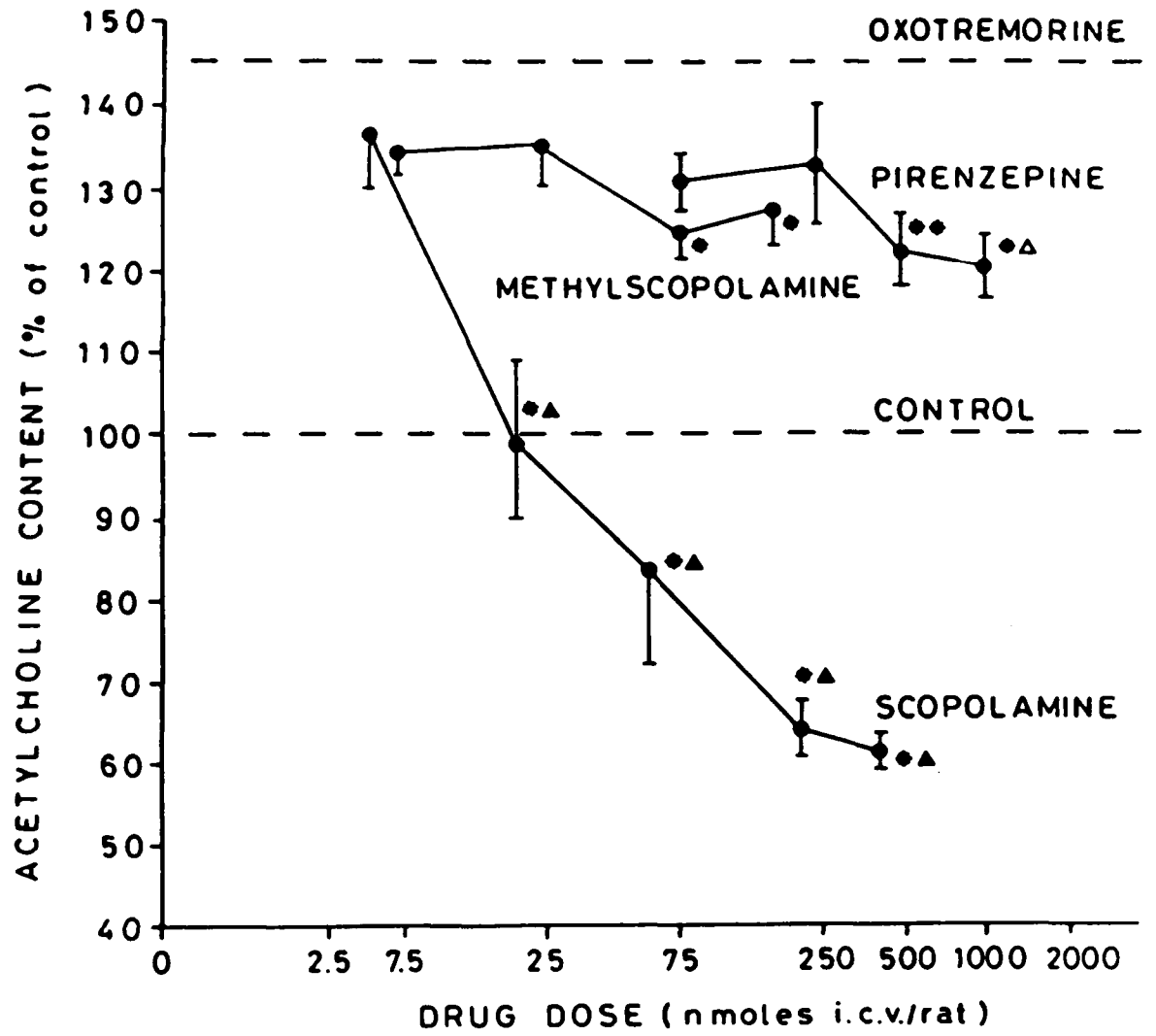
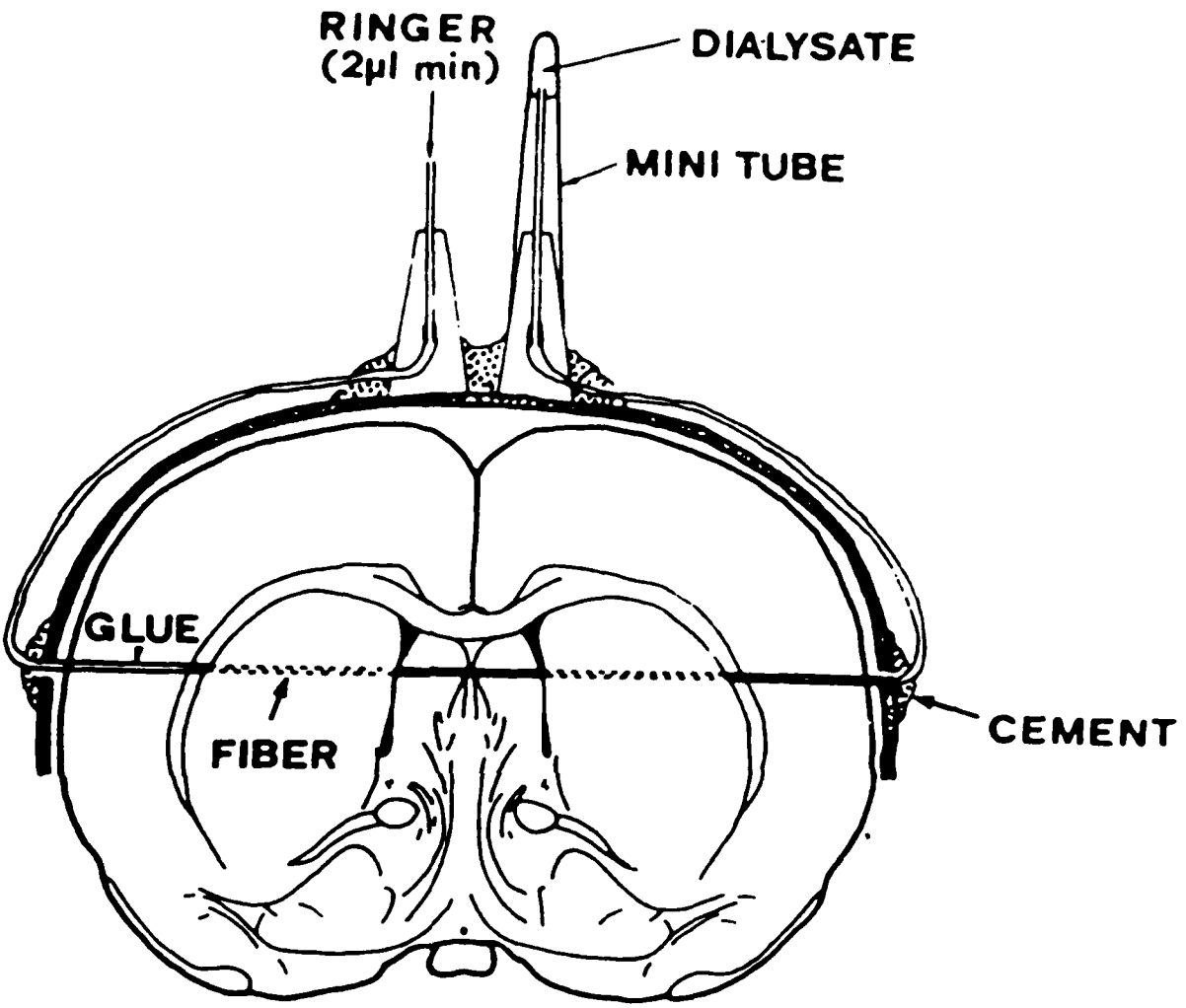


FIG. 3 -

Dose-response curves of scopolamine, N-methylscopolamine and pirenzepine in antagonizing the increase in striatal acetylcholine induced by oxotremorine. Oxotremorine was administered intraperitoneally to rats at the dose of 1 μmole/g, the minimum dose yielding the maximal increase. The selective and non-selective muscarinic receptor antagonists were administered intracerebroventricularly 10 min before the administration of oxotremorine and the rats were microwaved 20 min after oxotremorine injection. The abscissa shows the drug doses on a logarithmic scale (please see Legend to Fig.2) and the ordinate gives the content of acetylcholine as a percentage of the control value in the presence of oxotremorine. The vertical bars represent the S.E.M. of 6-8 rats per point. *=$p<0.01$; **=$p<0.001$ vs the saline treated group;Dunnett's test.

FIG. 4



Schematic diagram of a trans-striatal dialysis preparation in an awake rat.

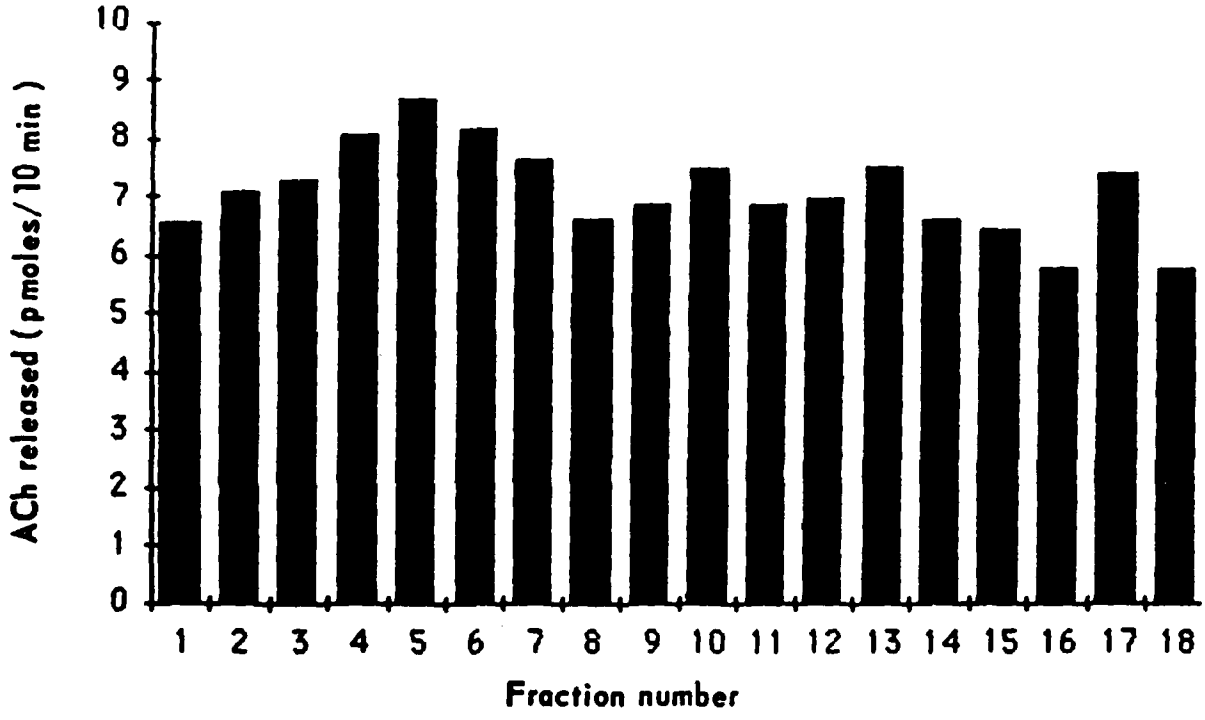


FIG. 5

Release of acetylcholine from rat striatum in vivo determined by the trans-striatal dialysis technique coupled with radioenzymatic assay ; effect of physostigmine. The striata were perfused with Krebs solution (as depicted in Fig. 4) containing 10 μM physostigmine (please see Methods). Perfusates were collected every 10 min over a period of 200 min. The abscissa shows the fraction number and the ordinate shows the amount of acetylcholine released expressed as p moles/10 min. A relatively constant release of acetylcholine was obtained over the entire period of the experiment. Similar results were obtained when a lower concentration of physostigmine (1 μM) was added to the perfusion fluid. The columns show the results of a typical experiment which was run in triplicate. In the absence of physostigmine, no acetylcholine was detected .

ON THE FUNCTIONAL ROLE OF MUSCARINIC RECEPTOR SUBTYPES IN RAT STRIATUM AND HIPPOCAMPUS: AN IN VIVO STUDY.

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Limited are the in vivo studies correlating the multiple muscarinic cholinergic receptor subtypes in the brain with functional parameters. The aim of this study was to clarify the nature of the muscarinic receptor involved in the feedback regulation of cholinergic neurotransmission in striatum and hippocampus using, as a functional parameter, the in vivo effectiveness of typical and atypical muscarinic receptor antagonists administered i.c.v. in altering striatal acetylcholine (ACh) content. In parallel, the selectivity of the various agents was determined in competition experiments against (^{3}H)QNB in membrane preparations from the two areas.

It was found that in striatum, the classical antagonist scopolamine (SCOP), which displays uniformly high binding affinity for M-1 and M-2 receptors, produced a 50% decrease in the ACh level at the peak dose of 75 nmoles and blocked the OTMN-induced increase in ACh content. Instead, its hydrophilic quaternary analog, N-methylscopolamine (NMS), induced at 30 min a decreasing effect of only about 20% from 5-150 nmoles and it did not antagonize OTMN even at the highest dose used. However, when NMS was allowed to act for longer periods of time (4-16 hr) it was able to produce an ACh lowering effect similar to that induced by SCOP within 15-30 min. Pirenzepine (PZ) the antagonist at M-1 receptors, at 30 min, did not alter striatal ACh content up to the i.c.v. dose of 1000 nmoles/rat and was not able to antagonize the ACh-accumulating effect of OTMN. However, when a time course study was performed with PZ at the non-M-1-selective dose of 250 nmoles, the drug was found to produce a large decrease within 4-16 hr, similar to that of NMS.

In the hippocampus, SCOP (75 nmoles), NMS (75 nmoles) and PZ (250 nmoles) decreased ACh content to the same extent at all times studied.

In conclusion, it is tentatively suggested that the muscarinic receptors mediating the feedback regulation of cholinergic neurotransmission are of the M-2 subtype. The data further imply that the receptors in the striatum, differently from those in the hippocampus, are not fully accessible to hydrophilic receptor antagonists. Binding data from competition experiments support this latter hypothesis.(Supported by AFOSR Grant No. 85-0025)

DETERMINATION OF IN VIVO ACETYLCHOLINE RELEASE BY TRANS-STRIATAL DIALYSIS COUPLED TO A RADIOENZYMATIC ASSAY: EFFECT OF DRUGS:

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By using a new technique, intracerebral dialysis in combination with a sensitive and specific radioenzymatic method it was possible to recover and measure endogenous extracellular acetylcholine(ACh) from the striatum of freely moving rats. The procedure used to insert the dialysis tube was essentially the same as already described by others (1,2). A thin dialysis tube (AN 69 membrane, DASCO) with a molecular weight cut-off >15,000 was inserted transversally through both caudate nuclei. The tube was perfused with a Ringer solution pH 6.1 (NaCl 147.2 mM; $CaCl_2$ 3.4 mM; KCl 4.0 mM) containing physostigmine sulphate 10 µM at a constant rate of 2 µl /min. The perfusates were collected at 10 min intervals in small replaceable test tubes containing 5 µl of 0.5 mM HCl to avoid ACh hydrolysis.

The biochemical procedure for the measurement of extracellular ACh levels consists in the isolation of ACh by low voltage paper electrophoresis and in the conversion of the residual contaminating choline or other choline analogs to phosphorylated products in the presence of choline phosphokinase and ATP. The ACh is then quantitatively hydrolyzed and the resultant choline is enzymatically acetylated with tritiated acetylcoenzyme A of high specific activity and counted after extraction into toluene based phosphor by liquid-liquid cation exchange chromatography (3). The results obtained show that without AChE inhibitor, ACh spontaneous release was 0.09 ±0.01 pmol/min/striatum, a value which is at the threshold of the method. In the presence of 1 µM and 10 µM of physostigmine the ACh levels in superfusates were found to be 0.45 ±0.02 and 0.83±0.03 pmol/min/ 2 striata, respectively. The latter concentration of the AChE inhibitor was used in all experiments. In basal conditions the output of ACh is stable over at least 5 hr. Increase of the K^+ concentration in the Ringer to 100 mM produced a sharp, reversible 87% increase of ACh output. Both the basal and K^+ stimulated release were Ca^{++} dependent. In addition scopolamine (0.5 mg/kg. s.c.), a muscarinic receptor antagonist, was shown to produce a long-lasting enhancement of ACh release by about 60% whereas oxotremorine (1mg/kg. i.p.), a muscarinic agonist reduced transiently the ACh release(44%). Hemicholinium-3 (20 µg i.c.v), the choline uptake inhibitor, also reduced ACh output to 29.7% of basal values. Thus, classical tests known to interfere with the synthesis, release and metabolism of ACh produce the expected changes in the release of ACh providing evidence for the physiological suitability of the method. (Supported by AFOSR Grant No. 85-0025)

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ACUTE ADAPTIVE CHOLINERGIC MECHANISMS IN CNS TRIGGERED BY ANTICHOLINESTERASE INSULT.

H. Ladinsky, F. Fiorentini, P. Cicioni, S. Consolo. Mario Negri Institute for Pharmacological Research, Milan, Italy.

The objective of this study was to determine whether the CNS resorts to acute adaptive mechanisms to reduce cholinergic tone in an attempt to fend off the toxic effects of an anticholinesterase poison. The effect of dichlorvos on rat brain ACh content was characterized. Optimal treatment (20 mg/kg, p.o., 30 min) increased ACh contents in hemispheric regions but not in cerebellum or midbrain-hindbrain. Peak increases were attained within 30 min in striatum, hippocampus and cortex but fall-off time was 5x slower in striatum denoting that AChE influences cholinergic activity differently in these brain regions. Our approach was to attempt to identify whether the accumulation of ACh in brain is extraneuronal or intraneuronal. Results with atropine, reserpine, inhibitors of monoamine synthesis and certain lesions suggest that much of the ACh buildup occurs intraneuronally secondarily to muscarinic receptor stimulation by the protected synaptic ACh through the activation of inhibitory feedback loops involving monoamines. Supported by AFOSR Grant No. 82-0306A.

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