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Chronic Administration of Cholinergic Agents: Effects on Behavior and Calmodulin

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LOULLIS, C. C., R. L. DEAN, A. S. LIPPA, L. R. MEYERSON, B. BEER AND R. T. BARTUS. *Chronic administration of cholinergic agents: Effects on behavior and calmodulin.* PHARMACOL BIOCHEM BEHAV **18**(4) 601–604, 1983.—Rats were implanted subcutaneously with Alza® pumps and 0.9% saline, physostigmine, or scopolamine were continuously infused for 15 days. Twenty-four hours after removal of the pumps all animals were trained on a single trail passive avoidance task. Twenty-four hours after training they were tested for retention. Following behavioral testing, animals were sacrificed, brain regions dissected, frozen and stored (−20°C) for calmodulin determinations. Animals which had previously received chronic infusions of scopolamine performed significantly better than controls, while those which previously received chronic infusions of physostigmine performed significantly worse during the retention test. No significant differences in calmodulin levels (soluble or particulate) were detected across brain regions or drug groups. These results indicate that continuous chronic infusion of drugs which can facilitate or inhibit CNS cholinergic activity can induce performance changes on a learning task opposite to those resulting following the acute administration of these same drugs.

Cholinergic agents Scopolamine Physostigmine Calmodulin Passive avoidance Retention

DATA from many laboratories demonstrate that changes in cholinergic neurotransmission, induced through pharmacological and/or precursor manipulation, can have profound effects on performance or retention of learning tasks. In general, administration of anticholinergics impairs retention, presumably by blocking the action of Ach at postsynaptic receptor sites, while that of cholinomimetics or acetylcholinesterase inhibitors enhances retention by mimicking the action of ACh or increasing ACh synaptic concentrations at the receptor (for review see [2]).

Changes in retention would also be expected following modification of muscarinic receptor sensitivity, since the action of endogenous ACh will be enhanced or diminished by an increase or decrease in receptor sensitivity. Recent data suggest that chronic administration of cholinergic and anticholinergic drugs can provide a means for bringing about such changes in muscarinic receptor sensitivity. More specifically, when cholinergic antagonists are administered chronically, an increase in muscarinic receptor density has been observed [3, 17, 21], whereas chronic administration of acetylcholinesterase inhibitors or cholinergic agonists induced a decrease in muscarinic receptor density [7, 8, 17–21]. On the basis of these data, it would be predicted that chronic administration of an anticholinergic drug would, following its elimination, result in enhancement of retention; and that of a cholinomimetic or acetylcholinesterase inhibiting drug would result in impairment of retention.

The receptor sensitivity changes which occur following chronic drug administration may also be associated with changes in post receptor coupling. An important component of these processes is calmodulin (CaM)—a small, thermostable, acidic calcium binding protein [5,6] found in brain [23] and other tissue [13] and present at central synapses [9,23]. In vitro and in vivo experiments in the dopaminergic system suggest that cellular changes in the ratio of soluble (or cytosolic) versus membrane-bound CaM are correlated with receptor responsiveness [10,12]. Thus in striatum, long term stimulation of the dopaminergic receptor is associated with an increase in soluble CaM levels and receptor subsensitivity, whereas long term blockade of the same receptor is associated with an increase in membrane bound calmodulin and receptor supersensitivity. To our knowledge there are no reported data regarding levels of CaM following chronic administration of cholinergic drugs. One in vitro study indicates, however, that the loss of muscarinic receptors under phosphorylating conditions is dependent on CaM [4]. Therefore, it is likely that CaM plays a pivitol role in membrane receptive/coupling events.

The present study examines the effects of chronic administration of cholinergic agents upon the retention of a learning task, forty-eight hours following the elimination of these agents. The effects of these same treatments on possible changes in the levels of soluble and membrane-bound CaM are also examined.

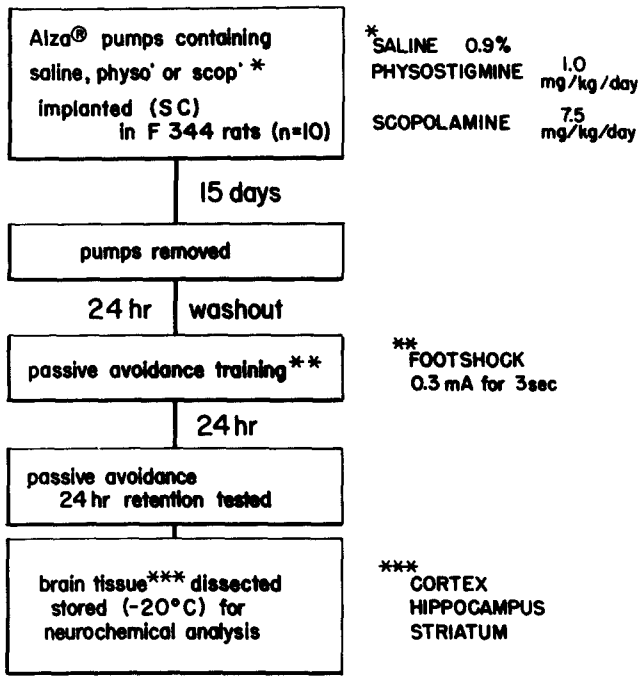

FIG. 1. A flow diagram of the methods.

METHOD

Subjects

Thirty, 7-month old Fischer 344 male rats (Harland Industries, IN) were used. Their mean body weight prior to the beginning of the experiment was 341±18 S.D. g. Animals were housed in pairs in our colony under a constant light-dark cycle (light 600–1800 hr) and were allowed to adapt to their home cages for at least 15 days. Food and water were available ad lib throughout the experiment.

PROCEDURE

Chronic Drug Infusion

Following adaptation, animals were divided into three groups (n=10) and implanted subcutaneously with Alza® pumps (Alza Corp., CA) containing either 0.9% saline, physostigmine (1 mg/kg/day) or scopolamine (7.5 mg/kg/day) based upon a mean osmotic infusion rate of 0.493 ml/hr. These agents were continuously delivered for 15 days and infusion was terminated by removal of the pumps.

Behavioral Testing

Twenty-four hours following the removal of the pumps, all animals were trained on a single trial passive avoidance task. The passive avoidance apparatus was modified from Bartus *et al.* [1] and consisted of two chambers, a smaller lighted and larger dark compartment. Access from one to the other compartment could be controlled through a vertically moving trap door.

On the training day, each animal was placed in the lighted compartment, the trap door was raised and when the animal fully entered the dark compartment, a 0.3 mA footshock was delivered for 3 seconds. The latency in sec to enter the dark compartment was recorded and the animal was removed from the chamber and returned to its home cage. Twenty-four hours after training animals were tested for retention in the exact manner as above except that no footshock was delivered when the animal entered the dark compartment. The latency in sec to enter the dark compartment was recorded. A 600 sec maximum latency criterion was selected.

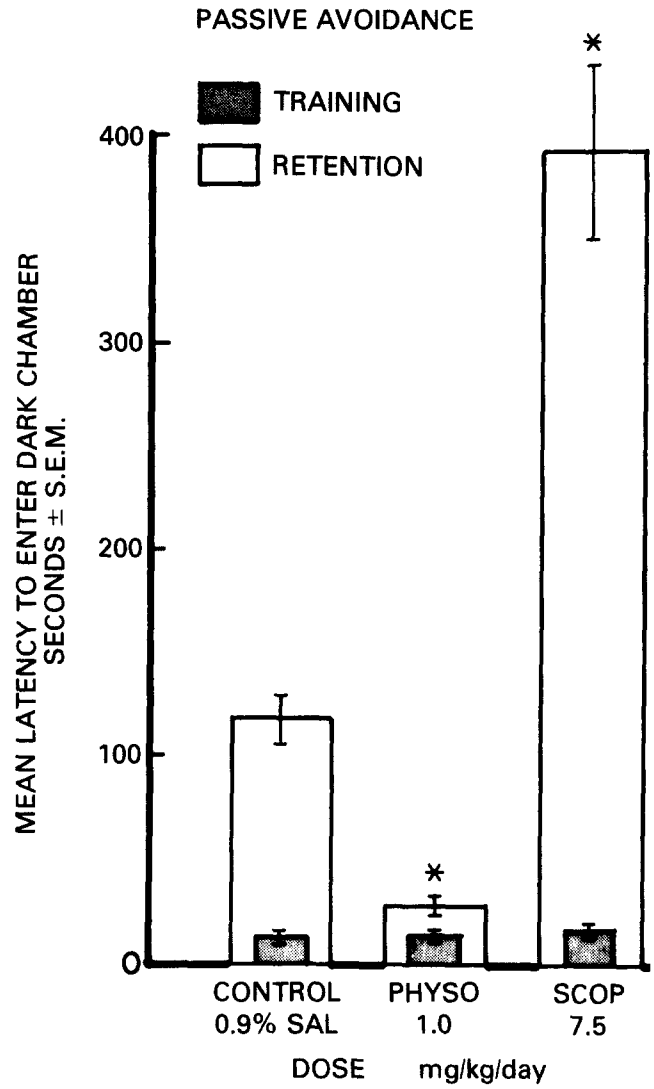

FIG. 2. The training and retention latencies for the control, chronically treated physostigmine and scopolamine groups are illustrated. All animals were trained 24 hours and tested 48 hours following pump removal. Asterisks indicate significant differences from control ($p<0.05$).

Dissection

Following behavioral testing, all animals were sacrificed by decapitation and the cortex, hippocampus and striatum were dissected on ice, quickly frozen and stored (−20°C) for CaM determinations.

TABLE 1

CaM LEVELS FOLLOWING CHRONIC DRUG TREATMENT

Group	Fraction	Striatum	μg/mg protein Hippocampus	Cortex
Control	Soluble	3.40 ± 0.27	1.62 ± 0.17	1.58 ± 0.15
	Membrane-bound	2.75 ± 0.32	1.57 ± 0.15	1.10 ± 0.11
	Total	2.34 ± 0.20	1.18 ± 0.20	0.81 ± 0.06
Physostigmine	Soluble	3.20 ± 0.24	1.61 ± 0.13	1.57 ± 0.23
	Membrane-bound	2.98 ± 0.35	1.68 ± 0.15	1.14 ± 0.15
	Total	2.21 ± 0.16	1.25 ± 0.22	0.95 ± 0.09
Scopolamine	Soluble	3.40 ± 0.23	1.47 ± 0.11	1.60 ± 0.14
	Membrane-bound	2.32 ± 0.15	1.53 ± 0.20	1.03 ± 0.09
	Total	2.29 ± 0.18	1.13 ± 0.20	0.79 ± 0.07

Values are means ± S.E.M.

Tissue Extraction and Radioimmunoassay for Calmodulin

Two 100 μl aliquots from a 0.32 M sucrose, 1:10 (w/v), homogenate were sampled. The first aliquot was used in order to determine total tissue CaM. It was diluted 1:10 (v/v) with extraction buffer (15 mM Tris buffer, pH 7.4, containing 0.1% Lubrol PX and 1 mM EGTA). The second aliquot was used in order to determine soluble and membrane-bound CaM.

This aliquot was subjected to centrifugation at 100,000 ×g for 30 min and the resultant supernatant containing soluble CaM was diluted 1:10 (v/v) with the extraction buffer. The pellet fraction was resuspended in 1 ml of the extraction buffer and homogenized for 30 sec using a polytron (Tekmar Co., OH) at 40% of maximum speed. All three fractions were heat inactivated (5 min at 90°C) and rapidly cooled in a bath containing a mixture of dry ice and methanol. All fractions were subjected to centrifugation at 10,000 ×g for 30 min and the resulting supernatants were appropriately diluted and their CaM contents determined by Radioimmunoassay (CAABCO Inc., TX).

Proteins for each fraction were determined (Bio-Rad protein assay, Bio-Rad, CA) from aliquots taken following the addition of the extraction buffer.

Statistical Analysis

The behavioral and CaM data were analyzed by one way analyses of variance. Following a significant overall F value, a two-tailed Dunnett *t*-statistic was used to test for significance between control and drug treatment means.

RESULTS

Results of the passive avoidance test are presented in Fig. 2. The mean latencies, in sec, to enter the dark chamber for control, physostigmine and scopolamine groups, from the training day (24 hr following drug elimination) are depicted by stippled bars. The mean latencies from the retention test (24 hr later) are depicted by the open bars. There were no significant differences in latency between groups on the training day. On the retention day, however, animals which had previously received physostigmine, showed significantly decreased latencies and those which received scopolamine significantly increased latencies to enter the dark chamber, $F(2,27)=5.49$, $p<0.01$.

No significant differences across drug treatments in any of the brain regions examined were observed in total, membrane-bound CaM, soluble CaM or the ratio of soluble to membrane-bound CaM. These data are presented in Table 1.

DISCUSSION

The results of the behavioral data from this study indicate that chronic infusion of drugs known to facilitate or inhibit cholinergic activity can induce robust changes in behavior which can persist beyond metabolism and elimination of the drugs. Available pharmacokinetic data [14,22] indicate that both physostigmine and scopolamine would be excreted and biotransformed by the time the animals were tested. It is therefore very unlikely that changes in behavior can be directly attributed to the in vivo presence of these drugs. Interestingly, these behavioral effects are opposite to those which occur following their acute administration. These results can be most parsimoniously accounted for by the changes which others have reported in muscarinic receptor sensitivity following chronic cholinergic stimulation or blockade [3, 7, 8, 17–21]. According to these studies physostigmine induces an increase in ACh concentrations at the synapse followed by a decrease in receptor sensitivity. Accordingly, upon its metabolism and elimination, therefore, the effect of endogenous ACh would be diminished and an impairment in retention expected, as we report here. Similarly, in scopolamine-treated animals, chronic blockade of the receptor induces an increase in receptor sensitivity [3,21]. Upon its metabolism and eliminiation, therefore, the effect of endogenous Ach would be facilitated, with a concomitant enhancement in retention, as we also observed.

The lack of significant changes in CaM, in the face of apparent alterations in receptor density [3, 7, 8, 17–21] and behavioral measures (this study) suggest that calmodulin may not play a role in cholinergic receptor modulation. However, alternative interpretations do exist and could be related to differences in time parameters. In our study, levels of CaM were determined at 48 hour post drug removal. It is possible that changes in CaM following cholinergic stimulation persist for only a few hours past drug removal while the

changes in receptor density persist for at least 48 hours. This raises the possibility that the time course of the CaM changes is different following cholinergic versus dopaminergic stimulation. This possibility cannot be evaluated on the basis of the available data. Alternatively, changes in CaM, following chronic drug stimulation of the cholinergic system, may be more subtle than those occurring in the dopaminergic system and thus more difficult to demonstrate with present assaying techniques.

Another point of possible interest is the distribution of CaM across brain regions. The highest levels are found in the striatum while the hippocampus and cortex contain approximately the same amounts. These findings raise the possibility of differences in CaM function across brain regions.

Clearly, further in vivo and in vitro studies are necessary in order to clarify the role of CaM following cholinergic and other drug stimulation as related to receptor responsiveness.

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