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Long-Term Suppression in Mice of the Development of Complementary Memory Storage Sites: Effect of a Muscarinic Antagonist

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FLEXNER, L. B., J. B. FLEXNER AND A. C. CHURCH. *Long-term suppression in mice of the development of complementary memory storage sites: Effect of a muscarinic antagonist.* PHARMACOL BIOCHEM BEHAV **39**(3) 689–694, 1991.—Bitemporal injections of puromycin consistently induce amnesia of aversive maze-learning in mice when administered within 3 days of training. These bitemporal puromycin injections lose their amnestic effectiveness, if the latency between training and injection is extended beyond 6 days. Consistent with other evidence, we conclude that in our experimental paradigm, complementary memory storage sites normally develop in additional cerebral areas during the 6 days following training. Previous experiments have indicated that the central adrenergic system is critically involved in this process. We now present evidence that the central cholinergic system is also critically involved. This conclusion is based upon our results with the muscarinic receptor antagonists, scopolamine and methyl scopolamine.

Memory Muscarinic receptors Scopolamine Methyl scopolamine Puromycin Long-term potentiation Hippocampus T-maze

DAMAGE to the hippocampal area of man (12, 16, 21), monkey (13), cat (29) and rodents (9, 11, 14, 17) has been found to severely impair recent memory while remote memory is largely spared. This observation has led to the concept that memory storage sites which appear to be initially limited to the hippocampal area, increase with time to involve additional cerebral areas. Consistent with this view is the finding that bitemporal intracerebral injections of the amnesic agent, puromycin, that affect the hippocampal-entorhinal area, consistently induce amnesia in mice if administered within 3 days of aversive Y-maze training. Production of amnesia at times longer than 6 days after training requires a combination of bitemporal plus biventricular plus bifrontal injections (T + V + F) of the antibiotic that affect widespread neocortical sites in addition to the hippocampal-entorhinal area (9).

Using this change in sensitivity to puromycin, we have found that the central adrenergic system is essential for the development of complementary memory sites (2–6). The experiments reported here were designed to test the effect of subcutaneous (SC) injections of the antimuscarinic agent, scopolamine (SCO), on the increase in memory storage sites. Specifically we have investigated: 1) the effect of SCO dose on the basic amnesic properties of puromycin; 2) the effect of SCO dose, administered post- or pretraining, on the normal increase in memory storage sites; 3) the duration of SCO's effect on storage sites; 4) the degree and duration of cerebral muscarinic receptor blockade following administration of SCO; and 5) the effect on the increase in memory storage sites of methyl SCO, a muscarinic receptor antagonist that fails to cross the blood-brain barrier.

METHOD

Drug Injection

The intracerebral injection technique has been fully described (3). Briefly, mice were lightly anesthetized with sodium hexobarbitone (Evipal, 150 mg/kg, IP). Bitemporal injections were made at a depth of 2 mm from the surface of the skull through needle holes in the skull located just above the angle between the caudal sutures of the parietal bones and the origins of the temporal muscle. Each bitemporal injection of puromycin contained 90 μg of puromycin dihydrochloride (ICN Pharmaceuticals) dissolved in 12 μl of distilled water and brought to pH 7 with NaOH. With bitemporal plus biventricular plus bifrontal (T + V + F) injections, each injection contained 30 μg per 12 μl of distilled water. It should be noted that a total of 180 μg of puromycin was used in both the 2 and 6 injection protocols. A peptidyl conjugate of puromycin that acts at synaptic sites in the

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nervous system appears to be the amnesic agent (7).

SCO and its methyl bromide derivative (Sigma) were dissolved in water and 0.1–0.2 ml was injected subcutaneously. All mice survived this treatment in excellent condition.

Animals and Behavioral Procedures

The Swiss-Webster mice of our closed colony were maintained, bred and tested by a single investigator (J.B.F.). In all the animal handling procedures, an effort was made to keep nonspecific stress to a minimum. As previously detailed (3), male and female mice (6–9 months, 30–35 g) were randomly selected and then trained in a single session in a Y-maze to a criterion of 9 out of 10 correct responses. Intermittent footshock, manually applied (0.2–0.4 mA from a DC source; 2 seconds on, 2 seconds off), was given for failure to move from the stem of the Y within 5 seconds and for errors of left-right discrimination. Shock was adjusted with individual mice to the minimal level (not less than 0.2 mA) that produced the desired behavioral response. The shock levels were calibrated by measurements of current taken directly from the source. After entering the correct arm of the maze and remaining there for 10 seconds, the mouse was allowed to climb up a ladder and was returned to its home cage for 30 seconds before starting the next trial. Without knowledge of the specific treatment given each mouse, the same procedure was used in testing for retention of memory of the training experience. The retention tests were delayed, however, for 10–15 days following puromycin. This waiting period ensured that the mice had recovered from the acute effects of the puromycin (lethargy, aphagia, adipsia, excitability on handling but no convulsions). Thus, the mice ran normally in the maze and, as previously noted, 90% of the testing errors were errors of discrimination.

Total errors were the sum of failures to make a choice within 5 seconds plus incorrect choices, i.e., all mistakes were added until the mouse performed correctly in 9 out of 10 consecutive trials.

The Mann-Whitney U-test was used for statistical comparisons between groups; median scores are used in presentation of the data with the range of variation shown in the figure legends.

Receptor Binding Assays

We have used an ex vivo receptor-binding assay largely as described (18). In this assay subjects are treated in vivo with a receptor ligand and the degree of receptor occupancy is then assessed in vitro.

Mice were sacrificed by cervical dislocation. The cerebral hemispheres were rapidly removed, frozen in liquid nitrogen, and stored at −70°C. Subsequently, the tissue was homogenized in 8 volumes of ice-cold 50 mM Na-K phosphate buffer (pH 7.4 at 27°C) with 10 strokes of a motor-driven Teflon/glass homogenizer. The crude membranes were then used immediately without washing. Between 3 and 4 mg of the membranes were incubated at 27°C with 1 nM [^{3}H]quinuclidinyl benzilate (QNB) (Amersham; 42 Ci/mmol) for 1 h in a final volume of 1 ml of buffer. Samples were filtered under reduced pressure with Whatman GF/B filters and then washed 3 times with 4 ml of ice-cold assay buffer. Specific binding (20) was defined as the difference in the amount of [^{3}H]QNB bound in the absence and presence of 1 mM atropine sulfate (Sigma). All samples were run in duplicate.

An estimate was made of the loss of SCO bound to receptors that occurred during homogenization and subsequent incubation. Dissociation of SCO from receptors during homogenization was estimated by measuring the gain in QNB binding of an aliquot treated with 20 strokes over that of a second aliquot treated with 10 strokes of the homogenizer. Loss during incubation was estimated by measuring the increase in QNB binding of an aliquot of a homogenate that was preincubated for 1 h before adding QNB over that of an aliquot to which QNB was added at the start of the routine incubation period of 1 h. Total gain of specifically bound QNB measured in these ways was 17% of the standard value (n=4). Routine assay values of QNB binding were correspondingly corrected.

EXPERIMENTS

In all of the following experiments, mice received a single subcutaneous injection of SCO or methyl SCO. In initial experiments, a dose of 3 mg/kg of SCO was used because many other studies of learning and memory also used this dose. As the dose response data from Experiment 2 shows, 3 mg/kg of SCO did not significantly affect memory spread. A lower dose (1.5 mg/kg) was, however, effective. In an effort to gain insight into the reason for this difference, several experiments were conducted with these two doses of SCO.

Experiment 1

Rationale. This experiment was designed to test whether SCO produces effects that would interfere with our tests of memory spread. These tests consisted of 4 groups of experimental controls.

1) The first control study was conducted to determine whether SCO affects learning and/or relearning of the Y-maze. In this test, mice were treated with SCO or saline, 1 day later trained to criterion in the Y-maze and then, after 7 days, tested for memory of the maze training. Untreated mice routinely exhibit excellent recall under these conditions.

2) The second control study was run to determine if, after treatment with SCO, bitemporal injections of puromycin induce amnesia within 3 days after learning. Mice were treated with SCO 1 day after maze training and 1 day later were injected bitemporally with puromycin. Untreated mice consistently show complete amnesia of the training experience when tested in this way.

3) The third set of control experiments was conducted to determine if, after treatment with SCO, bitemporal injections of puromycin fail to induce amnesia when administered 6 or more days after learning. In untreated mice, imposition of a delay between learning and bitemporal puromycin greater than or equal to 6 days results in a loss of puromycin's action with respect to memory. Mice in groups 3a and 3b were injected with SCO 9 days after training to allow ample time for the development of additional memory storage sites. Ten days after SCO treatment, puromycin was injected bitemporally. This interval between drug treatment and puromycin equalled the delay used in the other behavioral tests described below.

4) The fourth group of controls determined if SCO alters the sensitivity of the brain to puromycin in such a way that the 6 injection protocol with puromycin (T+V+F) is no longer able to induce amnesia 6 or more days after learning. The T+V+F protocol consistently produces amnesia in untreated controls under these conditions.

Results. The results of the tests listed above are presented in Table 1.

1) As shown in group 1 of Table 1, there was no statistically significant difference between the training and testing scores of the SCO and saline groups ($p=0.50$).

TABLE 1

LACK OF EFFECT OF SCOPOLAMINE (1.5 AND 3.0 mg/kg) ON RELEARNING AND ON THE ACTION OF PUROMYCIN

Drug Treatment and Timing	N	Median Errors		
		Train	Test	Retention
1. Effect on Learning/Relearning				
a. SCO (1.5 mg/kg)→Train→Test (1, 7)	4	5.5	0.0	NA
b. SCO (3.0 mg/kg)→Train→Test (1, 7)	4	5.0	0.0	NA
c. Saline→Train→Test	4	5.0	0.0	NA
2. Effect on Action of Puromycin: SCO Before Development of Additional Storage Sites				
a. Train→SCO (1.5 mg/kg)→Puro (1, 1)	4	6.0	9.0	–
b. Train→SCO (3.0 mg/kg)→Puro (1, 1)	4	5.0	9.5	–
3. Effect on Action of Puromycin: SCO After Development of Additional Storage Sites				
a. Train→SCO (1.5 mg/kg)→Puro (9, 10)	5	6.0	1.0	+
b. Train→SCO (3.0 mg/kg)→Puro (9, 10)	4	5.0	1.5	+
c. Train→SCO (1.5 mg/kg)→Puro (11, 10) T+V+F	5	5.5	11.0	NA
d. Train→SCO (3.0 mg/kg)→Puro (9, 10) T+V+F	4	5.0	10.0	NA

Time between procedures is indicated (in days) over arrows. Puromycin was injected bitemporally except as indicated. NA = not applicable.

2) As shown in group 2 of Table 1, puromycin induced profound amnesia of the maze training in mice treated with either 1.5 or 3.0 mg/kg of SCO.

3) The results of the third series of controls are given in groups 3a and 3b of Table 1. Consistent with past results in untreated mice, bitemporal puromycin failed to induce amnesia when a sufficient latency was introduced between training and the puromycin administration.

4) As shown in groups 3c and 3d of Table 1, SCO treatment did not interfere with the amnesic actions of the T+V+F puromycin protocol. Again, these results are consistent with past results in untreated mice.

Experiment 2

Rationale and results. The purpose of this experiment was to determine the effect of increasingly low doses of SCO on the spread of memory storage sites. The schedule of training, drug treatment and testing was identical to that used with adrenergic ligands (3, 5, 6); i.e., drug was given 2 days after training and then, to allow ample time for the normal process of memory spread, puromycin was bitemporally injected 10 days later. As has been mentioned, we used a dose of 3 mg/kg in our first experiments. As shown in Fig. 1, bitemporal puromycin failed to induce amnesia in mice receiving 3, 2, 0.5, or 0.0 mg/kg (median savings respectively +92%, +100%, +86%, and +85%). By contrast, bitemporal puromycin induced profound amnesia in the 14 mice that received 1.5 mg/kg of SCO (savings −40%). Seven of the mice treated with 1.0 mg/kg of SCO were also amnesic following bitemporal puromycin (savings −50%), whereas memory was present following puromycin in the remaining 7 mice of the group (savings +73%).

Experiment 3

Rationale and results. The purpose of this experiment was to compare the ex vivo binding data following a high (but behaviorally ineffective) dose of SCO with that which follows a lower (but behaviorally effective) dose of SCO. As shown in Fig. 2, 15 min after treatment with 3 mg/kg of SCO, specific binding of 1 nM [^{3}H]QNB in preparations of the cerebral hemispheres was completely blocked. Full recovery to normal levels of binding was observed 4 h after treatment. By contrast, the lower dose of SCO (1.5 mg/kg) blocked specific binding of [^{3}H]QNB by only 50% at 15 min after treatment while full recovery to normal binding levels was evident by 2 h after treatment.

Experiment 4

In Experiment 2, posttraining administration of SCO (1.5 mg/kg) prolonged the normal period during which bitemporal injections of puromycin induced amnesia. The purpose of the present experiment was to determine if a single administration

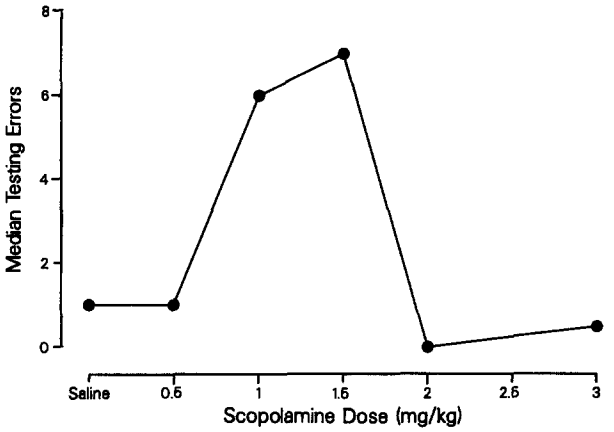

FIG. 1. Dose response curve of SCO's effect on the development of additional memory storage sites. Mice were injected subcutaneously with SCO 2 days after training, with bitemporal puromycin 10 days later and then tested for retention about 12 days after puromycin. Fourteen mice in both the 1.0 and 1.5 mg/kg groups; 6 mice in all other groups. Range of variation of testing errors at consecutive points: (0–1), (1–3), (0–15), (4–14), (0–1), and (0–2). See text for additional details.

of SCO produces a long-lasting effect, i.e., to determine if spontaneous recovery of memory spread eventually occurs. The results are presented in Table 2.

With SCO being given 2 days after training, puromycin was injected bitemporally from 20 to 60 days later. Group 1b of Table 2 shows that bitemporal puromycin (with one exception) consistently induced profound amnesia 20 and 30 days after SCO ($p<0.001$, groups 1b vs. 1a). This high degree of puromycin-induced amnesia was significantly reduced ($p=0.004$, groups 1c vs. 1b) at 60 days; 6 of the 12 mice in this group had high savings scores (median +76%) while the remaining 6 mice were amnesic (median savings −21%).

Analogous results were obtained when treatment with SCO

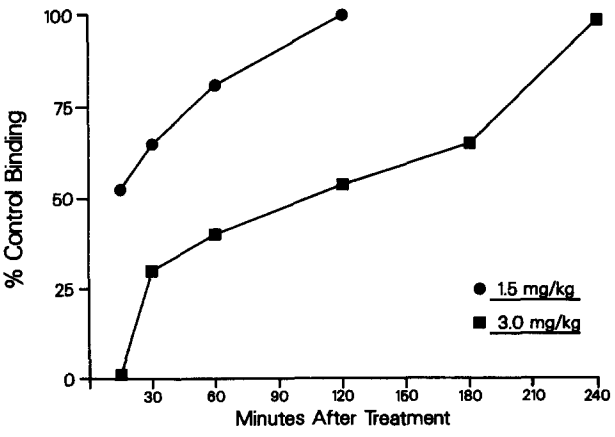

FIG. 2. Effect of a single subcutaneous dose of SCO (1.5 or 3.0 mg/kg) on specific [^{3}H]QNB (1 nM) binding by homogenates of the cerebral hemispheres. The values presented are medians (n=3 or 4); median binding for controls=28.5 pmol/g tissue (n=6). Range of variation in % control at consecutive points following a) 1.5 mg/kg of SCO; (47–53), (64–72), (74–83), and (89–102); b) 3.0 mg/kg of SCO; (0.7–2.2), (29–30), (37–50), (50–62), (55–74), and (97–102).

preceded training by 20, 30, or 60 days (groups 2a and 2b). The testing performance of these groups did not differ significantly from that of the corresponding groups in which treatment with SCO followed training. Kruskal-Wallis one-way analysis of variance was performed on the training scores of these groups. The test confirmed previous findings (3) that initial-training scores were not associated with subsequent testing scores ($p>0.1$).

Experiment 5

The purpose of this experiment was to test whether pharmacologic blockade of the muscarinic receptors found in the periphery would also affect memory spread. For this purpose we used scopolamine methyl bromide (methyl SCO) because this compound fails to cross the blood-brain barrier. The schedule of training, drug treatment and testing was identical to that used with SCO. In 9 mice methyl SCO was injected subcutaneously (1.5 mg/kg) 2 days after training and then, to allow ample time for the spread of memory storage sites, puromycin was injected bitemporally 10 days later. The median-testing savings in these mice was 100% and the training errors were normal (7.0).

From these findings it is evident that there was a highly significant difference ($p<0.001$) in the effects of SCO (1.5 mg/kg) versus the same dose of methyl SCO. It is apparent that SCO potently inhibited memory spread whereas its methylated derivative was without effect. We conclude that blockade of central muscarinic receptors is essential for inhibition of memory spread.

DISCUSSION

Our experience with SCO leads to the following conclusions:

1) In the absence of puromycin, SCO, under the present experimental conditions, had no effect on the learning of the Y-maze or on its relearning.

2) SCO had no effect on the memory disrupting properties of puromycin.

3) Both high and low doses of SCO, administered 2 days after training, had no effect on the increase of memory storage sites. An intermediate dose, however, given either before or after training inhibited this increase with some few exceptions, for at least 30 days and in about half the mice for at least 60 days. The observation that the high dose of SCO (3 mg/kg) blocked nearly all muscarinic-binding sites 15 min after its administration suggests that its failure to inhibit memory spread may be due to an excessive concentration. Similar observations have been made, for example, with cholinergic agonists (10) and in studies of the metabolic effects of adrenergic blocking agents. Thus, dichloroisoproterenol stimulates phosphorylase activity (probably through the activation of adenylate cyclase) at low concentrations but "at higher concentrations the drug inhibits its own action" (1). Efforts to understand the molecular basis of results of this kind have led to several hypotheses [for review (15)].

4) By contrast, the methyl analog of SCO, injected subcutaneously 2 days after training in a dose equal to the effective dose of SCO, had no effect on memory spread.

5) The consistent presence of memory for long periods of time in mice treated with SCO following training (but not challenged with puromycin; Table 2, groups 1a and 1d) suggests that the hippocampus (or nearby temporal regions) is capable of not only short-term memory but also long-term memory as well.

As noted above, the development of complementary memory storage sites appears to be critically dependent on the central adrenergic system. We may now state that this development appears to be critically dependent on both the central adrenergic and the central muscarinic systems. The conclusion that the central systems are involved is based upon the failure of subcutane-

TABLE 2

DURATION OF SCOPOLAMINE'S (SCO, 1.5 mg/kg) INHIBITION OF THE DEVELOPMENT OF ADDITIONAL MEMORY SITES

Drug Treatment and Timing	N	Median Errors		
		Train	Test	Retention
1. SCO after Training				
2 20				
a. Train→SCO-→Saline	8	5.5	0.0	NA
2 20–30				
b. Train→SCO----→Puro	15	5.5	8.5	(±)
Retained Memory	1	5.0	0.0	+
Amnesic	14	5.0	8.0	−
2 60				
c. Train→SCO-→Puro	12	5.5	3.0	(±)
Retained Memory	6	6.0	2.0	+
Amnesic	6	5.0	8.0	−
2 60				
d. Train→SCO-→Saline	8	5.5	1.0	NA
2. SCO before Training				
20–30 10				
a. SCO-----→Train--→Puro	12	6.5	8.5	(±)
Retained Memory	2	6.5	1.0	+
Amnesic	10	7.0	9.5	−
60 10				
b. SCO-→Train-→Puro	12	5.5	6.0	(±)
Retained Memory	4	5.0	0.5	+
Amnesic	8	6.0	8.0	−

ous injections of a beta-adrenergic receptor antagonist (2) and of methyl SCO, both of which fail to cross the blood-brain barrier, to affect the development of complementary sites. The possibility remains, however, that changes in both the central and peripheral neurotransmitter systems, are required for inhibition of memory spread. This possibility should be tested by intracerebral injections of the appropriate drugs. As has been explained (8), the intracerebral approach is impractical in our experiments; when puromycin injections follow other intracerebral injections, puromycin loses its consistent effectiveness for a long period of time. This appears to be due to an increased loss of the antibiotic from the brain as shown by accelerated turnover of tritiated puromycin and by a reduced level of inhibition of protein synthesis.

As mentioned above, numerous observations have led to the conclusion that essential memory storage sites, initially limited to the hippocampal area, increase with time to involve additional cerebral areas. This conclusion is directly supported by experiments in which cats were trained in a 1-way active-avoidance task (19). At 3 h after training, the cats received a combined entorhinal cortex-fornix lesion (hippocampal isolation) which resulted 3–4 weeks later in a large, statistically significant ($p = 0.002$) retention deficit compared to the unoperated controls. If, however, hippocampal isolation in the cats was delayed until 8 days after training, there was no difference between their savings and those of the controls. Thus, development of an independent forebrain engram required the presence of the hippocampal area for approximately 8 days after learning. Similarly, studies in the monkey (13) have led to this statement: "The conclusion seems inescapable that the older memories were stored upstream from both the limbic system and the medial thalamus, presumably within the cortical areas on which the limbic system and medial thalamus have the greatest influence." It appears that an analogous process of memory spread occurs in mice under the training conditions we have employed, and that components of both the adrenergic and cholinergic systems of the brain are required. Further experiments are needed to increase our understanding of this fundamental process.

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