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Chronic Scopolamine Treatment and Brain Cholinergic Function

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MARKS, M. J., M. F. O'CONNOR, L. D. ARTMAN, J. B. BURCH AND A. C. COLLINS. *Chronic scopolamine treatment and brain cholinergic function.* PHARMACOL BIOCHEM BEHAV **20**(5) 771–777, 1984.—Scopolamine was either continuously infused or injected once daily into C3H mice. Chronic infusion resulted in mice that were supersensitive to the hypothermia and tremor produced by the muscarinic agonist, oxotremorine. Chronic scopolamine infusion did not alter brain acetylcholinesterase (AChE) or choline acetyltransferase (ChAT) activities but it did produce an increase in brain muscarinic receptors, as measured by quinuclidinyl benzilate (QNB) binding. The maximal increase in QNB binding was seen at the 0.2 mg/kg/hr dose. Further increase in dose resulted in a return to control QNB binding in all brain regions studied except cortex. These animals were still supersensitive to oxotremorine, suggesting a dissociation between receptor number and response to agonist. Animals injected once daily for 10 days with 5 mg/kg exhibited an increase in QNB binding while no increase was seen at 20 mg/kg/day. Chronic oxotremorine infusion resulted in tolerance to the hypothermia-producing effects of oxotremorine. This was accompanied by a decrease in brain QNB binding. Coinfusion of scopolamine with oxotremorine blocked both the tolerance development and receptor changes. These experiments demonstrate that chronic scopolamine treatment can elicit an increase in brain muscarinic receptors which is accompanied by supersensitivity to agonists. However, this effect is not clearly dose related, and a strict relationship between receptor number and agonist response does not exist.

Scopolamine Oxotremorine Muscarinic Cholinergic receptors Supersensitivity Tolerance Acetylcholinesterase Choline acetyltransferase

THE relationship between tolerance development and changes in the number of muscarinic cholinergic receptors upon chronic administration of agonists or antagonists has been investigated with increasing frequency recently. The effects of chronic stimulation of muscarinic receptors have been studied primarily using acetylcholinesterase (AChE) inhibitors to increase the level of the endogenous neurotransmitter [4, 5, 7, 13]. Receptor numbers decrease after these treatments. A decrease in receptor number has also been observed in hybrid neuronal-glial cells cultured in the presence of various muscarinic agonists [12, 15, 17]. The effects of chronic blockade of muscarinic receptors have been studied in animals receiving daily injections of atropine [16,18] or scopolamine [2,9]. Under these conditions, receptor levels were observed to increase. However, incubation with antagonists had no effect on the number of muscarinic receptors in hybrid neuronal-glial cells [15]. With the exception of the latter study, the general pattern which has emerged is that chronic agonist administration results in a decreased number of muscarinic receptors while chronic antagonist administration increases the number of muscarinic receptors.

In the present investigation, a slightly different approach has been employed to study the development of tolerance and its relationship to neurochemical changes. The administration of cholinergic agents is achieved by continuous infusion through cannulae implanted in the jugular veins of mice. This method of administration allows drug levels to be maintained at relatively constant concentrations and permits ready adjustment of dosage levels. We have used this technique to examine the effects of the muscarinic agonist, oxotremorine, on several components of the cholinergic system [10,11]. We have observed that muscarinic receptor numbers decrease linearly, in five mouse brain regions, with increased dose of oxotremorine. No effect on AChE activity was observed following any dose of oxotremorine, while slight decreases may have occurred in choline acetyltransferase (ChAT) activity. Physiological and behavioral tolerance developed accompanying chronic oxotremorine infusion [10,11]. This tolerance is dose related. As infusion rates were increased, the dose-response curves for oxotremorine effects on body temperature and rotarod performance shifted increasingly to the right [10]. Considerable tolerance developed before a decrease in receptor number was seen. No changes in the K_i for inhibition of QNB binding by oxotremorine or the ratio of high and low affinity agonist binding

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sites were observed [10]. Thus, the relationship between receptor number and sensitivity to agonists is not clear.

In the present study, chronic infusion of scopolamine was used to study the effects of the administration of a muscarinic antagonist on muscarinic receptors, AChE and ChAT. The effects of chronic scopolamine infusion were also compared with those obtained with chronic scopolamine injection. In addition, sensitivity to the hypothermia- and muscle tremor-producing actions of oxotremorine was assessed in animals which had been treated chronically with scopolamine in order to further explore the relationship between muscarinic receptor number and sensitivity to muscarinic agonists.

METHOD

Animals

Before use in these experiments, female mice of the C3H/Ibg strain, obtained from the breeding colony at the Institute for Behavioral Genetics, Boulder, CO, were housed in small groups (2–5) and had free access to chow and water. The lighting cycle was 12 hr light/dark with the light period from 7:00 a.m. to 7:00 p.m.

Cannula Implantation

Cannulae made of silastic tubing were implanted in the right jugular veins of anesthetized mice (chloral hydrate, 125 mg/kg, and sodium pentobarbital, 60 mg/kg) using the method of Barr *et al.* [1]. Mice were allowed to recover for 2 days after the surgery before infusion was initiated. Following surgery, animals were singly housed.

Drug Infusion

After the recovery period, the implanted cannula was connected to polyethylene tubing attached to a Harvard infusion pump. Saline was infused into the animals for 2 days after which time drug infusions were started. Scopolamine solutions were initially infused at a rate of 0.1 mg/kg/hr and were increased daily by 0.1 mg/kg/hr until final rates of 0.2, 0.5, or 1.0 mg/kg/hr were attained. Animals in each infusion group were maintained at the final infusion rate for 5 days before testing.

Drug Injections

Alternatively, mice were chronically treated with scopolamine (0 mg/kg, 5 mg/kg, and 20 mg/kg) by intraperitoneal injection of the appropriate drug solution at 4:00 p.m. every day for 10 days. In addition, one group of animals was injected once with saline or 20 mg/kg scopolamine at 4:00 p.m.

Tolerance or Supersensitivity Test

Mice were removed from the chronic infusion apparatus 18 hr before testing to allow metabolism of the drug. Upon removal, cannulae were checked to confirm that they were still free-flowing. Testing was conducted at 9:00 a.m. the next morning by injection of 0.1 mg/kg oxotremorine. Body temperatures were measured at 20-min intervals, and the presence of tremor, if any, was noted. A Digitec 5810 rectal probe (YSI, Yellow Springs, OH) inserted 2.5 cm was used to measure body temperature with an accuracy of ±0.1 degrees. The effect of oxotremorine on body temperature was quantified by calculating the area under the time-temperature curve for each animal. Changes in sensitivity to muscarinic agents accompanying chronic infusion were assessed by measuring oxotremorine response because we have found that testing for agonist effects is more reliable than is testing for antagonist effects.

Biochemical Assays

Tissue preparation. Mice were sacrificed by cervical dislocation after completion of the tolerance test. Brains were removed, rinsed with 50 mM potassium phosphate, pH 7.4, and dissected into seven regions. These regions were: cerebral cortex, cerebellum, hindbrain (pons-medulla), striatum, hippocampus, hypothalamus, and midbrain. The region designated "midbrain" was the portion of the midbrain area remaining after removal of striatum, hippocampus, and hypothalamus. Homogenates (4% w/v) were made in 50 mM potassium phosphate buffer, pH 7.4.

Acetylcholinesterase. AChE activity was measured using a modification of the method of Ellmann *et al.* [6] as described previously [11].

Choline acetyltransferase. ChAT activity was measured using a modification of the method of Schrier and Schuster [14] as described previously [11].

Muscarinic receptor assays. [^{3}H]quinuclidinyl-N-benzilate (QNB) binding was determined using a slight modification of the method of Yamamura and Snyder [19]. Homogenate was added to 10 ml of potassium phosphate buffer, 20 mM, pH 7.4. Incubation was initiated by addition of [^{3}H]-L-QNB (New England Nuclear, 40.2 Ci/mmol). Five QNB concentrations between 10 pM and 175 pM were used. Samples were incubated at 37° for 40 min at which time particulate protein and bound QNB were collected by filtration on Whatman GFA glass fiber filters. The filters were washed with four 5-ml portions of ice-cold buffer. The washed filters were placed in Nalgene filmware bags and 3 ml of scintillation cocktail were added. The filters were mechanically crushed and counted at 20% efficiency. Receptor concentrations during the incubation did not exceed 15 pM. Binding constants were determined from Scatchard plots fitted by the method of least squares.

Protein was measured by the method of Lowry *et al.* [8].

Data analysis. Comparisons of binding data, enzymatic activities, and hypothermic responses as a function of treatment dose were made using one-way analysis of variance followed by Tukey's b post hoc tests at a significance level of $p<0.05$. Data on oxotremorine-induced body tremor were analyzed using chi-square tests. In addition, binding data were subjected to trend analysis after multiple analysis of variance.

RESULTS

Body weights and body temperatures were measured at the conclusion of the treatment period to provide information on the general health of the mice. While the body weights of treated animals appeared to be slightly lower than those of control animals, these differences were not significant, $F(5,49)=2.03$, $p>0.05$. In addition, no significant differences in basal body temperature were found, $F(5,49)=1.00$, $p>0.05$. These results suggest that mice chronically treated with scopolamine are not severely debilitated.

Tests for tolerance of mice to scopolamine are difficult since the effects of this drug are subtle and not easily measured. Alternatively, treated mice were tested for supersen-

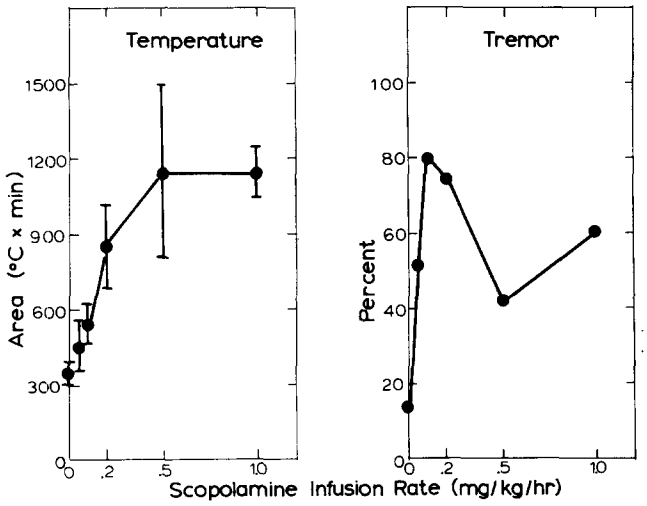

FIG. 1. Effect of acute oxotremorine injection on the responses of scopolamine-infused mice. Mice were injected IP with 0.1 mg/kg oxotremorine and monitored for the effects on body temperature and body tremor. Temperatures were measured before injection and at 20-min intervals thereafter until return to normal. Total drug effect was calculated as the area under the time-temperature curve. Temperature data are the mean±S.E.M. determined for 3–9 mice. Tremor data are the percentage of animals (7–14 per group) showing body tremor 20 min after injection.

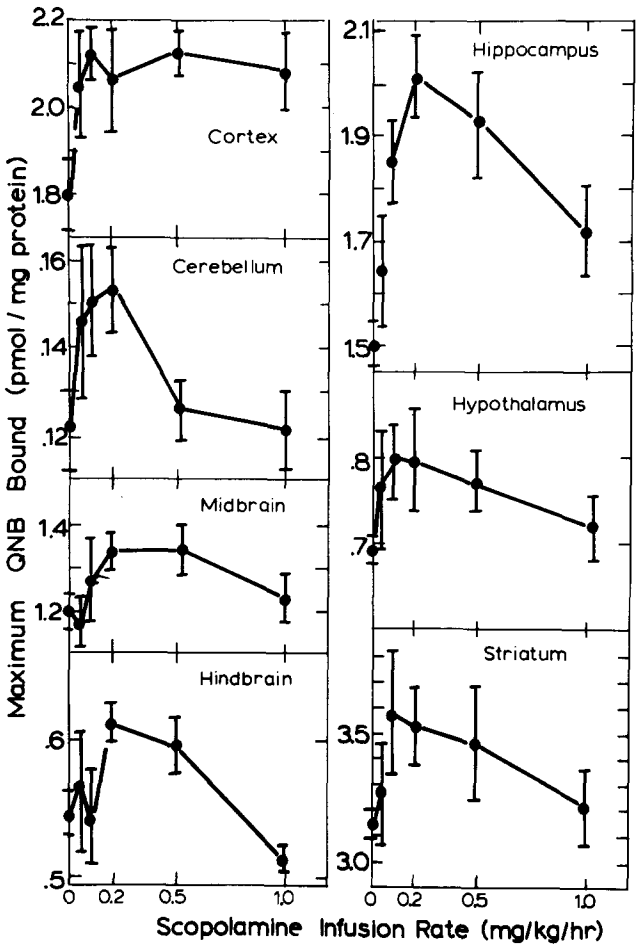

FIG. 2. Effect of chronic scopolamine infusion on maximal [^{3}H]-L-QNB binding. B_{max} values (pmol/mg protein) were calculated from Scatchard plots. Results are the mean±S.E.M. determined for 9–24 mice at each dose.

sitivity to the effects of the muscarinic cholinergic agonist, oxotremorine. Two effects of oxotremorine were measured: hypothermia and body tremor. Scopolamine infusion had little effect on the maximum temperature decrease occurring after injection of 0.1 mg/kg of oxotremorine, perhaps indicating the attainment of a floor effect. However, the duration of the hypothermia was prolonged such that the area under the time-temperature curve increased progressively as a function of scopolamine infusion rate (Fig. 1). Analysis of these data indicated significant differences among the treatment groups, $F(5,27)=6.88$, $p<0.001$. The post hoc test revealed that mice treated with either 0.2, 0.5, or 1.0 mg/kg/hr scopolamine had significantly greater areas under their time-temperature curves than did control mice, while the areas for animals infused with 0.05 or 0.1 mg/kg/hr scopolamine did not differ from those of controls. The percentage of animals in each scopolamine treatment group that exhibited oxotremorine-induced body tremor is shown in Fig. 1. Scopolamine treatment appeared to increase this percentage, and a chi-square test of the data, $\chi^2(5)=12.78$, $p<0.05$, indicated differences among the treatment groups. Further analysis revealed that all of the following treatment groups differed from control: 0.05, 0.1, 0.2, and 1.0 mg/kg/hr scopolamine. However, the percentage of animals with body tremor in the group infused with 0.5 mg/kg/hr scopolamine was not significantly different from that of controls. No significant differences among the groups treated with any nonzero dose of scopolamine were evident, $\chi^2(4)=2.74$, $p>0.05$.

Since scopolamine functions primarily as a muscarinic cholinergic antagonist, the effects of chronic infusion with this drug on various neurochemical components of the cholinergic system in the mouse brain were assessed. The activities of the enzymes AChE and ChAT in seven brain regions of mice chronically treated with 0.2, 0.5, or 1.0 mg/kg/hr scopolamine did not differ from those of saline-infused mice (data not shown).

The effect of chronic scopolamine infusions on the maximum amount of muscarinic receptor binding measured using [^{3}H]-L-QNB as the ligand is shown in Fig. 2. This treatment may have led to a biphasic change in receptor levels with an increase in binding observed after treatment with relatively low doses, with a return of binding to control levels after treatment with higher drug doses. The changes in receptor number were rather small, and a significant effect of drug treatment, as analyzed by ANOVA, was found for only two brain regions: cortex, $F(5,76)=4.73$, $p<0.01$, and hippocampus, $F(5,77)=5.96$, $p<0.001$. No significant differences ($p>0.05$) were noted for cerebellum, $F(5,77)=1.86$, midbrain, $F(5,76)=2.03$, hindbrain, $F(5,77)=1.81$, hypothalamus, $F(5,77)=0.60$, or striatum, $F(5,77)=1.63$.

Post hoc tests were run among the treatment groups for the two regions that displayed significant overall drug effects. This test in cortex indicated that treatment with either 0.05 (17.8% increase), 0.2 (18.3%), or 0.5 (18.3%) mg/kg/hr scopolamine resulted in increased QNB binding over that of saline-treated animals. Cortical QNB binding in mice treated with 0.1 or 1.0 mg/kg/hr scopolamine did not differ from that

of controls. The post hoc test for QNB binding in hippocampus indicated that ligand binding after treatment with 0.1 (23.3% increase), 0.2 (34.7%), or 0.5 (28.7%) mg/kg/hr scopolamine was higher than that after treatment with saline. Infusion of scopolamine at 0.05 or 1.0 mg/kg/hr had no significant effect on binding.

Inspection of the data in Fig. 2 suggested the possibility of a biphasic effect of chronic scopolamine infusion on QNB binding, i.e., in most brain regions binding appeared to increase at the lower infusion doses and return to control levels at the higher infusion rates. Therefore, the binding data were further analyzed by trend analysis after multiple analysis of variance in order to extract any higher order (quadratic or cubic) effects. Significant higher order effects were seen for five brain regions: cortex, hindbrain, midbrain, hippocampus, and striatum. Quadratic effects were seen for cortex, $F(1,76)=7.27$, $p<0.01$, midbrain, $F(1,76)=7.39$, $p<0.01$, hippocampus, $F(1,76)=18.39$, $p<0.001$, and striatum, $F(1,76)=6.39$, $p<0.05$. Cubic effects were seen for hindbrain, $F(1,76)=7.02$, $p<0.01$, and hippocampus, $F(1,76)=5.73$, $p<0.05$. Neither cerebellum nor hypothalamus showed a quadratic or cubic effect.

The effects of scopolamine treatment on the B_{max} values for QNB binding (Fig. 2) were calculated from Scatchard plots of the binding data as illustrated for hippocampus in Fig. 3. Scopolamine treatment may have had a modest effect on the K_D values for QNB in this brain region (Fig. 3, inset B). Analysis of the K_D values for all seven brain regions indicated that drug treatment had no significant effect on the K_D values for cortex, cerebellum, hindbrain, or hypothalamus, but overall effects were found for midbrain, hippocampus, and striatum. Post hoc tests in the three regions showing significant overall effects indicated that for midbrain the K_D for QNB of animals treated with 0.2 mg/kg/hr scopolamine (24.2±1.0 pmol) was greater than the K_D for all other treatment groups (average K_D=18.6 pmol). The K_D for QNB binding in hippocampus in animals treated with 0.2 mg/kg/hr scopolamine (23.8±1.6 pmol) was greater than the K_D for mice infused with saline (16.4±1.1 pmol), or 0.5 (15.5±1.4 pmol) or 1.0 (16.6±1.6 pmol) mg/kg/hr scopolamine. For striatum the K_D for QNB of animals treated with 0.2 mg/kg/hr scopolamine (25.4±1.3 pmol) was greater than the K_D in mice infused with either 0.5 (18.8±2.1 pmol) or 1.0 (18.1±1.7 pmol) mg/kg/hr scopolamine. No systematic dose-dependent changes in K_D occurred. The K_D for QNB in mice treated with 0.2 mg/kg/hr scopolamine was the only value found to be different from the K_D values for any other treatment group. These differences, while significant, were not substantial in that the K_D values for QNB in the 0.2 mg/kg/hr treatment group were only 20%–40% higher than the K_D values for the control group.

The pattern of receptor change after scopolamine infusion is biphasic: at low doses binding increases, while at higher doses binding decreases to control levels. One explanation for this decrease observed at high treatment levels is that the scopolamine has not been metabolized in the 18-hr period between the time the mice are removed from the infusion chambers and when they are assayed for QNB binding. Two experiments suggest this to be unlikely. First, the biological half-life of scopolamine, measured by the disappearance of the blockade of oxotremorine's effects in animals injected with scopolamine, was 40 min (data not shown). This short half-life would assure that complete metabolism of scopolamine had occurred in the 18 hr between removal from the treatment cages and assay for QNB binding. Second, a

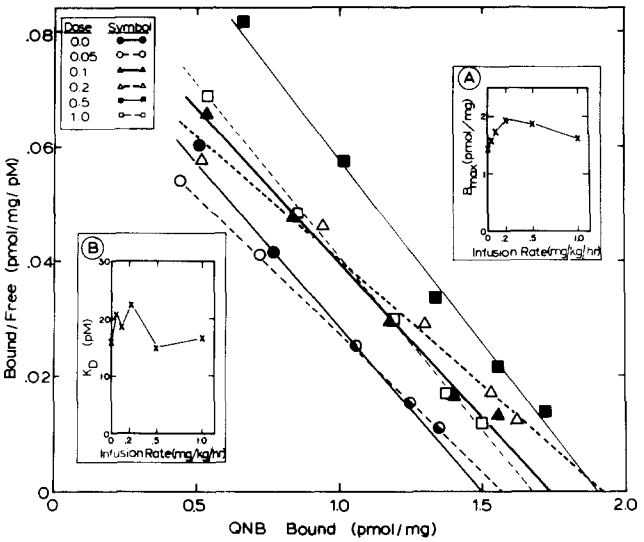

FIG. 3. Scatchard plots of hippocampal QNB binding after chronic scopolamine infusion. Mean values (n=9–24) for QNB binding in hippocampus after chronic infusion with one of six scopolamine doses are shown. The effect of infusion on B_{max} (Inset A) and K_D (Inset B) is also illustrated.

group of mice was injected IP with 20 mg/kg of scopolamine (a dose comparable to the 24 mg/kg/day infused into the mice at the highest treatment dose) and was assayed for QNB binding 18 hr after the injection. The amount of QNB binding in the seven brain regions of these animals was compared to that of a group of mice injected with saline 18 hr prior to assay. No differences between saline- and scopolamine-injected animals were found (data not shown). This result indicated that no residual scopolamine remained to interfere with the receptor assays.

To determine if the method of drug administration affected the response of the mice to chronic scopolamine treatment, mice were injected with scopolamine daily for 10 days. Three drug doses were used: 0 mg/kg (saline), 5 mg/kg, and 20 mg/kg. The results of this experiment are given in Table 1. Chronic scopolamine injection had no effect on the K_D for QNB in any brain region (data not shown), but changes in B_{max} were seen. In midbrain, hippocampus, and striatum significant overall effects of scopolamine injection were found. In midbrain and hippocampus QNB binding in mice chronically injected with 20 mg/kg scopolamine was less than that of mice injected with 5 mg/kg scopolamine. However, binding for neither treatment group differed from that for controls. For striatum, on the other hand, binding for animals treated with 20 mg/kg scopolamine was less than that for either controls or mice treated with 5 mg/kg scopolamine. These results indicate that no regular pattern of dose-dependent change in QNB binding occurred upon chronic scopolamine injection, a result similar to that found for animals chronically infused with the drug.

The data obtained from the chronic injection study were also analyzed for possible biphasic effects. Significant quadratic effects were seen for midbrain, $F(1,30)=4.27$, $p<0.05$, and striatum, $F(1,30)=4.63$, $p<0.05$. The quadratic effect for hippocampus, $F(1,30)=4.03$, $p=0.054$, was not statistically significant. This suggests a trend similar to that seen for the chronic infusion studies.

TABLE 1

EFFECTS OF SCOPOLAMINE INJECTION ON QNB BINDING

Treatment	Brain Region						
	Cortex	Cerebellum	Midbrain	Hindbrain	Hippocampus	Hypothalamus	Striatum
Saline	1.48 ± 0.08	0.10 ± 0.01	0.99 ± 0.09	0.60 ± 0.03	1.60 ± 0.08	0.69 ± 0.03	2.70 ± 0.17
5 mg/kg scopolamine	1.76 ± 0.13	0.11 ± 0.01	1.19 ± 0.11	0.61 ± 0.04	1.81 ± 0.11	0.75 ± 0.03	3.01 ± 0.20
20 mg/kg scopolamine	1.52 ± 0.14	0.10 ± 0.01	0.79 ± 0.05	0.55 ± 0.04	1.44 ± 0.08	0.69 ± 0.02	2.16 ± 0.07†
	F(2,31)= 1.54	F(2,31)= 0.21	F(2,31)= 5.12*	F(2,31)= 0.66	F(2,31)= 3.79*	F(2,31)= 1.48	F(2,30)= 6.54*

Mice were injected IP daily for 10 days with saline, 5 mg/kg scopolamine, or 20 mg/kg scopolamine. Eighteen hours after the final injection, the seven brain regions were assayed for QNB binding and the K_D and B_{max} (pmol/mg protein) were determined from Scatchard plots. Values for the B_{max} are mean ± SEM of 10–13 mice per treatment group. F values were obtained using one-way ANOVA for each brain region.

*$p<0.05$, one-way ANOVA.

†$p<0.05$, Significantly different from control, Tukey's b post hoc test.

TABLE 2

EFFECTS OF CHRONIC INFUSON OF SCOPOLAMINE AND OXOTREMORINE

Drug Infusion Rate (mg/kg/hr)	Physiological Response		Maximal QNB Binding (B_{max}) in Each Brain Region						
	Tremor	Body Temperature (Area: °C × min)	Cortex	Cerebellum	Midbrain	Hindbrain	Hippo-campus	Hypo-thalamus	Striatum
Saline	2/14	348± 44	1.68±0.07	0.12±0.01	1.16±0.04	0.53±0.02	1.45±0.06	0.68±0.03	3.02±0.11
1.0 oxotremorine	0/12	−19± 11	1.44±0.09	0.09±0.01	0.77±0.06†	0.35±0.03†	1.17±0.06†	0.41±0.03†	2.56±0.15†
0.5 scopolamine	3/7	1149±365	2.08±0.07*	0.12±0.01	1.31±0.06	0.58±0.03	1.88±0.11*	0.76±0.04	3.42±0.21
1.0 oxotremorine and 0.5 scopolamine	0/7	308± 89	2.10±0.11	0.12±0.01	1.24±0.07	0.53±0.03	1.80±0.05	0.73±0.05	3.31±0.14
	F(3,36)= 3.43	F(3,18)= 15.78‡	F(3,53)= 12.21‡	F(3,48)= 1.33	F(3,53)= 17.14‡	F(3,53)= 16.21‡	F(3,53)= 17.57‡	F(3,48)= 9.78‡	F(3,53)= 5.39‡

Mice were infused with the indicated drug solutions. After completion of the treatment period, animals were removed from the infusion chambers and, 18 hr later, were injected with 0.1 mg/kg oxotremorine. The number of mice with body tremor and the areas under the time-temperature curve were calculated. After the tolerance tests, the brains were assayed for QNB binding. Results presented are B_{max} (pmol/mg protein) values for each group (N=11–24). F values were obtained using a one-way ANOVA for each brain region.

*B_{max} values significantly higher than control, $p<0.05$ by Tukey's b test.

†B_{max} values significantly lower than control, $p<0.05$ by Tukey's b test.

‡$p<0.05$.

Since scopolamine is a muscarinic antagonist and blocks the acute effects of the muscarinic agonist, oxotremorine, the effect of simultaneous treatment with both of these agents was investigated. The results of this study are shown in Table 2. No significant differences in body tremor occurred between control mice and those in any of the treatment groups after a challenge dose of oxotremorine. However, a greater incidence of tremor was seen in mice treated with scopolamine alone than in mice treated with either oxotremorine alone or oxotremorine and scopolamine together. Drug treatment markedly affected the hypothermic response elicited by a challenge dose of oxotremorine. Mice chronically treated with oxotremorine were vitually unaffected by an acute injection of the drug, while mice chronically treated with scopolamine were hyperresponsive. The hypothermic response of both of these groups was significantly different from that of controls. Mice treated chronically with both drugs showed a hypothermic response nearly identical to that of controls.

Muscarinic receptor levels in brain regions of mice infused with oxotremorine, scopolamine, or a combination of these drugs are compared to control values in Table 2. As noted above, and confirmed with an independent analysis of variance, the cortices and hippocampi of mice infused with 0.5 mg/kg/hr scopolamine had greater QNB binding than did those of controls. Alternatively, the QNB binding in animals infused with oxotremorine alone was significantly less than that of control in midbrain (34%), hindbrain (34%), hippocampus (19%), hypothalamus (40%), and striatum (15%). Animals infused with a combination of oxotremorine and

scopolamine demonstrate maximal QNB binding that is significantly different from that of mice infused with oxotremorine alone in every brain region examined except cerebellum. We have previously shown cerebellar QNB binding to be unaffected by oxotremorine infusion [10,11]. The levels of QNB binding in all brain regions in those mice receiving both drugs were not significantly different from those measured in mice receiving only scopolamine. No treatment affected the K_D for QNB (data not shown).

DISCUSSION

Chronic infusion with a muscarinic antagonist, scopolamine, resulted in animals that were supersensitive to a challenge dose of oxotremorine, a muscarinic agonist, as measured by body temperature changes and tremor. The hypothermic response to the challenge dose of oxotremorine increased in a dose-dependent fashion along with infusion dose up to 0.5 mg/kg/hr. The maximal supersensitivity to oxotremorine-induced tremor was seen at a lower infusion dose (0.1 mg/kg/hr). Thus, it appears as though supersensitivity develops at different rates for different drug responses. The response to oxotremorine was measured at a single dose of agonist. Therefore, it is not yet possible to identify whether the supersensitivity arose from a shift to the left of the dose-response curves or a change in the maximal response after agonist treatment. We have demonstrated in a previous study of the tolerance to oxotremorine obtained after chronic infusion of this agonist that both changes occur [10]. Tolerance to drug effects on rotarod performance arose from shifts to the right in the dose-response curves, while tolerance to the effects on body temperature involved both a shift to the right and change in the slopes of the curves. The scopolamine-induced oxotremorine supersensitivity does not, in all likelihood, arise because of changes in ChAT or AChE activity. No changes in the activity of either ChAT or AChE were detected in animals that had been infused with any of three concentrations of scopolamine. Changes in muscarinic receptors appear to be a more plausible cause. Significant changes in the number of muscarinic receptors were observed in two brain regions after scopolamine treatment. However, the receptor changes were not strictly dose dependent. In fact, the most pronounced increases were observed in those animals treated with a relatively low scopolamine infusion rate (0.2 mg/kg/hr), the dose that elicited maximal changes in sensitivity to oxotremorine-induced tremor. With an additional increase in the infusion rate of antagonist, the number of QNB binding sites declined. Sensitivity to oxotremorine-induced tremor also declined in animals treated with these higher doses. Animals infused with the highest dose of scopolamine had receptor levels which did not differ from those of controls, but these same animals were supersensitive to oxotremorine-induced tremors. Thus, the association between receptor number and tremors decreases at the highest infusion rates. The association between increase in oxotremorine-induced hypothermia and receptor number also declines at the higher infusion doses in that receptor numbers return to control levels at higher infusion doses while hypothermic response does not.

Several mechanisms, operating independently or in combination, may explain the lack of correlation between response and receptor number. One possibility is that metabolism of scopolamine may have been induced resulting in decreased drug concentration at the high infusion doses. Another possibility is that one of the three binding sites for agonists [3] responds to chronic drug treatment with a dose-dependent increase, while the other agonist binding sites are unaffected or are decreased upon scopolamine treatment. A third possibility is that a neurochemical mechanism other than changes in the receptor itself (e.g., changes in coupling mechanisms or alterations of other neurotransmitter systems interacting with muscarinic receptors) becomes important when high concentrations of antagonist are present. For example, Richelson [12] has noted that desensitization of muscarinic receptor-mediated cyclic GMP formation precedes changes in QNB binding in cultured nerve cells incubated with muscarinic agonists. This may explain our observation [10] that tolerance to oxotremorine develops before a down-regulation of receptors is seen. At the higher scopolamine infusion dose, it may be that coupling processes are altered in sensitivity, thereby allowing receptors to return to control levels, while supersensitivity is maintained.

The magnitude of the receptor change in all cases was quite small, and the changes seen were primarily changes in B_{max}. While modest, but statistically significant, changes in K_D were seen in three brain regions, these were observed at only one infusion dose. No clear-cut trend in K_D changes was evident.

The largest percentage increase in B_{max} (35%) was noted in hippocampus after treatment at a scopolamine dose of 0.2 mg/kg/hr. Only two regions, cortex and hippocampus, displayed significant changes in QNB binding after chronic scopolamine treatment. The failure to detect changes in the other brain regions may have occurred, in part, as a result of the limits of resolution of the assay. Due to variance in measuring B_{max}, changes in QNB binding exceeding 15% of basal levels were required before significance was attained. Since 18 hr elapsed between the removal of mice from the chronic infusion cages, loss of muscarinic receptors and of supersensitivity to oxotremorine may have occurred. If this did occur, the magnitude of the scopolamine-induced changes would have been underestimated.

While the changes in the B_{max} values for QNB after chronic infusion (Fig. 2) and chronic injection (Table 1) were qualitatively similar, quantitative differences in these changes did occur. An apparent biphasic pattern of receptor change as drug dosage was increased was seen following either chronic infusion or injection. The magnitude of receptor changes after IP administration was smaller than that attained after infusion. Since the changes were biphasic, the lowest dose used in our study (5 mg/kg) may already have exceeded that dosage necessary for maximum receptor change in mice. Thus, the regulation of muscarinic receptor number by antagonists may depend both on the average concentration and on the peak concentration of the drug at the receptor site.

The biphasic response of the muscarinic receptors seen in most brain regions to chronic scopolamine infusion was unexpected. *A priori* one might expect a dose-dependent increase in binding or perhaps an increase to a constant high level of binding as antagonist dose was increased. A completely satisfying explanation for the biphasic response is not yet available. However, the fact that the biphasic response was not seen in cortex suggests that more than one mechanism for controlling receptor number may exist or brain regions differ in sensitivity if a single mechanism is involved. It seems unlikely that the general health of the mice has deteriorated with drug treatment, thereby resulting in a generalized loss of brain material. Not only were the drug-treated animals similar to controls in both body weight and

baseline body temperature, but also the activities of two cholinergic enzymes in the brains of the treated animals were unaffected by chronic drug treatment. It is also unlikely that residual scopolamine remained bound to the receptor and caused the measurement of either artifactually low B_{max} values or artifactually high K_D values, because injection of a large amount of scopolamine had little effect on either of these binding parameters 18 hr after injection.

Others have demonstrated that muscarinic receptors in the brains of animals increase after chronic antagonist treatment [2, 9, 16, 18]. These increases were seen in rats injected once [2, 9, 18] or twice [16] daily with varying doses (0.2 to 100 mg/kg) of scopolmaine [2,9] or atropine [16,18]. If these studies are compared, the largest increases in QNB binding were seen in those animals injected with the lowest doses. This agrees with our observation. Thus, the increase seen at low doses may be reversed at high doses when the receptor should be occupied for a greater proportion of the time. This might explain why we failed to detect an increase in QNB binding in those animals treated with the highest infusion doses and in those animals injected once daily with the 20 mg/kg dose.

Studies carried out in tissue culture have demonstrated that chronic treatment with muscarinic agonists results in a decrease in QNB binding [12, 15, 17]. This decrease could be blocked by simultaneous treatment with agonist and antagonist [15]. Chronic antagonist treatment alone, however, had no effect on QNB binding in these cultured cells. This result suggests that receptor stimulation by either exogenous agonist or endogenous agonist (presumably absent in cultured cells lacking synaptic contacts) is required before antagonist treatment can affect receptor number. The results of the current study also demonstrate that simultaneous treatment with scopolamine and oxotremorine prevents the tolerance to oxotremorine and the down-regulation of muscarinic sites observed when mice are infused with oxotremorine alone. This suggests that tolerance to oxotremorine arises as a direct result of changes in muscarinic receptors, not as a result of changes in other receptor systems that result independently of actions on muscarinic systems.

The results provided in the present study argue that the response of muscarinic receptors in mouse brain to chronic antagonist treatment is more complex than a simple up-regulation of these receptors and that a strict relationship between antagonist dose and receptor number does not exist. Additional studies will be required to determine what mechanisms in addition to receptor up-regulation may be controlling the development of tolerance to antagonists (as measured by supersensitivity to the effects of agonists), as well as to determine why a biphasic change in total muscarinic binding occurs upon antagonist treatment.

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REFERENCES

1. Barr, J. E., D. B. Holmes, L. M. Ryan and S. K. Sharpless. Techniques for the chronic cannulation of the jugular vein in mice. *Pharmacol Biochem Behav* **11:** 115–118, 1979.
2. Ben-Barak, J. and Y. Dudai. Scopolamine induces an increase in muscarinic receptor level in rat hippocampus. *Brain Res* **193:** 309–313, 1980.
3. Birdsall, N. J. M., A. S. V. Burgen and E. C. Hulme. The binding of agonists to brain muscarinic receptors. *Mol Pharmacol* **14:** 732–736, 1978.
4. Costa, L. G., B. W. Schwab, H. Hand and S. D. Murphy. Reduced (^{3}H) quinuclidinyl benzilate binding to muscarinic receptors in disulfoton-tolerant mice. *Toxicol Appl Pharmacol* **60:** 441–450, 1981.
5. Ehlert, F. J., N. Kokka and A. S. Fairhurst. Altered (^{3}H) quinuclidinyl benzilate binding in the striatum of rats following chronic cholinesterase inhibition with diisopropylfluorophosphate. *Mol Pharmacol* **17:** 24–30, 1980.
6. Ellman, G. L., K. D. Courtney, V. Andres, Jr. and R. M. Featherstone. A new and rapid colorimetric determination of cholinesterase activity. *Biochem Pharmacol* **7:** 88–95, 1961.
7. Gazit, H., I. Silman and Y. Dudai. Administration of an organophosphate causes a decrease in muscarinic levels in rat brain. *Brain Res* **174:** 351–356, 1979.
8. Lowry, O. H., N. H. Rosebrough, A. L. Farr and R. J. Randall. Protein measurement with Folin phenol reagent. *J Biol Chem* **193:** 265–275, 1951.
9. Majocha, R. and R. J. Baldessarini. Increased muscarinic receptor binding in rat forebrain after scopolamine. *Eur J Pharmacol* **67:** 327–328, 1980.
10. Marks, M. J., L. D. Artman and A. C. Collins. Quantitation of tolerance development after chronic oxotremorine treatment. *Pharmacol Biochem Behav* **19:** 103–113, 1983.
11. Marks, M. J., L. D. Artman, D. M. Patinkin and A. C. Collins. Cholinergic adaptations to chronic oxotremorine infusion. *J Pharmacol Exp Ther* **218:** 337–343, 1981.
12. Richelson, E. Desensitization of muscarinic receptor-mediated cyclic GMP formation by cultured nerve cells. *Nature* **272:** 366–368, 1978.
13. Schiller, G. D. Reduced binding of (^{3}H)-quinuclidinyl benzilate associated with chronically low acetylcholinesterase activity. *Life Sci* **24:** 1159–1164, 1979.
14. Schrier, B. K. and L. Shuster. A simplified radiochemical assay for choline acetyltransferase. *J Neurochem* **14:** 977–985, 1967.
15. Shifrin, G. S. and W. C. Klein. Regulation of muscarinic acetylcholine receptor concentration in cloned neuroblastoma cells. *J Neurochem* **34:** 993–999, 1980.
16. Takeyasu, K., S. Uchida, Y. Noguchi, N. Fujita, K. Saito, F. Hata and H. Yoshida. Changes in brain muscarinic acetylcholine receptors and behavioral responses to atropine and apomorphine in chronic atropine-treated rats. *Life Sci* **25:** 585–592, 1979.
17. Taylor, J. E., E. El-Fakahany and E. Richelson. Long-term regulation of muscarinic acetylcholine receptors on cultured nerve cells. *Life Sci* **25:** 2181–2187, 1979.
18. Westlind, A., M. Grynfarb, B. Hedlund, T. Bartfai and K. Fuxe. Muscarinic supersensitivity induced by septal lesion or chronic atropine treatment. *Brain Res* **225:** 131–141, 1981.
19. Yamamura, H. I. and S. H. Snyder. Muscarinic cholinergic binding in rat brain. *Proc Natl Acad Sci USA* **71:** 1725–1729, 1974.