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The Effects of Chronic Oxotremorine Treatment on Spatial Learning and Tolerance Development in Mice

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WEHNER, J. M. AND M. UPCHURCH. *The effects of chronic oxotremorine treatment on spatial learning and tolerance development in mice.* PHARMACOL BIOCHEM BEHAV 32(2) 543–551, 1989.—C57BL mice were treated with 0.5 mg/kg/hr oxotremorine through an implanted subcutaneous cannula for 6 days. Tolerance to oxotremorine was evaluated after treatment by constructing cumulative dose-response curves and measuring body temperature and rotarod performance. At 2 hr after removal, mice exhibited a 15-fold tolerance as measured by body temperature and a 4-fold tolerance as measured by rotarod performance. This tolerance as measured by body temperature was lost by two days after removal from treatment. Immediately after treatment, ^{3}H-QNB binding was reduced in cortex, hippocampus, midbrain, hindbrain, and hypothalamus. Receptors returned to normal within 4 to 8 days after cessation of treatment depending on the brain region. Spatial learning was examined using the Morris water task. Mice that began their training in this task 1 day after they were removed from oxotremorine treatment were impaired in their spatial ability as evidenced by a lack of preference for the trained site during a probe trial. Mice that began their training 2 days after cessation of oxotremorine treatment showed no evidence of impairment in spatial learning. These results suggest that a loss of muscarinic receptors after oxotremorine treatment can be dissociated from tolerance loss and spatial learning deficits.

Spatial learning   Muscarinic receptors   Tolerance development

PERTURBATIONS of the cholinergic system have been demonstrated to interfere with learning and memory processes in both humans and animals (1, 2, 5, 6, 12). These behavioral effects have been dependent on the learning paradigm as well as the dosage and treatment schedule of the specific cholinergic agent. Experimental lesions of cholinergic processes have implicated the cortex and hippocampus as regions mediating the acquisition and retention of spatial learning (11, 16, 18, 19, 38–40). Furthermore, loss of cholinergic neurons in aging and in Alzheimer's disease has suggested a crucial role of cholinergic neurons in learning and memory (6, 7, 33).

Chronic treatment with cholinergic agonists or organophosphorus anticholinesterases causes a reduction of muscarinic cholinergic receptors (8, 24–26, 32, 37, 42), but some of the behavioral effects of such treatments have been equivocal. For example, studies of organophosphate effects on learning in rodents have indicated no effect on the passive avoidance paradigm (5), while memory loss is a symptom of chronic organophosphate poisoning in humans (28,41). We have recently demonstrated that treatment of C57BL mice with DFP for 12 days not only produced a reduction in muscarinic receptors, but also produced a deficit in acquisition of spatial learning in the Morris water task (29), provided that treatment was prior to training (42). DFP does not impair retention of spatial learning (42).

The study of DFP effects indicated that the Morris water task provided a sensitive and reliable method to assess perturbations of cholinergic receptors and that spatial learning could be studied in C57BL mice. The study also indicated that DFP-pretreated mice were unable to learn a new platform position in a reversal test using the Morris water task even after their cortical and hippocampal receptors had returned to normal levels (42). Because DFP causes a reduction in both muscarinic and nicotinic cholinergic receptors (8,36), without producing a clear-cut development of tolerance to muscarinic agents (37), we sought to define more clearly the role of muscarinic receptor function in spatial learning. Marks *et al.* (24–26) have shown that chronic treatment with oxotremorine induces a down-regulation of muscarinic receptors as measured by ^{3}H-QNB, but has no effect on nicotinic receptors as measured by either ^{3}H-nicotine or ^{125}I-alpha-bungarotoxin binding (23). This treatment does not produce significant alteration of other components of the cholinergic system such as choline acetyltransferase (ChAT) activity, acetylcholinesterase (AChE)

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activity (25), or high affinity choline uptake (25). Thus, chronic oxotremorine treatment can be used to examine the effects of a selective alteration in muscarinic receptors.

In previous studies oxotremorine was administered via infusion through a jugular cannula (24–26). In present study, we administered oxotremorine through a subcutaneous cannula, thus providing a less traumatic means of surgical implantation. We studied the effects of muscarinic receptor down-regulation on spatial learning, and compared these effects with development and loss of tolerance to oxotremorine. Our experiments indicate that this method of chronic treatment results in loss of muscarinic receptors, development of tolerance, and a temporary impairment of spatial learning.

METHOD

Animals

Female C57BL/6Ibg mice, 60–90 days of age, were obtained from the breeding colonies at the Institute for Behavioral Genetics, University of Colorado, Boulder, CO. They were maintained on a 12:12 light:dark cycle (lights on at 0700 and off at 1900) with food and water available ad lib.

Chemicals

Oxotremorine was purchased from Sigma. [^{3}H]-Quinuclidinylbenzilate ([^{3}H]-QNB, sp.act.=34.7 Ci/mmol) was obtained from NEN.

Chronic Drug Treatments

Continuous infusion of oxotremorine was accomplished by inserting a silastic tubing subcutaneously at the back of the neck while animals were anesthetized by pentobarabital (50 mg/kg) and chloral hydrate (100 mg/kg). The operation was completed in approximately 10 min, with full recovery within 24 hr. Upon recovery, mice were housed singly in a chronic infusion chamber. The silastic tubing was connected to thermoplastic tubing which was connected to a 1-ml syringe. The syringe was placed in a Harvard infusion pump and oxotremorine was administered at a flow rate of 0.035 ml/hr. Syringes were refilled every 24 hr. Mice were administered sterile saline for 24 hr, then oxotremorine was given in doses that increased every 24 hr, beginning at 0.1 mg/kg/hr, followed by 0.2 mg/kg/hr, 0.3 mg/kg/hr, 0.4 mg/kg/hr and finally 0.5 mg/kg/hr. They were maintained on 0.5 mg/kg/hr for six days. Control mice were maintained on saline for the entire infusion period. All animals had access to food and water ad lib.

Three types of experiments were conducted. In the first set of experiments tolerance testing and receptor analyses were performed. Some animals were tested for tolerance either 2 hr (24) or 18 hr after cessation of infusion. Other treated mice were removed from the infusion chamber, housed singly, and tested for tolerance development either 2, 4, or 8 days after cessation of treatment. Mice were sacrificed for receptor analysis at various times after treatment. In the second set of experiments, mice were tolerance tested 2 hr after treatment and then began spatial learning training 24 hr after cessation of oxotremorine treatment. A separate group of mice was tolerance tested at 18 hr after cessation of treatment and then began spatial learning at 48 hr after removal from treatment. Saline-infused control mice that had been treated for an identical period of time were included in each type of analysis.

Assessment of Tolerance

Cumulative dose-response curves were obtained for rotarod performance and depression of body temperature in order to evaluate tolerance to oxotremorine. After removal from the infusion apparatus animals were trained on the rotarod (Ugo Basile Co., Milan, Italy) as described by Marks *et al.* (24). Two hours after training, saline infused mice were injected intraperitoneally (IP) every fifteen minutes with 0.04 mg/kg oxotremorine in order to establish a dose-response curve in the range of 0.04–1.6 mg/kg. Oxotremorine-infused mice were tested in the same fashion except the oxotremorine challenge doses ranged from 0.1–3.0 mg/kg depending on the day of withdrawal from infusion.

Body temperature was measured using a rectal thermometer (Digitec 5810, Yellow Springs Instrument Co., Yellow Springs, OH) and mice were tested for 100 sec on the rotarod. Tolerance data were evaluated for body temperature depression by establishing a dose-response curve for each individual mouse (log dose vs. body temperature). Linear regression analysis was performed and the dose producing a depression in body temperature to 35°C ($ED_{35°}$) was calculated as well as the slope of the line. The mean $ED_{35°}$ and the mean slope with 95% confidence levels were calculated for basis of comparison.

The mice were tested for 100 sec on the rotarod. Using this task, the four doses tested did not always produce a linear decrease in performance and thus prevented an individual assessment of each mouse to determine an ED_{50}. For this reason, the mean response of all mice in a specific condition was analyzed by calculating a mean ED_{50} and slope. This analysis does prevent statistical analysis of ED_{50} values for rotarod data at the various days after removal from treatment, but the data are provided here in order to 1) demonstrate that tolerance was observed for more than one measure and 2) allow comparison with previously published pharmacological studies of tolerance to oxotremorine (24). In one case where a common dose of oxotremorine (0.125 mg/kg) was administered as a challenge dose, a comparison was made between the rotarod scores by analysis of variance (ANOVA).

Assessment of Spatial Learning

The Morris water task was used as a test of spatial learning (23). This test has been adapted for mice in our laboratory and was performed exactly as described previously (42,43).

Apparatus

Details of the apparatus and the characteristics of the testing room are provided elsewhere (42,43). The animals were required to find a clear Plexiglas platform with a surface area 10.5 cm square in a galvanized iron pool 122 cm in diameter. The water in the pool was made opaque with nontoxic Crayola powder paint. Water temperature was maintained at 28°C with an aquarium heater that was removed during testing. Behavior was video-recorded with a camera mounted on the ceiling over the pool.

Training Protocol

Details of the training are provided elsewhere (42). On the first day of acquisition, the mouse was given a pretraining session in which it was taught how to climb onto the platform

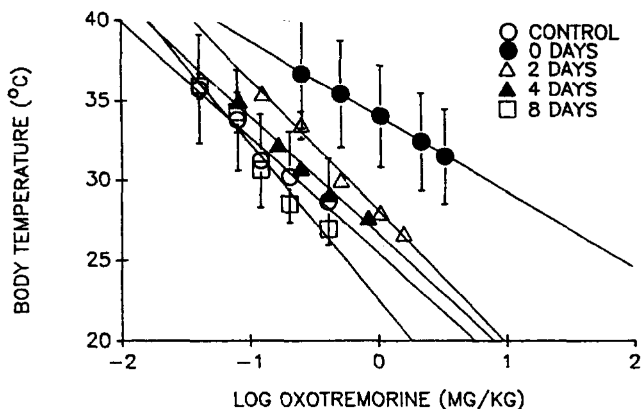

FIG. 1. Log dose-response curves for oxotremorine effects on body temperature. Chronically infused mice were tested for the hypothermic effects of oxotremorine using the successive injection technique at various times during withdrawal from infusion. Each point represents the mean±95% confidence level, N=6–20.

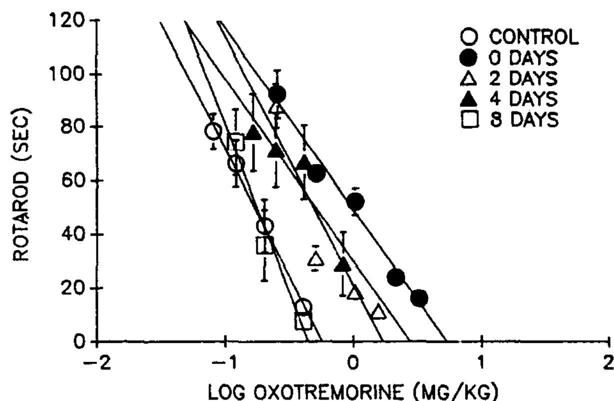

FIG. 2. Log dose-response curves for oxotremorine effects on rotarod performance. Chronically infused mice were tested for effects on the rotarod using the successive injection technique during withdrawal. Each point represents the mean±95% confidence level, for N=6–20.

from the water. Immediately following pretraining, acquisition training began.

Trials were given in blocks of four, with the animal starting once from each of four possible start locations during a block of trials. The order of start locations was random. The mouse was given 60 sec to find the platform and 60 sec to rest on it between trials. Latency to find the platform was recorded for each trial. If the animal failed to find the platform, a latency of 61 sec was recorded for that trial. The animal was returned to its home cage between blocks of trials. Three blocks of trials were given per day, with a 1 to 2 hr interval between each block. One to 2 hr after its final acquisition trial, the mouse was returned to the pool for a 60 sec probe trial during which the platform was not present. The observer used a videotape of this trial to count the number of times the animal crossed the site where the platform had been located, as well as the number of times the animal crossed other possible platform sites to which it had not been trained. Time spent searching each quadrant of the pool was also measured. Twelve reversal trials, in which the mouse was trained to swim to a site opposite to the original platform location, were given the following day.

Data from the acquisition trials, retention trials, and reversal trials were analyzed using mixed-model, between-within (treatment by trial) ANOVA. For the probe trial data, mixed-model two-way ANOVAS (treatment by platform site or quadrant) were used to measure platform site crosses. The Newman-Keuls post hoc test with corrections for between-within analyses was used for a more detailed analysis of probe trial results.

Receptor Analysis

On days 0, 2, 4, and 8 after treatment, mice were sacrificed by cervical dislocation. Brains were removed and dissected on ice into cortex, midbrain, hindbrain, hippocampus, striatum, and hypothalamus. Dissected brain tissue was frozen at −70° until receptor assays were performed. Homogenates were prepared in 10 volumes 50 mM Na^+/K^+ phosphate buffer, pH=7.4. Membranes were prepared as described previously (23,42) using washing procedures to eliminate any residual drug in the membranes.

[^{3}H]-QNB binding was measured by a modification of the method of Yamamura and Snyder (46) as previously described (23–26, 42). The final membrane pellet was resuspended and assayed in a final volume of 10.1 ml in 50 mM phosphate buffer pH=7.4 for 45 min at 37°C. For cortex, 5 concentrations of ^{3}H-QNB varying from 10–250 pM were used to determine B_{max} and K_d. Binding in the other five brain regions was determined at the highest ^{3}H-QNB concentration. Protein concentrations were determined by the method of Lowry *et al.* (21) using bovine serum albumin as a standard. Protein concentrations were cortex, 30–40 μg; midbrain, 90–110 μg; hindbrain, 100–150 μg; hippocampus, 30–40 μg; striatum, 30–40 μg; and hypothalamus 40–60 μg.

Receptors were analyzed using the EBDA program to determine B_{max} and K_d (22). Statistical analyses were performed using ANOVA techniques followed by post hoc analysis using the Student's *t*-test.

RESULTS

Behavioral Tolerance

Previous studies involving administration of the muscarinic agonist oxotremorine indicated that tolerance developed after chronic infusion through the jugular vein of mice (23–26). We have modified that technique to allow infusion of many drugs through a subcutaneous cannula implanted through a slit in the skin on the back of the mouse's neck. This method allows for less traumatic surgery. Because of this different route of drug administration and probably differential drug distribution, it was important to determine whether subcutaneously-infused mice could become tolerant to the effects of oxotremorine. C57BL mice were infused with 0.5 mg/kg/hr oxotremorine for 6 days. After removal from infusion, animals were trained on the rotarod and then examined for the effects of challenge doses of oxotremorine on both body temperature and rotarod by constructing cumulative dose-response curves. The results of tolerance testing are shown in Figs. 1 and 2. Those mice treated with 0.5 mg/kg/hr oxotremorine for 6 days exhibited approximately 15-fold tolerance to the hypothermic effects of oxotremorine (Fig. 1) and 4-fold tolerance to the effects of oxotremorine on rotarod performance (Fig. 2).

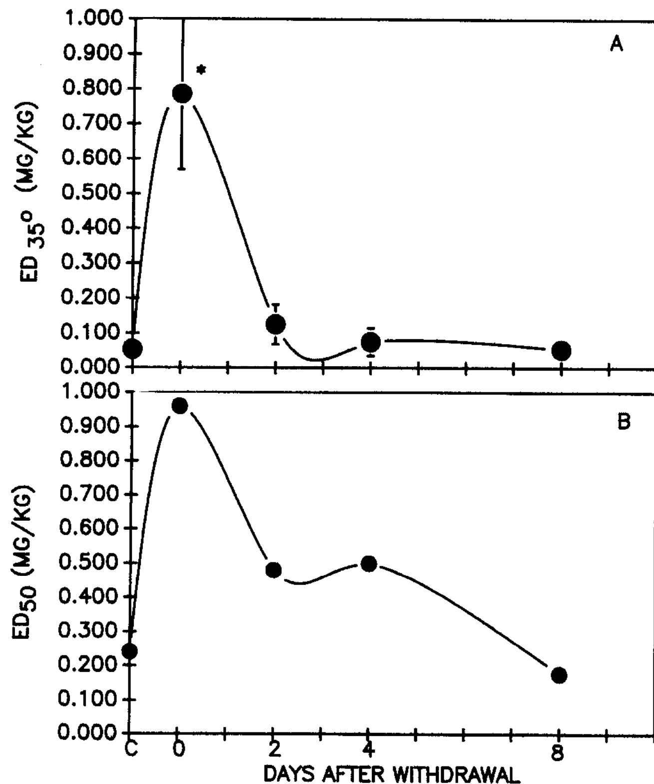

FIG. 3. Effect of withdrawal time on ED_{35}°/ED_{50} values for oxotremorine effects. (A) ED_{35}°, each point represents the mean±95% confidence levels, N=6–20; *$p<0.05$; (B) ED_{50}, each point represents the population mean for a sample of 6–20.

In order to determine the time course of tolerance loss, mice were infused for 6 days, removed from infusion and tested at 0, 1, 2, 4, and 8 days after withdrawal from oxotremorine (Figs. 1 and 2). Figure 3 shows the ED_{35}°s for temperature depression and ED_{50}s for impairment of rotarod performance at each time tested during withdrawal. There was a significant alteration when mice were tested within 2 hours (day 0 of withdrawal) after removal from oxotremorine infusion, ED_{35}°=0.79±0.218 mg/kg as compared to ED_{35}°=0.053±0.013 mg/kg for saline-infused controls. At 24 hr after treatment there was still a significant 3–5-fold tolerance, with treated mice having an ED_{35}°=0.19±0.02 mg/kg. By 48 hr after cessation of treatment, this group had an ED_{35}°=0.125±0.057 which was not significantly different from control. The analysis of slopes of the dose-response curves for body temperature indicated that none were significantly different from control except at day 0 of withdrawal.

As with body temperature, tolerance to impairment of rotarod performance was progressively lost after withdrawal from oxotremorine infusion. On day 0 of withdrawal from oxotremorine infusion, the mean ED_{50} was 0.96 mg/kg as compared to 0.24 mg/kg for saline-infused mice. The results of the analysis of tolerance indicated that body temperature depression was a more reliable indicator of tolerance development. The lack of a gradual linear reduction in rotarod performance in each individual mouse over the four increasing doses of oxotremorine challenge prevented an accurate dose-response analysis that is needed to derive individual ED_{50}s, i.e., for some animals only two of the four doses of oxotremorine caused a reduction from the 100 sec criteria. A comparison of rotarod response for saline-treated, oxotremorine-treated at day 2 and day 8 of withdrawal after the oxotremorine challenge dose of 0.125 mg/kg indicated that there were significant differences between these three groups, $F(2,33)=4.00$, $p<0.05$. Post hoc analysis indicated that saline-treated mice were more affected than those tested at day 2 ($p<0.05$) of withdrawal but not at day 8. Because a method of tolerance prescreening for each mouse was needed prior to examination of spatial learning ability, body temperature depression was used as the measure of tolerance in all subsequent phases of the study.

Muscarinic Receptors

Previous studies have indicated that oxotremorine induces a dose-dependent loss of muscarinic receptors in brain (23–26). To determine whether the loss of tolerance to oxotremorine correlated with recovery of receptors, ^{3}H-QNB binding was analyzed in six brain regions. Cortex and hippocampus were of particular interest because these regions of the brain are thought to mediate spatial learning (11, 18, 19, 31, 35, 39, 44). Both cortical and hippocampal muscarinic receptors were decreased as a result of subcutaneous infusion with oxotremorine (Fig. 4). It was also of interest to compare the time course of recovery of muscarinic binding in these regions with the effects on spatial learning. Figure 4 shows the loss of receptors and recovery after withdrawal from infusion. In all brain regions there were no significant differences in ^{3}H-QNB binding of saline-infused animals across time so that saline-treated mice from the various times of treatment were combined.

In cortex, there was a significant effect of oxotremorine treatment on muscarinic receptor number, $F(1,55)=15.0$, $p<0.001$, but no significant effect of withdrawal time after treatment. Post hoc analysis revealed that at 0 and 2 days after withdrawal oxotremorine-treated mice had a significantly lower number of receptors at day 0 and day 2 after withdrawal, but by 4 days after treatment receptors had returned to normal levels. There were no significant effects on K_d for ^{3}H-QNB as a result of oxotremorine treatment. A similar pattern was observed for loss of muscarinic receptors in the hippocampus. There was a significant effect of oxotremorine treatment, $F(1,55)=20.4, p<0.001$, but no effect of days during withdrawal. Post hoc analysis revealed that oxotremorine-treated mice had a significantly lower number of muscarinic receptors at 0 and 2 days after treatment, but not at 4 and 8 days after withdrawal.

In midbrain, hindbrain, and hypothalamus there were significant losses of receptors as a function of treatment, $F(1,51)=25.9$, $p<0.001$; $F(1,48)=28.06$, $p<0.001$; and $F(1,54)=17.1$, $p<0.001$, respectively. There was no significant effect of time of withdrawal after treatment. In midbrain, receptors were lower than in saline-infused mice at every day tested during withdrawal except at day 8 when muscarinic receptors had returned to within normal range. In hindbrain, muscarinic receptors had returned to normal in oxotremorine-treated mice by day 4 of withdrawal. In hypothalamus, muscarinic receptors in oxotremorine-infused mice had returned to within normal range by 8 days after withdrawal from infusion. In striatum, there was no significant effect of oxotremorinė treatment on muscarinic receptor number, $F(1,52)=2.21$, n.s.

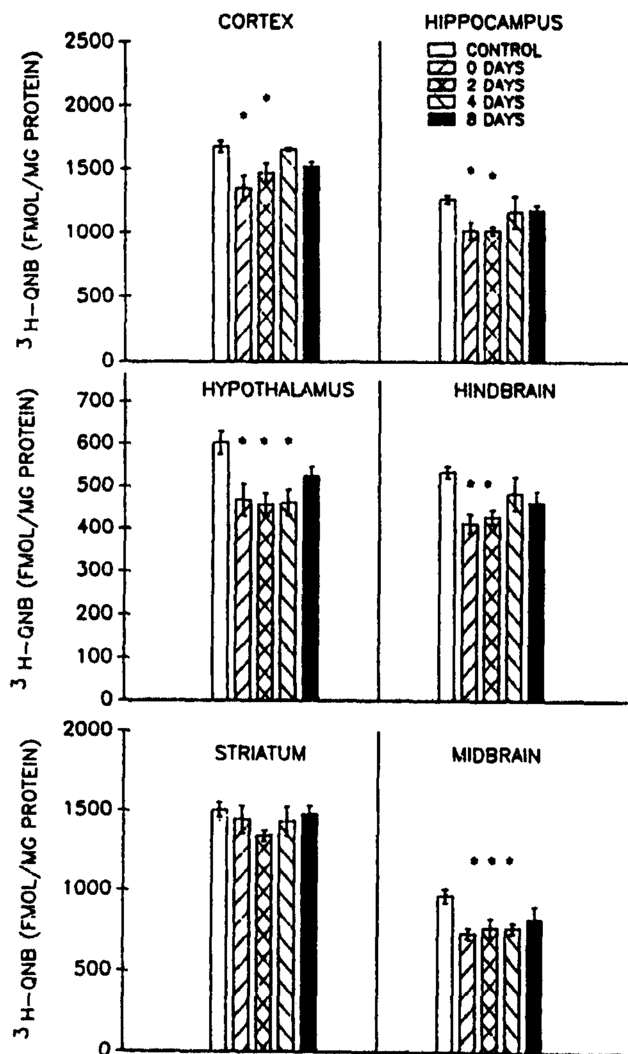

FIG. 4. Effect of oxotremorine on ^{3}H-QNB binding in brain regions as a function of time during withdrawal. Values significantly different from saline controls are indicated with * for $p<0.05$.

Spatial Learning Testing

In order to test the hypothesis that muscarinic receptor number is important in regulating an animal's ability to undergo spatial learning, mice were tested in two groups. The first group began training at 24 hr after cessation of treatment; at this time mice exhibited both tolerance and decreased muscarinic receptors in cortex and hippocampus. The second group began training at 48 hr after cessation of treatment; at this time mice still had reduced muscarinic receptors, but were not tolerant. All mice used for the Morris water task were tolerance tested the day before the beginning of acquisition training. A blind testing protocol was used so that the individual testing for tolerance development was not the same as the individual studying the Morris water task. In all cases, animals that did not develop tolerance to oxotremorine due to removal of their cannula or other interruption in the infusion protocol were not impaired in the spatial learning test.

Prior to our evaluation of spatial learning, some oxotremorine-treated mice were tested for their ability to locate a visible platform in order to evaluate whether oxotremorine treatment affected visual acuity and would thereby prevent mice from forming a spatial map of the room. There was no evidence of visual impairment as a result of oxotremorine treatment.

Oxotremorine treatment produced a spatial learning impairment in animals that began their training 24 hr after removal from infusion. Analysis of latency to locate the hidden platform on each day of acquisition training indicated that oxotremorine treatment had a significant effect on the first day of training, $F(1,16)=6.80, p<0.05$. There was also a significant effect of trial on latencies during the first day of testing, $F(11,176)=6.76, p<0.001$. By the second and third days of training there were no significant effects of oxotremorine treatment on the latency to reach the invisible platform. These data are summarized in Table 1.

In the probe trial (performed on the third day of training or a total of four days postdrug treatment, Fig. 5), oxotremorine-treated mice were significantly impaired as compared to saline-infused mice in their preference for crossing the trained site when the platform was removed, as evidenced by a significant site-drug interaction, $F(3,48)=4.42, p<0.01$. Saline-infused mice exhibited a significant effect of site in the probe trial, $F(3,30)=9.10, p=0.001$, with post hoc analysis revealing a preference for the trained site over the other three sites ($p<0.01$). Oxotremorine-treated mice did not show a significant preference for the trained site, $F(3,18)=3.10, p=0.052$. Post hoc analysis indicated that any preference that the oxotremorine mice showed was not for the trained site, but rather for that to the right of the trained site ($p<0.05$). Oxotremorine treatment also caused an impairment as measured by search time (Table 1), such that mice did not show a preference for the trained quadrant, $F(3,18)=2.53, p=0.09$. Saline-treated mice, however, did show such a preference, $F(3,30)=6.54, p<0.01$. There was no effect of drug treatment on reversal training, $F(1,16)=0.05$, n.s., nor was there a drug by trial interaction, $F(11,176)=0.99$, n.s. Within treatment groups, however, oxotremorine-treated mice failed to show a trial effect during reversal, $F(11,66)=0.90$, n.s., while saline-treated animals showed a strong trial effect, $F(11,110)=3.15, p<0.001$.

When acquisition training was begun at 48 hr after the removal from oxotremorine, there no longer was an effect of oxotremorine on any spatial learning parameter (Table 1, Fig. 5). There were no significant differences between saline- and oxotremorine-treated mice in latency to find the hidden platform, $F(1,8)=0.05$, n.s., during the first day of acquisition training, but there was a significant trial effect, indicating that mice were improving over the first day in their ability to locate the platform, $F(11,88)=3.94, p<0.001$. Two-way ANOVAs revealed that there was no effect of drug treatment on site preference as examined by the number of site crossings during the probe trial, $F(1,8)=0.39$, n.s., nor was there a significant drug by site interaction, $F(3,24)=0.44$, n.s. Site did have a significant effect, $F(3,24)=5.00, p<0.01$, such that mice more frequently crossed the trained site than other sites (post hoc analysis, $p<0.05$). Search time was also unaffected by oxotremorine treatment, $F(1,8)=0.41$, n.s., Table 1, but again there was a significant effect of quadrant, such that mice spent more of the total time in the quadrant containing the trained site, $F(3,24)=4.77, p<0.01$.

TABLE 1

ACQUISITION LATENCY AND PROBE TRIAL SEARCH TIME RESULTS (MEAN ± S.E.M.)

	Total Latency			
	24-Hr Postinfusion Group		48-Hr Postinfusion Group	
	Saline	Oxotremorine	Saline	Oxotremorine
Day 1	331 ± 35.8*	463 ± 29.0	392 ± 64.0	376 ± 28.4
Day 2	236 ± 46.4	261 ± 27.6	182 ± 38.1	152 ± 28.2
Day 3	208 ± 58.7	143 ± 19.0	146 ± 24.4	127 ± 24.9
Day 4	207 ± 22.2	214 ± 16.9	154 ± 22.4	210 ± 22.4
	Probe Trial Search Times			
	24-Hr Postinfusion Group		48-Hr Postinfusion Group	
Quadrant	Saline	Oxotremorine	Saline	Oxotremorine
Trained	27.9 ± 4.42	17.4 ± 2.04	21.6 ± 3.53	22.2 ± 3.37
Left	8.6 ± 1.24‡	12.6 ± 1.67	11.5 ± 4.42	13.7 ± 1.82
Right	16.6 ± 3.64†	18.9 ± 2.54	20.7 ± 6.51	18.4 ± 1.83
Opposite	8.2 ± 2.21‡	10.7 ± 2.22	7.9 ± 1.52	8.2 ± 2.72†

*$p<0.05$ compared to oxotremorine-treated mice.
†Differs from trained quadrant, $p<0.05$; ‡Differs from trained quadrant, $p<0.01$.

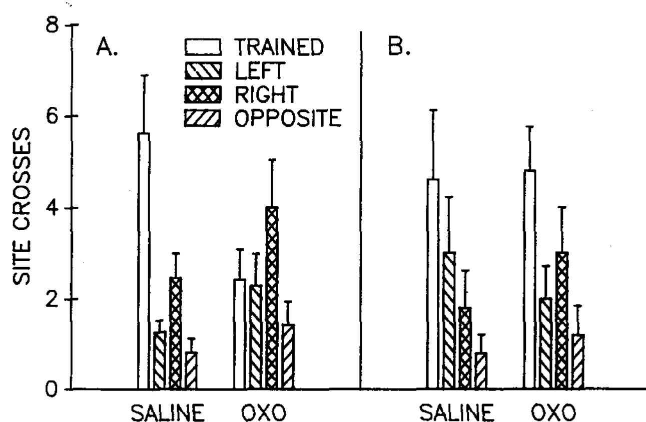

FIG. 5. Effect of oxotremorine on spatial learning site crossings using a probe trial test. Each value is the mean±SEM, N=5–6. (A) Mice began acquisition training at 24 hr after removal from oxotremorine or saline; probe trials were conducted after a total of 4 days posttreatment. (B) Mice began acquisition training at 48 hr after removal from oxotremorine or saline; probe trials were conducted after a total of 5 days posttreatment.

DISCUSSION

As reported previously, a reduction in muscarinic receptors and a development of tolerance are seen after chronic exposure to oxotremorine by intravenous (IV) infusion (23–26). Our results indicate that subcutaneous infusion of oxotremorine also produces a loss of muscarinic receptors and development of tolerance. The subcutaneous infusion technique provides a reliable, easy way to administer drugs over a period of days. Although the degree of tolerance as measured by body temperature and rotarod performance is less than that observed by Marks *et al.* (24) using the IV route of administration, comparable losses of muscarinic receptors were observed in our study. In the same study, Marks *et al.* (24) showed that behavioral tolerance to oxotremorine does not appear to result from metabolic tolerance since the biological half-life of oxotremorine in treated mice was similar to that in control mice. Furthermore, the loss of QNB binding was not accompanied by a change in the K_I of the receptor for the agonist, carbamylcholine. Their results support the hypothesis that development of tolerance to oxotremorine is associated with a loss of receptor sites and not a change in the receptor itself.

The results of the present study suggest that while a loss of receptors may underlie tolerance development, there may be some dissociation between receptor numbers and the maintenance of tolerance. When the time course for the recovery of receptors during withdrawal from oxotremorine was compared to that for loss of tolerance as measured by body temperature depression, it did not perfectly mimic the loss of tolerance. This suggests that either there is a dissociation between changes in receptor number and behavioral tolerance, or that the wide variation in measurement of behavioral tolerance may obscure detecting the exact time course of return to the normal state. The idea that there may not be a complete association of receptor loss with tolerance development is supported by the results of other recent studies. Smolen *et al.* (37) demonstrated that loss of muscarinic receptors as a result of agonist-induced down-regulation may not totally explain tolerance development. In their study they produced graded changes in ^{3}H-QNB binding following various DFP treatments, but did not observe parallel changes in drug response. Lim *et al.* (20) have also reported such a dissociation between receptor changes and behavioral tolerance.

The analysis of spatial learning in mice chronically treated with oxotremorine indicated that a temporary impairment of

spatial learning occurred. Specifically, mice that began their training at a time when they were still tolerant to the drug showed a spatial impairment in a probe trial given two days later. Mice that began their training at a time when they were no longer tolerant to the drug were not impaired in the spatial learning task. It should be noted that both groups of mice began their training at times when muscarinic binding was reduced in the cortex and hippocampus, and that both groups were given their probe trials after hippocampal and cortical muscarinic binding had returned to normal levels. The time course of the spatial impairment appeared to parallel that of tolerance loss rather than that of the muscarinic receptor recovery.

Recently, several studies have indicated that spatial learning may be mediated by M_1 muscarinic receptors (4, 13, 27). We cannot infer the respective roles of M_1 and M_2 muscarinic receptor subtypes in behavioral tolerance or impairment or spatial learning from our results because we measured total muscarinic receptors. Although oxotremorine is a relatively weak agonist at M_1 receptors (10), Marks *et al.* (26) showed a reduction of both subtypes when they performed ^{3}H-pirenzepine and ^{3}H-QNB binding in animals chronically treated with oxotremorine. It thus appears that constant high levels of oxotremorine might cause a reduction in both subtypes of muscarinic receptors and that we may have modified both sites in our study. The role of the muscarinic receptor subtypes in mediation of spatial learning is currently being assessed in our laboratory.

The impairment produced by oxotremorine did not appear to be as severe as that produced by chronic DFP treatment (42). In addition to showing a lack of search preference in the probe trial, DFP-treated C57BL mice exhibited significant increases in latency to find the hidden platform and in the number of trials required to reach criterion. Furthermore, DFP-pretreated mice continued to show deficits when tested for their memory of the platform's original location (retention) and for their ability to learn a new platform location (reversal). Since retention and reversal were tested 16 days after the final DFP injection, at a time when muscarinic binding had returned to normal levels, it appeared that the deficits produced by DFP treatment were relatively long lasting.

There are several possible explanations for the differences in the magnitude and duration of the impairment produced by DFP and oxotremorine on spatial learning. Although both drugs are thought to produce an agonist-induced down-regulation of receptors, they have different mechanisms of action. DFP elevates acetylcholine levels through irreversible inhibition of AChE (17) and may be neurotoxic, while oxotremorine acts as a direct agonist at the muscarinic receptor (46). Because of these different mechanisms of action, DFP treatment reduces binding at both muscarinic and nicotinic sites (8,36), while chronic oxotremorine treatment results in a selective decrease in muscarinic binding (23). At present, we cannot rule out the possibility that the differential effects on nicotinic binding could contribute to the differences observed in the degree of impairment.

It is also difficult to dismiss a role of the muscarinic system in spatial learning, since numerous lesion and chemical treatment studies support such a role (2, 11, 15, 18, 19, 40). Research conducted in this laboratory indicates that the muscarinic receptor down-regulation produced by DFP treatment is longer lasting than that produced by chronic oxotremorine treatment (42). It is possible that differences between the two drug effects could be related to the different time courses of receptor recovery. The results of the current experiment indicate, however, that the spatial impairment may not be related to the change in muscarinic binding alone. A more tenable hypothesis is that the loss of muscarinic receptors may be related to an impairment of spatial learning, but that changes in other aspects of cholinergic function, such as acetylcholine release, choline uptake, ChAT activity, or receptor-coupling mechanisms in the cell membrane may also be likely to play a role in the impairment of spatial learning and the expression of tolerance to oxotremorine. It is unlikely that changes in choline uptake or in ChAT activity could have accounted for the spatial learning deficit seen in our laboratory, as these neurochemical characteristics are not altered by chronic oxotremorine or DFP treatment (24, 25, 34, 37). Possible changes in other characteristics of cholinergic neurons remain to be investigated.

Finally, it is known that DFP alters neurotransmitter systems other than the cholinergic system (17) and that manipulation of the hippocampal glutamatergic function alters spatial learning ability in rats (30). It is possible that the differences between the effects of oxotremorine and DFP on spatial learning ability may be related to the differential effects of these drugs on noncholinergic systems.

Both the present study and our previous study of DFP effects suggest that spatial learning in the Morris water task is critically dependent on normal function of brain cholinergic systems during early acquisition. In each case, an abnormality of cholinergic function present early in training resulted in a spatial deficit that was manifested at a later time when cholinergic function was apparently normal.

Several recent studies have suggested that brain cholinergic systems are involved in the integration of spatial information into a response strategy. In general, treatments with muscarinic antagonists seem to produce the greatest spatial deficits when animals must acquire a spatial reference memory task under the influence of these drugs (39,45) or when animals must use spatial working memory to perform the task successfully (3,9). Animals overtrained in spatial reference memory task are relatively resistant to the effects of muscarinic antagonists [(3,45), but also see (14) for conflicting data]., The common factor in these experiments appears to be that anticholinergic agents are most capable of disrupting performance when animals are required to learn new spatial information or to modify their existing spatial knowledge. Our data extend these findings by indicating that a temporary abnormality of cholinergic function can disrupt spatial learning ability and that the learning deficit resulting from abnormal cholinergic function early in training will be manifested even if animals are given additional training at a time when cholinergic function appears normal.

In summary, treatment of C57BL mice with chronic, subcutaneous oxotremorine resulted in decreased ^{3}H-QNB binding in cortex, hippocampus, hindbrain, and hypothalamus. Behavioral tolerance developed but was rapidly lost before the return of muscarinic receptors to normal levels. Spatial learning was impaired in animals that began training at a time when they exhibited behavioral tolerance to the drug. Animals that began their training after loss of tolerance did not show a spatial deficit. These results suggest that the state of cortical and/or hippocampal cholinergic function is important in acquisition of spatial learning, but that there may be a dissociation between muscarinic receptor number and the behavioral effects of chronic oxotremorine treatment. The results also indicate that disruption of cholinergic function during early acquisition of a spatial task can have relatively long-lasting effects on the exhibition of spatial learning.

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