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Tolerance, Cross-Tolerance, and Receptors After Chronic Nicotine or Oxotremorine

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MARKS, M. J. AND A. C. COLLINS. *Tolerance, cross-tolerance, and receptors after chronic nicotine or oxotremorine.* PHARMACOL BIOCHEM BEHAV 22(2) 283–291, 1985.—Saline, 8.0 mg/kg/hr nicotine, or 1.0 mg/kg/hr oxotremorine was continuously infused into the jugular veins of DBA female mice. After 10 days of treatment, respiratory rate, Rotarod performance, Y-maze crossings, Y-maze rears, heart rate, and body temperature were measured after challenge with 2.0 mg/kg nicotine or saline or 0.2 mg/kg oxotremorine. Nicotine-infused mice were tolerant to the effects of nicotine for all six tests and oxtremorine-infused mice were tolerant to the effects of oxotremorine for all six tests and to the effects of nicotine on heart rate and body temperature. Oxotremorine infusion reduced the B_{max} for [^{3}H]-L-QNB binding, but had no effect on B_{max} for either [^{3}H]-DL-nicotine or [^{125}I]-α-BTX binding. Conversely nicotine infusion did not alter the B_{max} for [^{3}H]-L-QNB binding, but increased the B_{max} for both [^{3}H]-DL-nicotine and [^{125}I]-α-BTX binding. These results indicate that tolerance developed to the effects of two cholinergic agents, nicotine and oxotremorine, and that some cross-tolerance to the effects of nicotine occurred in oxotremorine-treated mice. Treatment with oxotremorine caused down-regulation of muscarinic receptors, while treatment with nicotine caused up-regulation of nicotinic receptors. Although some cross-tolerance to the effects of nicotine occurred in oxotremorine-treated mice, this did not appear to result from changes in nicotinic receptors.

Nicotine Oxotremorine Cholinergic drugs Nicotinic receptors Muscarinic receptors
α-Bungarotoxin Quinuclidinyl benzilate Tolerance Cross-tolerance Chronic drug treatment

TWO major classes of cholinergic receptors, muscarinic and nicotinic, have long been recognized [8] and the interaction between these classes of cholinergic receptors in the autonomic nervous system is well established [23]. In addition, agents which act by inhibiting acetylcholinesterase activity (anti-cholinesterases) elicit effects on both of these cholinergic receptor types through the common mechanism of increasing acetylcholine levels. Since interactions between nicotinic and muscarinic cholinergic receptor systems are known to occur, it seems reasonable to suggest that chronic treatment with drugs of one class may affect the responses of an organism to drugs of the other class. One clinical aspect of this type of interaction may occur in smokers who are tolerant to the effects of nicotine, another may arise in those persons, such as agricultural workers, chronically exposed to anticholinesterases.

The effects of chronic treatment with cholinergic agents on the muscarinic receptor system have been studied in some detail. Chronic injections of irreversible anticholinesterases decrease the number of muscarinic receptors [6, 9, 10, 12, 28, 32, 36]. In addition, chronic injection of oxotremorine results in tolerance to the effects of an acute injection of the drug [16], and decreases in the number of muscarinic receptors in the brain [4]. We have demonstrated that chronic infusion of oxotremorine also elicits a decrease in the number of brain muscarinic receptors (down-regulation). The sensitivity of chronically infused mice to oxotremorine-induced hypothermia decreased 80-fold and to oxotremorine-induced debilitation of rotarod performance decreased 25-fold [17]. Chronic application of carbamylcholine to the spinal cord also results in a down-regulation of muscarinic receptors [34]. The results of these studies are all consistent: Chronic treatment with either direct or indirect acting muscarinic agonists leads to a down-regulation of muscarinic receptors and also (when measured) to the development of tolerance to the effects of those agents.

Tolerance also develops to the effects of nicotine with chronic treatment. The effects of nicotine on locomotor activity of rats [33, 34] and mice [13] decrease in animals chronically injected with the drug. Chronic ingestion of nicotine in drinking water results in tolerance to locomotor effects of nicotine in rats [11]. A similar effect has been seen in mice chronically exposed to tobacco smoke [2]. Tolerance to the effects of nicotine also develops with chronic infusion of the agent [20]. Nicotine treatment also has effects on nicotinic receptors. Chronic ingestion of nicotine in drinking water results in a decrease in the number of d-tubocuraranine bind-

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ing sites in rat midbrain [11]. On the other hand, chronic nicotine injection increases the binding of [^{3}H]-acetylcholine in rat brain cortex [29]. The properties of the [^{3}H]-acetylcholine binding site are consistent with those expected for a nicotinic cholinergic receptor [30]. We have also noted that chronic nicotine infusion increases the binding of [^{3}H]-DL-nicotine in several regions of mouse brain and also increases the binding of [^{125}I]-α-bungarotoxin (α-BTX) in hippocampus [20]. The properties of the [^{3}H]-DL-nicotine binding site are similar to those of the [^{3}H]-acetylcholine binding site and [^{3}H]-DL-nicotine binding has many properties that are consistent with those expected for a nicotinic, cholinergic receptor [21, 27]. However, whether this site is, indeed, cholinergic remains in question [1,31].

The present study was undertaken to determine whether interactions occur between nicotinic and muscarinic systems in mice chronically infused with either nicotine or oxotremorine, agonists for nicotinic and muscarinic cholinergic receptors, respectively. Chronically infused mice were tested for tolerance to the treatment drug and for cross-tolerance to the other drug using a multifactorial test battery. In addition, the brains of the chronically infused animals were dissected and assayed for the binding of [^{3}H]-L-quinuclidinyl benzilate (QNB), a potent muscarinic antagonist [37], and for the binding of [^{3}H]-DL-nicotine [21, 27] and [^{125}I]-α-BTX [19,21], two ligands that appear to label different populations of nicotinic receptors [21, 27, 30].

METHOD

Materials

The radiolabeled compounds [^{3}H]-DL-nicotine, (N-methyl, ^{3}H, specific activity 71.2 Ci/mmol), [^{3}H]-L-quinuclidinyl benzilate (QNB) (benzilic-4, 4-, ^{3}H, specific activity, 33.1 Ci/mmol), [^{125}I]-α-bungarotoxin (αBTX) (tyr, ^{125}I, original specific activity, 15.2 μCi/μg) were purchased from New England Nuclear, Boston, MA.

Polyethylenimine, L-nicotine, oxotremorine, HEPES, TRIS, and TRIS hydrochloride were purchased from Sigma Chemical Co., St. Louis, MO. The nicotine was redistilled periodically. Toluene was obtained from J. T. Baker Chemical Co., Phillipsburg, NJ; 2,5-diphenyloxazole from Fisher Chemical Co., Fairlawn, NJ; and Triton X-100 from Research Products International, Mount Prospect, IL. Glass fiber filters were purchased from Boehringer-Mannheim Corp., Indianapolis, IN.

Animals

Female DBA/2lbg mice were used in these studies and were obtained from the breeding colony at the Institute for Behavioral Genetics, Boulder, CO. All mice were weaned at 25 days of age and were housed with 2–5 female littermates until surgery. Mice were permitted free access to food (Wayne Lab Blox) and water. A 12 hr light/12 hr dark cycle (lights on 7:00 am–7:00 pm) was used.

Surgery

A cannula constructed of silastic tubing was inserted into the right jugular vein of the mouse [3] under anesthesia (pentobarbital, 50 mg/kg, and chloral hydrate, 70 mg/kg). The cannula contained sterile saline and 3 g/l sodium citrate as an anticoagulant. Mice were allowed to recover from the effects of the surgery for two days after which time the animals were transferred to the chronic infusion chambers.

Chronic Drug Infusion

Two days after surgery, the cannula was attached to thermoplastic tubing which was connected to a 1-ml syringe mounted on a Harvard Infusion Pump. The rate of infusion was 35 μl/hr. Sterile saline was administered for 2 days after which time drug treatment began. Three treatments were used: saline (no drug), nicotine (8.0 mg/kg/hr), and oxotremorine (1.0 mg/kg/hr). Final treatment doses were attained by increasing the drug dosage daily. Initial treatment rates were 1.0 mg/kg/hr for nicotine and 0.1 mg/kg/hr for oxotremorine and the final dosage rates were achieved after 7–10 days. Treatment was continued for 10 days at the final dose. Saline infusion was continued throughout the treatment period for the control animals. Mice were weighed periodically and the concentrations of drug solutions were adjusted for change in weight so that dose could be maintained.

Tolerance Tests

A multifactorial test battery was used to assess the responses of the mice to nicotine (2.0 mg/kg) and oxotremorine (0.2 mg/kg), as well as to establish the baseline performance of the animals. Five separate tests were included in the battery: respiration, Rotarod performance, Y-maze, heart rate, and body temperature. Two types of results were obtained from the Y-maze: line crossings and rears. All mice were tested for their responses to the acute effects of 2.0 mg/kg nicotine, saline, and 0.2 mg/kg oxotremorine. The tests were conducted on three successive days. Testing began 2 hr after the mice were removed from the infusion cages. The effects of nicotine were determined on day 1, the effects of saline were determined on day 2, and the effects of oxotremorine were determined on day 3. Immediately after each day of testing the mice were placed back in the infusion chamber and drug infusion was reinitiated. It has previously been found that this test order minimizes any test day by response interaction (unpublished observations). In addition, the responses of mice tested by saline injection on three successive days (to model an animal that was tolerant to both nicotine and oxotremorine) did not change over the three-day test period (unpublished observations). Each test in the battery was conducted as follows:

Respiratory rate. A Respiration Rate Monitor (Columbus Instruments, Columbus, OH) was used to measure respiratory rate. Mice were placed in a glass jar, the floor of which was covered with aspen shavings. A closed system was created by placing a lid, in which a pressure transducer was mounted, on the jar. Respiratory rate was measured by recording the number of breaths/min over a 1-min period. Five separate readings, 15 sec apart, were made. Recording was begun 1 min after the lid was put in place. These measurements were made 1 min after the injection of nicotine and 16 min after the injection of saline or oxotremorine.

Rotarod performance. Mice had previously been trained to walk on the Rotarod (Ugo Basile Co., Milan, Italy) for 100 sec. Rotation speed was 8 rpm. After the measurement of the respiratory rate, the mouse was placed on the rotating rod until 100 sec had elapsed or until the animal fell from the device, in which case the time was recorded. The Rotarod test was conducted 2.5 min after nicotine injection or 17.5 min after saline or oxotremorine injection.

Y-maze activity. The maze is a symmetrical Y-shaped runway. Each arm of the maze is 26 cm long, 6.1 cm wide, and 10.2 cm high. The arms are subdivided into two equal

sections. The maze is constructed of black acrylic plastic with covers of red translucent acrylic plastic. Illumination is with red light. The mouse to be tested was placed in the center of the maze and movement from one section to another was recorded for 3 min. The number of rearings occurring in the test period was also recorded. This test was conducted 4.5 min after nicotine injection and 19.5 min after saline or oxotremorine injection.

Heart rate. After the Y-maze test was completed, the mouse was placed in a restrainer and needle electrodes were inserted through the skin. One was placed immediately behind the left foreleg and the other immediately in front of the right hindleg. The electrodes were connected through a preamplifier to an E and M physiograph (Narco Biosystems, Houston, TX). Heart rate was monitored for 6 sec and the rate was estimated by counting the number of QRS complexes. Heart rate was measured 8.5 min after the injection of nicotine and 23.5 min after the injection of saline or oxotremorine.

Body temperature. Temperature was measured with a rectal probe (Bailey Instruments, Saddle Brook, NJ). The probe was lubricated with peanut oil before it was inserted 2.5 cm into the rectal cavity. Body temperature was measured 15 min after injection of nicotine and 30 min after injection of saline or oxotremorine.

The timing of the tests was determined from the results of time course studies for the effects of nicotine [19] and oxotremorine [17,18] which we have previously assessed on several of the components of the test battery.

Prior to the conducting of the tolerance tests, mice were removed from the treatment chambers and their cannulas were tested for free flow. Two hr elapsed between the removal of the mice from the infusion chambers and the initiation of the tolerance testing to allow the nicotine and oxotremorine to be metabolized. During this time period, the mice were trained on the Rotarod.

The drug doses used in the tolerance tests were 2.0 mg/kg nicotine and 0.2 mg/kg oxotremorine. Drugs were dissolved in sterile saline and injected (0.01 mg/g body weight) intraperitoneally. The doses used gave maximal, or near maximal, responses in naive mice for each test.

Biochemical Measurements

The binding of radiolabeled QNB, nicotine, and α-BTX to whole particulate fractions of mouse brain regions was measured using filtration assays described in detail elsewhere [19, 20, 21]. The methods used for tissue preparation and ligand binding will be summarized briefly below.

Tissue preparation. After the completion of the third tolerance test, the mouse was killed by cervical dislocation and its brain was removed and dissected into 7 regions: cortex, cerebellum, hindbrain (pons-medulla), hypothalamus, hippocampus, striatum, and midbrain (midbrain areas remaining after removal of hypothalamus, hippocampus, and striatum). The cerebellum was discarded owing to its low level of cholinergic activity. The tissue pieces were placed in 10 vol of HEPES-buffered Ringer's solution (NaCl, 118 mM; KCl, 4.8 mM; $CaCl_2$ 2.5 mM; $MgSO_4$, 1.2 mM; HEPES, 20 mM; pH adjusted to 7.5 with NaOH) and frozen at −70°C. On the day of assay, the samples were thawed and homogenized with a glass-teflon homogenizer. The particulate fraction was collected by centrifugation for 20 min at 18000 g. The pellet was resuspended in 20 vol of water and incubated on ice for 60 min. After this incubation, the sample was centrifuged for 20 min at 18000 g. The resulting pellet was resuspended in buffer for use in the ligand binding assays. The method outlined above is similar to that employed by Romano and Goldstein [27] to prepare rat brain for nicotine binding. Prior to each of the three centrifugation steps, the homogenates were incubated for 5 min at 37°C. These incubations were included to promote the dissociation of drugs, with which the animals had been treated, from the tissue. This method has been shown to remove nicotine from mouse brain tissue [20].

[^{3}H]-L-QNB binding. The binding of [^{3}H]-L-QNB to brain tissue was measured using a modification of the method of Yamamura and Snyder [37] as described previously [18,21]. Aliquots of brain tissue (adjusted for region such that tissue receptor concentration was less than $0.25 \times K_D$) were pipeted into 10 ml of HEPES-buffered Krebs-Ringer buffer. Binding was initiated by the addition of [^{3}H]-L-QNB. The binding reaction was run for 60 min at 37°C. A single concentration of ligand was used to assay binding in 5 of the brain regions. The average QNB concentration was 138 pM. Binding to cortex was measured at 5 QNB concentrations and the binding parameters (K_D and B_{max}) for this brain region were determined from Scatchard plots of the data. The binding reaction was terminated by filtration of the samples onto Boehringer-Mannheim glass fiber filters. The filters were washed three times with 3-ml aliquots of ice-cold buffer. The vacuum was −25 to −50 torr. Blanks obtained using 10^{-6}M atropine, 10^{-4}M oxotremorine, or no tissue were equivalent. The no tissue blank was used primarily.

[^{3}H]-DL-nicotine binding. The binding of [^{3}H]-DL-nicotine was measured using a modification of the method of Romano and Goldstein [27] as described previously [19, 20, 21]. Binding was measured using 100–600 μg of protein. Final incubation volume was 250 μl. HEPES-buffered Krebs-Ringer solution was used as the buffer. In addition, 500 mM TRIS (pH 7.5 at 37°) was included to reduce the nonspecific binding. The binding was conducted in 12×75 mm polypropylene tubes at 37°C. The reaction was initiated by the addition of the labeled ligand and continued for 5 min. The binding was terminated by addition of 3 ml of ice-cold buffer followed immediately by filtration of the samples onto Boehringer-Mannheim glass fiber filters which had been soaked in buffer containing 0.5% polyethylenimine to reduce nonspecific binding [27,30]. The filters were subsequently washed four more times with 3-ml aliquots of ice-cold buffer. The vacuum was −50 to −100 torr. All filtrations and washes were conducted in a 4° cold room using apparatus cooled to 4°. Blanks were obtained by including 1×10^{-5}M L-nicotine in the incubations. A single concentration of radiolabeled nicotine (25.0 nM) was used for these assays in all brain regions. In addition, the affinity of nicotine for the binding site in cortex was estimated by displacement of [^{3}H]-DL-nicotine binding by nonlabeled L-nicotine. Since the binding is stereospecific [1, 21, 27], the displacement data were used to calculate the K_D (for L-nicotine) and the B_{max} for ligand binding after conversion of the results to a form suitable for use in Scatchard plots.

[^{125}I]-α-BTX binding. The binding of [^{125}I]-α-BTX was measured as described previously [19, 20, 21]. Binding was measured using 50–300 μg of protein in a final volume of 500 μl of HEPES buffered Krebs-Ringer solution. The reaction was initiated by the addition of [^{125}I]-α-BTX and continued for 2.5 hr at 37°C. At the completion of the incubation, samples were diluted with 3 ml of ice-cold buffer and filtered on Boehringer-Mannheim filters which had been soaked in buf-

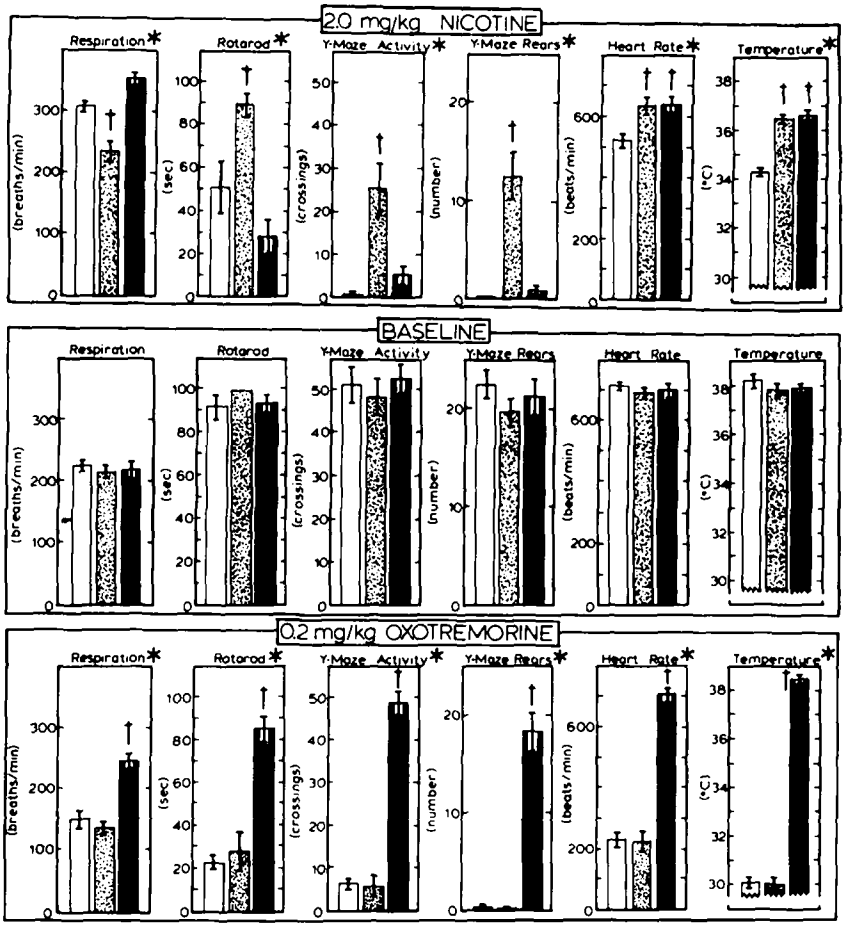

FIG. 1. Responses of chronically treated mice to acute injections of nicotine, saline, and oxotremorine. DBA female mice which had been chronically infused with saline (open box), 8.0 mg/kg/hr nicotine (dotted box) or 1.0 mg/kg/hr oxotremorine (closed box) were tested on three successive days for the effects of 2.0 mg/kg/hr nicotine, saline, and 0.2 mg/kg oxotremorine on each of the six responses as described in the methods. Each value represents the mean±S.E.M. of 11–13 mice for each chronic drug treatment. *Overall effect of treatment, one way ANOVA, $p<0.05$. †Different from saline-infused control, Tukey's b post hoc test, $p<0.05$.

fer containing 0.5% polyethylenimine to reduce the blank. The filters were then washed 4 times with 3-ml aliquots of ice-cold buffer. Vacuum pressure was −50 to −100 torr. Samples containing 1×10^{-3}M nicotine served as the blanks. For 5 brain regions a single concentration of [^{125}I]-α-BTX (1.02 nM) was used. Four additional concentrations of ligand were used with cortex to calculate K_D and B_{max} from Scatchard plots.

Scintillation counting. After the samples were washed, the glass fiber filters were placed in 10-ml Nalge filmware bags, and 2.5 ml of scintillation fluid (toluene, 1.35 liters; Triton X-100, 900 ml; 2,5-diphenyloxazole, 10.5 g) were added. After the bags were sealed, the filters were crushed and radioactivity was determined on a Beckman 7000 liquid scintillation spectrometer. Tritium was counted at 22% efficiency, and ^{125}I was counted at 40% efficiency.

Protein assay. Protein was measured using the method of Lowry *et al.* [15] with bovine serum albumin as the standard.

Data analysis. The results of both the tolerance tests and binding assays were analyzed using one-way analysis of variance (ANOVA) as a function of drug treatment. The tolerance tests were analyzed independently and the binding data were analyzed separately for each ligand in each brain region. The results of those analyses where a significant effect of treatment was detected were further examined using Tukey's b post hoc test. Significance was set at the $p<0.05$ level. Binding constants (K_D and B_{max}) were calculated by linear regression analysis of Scatchard plots of the binding data. The lines obtained were tested for parallelism (indicating equal slope and therefore equal K_D values) and for superimposability (both K_D and B_{max} values equal) using *t*-tests. Since multiple comparisons between control and treated mice were made, significance levels for these tests were set at $p<0.025$.

RESULTS

The results displayed in Fig. 1 are the responses of DBA mice which had been chronically infused with saline, 8.0 mg/kg/hr nicotine, or 1.0 mg/kg/hr oxotremorine to acute

TABLE 1

EFFECTS OF CHRONIC DRUG INFUSION ON CORTICAL RECEPTOR BINDING

	[^{3}H]-DL-nicotine		[^{125}I]-α-BTX		[^{3}H]-L-QNB	
Drug Infused	B_{max}*	K_D	B_{max}*	K_D	B_{max}*	K_D
Saline	32.3 ± 4.4	17.6 ± 6.7	13.7 ± 2.0	0.42 ± 0.11	2176 ± 134	0.025 ± 0.003
Nicotine	65.1 ± 6.5†	17.7 ± 5.0	23.4 ± 6.1†	0.36 ± 0.14	2049 ± 138	0.020 ± 0.003
Oxotremorine	36.9 ± 4.5	21.8 ± 6.2	14.6 ± 2.8	0.30 ± 0.06	1593 ± 106†	0.023 ± 0.003

K_D (nM) and B_{max} (fmol/mg protein) were calculated by linear regression analysis of Scatchard plots of binding data obtained using cerebral cortical tissue. Six to eight mice per treatment group were used. Results are mean ± S.D.

*$p<0.05$, overall significant effect of treatment, one-way ANOVA.

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

challenge doses of saline, 2.0 mg/kg nicotine, and 0.2 mg/kg oxotremorine. The effects of the acute doses were measured using 6 tests: respiratory rate, Rotarod performance, Y-maze activity, Y-maze rears, heart rate, and body temperature. All of these parameters are affected by acute administration of nicotine or oxotremorine to naive mice. Each response was analyzed separately using one-way ANOVA comparing treatment groups.

The responses of all the treatment groups after saline injection were identical. (Respiration, $F(2,35)=0.26$; Rotarod, $F(2,31)=0.87$; Y-maze crossings, $F(2,35)=0.15$; Y-maze rears, $F(2,35)=0.52$; heart rate, $F(2,34)=0.86$; and body temperature, $F(2,35)=1.73$; in all cases $p>0.10$.) These results indicate that chronic treatment with 8.0 mg/kg/hr nicotine or 1.0 mg/kg/hr oxotremorine did not markedly affect the basal physiological and behavioral responses of the mice when measured two hours after removal from the infusion cages.

In contrast, the responses after administration of a dose of 2.0 mg/kg nicotine differed among treatment groups in each of the six tests (Respiration, $F(2,35)=20.92$; Rotarod, $F(2,34)=10.35$; Y-maze crossings, $F(2,34)=10.22$; Y-maze rears, $F(2,34)=19.67$; heart rate, $F(2,35)=16.10$; body temperature, $F(2,35)=36.58$; in all cases $p<0.001$). Since differences were found for all six tests, the groups were further compared using Tukey's b post hoc test at a significance level of $p<0.05$. These analyses revealed that the responses of mice challenged with the 2.0 mg/kg nicotine dose were reduced in those animals infused with 8.0 mg/kg/hr nicotine. Reduced responses, relative to saline-infused controls, were seen for respiration, Rotarod, Y-maze crossings, and Y-maze rears. Those mice chronically infused with 1.0 mg/kg/hr oxotremorine did not differ from controls in any of these four tests. On the other hand, the responses of mice to 2.0 mg/kg nicotine were reduced in animals infused with either 8.0 mg/kg/hr nicotine or 1.0 mg/kg/hr oxotremorine for the heart rate and body temperature tests. These results indicate that mice infused with 8.0 mg/kg/hr nicotine are tolerant to the effects of an acute challenge dose of 2.0 mg/kg of nicotine for each of the 6 responses measured and, in addition, that mice treated with 1.0 mg/kg/hr oxotremorine are cross-tolerant to the effects of nicotine on both heart rate and body temperature.

The responses of the mice in the three treatment groups after challenge with an acute 0.2 mg/kg dose of oxotremorine were significantly different for all six tests (respiration, $F(2,33)=36.61$; Rotarod, $F(2,31)=33.52$; Y-maze crossings, $F(2,33)=84.47$; Y-maze rears, $F(2,33)=34.49$; heart rate, $F(2,33)=126.27$; and body temperature, $F(2,33)=301.41$; in all cases $p<0.001$). Since differences were found for each test, the groups were further compared using Tukey's b post hoc test at a significance level of $p<0.05$. These analyses revealed that the responses of mice treated with 1.0 mg/kg/hr oxotremorine were significantly different from those of saline-infused mice for all tests, while those of mice treated with 8.0 mg/kg/hr nicotine did not differ from the responses of saline-infused mice in any of the tests. These results indicate that mice chronically infused with 1.0 mg/kg/hr oxotremorine are tolerant to the effects of a challenge dose of 0.2 mg/kg of oxotremorine, but that mice infused with 8.0 mg/kg/hr nicotine are not cross-tolerant to these acute effects of oxotremorine.

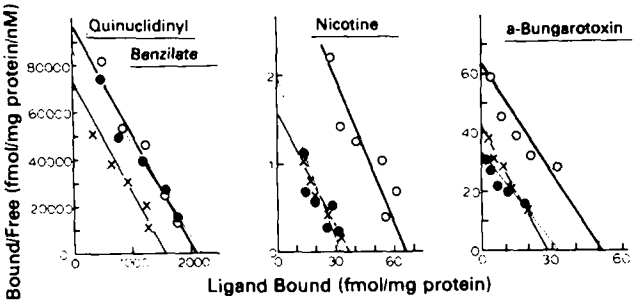

FIG. 2. Scatchard plots for [^{3}H]-L-QNB, [^{3}H]-DL-nicotine, and [^{125}I]-α-BTX binding to cerebral cortex after chronic drug treatment. Saturation curves were constructed for the ligands indicated using cortex tissue obtained from mice chronically treated with either saline (●), 8.0 mg/kg/hr nicotine (○) or 1.0 mg/kg/hr oxotremorine (X). Each point is the mean of results from 5–7 mice per group.

The effect of chronic treatment with saline, 8.0 mg/kg/hr nicotine, and 1.0 mg/kg/hr oxotremorine on the equilibrium binding kinetics of [^{3}H]-L-QNB, which binds to muscarinic cholinergic receptors [38], and of [^{3}H]-DL-nicotine and [^{125}I]-α-BTX, which appear to bind to two different types of nicotinic receptors [1, 21, 27], were also measured. The Scatchard plots for the binding of these three ligands to cerebral cortical particulate fractions are shown in Fig. 2. Table 1 presents a summary of the maximal binding (B_{max}) and dissociation constants (K_D) calculated from the Scatchard plots presented in Fig. 2. Results presented are mean±S.D. of values calculated from the Scatchard plots. Chronic nicotine infusion resulted in significant increases in maximal [^{3}H]-DL-nicotine (101.5%) and [^{125}I]-α-BTX (60.3%) binding.

No changes in K_D were detected. Nicotine infusion did not alter either parameter for [^{3}H]-L-QNB binding. Chronic oxotremorine infusion, on the other hand, decreased maximal [^{3}H]-L-QNB binding (26.8%) but was without effect on either [^{3}H]-DL-nicotine or [^{125}I]-α-BTX binding. Similarly, no effect was seen on the K_D value for any of the ligands tested. These results indicate that chronic treatment with nicotine increased the binding of nicotinic ligands and that chonic treatment with oxotremorine significantly decreased the binding of the muscarinic ligand in cerebral cortex. However, treatment with nicotine had no effect on muscarinic receptor binding and treatment with oxotremorine had no effect on nicotinic receptor binding. In no case did chronic drug treatment affect the K_D for any of the three ligands: All changes observed were in the number (B_{max}) of binding sites.

To determine if the pattern of receptor change after chronic drug treatment occurred in brain areas other than cortex, five other brain regions were assayed for ligand binding as well. In these assays a single concentration of radiolabeled ligand was used. The results shown in Fig. 3 are those for the binding of [^{3}H]-L-QNB in the various brain regions. In each of these regions, a significant overall effect of drug treatment was indicated (cortex, $F(2,22)=6.32$, $p<0.01$; midbrain, $F(2,24)=12.63$, $p<0.001$; hindbrain, $F(2,24)=19.06$, $p<0.001$; hippocampus, $F(2,24)=19.06$, $p<0.001$; striatum, $F(2,24)=5.13$, $p<0.05$; and hypothalamus, $F(2,24)=4.06$, $p<0.05$). The post hoc tests indicated that only those animals infused with 1.0 mg/kg/hr oxotremorine differed significantly from saline-treated mice. The amount of [^{3}H]-L-QNB binding was found to be significantly reduced in all regions except hypothalamus. The pattern of change in [^{3}H]-L-QNB binding in all of the brain is similar—only chronic oxotremorine infusion affected receptor levels.

Figure 4 presents the effects of drug treatment on the binding of [^{3}H]-DL-nicotine in six brain regions. Significant effects of drug treatment were indicated in five of the regions (cortex, $F(2,38)=13.59$, $p<0.001$; midbrain, $F(2,38)=6.79$, $p<0.01$; hindbrain, $F(2,38)=5.26$, $p<0.001$; hippocampus, $F(2,18)=14.54$, $p<0.01$; and hypothalamus, $F(2,17)=7.08$, $p<0.01$), while no significant effect of drug treatment was seen for striatum, $F(2,17)=2.09$, $p>0.05$. The subsequent post hoc tests revealed that the binding of [^{3}H]-DL-nicotine was significantly greater in mice treated with 8.0 mg/kg/hr nicotine than in control mice in each of the 5 regions. In no case was the binding of [^{3}H]-DL-nicotine altered in those mice treated with 1.0 mg/kg/hr oxotremorine.

Figure 5 illustrates the effects of chronic drug treatment on [^{125}I]-α-BTX binding in 6 brain regions. Overall significant effects of treatment were indicated for cortex, $F(2,18)=14.54$, $p<0.001$, and hippocampus, $F(2,18)=3.77$, $p<0.05$, but not in the other four brain regions (midbrain, $F(2,18)=0.49$; hindbrain, $F(2,18)=2.10$; striatum, $F(2,18)=0.81$; and hypothalamus, $F(2,18)=1.32$; in all cases $p>0.05$). Subsequent post hoc tests showed that the binding of [^{125}I]-α-BTX was higher in cortex and hippocampus from mice infused with 8.0 mg/kg/hr nicotine than it was in saline-infused mice. The binding of [^{125}I]-α-BTX was unaffected by chronic oxotremorine treatment.

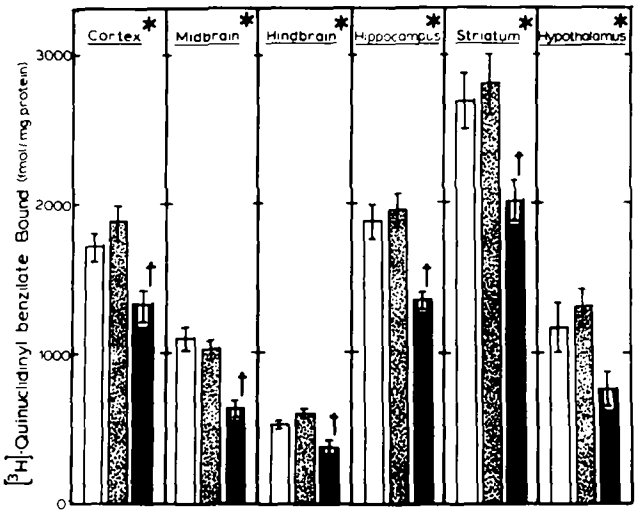

FIG. 3. Binding of [^{3}H]-L-QNB to brain regions of chronically treated mice. Specific [^{3}H]-L-QNB binding was determined using a single ligand concentration (138±11 pM) for each of the six brain regions obtained from mice infused with saline (open box), 8.0 mg/kg/hr nicotine (dotted box) or 1.0 mg/kg/hr oxotremorine (closed box). Results are mean±S.E.M. for 8–10 individuals per treatment. *Significant overall effect of treatment within brain region, one-way ANOVA, $p<0.05$. †Significantly different from binding in saline-treated mice, Tukey's b post hoc test, $p<0.05$.

DISCUSSION

The present study examined the effects of oxotremorine and nicotine on six different behavioral or physiological measures. These agents elicited similar effects on five of the measures in saline-treated mice. Only for respiration was a difference in response observed: Nicotine increased while oxotremorine decreased respiration rate. This suggests there is not an obligatory relationship between the actions of these drugs, i.e., nicotine does not elicit all of its actions, for example, by increasing the release of acetylcholine which then acts at muscarinic receptors.

Further evidence for a dissociation between nicotinic and muscarinic actions comes from the tolerance studies. Chronic oxotremorine-treated animals were virtually unaffected by the 0.2 mg/kg challenge dose of oxotremorine, i.e., they were tolerant to oxotremorine. However, when challenged with 2.0 mg/kg nicotine, oxotremorine-infused animals were not tolerant to nicotine for the respiration, Rotarod, and Y-maze tests. However, cross-tolerance was seen for heart rate and temperature, i.e., oxotremorine-treated animals were tolerant to the effects of both nicotine and oxotremorine. These results argue that oxotremorine and nicotine influence respiration, Rotarod performance, and Y-maze activity and rearing by different mechanisms or systems, while similar mechanisms or systems may be affected by the two drugs for their effects on heart rate and body temperature. The selectivity of cross-tolerance suggests that muscarinic-nicotinic interactions occur in localized regions of the central and peripheral nervous systems and that, even though these two drugs may elicit similar responses, this similarity of response need not be due to an identity of site of action.

Peripheral mechanisms are likely to regulate the interactions between nicotine and oxotremorine on heart rate [14] although central mechanisms may also be important [5]. For example, nicotine probably stimulates autonomic ganglia which leads to an increase in vagal release of acetylcholine to slow the heart. The cross-tolerance seen between nicotine and oxotremorine in oxotremorine-infused animals may be

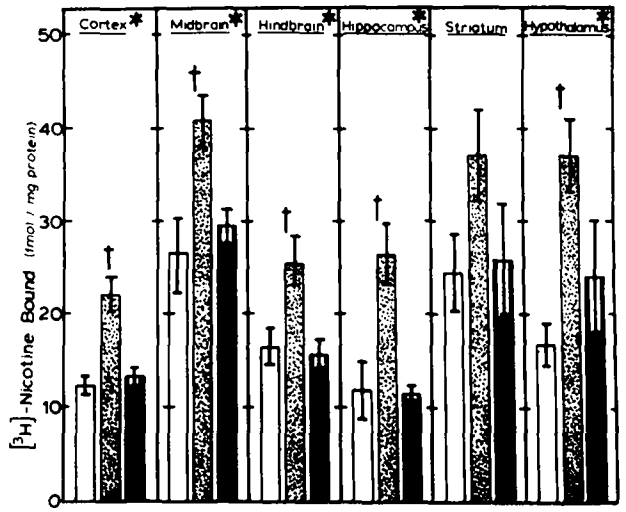

FIG. 4. Binding of [^{3}H]-DL-nicotine to brain regions of chronically treated mice. Specific [^{3}H]-DL-nicotine binding was determined using a single ligand concentration (25±1.4 nM) for each of six brain regions from mice infused with saline (open box), 8.0 mg/kg/hr nicotine (dotted box), or 1.0 mg/kg/hr oxotremorine (closed box). Results are mean±S.E.M. for 13–15 individuals for cortex, midbrain, and hindbrain and for 6–7 individuals for the other three regions for each treatment. *Significant overall effect of treatment within brain region, one-way ANOVA, $p<0.05$. †Significantly different from binding in saline-treated mice, Tukey's b post hoc test, $p<0.05$.

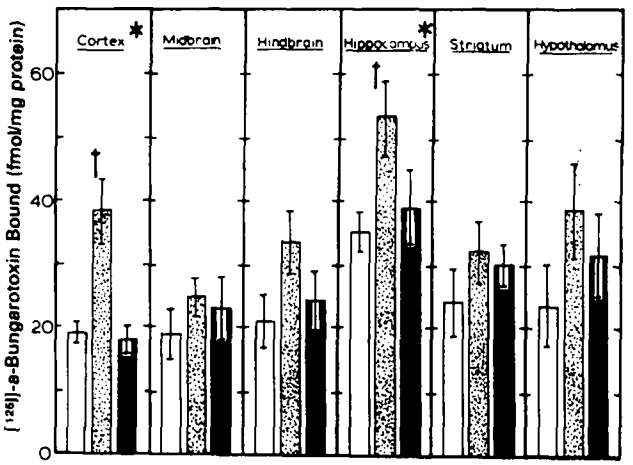

FIG. 5. Binding of [^{125}I]-α-BTX to brain regions of chronically treated mice. Specific [^{125}I]-α-BTX binding was determined using a single ligand concentration (1.02±0.08 nM) for each of six brain regions obtained from mice treated with saline (open box), 8.0 mg/kg/hr nicotine (dotted box) or 1.0 mg/kg/hr oxotremorine (closed box). Results are mean±S.E.M. for 5–8 individuals per treatment. *Significant overall effect of treatment within brain region, one-way ANOVA, $p<0.05$. †Significantly different from binding in saline-treated mice, Tukey's b post hoc test, $p<0.05$.

due to decreased sensitivity to the acetylcholine released following nicotine administration. Since the hypothalamus is important in regulating body temperature [7], an interaction between nicotinic and muscarinic systems in hypothalamic thermoregulatory pathways may also occur.

Animals chronically infused with nicotine were tolerant to the actions elicited by the 2.0 mg/kg challenge dose of nicotine. This tolerance, however, was not complete for most of the measures. In addition, these mice responded to the 0.2 mg/kg oxotremorine dose to the same degree as did the saline-infused animals, i.e., no evidence for cross-tolerance was obtained. Thus, cross-tolerance is test specific and unidirectional.

A potential explanation for the unidirectional nature of the cross-tolerance may relate to the magnitude of tolerance elicited by oxotremorine and nicotine. We [17] have previously demonstrated that chronic oxotremorine infusion at 1 mg/kg/hr elicits a 24-fold increase in the dose required to inhibit Rotarod performance by 50% and an 80-fold increase in the oxotremorine dose required to depress body temperature to 35°. On the other hand, in a study of the effects of chronic nicotine infusion we observed only a 2–5 fold decrease in sensitivity to several nicotine effects [20]. The unidirectional nature of the cross-tolerance may relate to this difference in magnitude of tolerance. Perhaps, bidirectional cross-tolerance would be observed at higher nicotine doses. Our experience suggests, however, that mice may not survive treatment with higher nicotine doses. Interestingly, the degree of tolerance to nicotine on Y-maze activity in the present study is not significantly different, and may even be less, than that achieved by chronic injection of lower nicotine doses into rats [33,34] and mice [13].

It is unlikely that the tolerance or cross-tolerance observed in the present study is due to altered metabolism. If cross-tolerance were due to altered metabolism, it should have been seen for every measure. Nicotine tolerance does not seem to arise from altered metabolism since several studies have demonstrated that chronic treatment of mice with nicotine by injection [13], by administration of tobacco smoke [2], or by constant infusion [20] has little effect on the metabolism of this drug. In addition, chronic infusion of oxotremorine does not alter the biological half-life for disappearance of the effects of this compound [17]. Thus, the tolerance which occurs following chronic infusion of nicotine or oxotremorine is probably due to altered tissue sensitivity.

Receptor changes may well influence the tolerance. Chronic infusion of either oxotremorine or nicotine affected the numbers of binding sites for muscarinic or nicotinic receptors. The effects were selective. The infusion of oxotremorine, a muscarinic agonist, resulted in a decrease in the number of binding sites for [^{3}H]-L-QNB only. This change in muscarinic receptors after chronic oxotremorine treatment is consistent with previous observations that chronic treatment with anti-cholinesterases [6, 9, 10, 12, 28, 32, 36] or muscarinic agonists [4, 17, 18, 35] resulted in down-regulation of these receptors and is consistent with the results expected for chronic agonist treatment. The infusion of nicotine, a nicotinic agonist, resulted in an increase in the number of binding sites for [^{3}H]-DL-nicotine in 5 of 6 brain regions, as well as an increase in [^{125}I]-α-BTX binding in cortex and hippocampus. These two binding sites have properties expected of nicotinic receptors [1, 21, 24, 26, 27]. The increase in [^{3}H]-DL-nicotine binding after chronic nicotine treatment is consistent with that observed previously for the binding of this ligand [20] in mice and for the binding of [^{3}H]-acetylcholine (which has properties very similar to those for the [^{3}H]-DL-nicotine binding site [30]) after chronic injection of nicotine in rats [29]. The mechanism responsible for this

up-regulation is not yet known although it has been postulated that the increase in binding may arise from chronic receptor desensitization [20]. An increase in [^{125}I]-α-BTX binding accompanying chronic nicotine treatment has also been noted previously in hippocampus [20]. The higher infusion rate used in the present study resulted in significantly higher binding in cortex and hippocampus. The increase in [^{125}I]-α-BTX binding was less pronounced than was the increase in [^{3}H]-DL-nicotine binding. This difference in response may have arisen as a consequence of the 40-fold difference in affinity of nicotine for these two sites [21], such that changes in the number of binding sites labeled with [^{3}H]-DL-nicotine occur at lower doses of nicotine than do changes in the number of sites labeled with [^{125}I]-α-BTX. The fact that both binding sites respond to nicotine treatment suggests that they are both involved in mediating nicotine's effects in vivo. Chronic oxotremorine infusion had no effect on either of these nicotinic receptors in any brain region, nor did chronic nicotine infusion affect the muscarinic receptors, an observation which has been previously reported [20,29].

The changes which occur in the ligand binding in cortex are changes in the B_{max} for the ligands. The K_D values are unaffected. Owing to limitations of tissue, saturation curves could not be constructed for the other brain regions using tissue from a single mouse. The K_D for each ligand is the same in the six brain regions [18, 19, 20, 21, 22]. In addition, previous studies have shown that chronic oxotremorine infusion had no effect on the K_D for [^{3}H]-QNB [17,18] and that chronic nicotine infusion did not alter the K_D for any of the ligands used in this study [20]. It seems likely, therefore, that any changes in ligand binding that occurred in regions other than cortex reflect changes in the B_{max} values for that ligand.

Although tolerance to the effects of nicotine on body temperature was noted in mice chronically infused with oxotremorine, treatment with this drug had no effect on two nicotinic receptors in hypothalamus. This result indicates that the cross-tolerance to the hypothermic effects of nicotine observed after chronic oxotremorine treatment does not arise from a direct effect of oxotremorine on these nicotinic receptors. Whether other nicotinic sites (i.e., d-tubocuraranine binding [25]) are important in mediating the effects of nicotine and are altered by chronic oxotremorine or nicotine treatment remains to be determined. Alternatively, the cross-tolerance may arise from mechanisms not directly related to nicotinic receptors but by the relationship of these receptors to muscarinic neurons rendered subsensitive by chronic oxotremorine infusion, (i.e., nicotinic receptors presynaptic to or nicotinic neurons proximal to muscarinic receptors). If only a few muscarinic receptors interact with nicotinic sites (a likely possibility considering the fact that muscarinic receptors greatly outnumber nicotinic receptors in mouse brain), the absence of measurable cross-tolerance to oxotremorine after chronic nicotine infusion is easily envisioned.

The results presented in this study suggest that cross-tolerance to some of the effects of nicotine occur in mice chronically treated with the muscarinic agonist oxotremorine. Since the observation of cross-tolerance is test specific and unidirectional, a generalized muscarinic-nicotinic interaction is unlikely to underlie all of the effects of nicotine. Since some cross-tolerance to the effects of nicotine occur after chronic treatment with oxotremorine, the possibility that tolerance to nicotine arising after treatment with agents that have generalized cholinergic effects, such as anticholinesterases, may arise, in part, from the effects of these agents on muscarinic systems must be considered.

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