Pharmacology Biochemistry & Behavior, Vol. 27, pp. 723–732. © Pergamon Journals Ltd., 1987. Printed in the U.S.A. 0091-3057/87 $3.00 + .00

Genetic Influences on Tolerance Development With Chronic Oxotremorine Infusion

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Received 5 November 1986

MARKS, M. J., E. ROMM AND A. C. COLLINS. *Genetic influences on tolerance development with chronic oxotremorine infusion.* PHARMACOL BIOCHEM BEHAV 27(4) 723–732, 1987.—Mice of four inbred strains (BALB, C57BL, DBA and C3H) were administered either saline or oxotremorine, a muscarinic agonist, at a dose of 0.5 mg/kg/hr by constant infusion through cannulas implanted in the right jugular veins. Chronic treatment resulted in the development of tolerance to the effects of oxotremorine both on rotarod performance and on body temperature. For drug-treated BALB mice, the dose-response curves for both measures were parallel to those for saline-treated mice, while for DBA and C3H mice the slopes of the dose-response curves were significantly less for treated mice than they were for controls. The equi-effective doses for the drug-treated animals were at least 8-fold greater than those for saline-treated mice. Drug treatment resulted in a significant decrease in the total number of muscarinic receptors in cortex as measured by the binding of [^{3}H]quinuclidinyl benzilate (QNB) without effect on the K_D for this ligand. Similarly, drug treatment did not affect the affinity of carbamylcholine as an inhibitor of QNB binding, but did significantly decrease the levels of both the high- and low-affinity agonist binding sites in cortex. The number of M1 muscarinic receptors measured by high affinity [^{3}H]pirenzepine (PZ) binding was also significantly decreased in cortex without effect on the K_D. The experiments were extended to five other brain regions. Full saturation curves were not constructed, however. Oxotremorine treatment significantly reduced QNB binding in every brain region. While the binding to agonist affinity states measured by carbamylcholine inhibition of QNB binding and M1 receptor levels measured by high affinity PZ binding tended to decrease with oxotremorine treatment not all changes were statistically significant. The changes in muscarinic receptor subtype levels induced by oxotremorine infusion did not differ among the strains. The results demonstrate that chronic treatment with a muscarinic agonist results in substantial tolerance to the effects of the drug in all four mouse strains. Although some differences in tolerance development exist, these differences are not readily explained by differences in the number or affinity states of brain muscarinic receptors.

Tolerance development Chronic oxotremorine infusion Brain muscarinic receptors

CHRONIC treatment with muscarinic cholinergic drugs is accompanied by changes in response to the drug: Agonist treatment results in tolerance to the effects of the agonist [16–18, 21], while antagonist treatment results in heightened sensitivity to agonists [22,30]. Brain muscarinic receptors respond to chronic treatment with receptor agonists or antagonists. Receptor numbers decrease with treatment with either direct-acting [2, 17, 18, 21, 31] or indirect-acting [6, 8–10, 25, 32] agonists or increase with treatment with antagonists [1, 2, 22, 26, 30], and it has been suggested that these changes in receptor number underlie the changes in drug response.

Several muscarinic receptor types have been indentified. The antagonist, quinuclidinyl benzilate (QNB), binds to brain tissue with high affinity and the binding properties of this ligand correspond to those expected for a single site [36,39]. However, inhibition of antagonist binding by agonists reveals high and low affinity agonist binding states [3, 4, 38]. While most antagonists interact with muscarinic receptors with kinetics representing simple mass action [13, 36, 39], the synthetic antagonist, pirenzepine (PZ), displays considerable selectivity [11–13, 36, 37]. The sites to which PZ selectively binds and exerts its effects have been termed M1 receptors; the lower affinity PZ sites are called M2 receptors. The existence of subtypes of muscarinic receptors, of multiple agonist affinity states on these receptors, and of different receptor coupling mechanisms probably all contribute to the in vivo responses to muscarinic agents.

Genetic factors influence the responsiveness of mice to both nicotinic and muscarinic cholinergic drugs. Differences in responsiveness to nicotine have been observed for several behavioral and physiological tests, including locomotor activity, respiratory rate, heart rate, and body temperature [19,24]. Differences in responsiveness to the

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anticholinesterase, diisopropylfluorophosphate, have also been observed for many of the same behavioral and physiological tests [29] and differences in response to the muscarinic agonist, oxotremorine, have been noted for locomotor activity and body temperature [23]. These studies demonstrate that genotype is an important factor influencing acute responsiveness to cholinergic agents.

Genotype also influences the ability of mice to develop tolerance to the effects of cholinergic drugs with chronic treatment. The development of tolerance to the effects of nicotine differs among four inbred strains: Those strains initially more sensitive to the effects of nicotine develop more tolerance than do those strains that are initially less sensitive [25]. In contrast, the rate of development of tolerance to the effects of diisopropylfluorophosphate is faster in a rat line selected for resistance to the effects of this anticholinesterase than it is in a line selected for sensitivity [27]. These results suggest that genetic factors are important in influencing tolerance development to cholinergic agents.

The present study was undertaken to determine the role of genotype in the ability of mice of four mouse strains (BALB, C57BL, DBA and C3H) to develop tolerance to the effects of the muscarinic agonist, oxotremorine. In addition, the influence of genotype on the ability of muscarinic receptors to respond to the chronic treatment has also been examined.

METHOD

Materials

The radioisotopes L-[^{3}H]QNB (29.4 Ci/mmol) and [^{3}H]PZ (82.3 Ci/mmol) were purchased from New England Nuclear (Boston, MA). Oxotremorine, atropine sulfate, carbamylcholine chlorine, bovine serum albumin and polyethylenimine were obtained from Sigma Chemical Co. (St. Louis, MO). Glass fiber filters were purchased from Boehringer-Mannheim (Indianapolis, IN). Budget Solve scintillation fluid was purchased from Research Products International (Mt. Prospect, IL). Nonradioactive PZ was a generous gift of Boehringer-Ingleheim.

Mice

Sixty to ninety day old female mice of four inbred strains (BALB/cByJ, C57BL/6Ibg, DBA/2Ibg, and C3H/2Ibg) were used. The mice were bred in the colony of the Institute for Behavioral Genetics, Boulder, CO. Before the experiments were begun, the animals were housed with female littermates and permitted free access to food (Wayne Lab Blox) and water. The light cycle was 12 hr light/12 hr dark (lights on 7 a.m. to 7 p.m.).

Chronic Drug Treatment

Oxotremorine, final dose 0.5 mg/kg/hr, was administered by intravenous infusion through a cannula implanted in the right jugular vein. Anesthesia used for the surgery was pentobarbital (approximately 40 mg/kg)/chloral hydrate (approximately 80 mg/kg); actual dosage varied with strain of mouse. After a one day recovery period, each mouse was placed in a cage, and its cannula was attached to tubing connected to a syringe mounted on a Harvard Infusion Pump. The mice were infused initially with saline. For control mice, the saline infusion was contined throughout the treatment period. For treated mice, the oxotremorine infusion was begun after one day of saline treatment. The final dose of 0.5 mg/kg/hr was attained by starting treatment at 0.1 mg/kg/hr and increasing the dosage by 0.1 mg/kg/hr daily. Treatment continued at the final dose for 8 days. This method of oxotremorine treatment has been described previously [17, 18, 21].

Tolerance Test

The tests used to assess responsiveness to oxotremorine were rotarod performance and body temperature. Tolerance was assessed by constructing dose-response curves in each individual animal. This was accomplished by injecting the animal with oxotremorine and testing its ability to walk on a rotarod (8 rpm) and by measuring its body temperature 15 min after injection. Immediately thereafter the mouse was injected with another dose of oxotremorine and rotarod performance and body temperature assessed as described above. This procedure was repeated until a cumulative oxotremorine dose-response curve had been constructed for each animal. This usually involved a minimum of four drug injections. We have previously reported that these dose-response curves are identical to the dose-response curves constructed by giving individual animals different doses of oxotremorine [17]. Dosage was adjusted depending on strain and treatment. Animals were pretrained so that in a drug-free state the animal could negotiate the rotarod for 100 sec. Body temperature was measured using a Bailey Instruments rectal probe. Details of the test method have been published previously [17].

Tissue Preparation

At the completion of the tolerance test, each mouse was sacrificed by cervical dislocation, its brain was removed and dissected into seven regions: cerebral cortex, cerebellum, hindbrain (pons-medulla), hippocampus, hypothalamus, striatum, and midbrain (areas remaining after dissection, primarily thalamus). Cerebellum was discarded owing to low cholinergic activity. The brain regions were homogenized in 10 volumes of 10 mM potassium phosphate buffer, pH=7.4. The whole particulate fraction was collected by centrifugation at 18000 × g for 20 min. The pellet was washed twice by resuspension in phosphate buffer and recentrifugation. The final pellet was resuspended to a protein concentration of approximately 1 mg/ml in 10 mM potassium phosphate buffer, pH 7.4.

L-[^{3}H]QNB Binding

The binding of L-[^{3}H]QNB was measured using a modification of the method of Yamamura and Snyder [39] as described previously [18,20]. Incubations were conducted at 22° for 2 hr in 10 ml of 10 mM potassium phosphate buffer (pH 7.4) and were terminated by filtration over glass fiber filters. Saturation curves were constructed for cortical tissue using six ligand concentrations. Binding in the other five regions was measured at a single concentration of L-[^{3}H]QNB (77 pM). Blanks were determined by measuring binding in the presence of 1 μm atropine or by omitting protein with identical results.

Carbamylcholine Inhibition of L-[^{3}H]QNB Binding

Agonist affinity states of muscarinic receptors were estimated by including carbamylcholine chloride in the incubation. The concentrations of carbamylcholine chloride used varied between 10^{-9} M and 10^{-3} M. Proportions of the two affinity states and the IC_{50} values were calculated using a two

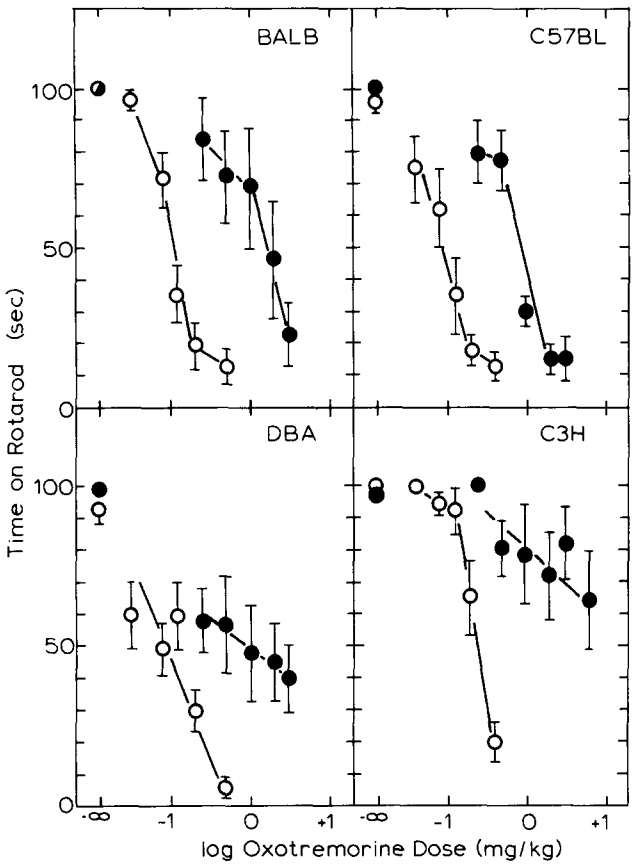

FIG. 1. Dose-response curves for oxotremorine effects on rotarod performance. Dose-response curves for rotarod performance were constructed using the sequential injection method for both control (○) and oxotremorine-infused (●) mice. Each point represents mean±SEM for 8 individual animals.

site model as described previously [18]. The method of Cheng and Prussoff [5] was used to estimate the K_i values.

[^{3}H]PZ Binding

The binding of the M1 selective antagonist, [^{3}H]PZ, was measured using a modification of the method of Watson *et al.* [37]. Incubations were conducted in 1 ml volumes of 10 mM potassium phosphate buffer (pH 7.4) for 1 hr at 22°. The binding reaction was terminated by filtration of the samples over glass fiber filters that had been soaked in 0.5% polyethylenimine. Concentrations of [^{3}H]PZ ranging from 0.5 nM to 120 nM were used for the saturation curves. Concentrations of 2 nM and above were achieved by mixing radioactive and nonradioactive PZ. The proportions of the two PZ binding sites were calculated by iteration of the binding data. Blanks were determined by including 1 μM atropine in the assays.

Scintillation Counting

After filtration, the washed glass fiber filters were placed in 7 ml scintillation vials and 2.5 ml of Budget Solve was added. The vials were mechanically shaken for 1 hr and tritium was determined using a Beckman 1800 Liquid Scintillation Spectrometer. Counting efficiency was 45%.

Protein Measurement

Protein was measured using the method of Lowry *et al.* [14] with bovine serum albumin as the standard.

Statistical Analysis

Dose-response curves were analyzed by linear regression and lines were compared using *t*-tests [7]. Binding data were analyzed by Analysis of Variance followed by Duncan's New Multiple Range *post hoc* test.

RESULTS

Tolerance Tests

Dose-response curves for the effect of oxotremorine on rotarod performance are shown in Fig. 1. The slopes and ED_{50} values for these curves are summarized in Table 1. No significant differences in the slopes of the lines were observed. The ED_{50} values for BALB, DBA and C57BL mice were very similar; the ED_{50} for C3H mice was 2–3-fold greater than that of the other three strains.

Chronic oxotremorine infusion resulted in a parallel shift to the right of the dose-response curves for oxotremorine effects on rotarod performance for both BALB and C57BL mice. The ED_{50} value for drug-treated BALB mice was about 15-fold greater and the ED_{50} value for C57BL mice was about 8-fold greater than were the ED_{50} values for saline-treated mice of these two strains. The slopes for the dose-response curves for drug-treated DBA and C3H mice were significantly less than those for the saline-treated mice of these two strains. Consequently, the ED_{50} values for animals of these strains reflect a change both in slope and intercept of the dose-response curves. The ED_{50} value for treated DBA mice was approximately 10-fold higher than was the ED_{50} value for the saline-treated DBA mice. No calculation of an ED_{50} value for C3H mice was made because the highest dose of oxotremorine did not impair rotarod performance by 50%. The change in ED_{50} for C3H mice exceeded 20-fold.

Dose-response curves for the effect of oxotremorine on body temperature are shown in Fig. 2. The slopes and $ED_{-3.0}$ (dose required to lower temperature by 3.0°) are summarized in Table 1. The slopes of these curves for control animals do not differ. Because the lines are parallel, the $ED_{-3.0}$ values directly reflect the relative sensitivity of mice of the four strains. As was the case for rotarod performance, C3H mice were the least sensitive of the four strains. DBA mice were the most sensitive.

Chronic oxotremorine treatment had a differential effect on the slopes of the dose-response curves for the four strains. While the slope of the curve for BALB mice was decreased slightly with chronic treatment (83% of control), this decrease was not significant. The slopes of the curves for the other three strains were significantly lower than the corresponding control values (C57BL 63% of control, DBA mice 52% of control, C3H mice 36% of control). The chronic treatment markedly increased the $ED_{-3.0}$ values for all four strains (BALB 20-fold, C57BL 34-fold, DBA 15-fold, and C3H 46-fold increases, respectively). These changes do not result from simple shifts to the right of the dose-response curves inasmuch as the slopes of the lines were also affected by chronic oxotremorine infusion.

Muscarinic Receptors in Cerebral Cortex

The brains of the chronically treated mice were analyzed

TABLE 1
TOLERANCE TEST PARAMETERS

Strain	Rotarod		Body Temperature	
	Saline	Oxotremorine	Saline	Oxotremorine
	ED_{50} (mg/kg)		$ED_{-3.0°}$ (mg/kg)	
BALB	0.104±0.020	1.68±0.23*	0.030±0.008	0.61 ± 0.08*
C57BL	0.098±0.012	0.81±0.30*	0.034±0.004	1.16±0.35*
DBA	0.085±0.055	0.90±0.35*	0.025±0.005	0.38±0.05*
C3H	0.240±0.062	>5.0*	0.041±0.005	1.90±1.05*
	Slope			
BALB	−116.2±14.1	−93.6±12.5	−7.67±0.79	−6.33±0.37
C57BL	−109.0±15.6	−105.0±31.4	−6.84±0.35	−4.28±0.60
DBA	−53.7±16.0	−16.8± 2.8*	−6.37±0.47	−3.34±0.16
C3H	−113.6±20.6	−19.3± 7.4*	−6.75±0.35	−2.44±0.46

Slopes and ED values were calculated from log dose-response curves. Values represent mean ± SD. Those values marked (*) differ significantly from the corresponding control value ($p<0.05$, t-test).

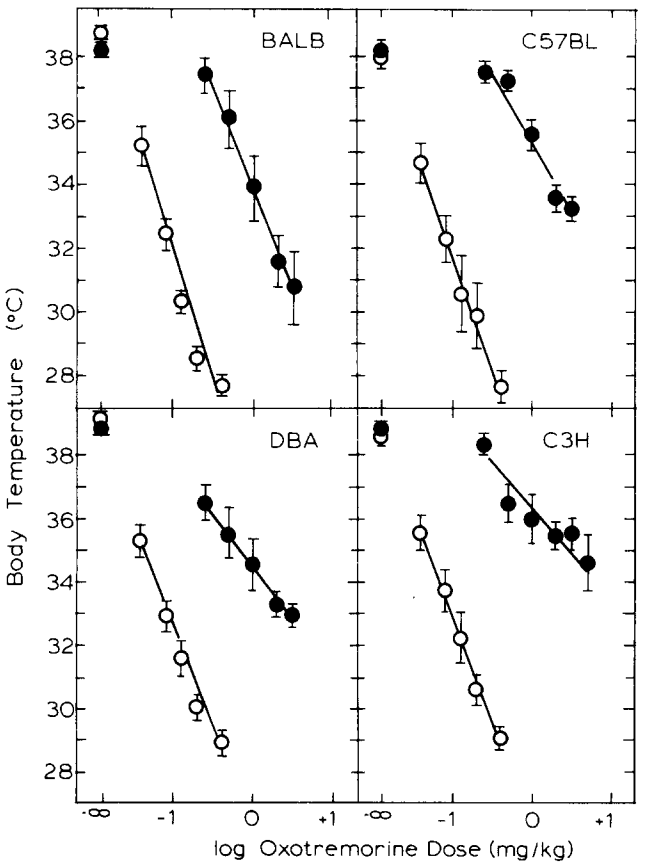

FIG. 2. Dose-response curves for oxotremorine effects on body temperature. Dose-response curves for body temperature were constructed by sequential injection of oxotremorine to both control (○) and oxotremorine-infused (●) mice. Each point represents the mean±SEM for 8 individual animals.

for total muscarinic receptor levels (L-[^{3}H]QNB binding), the agonist affinity states of the total muscarinic receptor population (carbamylcholine inhibition of L-[^{3}H]QNB binding), and for the levels of M1 receptor subtype ([^{3}H]PZ binding). The results shown in Fig. 3 are Scatchard plots for L-[^{3}H]QNB binding to cerebral cortex of both saline- and oxotremorine-infused mice. Neither the K_D nor the B_{max} differed among these mouse strains in this brain region. Chronic oxotremorine had no effect on the K_D value for L-[^{3}H]QNB in any strain (overall average K_D was 6.7 pM, values for the individual treatment groups are given in the legend to Fig. 3), but drug treatment resulted in a significant decrease in the B_{max} for this ligand for all four strains. This change did not differ among the strains (see legend to Fig. 3 for values).

Figure 4 presents curves for the inhibition of L-[^{3}H]QNB by the agonist, carbamylcholine. The inhibition curves are all shallow indicating that the inhibition does not reflect simple mass action. The inhibition curves can be resolved into two components with different inhibition constants for carbamylcholine, one approximately 5 nM (IC_{50} about 60 nM) and the other about 2 μM (IC_{50} about 25 μM). Neither inhibition constant differed among the strains, nor was either inhibition constant affected by chronic oxotremorine treatment. Chronic treatment decreased the binding exhibited by both affinity states, but the amount of decrease was the same for all four strains. The treatment was equally effective in lowering the binding of both affinity states (see Table 3 for values).

The curves presented in Fig. 5 are Scatchard plots for the binding of [^{3}H]PZ to cortical tissue. In contrast to the linear Scatchard plots obtained for L-[^{3}H]QNB binding, the Scatchard plots for [^{3}H]PZ binding are curvilinear. The curvilinear plots can be resolved into two linear components. However, owing to limitations in the reliability of the data only the high affinity (average K_D 2.1 nM, values for each treatment group are given in the legend to Fig. 5) binding site can be reliably estimated. High affinity [^{3}H]PZ binding did not differ among the strains. The B_{max} for this high affinity

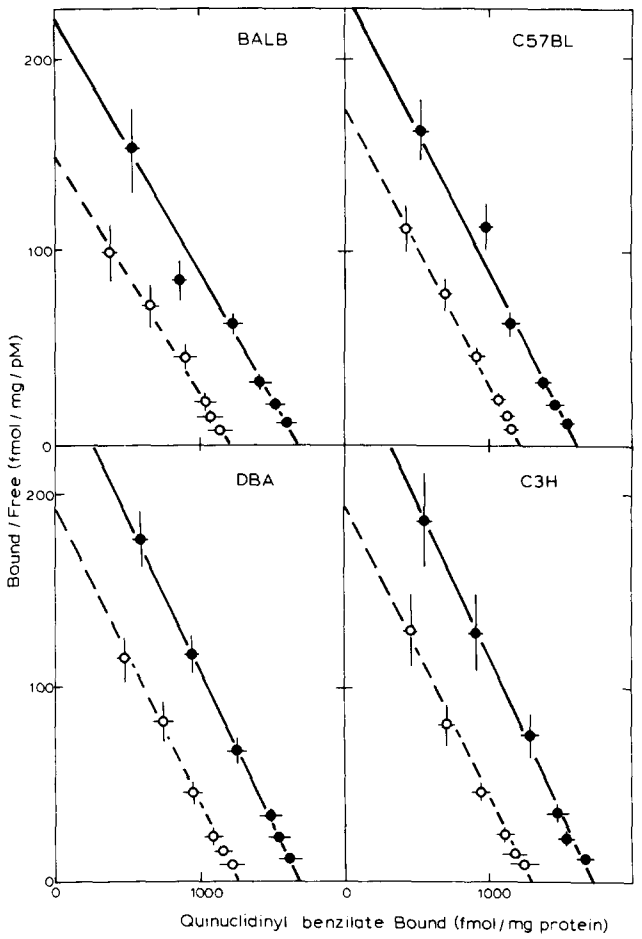

FIG. 3. Scatchard plots for L-[^{3}H]QNB binding in cerebral cortex. The binding of L-[^{3}H]QNB was measured both for control (●) and oxotremorine-treated (○) mice. Points represent mean±SEM of eight separate experiments. While oxotremorine treatment had no significant effect on the K_D for L-[^{3}H]QNB (K_D values in pM are—BALB: 7.6±1.6 and 7.9±0.6; C57BL: 6.1±0.8 and 6.9±0.2; DBA: 6.2±1.0 and 6.7±0.6; and C3H: 5.6±1.0 and 6.5±0.6 pM for saline and oxotremorine-treated mice of each strain, respectively), while the B_{max} for this ligand was significantly reduced in each strain (B_{max} values in pmol/mg protein—BALB: 1.67±0.12 and 1.21±0.04; C57BL: 1.59±0.08 and 1.22±0.18; DBA: 1.70±0.18 and 1.27±0.18; C3H: 1.64±0.10 and 1.28±0.06 pmol/mg for saline and oxotremorine-treated animals, respectively.

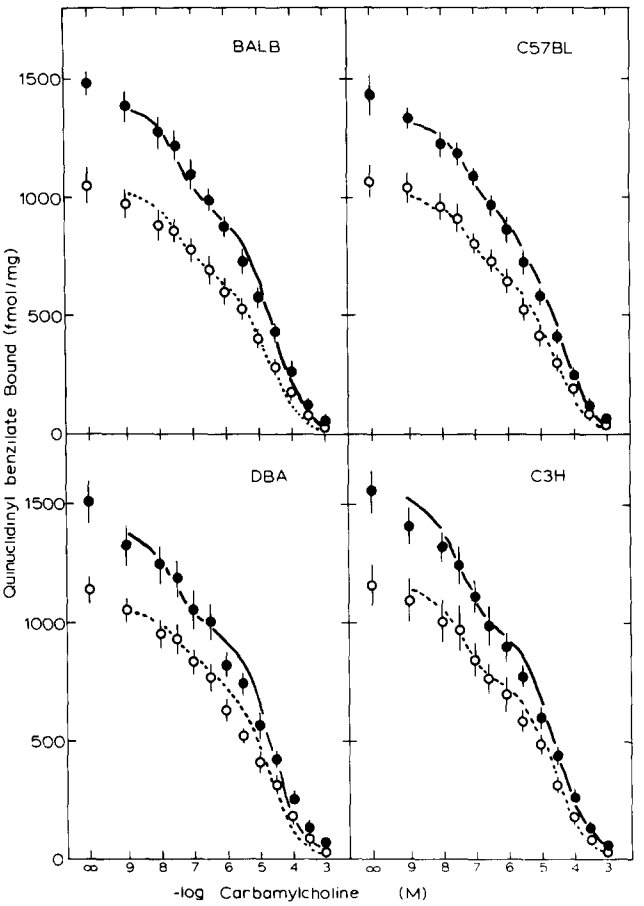

FIG. 4. Inhibition of L-[^{3}H]QNB binding by carbamylcholine in cerebral cortex. The binding of L-[^{3}H]QNB was measured in the presence of the indicated concentrations of carbamylcholine. Points represent mean±SEM of 8 separate experiments. The curves represent the best fit of the data to a 2-site model. (● present values for saline-infused animals and ○ are the values obtained with oxotremorine-infused animals.)

binding site was reduced by chronic oxotremorine treatment in all four strains. The K_D was unaffected. The B_{max} values for cortex are summarized in Table 4 along with the values for five other brain regions.

Muscarinic Receptors in Six Brain Regions

Table 2 presents the binding of L-[^{3}H]QNB in six brain regions of the four mouse strains. The absolute amount of muscarinic receptors differs among the brain regions in control mice, $F(5,168)=318.56$. The regions can be divided into four groups: (1) hindbrain (lowest binding); (2) midbrain and hypothalamus; (3) cortex and hippocampus; and (4) striatum (highest binding). No significant difference in the binding among the four strains was detected. Oxotremorine treatment resulted in a significant decrease in the binding in each brain region, $F(1,48)$ for all regions: cortex=48.83; midbrain=78.14; hindbrain=58.15; hippocampus=104.21; striatum=20.66; hypothalamus=59.69. The results were examined to determine if the percentage change in each brain region was the same. This analysis indicated that there was no overall difference among the strains, but that there was a difference in the receptor lability in different regions, $F(5,168)=5.21$. The significance arose because the decrease in hypothalamus (average 41.3%) was greater than that in striatum (average 18.9%). The percentage decrease in the other four regions (range 26.3% to 34.5%) did not differ from either extreme.

The results presented in Table 3 summarize measurements of the agonist affinity states for total muscarinic receptors determined by inhibition of L-[^{3}H]QNB binding in the six brain regions. The high affinity binding varied among the brain regions, $F(5,168)=54.02$. The regions can be divided into three groups: (1) hindbrain, hippocampus, midbrain, and hypothalamus (least amount of high affinity agonist binding); (2) cortex; and (3) striatum (greatest amount of high affinity

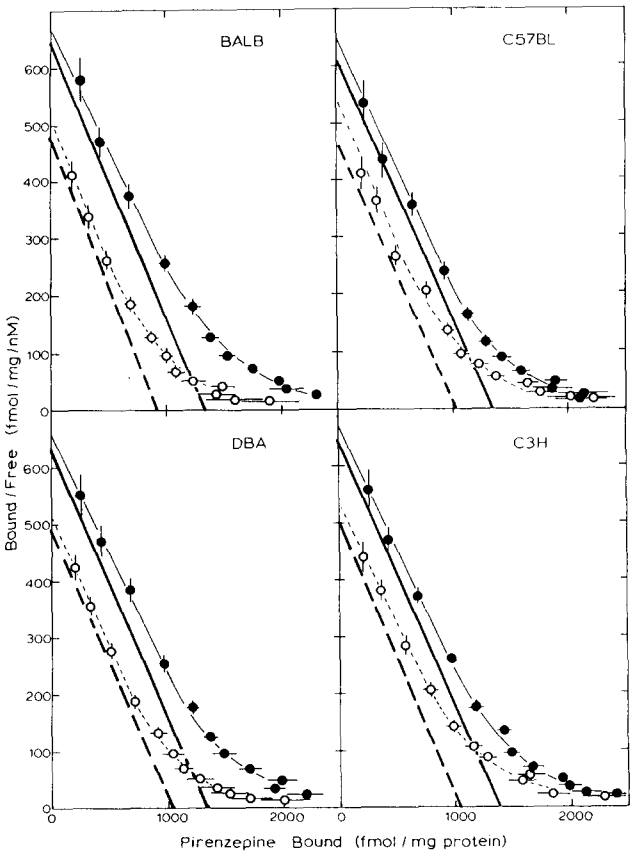

FIG. 5. Scatchard plots for [^{3}H]PZ binding in cerebral cortex. The binding of [^{3}H]PZ was measured for both control (●) and oxotremorine-infused (○) mice. Points represent mean±SEM of 8 separate experiments. The high affinity binding of [^{3}H]PZ was calculated by iteration and is represented by the dense lines. Chronic oxotremorine treatment did not significantly affect the K_D for high affinity binding (K_D values in nM—BALB: 2.13±0.16 and 1.94±0.18; C57BL: 2.17±0.13 and 2.25±0.40; DBA: 1.95±0.09 and 2.13±0.17; and C3H: 2.11±0.18 and 2.02±0.07 for control and oxotremorine-treated mice of each strain, respectively). The B_{max} values for the high affinity binding were significantly decreased by oxotremorine treatment. B_{max} values are summarized in Table 4.

TABLE 2

TOTAL QNB BINDING

	QNB Bound (fmol/mg protein)			
	BALB	C57BL	DBA	C3H
Cortex				
Saline	1599±70	1546±93	1628±97	1678±91
Oxotremorine	1131±89*	1148±64*	1226±69*	1254±103*
% Control	70.7±6.4	74.3±6.1	75.3±6.2	74.7±7.4
Midbrain				
Saline	780±58	706±40	696±31	703±28
Oxotremorine	446±22*	482±33*	528±48*	480±30*
% Control	57.2±5.1	68.3±6.1	75.9±7.7	68.2±5.1
Hindbrain				
Saline	417±37	521±32	458±48	463±37
Oxotremorine	281±23*	316±17*	325±23*	292±24*
% Control	67.4±8.1	60.7±5.0	71.0±7.6	63.1±7.2
Hippocampus				
Saline	1605±68	1611±97	1773±75	1663±75
Oxotremorine	1123±43*	1158±72*	1172±48*	1203±61*
% Control	70.0±4.0	71.9±6.2	66.1±3.9	72.3±4.9
Striatum				
Saline	2380±164	2197±136	2596±129	2505±91
Oxotremorine	1936±121*	1774±164*	2032±116*	2157±167
% Control	81.3±7.6	80.8±9.0	76.3±5.9	86.1±7.4
Hypothalamus				
Saline	775±26	838±84	886±123	817±58
Oxotremorine	478±32*	424±44*	497±18*	495±45*
% Control	61.6±4.6	56.5±7.7	56.1±8.1	60.6±7.0

The binding of L-[^{3}H]QNB (fmol/mg) was measured using 77 nM L-[^{3}H]QNB as described in the Method section. Values represent mean ± SEM for 8 measurements. Values marked (*) differ significantly from their corresponding control values ($p<0.05$, Duncan's test).

agonist binding). No significant differences among the strains were noted. When the percentage of high affinity agonist binding was analyzed, a slightly different pattern emerged. Significant differences among the regions were still noted, F(5,168)=35.78, but the regions divided into two groups: (1) hippocampus, cortex and striatum (low percentage of high affinity binding, 38.1% or less) and (2) midbrain, hindbrain and hypothalamus (high percentage of high affinity agonist binding, 54.2% or higher). No differences among the strains were noted. Chronic oxotremorine treatment resulted in a significant decrease in binding in all six regions, F(1,48) for all regions: cortex=43.13; midbrain=95.85; hindbrain=45.41; hippocampus=12.71; striatum=11.17; and hypothalamus=29.07. The percentage decreases in high affinity binding did not differ significantly among strains or regions.

The absolute amount of low affinity agonist binding also differed among the regions, F(5,168)=275.56. The regions can be divided into five groups: (1) hindbrain (lowest); (2) midbrain and hypothalamus; (3) cortex; (4) hippocampus; and (5) striatum (highest). When the percentage of total muscarinic receptors representing the low affinity state was analyzed a slightly different pattern emerged. The regions can be divided into two groups: (1) hindbrain, midbrain and hypothalamus (percentage of low affinity agonist sites less than 47%) and (2) cortex, striatum and hippocampus (percentage of low affinity agonist states greater than 60.5%). The percentage of the muscarinic receptors corresponding to the low affinity agonist state did not differ among the strains. Chronic oxotremorine infusion decreased the low affinity agonist state in all six brain regions, F(1,48) for all six regions: cortex=25.41; midbrain=13.79; hindbrain=10.58; hippocampus=49.99; striatum=10.29; and hypothalamus=29.81. No significant differences among the strains were observed. Analysis of the percentage change of these binding sites indicated that no regional or strain differences occurred.

Table 4 summarizes the results obtained for the binding of [^{3}H]PZ. The absolute amount of high affinity [^{3}H]PZ binding (M1 receptors) differed markedly among the six brain regions, F(5,168)=337.98. The regions can be divided into four

TABLE 3

AGONIST AFFINITY STATES

	High Affinity Binding (fmol/mg protein)				Low Affinity Binding (fmol/mg protein)			
Strain	BALB	C57BL	DBA	C3H	BALB	C57BL	DBA	C3H
Cortex								
Saline	575± 44	561± 30	642± 22	683± 55	1009± 61	981± 51	960± 85	980± 100
	(36.0± 3.1)	(36.3± 2.9)	(39.4± 2.7)	(40.7± 4.0)	(63.1± 4.5)	(63.4± 5.0)	(59.0± 6.3)	(58.4± 6.7)
Oxotremorine	439± 30*	416± 18*	453± 25*	492± 44*	685± 54*	734± 52*	761± 70*	762± 66
	(38.8± 3.8)	(36.2± 2.6)	(36.9± 2.9)	(39.2± 4.8)	(60.5± 6.3)	(63.9± 5.8)	(62.1± 6.7)	(60.8± 7.3)
% Control	76.3± 7.8	74.2± 5.1	70.6± 4.6	72.0± 8.7	67.9± 6.7	74.8± 6.6	79.3±10.1	77.8±10.4
Midbrain								
Saline	422± 17	390± 20	419± 29	415± 21	386± 59	345± 31	309± 16	307± 23
	(54.1± 4.6)	(55.2± 4.2)	(60.2± 5.0)	(59.0± 3.8)	(49.5± 8.4)	(48.9± 5.2)	(44.4± 3.0)	(43.7± 3.7)
Oxotremorine	212± 16*	232± 24*	313± 24*	255± 17*	251± 30*	262± 33*	235± 36*	239± 20*
	(47.5± 4.3)	(48.1± 5.4)	(59.3± 7.1)	(53.1± 4.9)	(56.3± 7.3)	(54.3± 7.3)	(44.5± 7.9)	(49.8± 5.2)
% Control	50.2± 4.3	59.5± 6.9	74.7± 7.7	61.4± 5.1	65.0±12.6	75.9±11.8	76.1±12.3	77.9± 8.7
Hindbrain								
Saline	270± 17	325± 21	334± 37	332± 32	169± 26	172± 28	138± 15	143± 13
	(64.7± 7.0)	(62.4± 5.6)	(72.9±11.1)	(71.7± 9.0)	(40.5± 7.2)	(33.0± 5.7)	(30.1± 4.6)	(30.9± 3.7)
Oxotremorine	174± 13*	203± 24*	240±18*	199±13*	117±18*	130± 15	98± 8*	110± 13
	(61.9± 6.9)	(64.2± 8.3)	(73.8± 7.6)	(68.1± 7.1)	(41.6± 7.3)	(41.1± 5.2)	(30.3± 3.3)	(37.7± 5.4)
% Control	64.4± 6.3	62.5± 8.4	72.0± 9.6	59.9± 7.0	69.2±15.1	75.6±15.1	71.0± 9.7	76.9±11.5
Hippocampus								
Saline	309± 44	288± 29	478± 58	373± 36	1300± 93	1304± 102	1312± 58	1295± 52
	(19.3± 2.9)	(17.5± 2.1)	(27.0± 3.5)	(22.4± 2.4)	(81.0± 6.7)	(80.9± 8.0)	(74.0± 4.5)	(77.9± 4.7)
Oxotremorine	291± 43	216± 53	251± 41*	220± 60*	814± 58*	981± 30*	918± 30*	975± 76*
	(25.9± 4.0)	(18.6± 4.7)	(21.4± 3.6)	(18.3± 5.1)	(72.4± 5.9)	(84.7± 5.9)	(78.3± 4.1)	(81.0± 7.5)
% Control	94.1±19.5	75.0±19.8	52.5±10.7	59.0±17.6	62.6± 6.3	75.2±10.0	70.0± 3.8	75.3± 6.6
Striatum								
Saline	680±116	744± 72	949±100	963± 77	1758± 181	1483± 136	1679± 77	1596± 32
	(28.6± 4.1)	(33.8± 3.9)	(36.6± 4.2)	(38.4± 3.4)	(73.9± 9.2)	(67.5± 7.5)	(64.7± 4.4)	(63.7± 2.6)
Oxotremorine	553± 71	621± 45	741± 66	668± 69*	1410± 127*	1206± 135	1316± 63*	1495± 137
	(28.6± 4.1)	(35.0± 4.1)	(36.5± 3.9)	(31.9± 4.0)	(72.8± 8.0)	(68.0± 9.9)	(64.8± 4.8)	(69.3± 8.3)
% Control	81.3±17.4	75.0±19.8	78.1±10.8	69.4± 9.1	80.2±11.0	81.3±11.8	78.4± 5.2	93.6± 8.8
Hypothalamus								
Saline	398± 58	474± 53	482± 68	447± 52	380± 46	381± 58	422± 62	375± 13
	(51.4± 7.7)	(56.6± 8.5)	(54.4±10.7)	(54.7± 7.5)	(49.0± 6.2)	(45.5± 8.3)	(47.6± 9.6)	(45.9± 3.6)
Oxotremorine	246± 26*	276± 29*	262± 31*	288± 48*	247± 33*	242± 22*	244± 16*	227± 26*
	(51.5± 6.4)	(56.0± 6.9)	(52.7± 6.5)	(58.2±11.0)	(51.6± 7.7)	(49.1± 5.5)	(49.0± 3.7)	(45.9± 6.7)
% Control	61.8±12.8	58.2± 8.9	54.4±10.0	64.4±13.1	65.0±11.7	63.5±11.3	57.8± 9.3	60.5± 7.2

High- and low-affinity agonist binding sites were determined by inhibition of L-[^{3}H]QNB binding with carbamylcholine and fitting the resulting data to a two-site model as described in the Method section. Binding is expressed as fmol/mg. Values represent mean±SEM of 8 determinations. Values in parentheses are the percentages of total L-[^{3}H]QNB binding corresponding to each agonist affinity state. Results marked (*) differ significantly from the corresponding controls ($p<0.05$, Duncan's test).

TABLE 4
HIGH AFFINITY PIRENZEPINE BINDING

	PZ Bound (fmol/mg protein)			
	BALB	C57BL	DBA	C3H
Cortex				
Saline	1231±45	1157±41	1251±73	1235±75
Oxotremorine	906±49*	937±40*	943±56*	1003±27*
% Control	73.6±4.8	81.0±4.5	75.3±6.3	81.2±7.3
Midbrain				
Saline	374±104	217±18	316±51	295±55
Oxotremorine	301±73	163±37	256±68	262±32
% Control	80.5±29.6	75.1±18.2	81.0±25.2	88.8±19.8
Hindbrain				
Saline	37±5	49±7	37±5	41±6
Oxotremorine	35±3	36±5	36±4	35±7
% Control	94.6±15.1	73.5±14.6	97.3±17.0	85.4±21.2
Hippocampus				
Saline	1311±57	1381±92	1396±73	1425±25
Oxotremorine	897±50*	986±57*	1063±29*	947±73*
% Control	68.4±4.8	71.4±6.3	76.1±4.5	66.5±6.2
Striatum				
Saline	1587±132	1344±91	1640±133	1629±90
Oxotremorine	1471±77	1265±79	1413±71	1609±110
% Control	92.7±9.1	94.0±8.7	86.2±7.3	98.8±8.7
Hypothalamus				
Saline	307±29	354±92	380±92	310±37
Oxotremorine	199±19*	267±42*	199±24*	181±18*
% Control	64.8±8.7	75.4±22.9	52.4±14.2	58.3±9.1

High affinity [^{3}H]PZ binding was calculated from Scatchard plots and the values shown represent mean±SEM for 8 determinations. Values marked (*) differ significantly from the corresponding controls ($p<0.05$, Duncan's test).

groups: (1) hindbrain (lowest); (2) midbrain and hypothalamus; (3) cortex and hippocampus; and (4) striatum (highest). The amount of M1 receptor in striatum is almost 40-fold higher than that in hindbrain. The percentage of total muscarinic receptors represented by the M1 subtype also shows wide regional variation, $F(5,168)=68.24$. The regions can be subdivided into three groups: (1) hindbrain (lowest, 7%); (2) midbrain and hypothalamus (about 37%); and (3) cortex, hippocampus and striatum (highest, over 58%). In general the regions with highest total muscarinic receptors also have the highest percentage of M1 receptors. No differences among the strains in either absolute binding or percentage binding were noted. The binding of [^{3}H]PZ tended to decrease in all regions after chronic oxotremorine infusion, but the decrease was significant in only three of the six regions, cortex, $F(1,56)=52.36$, hippocampus, $F(1,56)=75.88$ and hypothalamus, $F(1,58)=9.15$. Analysis of the percentage changes did not reveal any differences among the regions. The four mouse strains did not differ in their response to oxotremorine infusion.

DISCUSSION

Modest differences among four inbred mouse strains in their behavioral responses after the acute administration of the muscarinic, cholinergic agonist, oxotremorine, have been observed. Mice of the C3H strain were less sensitive than those of either BALB, C57BL, or DBA strains both for rotarod performance and body temperature. Chronic treatment with oxotremorine resulted in substantial tolerance to the effects of this drug in all four strains, with C3H mice as good, or better, at developing tolerance than the other three strains. This result contrasts with that obtained after nicotine treatment in which C3H mice developed less tolerance than did mice of the other three strains [25]. This difference in result demonstrates that C3H mice are not incapable of developing tolerance to any cholinergic agent, and suggests that mice with a higher acute threshold of effect do not necessarily develop less tolerance than mice with lower thresholds.

Chronic oxotremorine treatment affected the slopes of some of the dose-response curves as well as the ED_{50} values. Differences among the strains were also noted. Chronic treatment did not affect the slope of either dose-response curve for BALB mice, while it significantly decreased slopes for both curves for DBA and C3H mice. The underlying reasons for these differences remain unknown, but changes in metabolism, receptor levels, or receptor coupling mechanisms may explain the altered shape of the dose-response curves and the differences among the strains.

Gross differences in the response of muscarinic receptor levels to chronic treatment seem an unlikely explanation for the strain differences in tolerance development. While differences in all parameters of muscarinic receptor binding among the six brain regions were apparent, no striking differences among the four strains were found. The wide variation in regional muscarinic binding including total number of muscarinic receptors, the proportion of high and low agonist affinity states measured by carbamylcholine inhibition, and in the number of M1 muscarinic receptors observed in this study is consistent with previous reports of regional distributions of muscarinic receptors [4, 12, 15, 17, 31, 32, 37]. Chronic oxotremorine treatment resulted in a decrease in the total number of muscarinic receptors and the proportion of the two agonist affinity states was reduced to the same extent. This result is consistent with that observed for six brain regions in a single mouse strain after chronic oxotremorine treatment [17] and in rat striatum after chronic anticholinesterase treatment [9]. While K_D and B_{max} values for QNB binding were not measured in every brain region in the present study, chronic oxotremorine treatment did not affect the K_D for QNB in any brain region of C3H mice [17,18]. Likewise, oxotremorine treatment did not affect the K_D for QNB or PZ binding in cortex as determined in the present study. Therefore, it seems unlikely that K_D values in other brain regions would be altered by chronic oxotremorine infusion. The effect of chronic oxotremorine infusion on these receptors did not differ among the strains, suggesting that the underlying mechanisms controlling the down-regulation of these receptors is similar. It should be noted that the proportion of agonist affinity states of high and low affinity is dependent on the agonist used to inhibit ligand binding as well as the composition of the buffer used in the experiments [38]. The relatively large differences between the K_I values at the two agonist affinity states displayed by carbamylcholine improves the ability for resolution of these affinity states. Use of an agonist other than carbamylcholine may have altered the results to some extent. However, in a previous study using C3H mice, the proportion of high and low affinity agonist binding states measured with carbamylcholine did differ significantly from the proportions measured with oxotremorine [17].

The direct measurement of M1 muscarinic receptors with [^{3}H]PZ binding extends the information on the effects of chronic muscarinic agonist treatment on this receptor subtype. Chronic oxotremorine treatment affected M1 receptors in the same way as it did the total muscarinic receptors, but the M1 subtype may have been slightly less labile than total muscarinic receptors and by difference, therefore, M2 receptors. In three brain regions (cortex, hippocampus and hypothalamus; the three regions showing significant effect of drug treatment) the percentage change of M1 receptors was similar to that observed for total muscarinic receptor binding. In the three other brain regions (midbrain, hindbrain, and striatum; the three regions failing to show significant effects of drug treatment) the percentage change of M1 receptors tended to be less than that for total muscarinic receptors. Although these differences are not robust they suggest that M1 receptors in some brain regions may be less affected by chronic oxotremorine treatment than M2 receptors. However, measurement of low affinity PZ binding has proved to be too variable in our hands to be a trustworthy measure of M2 receptors. Alternate methods such as inhibition of QNB binding by PZ may prove more reliable. Direct measurement of M2 receptors, rather that measurement as the difference between total muscarinic receptors and M1 receptors, would be the most satisfactory means with which to investigate the effects of oxotremorine treatment on these receptor subtypes.

In summary, the results reported here suggest that mouse strains differ in sensitivity to an acute challenge dose of oxotremorine but these differences cannot be readily explained by differences in the number or affinity of muscarinic receptors or receptor subtypes. Similarly, mouse strains differ in their ability to develop tolerance to oxotremorine but these differences cannot likely be explained by differences in receptor changes. Therefore, other factors such as strain differences in oxotremorine metabolism or receptor coupling may underlie these genetically determined differences in sensitivity to oxotremorine.

ACKNOWLEDGEMENT

This work was supported by Grant DA 03194 from the National Institute on Drug Abuse.

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