Pharmacology Biochemistry & Behavior, Vol. 26, pp. 173–182, 1987. © Pergamon Journals Ltd., 1987. Printed in the U.S.A. 0091-3057/87 $3.00 + .00

Cross-Tolerance Between Muscarinic Agonists: Role of Muscarinic Receptors

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Received 1 July 1986

COLLINS, A. C., T. N. SMOLEN, A. SMOLEN AND L. J. MEDHURST. *Cross-tolerance between muscarinic agonists: Role of muscarinic receptors.* PHARMACOL BIOCHEM BEHAV **26**(1)173–182, 1987.—In order to explore the relationship between response to muscarinic agonists and brain muscarinic receptors, two mouse strains that differ in acute sensitivity (DBA and C3H) were injected chronically with DFP or infused with oxotremorine. Chronic DFP-treated DBA mice were not tolerant to DFP's effects on any measure, but they were cross-tolerant to the effects of oxotremorine on heart rate and body temperature. DFP-treated C3H mice were not tolerant to DFP or cross-tolerant to oxotremorine on any measure. Oxotremorine infusion resulted in tolerance to oxotremorine in both mouse strains, and chronically infused DBA mice were cross-tolerant to DFP on five of the six measures. Oxotremorine-infused C3H mice were cross-tolerant to DFP on two of the measures. These results suggest that genetic factors influence the development of tolerance or cross-tolerance. These genetic factors do not seem to be related to changes in brain QNB binding. Both mouse strains showed comparable changes in QNB binding following chronic DFP and oxotremorine with DFP eliciting reductions in QNB binding in striatum and hippocampus and oxotermorine eliciting reductions in nearly every brain region. However, tolerance and cross-tolerance did not seem to correlate with changes in binding which suggests that the relationship between receptor changes and responses to muscarinic agonists must be examined further.

Organophosphates Oxotremorine Muscarinic Cholinergic receptors Tolerance Genetics
Respiration rate Heart rate Hypothermia Locomotor activity

A number of recent studies have demonstrated that chronic treatment of laboratory animals with organophosphate acetylcholinesterase (AChE) inhibitors results in a decrease in the number of brain muscarinic receptors, as measured by (^{3}H)-L-quinuclidinyl benzilate (QNB) binding [3, 4, 6–8, 27, 28, 32, 33], and several other studies have demonstrated that chronic organophosphate treatment results in tolerance to some of the actions of the organophosphates [2, 5, 19, 20, 25, 26]. Subsequently, it has been suggested that this reduction in QNB binding is responsible for the tolerance to organophosphates which is consistent with the observations that chronic treatment with the muscarinic agonist, oxotremorine, results in tolerance to oxotremorine, and a reduction in the number of brain QNB binding sites [11,12].

Recently, we have investigated the effects of chronic treatment of DBA mice with the organophosphate diisopropylfluorophoshphate (DFP) on brain muscarinic receptors, and attempted to correlate any changes in receptor number with tolerance to DFP or cross-tolerance to oxotremorine [29]. Although DFP treatment elicited sizable reductions (as great as 40–50%) in the number of QNB binding sites in striatum, cortex, and hippocampus, no tolerance to DFP was detected as assessed by measuring the effects of DFP on respiration rate, heart rate, body temperature, Y-maze activity and tremor. Rather, supersensitivity to the effects of DFP on respiratory rate, heart rate, and body temperature was observed. The animals did exhibit some tolerance to DFP in that, as treatment proceeded, the excess salivation, lacrimation, and diarrhea elicited by DFP disappeared. The animals were also slightly cross-tolerant to oxotremorine, but the cross-tolerance was minimal when compared to the 35–80 fold tolerance that we have detected in mice that had been infused chronically with oxotremorine [11,12]. This was a curious finding in that the muscarinic receptor changes elicited by chronic DFP treatment were generally greater than those elicited by chronic oxotremorine infusion. These results raise questions concerning the meaning of changes in muscarinic receptor numbers following chronic organophosphate treatment.

Our studies of the effects of chronic DFP treatment were carried out using DBA/2Ibg mice [29] whereas our studies of chronic oxotremorine infusion [11,12] have been carried out, for the most part, in C3H/Ibg mice. In view of the finding that mouse strains differ in acute sensitivity to DFP [30,31]

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and oxotremorine [14], it seems possible that our failure to detect marked tolerance to DFP may have been due to some genetic factor that regulates tolerance development. Along these lines, two rat lines have been selectively bred by Russell *et al.* for differences in response to an acute challenge dose of DFP [21]. These rat lines also differ in the ability to develop tolerance following chronic treatment with DFP [22]. Perhaps DBA mice cannot develop tolerance to DFP whereas other mouse strains might. Therefore, we assessed the development of tolerance to DFP and oxotremorine, as well as cross-tolerance between these agents, in both DBA and C3H mice. In addition, the relationship between changes in brain muscarinic receptors and tolerance and cross-tolerance was explored.

METHOD

Materials

The radiolabeled compound L-[^{3}H]QNB (benzilic-4,4'-^{3}H, specific activity 30.2 Ci/mmol) was obtained from New England Nuclear Corporation (Newton, MA). Diisopropylfluorophosphate (DFP) and bovine serum albumin were purchased from Sigma Chemical Co. (St. Louis, MO). Glass fiber filters and Hepes were purchased from Boehringer-Mannheim (Indianapolis, IN). Toluene was obtained from 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). Inorganic compounds were reagent grade.

Mice

Male mice of the DBA/2Ibg and C3H/Ibg strains were used in this study. These strains have been maintained in the breeding colony at the Institute for Behavioral Genetics for at least 20 generations, and were selected for the studies presented here because earlier studies from our laboratory have demonstrated that they differ in acute response to DFP [30,31] and oxotremorine [14]. The mice were between 60 and 90 days old at the time of testing, were maintained on a 12/12 light/dark cycle, and were given free access to food (Wayne Lab Blox) and water.

Chronic Oxotremorine Treatment

The continuous infusion of oxotremorine was accomplished using the method described previously [11,12]. Briefly, this method consists of inserting a silastic cannula into the right jugular vein while the animals are anesthetized with pentobarbital (45 mg/kg) and chloral hydrate (63 mg/kg). Two days after surgery, the animals were placed singly in infusion chambers (15×15×25 cm), and each cannula was attached to thermoplastic tubing that was connected to a 1-ml syringe mounted on a Harvard infusion pump. The flow rate was set at 35 μl/hr. The mice were infused for 2 days with saline before oxotremorine treatment was initiated. On the morning of the third day, oxotremorine treatment was started at the rate of 0.1 mg/kg/hr. The dose was increased by 0.1 mg/kg/hr each day until the final infusion rate, 0.5 mg/kg/hr, was achieved. Animals were maintained at this final infusion dose for 8 days. Chronic saline-infused animals served as the controls.

Chronic DFP Treatment

DFP was prepared in saline and injected intraperitoneally. DFP is frequently administered in an oil vehicle, but it is stable for several hours in saline [9]. The saline solution was easier to administer, and was received better by the animals. Solutions were injected within 1 hr after preparation. Animals were injected once every 5 days for 1 month with a 4 mg/kg dose of DFP. This dosing schedule was used because preliminary studies suggested that it elicited changes in QNB binding that were similar to those obtained with the chronic oxotremorine treatments used in these studies. Saline-injected animals served as controls.

Tolerance Tests

Tolerance to DFP and cross-tolerance to oxotremorine were measured using a test battery consisting of the following tests: respiratory rate, Y-maze activity (both line crossings and rears), heart rate, body temperature, and rotarod performance. All tests were conducted on each individual. We have demonstrated in previous experiments that no significant inter-test interactions occur [15].

Tolerance and cross-tolerance were assessed in slightly different ways for each group. For those animals treated chronically with DFP, the animals were challenged with 0.1 mg/kg dose of oxotremorine 5 days after receiving the last DFP dose. The test battery was run 15 min later. The next day these animals were challenged with saline and tested 15 min later. The day after that the animals were challenged with a 4 mg/kg dose of DFP, and the test battery was run 2 hours later. Challenge doses of oxotremorine and DFP, and test times, were chosen from results obtained in earlier studies of the genetic influences on acute responses to oxotremorine and DFP in several mouse strains including the DBA and C3H [14, 30, 31]. Saline-treated controls were tested in an identical fashion.

The oxotremorine-infused animals were separated into two different groups. In one of these groups, animals were challenged with a 0.2 mg/kg dose of oxotremorine 2 hr after chronic infusion had been terminated. This time was sufficient to allow for the total elimination of oxotremorine from the animal [11,12]. The test battery was run 15 min after oxotremorine challenge. The next day these animals were challenged with saline (tested 15 min after injection), and the day after this the animals were challenged with 4 mg/kg DFP. Once again, DFP-challenged animals were tested 2 hr after injection. The second oxotremorine-infused group was tested differently in that the animals were challenged only with 4 mg/kg DFP 2 hr after oxotremorine infusion was ended. This group was included because we have no data concerning the rate of return to control of brain muscarinic receptors following termination of chronic oxotremorine infusion. Unpublished data from our laboratory indicate that QNB binding remains significantly depressed from control for a minimum of 15 days after termination of chronic DFP injection. It is unknown whether the time course of recovery of QNB receptors would be similar following chronic oxotremorine.

The tests were run in the following order:

Respiratory rate. Respiration was measured using a Respiration Monitor (Columbus Instruments, Columbus, OH). Five individual readings of respiration rate were made over a 1-min time period and averaged.

Y-maze. After completion of the respiration test, the mouse was transferred to a Y-maze. Both line crossings and rears were recorded during a 3 minute test session.

Heart rate. After the Y-maze test was completed, the

mouse was placed in a restrainer and needle electrodes were inserted through the skin. The electrodes were connected through a preamplifier to an E & M physiograph (Narco Biosystems, Houston, TX). Heart rate was monitored for 6 sec.

Body temperature. Body temperature was measured with a rectal thermometer (Bailey Instruments, Saddlebrook, NJ).

Rotarod performance. This test was carried out only on chronic oxotremorine-infused animals. After removing the animals from the chronic infusion chambers, they were trained to walk on the rotarod (Ugo Basile Co., Milan, Italy). Rotation speed was 8 rpm. When a mouse could stay on the rotarod for 100 sec at that speed it was considered to have reached criterion. This training was completed within 90 min after the animal had been removed from the infusion apparatus.

A complete description of these tests has been published previously [15].

Tissue Preparation

After completion of the tolerance test, the mouse was killed by cervical dislocation and its brain was removed, the blood rinsed off, and the brain dissected into six regions: cortex, hindbrain (pons-medulla), hypothalamus, hippocampus, striatum, and midbrain (tissue remaining after removal of all of the other areas, contains primarily thalamus). The cerebellum was discarded because it has a 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 quickly frozen at −70°. On the day of assay, the samples were thawed and homogenized with a glass-teflon homogenizer. The particulate fraction was prepared using the method of Romano and Goldstein [24].

[^{3}H]-L-QNB Binding

The binding of [^{3}H]-L-QNB was measured using a modification of the method of Yamamura and Snyder [34] as described previously [13]. A single concentration (150 pM) of ligand was used to assay binding in these studies. Previous studies from our laboratory have demonstrated that chronic oxotremorine infusion and DFP injection elicit changes in the number, but not the affinity, of brain muscarinic receptors [11, 12, 29]. Therefore, binding assays using a single ligand concentration are sufficient to provide data concerning the effects of chronic drug treatment on the number of brain QNB binding sites. Blanks were obtained by omitting protein from the assays or by adding 1 μM atropine. These methods give identical results.

Protein Assay

Protein was measured using the method of Lowry *et al.* [10] with bovine serum albumin as the standard.

Scintillation Counting

After the samples were washed, the glass fiber filters were placed in polypropylene scintillation vials (7 ml) and 2.5 ml of scintillation fluid (toluene, 1.35 l; Triton X-100, 0.9 l; 2,5-diphenyloxazole, 10.5 g) were added. The samples were mechanically shaken for 30 min and radioactivity was determined on an LS 1800 liquid scintillation spectrometer (Beckman Instruments, Fullerton, CA). Tritium was counted at 40% efficiency.

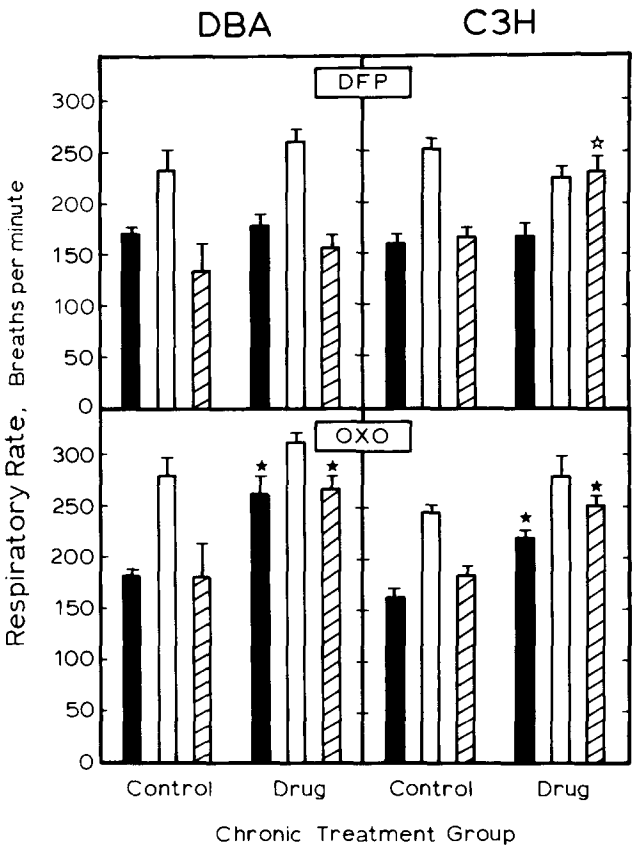

FIG. 1. Effects of chronic DFP injection or oxotremorine infusion on the respiration rate of DBA and C3H mice. The upper panels present the effects of oxotremorine, saline, and DFP challenge on the respiratory rates of animals that were injected chronically with saline (control) or DFP (drug) and challenged with 0.1 mg/kg oxotremorine (shaded column) on the fifth day after the last chronic injection, with saline (open column) the next day, and with 4 mg/kg DFP (striped column) the day after that. The lower panels present the responses elicited by oxotremorine, saline, and DFP in saline-(control) and oxotremorine-infused (drug) DBA and C3H mice. Infused animals were challenged with 0.1 mg/kg oxotremorine 2 hr after termination of infusion, with saline the day after that, and with 4 mg/kg DFP the day after that. Respiration rate was measured as described in the Method section. Each point represents the mean±S.E.M. of 11 animals for chronic DFP-treated DBA mice, and 5 for C3H mice; for chronic saline-injected DBA mice n=12, and n=5 for C3H injection controls. For the infusion experiments, n=8 for the DBA saline- and oxotremorine-infused groups, and n=6 for the C3H saline- and oxotremorine-infused groups. A (☆ or ★) indicates that chronic DFP-injected or oxotremorine-infused animals have a significantly different response than do chronic saline-treated controls ($p<0.05$ or <0.01, respectively).

Data Analysis

Results of the tolerance tests and biochemical assays were analyzed using one-way Analyses of Variance (ANOVA). One-way, rather than three-way, ANOVAs were used because these simpler analyses focus on the major concern of our study: does chronic drug treatment result in tolerance or cross-tolerance?

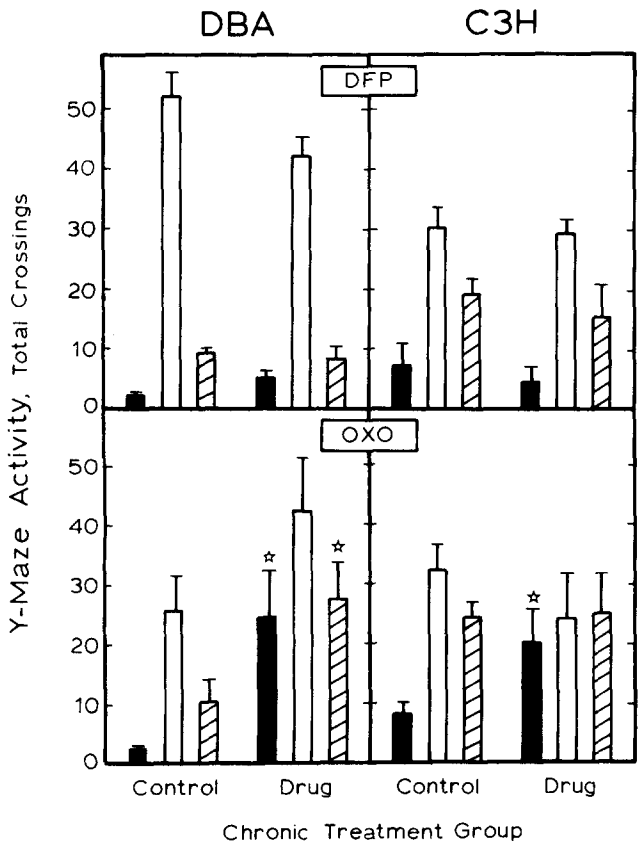

FIG. 2. Effects of chronic DFP injection or oxotremorine infusion on Y-maze activity (total number of crosses) of DBA and C3H mice. The chronic treatment and drug challenge doses are identical to those given in the legend to Fig. 1 as are the number of animals in each group. Y-maze crosses were measured as described in the Method section. A (☆ or ★) indicates that chronic DFP-injected or oxotremorine-infused animals have a significantly different response than do chronic saline-treated controls ($p<0.05$ or <0.01, respectively).

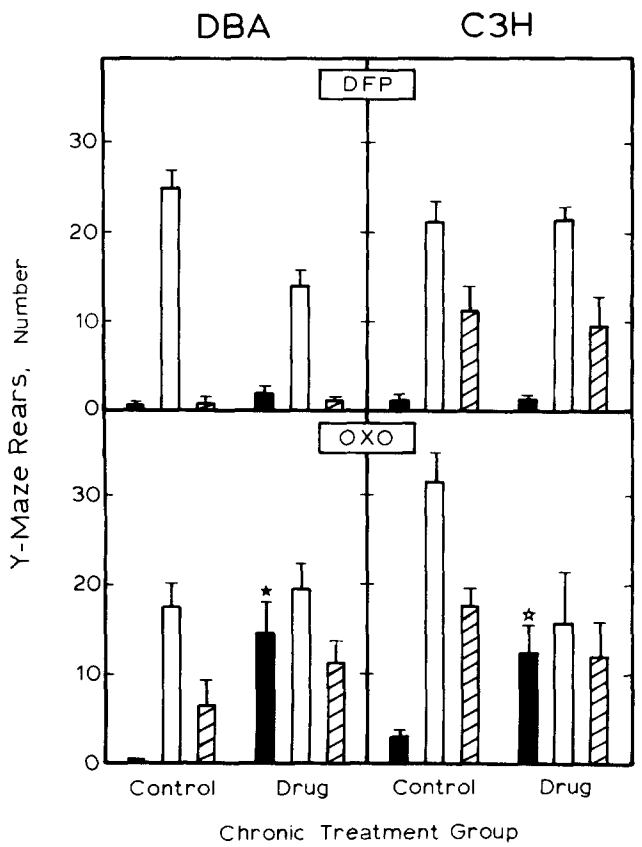

FIG. 3. Effects of chronic DFP treatment or oxotremorine infusion on Y-maze activity (rearing) in DBA and C3H mice. The chronic treatment and drug challenge doses are identical to those given in the legend to Fig. 1 as are the number of animals in each group. Y-maze rears were measured as described in the Method section. A (☆ or ★) indicates that chronic DFP-injected or oxotremorine-infused animals have a significantly different response than do chronic saline-treated controls ($p<0.05$ or <0.01, respectively).

RESULTS

Figures 1–6 present the effects of chronic oxotremorine infusion or DFP injection on the responses of DBA and C3H mice to challenge injections with oxotremorine, DFP, and saline. The results reported in the upper panels are those obtained in DBA and C3H mice that had been chronically injected with saline or DFP, and the lower panels present the responses of DBA and C3H mice to oxotremorine, saline, and DFP in chronic saline-infused and chronic oxotremorine-infused animals.

Figure 1 presents the results for the respiration test. Both oxotremorine and DFP elicited decreases in respiration rate in chronic saline-injected DBA and C3H mice (upper panels). Chronic DFP treatment resulted in tolerance to the effects of DFP on respiration rate in C3H, $F(1,7)=11.27$, $p<0.05$, but not DBA mice. No evidence of cross-tolerance to oxotremorine was seen in either mouse strain. The lower panels show that oxotremorine and DFP challenge elicited decreases in respiration rate in chronic saline-infused DBA and C3H mice. However, unlike the results obtained with chronic DFP-treated animals, chronic oxotremorine-infused animals were tolerant to the effects of oxotremorine [DBA, $F(1,14)=35.73$, $p<0.001$; C3H, $F(1,12)=59.66$, $p<0.001$], and cross-tolerant to the effects of DFP [DBA, $F(1,14)=10.10$, $p<0.01$; C3H, $F(1,12)=48.56$, $p<0.001$].

Figure 2 presents the results obtained with the Y-maze crosses test. Both DFP and oxotremorine challenge elicited decreases in Y-maze locomotor (crosses) activity in both mouse strains. Chronic DFP injection did not result in tolerance to the effects of DFP or cross-tolerance to the effects of oxotremorine in either mouse strain (Fig. 2, upper panels). However, chronic oxotremorine infusion (Fig. 2, lower panels) resulted in tolerance to the effects of oxotremorine [DBA, $F(1,14)=7.92$, $p<0.05$; C3H, $F(1,12)=6.06$, $p<0.05$]. Only the oxotremorine-infused DBA mice were cross-tolerant to the effects of DFP, $F(1,14)=5.64$, $p<0.05$.

Figure 3 presents the results obtained for the Y-maze rears test. Both drugs elicit decreases in Y-maze rearing activity in chronic saline-treated animals. Chronic DFP-treated animals were not tolerant to DFP or cross-tolerant to oxotremorine (upper panels) whereas chronic oxotremorine-

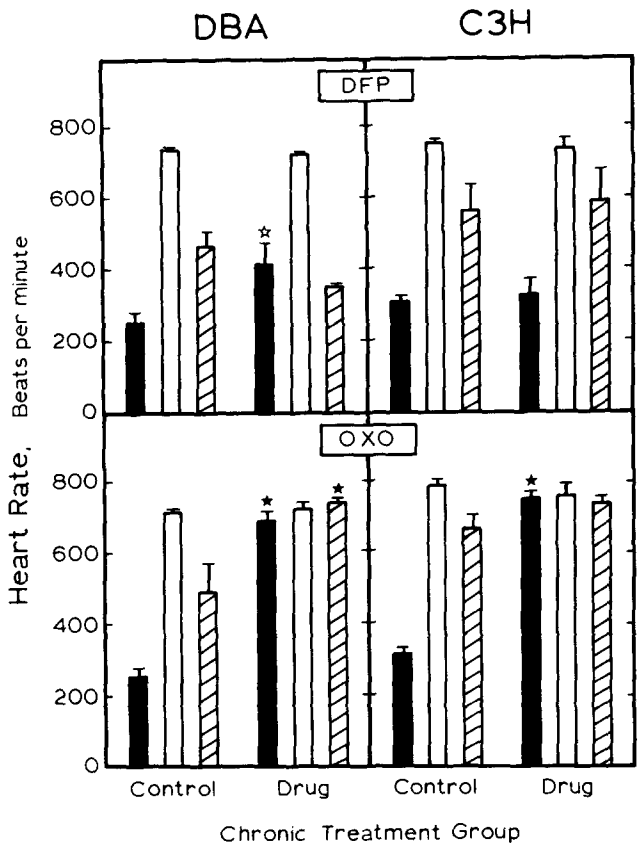

FIG. 4. Effects of chronic DFP treatment or oxotremorine infusion on heart rate of DBA and C3H mice. The chronic treatment and drug challenge doses are identical to those given in the legend to Fig. 1 as are the number of animals in each group. Heart rate was measured as described in the Method section. A (☆ or ★) indicates that chronic DFP-injected or oxotremorine-infused animals have a significantly different response than do chronic saline-treated controls ($p<0.05$ or <0.01, respectively).

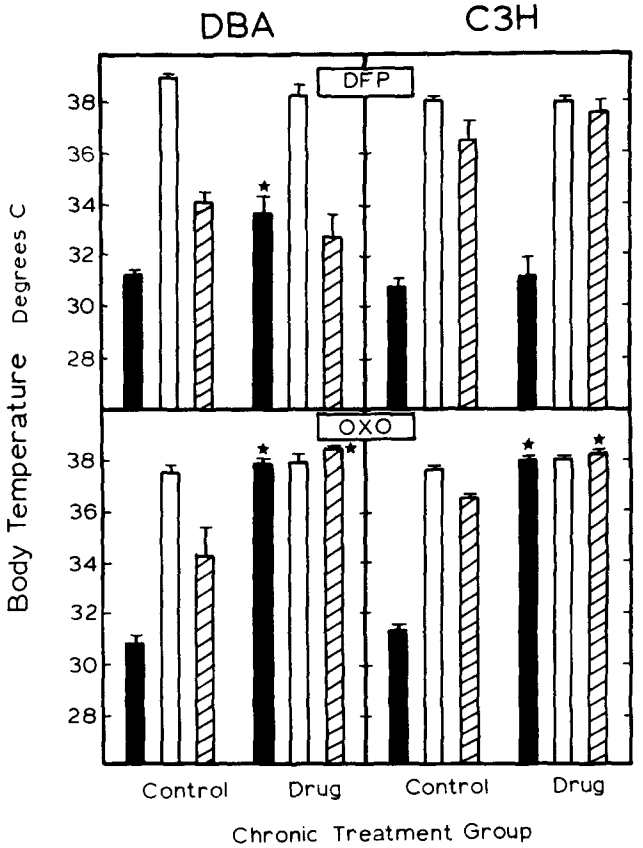

FIG. 5. Effects of chronic DFP treatment or oxotremorine infusion on the body temperature of DBA and C3H mice. The chronic treatment and drug challenge doses are identical to those given in the legend to Fig. 1. Body temperature was measured as described in the Method section. A (☆ or ★) indicates that chronic DFP-injected or oxotremorine-infused animals have a significantly different response than do chronic saline-treated controls ($p<0.05$ or <0.01, respectively).

infused animals were tolerant to oxotremorine (lower panels) [DBA, $F(1,14)=13.60, p<0.05$; C3H, $F(1,12)=5.97, p<0.05$]. Chronic oxotremorine-infused mice of both strains were not cross-tolerant to DFP.

Figure 4 presents the results obtained with the heart rate test. Acute challenge with both oxotremorine and DFP decreased heart rate, and chronic DFP injection did not result in tolerance to DFP in either mouse strain. C3H mice that had been chronically injected with DFP did not manifest cross-tolerance to oxotremorine, but cross-tolerance was observed in DBA mice, $F(1,21)=7.53$, $p<0.05$ (upper panels). Chronic oxotremorine-infused mice were tolerant to oxotremorine's heart rate-decreasing effects [DBA, $F(1,14)=126.59$, $p<0.001$; C3H, $F(1,12)=421.25$, $p<0.001$], and oxotremorine-infused DBA mice were also cross-tolerant to DFP, $F(1,14)=9.10$, $p<0.01$ (lower panels). It is not clear whether oxotremorine-infused C3H mice were cross-tolerant to the heart rate-decreasing effects of DFP because the 4 mg/kg dose of DFP did not elicit a profound reduction in heart rate in this strain.

Acute challenge of saline-treated or saline-infused mice with oxotremorine or DFP resulted in a decrease in body temperature (Fig. 5). Chronic DFP-treated DBA mice were not tolerant to the effects of DFP on body temperature, but a significant reduction in the response to oxotremorine was seen, $F(1,21)=12.00, p<0.01$. C3H mice were not tolerant to DFP's hypothermic actions nor were they cross-tolerant to the hypothermic effects elicited by oxotremorine. Chronic oxotremorine infusion (Fig. 5, lower panels) resulted in tolerance to the actions of oxotremorine [DBA, $F(1,14)=452.58$, $p<0.001$; C3H, $F(1,12)=717.17$, $p<0.001$], and cross-tolerance to the actions of DFP [DBA, $F(1,14)=11.92$, $p<0.01$; C3H, $F(1,12)=18.88$, $p<0.001$].

Figure 6 presents the effects of oxotremorine and DFP on the rotarod test. A challenge dose of oxotremorine uniformly resulted in a decreased ability of saline-treated DBA and C3H mice to perform on the rotarod. DFP treatment also decreased rotarod performance in DBA mice, but decreased performance was seen only in C3H mice that had been chronically injected. For some unknown reason, saline-infused C3H mice were resistant to the rotarod-impairing effects of DFP. Chronic DFP treatment did not result in

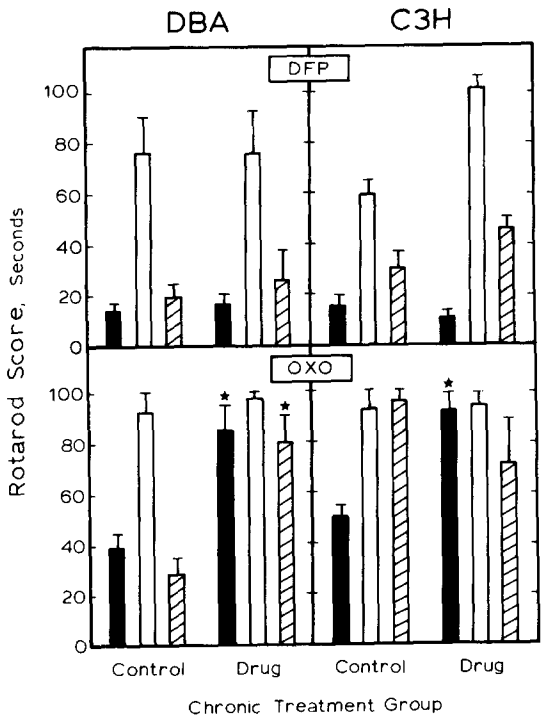

FIG. 6. Effects of chronic DFP treatment or oxotremorphine infusion on the rotarod performance of DBA and C3H mice. The chronic treatment and drug challenge doses are identical to those given in the legend to Fig. 1 as are the number of animals in each group. Rotarod performance was measured as described in the Method section. A (☆ or ★) indicates that chronic DFP-injected or oxotremorine-infused animals have a significantly different response than do chronic saline-treated controls ($p<0.05$ or <0.01, respectively).

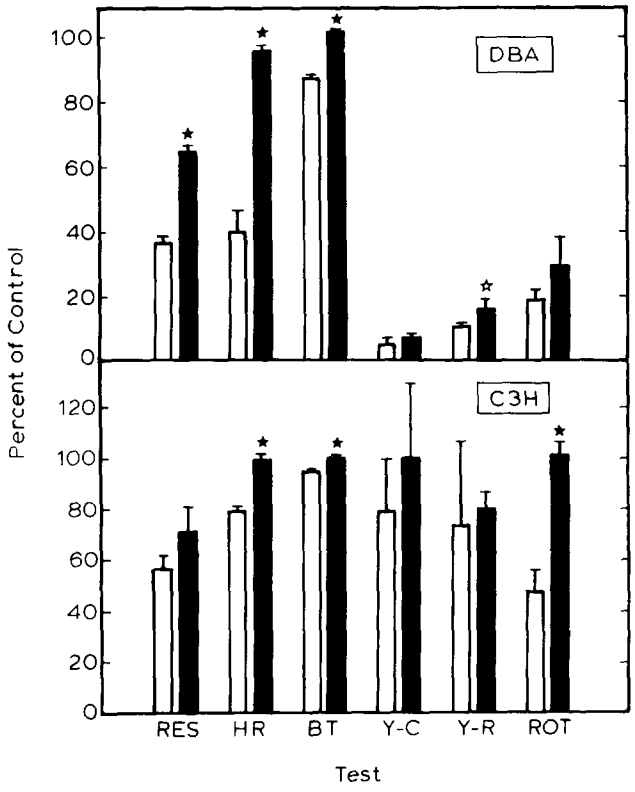

FIG. 7. Effects of DFP challenge on respiratory rate (RES), heart rate (HR), body temperature (BT), Y-maze crosses (Y-C), Y-maze rears, (Y-R), and rotarod performance (ROT). DBA and C3H animals were infused chronically with saline (open column) or oxotremorine (shaded column), as described in the Method section, and challenged with 4 mg/kg DFP 2 hr after termination of infusion. Each point represents the mean±S.E.M. of 4 animals. All data are presented in terms of % of Control where the control values were (mean±S.E.M.) 312±6 (RES), 723±13 (HR), 37.9±0.4 (BT), 42±9 (Y-C), 19±3 (Y-R), and 97±3 (ROT) for DBA mice, and 280±22 (RES), 757±36 (HR), 38.0±0.2 (BT), 24±8 (Y-C), 15±6 (Y-R), and 94±5 (ROT) for C3H mice. A (☆ or ★) indicates the chronic oxotremorine-infused animals are cross-tolerant to DFP ($p<0.05$ or <0.01, respectively).

tolerance to DFP or cross-tolerance to oxotremorine on the rotarod in either mouse strain (Fig. 6, upper panels), but tolerance to oxotremorine was detected in chronic oxotremorine-infused animals (lower panels) [DBA, $F(1,14)=16.05$, $p<0.005$; C3H, $F(1,12)=22.28$, $p<0.001$]. Oxotremorine-infused DBA mice were cross-tolerant to DFP, $F(1,14)=11.91$, $p<0.01$. It should be noted that chronic infusion alone apparently affected the response of C3H mice to DFP on the rotarod test. Therefore, the failure to detect a difference in response to DFP between saline-infused and oxotremorine-infused C3H mice does not mean that this strain does not develop cross-tolerance between oxotremorine and DFP.

All of the chronic infusion data reported to this point were obtained using a protocol where the animals were challenged with oxotremorine 2 hr after termination of chronic infusion. The next day the animals were tested following saline injection, and the third day the animals were tested following DFP challenge. Using this protocol, cross-tolerance was found between oxotremorine and DFP in five out of the six measures in oxotremorine-infused DBA mice and only two out of the six measures in oxotremorine-infused C3H mice. Such a circumstance might occur if the two mouse strains differ in the rate of loss of cross-tolerance. Therefore, a group of oxotremorine-infused mice was challenged with DFP 2 hr after termination of saline or oxotremorine infusion. The results of this experiment are reported in Fig. 7. Note that all of the data reported in this figure are expressed in terms of percent of control where the control values used were obtained from saline-infused mice. Chronic oxotremorine-infused DBA mice showed statistically significant cross-tolerance to the effects of DFP on four of the six measures [respiratory rate, $F(1,6)=82.75$, $p<0.01$; heart rate, $F(1,6)=62.66$, $p<0.01$; body temperature, $F(1,6)=116.75$, $p<0.01$; and Y-maze rearing activity, $F(1,6)=13.65$, $p<0.05$], and a suggestion of cross-tolerance was seen for the rotarod test; i.e, cross-tolerance was clearly evident for four of the six measures, and a suggestion of cross-tolerance was seen for a fifth measure. These results compare favorably with the results reported in Figs. 1–6 for DBA mice. Statistically significant cross-tolerance to the effects of DFP was seen with

three of the six tests in chronic oxotremorine-infused C3H mice in that oxotremorine-infused animals were cross-tolerant to the effects of DFP on heart rate, $F(1,6)=38.50$, $p<0.01$, body temperature, $F(1,6)=22.61$, $p<0.01$, and rotarod performance, $F(1,6)=33.92$, $p<0.01$. The failure to detect cross-tolerance for the Y-maze tests may be related to the fact that the 4 mg/kg DFP dose elicited marginal effects on these tests in the C3H strain. Thus, more evidence of cross-tolerance is seen in C3H mice if the interval between cessation of oxotremorine infusion and DFP challenge is decreased.

Figure 8 presents the effects of chronic DFP treatment (upper panels), and chronic oxotremorine infusion (lower panels) on brain QNB binding in six brain regions in the two mouse strains. Chronic DFP treatment elicited significant reductions in QNB binding in three brain regions in DBA mice: striatum, $F(1,17)=8.15$, $p<0.01$; cortex, $F(1,14)=7.65$, $p<0.05$; and hippocampus, $F(1,16)=9.41$, $p<0.01$. Similarly, significant reductions were seen in striatum, $F(1,9)=203.31$, $p<0.001$, and hippocampus, $F(1,9)=31.33$, of C3H mice. A statistically significant reduction in QNB binding was not observed in C3H cortex, but a modest reduction in hindbrain binding was seen, $F(1,9)=5.94$, $p<0.05$. Chronic oxotremorine infusion also resulted in a decrease in QNB binding (lower panels), but this decrease was more widespread than the decreases elicited by chronic DFP treatment. In DBA mice, significant decreases were seen in cortex, $F(1,12)=81.93$, $p<0.001$; hippocampus, $F(1,12)=11.85$, $p<0.01$; midbrain, $F(1,12)=20.72$; hypothalamus, $F(1,12)=22.59$, $p<0.001$; and hindbrain, $F(1,11)=8.16$, $p<0.05$; and in C3H mice significant decreases were seen in hippocampus, $F(1,11)=11.05$, $p<0.01$; midbrain, $F(1,11)=49.67$, $p<0.001$; hypothalamus, $F(1,11)=24.64$, $p<0.001$; and hindbrain, $F(1,11)=28.68$, $p<0.001$. The apparent reductions in QNB binding in the striata of both mouse strains were not statistically significant. Thus, chronic oxotremorine infusion elicited a reduction in brain QNB binding, but the greatest effects were elicited in regions other than the striatum which is the brain region that is affected to the greatest degree by chronic DFP treatment.

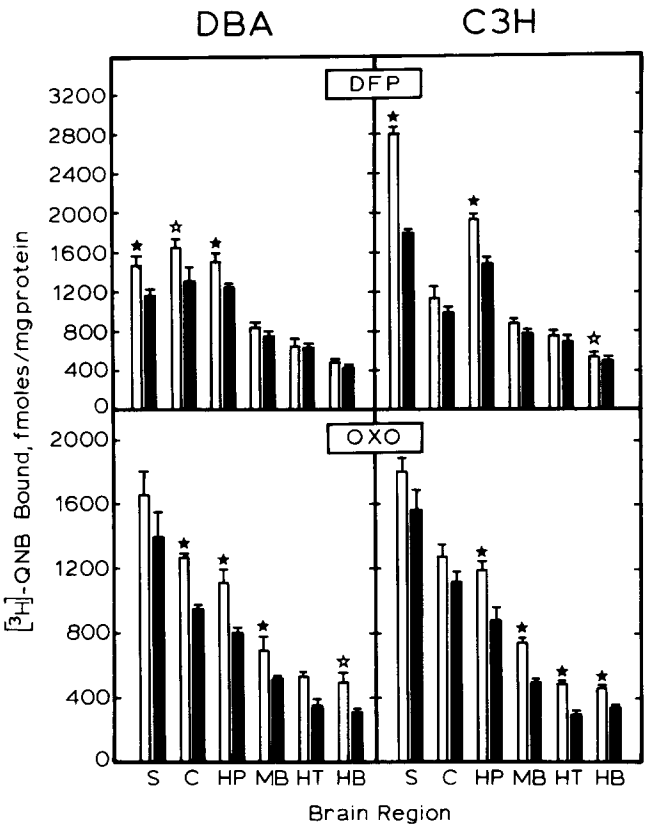

FIG. 8. Effects of chronic DFP injection (upper panels) or oxotremorine infusion (lower panels) on brain QNB binding in six brain regions of DBA and C3H mice. Mice were injected chronically with DFP or saline or infused chronically with oxotremorine or saline, as described in the Method section. QNB binding was measured in the striatum (S), cortex (C), hippocampus (HP), midbrain (MB), hypothalamus (HT), and hindbrain (HB) using 150 pM QNB. Since chronic DFP or oxotremorine treatment do not change the K_D for QNB [11, 12, 29], these results reflect the effects of chronic drug treatment on changes in the number of brain QNB binding sites. The results obtained with saline-treated animals are presented in the open columns, and the results obtained with chronic drug-treated (DFP or oxotremorine) are presented in the shaded columns. Each point represents the mean±S.E.M. of 9–11 animals for the DBA mice and 5–6 for the C3H mice. A (☆ or ★) indicates that the chronic drug-treated animals are significantly ($p<0.05$ or <0.01, respectively) different from the saline controls.

DISCUSSION

The results reported here are surprising in that although both chronic oxotremorine infusion and chronic DFP injection resulted in a decrease in the number of brain QNB binding sites, tolerance and cross-tolerance were observed only in the chronic oxotremorine-infused mice. We had anticipated finding tolerance to DFP since a number of other investigators have observed tolerance to organophosphates following chronic treatment [2, 5, 19, 20, 25, 26]. The vast majority of these studies have used rats, and it may be that species differences regulate tolerance to organophosphates. As noted previously, the two rat lines that have been bred for differential sensitivity to an acute dose of DFP [21] also differ in tolerance development [22], and this relative difference is test specific. For example, both sexes of the resistant line developed tolerance to the hypothermia-producing effects of DFP more rapidly than did the sensitive line, but for an operant drinking response females of both lines were tolerant after only three injections, while resistant males required seven injections to develop tolerance and sensitive males were not tolerant even after eight injections [22].

We have reported previously that inbred mouse strains differ in sensitivity to the lethal effects of DFP [31], and to the effects of DFP on the various tests used in the studies reported here [30]. In this regard, C3H mice were less sensitive to the actions of DFP than were DBA mice. A careful inspection of the results presented in Figs. 1–6 will reveal that the present study has replicated this finding. However, this difference in acute sensitivity did not seem to affect tolerance development in that neither DBA nor C3H mice developed appreciable tolerance to DFP. Genetic factors may regulate cross-tolerance following chronic DFP treatment in that DBA mice that had been injected chronically with DFP were cross-tolerant to the effects of oxotremorine on heart rate and body temperature. Chronic DFP-treated DBA mice were not cross-tolerant to the effects of oxotremorine on respiration rate, the two Y-maze tests, or the

rotarod test. Chronic DFP-treated C3H mice did not exhibit cross-tolerance to the effects of oxotremorine on any of the tests.

As noted above, the failure to detect tolerance to the effects of DFP was a surprising one in that numerous studies have demonstrated tolerance to organophosphates in the rat [2, 5, 19, 20, 25, 26] and Costa *et al.* [6] have observed tolerance to the antinociceptive effects of DFP in CD-1 mice. It may be that mice develop tolerance to only selected aspects of organophosphate action. Along these lines, it was apparent to us as we chronically injected the mice with DFP that the intensity of the salivation, lacrimation, and diarrhea elicited by DFP was decreasing. This tolerance was not total, however, and, as is apparent from the data, not all drug effects were altered by chronic treatment. One potential explanation for our failure to detect appreciable tolerance to DFP is that the animals were injected only once every 5 days. The rat studies [2, 5, 19, 20, 25, 26] and the Costa *et al.* study [6] that used CD-1 mice used treatment protocols where the animals were injected with organophosphates either on a daily basis or every other day. In preliminary studies we tried other injection schedules, but the mice, particularly the C3H mice, did not tolerate these schedules well. For example, only 60% of the C3H mice injected with DFP every 5 days survived this treatment, and when injected once every 4 days, only about 20% survived. This suggests an inability of this mouse strain to develop tolerance to the actions of DFP even though it is relatively insensitive to the actions elicited by a single dose.

As noted in Fig. 8, chronic DFP treatment resulted in significant reductions in QNB binding in striatum and hippocampus in both mouse strains. The strains differed slightly in that cortical QNB binding was significantly reduced only in DBA mice, and a significant reduction in QNB binding in hindbrain was seen only in C3H mice. Since neither mouse strain developed appreciable tolerance to DFP, it is difficult to argue that changes in QNB binding are related in a cause-effect fashion to alterations in response (tolerance) to DFP.

DBA mice that had been treated chronically with DFP were cross-tolerant to the effects of oxotremorine on heart rate and body temperature, but no such cross-tolerance was seen in C3H mice. Since both chronic DFP treatment and chronic oxotremorine infusion elicited decreases in brain QNB binding, it would seem reasonable, based on the results obtained with DBA mice, to suggest that the cross-tolerance to oxotremorine is related to the decreased QNB binding. However, C3H mice did not exhibit cross-tolerance even though chronic DFP treatment resulted in a reduction in QNB binding. This result also suggests that the relationship between DFP-induced decreases in QNB binding and changes in response to cholinergic agonists is not totally straight forward.

Chronic oxotremorine infusion resulted in tolerance to the actions of oxotremorine in all six of the behavioral and physiological measures in both mouse strains. DBA mice that had been infused chronically with oxotremorine were cross-tolerant to the effects of DFP on five of the six measures whereas C3H mice were cross-tolerant to DFP on two (respiration rate and body temperature) of the measures when challenged with DFP 2 days after termination of chronic oxotremorine infusion. When challenged 2 hr after termination of infusion, DBA mice were cross-tolerant to the effects of DFP on five of the six measures (only the Y-maze crosses test failed to detect any cross-tolerance) whereas C3H mice were cross-tolerant on three of the measures (heart rate, body temperature, and rotarod). The C3H mice may also have been cross-tolerant to DFP for the Y-maze tests, but the DFP dose used did not elicit a robust effect which serves to minimize any possibility of detecting tolerance. A comparison of the results reported in Figs. 1–6 with those reported in Fig. 7 suggests that oxotremorine-infused C3H mice develop cross-tolerance to DFP, but they lose this cross-tolerance more rapidly than do DBA mice.

Both of the mouse strains exhibited decreases in QNB binding following chronic oxotremorine infusion. QNB binding was reduced in five of the six brain regions in DBA mice, and in only four of the six regions in C3H mice. It may be that this modest difference underlies the apparent strain difference in cross-tolerance development, but we doubt this because earlier, more extensive, studies from our laboratory of the effects of chronic oxotremorine infusion on QNB binding in C3H mice indicated that statistically significant reductions in QNB binding are achieved in cortex in C3H mice that are tolerant to oxotremorine [11,12]. The results obtained from the chronic oxotremorine studies demonstrate, however, that reductions in QNB binding may result in a reduction in some of the effects elicited by organophosphates.

When the effects of chronic DFP and oxotremorine treatment on brain QNB binding are compared, it is obvious that identical effects on QNB binding are not achieved. DFP treatment was somewhat specific in that significant reductions in binding were detected primarily in the striatum and hippocampus. Cortical and hindbrain binding was reduced in a genotype-dependent fashion; i.e., cortical binding was decreased in DBA mice and hindbrain binding was decreased in C3H mice. Chronic oxotremorine infusion did not elicit significant reductions in QNB binding in the striatum in either mouse strain. This difference in drug action might be explained by the suggestion that oxotremorine may be selective for only one of the subclasses of muscarinic receptors [16], but we should note that our earlier analyses of the effects of chronic oxotremorine infusion provided evidence which indicates that both M_1 and M_2 receptors are down-regulated by chronic oxotremorine infusion [15]. Alternatively, it may be that oxotremorine elicits a decrease in QNB binding by classical down-regulation whereas DFP elicits a decrease in QNB binding in another way. DFP might elicit a decrease in QNB binding partly by classical agonist-induced down-regulation, and partly by neurotoxic actions. It has been clearly demonstrated that organophosphates elicit a neuropathy in humans and the chicken [1]. This neuropathy is largely restricted to the peripheral nervous system, and mice do not appear to be very sensitive to it. Recently, several studies have suggested that organophosphates, particularly those that elicit seizures, may cause damage to the central nervous system [17, 18, 23]. While we did not see obvious seizures in our animals, it may be that the failure of DBA and C3H mice to develop appreciable tolerance to DFP is a subtle manifestation of a neurotoxic action. Alternatively, it may be that many of DFP's actions are mediated by nicotinic systems and that chronic DFP treatment does not affect nicotinic responses. Clearly, these possibilities warrant a more rigorous examination.

It should be noted that the decreased number of QNB binding sites reported here likely represents a decrease in the number of binding sites since previous studies from our laboratory have demonstrated that chronic oxotremorine infusion and chronic DFP treatment elicit decreases in B_{max} and not

changes in K_D [11, 12, 29]. We have also demonstrated that DBA and C3H mice do not differ in maximal QNB binding in any of the six brain regions that we have examined [14].

Chronic DFP treatment did not change QNB binding in midbrain, hindbrain, and hypothalamus whereas chronic oxotremorine infusion resulted in decreased QNB binding in nearly every brain region examined. This may explain the observation that chronic oxotremorine infusion resulted in tolerance and cross-tolerance for the various tests whereas chronic DFP injection did not. It may be that the primary control of the various tests that we measured lies in brain regions or tissues (for example, heart) that have QNB binding sites that are affected by chronic oxotremorine infusion, but not by chronic DFP treatment. However, other investigations that have reported tolerance to organophosphates have also observed changes in QNB binding only in brain regions such as striatum, cortex, and hippocampus [3, 4, 6, 8, 33]. Therefore, it seems reasonable to suspect that some explanation other than differences in sites affected underlies the difference in tolerance development.

In summary, the results reported here demonstrate that both chronic oxotremorine and chronic DFP treatments elicit a reduction in the number of brain QNB binding sites. Since only chronic oxotremorine treatment resulted in tolerance and cross-tolerance, it seems reasonable to suspect that these two muscarinic agonists alter QNB binding via different mechanisms or at different sites. Neuroanatomical studies are required to resolve these issues.

ACKNOWLEDGEMENTS

The authors wish to thank Elena Romm for technical assistance with some of the experiments. This work was supported by AFOSR 82-0300.

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