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Dissociation of Decreased Numbers of Muscarinic Receptors From Tolerance to DFP

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SMOLEN, T. N., A. SMOLEN AND A. C. COLLINS. *Dissociation of decreased numbers of muscarinic receptors from tolerance to DFP.* PHARMACOL BIOCHEM BEHAV **25**(6) 1293–1301, 1986.—Several studies have demonstrated that chronic treatment with organophosphates, such as DFP, elicits a decreased number of brain muscarinic receptors (measured by the binding of QNB) which has been presented as an explanation for tolerance to the organophosphates. The purpose of the studies presented here was to assess whether graded changes in QNB binding could be attained following different methods of chronic DFP treatment, and whether tolerance to DFP paralleled these changes. Male DBA mice were injected with DFP every 4 days or 2 days for 30 days or daily for 14 days. The animals were subsequently challenged with DFP or the muscarinic agonist, oxotremorine, and respiratory rate, heart rate, body temperature, Y-maze activity and rearing were recorded. Chronic DFP-treated animals were supersensitive to the effects of DFP on respiratory rate, heart rate, and body temperature whereas a modest tolerance to the effects of oxotremorine on respiratory rate, heart rate, and body temperature was seen. Neither tolerance nor supersensitivity were observed for the effects of DFP and oxotremorine on the Y-maze measures. Chronic DFP treatment elicited reduced binding of QNB in striatum, cortex, and hippocampus with the group that had been treated every other day exhibiting the greatest changes. The changes in drug response did not parallel changes in QNB binding which raises questions as to the cause of the reduction in binding.

Organophosphates Muscarinic Cholinergic Neurotoxicity Tolerance Respiration rate Heart rate Hypothermia Locomotor activity

CHRONIC drug administration frequently results in tolerance to the behavioral and physiological effects of the administered drug. Certainly this appears to be the case with drugs that affect muscarinic, cholinergic receptors. Earlier studies from our laboratory have demonstrated that chronic infusion of mice with the muscarinic agonist, oxotremorine, results in a marked reduction in response to challenge doses of this agent [15,16]. The degree of tolerance increased with the dose of chronically infused oxotremorine and at its maximum, the doses required to elicit the same responses as measured in naive animals were 35–80 fold greater. These studies also demonstrated that chronic oxotremorine infusion resulted in a decrease in the number of brain muscarinic receptors, as measured by ^{3}H-quinuclidinyl benzilate (QNB) binding. However, substantial tolerance was seen before reduced QNB binding was detected which suggested that changes in some other biochemical process explain early stages of tolerance to oxotremorine.

Tolerance to the effects of the irreversible acetylcholinesterase (AChE) inhibitors, the organophosphates, has also been described. Early studies noted that chronic organophosphate treatment resulted in tolerance to the effects of challenge doses of the cholinergic agonist, carbachol, on the tone of the ileum [1, 24, 25], as well as heart rate and blood pressure [11]. Tolerance develops very rapidly to the hypothermia-producing effects of the organophosphate, diisopropylfluorophosphate (DFP), in that rats given three injections of DFP no longer responded with hypothermia [29]. Tolerance to the effects of DFP on fluid consumption [2, 27, 38], fixed ratio responding [28,37], and antinociception [6] have also been described.

Many investigators have studied the effects of chronic organophosphate treatment on brain muscarinic receptors (see, for examples [3, 4, 7, 8, 10, 39, 41, 44, 45]). Without exception, all of these studies have demonstrated that chronic inhibition of brain AChE activity results in a decrease in the number of brain muscarinic receptors which has led to the suggestion that tolerance to organophosphates is due to the reduction in brain muscarinic receptors. Since other studies have demonstrated that chronic organophosphate treatment does not alter choline acetyltransferase activity [36] choline uptake [34,35], or acetylcholine synthesis [34] it seems logical to assume that the often observed decrease in muscarinic receptors explains tolerance.

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We have been investigating the influence of genotype on the acute sensitivity of mice to a number of cholinergic agents including oxotremorine [20], nicotine [18], and DFP [42,43]. The responses that we commonly measure include body temperature, heart rate, respiratory rate, Y-maze activity (locomotor activity and rearing), rotarod performance, and acoustic startle response; and these have been incorporated into a test battery where all of these can be measured in a single animal following a single dose of the drug [21]. Strain differences were found for all of these drugs with the relative strain sensitivity to DFP being nearly identical to the relative strain sensitivity to oxotremorine [42] which may indicate that the major control of the responses to DFP involves the activation of brain muscarinic receptors. Certainly, such a conclusion is consistent with the observation that muscarinic receptors represent approximately 90% of the total brain cholinergic receptors in the mouse [19]. Curiously, however, the mouse strains that we have been studying do not differ in the number of affinity of brain muscarinic receptors as measured in seven brain regions [20]. Therefore, we have suggested that some factor other than the number of brain muscarinic receptors explains strain differences in response to oxotremorine and DFP. However, Overstreet *et al.* [31] have reported that the two rat lines that have been selectively bred for differences in sensitivity to an acute dose of DFP [30] have different numbers of muscarinic receptors in the striatum and hippocampus with the sensitive line having a greater number of QNB binding sites. This observation led these investigators to suggest that the acute response to DFP may be regulated by the number of brain muscarinic receptors. Thus, in the rat it seems as though genetically-determined acute sensitivity to DFP may be regulated by muscarinic receptor numbers whereas in the mouse such a relationship has not been established.

The studies reported here comprise an attempt to establish the relationship between the development of tolerance to DFP and potential changes in the number of brain muscarinic receptors in the mouse. As noted previously, a number of investigations have demonstrated that chronic treatment with organophosphates results in a decrease in the number of brain muscarinic receptors while other studies have suggested that tolerance to organophosphates may be related to this decrease in muscarinic receptor number. Unfortunately, none of these studies have attempted to alter the number of muscarinic receptors in a dose-related way. If tolerance to organophosphates is related to changes in the number of brain muscarinic receptors, greater tolerance should be seen with larger changes in receptor numbers. Therefore, mice were treated chronically with DFP using three different treatment protocols in an attempt to elicit different levels of receptor changes. Tolerance to DFP and cross-tolerance to oxotremorine were assessed, and correlations with brain QNB binding were sought in an attempt to ascertain whether tolerance development paralleled any changes in brain muscarinic receptors.

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), bovine serum albumin, acetylthiocholine, and polyethylenimine 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 strain were used in this study. This strain has been maintained in the breeding colony at the Institute for Behavioral Genetics for at least 20 generations, and were used in the studies described here because they are neither very sensitive nor very resistant to the effects elicited by an acute dose of DFP [42,43]. The mice were between 60 and 90 days old at the time of testing, were maintained on a 12/12 light/dark cycle, and given free access to food (Wayne Lab Blox) and water.

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 [12]. The saline solution was easier to administer, and was received better by the animals. Solutions were injected within 1 hr after preparation. Three treatment groups were developed: animals injected once every 4 days for 1 month with a 4 mg/kg dose of DFP, animals injected every other day for 1 month with a 2 mg/kg DFP dose, and animals injected with a 4 mg/kg DFP dose followed by 1 mg/kg thereafter for a total of 14 days. Three treatment schedules were used in an attempt to alter brain muscarinic receptors in a differential fashion.

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, and body temperature. All tests were conducted on each individual. We have demonstrated in previous experiments that no significant inter-test interactions occur [21].

Tolerance to DFP and cross-tolerance to oxotremorine were assessed in a slightly different fashion for each group. The 4-day treatment group was challenged with a 0.1 mg/kg dose of oxotremorine 4 days after the last DFP dose. The next day these animals were tested following a saline injection, and the day after that the animals were challenged with a 4 mg/kg dose of DFP. With the 2-day group, animals were challenged with saline 1 day after the last DFP injection and with either oxotremorine (0.1 mg/kg) or DFP (4 mg/kg) on the next day. Those animals that had been injected once daily with DFP for 14 days were challenged either with oxotremorine (0.1 mg/kg) or DFP (4 mg/kg) on day 15. The next day these animals were challenged with saline. The 4 mg/kg dose of DFP and the 0.1 mg/kg dose of oxotremorine were chosen as the challenge doses because our earlier dose-response analyses of the effects elicited by acute doses of these agents [20, 42, 43] indicated that these doses elicited readily measurable effects, but these effects were not maximal. This should facilitate the measurement of tolerance. Each treatment group had saline-treated controls that were tested in an identical fashion.

The following tests were run 15 min after oxotremorine treatment, 120 min after DFP challenge, and 15 min after saline challenge. A detailed description of these tests has been published [21].

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-min 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, Saddlebrrok, NJ).

The timing of these tests was determined from the results of time course studies for the effects of oxotremorine [20] and DFP [42] on the individual components of the test battery.

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 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 [33].

^{3}H-L-QNB Binding

The binding of ^{3}H-L-QNB was measured using a modification of the method of Yamamura and Snyder [46] as described previously [19]. A single concentration of ligand (147±8 pM) was used to assay binding in five of the brain regions. Binding to cortex was measured at six QNB concentrations (10–150 pM), and the binding parameters (K_D and B_{max}) for this brain region were determined from Scatchard plots of the data. Approximate amounts of protein in the binding assays were: 20 μg for cortex, striatum, hippocampus and hypothalamus; 100 μg for midbrain and hindbrain. Blanks were obtained by omitting protein from the assays or by adding 1 μM atropine. These methods give identical results.

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.

Brain Acetylcholinesterase Activity

Brain AChE activity was measured using a modification of Ellman's method [9], as described previously [20]. Tissue homogenates were diluted (1:5 to 1:40) in 0.05% Trition X-100 in 50 mM potassium phosphate, pH 7.4. For Km determinations, 5 concentrations (31–500 μM) of the substrate, acetylthiocholine, were used. A saturating concentration (500 μM) was used for other determinations. Blanks contained the specific AChE inhibitor BW 254 C51 (10 μM).

Protein Assay

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

Data Analysis

All kinetic analyses were conducted by linear regression of Scatchard plots of the data. Results of the tolerance tests and biochemical assays were analyzed using analysis of variance (ANOVA). In those groups where significant overall effects were indicated, group means were compared with Duncan's test.

RESULTS

Figures 1–5 present the responses of mice that had been treated chronically with DFP or saline, and were subsequently challenged with 4 mg/kg DFP, 0.1 mg/kg oxotremorine or saline. Each figure is separated into three panels. The left-hand panel in each figure presents the effects elicited by a DFP challenge, the middle panel presents the effects elicited by oxotremorine challenge, and the right-hand panel presents the effects elicited by a saline challenge. Solid bars present the results obtained with chronic DFP treated animals, and open bars present the results obtained with animals chronically injected with saline. Each panel includes a horizontal shaded area that presents the mean± S.E.M. of the responses observed in naive control animals.

Figure 1 presents the effects of DFP, oxotremorine, and saline on respiratory rate in DBA male mice that had been chronically treated with DFP or saline. Chronic DFP treatment did not result in tolerance to the effects of DFP on respiration rate in that a 4 mg/kg DFP challenge elicited the same reduction in respiratory rate in the chronic DFP-treated animals as it did in the chronic saline-treated animals. Chronic DFP treatment did result in a reduced response to oxotremorine, however. The tolerance to oxotremorine was statistically significant in the 4-day treatment group, less in the 2-day group, and no tolerance was seen in the 1-day group. Chronic DFP-treated and chronic saline-treated animals did not differ from one another in response to a saline challenge in the 4-day and 1-day treatment groups. The 2-day groups were not tested this way.

Tolerance to the actions of DFP was not seen for the heart rate test either (Fig. 2). All of the chronic DFP-treated groups were supersensitive to a challenge dose of DFP when compared with the chronic saline-treated controls. In contrast, all of the chronic DFP-treated animals were tolerant to the effects of a challenge dose of oxotremorine, but the degree of tolerance to oxotremorine's actions differed among the various treatment groups. The greatest tolerance was seen in the 4-day treatment group, and the least tolerance in the 1-day treatment group. Chronic DFP or saline treatment did not alter the response to saline.

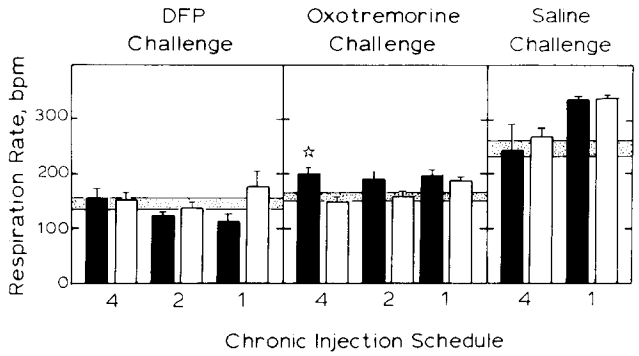

FIG. 1. The effect of DFP, oxotremorine (OXO), or saline (SAL) on respiration rate: Male DBA mice were injected with DFP (4 mg/kg) or physiological saline (0.01 ml/g b.wt.) for one month (4 and 2 day intervals) or two weeks (1 day interval). Following chronic DFP (solid bars) or SAL (open bars) treatment the mice were tested for tolerance to DFP and cross-tolerance to OXO: different groups of mice were challenged with DFP, OXO or SAL (4 mg/kg, 0.1 mg/kg, physiological SAL, respectively) 4, 2, or 1 day after the last chronic drug treatment and their respiration rate measured two hours later. Each bar represents the mean±SEM of 5–11 mice. The horizontal shaded area represents the mean±SEM of the response observed in naive control animals (n=12). ☆Significantly different from chronic saline control, $F(1,20)=11.24$, $p<0.05$.

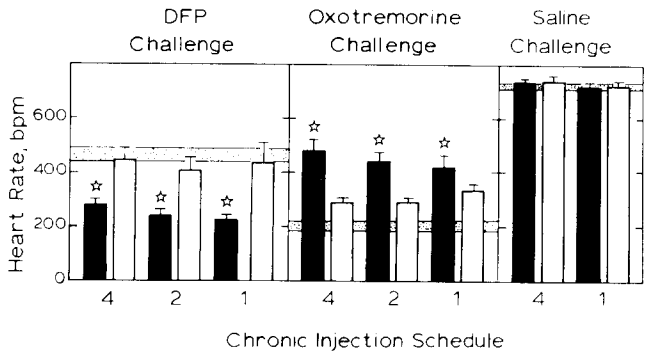

FIG. 2. The effect of DFP, OXO, or SAL on heart rate. The protocol used was identical to that outlined in Fig. 1. Each bar represents the mean±SEM of the response observed in naive control mice (n=12). ☆Significantly different from chronic saline control, $p<0.05$ [4-day schedule: DFP challenge $F(1,11)=10.64$, OXO challenge $F(1,12)=19.96$; 2-day schedule: DFP challenge $F(1,19)=12.02$, OXO challenge $F(1,20)=21.55$; 1-day schedule: DFP challenge $F(1,10)=13.07$, OXO challenge $F(1,10)=9.39$].

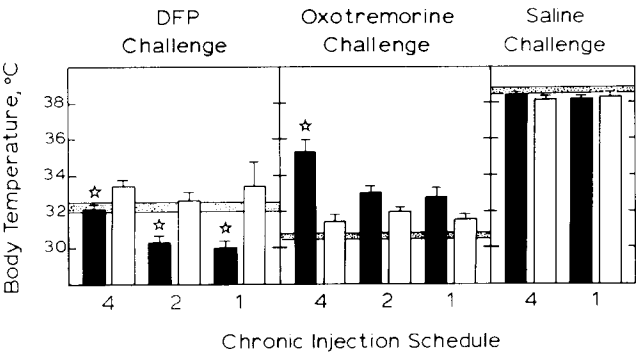

FIG. 3. The effect of DFP, OXO, or SAL on body temperature. The protocol used was identical to that outlined in Fig. 1. Each bar represents the mean±SEM of 5–11 mice. The horizontal shaded area represents the mean±SEM of the response observed in naive control animals (n=12). ☆Significantly different from chronic saline control, $p<0.05$ [4-day schedule: DFP challenge $F(1,11)=6.17$, OXO challenge $F(1,12)=18.53$; 2-day schedule: DFP challenge $F(1,19)=17.35$, OXO challenge $F(1,20)=13.37$; 1-day schedule: DFP challenge $F(1,10)=8.89$, OXO challenge $F(1,11)=4.9$].

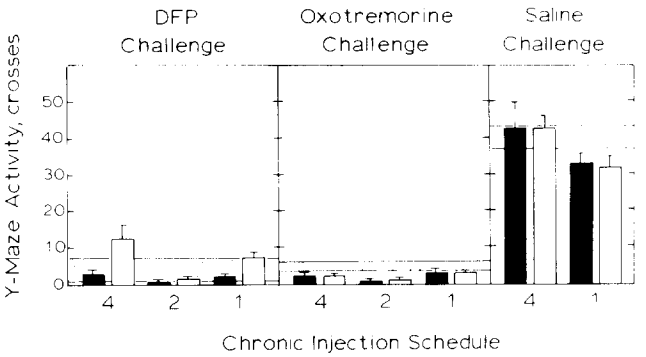

FIG. 4. The effect of DFP, OXO, or SAL on Y-maze activity. The protocol used was identical to that outlined in Fig. 1. Each bar represents the mean±SEM of 5–11 mice. The horizontal shaded area represents the mean±SEM of the response observed in naive control mice (n=12).

Chronic DFP treatment also resulted in supersensitivity to the effects of DFP on body temperature (Fig. 3). Statistically significant increases in DFP-induced hypothermia were seen in the 4-day, 2-day, and 1-day treatment groups. As was the case with the heart rate test, the DFP-treated animals were cross-tolerant to the effects of oxotremorine, with the 4-day treatment group exhibiting statistically significant cross-tolerance to oxotremorine.

Figures 4 and 5 present the results obtained with the Y-maze tests. No evidence for tolerance to the effects of DFP or cross-tolerance to the effects of oxotremorine on Y-maze crosses (Fig. 4) or rears (Fig. 5) was seen.

Figure 6 presents typical Scatchard analyses of QNB binding in cortex from saline-treated controls, and in the 4-day and 1-day treatment groups. The slopes of the plots obtained from saline- and DFP-treated animals are virtually identical which indicates that K_D values were not altered by chronic DFP treatment. The K_D for QNB binding in the

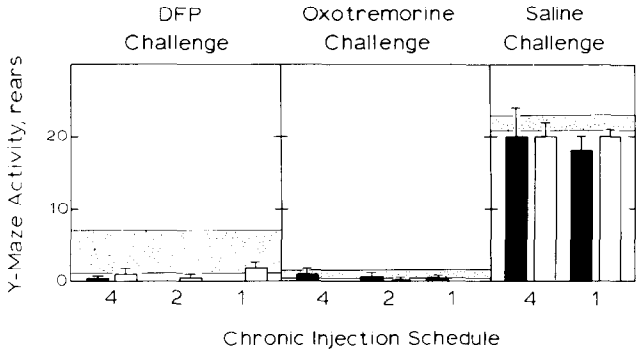

FIG. 5. The effect of DFP, OXO, or SAL on Y-maze rearing. The protocol used was identical to that outlined in Fig. 1. Each bar represents the mean±SEM of 5–11 mice. The horizontal shaded area represents the mean±SEM of the response observed in naive control mice (n=12).

4-day treatment group was 27.3±3.0 pM in DFP-treated animals and 26.6±5.1 pM in saline-injected animals. Nearly identical values were obtained for DFP-treated animals in the 1-day treatment group (24.9±2.5 pM). Therefore, any changes in binding presumably represent changes in the number of QNB binding sites.

Figure 7 presents the effects of chronic DFP treatment on QNB binding in the six brain regions for the 4-day treatment group. DFP treatment did not change the number of QNB binding sites in midbrain, hypothalamus, and hindbrain. Modest reductions in QNB binding were seen in striatum (21.1%), cortex (17.1%), and hippocampus (16.2%). Figure 8 presents the results obtained with the 2-day treatment group. As was the case with the 4-day group, DFP treatment did not elicit a change in QNB binding in midbrain, hypothalamus, and hindbrain. Marked reductions in binding were seen in striatum (45.0%), cortex (27.1%), and hippocampus (28.4%) when compared to saline-treated controls. Figure 9 presents the results obtained with the 1-day treatment groups. Once again, QNB binding was not changed in midbrain, hypothalamus, and hindbrain, but decreased binding was detected in cortex (12.1%) and hippocampus (19.5%). Only striatum showed a marked (33%) reduction in QNB binding.

Table 1 presents the acetylcholinesterase activity measured in these various treatment groups. Significant reductions from control were seen in all brain regions in each group.

DISCUSSION

The most notable finding in the present study is that chronic DFP treatment of DBA mice did not result in tolerance to DFP's effects on respiratory rate, heart rate, body temperature, and the two Y-maze activities even though QNB binding was reduced in several brain regions. It should be noted, however, that other signs of tolerance were seen in that the chronic DFP-treated mice showed minimal signs of parasympathetic nervous system hyperactivity (salivation, lacrimation, diarrhea) after several injections. Surprisingly, supersensitivity to DFP's actions was seen for two of the tests (heart rate, and body temperature). This supersen-

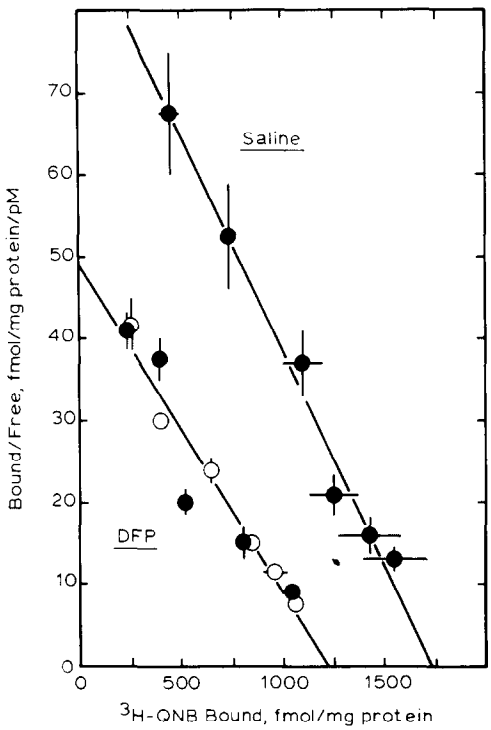

FIG. 6. Scatchard analysis of ^{3}H-QNB binding in cortex. Cortical membranes were prepared from chronic DFP- (4-day ●; 1-day ○) or saline-treated animals (●). Membranes were incubated with ^{3}H-QNB (10–150 pM) and binding determined as described in the Method section.

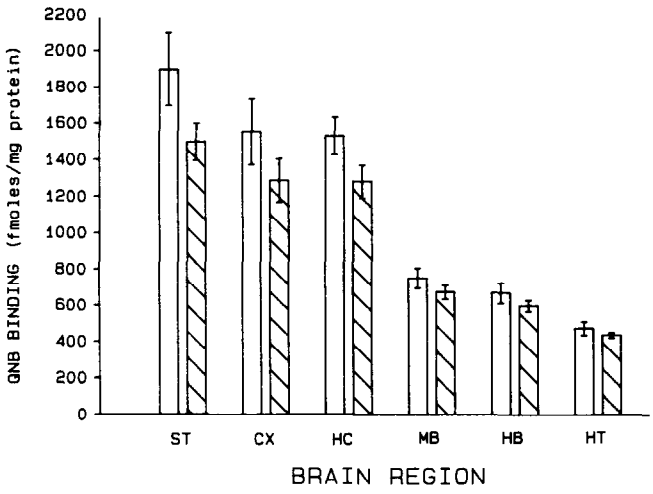

FIG. 7. ^{3}H-QNB binding in DBA mouse brain following a 4-day injection schedule. The mice were injected once every 4 days for one month with a 4 mg/kg dose of DFP (slashed bars) or physiological saline (open bars). Each graph represents the mean±SEM of 5–6 separate determinations. ^{3}H-QNB binding is expressed in fmoles/mg protein and was conducted using a single ligand concentration (147 pM). There were no significant differences between DFP and saline-treated mice. Striatum (ST), $F(1,9)=3.49$; cortex (CX), $F(1,10)=1.54$; hippocampus (HC), $F(1,10)=3.20$; midbrain (MB), $F(1,10)=1.27$; hindbrain (HB), $F(1,10)=0.82$; hypothalamus (HT), $F(1,10)=1.21$.

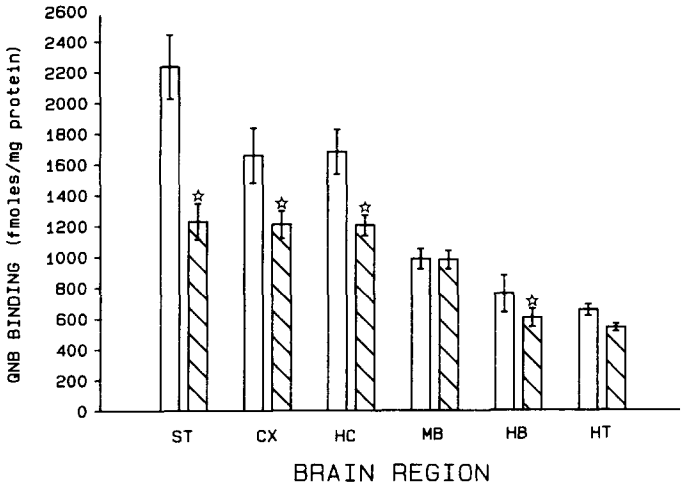

FIG. 8. ^{3}H-QNB binding in DBA mouse brain following a 2-day injection schedule. The mice were injected once every other day for one month with a 2 mg/kg dose of DFP (slashed bar) or physiological saline (open bar). Each graph represents the mean±SEM of 7–11 separate determinations. ^{3}H-QNB binding is expressed in fmoles/mg protein and was conducted using a single ligand concentration (147 pM): ☆Significantly different from chronic saline treated mice, $p<0.05$. Striatum (ST), $F(1,14)=20.51$; cortex (CX), $F(1,16)=6.29$; hippocampus (HC), $F(1,15)=10.85$; midbrain (MB), $F(1,18)=0.003$, hindbrain (HB), $F(1,18)=7.22$; hypothalamus (HT), $F(1,18)=1.49$.

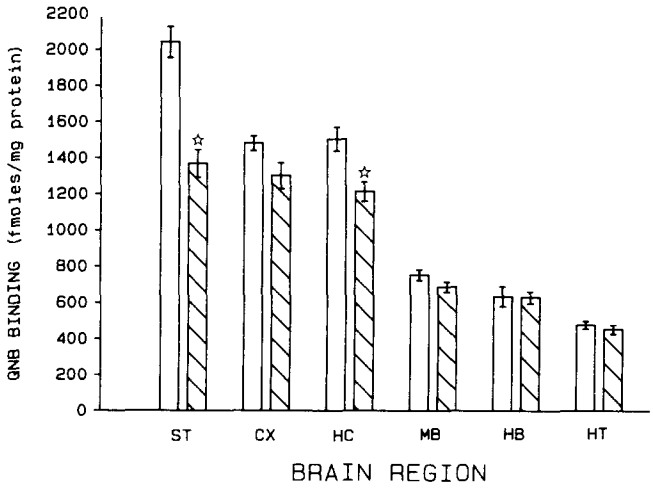

FIG. 9. ^{3}H-QNB binding in DBA mouse brain following a 1-day injection schedule. The mice were injected with 4 mg/kg dose of DPF followed by daily injections of 1 mg/kg DFP (slashed bar) or physiological saline (open bar) for a total of 14 days. Each graph represents the mean±SEM of 9–14 separate determinations. ^{3}H-QNB binding is expressed in fmoles/mg protein and was conducted using a single ligand concentration (147 pM). ☆Significantly different from chronic saline treated mice, $p<0.05$. Striatum (ST), $F(1,19)=33.35$; cortex (CX), $F(1,23)=4.08$; hippocampus (HC), $F(1,20)=11.87$; midbrain (MB), $F(1,23)=2.42$; hindbrain (HB), $F(1,23)=0.49$; hypothalamus (HT), $F(1,22)=0.10$.

TABLE 1

ACHE ACTIVITY FOLLOWING CHRONIC DFP TREATMENT

	Brain Region					
	Cortex	Midbrain	Hindbrain	Hippo-campus	Striatum	Hypo-thalamus
Control	10.9 ± 1.0	9.9 ± 1.6	6.7 ± 0.6	6.9 ± 0.5	75.2 ± 6.9	8.1 ± 0.8
4-day	0.9 ± 6.2	1.1 ± 0.2	1.1 ± 0.2	0.4 ± 0.1	1.5 ± 0.4	0.9 ± 0.3
2-day	0.4 ± 0.1	0.5 ± 0.2	0.4 ± 0.1	0.6 ± 0.3	1.0 ± 0.3	0.8 ± 0.3
1-day	1.9 ± 0.2	2.2 ± 0.1	2.5 ± 0.1	1.4 ± 0.2	4.7 ± 0.4	2.4 ± 0.1

Enzyme activity was measured as described in the Method section, and is expressed as mean ± SEM in μmoles/mg protein/hr. N=6 animals in each group. Enzyme activity was measured 4 days, 2 days, or 1 day following the last treatment with DFP.

sitivity increased as the interval between DFP challenge decreased.

Chronic DFP-treated animals exhibited a modest tolerance to oxotremorine for some of the measures (respiration, heart rate, and body temperature), but this tolerance did not parallel the changes in brain QNB binding, i.e., no relationship between tolerance to oxotremorine and changes in QNB binding was observed. The degree of tolerance to oxotremorine was minimal compared to the tolerance achieved following chronic oxotremorine infusion [15,16].

A major goal of the studies reported here was to achieve, using different DFP-treatment protocols, differential reductions in the number of QNB binding sites. This was achieved with the mice that had been injected with 2 mg/kg every other day for one month showing more dramatic changes in QNB binding than did the other two treatment groups. For each of the treatment groups, chronic DFP treatment affected a reduction in QNB binding in some, but not all, brain regions. Those regions that showed reliable changes were those that had the greatest number of QNB binding sites, i.e., striatum, cortex, and hippocampus. This reduction in QNB binding represents a decrease in the number of muscarinic receptors since the K_D for QNB binding was not altered by chronic DFP treatment. Ours is not the first study to note that chronic organophosphate treatment does not elicit decreases in QNB binding in all brain regions. Churchill *et al.* [3,4] treated rats chronically with soman or DFP and, using quantitative autoradiographic techniques, observed

large decreases in QNB binding in cortex, caudate-putamen, and the hippocampus while minor or no decreases were seen in hypothalamus, reticular formation, pontine nuclei, inferior colliculus, and cerebellum. Yamada *et al.* [45] examined the effects of chronic DFP treatment on QNB binding in guinea pig brain, and observed significant decreases in QNB binding in striatum, cortex, and hippocampus. Clearly, our results with mice replicate these findings. Several other studies have also demonstrated regional specificity in muscarinic receptor changes following chronic organophophate treatment [7,10].

As mentioned previously, a number of investigations have demonstrated that chronic DFP treatment results in tolerance to the effects of DFP, and it has been suggested that these changes in QNB binding may explain tolerance to organophosphates. Costa *et al.* [7] studied the time course of disulfoton-induced changes in QNB binding in Charles River CD-1 mice, and noted that these changes paralleled the development of tolerance to disulfoton as measured by the reversal of disulfoton-induced decreases in body weight. None of the other studies that have asserted that tolerance develops to organophosphates have attempted to assess the relationship between receptor changes and tolerance by using any more than one treatment protocol or drug dose. Therefore, these investigators made conclusions based on a correlation between two points. Our results suggest that the relationship between tolerance to DFP and receptor changes must be examined more closely.

Our failure to detect tolerance to the effects of DFP on any of the measures that we made might be explained by the suggestion that these responses are regulated by muscarinic receptors in brain regions or tissues (e.g., heart) where muscarinic receptors are not affected by chronic DFP treatment. Clearly, a finer anatomical study is required to assess thoroughly the relationship between changes in QNB binding and tolerance, if it exists, to DFP. The suggestion that chronic DFP treatment elicits changes in QNB binding in brain nuclei that are not involved in the regulation of the responses that we measured is not consistent with the observation that these same mice are cross-tolerant to the effects of oxotremorine on some of these measures. If it is assumed that DFP-induced decreases in respiratory rate, heart rate, and body temperature are mediated via effects on muscarinic receptors, it is difficult to argue on the basis of muscarinic receptor numbers that mice should be supersensitive to DFP and tolerant to oxotremorine. One possible explanation for our observation that chronic DFP-treated mice were supersensitive to DFP and tolerant to oxotremorine is that some or all of the responses that we measured may be regulated to a significant degree by brain nicotinic receptors. Indeed, it has been demonstrated that chronic treatment with organophosphates results in a decrease in the number of brain ^{3}H-nicotine [5], and ^{3}H-acetylcholine [40] binding sites. Such an effect would be expected to result in tolerance to nicotinic agonists. However, it should be noted that tolerance to nicotine parallels in a dose-response and time-course fashion an up-regulation of brain ^{3}H-nicotine binding sites [17,22]. Therefore, the relationship between organophosphate-induced decreases in brain nicotinic receptors and response to nicotinic agonists remains unclear.

One potential explanation for our failure to detect measurable tolerance to DFP's actions may be found in genetic influences on drug response. For example, two rat lines have been selectively bred for differences in acute sensitivity to DFP [30]. Interestingly, these two rat lines also differ in tolerance development following chronic treatment [37]. Therefore, it may be tolerance to DFP actions can occur only in certain genetic stocks, and it may be that the DBA mouse strain is one of those genetic stocks that does not develop tolerance to organophosphates. Whatever the case may be, the results reported here clearly demonstrate that tolerance need not develop following chronic DFP treatment, and that changes in muscarinic receptors are not necessarily an underlying cause of tolerance to DFP.

Several recent studies have indicated that chronic treatment with organophosphates results in a neurotoxicity [23, 26, 32]. Most of these studies have used soman, and it has been suggested that the neurotoxicity arises because of hypoxia that accompanies soman-induced seizures [26]. We did not observe seizures following DFP treatment, but it may be that chronic treatment resulted in two effects on brain muscarinic receptors: a classical agonist-induced down-regulation of QNB binding sites that explains the tolerance to oxotremorine, and a neurotoxic action that damaged neurons containing QNB binding sites. Destruction of these neurons might result in enhanced responsiveness to DFP.

A comparison of the effects of chronic DFP treatment on brain QNB binding sites with our earlier studies of the effects of chronic oxotremorine infusion on these same sites [15,16] reveals that chronic DFP treatment elicited, for the most part, more dramatic decreases in brain QNB binding. However, DFP treated mice were only marginally tolerant to the effects of oxotremorine whereas oxotremorine-treated mice exhibited 35–80 fold tolerance to the effects of oxotremorine on body temperature and rotarod performance [15]. Chronic oxotremorine treatment elicited changes in QNB binding in striatum, cortex, and hippocampus plus midbrain and hindbrain, but marked tolerance was seen before significant reductions in receptor numbers occurred [16]. These findings are not consistent with the observation that DFP treatment elicited greater changes in QNB binding but only marginal tolerance to oxotremorine. Taken together, these data suggest that further explanations must be sought regarding the meaning of changes in brain QNB binding following chronic organophosphate treatment.

In conclusion, the studies reported here have demonstrated that chronic treatment with DFP does not necessarily result in tolerance to the effects of DFP although tolerance to the muscarinic agonist, oxotremorine was seen. DFP treatment also elicited a decrease in the number of brain muscarinic receptors, but this decrease does not correlate well with changes in response to either DFP or oxotremorine. The possibility that some of the loss in muscarinic receptors is due to neurotoxic actions of DFP must be considered.

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