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Species Differences in Diisopropylfluorophosphate-Induced Decreases in the Number of Brain Nicotinic Receptors

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VAN DE KAMP, J. L. AND A. C. COLLINS *Species differences in diisopropylfluorophosphate-induced decreases in the number of brain nicotinic receptors.* PHARMACOL BIOCHEM BEHAV 42(1) 131-141, 1992.—DBA and C3H mice were injected chronically with 2.0 mg/kg diisopropylfluorophosphate (DFP) every other day for 2 or 4 weeks. Although acetylcholinesterase (AChE) activity and muscarinic receptor numbers {[^{3}H] quinuclidinyl benzilate (QNB) binding} were decreased in DFP-treated DBA and C3H mice, the number of nicotinic receptors {L-[^{3}H]nicotine and α-[^{125}I]bungarotoxin (BTX) binding} was unchanged by chronic DFP treatment. Sprague-Dawley rats injected chronically with lower doses of DFP than were used in mice exhibited a greater reduction in AChE activity, as well as accompanying decreases in [^{3}H]QNB and [^{3}H]nicotine binding. Neither species exhibited changes in α-[^{125}I]BTX following chronic DFP injection. The effects of chronic DFP treatment on sensitivity to DFP and to nicotine were also assessed in the two mouse strains using a battery of behavioral and physiological tests that included rotarod performance, Y-maze crossing and rearing activity, heart rate, and body temperature. No tolerance to DFP was observed in either mouse strain after 2 weeks of treatment. Following 4 weeks of treatment, DFP-treated DBA mice exhibited modest tolerance to the effect of DFP on body temperature. C3H mice did not survive the 4-week treatment. Some evidence for reduced sensitivity to nicotine's effects was detected in the DFP-treated DBA mice, but cross-tolerance to nicotine was not observed in the DFP-injected C3H mice. Because chronic DFP treatment did not evoke a change in the number of brain nicotinic receptors, the reduced sensitivity to some of nicotine's effects seen in DBA mice must be due to some factor other than receptor downregulation.

Acetylcholinesterase Nicotine Nicotinic receptors DFP Muscarinic receptors Genetics Tolerance

ACETYLCHOLINESTERASE (AChE) is largely responsible for the inactivation of acetylcholine (ACh), and agents that inhibit this enzyme are of economic value as insecticides and pesticides. The most valuable agents are the organophosphates, which are irreversible inhibitors of AChE. Comparatively little is known concerning the long-term effects of these agents, but there is reason for concern since some humans who have been exposed to organophosphates suffer a variety of intellectual and psychiatric disorders that may be permanent (16,41,42). The causes of these disorders are unknown.

One of the well-documented effects of chronic exposure to organophosphates is that the number of brain muscarinic cholinergic receptors is decreased following chronic treatment. These changes have been detected using L-[^{3}H]-quinuclidinyl benzilate ([^{3}H]QNB) as a ligand (45), and many studies have demonstrated that chronic organophosphate treatment results in a decrease in the number of brain [^{3}H]QNB binding sites [see (4,5,10,11,18,44) for examples]. Reductions in [^{3}H]QNB binding are largely restricted to the cortex, striatum, and hippocampus (4,5,44).

The number of brain nicotinic receptors may also be reduced following chronic organophosphate treatment. Schwartz and Kellar (39) demonstrated that chronic injection of rats with diisopropylphosphofluoridate (DFP) results in a decrease in the number of brain [^{3}H]ACh binding sites. Similarly, Costa and coworkers noted that chronic treatment of rats with disulfoton (8) and mice with DFP (9) results in a decrease in the number of brain [^{3}H]nicotine binding sites. These studies were investigating effects of chronic treatment on the same nicotinic receptor subtype since [^{3}H]ACh and [^{3}H]nicotine appear to bind to identical sites (6,27).

Brain nicotinic receptors have also been studied using

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α-[^{125}I]bungarotoxin (α-[^{125}I]BTX) as a ligand (20,31,33), but potential effects of chronic organophosphate treatment on these sites have not been measured perhaps because several electrophysiological studies, carried out 10–15 years ago, reported that α-BTX did not inhibit cholinergic transmission in the autonomic and central nervous systems (3,14,30). Recent studies, however, utilizing the frog oocyte expression system, demonstrated that the α-BTX binding protein is responsive to nicotinic agonists including acetylcholine (12). This, coupled with the observation that chronic nicotine treatment results in an upregulation of α-[^{125}I]BTX binding (22,25,26), suggests that the α-BTX binding site is functional.

Several studies, utilizing rats, have suggested that the decrease in [^{3}H]QNB binding elicited by chronic organophosphate treatment may underlie the development of tolerance to organophosphates (2,28,29,34), but recent studies from our laboratory have demonstrated that decreases in [^{3}H]QNB binding can be obtained in DBA mice without the development of tolerance to the organophosphate, DFP (44). Chronic DFP-treated mice did exhibit, however, a modest reduction in sensitivity to the muscarinic agonist, oxotremorine. These changes in sensitivity did not parallel changes in [^{3}H]QNB binding. A subsequent study compared the effects of chronic DFP treatment using DBA and C3H mice, which differ in sensitivity to an acute challenge dose of DFP (43). Neither strain developed tolerance to DFP following several chronic treatment regimens (7), but chronic DFP-treated DBA mice were cross-tolerant to oxotremorine. C3H mice, in contrast, did not exhibit a decreased sensitivity to oxotremorine following chronic DFP treatment.

The studies reported here were conducted to ascertain whether genetic factors regulate the effects of chronic DFP treatment on brain nicotinic receptors, as measured by both [^{3}H]nicotine and α-[^{125}I]BTX binding. Potential changes in [^{3}H]QNB binding and in sensitivity to DFP and to nicotine were also assessed. DBA and C3H mice were used because of our earlier observations that suggested the two strains differ in initial sensitivity to DFP (43) and in the development of cross-tolerance to oxotremorine (7). In addition, the effects of chronic DFP treatment on cholinergic receptor binding were determined using Sprague-Dawley rats as a comparison group.

METHOD

Animals

Male mice of the DBA/2IBG and C3H/2IBG strains and male Sprague-Dawley rats were used in this study. The mouse strains have been maintained in the breeding colony at the Institute for Behavioral Genetics for at least 20 generations. Mice were housed three to five per cage with like-sexed littermates. Rats were housed three per cage in a separate room. Both rooms were maintained at 21 ± 2°C under a 12L:12D photoperiod. Food and water were available ad lib. Animals were 60–90 days of age when tested. All testing was conducted between 9:00 a.m. and 4:00 p.m.

Materials

L-[^{3}H]Nicotine (*N*-[methyl^{3}H], initial specific activity 78.4 Ci/mmol) was obtained from Amersham Corporation (Arlington Heights, IL). L-[^{3}H]QNB (benzilic-4,4′-^{3}H, initial specific activity 30.0 Ci/mmol), and α-[^{125}I]BTX (Tyr-^{125}I, initial specific activity 130.7–140.8 Ci/mmol) were obtained from New England Nuclear Corporation (Newton, MA). [^{3}H]Nicotine was repurified by thin-layer chromatography to reduce the blank and eliminate what appears to be a low-affinity binding artifact (38). It was stored frozen with a fourfold excess of mercaptoacetic acid (37).

L-Nicotine, DFP, bovine serum albumin, polyethylenimine, Tris-HCl, Triton X-100, acetylthiocholine, mercaptoacetic acid, and the specific AChE inhibitor, BW 254 C51, were purchased from Sigma Chemical Company (St. Louis, MO). L-Nicotine was periodically redistilled. Glass fiber filters and HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethane sulfonic acid, were purchased from Boehringer Mannheim (Indianapolis, IN). Scintillation fluid (Safety Solve) and polypropylene scintillation vials were obtained from Research Products International (Mount Prospect, IL). Inorganic compounds were reagent grade.

Chronic DFP Treatment

DFP was prepared in saline and injected intraperitoneally. DFP is frequently administered in an oil vehicle, but is stable for several hours in saline (17). The saline solution was easier to administer and seemed to cause less discomfort to animals than did an oil injection. The solution was injected within 1 h after preparation. DBA and C3H mice were injected every other day for 2 weeks with a 2.0-mg/kg dose of DFP for a total of eight injections. In addition, another group of DBA mice was injected every other day for 4 weeks with a 2.0-mg/kg dose of DFP for a total of 16 injections. Sprague-Dawley rats were injected subcutaneously with 1.0 mg/kg DFP on the first day, 0.4 mg/kg DFP on the second day, and 0.2 mg/kg DFP on the fourth, sixth, eighth, and tenth days of treatment. Control rats received an injection of saline on the same schedule.

Tolerance Tests

Responses to DFP and cross-tolerance to nicotine were assessed in mice treated with DFP for 2 weeks using a test battery consisting of the following tests: rotarod performance, Y-maze activity (both line crossings and rears), heart rate, and body temperature. All tests were conducted on each individual because previous studies from our laboratory demonstrated that no significant intertest interactions occur (24). The same test battery was used with the exception of rotarod performance to assess sensitivity to DFP and nicotine in DBA mice treated with DFP for 4 weeks. Each treatment group included controls that were tested following saline injection.

Testing was begun 2 min after saline or nicotine challenge in the first experiment. In the second experiment, testing was begun 4 min after saline or nicotine challenge because rotarod performance was excluded. In both experiments, testing was begun 120 min after DFP challenge. A detailed description of these tests has been published (24).

Rotarod test. Mice were trained to walk on a 5-cm diameter rod (Rotarod, Ugo Basile Co., Milan, Italy) at a speed of 8 rpm until they were capable of remaining on the apparatus for three successive 100-s test periods. Once trained, their performance remained stable for several days. Mice were injected with saline or the challenge drug and placed on the rotating rod for 100 s or until they fell off.

Y-maze test. After completion of the rotarod test, mice were transferred to a Y-maze. The maze has three arms 26 cm long, 6.1 cm wide, and 10.2 cm high. Each arm is subdivided into two equal sections and line crossings and rears (animal stands up on its hind legs) were recorded during a 3-min test period.

Heart rate. After completion of the Y-maze test, mice were

placed in a restrainer and needle electrodes were inserted through the skin immediately in front of the left forelimb and right hindlimb. The electrodes were connected through a preamplifier to an E&M physiograph (Narco Biosystems, Houston, TX). Heart rate was monitored for 6 s.

Body temperature. Body temperature was measured with a rectal thermometer (Bailey Instruments, Saddlebrook, NJ). Saline- and DFP-challenged mice were measured immediately following the heart rate measurement, whereas nicotine-challenged animals were measured 15 min after injection.

The timing of the tests was determined from the results of time course studies for the effects of nicotine (24) and DFP (43).

Biochemical Measurements

The binding of radiolabeled QNB, nicotine, and α-BTX to crude membrane fractions of mouse and rat brain regions was measured using filtration assays described in detail elsewhere (27). The methods used for tissue preparation and ligand binding will be summarized briefly below.

Tissue preparation. After completion of the tolerance tests, mice were sacrificed by cervical dislocation. Rats were sacrificed by carbon dioxide inhalation 24 h after the last injection. The brain was removed, rinsed with distilled water, and dissected into seven regions (mice): cortex, cerebellum, hindbrain (pons-medulla), hypothalamus, hippocampus, striatum, and midbrain (tissue remaining after removal of all other areas; contains primarily thalamus). In rats, the colliculi were also dissected out.

Tissue pieces were placed in 10 volumes of ice-cold Krebs-Ringer's HEPES (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 samples were homogenized with a glass-Teflon homogenizer. The tissue preparation method was essentially that of Romano and Goldstein (37). The particulate fraction was collected by centrifugation for 20 min at 18,000 × *g*. The buffer was discarded and the pellet was resuspended in 20 volumes of distilled water and incubated on ice for 60 min. After this incubation, samples were centrifuged for 20 min at 18,000 × *g*. The buffer was discarded and the pellet was resuspended in 10 volumes of Krebs-Ringer's HEPES and centrifuged for 20 min at 18,000 × *g*. The buffer was discarded and 10 volumes of fresh buffer were added. Samples were then frozen at −70°C until assay. Prior to each centrifugation, samples were incubated at 37°C for 5 min to promote the dissociation of drugs with which animals had been treated from the tissue. This method has been shown to remove nicotine from mouse brain tissue (25).

[^{3}H]QNB binding. The binding of [^{3}H]QNB to brain tissue was measured using a modification of the method of Yamamura and Snyder (45) as described previously (23). Aliquots of brain tissue (adjusted for region such that tissue receptor concentration was less than 0.25 × K_d) were pipetted into 10 ml HEPES-buffered Krebs-Ringer's buffer. Binding was initiated by the addition of [^{3}H]QNB. The binding reaction was run for 60 min at 37°C. A single concentration of ligand was used to assay binding in six of the brain regions. The average [^{3}H]QNB concentration was 98 ± 3 pM. Binding to cortex was measured at six [^{3}H]QNB concentrations and the binding parameters (K_d and B_{max}) for this brain region were determined from Scatchard plots of the data. The binding reaction was terminated by filtration of the samples onto glass fiber filters. The filters were washed three times with 3-ml aliquots of ice-cold buffer. The vacuum was −25 to −50 torr. Nonspecific binding was determined with a no-tissue blank.

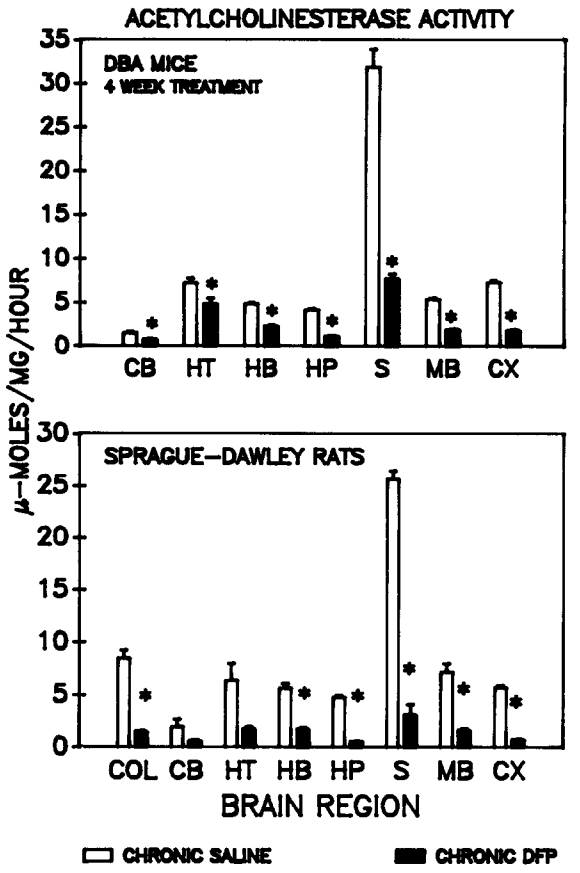

FIG. 1. Effect of chronic DFP treatment on AChE activity. AChE activity was measured in seven brain regions from DBA mice injected IP every other day for 4 weeks with either saline or 2.0 mg/kg DFP and in eight brain regions from Sprague-Dawley rats injected SC every other day for 10 days with either saline or 0.2 mg/kg DFP. Each bar represents the mean ± SEM of three to nine animals. Brain regions are designated by the following abbreviations: COL, colliculi; CB, cerebellum; HT, hypothalamus; HB, hindbrain; HP, hippocampus; S, striatum; MB, midbrain; CX, cortex. *Values differing significantly between chronic saline- and DFP-treated animals ($p < 0.05$).

L-[^{3}H]Nicotine binding. The binding of [^{3}H]nicotine was measured using a modification of the method of Romano and Goldstein (37) as described previously (27). Binding was measured using 100-600 μg protein. Final incubation volume was 250 μl. HEPES-buffered Krebs-Ringer's solution was used as the buffer. In addition, 500 mM Tris (pH 7.5 at 37°C) was included to reduce the nonspecific binding. The binding was conducted in 12 × 75 mm polypropylene tubes at 4°C. The reaction was initiated by the addition of labeled ligand. Incubation time was 90 min. Binding was terminated by addition of 3 ml ice-cold buffer followed immediately by filtration of the samples onto glass fiber filters that had been soaked in buffer containing 0.5% polyethylenimine to reduce nonspecific binding (40). Filters were subsequently washed four more times with 3-ml aliquots of ice-cold buffer. The vacuum was −50 to −100 torr. All filtrations and washes were conducted in a 4°C room using apparatus cooled to 4°C. Blanks were obtained by including 10 μM L-nicotine in the incubations.

In DBA and C3H mice treated for 2 weeks with DFP and in Sprague-Dawley rats, a single concentration of radiolabeled

nicotine (approximately 5 nM) was used in all brain regions except the cortex. The binding parameters (K_d and B_{max}) in cortex were estimated by displacement of [^{3}H]nicotine by nonlabeled L-nicotine. The displacement data were used to calculate the K_d and B_{max} for ligand binding after conversion of the results to a form suitable for use in Scatchard plots. In DBA mice treated with DFP for 4 weeks, the binding parameters in all brain regions were estimated using displacement data.

α-[^{125}I]BTX binding. The binding of α-[^{125}I]BTX was measured as described previously (23). Binding was measured using 50–300 μg protein in a final volume of 500 μl HEPES-buffered Krebs-Ringer's solution. The reaction was initiated by the addition of α-[^{125}I]BTX and continued for 3 h at 37°C. At the completion of the incubation, samples were diluted with 3 ml ice-cold buffer and filtered on glass fiber filters that had been soaked in buffer containing 0.5% polyethylenimine to reduce the blank. Filters were then washed four times with 3-ml aliquots of ice-cold buffer. Vacuum pressure was −50 to −100 torr. Samples containing 1 mM L-nicotine served as blanks.

A single concentration of α-[^{125}I]BTX (approximately 1.2 nM) was used in all brain regions except cortex in DBA and C3H mice treated with DFP for 2 weeks and in Sprague-Dawley rats. Binding to cortex was measured at five additional concentrations of ligand to calculate K_d and B_{max} from Scatchard plots. In DBA mice treated with DFP for 4 weeks, five ligand concentrations were used to measure binding in all brain regions. Binding parameters were calculated from Scatchard plots for all seven brain regions.

Protein assay. Protein was measured using the method of Lowry et al. (19) with bovine serum albumin as the standard.

Radioactivity assessment. After tritium samples were washed, the glass fiber filters were placed in polypropylene scintillation vials (7 ml) and 2.5 ml scintillation fluid (Safety Solve) was added. 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 45% efficiency. After the radiolabeled iodinated samples were washed, the glass fiber filters were placed in 12 × 75 mm borosilicate glass tubes. Radioactivity was determined on a Packard Minaxi Auto-Gamma 5000 series gamma counter at 80% efficiency.

AChE activity. Brain AChE activity was measured in DBA mice treated with DFP or saline for 4 weeks and in Sprague-Dawley rats using a modification of the method of Ellman et al. (15) as described previously (21). Tissue homogenates were diluted (1 : 5 to 1 : 40) in 0.05% Triton X-100 in 50 mM potassium phosphate, pH 7.4. A saturating concentration (500 μM) of the substrate, acetylthiocholine, was used. Blanks contained the specific AChE inhibitor BW 254 C51 (10 μM).

Data Analysis

AChE activity and radioligand binding levels were analyzed by one-way analysis of variance (ANOVA) as a function of chronic treatment, with each brain region analyzed separately. Binding constants (K_d and B_{max}) were calculated by linear regression analysis of Scatchard plots of the binding

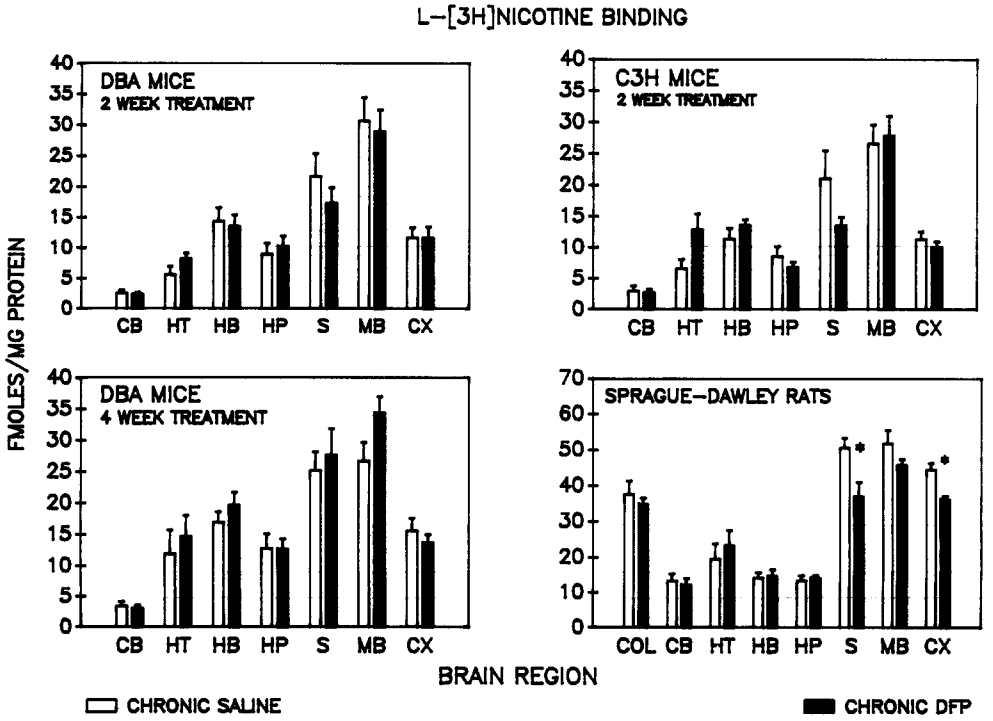

FIG. 2. Effect of chronic DFP treatment on L-[^{3}H]nicotine binding. L-[^{3}H]Nicotine binding was measured in seven brain regions from DBA and C3H mice injected IP every other day for 2 or 4 weeks with either saline or 2.0 mg/kg DFP and in eight brain regions from Sprague-Dawley rats injected SC every other day for 10 days with either saline or 0.2 mg/kg DFP. The whole particulate fraction of each brain region was incubated for 90 min at 4°C with L-[^{3}H]nicotine. Each bar represents the mean ± SEM of six to nine animals. Brain regions are designated by the same abbreviations used in Fig. 1. *Values differing between chronic saline- and DFP-treated animals ($p < 0.05$).

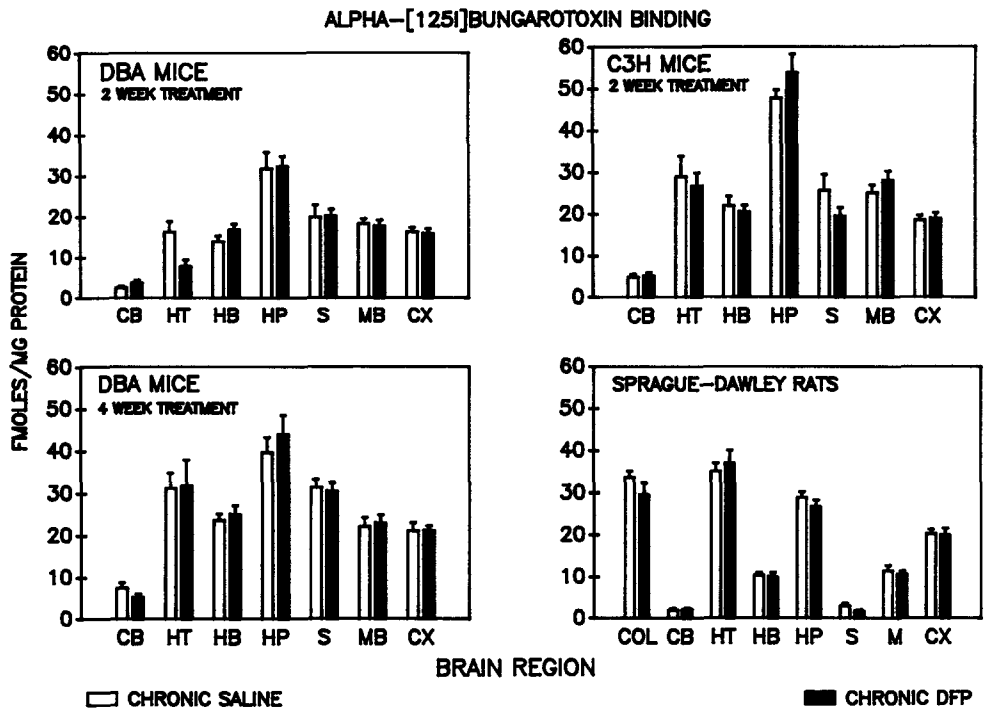

FIG. 3. Effect of chronic DFP treatment on α-[^{125}I]BTX binding. α-[^{125}I]BTX binding was measured in seven brain regions from DBA and C3H mice injected IP every other day for 2 or 4 weeks with either saline or 2.0 mg/kg DFP and in eight brain regions from Sprague-Dawley rats injected SC every other day for 10 days with either saline or 0.2 mg/kg DFP. The whole particulate fraction of each brain region was incubated for 3 h at 37°C with α-[^{125}I]BTX. Each bar represents the mean ± SEM of 8–10 animals. Brain regions are designated by the same abbreviations used in Fig. 1. No significant differences between chronic saline- and DFP-treated animals were observed.

data. The lines obtained were tested for parallelism. In addition, the K_d and B_{max} for each ligand were analyzed by one-way ANOVA as a function of chronic treatment. The results of each tolerance test were analyzed using two-way ANOVA as a function of chronic treatment and acute challenge dose. The results of those analyses where a significant effect of treatment was detected were further examined using the Newman-Keuls posthoc test. The dose-response curves were also analyzed using a regression line comparison program. The program sequentially tested for differences in homogeneity of variance (an F-test), slope (a t-test), and whether the lines are superimposable (a t-test). The drug doses required to elicit a standard effect (effective doses) were also calculated from this analysis. The effective doses that were calculated include: the doses required to reduce rotarod performance, Y-maze crosses, and Y-maze rears by 50% (ED_{50}), heart rate by 100 beats per minute (ED_{-100}), and body temperature by 2°C (ED_{-2}). Significance was set at the $p < 0.05$ level for all analyses.

RESULTS

AChE activities measured in seven brain regions from DBA mice treated for 4 weeks with saline or DFP and in eight brain regions from Sprague-Dawley rats treated for 10 days with DFP are presented in Fig. 1. Significant reductions in AChE activity were observed in all brain regions except the cerebellum and hypothalamus in rats. AChE activity was reduced by 33–76% in DFP-treated DBA mice and by 70–88% in DFP-treated Sprague-Dawley rats. Although mice showed consistently less inhibition than did rats, the pattern of change was virtually identical. The correlation of percentage of inhibition of AChE activity across the eight regions tested in the two species was 0.840 ($p < 0.01$).

Figure 2 presents the effect of chronic DFP treatment on [^{3}H]nicotine binding in seven brain regions from DBA and C3H mice and in eight brain regions from Sprague-Dawley rats. No significant differences in binding were observed in DFP-treated C3H and DBA mice regardless of the length of treatment. DFP-treated Sprague-Dawley rats displayed significant decreases in the number of [^{3}H]nicotine binding sites in striatum, $F(1, 14) = 7.95$, $p < 0.05$, and cortex, $F(1, 7) = 19.90$, $p < 0.01$).

The effects of chronic DFP treatment on α-[^{125}I]BTX binding are illustrated in Fig. 3. No effect of chronic DFP treatment was detected in DBA and C3H mice or in Sprague-Dawley rats.

Figure 4 depicts the effect of chronic DFP treatment on [^{3}H]QNB binding in seven brain regions from DBA and C3H mice and in eight brain regions from Sprague-Dawley rats. Binding was significantly reduced in the hippocampus, $F(1, 16) = 13.84$, $p < 0.01$, striatum, $F(1, 18) = 13.48$, $p < 0.01$, and cortex, $F(1, 18) = 5.71$, $p < 0.05$, of DBA mice treated with DFP for 2 weeks, whereas 4 weeks of treatment resulted in significant reductions in the binding of hindbrain, $F(1, 16) = 12.96$, $p < 0.01$, hippocampus, $F(1, 16) = 15.86$, $p < 0.001$, and striatum, $F(1, 16) = 8.08$, $p < 0.01$. DFP-treated C3H mice also exhibited significant de-

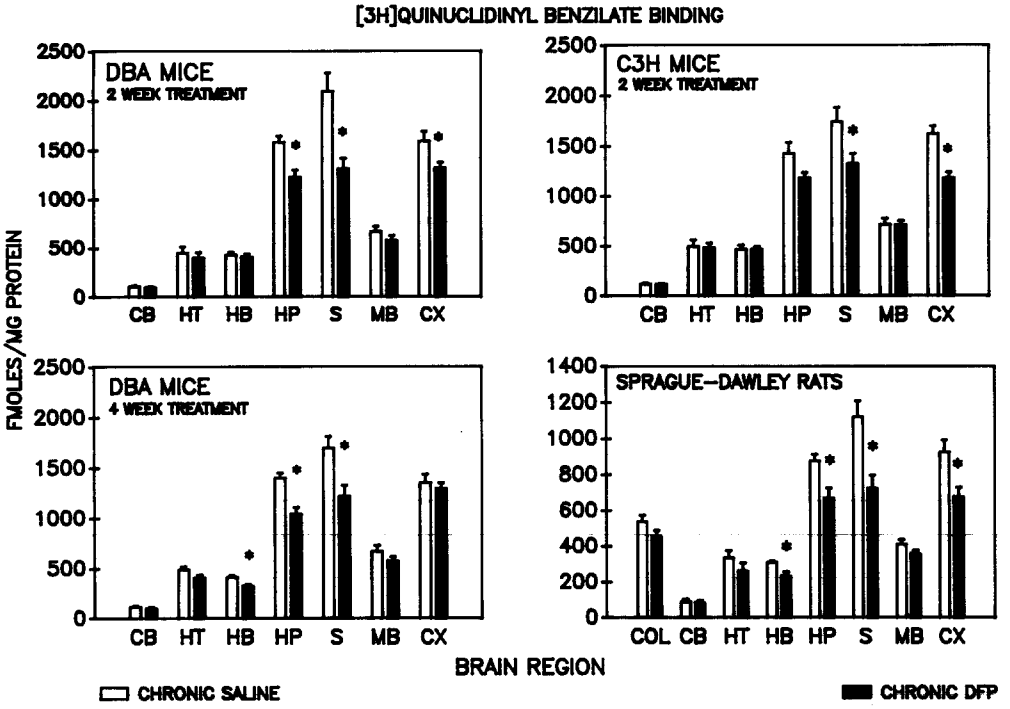

FIG. 4. Effect of chronic DFP treatment on [^{3}H]QNB binding. [^{3}H]QNB binding was measured in seven brain regions from DBA and C3H mice injected IP every other day for 2 or 4 weeks with either saline or 2.0 mg/kg DFP and in eight brain regions from Sprague-Dawley rats injected SC every other day for 10 days with either saline or 0.2 mg/kg DFP. The whole particulate fraction of each brain region was incubated for 60 min at 37°C with [^{3}H]QNB. Each bar represents the mean ± SEM of six to nine animals. Brain regions are designated by the same abbreviations used in Fig. 1. *Values differing between chronic saline- and DFP-treated animals ($p < 0.05$).

creases in binding in striatum, $F(1, 16) = 5.59, p < 0.05$, and cortex, $F(1, 17) = 21.08, p < 0.001$. Significant decreases in binding were observed in the hindbrain, $F(1, 14) = 10.15$, $p < 0.01$, hippocampus, $F(1, 14) = 9.82$, $p < 0.01$, striatum, $F(1, 14) = 12.25$, $p < 0.01$, and cortex, $F(1, 14) = 10.37$, $p < 0.01$ of DFP-treated Sprague-Dawley rats. Changes in [^{3}H]QNB binding across species were quite similar with the exception of cortex. The correlation of % decrease in [^{3}H]QNB binding between DBA mice and Sprague-Dawley rats is 0.464 (ns) if all brain regions are included, but $r = 0.92$, ($p < 0.01$) if the cortex is omitted.

Table 1 presents the effect of chronic DFP treatment on the binding parameters of the three radioligands in cortex. The K_d and B_{max} of [^{3}H]nicotine and α-[^{125}I]BTX binding in cortex were unchanged by DFP treatment in DBA and C3H mice and Sprague-Dawley rats. However, the difference in B_{max} values for L-[^{3}H]nicotine binding between control and DFP-treated rats just barely missed being statistically significant, $F(1, 8) = 5.25, p = 0.051$. The K_d of [^{3}H]QNB binding was unaffected by chronic DFP treatment; however, the B_{max} of [^{3}H]QNB binding was significantly reduced in C3H mice, $F(1, 17) = 16.83, p < 0.001$, DBA mice, $F(1, 12) = 12.17$, $p < 0.01$, and Sprague-Dawley rats, $F(1, 13) = 5.19, p < 0.05$, treated chronically with DFP. No significant differences in K_d and B_{max} of [^{3}H]nicotine and α-[^{125}I]BTX binding were obtained in cerebellum, hypothalamus, hindbrain, hippocampus, striatum, and midbrain in DBA mice treated with DFP for 4 weeks (data not shown).

Correlations between % decrease in AChE activity and binding were calculated for DBA mice and Sprague-Dawley rats to establish potential relationships between these measures. Nonsignificant correlations were obtained between % inhibition of AChE and % decrease in [^{3}H]QNB binding ($r = 0.15$ in DBA mice if all seven brain regions are used to calculate r; $r = 0.62$ if cortex is omitted; $r = 0.54$ in rats). Thus, % inhibition of AChE does not predict effects on QNB binding. Similarly, the correlation between % inhibition of AChE activity and changes in [^{3}H]nicotine binding in rat brain were not significant ($r = 0.51$).

Dose–response curves for DFP actions were constructed using DBA and C3H mice that had been injected for 2 weeks. Table 2 presents a comparison of the effects of DFP on chronic saline- and DFP-injected DBA and C3H mice as estimated by the calculation of the DFP doses required to elicit standard effects (values resembling the ED_{50}). Significant strain differences in the dose required to decrease Y-maze crossing activities by 50% were obtained for chronic saline-injected animals. Chronic DFP injection failed to elicit significant changes in any of the effective dose values for any response in either mouse strain. Thus, no tolerance to DFP was observed in either strain following treatment for 2 weeks. However, when DBA mice were treated with DFP for 4 weeks, tolerance to the effect of DFP on body temperature was observed, $F(1, 24) = 5.57, p < 0.05$ (Fig. 5). Despite several attempts, comparable data could not be obtained with the C3H strain; C3H mice did not survive the 4-week treatment.

Chronic DFP treatment of DBA mice for 2 and 4 weeks resulted in a modest reduction in the response to nicotine.

TABLE 1

EFFECT OF CHRONIC DFP TREATMENT ON THE CORTICAL BINDING PARAMETERS OF THREE RADIOLIGANDS

		L-[^{3}H]Nicotine		α-[^{125}I]BTX		[^{3}H]QNB	
Animal	Treatment	K_d	B_{max}	K_d	B_{max}	K_d	B_{max}
DBA mice, 2 weeks	SAL	5.58 ± 0.80	31.7 ± 2.5	0.44 ± 0.07	22.0 ± 4.2	6.44 ± 1.32	1722 ± 72
	DFP	5.01 ± 0.59	27.2 ± 1.8	0.54 ± 0.18	23.1 ± 3.0	9.34 ± 1.75	1358 ± 72*
DBA mice, 4 weeks	SAL	4.99 ± 0.94	34.7 ± 0.8	0.54 ± 0.07	31.7 ± 2.0	–	–
	DFP	5.57 ± 1.06	31.5 ± 1.9	0.60 ± 0.05	28.8 ± 1.8	–	–
C3H mice, 2 weeks	SAL	7.96 ± 1.31	33.0 ± 2.0	0.52 ± 0.09	27.1 ± 3.3	7.76 ± 1.25	1657 ± 99
	DFP	7.54 ± 1.39	28.2 ± 3.1	0.32 ± 0.04	20.8 ± 2.8	4.94 ± 1.17	1188 ± 49*
S.-D. rats, 10 days	SAL	2.11 ± 0.25	53.4 ± 7.6	0.67 ± 0.03	44.6 ± 2.8	14.74 ± 1.83	1156 ± 110
	DFP	2.06 ± 0.39	46.3 ± 4.1	0.67 ± 0.03	42.6 ± 2.5	16.03 ± 1.34	854 ± 95*

L-[^{3}H]Nicotine, α-[^{125}I]BTX, and [^{3}H]QNB binding in cortex were assessed in DBA and C3H mice injected IP every other day for 2 or 4 weeks with either saline or 2.0 mg/kg DFP and in Sprague-Dawley rats injected SC every other day for 10 days with either saline or 0.2 mg/kg DFP. The whole particulate fraction of cortical tissue was incubated with six concentrations of either L-[^{3}H]nicotine for 90 min at 4°C, α-[^{125}I]BTX for 3 h at 37°C, or [^{3}H]QNB for 60 min at 37°C. K_d (L-[^{3}H]nicotine and α-[^{125}I]BTX, nM; [^{3}H]QNB, pM) and B_{max} (all ligands, fmol bound/mg protein) for each radioligand were calculated from Scatchard plots. Values listed represent the mean ± SEM of three to eight separate determinations.

*Significant differences ($p < 0.05$).

Figure 6 presents the responses to nicotine of 4-week control and DFP-treated DBA mice (2-week data not shown). ANOVA detected significant changes in the effect of nicotine on heart rate [2-week treatment, $F(1, 47) = 4.43$, $p < 0.05$, 4-week treatment, $F(1, 50) = 7.65$, $p < 0.01$]. C3H mice, in contrast, did not develop cross-tolerance to nicotine following 2 weeks of chronic DFP treatment (data not shown). Potential evidence for cross-tolerance was also obtained from the line comparison analyses (Table 3). Chronic DFP treatment failed to alter any of the effective dose values in the less sensitive C3H mice, but evidence of reduced sensitivity to nicotine was seen for each of the measures except the rotarod test in the DBA mice; the nicotine dose-response curves generated for DFP-treated DBA mice were parallel but not superimposable to those obtained in the control DBA mice for the Y-maze crossing and rearing, heart rate, and body temperature tests.

The data presented in Table 3 also demonstrate that control DBA mice were more sensitive to nicotine effects than were C3H mice. Significantly lower effective dose values were obtained in the control DBA mice, in comparison to control C3H mice, for Y-maze crossing and rearing activities, heart rate, and body temperature; the dose-response curves obtained from the two strains were not superimposable.

The responses of DBA mice to the peripherally acting, quaternary amine, nicotinic agonist dimethylphenylpiperazinium (DMPP), were also measured to determine whether the cross-tolerance to nicotine seen in DBA mice was centrally or peripherally mediated. Heart rate and body temperature responses to DMPP were measured in DBA mice that had been injected for 4 weeks with saline or DFP. Chronic DFP treatment did not alter sensitivity to an acute challenge of 1.5 mg/kg DMPP as measured by either heart rate or body temperature. DMPP injection decreased heart rate from 757 ± 10 to 593 ± 24 beats per minute in chronic saline-treated mice and from 769 ± 13 to 605 ± 20 beats per minute in chronic DFP-treated mice. DMPP injection decreased the body temperature from 37.9 ± 0.4 to 36.1 ± 0.3°C in chronic saline-treated mice and from 38.2 ± 0.2 to 36.5 ± 0.3°C in chronic DFP-treated mice.

TABLE 2

EFFECT OF CHRONIC DFP TREATMENT ON EFFECTIVE DOSES FOR DFP ACTIONS

Animal	Treatment	Rotarod	Y-Maze Crosses	Y-Maze Rears	Heart Rate	Body Temp
DBA mice	SAL	–	2.48 ± 0.75	1.58 ± 0.23	2.10 ± 0.60	2.05 ± 0.31
	DFP	–	2.22 ± 0.38	2.01 ± 0.29	1.71 ± 0.41	2.87 ± 0.96
C3H mice	SAL	5.05 ± 1.45	–	3.92 ± 0.92*	2.60 ± 0.75	3.16 ± 0.70
	DFP	–	–	4.77 ± 1.09	2.44 ± 0.57	3.15 ± 0.44

Dose-response curves for each of the components of the test battery were analyzed by a computerized regression-like comparison program and parameters reflecting the sensitivity of each mouse strain for each treatment group were calculated. The following effective doses were calculated: rotarod, Y-maze crosses and Y-maze rears, ED_{50}, [the dose (mg/kg) required to reduce rotarod performance or the number of crosses or rears to 50% of saline levels]; heart rate, ED_{-100} [the dose (mg/kg) required to reduce heart rate by 100 beats/min]; and body temperature, ED_{-2} [the dose (mg/kg) required to lower body temperature by 2°C]. Values are the mean ± SEM calculated from the dose-response curves. (–), slope of the dose-response curve was not significantly different from zero; no value was calculated.

*Indicates dose-response curves generated from chronic saline-treated DBA and C3H mice were parallel but not superimposable, $p < 0.05$.

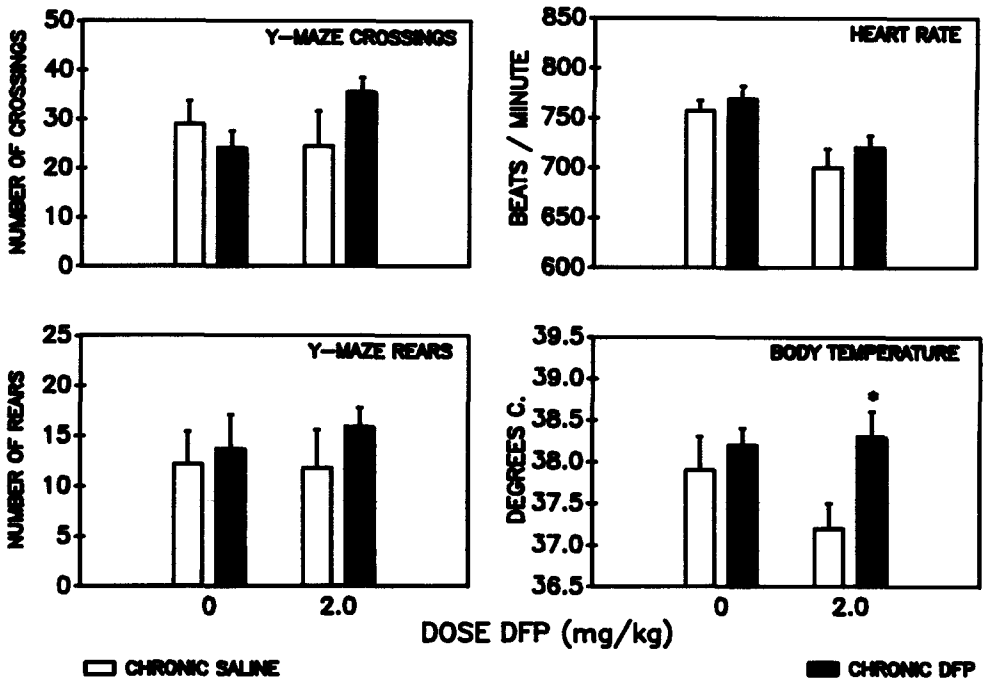

FIG. 5. Responses of DBA mice to DFP after 4 weeks of treatment. DFP sensitivity was assessed in DBA mice injected IP every other day for 4 weeks with either saline or 2.0 mg/kg DFP. Two days after the last injection, mice were tested for their response to DFP after acute IP administration of 0.0 or 2.0 mg/kg DFP. Each bar represents the mean ± SEM of six to eight animals. *Values differing significantly between chronic saline- and DFP-treated animals ($p < 0.05$).

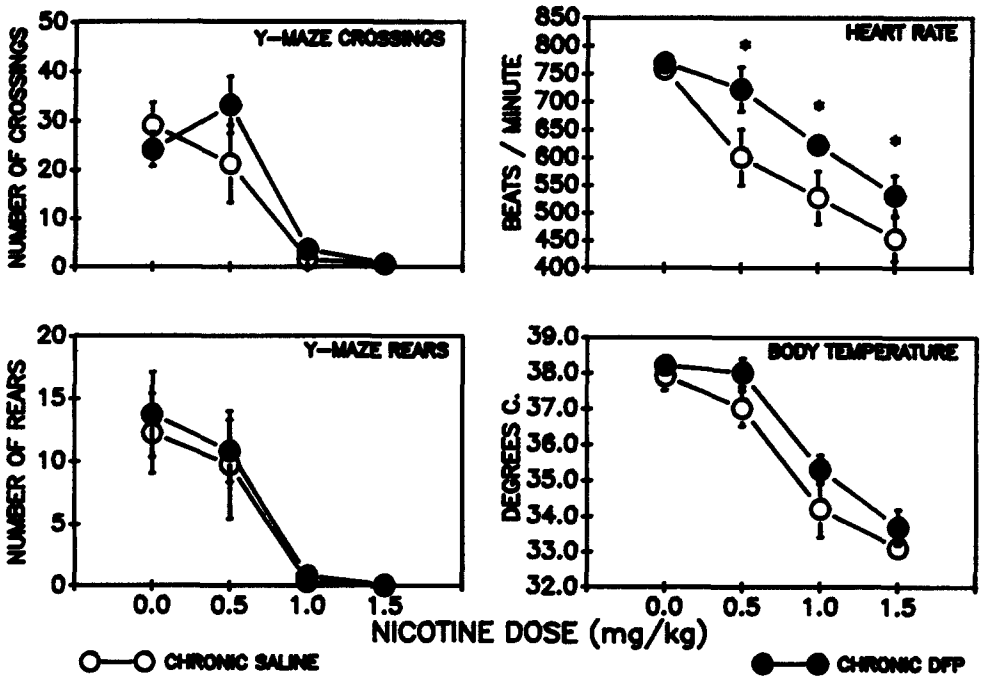

FIG. 6. Cross-tolerance of DBA mice to nicotine after 4 weeks of DFP treatment. Nicotine cross-tolerance was assessed in DBA mice injected IP every other day for 4 weeks with either saline or 2.0 mg/kg DFP. Two days after the last injection, mice were tested for their response to 0.0, 0.5, 1.0, or 1.5 mg/kg nicotine. The tests were run at the following times postinjection: Y-maze crossings and rears, 4 min; heart rate, 8 min; body temperature, 15 min. Each point represents the mean ± SEM of six to eight animals. *Values differing significantly between chronic saline- and DFP-treated animals ($p < 0.05$).

TABLE 3

EFFECT OF CHRONIC DFP TREATMENT ON EFFECTIVE DOSES FOR NICOTINE ACTIONS

Animal	Treatment	Rotarod	Y-Maze Crosses	Y-Maze Rears	Heart Rate	Body Temp
DBA mice	SAL	–	0.58 ± 0.08	0.49 ± 0.08	0.57 ± 0.07	0.76 ± 0.05
	DFP	–	0.90 ± 0.11*	0.80 ± 0.08*	0.80 ± 0.11†	1.06 ± 0.13*
C3H mice	SAL	–	1.41 ± 0.28‡	1.08 ± 0.16‡	1.38 ± 0.32§	1.45 ± 0.13§
	DFP	–	1.02 ± 0.10	0.90 ± 0.09	1.40 ± 0.30	1.28 ± 0.13

Dose-response curves for each of the components of the test battery were analyzed by a computerized regression-like comparison program and parameters reflecting the sensitivity of each mouse strain for each treatment group were calculated. The following calculations were made: rotarod, Y-maze crosses and Y-maze rears, ED_{50} [the dose (mg/kg) required to reduce rotarod performance or the number of crosses or rears to 50% of saline levels]; heart rate, ED_{-100} [the dose (mg/kg) required to reduce heart rate by 100 beats/min]; and body temperature, ED_{-2} [the dose (mg/kg) required to lower body temperature by 2°C]. Values are the mean ± SEM calculated from the dose-response curves. (–), slope of the dose-response curve was not significantly different from zero; no value was calculated.

*Dose-response curves generated from chronic saline- and DFP-treated mice were parallel but not superimposable.

†Dose-response curves generated from chronic DFP- and saline-treated mice were neither parallel nor superimposable.

‡Dose-response curves generated from chronic saline-treated DBA and C3H mice were parallel but not superimposable.

§Dose-response curves generated from chronic saline-treated DBA and C3H mice were neither parallel nor superimposable.

DISCUSSION

The studies reported here yielded several major findings. These include: 1) Chronic DFP treatment resulted in marked inhibition of brain AChE activity in both mice and rats, but the inhibition obtained in rats was greater even though smaller doses of DFP were given to the rat; 2) chronic DFP treatment failed to reduce [^{3}H]nicotine and α-[^{125}I]BTX binding in both mouse strains but decreases in [^{3}H]QNB binding were detected; 3) both [^{3}H]QNB and [^{3}H]nicotine binding were reduced by chronic DFP treatment in rats, but α-[^{125}I]BTX binding was not affected; 4) two weeks of chronic DFP treatment failed to elicit measurable tolerance to DFP in either mouse strain, but after 4 weeks of treatment DBA mice exhibited a modest reduction in sensitivity to the effects of DFP on body temperature; 5) inbred mouse strain differences in cross-tolerance to nicotine were detected with DBA mice exhibiting a modest reduction in sensitivity to nicotine's effects on several measures.

The observation that chronic DFP treatment failed to reduce the number of mouse brain [^{3}H]nicotine binding is surprising given that earlier studies using the rat (8,39) and CD-1 outbred mice (9) observed decreases in binding following chronic organophosphate treatment. However, it should be noted that a tendency toward reduced [^{3}H]nicotine binding was seen in striatum in both mouse strains after 2 weeks of DFP treatment, but this apparent difference was not statistically significant. A potential explanation for our failure to detect a change in radiolabeled nicotine binding may relate to the amount of AChE inhibition that was achieved. Even though mice were injected with higher doses of DFP and for a longer time period than were rats, brain AChE activity was affected less. Thus, more rigorous dosing with DFP may be necessary to elicit changes in nicotine binding in one or both of the inbred mouse strains we tested. Consistent with this notion, Costa and Murphy (9) found decreases in nicotine binding in a study that utilized CD-1 outbred mice. These mice were treated with 2 mg/kg DFP every day for 14 days. This dosing schedule is twice that used in our study. It does not seem likely that we will be able to resolve the dosing issue since C3H and DBA mice barely survived treatment with 2 mg/kg every other day, perhaps because both strains are highly inbred.

Neither mice nor rats exhibited a decrease in α-[^{125}I]BTX binding following chronic DFP treatment. As noted previously, several electrophysiological studies (3,14,30) noted that α-BTX does not block cholinergic transmission in the autonomic nervous system. The authors of these studies suggested, consequently, that the α-BTX binding site may not be functional. If true, this might explain why chronic DFP treatment did not evoke a change in this binding site in Sprague-Dawley rats or in either of the mouse strains tested.

Both mouse strains exhibited decreases in [^{3}H]QNB binding following chronic DFP treatment. Assuming the decrease in [^{3}H]QNB binding arose as a consequence of a classical agonist-induced downregulation of the muscarinic receptor, the decrease in [^{3}H]QNB binding provides reassurance that the treatment used resulted in increases in brain ACh levels. However, the observation that inhibition of AChE activity was not significantly correlated with decreases in [^{3}H]QNB binding argues that a direct relationship between enzyme inhibition and receptor regulation does not exist. Nonetheless, it may be that the C3H and DBA mice used in our study failed to exhibit decreases in [^{3}H]nicotine binding because synaptic AChE activity was not inhibited enough to elicit sustained increases in ACh concentrations. The failure to detect an effect of chronic DFP treatment on either [^{3}H]nicotine or α-[^{125}I]BTX binding using a treatment protocol that resulted in a decrease in [^{3}H]QNB binding might arise if AChE activity is affected more in those brain nuclei that express muscarinic receptors.

Chronic DFP-induced decreases in brain cholinergic receptors have been explained using an agonist-induced down-regulation model (18,39), but other alternatives must be considered, especially since two recent studies showed that continuous infusion with the reversible carbamate AChE in-

hibitor, physostigmine, elicited increases, rather than decreases, in [^{3}H]nicotine binding; physostigmine infusion did not alter [^{3}H]QNB binding in rat (13) or mouse (1) brain. The continuous infusion of mice with physostigmine (1) achieved inhibition of AChE comparable to that achieved by chronic DFP injections in the present study, yet different effects on receptor binding were achieved with these two drugs, which indicates that AChE inhibition is not necessarily the cause of receptor changes. One possible explanation for the differences obtained with reversible and irreversible AChE inhibitors is that organophosphates elicit neurotoxicity (28,32,36). Perhaps the reduction in receptor binding elicited by chronic DFP treatment arises as a consequence of toxic actions. At least in the mouse, it may be that neurons that possess [^{3}H]nicotine binding sites are less sensitive to these potential neurotoxic actions.

Chronic DFP treatment failed to elicit uniform tolerance to DFP in either mouse strain. This finding agrees with two earlier studies from our laboratory (7,44). Costa and Murphy (9) detected significant tolerance to the antinociceptive effects of DFP in outbred CD-1 mice, as well as cross-tolerance to oxotremorine- and nicotine-induced antinociception. Once again, it may be that we would have obtained evidence for tolerance to DFP if the inbred mice that were used could have survived a more vigorous DFP treatment such as that used by Costa and Murphy.

Our earlier studies to cross-tolerance to oxotremorine in chronic DFP-treated mice revealed that modest cross-tolerance developed in DBA, but not C3H, mice (7). Similar results were obtained in the present study in that only DBA mice showed any hint of cross-tolerance to nicotine. This strain did not, however, demonstrate any changes in nicotinic receptors. DBA mice develop tolerance to nicotine following intravenous infusion of nicotine, and the tolerance that develops is paralleled in a dose-dependent fashion by upregulation of the [^{3}H]nicotine binding site (22,26). Furthermore, the time courses of tolerance acquisition and loss parallel changes in this binding site (25). These findings argue that tolerance to nicotine may be explained by the upregulation of the [^{3}H]nicotine binding site. More recently, however, we (35) demonstrated that tolerance to nicotine can develop independently of receptor changes following chronic nicotine injection. The tolerance seen following chronic injection has many of the characteristics associated with what is referred to as "learned" tolerance and seems to be associated with preinjection elevations of plasma corticosterone, which allosterically inhibits binding to brain nicotinic receptors (35). It may be that the modest cross-tolerance to nicotine seen in chronic DFP-injected DBA is due to a variant of this learned tolerance.

In summary, the results reported here demonstrate that chronic DFP treatment will elicit decreases in [^{3}H]QNB binding in mouse brain at doses that failed to alter brain [^{3}H]nicotine and α-[^{125}I]BTX binding. A less rigorous DFP treatment protocol elicited decreases in rat brain [^{3}H]QNB and [^{3}H]nicotine binding. Differences in AChE inhibition may not account for the differences in effects of chronic treatment on brain cholinergic receptor numbers. Since chronic DFP treatment did not alter brain nicotinic receptors, the reduced sensitivity to nicotine seen only in DBA mice must be due to some factor other than receptor changes.

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