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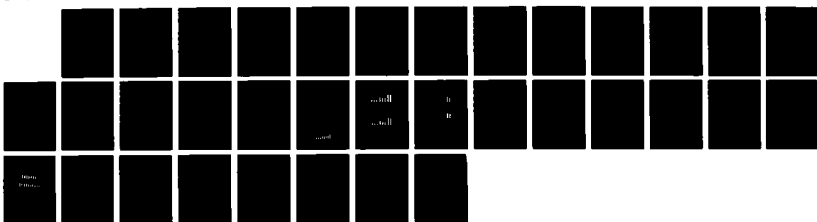
ORGANOPHOSPHATES: GENETICS RECEPTORS AND ANTIDOTES(U)
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FINAL PROGRESS REPORT

ORGANOPHOSPHATES: GENETICS RECEPTORS AND ANTIDOTES

(AFOSR 82-0300)

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Inbred mouse strains were found to differ in sensitivity to a number of behavioral and physiological effects elicited by DFP as well as in lethality. These differences were not easily explained in terms of differential inhibitors of acetylcholinesterase (AChE). Nicotine-induced seizures were studied as a model system for organophosphate-induced seizures. Nicotine-induced seizures seem to be regulated, in the mouse, by a limited (perhaps one) number of genes and these genes also seem to regulate the number of hippocampal nicotinic receptors. Acute studies with DFP indicated that brain AChE activity does not return to control levels in adult male mice but control levels are regained in reaggregate brain cultures and in DFP-treated mouse pups. Similarly, QNB binding did not return to control in striatum of DFP-treated mice. This, plus the absence of tolerance development, suggests that DFP may cause irreversible damage to mouse brain.					
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This report will summarize the progress made during the entire 3-year period of support from AFOSR 82-0300, but major emphasis will be paid to the work completed during the last year of funding. At the time of writing this report, five papers have appeared in the scientific literature that cite AFOSR 82-0300 for total or partial support. These are:

Miner, L.L., Marks, M.J. and Collins, A.C. Classical genetic analysis of nicotine-induced seizures and nicotinic receptors. Journal of Pharmacology and Experimental Therapeutics, 1984, 231: 545-554.

Smolen, A., Smolen, T.N., Wehner, J.M. and Collins, A.C. Genetically determined differences in acute responses to diisopropylfluorophosphate. Pharmacology Biochemistry and Behavior, 1985, 22: 623-630.

Miner, L.L., Marks, M.J. and Collins, A.C. Relationship between nicotine-induced seizures and hippocampal nicotinic receptors. Life Sciences 1985, 37: 75-83.

Wehner, J.M., Smolen, A., Smolen, T.N. and Murphy, C. Recovery of acetylcholinesterase activity after acute organophosphate treatment of CNS reaggregate cultures. Toxicology and Applied Pharmacology 1985, 5: 1104-1109.

Smolen, A., Smolen, T.N., Oh, E.I. and Collins, A.C. A strain comparison of physiological and locomotor responses of mice to DFP. Pharmacology Biochemistry and Behavior, 1986, 24: 1077-1082.

An additional five manuscripts have been submitted for possible publication, and two of these have already been accepted for publication. Two additional manuscripts are in preparation. These manuscripts are:

Miner, L.L., Marks, M.J. and Collins, A.C. Genetic analysis of nicotine-induced seizures and hippocampal nicotinic receptors in the mouse. Journal of Pharmacology and Experimental Therapeutics, in press.

Smolen, T.N., Smolen, A. and Collins, A.C. Dissociation of decreased numbers of muscarinic receptors from tolerance to DFP. Pharmacology Biochemistry and Behavior, accepted for publication.

Smolen, A., Smolen, T.N., Han, P.C. and Collins, A.C. Sex differences in the recovery of brain acetylcholinesterase activity following a single exposure to DFP. Neurobehavioral Toxicology, submitted.

Collins, A.C., Smolen, T.N. and Smolen, A. Cross-tolerance between muscarinic agonists: role of muscarinic receptors. Pharmacology Biochemistry and Behavior, submitted.

Murphy-Erdosh, C. and Wehner, J.M. The effects of acute and chronic DFP treatment on the cholinergic system of young mice. Pharmacology Biochemistry and Behavior, submitted.

Mitchell, D., Smolen, A. and Collins, A.C. Time course of recovery of brain muscarinic and nicotinic receptors following chronic DFP treatment. In preparation.

Smolen, A., Medhurst, L.J., Smolen, T.N. and Collins, A.C. Differences among inbred mouse strains in sensitivity to lethal effects arising from chronic DFP treatment. In preparation.

In addition, two abstracts have been published that describe work that was supported by AFOSR 82-0300. These are:

Wehner, J.M., Smolen, A. and Smolen, T.N. Inhibition of acetylcholinesterase (AChE) by diisopropylflourophosphate (DFP) in mouse CNS reaggregate cultures. Federation Proceedings 1984, 43: 349.

Collins, A.C., Smolen, T.N. and Smolen, A. Chronic DFP reduces brain QNB binding but elicits minimal tolerance. The Pharmacologist 1985, 27:171.

This summary progress report will highlight the findings that will be found in greater detail in these manuscripts, and we will discuss the results of other experiments that met with more limited success; i.e., questions were answered, but the results are largely negative and, therefore, will not likely be viewed as being publishable. Some of these "negative" findings can be made "positive" with additional studies, and we intend to do some of the necessary studies as time and resources allow.

The overall goals of our research effort changed somewhat as our investigations evolved. Concentrated efforts were made in several different areas:

- 1) The genetic regulation of nicotine-induced seizures and the role of brain nicotinic receptors in the regulation of seizure sensitivity.
- 2) The genetic regulation of acute responses/sensitivity to DFP.
- 3) The recovery of brain acetylcholinesterase (AChE) activity following treatment with DFP: studies of potential neurotoxicity.
- 4) The effects of chronic DFP treatment on brain cholinergic (muscarinic and nicotinic) receptors, and the role of these receptor changes in tolerance to the actions of DFP.
- 5) The synthesis of various nicotine derivatives and the testing of these agents as potential blockers of brain nicotinic receptors.

Progress made in each of these areas will be described, in turn.

Nicotine-induced seizures.

This project had a number of positive results, not the least of which is that these studies served as the basis for L.L. Miner's Ph.D. thesis. Dr. Miner successfully defended her thesis on 7-25-86.

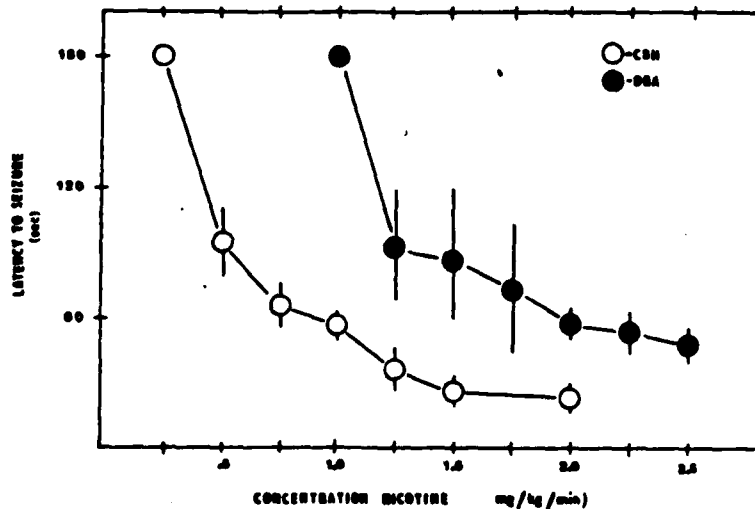
The rationale behind these studies relates to the fact that organophosphate AChE inhibitors induce convulsions under some circumstances. Since muscarinic agonists, such as oxotremorine, are very poor inducers of seizures whereas nicotine is a potent inducer of such seizures, it may be

organophosphates elicit seizures because of excess stimulation of brain nicotinic receptors. This rationale led to our investigation of nicotine-induced seizures and the genetic regulation of such seizures. The results indicate that nicotine-induced seizures may be regulated by a single gene and that seizure sensitivity may be regulated by the number of nicotinic receptors found in the hippocampus. We have demonstrated that mouse brain has two classes of nicotinic receptors that may be measured with L-³H)-nicotine or with (¹²⁵I)-bungarotoxin (BTX). Nicotine-induced seizures appear to be regulated by the number of hippocampal BTX binding sites.

In our published studies (Miner et al., 1984, 1985) we have demonstrated that, following the ip injection of nicotine, mice of the C3H strain are more sensitive to nicotine-induced seizures than are mice of the DBA strain. This assessment was made by constructing dose-response curves for nicotine-induced seizures in these two strains. A classical genetic cross analysis, which consists of generating F1, F2, and backcross (F1-x-C3H and F1-x-DBA) generations, revealed that seizures are regulated in such a way that a single gene could be responsible for their control. Furthermore, seizure sensitivity seemed to co-segregate with the number of hippocampal BTX binding sites.

These studies had a methodological problem. Seizure sensitivity could not be assessed for each individual animal following the ip injection of a single dose (the animal either did, or did not, convulse) whereas the BTX binding in each animal could be. Thus, our correlational analyses involved a comparison of parametric and nonparametric statistics. This problem could be overcome by using iv administration techniques. C3H and DBA mice were infused with nicotine until a seizure developed. Latency to seizure (time between initiation of infusion and the development of a seizure) should be a measure of the nicotine dose required to elicit a seizure in each animal. Figure 1 presents the results of an experiment where C3H and DBA mice were infused with nicotine at varying rates and time to seizure determined. Consistent with our ip experiments, C3H mice were more sensitive to nicotine-induced seizures. This difference was maintained at all rates of nicotine infusion.

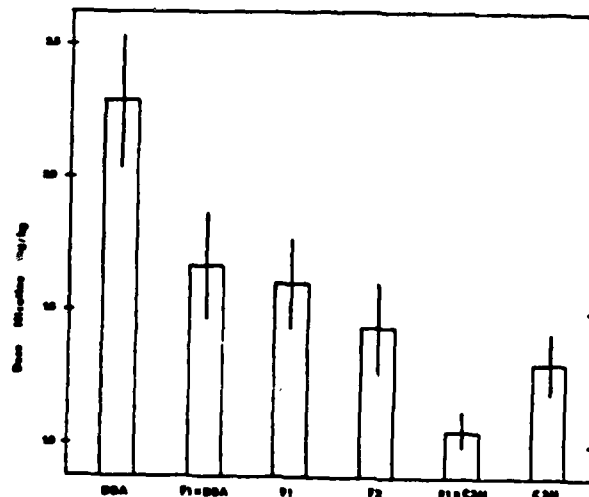
FIGURE 1



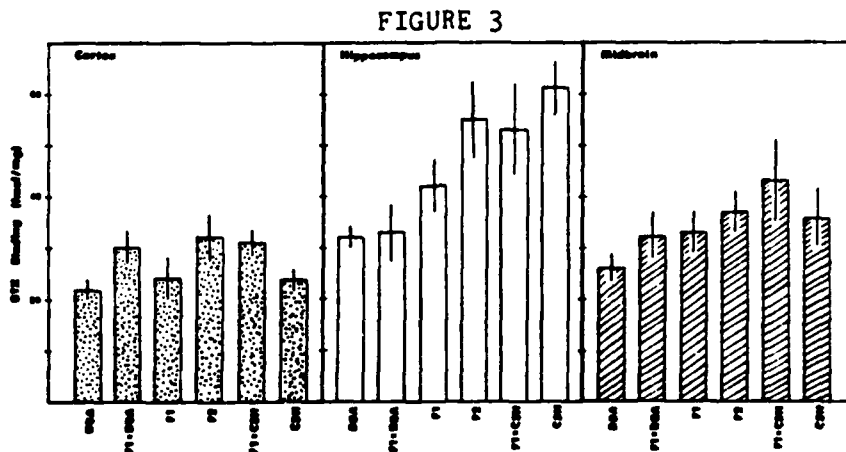
In addition to this quantitative difference in sensitivity, we generally observed that C3H mice developed full-blown seizures, even though the infusion was stopped as soon as a myoclonic jerk was seen, and these seizures usually ended in the animal's death. With DBA mice, seizures were quickly terminated and all of the animals survived. Thus, we observed differences in quantitative (dose required to elicit a seizure) and qualitative (severity and consequences) aspects of nicotine-induced seizures.

Figure 2 presents the results of the study of seizure sensitivity, following the iv infusion of 2 mg/kg/min nicotine, in C3H, DBA, F1, F2, and backcross animals. Seizure sensitivity segregated in a fashion that is consistent with nicotine sensitivity being regulated by a single gene with two alleles that are co-dominant (a result that is different than that seen following ip nicotine administration where dominance was seen) or by a small number of genes with additive effects.

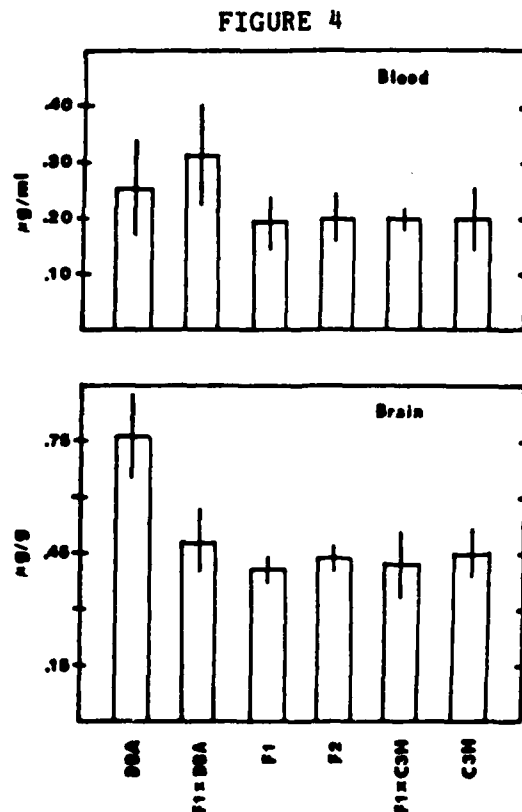
FIGURE 2



BTX binding was measured in three brain regions in these mice. Figure 3 presents the results of these measurements for cortex, hippocampus and midbrain. It should be obvious from a comparison of figures 2 and 3 that nicotine-induced seizure sensitivity correlates best with hippocampal BTX binding. We have also measured L-(³H)-nicotine binding in these animals. No differences were seen.

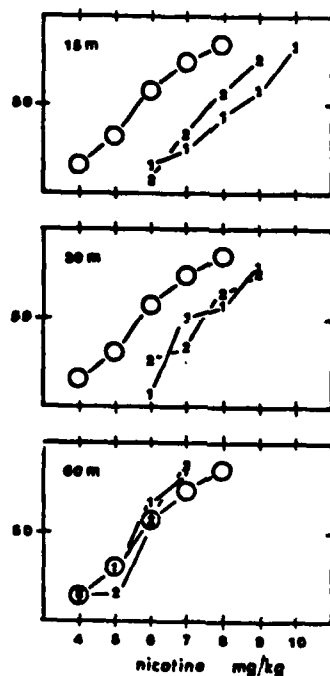


Differences in seizure sensitivity could be due to differences in the metabolism or distribution of nicotine. Therefore, we measured radiolabelled nicotine in the blood and brain of another group of animals at the time of clonic seizures. The results of these experiments are reported in Figure 4. While differences were found, they did not correlate with seizure sensitivity.

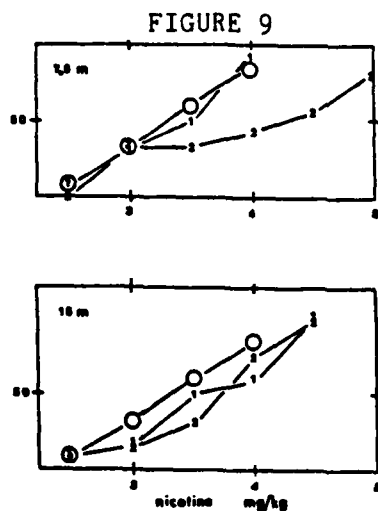


leading to depolarization and the generation of an action potential. Subsequently, the receptor undergoes a second conformational change. This new receptor form binds the agonist with very high affinity, but the receptor's ion channel is no longer functional. This process has been well described for the nicotinic receptor from *Torpedo californica* and mammalian skeletal muscle, but we do not know if this occurs for mouse brain nicotinic receptors. Furthermore, we have no idea as to the time scale of any desensitization process. Therefore, we attempted a simple experiment with C3H and DBA mice. Animals were pretreated with saline (controls) or nicotine (1 or 2 mg/kg; these are subseizure doses of nicotine) 7.5, 15, 30 or 60 min before challenge with a potential seizure-inducing dose of nicotine. Challenge doses were selected so that dose-response curves could be generated. Figure 8 presents the results of these experiments for DBA mice, and Figure 9 presents the results for C3H mice. As can be seen in Figure 8, DBA mice exhibit a profound shift to the right of the dose-response curves following nicotine pretreatment. This behavioral desensitization disappears over the time period between 15 and 60 min.

FIGURE 8



C3H mice exhibit little or no behavioral desensitization as can be seen from Figure 9. The effect seen in the C3H mice pretreated with 2 mg/kg nicotine at the 7.5 min time period may be due to a phenomenon resembling postictal depression (data not shown).



These results suggest that another factor, other than differences in the number of brain nicotinic receptors, that may influence seizure sensitivity is the ability of these receptors to desensitize. Such a phenomenon could influence the results of the studies being carried out under Dr. Wehner's AFOSR-supported research project. Therefore, studies are in progress to explore further the genetic regulation of nicotinic receptor desensitization.

Genetics of acute sensitivity

The results of these studies have been described in detail in two of our published manuscripts (Smolen et al., 1985, 1986) and were described in detail in earlier progress reports. Therefore, these results will not be discussed in detail here. Our primary observation has been that mouse strains differ in sensitivity to an acute challenge dose of DFP. This is true for a number of responses including lethality, body temperature, heart rate, locomotor activity (Y-maze crosses and rears), respiration rate, and rotarod performance. The relative strain sensitivities varied depending upon the test, and these differences could not be readily explained by differences in inhibition of brain AChE activity.

Recovery of brain AChE activity after acute DFP treatment

One of the major goals of our research efforts was to assess the effects of chronic DFP treatment on brain muscarinic and nicotinic receptors and to correlate any changes in these cholinergic receptors with tolerance to organophosphates. In order to do such studies, it was decided that we should have some knowledge of the time course of AChE inhibition following treatment with a single dose of DFP. Therefore, male and female DBA, C57BL, and C3H mice were injected with a single dose of DFP (6.33 mg/kg) and whole brain AChE activity was measured at varying time periods out to 40 days post injection. The results of this experiment are presented in Figure 10. Interestingly, control levels were not regained in male DBA and C57BL mice.

We speculate that this may be an indication of some irreversible effect of DFP; perhaps a single dose causes some damage that is irreversible or slowly reversible.

FIGURE 10

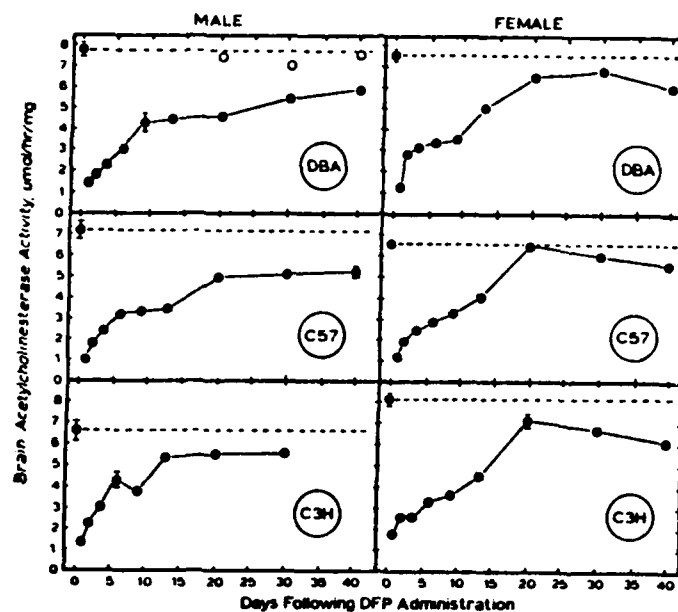


Table 1 presents the results of a subsequent study in DBA male mice where AChE activities were measured in seven brain regions 1, 13, and 30 days after treatment with a single (6.33 mg/kg) dose of DFP. Control values were not regained in cortex, midbrain, hippocampus, and hypothalamus at the 30 day time point. Therefore, brain regions differ in their ability to recover from an acute DFP challenge.

TABLE I

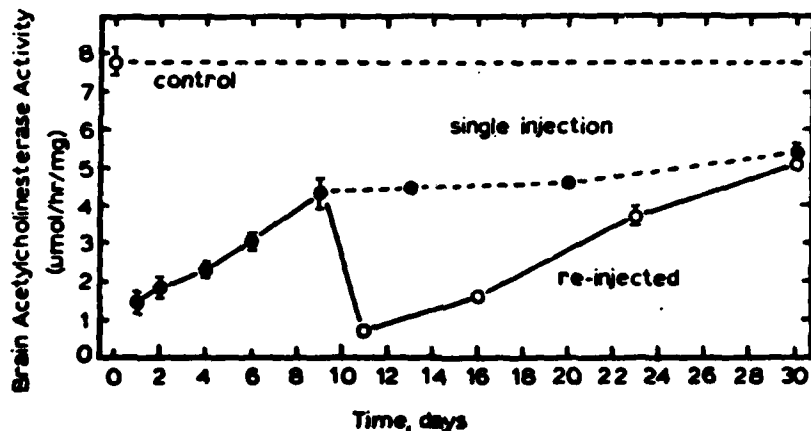
Regional Distribution of Acetylcholinesterase Activity in Brain Following
a Single Injection of Diisopropylfluorophosphate

DAY	Acetylcholinesterase Activity (umols/hr/mg protein)					
	C	M	H	P	S	T
CONTROL	7.55 ± .29	8.7 ± 1.03	5.78 ± .29	4.53 ± .18	15.20 ± 1.03	5.25 ± .52
1	0.94 ± .07*	1.42 ± .16*	1.52 ± .23*	.84 ± .07*	1.87 ± .29*	1.53 ± .15*
% Control	12.4	16.3	26.3	18.5	12.3	29.1
13	4.74 ± .23*	3.97 ± .14*	4.49 ± .40*	2.88 ± .09*	10.86 ± 1.24*	3.67 ± .26*
% Control	62.8	45.6	77.7	63.5	71.4	69.9
30	5.66 ± .47*	6.14 ± .57*	6.00 ± .97	4.06 ± .29*	14.38 ± 1.67	3.97 ± .23*
% Control	74.9	70.5	103.8	89.6	94.6	75.6

Male DBA mice were injected with DFP (6.33 mg/kg). At the times indicated AChE activity was measured in cortex (C), midbrain (M), hindbrain (H), hippocampus (P), striatum (S), and hypothalamus (T). The listed values represent the mean ± the standard error of the mean of 5-10 separate determinations. An * indicates significant differences from the saline-injected control group, $p < .05$.

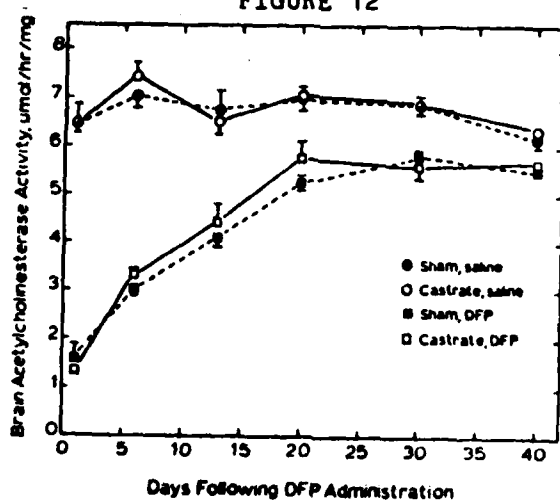
Figure 11 presents the results of an experiment where DBA male mice were injected with a second 6.33 mg/kg DFP dose 10 days after receiving a first injection and recovery of AChE activity measured. As can be seen from this figure, twice-injected animals recovered AChE activity to the same level as did once-injected animals. Thus, if neurotoxicity is the explanation for the failure to recover control levels of enzyme activity this neurotoxicity develops quickly, and perhaps maximally, following a single dose of DFP.

FIGURE 11



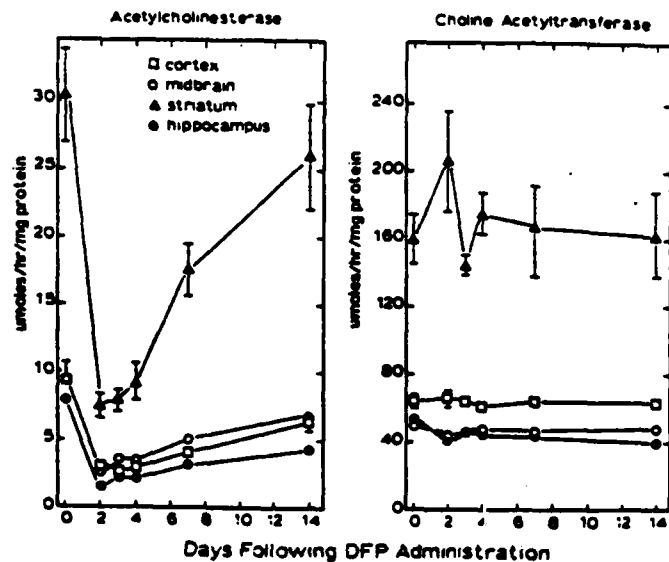
We observed a sex difference in recovery of AChE activity, following DFP, in DBA and C57BL mice. One such explanation could be an effect of sex hormones on DFP action or metabolism (neurotoxicity, if it explains the lack of recovery, could be due to a DFP metabolite). Therefore, a series of experiments was carried out where male DBA mice were castrated or sham operated, treated with saline or DFP, and the recovery of AChE activity measured. The results of this study are presented in Figure 12. No differences were observed between castrated and sham operated controls in recovery of AChE activity. Therefore, the mere presence of male sex hormones is not necessary to affect the recovery of AChE activity following DFP treatment.

FIGURE 12



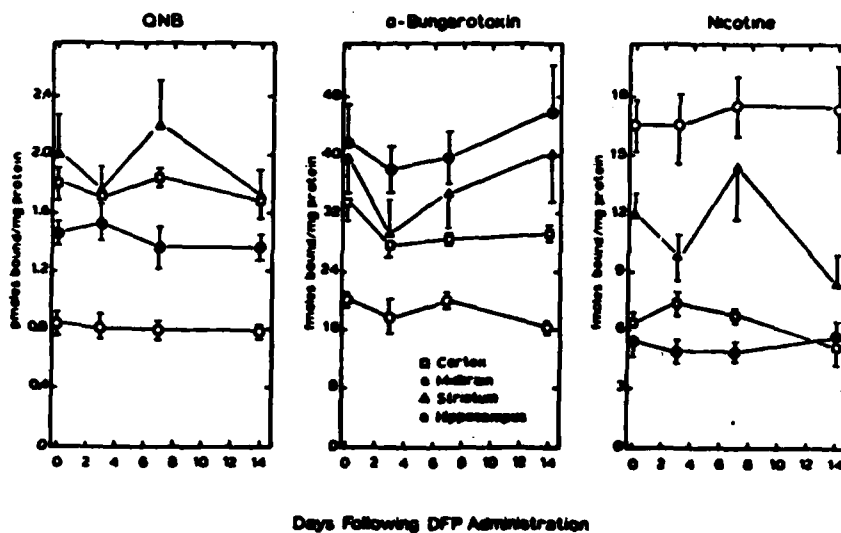
If neurotoxic actions are induced by a single dose of DFP, it seemed possible that other markers of cholinergic systems might be affected. Therefore, we measured the effects of a single (6.33 mg/kg) DFP dose on AChE and choline acetyltransferase activities in four brain regions of male DBA mice. The four regions studied were cortex, midbrain, striatum, and hippocampus. The results of these studies are presented in Figure 13. DFP affected, as expected, AChE activity which recovered partially during the 14 day observation period. Choline acetyltransferase activity was not affected by a single dose of DFP.

FIGURE 13



A similar study was carried out in which the effects of a single (6.33 mg/kg) DFP dose on the binding of quinuclidinyl benzilate (QNB, a ligand that measures brain muscarinic, cholinergic receptors), L-nicotine and BTX in cortex, midbrain, striatum, and hippocampus were measured. These results are presented in Figure 14. No significant effects were seen. Therefore, if neurotoxicity does develop following a single dose of DFP, this action is not detected by changes in any other cholinergic markers. Obviously, further experiments are required to provide an explanation for the failure to recover control levels of AChE activity in specific brain regions following a single dose of DFP.

FIGURE 14

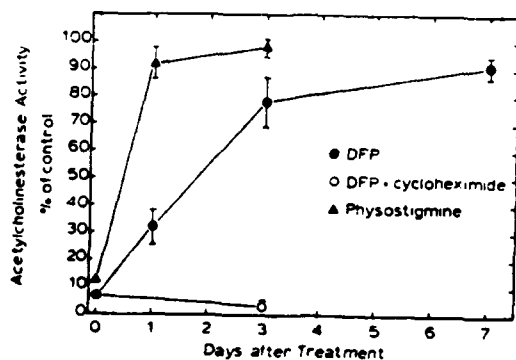


The failure to regain control levels of AChE activity following treatment with DFP might occur because of a neurotoxic action of DFP or a metabolite. The "active metabolite" hypothesis was tested by carrying out a collaborative study with Dr. Jeanne Wehner. Jeanne has used reaggregate cultures in her work for a number of years. Reaggregate cultures are produced by treating fetal mouse brain with trypsin to dissociate the cells and placing the cells in tissue culture flasks containing a suitable nutritive medium, generally Dulbecco's modified Eagle's medium containing 15% horse serum. The cells reaggregate into spheres in culture and serve as an ideal system to test hypotheses regarding toxic actions of drugs and chemicals.

We used reaggregate cultures prepared from fetal DBA brain. These cultures were treated with DFP (0.6 mg/L), or with the carbamate AChE inhibitor, physostigmine (0.5 mg/ml), or with cycloheximide plus DFP. The return of AChE activity was measured over the next 7 days. The results of this experiment are reported in Figure 15. Control levels of AChE activity were regained following physostigmine treatment within the first day or two whereas 7 days were required for the DFP-treated cultures. Cycloheximide treatment blocked the return of AChE activity in DFP-treated reagggregates indicating that protein synthesis is responsible for the regeneration of enzyme activity. These results are consistent with the suggestion that a metabolite of DFP may be responsible for the failure to regain control

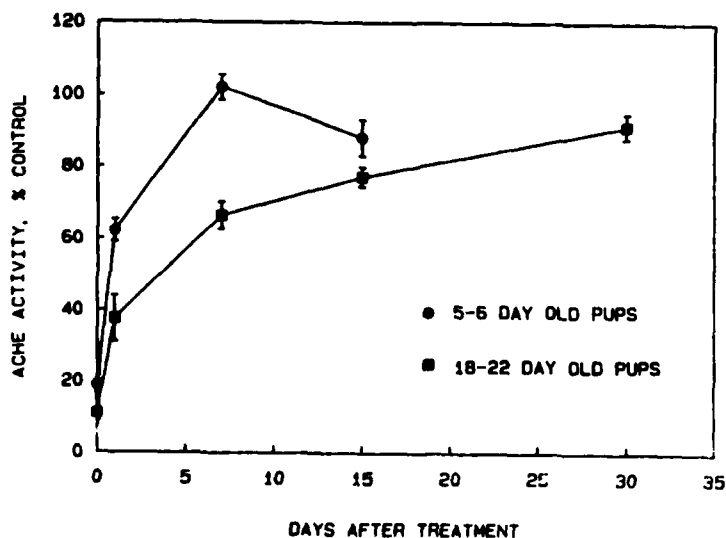
levels of AChE activity in adult, male DBA mice since reaggregate cultures presumably do not metabolize DFP and control levels of AChE activity were regained in these cultures. Alternatively, it may be that brain tissue obtained from immature animals is not affected by any potential neurotoxic actions of DFP.

FIGURE 15



In order to test the idea that age affects the ability of mice to regenerate control levels of AChE activity following a single dose of DFP Dr. Wehner injected 5-6 day old mouse pups with 3 mg/kg DFP and 18-22 day old pups with 8 mg/kg DFP. As can be seen in Figure 16, the 5-6 day old pups regenerated AChE activity much more rapidly than did the 18-22 day old pups. The 5-6 day old pups had control levels of AChE activity by 7 days after treatment, but the older animals still exhibited a modest, but statistically significant, depression of AChE activity 30 days after injection. These results suggest that immature mice are not sensitive to any neurotoxic actions of DFP. One possible explanation is that very young animals have not developed the capacity to form a neurotoxic metabolite of DFP.

FIGURE 16



In summary, we have demonstrated that sex, strain, brain region and age influence the ability of mouse brain to regain control levels of AChE activity following exposure to a single dose of DFP. The fact that other

cholinergic markers are not affected by a single dose of DFP suggests that if neurotoxicity is the cause of the failure to regain control levels of enzyme activity, this neurotoxic action is subtle.

Chronic treatment studies

Chronic treatment with psychoactive agents commonly leads to tolerance to the effects elicited by these agents. In the case of organophosphates, a number of studies have demonstrated that chronic treatment leads to a subtle tolerance and to changes in brain cholinergic receptors. Decreases in brain muscarinic receptors, as measured by QNB binding, and decreases in L-(³H)-nicotine binding have been described. These changes in brain cholinergic receptors, it has been suggested, are responsible for the tolerance that has been seen. The goals of our chronic treatment studies included attempting to test the hypothesis that changes in brain cholinergic receptors explain tolerance to DFP. In order to test this hypothesis thoroughly we designed protocols that we hoped would change brain cholinergic receptors to different degrees. We reasoned that if tolerance to DFP is due to changes in brain cholinergic receptors, greater tolerance should be seen with greater changes in receptors.

In the first series of experiments, male DBA mice were treated chronically with DFP or saline (controls) using one of three protocols: 4 mg/kg every 4 days for 1 month (the 4-day group); 2 mg/kg every other day for 1 month (the 2-day group); and 4 mg/kg on the first day followed by 1 mg/kg daily for a total of 14 days (the 1-day group). After chronic treatment was completed, the response of the animals to a challenge dose (4 mg/kg) of DFP was measured. In addition, any potential cross-tolerance to the muscarinic agonist, oxotremorine (0.1 mg/kg), was assessed as was the response to a saline challenge. Responses were measured using a standard battery of behavioral and physiological tests: respiration rate, heart rate, body temperature, Y-maze activity (crosses), and Y-maze rears. Figure 17 presents the results for the respiration test. In Figures 17-21 horizontal bars will be found in each panel. These bars represent the mean response (\pm the S.E.M.) of naive animals to a 4 mg/kg dose of DFP, a 0.1 mg/kg dose of oxotremorine, or saline, respectively. In addition, for all of these figures, solid bars represent the results obtained with chronic DFP-treated animals and open bars are the appropriate chronic saline-treated controls. As can be seen in Figure 17, both DFP and oxotremorine depress respiration. Chronic DFP-treated animals were not tolerant to the respiratory depressant effects of DFP. The 4-day group exhibited a modest cross-tolerance to the effects of a challenge dose of oxotremorine. This effect was not seen in any other group.

FIGURE 17

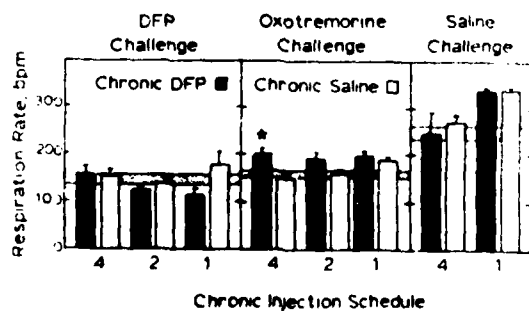


Figure 18 presents the results for the heart rate test. Chronic DFP-treated animals were supersensitive to the heart rate lowering effects of DFP, but were cross-tolerant to the depression in heart rate elicited by oxotremorine. This cross-tolerance was greatest in the 4-day group and least in the 1-day group.

FIGURE 18

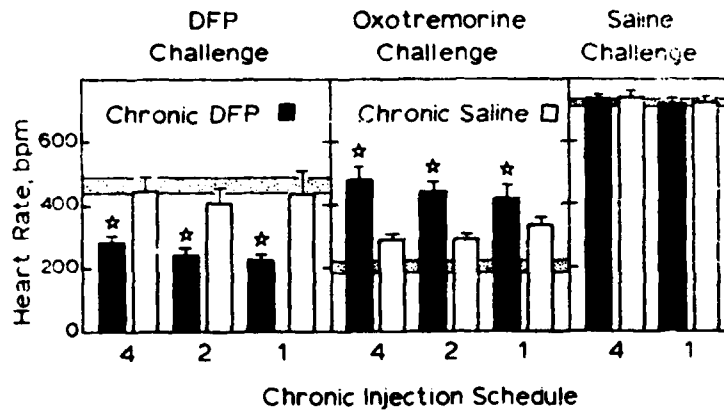
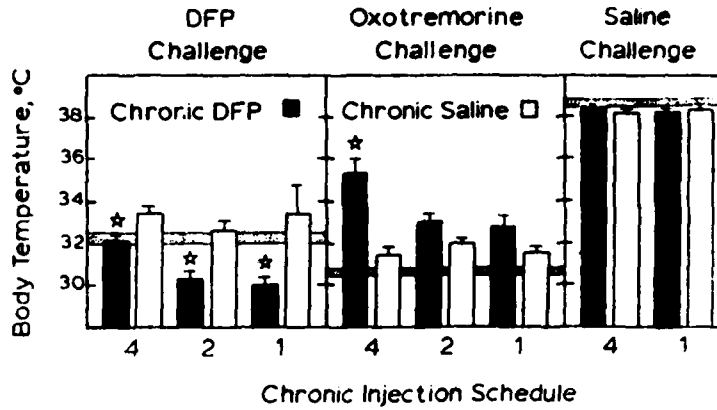


Figure 19 presents the results obtained for the body temperature test. Both DFP and oxotremorine challenge decrease body temperature. AS was the case for the heart rate test, chronic DFP-treated animals from all groups were supersensitive to the hypothermia-producing effects of DFP, and the 4-day group was cross-tolerant to oxotremorine.

FIGURE 19



Figures 20 and 21 present the results for the two Y-maze tests. Both DFP and oxotremorine challenge result in decreases in Y-maze crosses and rears. Chronic DFP-treated are no different from naive animals in their responses to DFP, oxotremorine or saline; i.e., no tolerance, cross-tolerance or supersensitivity developed.

FIGURE 20

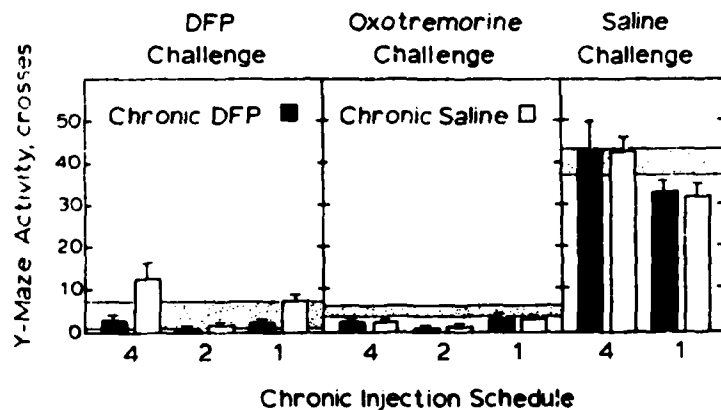
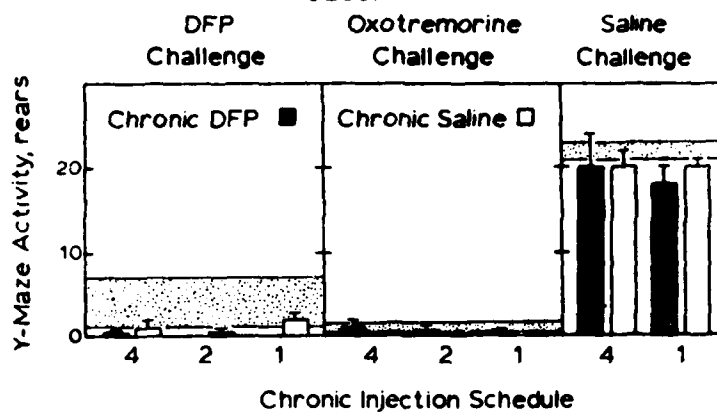


FIGURE 21



We also measured AChE activity and QNB binding in six brain regions from the chronically treated animals. The degree of AChE inhibition was similar in the three DFP-treated groups (data not shown). QNB binding was changed in several brain regions. These changes represent changes in maximal binding in that no changes in K_d were found (data not shown).

Figure 22 presents the effects of chronic DFP treatment on QNB binding in six brain regions (striatum, ST; cortex, CX; hippocampus, HC; midbrain, MB; hindbrain, HB; and hypothalamus, HT) in the 4-day treatment group. None of the apparent reductions in QNB binding were statistically significant.

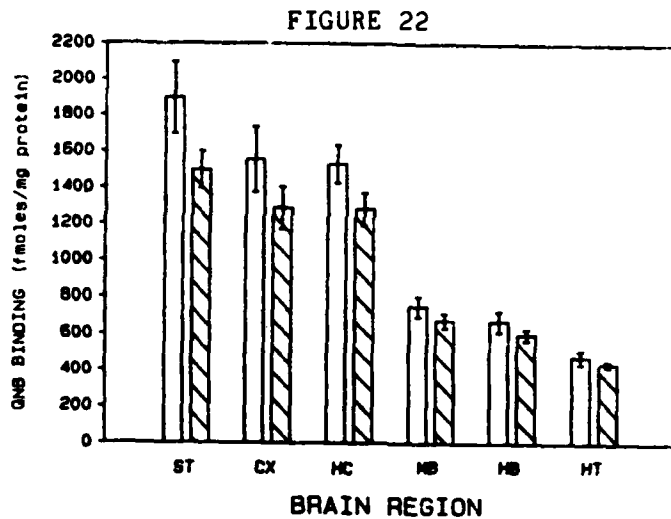


Figure 23 presents the results of DFP treatment on QNB binding in the 2-day treatment group. Statistically significant reductions in binding were seen in striatum, cortex, hippocampus, and hindbrain.

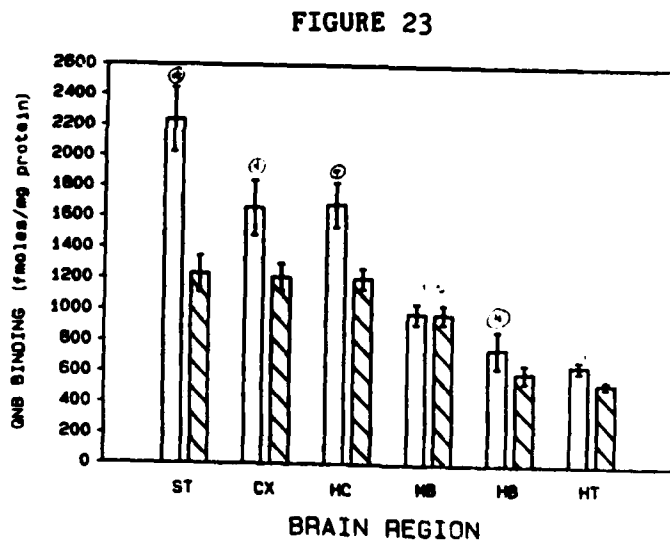
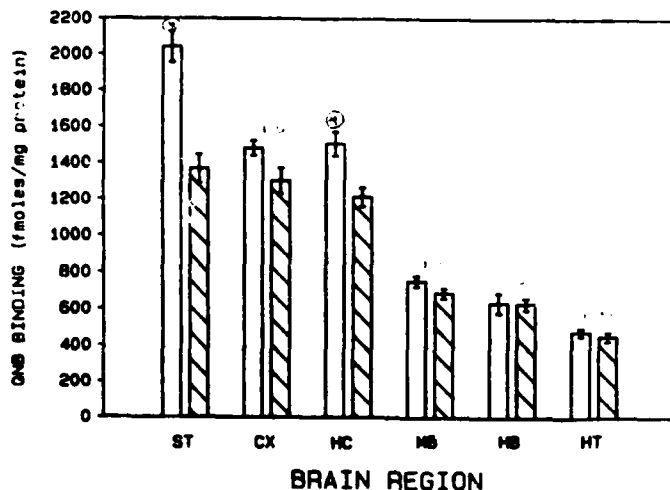


Figure 24 presents the results for the 1-day treatment group. Statistically significant reductions were detected in striatum and hippocampus.

FIGURE 24



In summary, we failed to detect significant tolerance to DFP in virtually every test, yet reductions in brain QNB binding were seen. These results suggest that the reductions in QNB binding are not necessarily the cause of tolerance. Other explanations for their meaning must be sought.

Several years ago we published three papers that demonstrated chronic infusion with oxotremorine resulted in tolerance to the actions of oxotremorine (hypothermia and rotarod impairment were measured), and in reductions in QNB binding. The tolerance observed was very large, 35-80 fold, and a substantial tolerance to oxotremorine could be detected before measurable changes in QNB binding were seen. In view of the ambiguity of the results obtained with chronic DFP treatment we carried out an experiment where DBA and C3H mice were chronically infused with oxotremorine or chronically injected with DFP. The mice were infused with oxotremorine at a rate of 0.5 mg/kg/hr for 8 days. Saline-infused animals served as the controls. DFP-treated animals were injected with 4 mg/kg every 5 days for 1 month. These protocols were developed because preliminary studies suggested that they affected similar reductions in QNB binding. After drug treatment was complete the animals were tested for tolerance and cross-tolerance.

Figure 25 presents the effects of chronic oxotremorine infusion and DFP treatment on the respiration rate of C3H and DBA mice. Figures 25-29 are arranged identically. The upper two panels present the results obtained in DBA (upper left-hand panel) and C3H (upper right-hand panel) mice that had been chronically injected with DFP or saline. The lower two panels present the results obtained with DBA (lower left-hand panel) and C3H (lower right-hand panel) that had been chronically infused with oxotremorine or saline. Animals were challenged with 0.1 mg/kg oxotremorine (solid bars), with saline the day after that (open bars), and with 4 mg/kg DFP the day after that (hatched bars). The animals were challenged 5 days after their last DFP injection and the day after oxotremorine infusion was terminated. These times were chosen because we wanted to make certain that no residual drug, or direct drug effect, would be present to confound the tolerance tests.

Consistent with our earlier study, DBA mice that had been chronically injected with DFP were not tolerant to DFP's respiratory depressant effects nor were they cross-tolerant to the actions of oxotremorine. Chronic DFP-treated C3H mice were tolerant to the respiratory depressant effects of DFP. Chronic oxotremorine-infused DBA and C3H mice were tolerant to the respiratory depressant effects of oxotremorine and they were cross-tolerant to DFP.

FIGURE 25

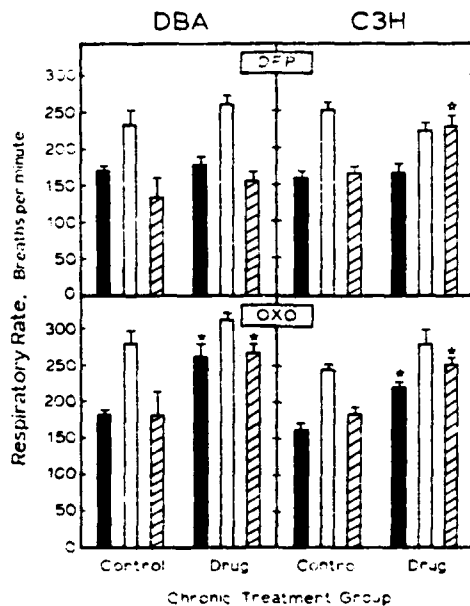


Figure 26 presents the results from the heart rate test. Chronic DFP-treated DBA mice were cross-tolerant to oxotremorine's cardiodepressant effects, and both tolerance to oxotremorine and cross-tolerance to DFP was seen in oxotremorine-infused DBA and C3H mice.

FIGURE 26

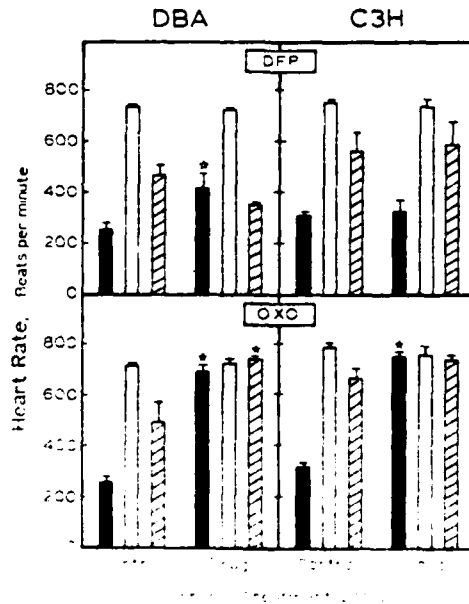


Figure 27 presents the results for the body temperature test. The results were virtually identical to that of the heart rate test. Only DBA mice treated chronically with DFP exhibited an altered drug response. These animals were cross-tolerant to oxotremorine, but they were not tolerant to DFP. Chronic oxotremorine infusion resulted in tolerance to oxotremorine and cross-tolerance to DFP in both mouse strains.

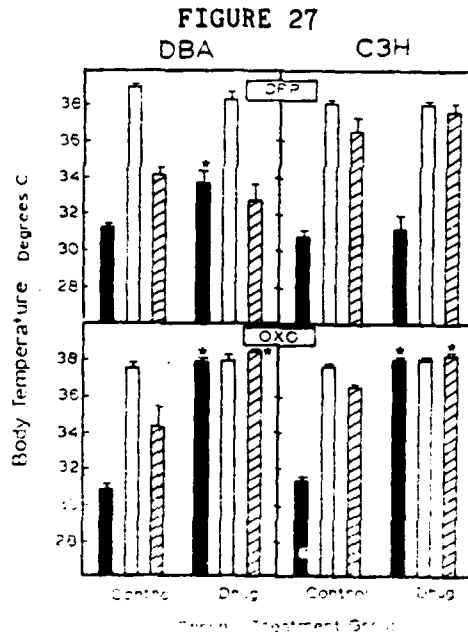


Figure 28 presents the results for the Y-maze crosses test. DFP treatment did not result in tolerance to DFP or cross-tolerance to oxotremorine in either mouse strain whereas oxotremorine-infused mice displayed tolerance to oxotremorine and cross-tolerance to DFP. Virtually identical results were obtained for the Y-maze rears test (Figure 29).

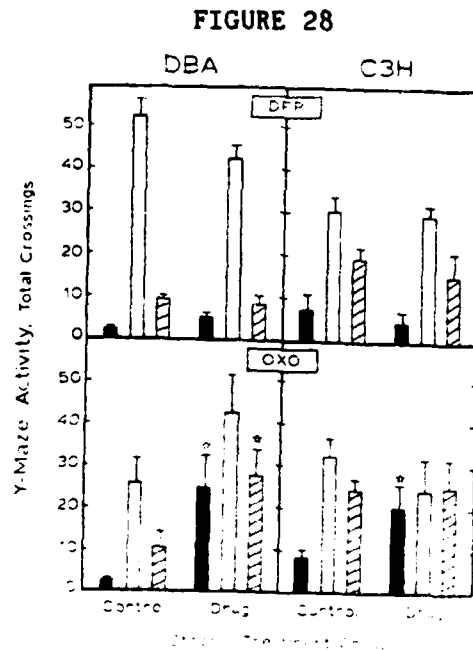
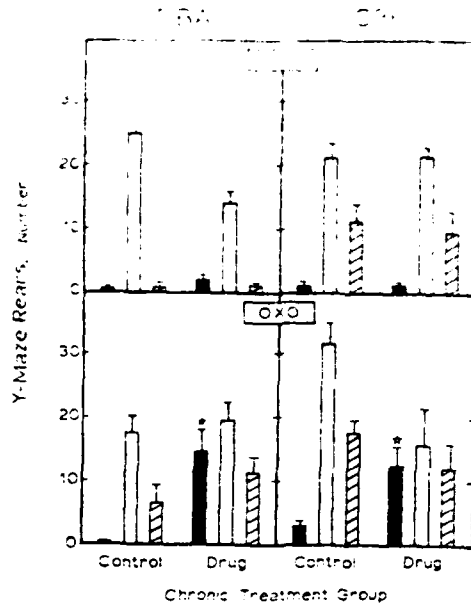


FIGURE 29



This series of experiments also used rotarod performance as a measure of drug response. Both DFP and oxotremorine impair the ability of mice to walk on the rotarod apparatus. The data reported in Figure 30 demonstrate that DFP-treated DBA and C3H mice are not tolerant to DFP's actions nor are they cross-tolerant to oxotremorine, but oxotremorine-infused animals are tolerant to oxotremorine and cross-tolerant to DFP.

FIGURE 30

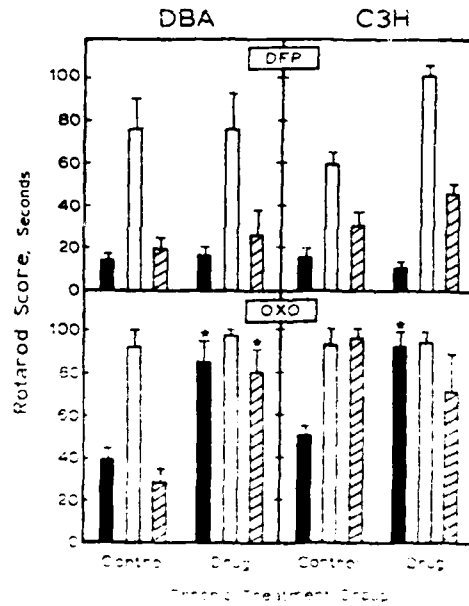
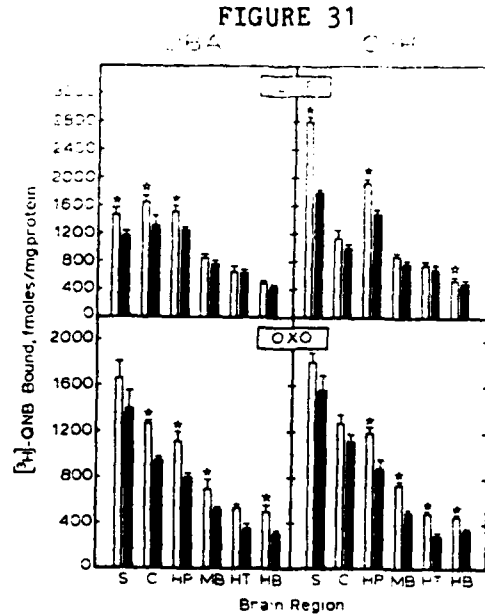


Figure 31 presents the QNB binding in these various treatment groups. QNB binding was measured in striatum (S), cortex (C), hippocampus (HP), midbrain (MB), hypothalamus (HT), and hindbrain (HB). Chronic DFP treatment elicited decreases in QNB binding in striatum, cortex, and hippocampus in DBA mice, and in striatum, hippocampus, and hindbrain in C3H mice. Virtually all brain regions, with the exception of striatum, showed reduced QNB binding in chronic oxotremorine-infused DBA and C3H mice.

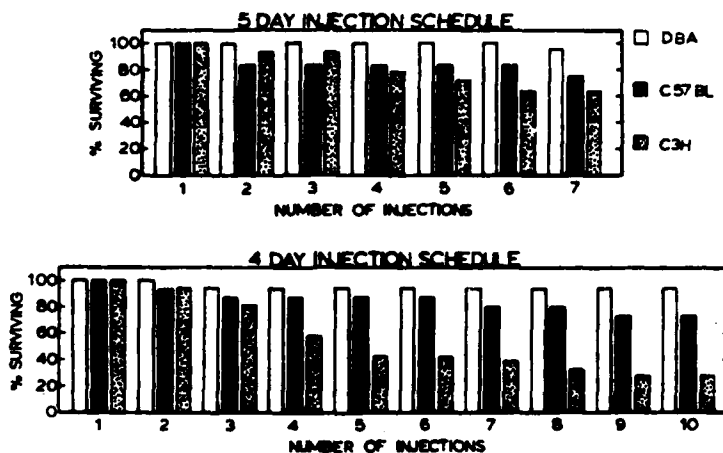


In summary, this series of experiments demonstrated that both DFP and oxotremorine can produce reductions in brain QNB binding, but these changes are not quite the same. DFP restricts its actions to only a few brain regions whereas oxotremorine's actions are more widespread. This may explain why oxotremorine-treated animals show a reduced response to DFP whereas DFP-treated animals do not. Whatever the case, these data demonstrate that changes in QNB binding can result in reduced response to DFP, but these data also indicate that DFP-induced decreases in QNB binding may not arise from a classic agonist-induced down regulation of receptors.

We also carried out a series of experiments that involved an assessment of the effects of chronic DFP treatment on brain nicotinic receptors, and tolerance to DFP and cross-tolerance to nicotine. No tolerance or cross-tolerance were seen. However, the nicotinic receptors were not altered by the treatment protocol that we used. This protocol involved injecting DBA, C3H, and C57BL mice with 4 mg/kg DFP every 5 days for a month. Five days after the last DFP treatment the animals were challenged with nicotine, and nicotine's effects on our test battery determined. The next day the animals were challenged with saline, and the day after that (7 days after the last chronic DFP dose) the response to DFP was determined. Immediately after DFP testing the animals were sacrificed, and the brains were removed for receptor (nicotine and BTX) measurement. As the reader will see from the results obtained in the study to be described next, 7 days is sufficient for brain nicotinic receptors to return to normal. Therefore, another treatment protocol must be devised to answer the question of what effect alteration of brain nicotinic receptors has on tolerance to DFP and cross-tolerance to nicotine.

In the course of carrying out these chronic DFP treatment studies, we had a considerable problem with lethality, especially in the C3H strain. Figure 32 presents a summary of the lethality seen in DBA, C57BL, and C3H mice that had been injected chronically with DFP on an every 4 or 5 day schedule. Clearly, strain differences are evident. These results suggest the possibility of strain differences in chronic toxicity resulting from DFP. At this stage, we have no idea as to the cause of these strain differences except our data indicate that AChE activity is affected identically in these three strains by the chronic treatment protocol.

FIGURE 32



As we proceeded with these experiments we realized that we had no idea as to the time course of recovery of brain muscarinic (QNB binding) and nicotinic (^3H -nicotine and ^{125}I -BTX) receptor numbers following chronic DFP treatment. Therefore, a study was carried out where DBA mice were injected every other day for 14 days with 4 mg/kg DFP. Animals were sacrificed at varying times after cessation of chronic treatment, brains removed and dissected into six regions, and QNB, nicotine, and BTX binding measured. The results of these experiments are presented in Figures 33-35. Figure 33 depicts the QNB binding results. Significant reductions in QNB binding were seen only in striatum. The reductions in QNB binding in cortex and hippocampus were marginal and control levels were regained quickly. However, striatal QNB binding was still significantly depressed from control even at the 28 day time point. Therefore, striatal binding may be permanently depressed; if not, the rate of recovery is extraordinarily slow. We are currently carrying out a series of studies where the recovery of QNB binding following chronic oxotremorine infusion is being measured. If QNB binding returns to control in oxotremorine-infused animals we would

interpret this result as suggesting that DFP may have toxic actions on the striatum.

FIGURE 33

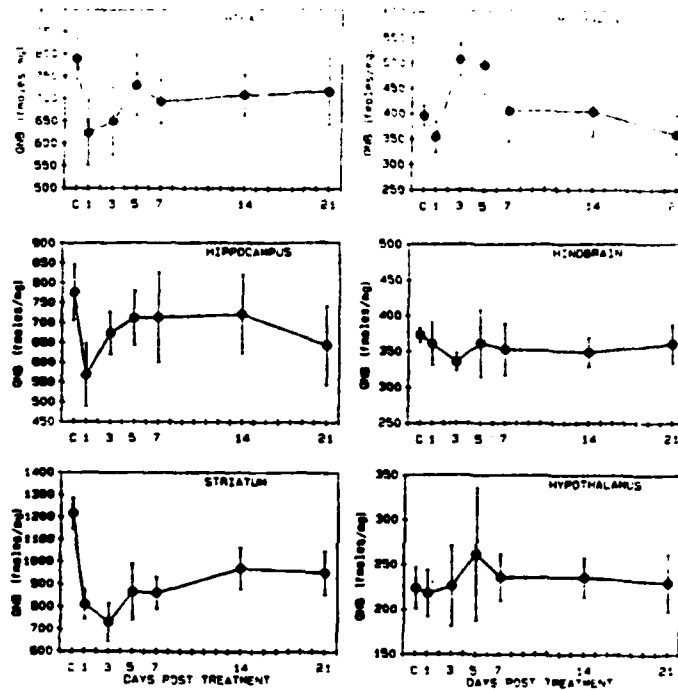


Figure 34 presents the effects of chronic DFP treatment on the binding of L-(³H)-nicotine binding. DFP treatment elicited reductions in binding in virtually every brain region. Unlike striatal QNB binding, nicotine binding recovered quickly such that control levels were regained within 5-7 days.

FIGURE 34

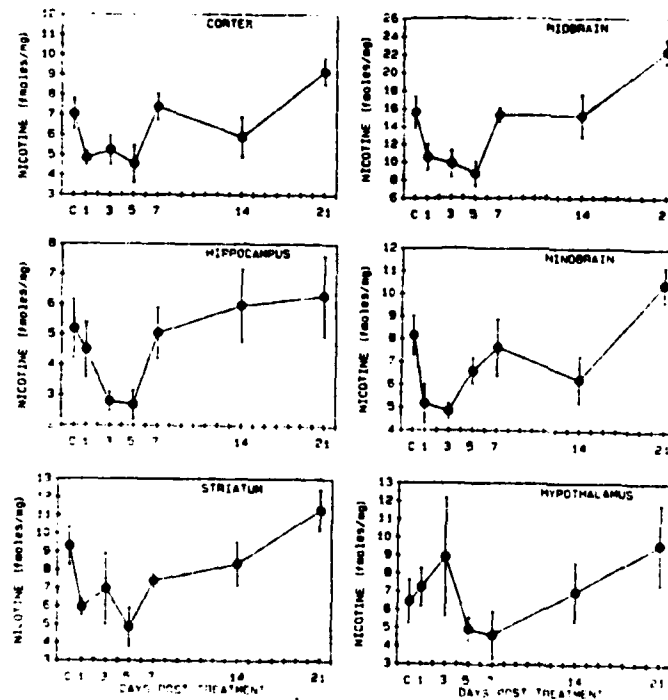
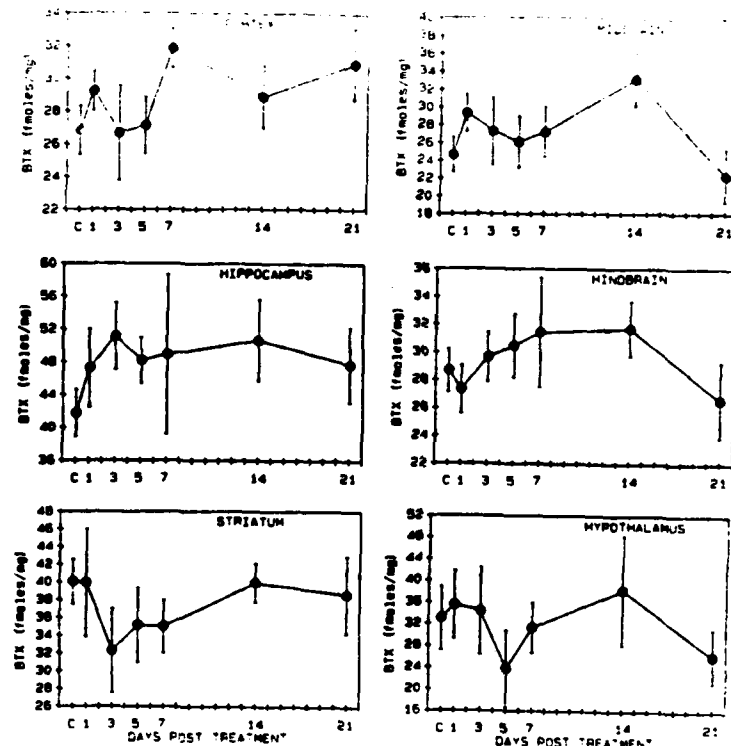


Figure 35 presents the effects, or lack thereof, of chronic DFP treatment on brain (125 I)BTX binding. No changes were seen. The literature is replete with studies that question whether the BTX binding site in brain is functional in adult rodent brain. The results of this study add to these concerns.

FIGURE 35

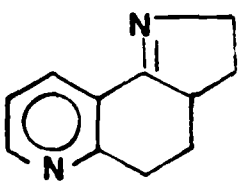
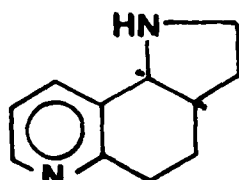
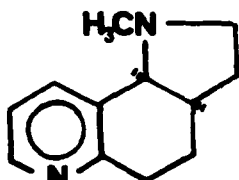
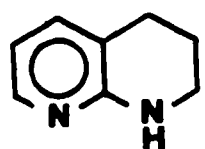
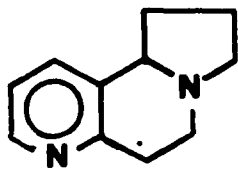
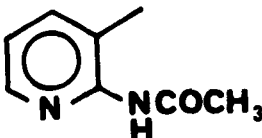
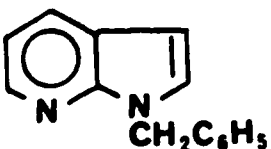
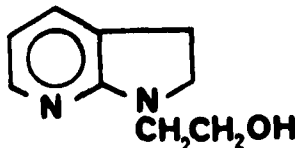


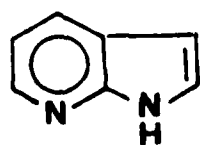
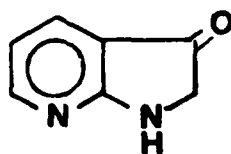
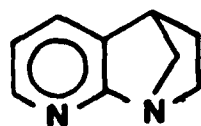
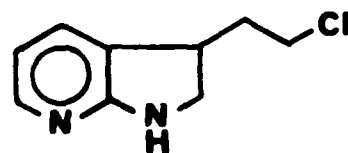
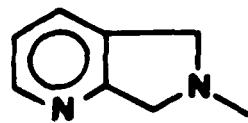
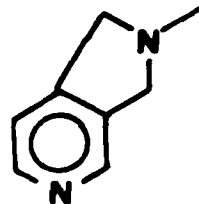
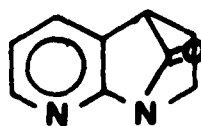
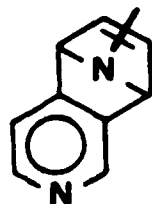
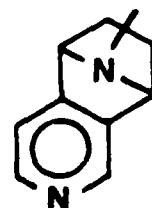
In overall summary, our results indicate that chronic DFP treatment changes brain QNB and nicotine binding. The observation that DFP-treated animals are not tolerant to DFP whereas oxotremorine treated animals are suggests that changes in QNB binding can result in tolerance to DFP, but DFP changes QNB binding with different results than does oxotremorine. The failure of striatal QNB binding to return to control levels following cessation of chronic DFP treatment may support the "DFP is neurotoxic" hypothesis.

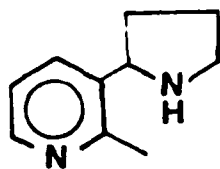
Synthesis and testing of nicotine analogues

Over the course of the entire grant period, a large number of nicotine analogues were synthesized. The structures of these compounds and their potency as inhibitors (IC_{50} values) of the binding of brain cholinergic receptor ligands is found in the accompanying table. Further testing was not carried out or was not successful because the compounds proved, for the most part, to be very weak inhibitors of binding, and they were very unstable chemically.

IC50 Values in M

	<u>NIC</u>	<u>BTX</u>	<u>QNE</u>
	1×10^{-7}	$\cdot 10^{-4}$	$525 \cdot 10^{-6}$
	1.4×10^{-7}	$>10^{-4}$	7.41×10^{-7}
	2.8×10^{-7}	$>10^{-4}$	1×10^{-6}
	7.08×10^{-5}	3×10^{-5}	1.26×10^{-4}
	4×10^{-8}	6×10^{-5}	3.8×10^{-4}
	2×10^{-6}	5×10^{-3}	2.24×10^{-4}
	9.85×10^{-3}	$>10^{-3}$	5×10^{-5}
	7×10^{-3}	$\cdot 10^{-3}$	1.6×10^{-3}

 7×10^{-5} $>10^{-3}$ 7×10^{-3}  1.5×10^{-2} $>10^{-2}$ $>10^{-3}$  4.68×10^{-4} 3×10^{-2} 2.3×10^{-6}  1.26×10^{-3} 6×10^{-4} 6.3×10^{-5}  1.78×10^{-5} 1.78×10^{-4} $>10^{-3}$  5.02×10^{-6} 2.82×10^{-3} 5.62×10^{-4}  $>10^{-3}$ $>10^{-3}$ 2×10^{-4}  1×10^{-5} 3.98×10^{-6} 1.78×10^{-4}  3.16×10^{-6} 3.98×10^{-6} 1.78×10^{-4}



$>10^{-2}$

$>10^{-2}$

2.24×10^{-4}

CONCLUSION

Our studies have made several new contributions to the literature. These include:

- 1) Acute and chronic responses are regulated by genetic factors in the mouse. This may mean that humans have differential sensitivity to the acute and chronic toxic effects elicited by organophosphates.
- 2) Acute and chronic exposure to DFP may result in neurotoxicity.
- 3) Chronic exposure to DFP does not necessarily result in tolerance, and changes in QNB binding are not necessarily a cause of tolerance. Changes in QNB binding may be a manifestation of neurotoxicity.

END

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