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## INTRODUCTION

This study is focused on the neurotoxic actions of the insecticides permethrin (PM) and chlorpyrifos (CPF) as they relate to the development of Parkinson's Disease (PD). These compounds possess properties that could damage the nigro-striatal system, which is the primary brain lesion in PD. The research is assessing the ability of each compound alone, or in combination, to directly induce neurochemical or neuropathological hallmarks of PD. In addition, since PD is hypothesized to have a multifactorial etiology, these compounds are also being tested for their ability to synergize the actions of the established Parkinsonian neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). This approach will determine any ability of the insecticides to accelerate or intensify idiopathic disease processes. Experiments are performed on the C57BL6 black mouse, which when given MPTP is a valid rodent model for the development of PD. For each treatment group, effects consistent with metabolic insult and changes in cholinergic and dopaminergic neurotoxicity in the striatum are measured. Cell stress in striatal nerve terminals is evaluated by measurements of mitochondrial function. Other neurochemical studies measure effects specific to the dopaminergic pathways in the striatum, including the amounts of dopamine and its metabolite 3,4-dihydroxyphenylacetic acid, as well as the ability of isolated nerve terminals to transport dopamine. Because of the anticholinergic effect of chlorpyrifos, we also measure acetylcholinesterase activity, and the density/function of muscarinic and nicotinic receptors following insecticide treatment. Neuropathology studies will determine any gross changes in immunocytochemical markers for glial fibrillary acid protein. Other antibody labeling studies will assess effects specific to the dopaminergic system, including antibody labeling of tyrosine hydroxylase and the dopamine transporter. These studies represent a unique combination of research approaches and will provide a comprehensive and integrated evaluation of the possible Parkinsonian or neurodegenerative effects of these insecticides.

## BODY

The experiments for the second year were, in part, completion of studies related to Objective #1, which is to characterize any effects on biomarkers of PD over a range of doses of PM and CPF. At the end of the last annual report, we mentioned that we had a number of frozen brain tissue samples from treated mice that would be used for additional radioligand binding studies, and this area is where we began work in year two. Similarly, we have performed only a portion of the experiments planned for the second year. The loss of postdoctoral scientists was the main factor limiting our ability to complete all of the scheduled experiments. Overall, the project lost 7 months of postdoctoral effort due to changes in employment. Although Dr. Daniel Karen filled the position vacated by Dr. Paul Harp on 1/1/00, the project was only at full employment for 5 months when Dr. Wen Li took a permanent job in the Virginia/Maryland Regional College of Veterinary Medicine in mid May. She was replaced by Dr. Jeff Gillette, who completed his doctoral work this past summer at the Dept. of Toxicology, North Carolina State University. Jeff joined the project in late August, and his CV is included in the appendix. Dan Karen then took a permanent position with Arcadis JSA, an environmental consulting firm in Long Beach, CA. I have had the good fortune to hire capable people for this project, but the downside is that they have had no trouble finding permanent positions in industry, government, and academia. The position just vacated by Dan Karen has been offered to Dr. Jing Shen, a Chinese M.D., who is currently working at the Tokyo Women's Hospital in the pediatric cardiology unit. We are currently trying to secure an appropriate visa for Dr. Shen. Because new people have joined the group every few months, we have also had to spend an inordinate amount of time in training, and will eventually require a no-cost grant extension to finish the work. As it became clear that the project was going to experience another round of turnover in personnel, I made a strategic decision to emphasize study of synergistic interactions, which was a main theme of the grant and was the major objective of year two. Moreover, we analyzed synergism only from the perspective of dopaminergic biomarkers, those more closely associated with Parkinsonism, and delayed work on cholinergic effects. Results for year two of the project are organized by alphabetical listing of objectives, as given in the amended grant proposal and in the annual report for year one.

Treatment of mice in year two for studies of insecticides alone, and insecticides with MPTP, was performed as described in the proposal, and is illustrated in Figures 1 and 2, respectively. We chose the following doses for these studies: 200 mg/kg PM (ip), 75 mg/kg CPF (sc), and 30 mg/kg MPTP (ip), which were all sublethal based on previous work on this or other projects (see last year's report). Treatment groups for the procedure given in Figure 1 were controls, PM, CPF, or PM+CPF, but these studies were limited in number. Because PM and CPF did not deplete dopamine when given alone, we emphasized the MPTP combination studies. Treatments for the procedure given in figure 2 were controls, MPTP, MPTP+PM, MPTP+CPF, or MPTP+PM+CPF.

One of the first observations we made was whether combination treatments of these toxicants at sublethal doses caused greater than expected mortality. No increased mortality was observed in treatments of PM+CPF. However, the MPTP synergism studies showed increased toxicity in combination treatments. A representative experiment is shown in Figure 3. There was some unexpected toxicity at doses of MPTP that previously gave no lethality. Controls and PM had no lethal effects (Fig. 3). The toxicity of MPTP+ CPF group was about additive, while that of MPTP+PM was a bit less than additive. The triple treatment group had lethality that was greater than additive and approached 60%. Replicates of this study at 30 and also surprisingly, 20 mg/kg MPTP, also showed some mortality. Thus, the neurochemical results for most of the rest of the report represent findings among survivors of combination treatments.

**a. Assess toxicant effects on dopamine titers and turnover by measuring the dopamine and 3,4-dihydroxyphenyl acetic acid (DOPAC) content of the striata from treated mice.**

**Methods:** Dopamine and DOPAC content of the striatum were determined by HPLC with electrochemical detection (Hall *et al.*, 1992). In all studies, mice were sacrificed by cervical dislocation, and the striata rapidly dissected and weighed. The striata are homogenized in 5% trichloroacetic acid (TCA) with 62.4 ng of 3,4-dihydroxybenzylamine (DHBA) as the internal standard, and stored at -70 °C until analyzed. The samples were thawed, centrifuged to pellet the membranes and the supernatant analyzed for dopamine and DOPAC by HPLC through a C<sub>18</sub> column. The mobile phase consisted of [90% aqueous (0.1 M sodium acetate, 2 mM heptanesulfonic acid, 0.33 mM EDTA, adjusted to pH 4 with glacial acetic acid)/10% methanol (v/v)], at a flow rate of 1 ml/min. Quantitation was determined from standard curves of peak height ratios (dopamine/DHBA) obtained from stock solutions containing dopamine at multiple levels and DHBA as the internal standard. Data were analyzed by analysis of variance (ANOVA) with Student-Newman-Keuls means separation test.

**Results and Discussion:** Our initial studies on dopamine depletion with insecticides+MPTP are shown in Figure 4. In this study, 30 mg/kg MPTP showed about 70% depletion. This depletion was significantly enhanced by concomitant treatment with CPF or PM. We observed no insecticide-dependent depletion of DOPAC in this or other studies (see Fig. 5). We then designed a large, complete study of the effects of toxicant treatment on dopamine titers (Figure 5). In this experiment, dopamine content was about 140 pmoles/mg striatum in controls, and as before, high doses of CPF (75 mg/kg) and PM (200 mg/kg) had no effect on dopamine content. There was, however, a significant reduction in dopamine by 30 mg/kg MPTP (*ca.* 90%); unfortunately, much more than our previous studies would have indicated (usually around 50% reduction; Bloomquist *et al.*, 1999; 70% in Fig. 4). Thus, it is not surprising that the insecticides showed no enhancement in this experiment, since the dopamine was essentially eliminated by the MPTP treatment. In the first year's report, we saw an elevation of DOPAC by CPF treatment (100 mg/kg), and consistent with this observation, there was a small, but not significant increase at 75 mg/kg CPF (Fig. 5). As mentioned above, there was no enhancement of depletion of DOPAC by insecticides, over and

above the effect of the MPTP (78% reduction, Fig. 5). We then tried an experiment at a dose of 20 mg/kg MPTP (Fig. 6). In this case, MPTP depleted dopamine about 65%, and we also saw a significant enhancement of dopamine depletion by the insecticides. Surprisingly, there was no greater effect in the triple treatment group above that obtained for each insecticide alone. Moreover, there was no effect on DOPAC titers. These findings suggest that the depletion of dopamine is not due to extensive cytotoxicity. The experiment shown in Figure 5 is also the one depicted in Figure 3, showing extensive mortality. Recall that the neurochemical analysis was done on the survivors, whereas the most heavily affected individuals probably died. Another study using 15 mg/kg MPTP showed no mortality in any of the groups, but these studies could not be analyzed in time for this report. An important additional consideration is the dose-response relationship of the insecticide effect. To date, we have only done these studies at high insecticide doses. In year three we will also begin to evaluate the persistence of these neurochemical effects by evaluating mice at variable times after the cessation of treatment. We also attempted studies to assess synergism between MPTP and PM at the 1.5 mg/kg dose of PM, where upregulation of transport occurs, but also had near complete depletion of dopamine by 30 mg/kg MPTP alone (data not shown). We will also repeat these experiments at 15 mg/kg MPTP.

**b. Assess effects on the density and kinetic properties of dopamine transporters in striatal synaptosomes from treated mice.**

**Methods:** For both dopamine uptake and radioligand binding studies, we prepared striatal synaptosomes *ex vivo* from treated mice using the methods of Bloomquist *et al.* (1994). For studies of dopamine uptake kinetics, synaptosomes were incubated with [<sup>3</sup>H]dopamine (Amersham Corp.) for 2 min. Transport was terminated by dilution with 3 ml of ice cold buffer and immediate vacuum filtration on glass fiber filters. The filters were then washed with cold buffer and the amount of radioactivity on the filters determined by liquid scintillation spectrometry. Nonspecific uptake was determined in Na<sup>+</sup>-free buffer following the method of Krueger (1990). For labeling of dopamine uptake sites, we used an equilibrium binding assay with the established uptake inhibitor [<sup>3</sup>H]GBR12935 (NEN Research). Binding employed incubation to equilibrium, followed by filtration, rapid washing, and liquid scintillation counting. Nonspecific binding was estimated with saturating concentrations of the closely related analog GBR12909. Uptake and radioligand binding data will be analyzed using InPlot<sup>TM</sup> (GraphPad Software, San Diego, CA).

**Results and Discussion:** We confirmed our observation in last year's report that PM treatment significantly alters maximal transport ( $V_{max}$ ) of dopamine with little effect on half maximal substrate affinity ( $K_m$ ). Figure 7 shows representative isotherms illustrating that the main effect of PM is a change in  $V_{max}$ . Kinetic data analysis confirmed no difference in  $K_m$  values (data not shown). Final replicated results for  $V_{max}$  determinations in groups of treated mice given PM is shown in Figure 8. These experiments were replicated at least twice over a broad range of doses, with the 1.5 mg/kg group replicated four times. The lowest doses of PM (0.2, 0.4, and 0.8 mg/kg) had no statistically significant effect on maximal dopamine uptake. An increase in maximal transport of dopamine was observed at 1.5 mg/kg, and this effect progressed into a depression of uptake at higher doses (Fig. 8).

The dose of PM where upregulation occurs (1.5 mg/kg) is miniscule compared to its LD<sub>50</sub>, and 200 mg/kg ip caused no lethality, no overt signs of toxicity, and only a slight effect in our behavioral studies (see below). Thus, 1.5 mg/kg is at least 2 orders of magnitude less than a nonlethal dose. It is also important to note that technical permethrin is a mixture of four stereoisomers, only one of which (1*R*, 3*R*, *cis*) is expected to have any toxic effects in mammals, based on studies of acute lethality (Casida *et al.*, 1983). If so, the true level of toxic isomer in these studies is 0.375 mg/kg.

The level of increased dopamine transport caused by PM (mean of four experiments: 33% above control) is about the same as that observed for cocaine, which raises expression 50% when given 5 times/day at 40 mg/kg (Miller *et al.*, 1993). In contrast to PD, schizophrenia results from dopamine

overactivity (Bowman and Rand, 1980) and dopamine uptake in postmortem brain preparations from schizophrenic patients was increased 74% over controls (Haberland and Hetey, 1987). Thus, the level of DAT overexpression in schizophrenia appears similar to that resulting from low dose exposures to PM. Moreover, psychiatric problems are known to result from OP exposure and disturbance of consciousness has been reported for pyrethroid intoxication in man (He *et al.*, 1988). We speculate that the dopamine transporter was up-regulated in response to increased levels of synaptic dopamine, which is consistent with findings we recently published on the structurally-related pyrethroid, deltamethrin (Kirby *et al.*, 1999).

Changes in uptake may be correlated with the binding density of ligands that label the DAT. Our first experiments with [<sup>3</sup>H]GBR12935 in PM-treated C57 mice (100 and 200 mg/kg) are shown in Figure 9. GBR binding was saturable in all cases, to a single site with  $K_d$  values in the low nanomolar range and with  $B_{max}$  of 80 pmol/mg protein, in controls. These values are similar to those reported by Horn (1990), who listed a  $K_d$  of 0.82 nM but a  $B_{max}$  of 5.5 pmole/mg protein for rat striatum, much less than we see in mouse. Recall that maximal dopamine uptake is reduced at these high doses of PM (Figs. 7,8), which is not reflected in a decrease in  $B_{max}$  for this ligand. This finding is in accord with DAT antibody labeling reported in the first report, which was not changed by treatment with 200 mg/kg PM. This observation suggests that other mechanisms besides a change in density of the DAT is responsible for the reduced uptake. One possibility is that the synaptosomes are able to transport the dopamine normally, but the synaptosomes are unable to sequester it due to enhanced leakage. Pyrethroids are known to stimulate secretion of transmitters (Kirby *et al.*, 1999), but in resting synaptosomes this effect usually requires some stimulus (*e.g.*, veratridine activation of sodium channels). There may also be a PM-induced effect on cytotoxicity or respiration, since reduced complex I activity (MTT assay-next section) was observed previously (last year's report).

Although there was no change in DAT ligand binding at high doses of PM, we would expect that levels of [<sup>3</sup>H]GBR12935 binding will increase in mice given 1.5 mg/kg PM. We have recently documented that the organochlorine insecticide heptachlor virtually doubles dopamine transport, and that antibody labeling of DAT in western blots of synaptosomal protein is increased by a similar amount (Miller *et al.*, 1999). Moreover, the dose-response curve for heptachlor has a shape similar to that reported here for PM. Thus, we were surprised by our finding of no increase in DAT expression at this dose using immunocytochemical labeling (biomarker f). If this latter finding is confirmed and no increase in DAT is revealed by [<sup>3</sup>H]GBR12935 binding, we will have to conclude that a change in activity of the DAT protein or a different isoform of the DAT is being induced by PM.

CPF at doses known to inhibit 10-90% cholinesterase activity (20-100 mg/kg) showed modest effects on dopamine uptake (Fig. 10). Uptake was slightly higher (but not statistically significant) at 25 and 50 mg/kg CPF. In contrast, uptake was significantly reduced (10%) at a dose of 100 mg/kg. The magnitude of this effect is similar to that of CPF on choline uptake, where it is reduced 16-20% (Liu *et al.*, 1995). Overall, these findings suggest that transporter activity/expression is a sensitive biomarker for toxicity in neurotransmitter systems, especially where the transporter is the primary mechanism for terminating transmitter action.

**c. Compare the extent of toxin-dependent actions on mitochondrial function in striatal synaptosomes by measuring thiazolyl blue dehydrogenase activity.**

**Methods:** The thiazolyl blue (MTT) assay kit from Sigma Chemical Co. (St. Louis, Missouri), which reports on the amount of dehydrogenase activity at mitochondrial complex 1 (Slater *et al.*, 1963) was used essentially as described in the proposal. We use synaptosomes instead of brain mitochondria in order to assess respiration of mitochondria in a cellular environment approximating what they experience *in vivo*, which we hypothesize will be altered by poisoning. Accordingly

**Results and Discussion:** Toxicity to nerve terminals *in vivo* should be reflected in reduced mitochondrial function *in vitro*. We have observed small, but statistically significant reductions in mitochondrial activity, which is also observed in PD (Schapira *et al.*, 1990) and other neurodegenerative diseases (Beal *et al.*, 1993). Our preliminary experiments using the MTT assay in synaptosomes from treated mice found that it does not follow typical Michaelis-Menten kinetics, since production of formazan product is linear with respect to concentration, up to about 0.5 mM, where the activity saturates and then declines at higher substrate concentrations (last year's report). Given this somewhat unusual dependence on substrate concentration, in subsequent experiments we emphasized effects at concentrations up to 0.55 mM, which is at or near maximal effect, which we expect to be better correlated with cytotoxicity or cell stress. We found statistically significant reductions (about 35%) of dehydrogenase activity with 75 mg/kg CPF and 200 mg/kg PM, but no increase in toxic effect when the two were combined (Fig. 11). Results of a larger experiment, including MPTP, are shown in Figure 12. Reduced activity was observed with CPF, PM, and MPTP (30 mg/kg). However, when combined with either CPF or PM, mitochondrial activity was slightly above controls, whereas the triple treatment group showed less activity than all the others, which was expected. In this case also, we are concerned that the responses reflect the fact that they were measured from survivors of the combined treatments. In addition, the MTT studies were done on synaptosomal membranes pooled from all the mice in a treatment group. This approach is the same as we used in the dopamine uptake studies, where membranes from multiple mice is required to run the uptake curves, which can then be subjected to statistical analysis. Since we have shown that a single substrate concentration causing maximal effect seems to be the best approach for comparing MTT reductase activity, we now plan to perform this assay on individual mice, so that the experimental variability in the assay will be related to the response of individual animals. In the data we have collected so far, the experimental variability is only due to sampling of the pooled membranes, which as shown in Figure 12 gives statistical significance for very small differences. Although the level of MTT reduction should be treatment-related, a more appropriate estimate of the experimental variability will result from this appropriate change in our experimental approach.

- d. **Search for anatomical evidence of general neurotoxicity within light microscopic preparations of the nigro-striatal system by examining glial fibrillary acidic protein (GFAP) immunoreactivity as a marker for gliosis.**
- e. **Search for anatomical evidence of neurotoxicity within specific dopaminergic neurochemical components of the nigro-striatal system using immunohistochemical staining for the catecholamine-synthesizing enzymes tyrosine hydroxylase (TH) and dopamine beta hydroxylase (DBH).**
- f. **Confirm whether functional changes in dopamine transport are due to fluctuating levels of the dopamine transporter (DAT) protein using immunohistochemical identification.**

**Methods:** These biomarkers are dealt with together since fixation, sectioning, staining and analysis of the tissue is the same for both. A portion of the time during this reporting period was spent training a half-time wage employee who will now be able to assist in this portion of the project. A time delay of a few weeks was encountered when the cryostat used for this portion of the project developed electrical problems. A minor modification to our tissue processing protocol involved a change from using the Vectastain avidin-biotin complex to use of the Vectastain Elite avidin-biotin complex in order to improve the intensity of immunolabeling. In the last progress report we detailed the procedure for an image analysis protocol that we developed when it was found that the originally proposed Sigma Scan Pro software was inadequate for our purposes. In that report, we also outlined a procedure for reliably locating specific fields within the striatum to be subjected to quantification of staining density. Using these methodologies, we have examined whether changes in DAT immunolabeling occur over a range of low doses of PM (0.8, 1.5 and 3.0 mg/kg) that

includes the concentration that we have shown to produce increased dopamine uptake in striatal synaptosomes. Changes in DAT immunolabeling were also examined for a high dose of PM (200 mg/kg) that we have shown to produce a reduction in dopamine uptake within striatal synaptosomes. Immunolabeling of the dopamine-synthesizing enzyme tyrosine hydroxylase (TH) was also examined for the high dose of PM (same mice used for DAT labeling) and for a dose of CPF that we have shown to produce significant behavioral effects. Some cases were eliminated from analysis due to inadequate staining intensity. The mice reported on below do not include those small numbers of PM and CPF animals that we reported on last year given their temporal separation from the current study groups and the minor change in staining procedure noted above.

Given the inherent variability of immunocytochemical procedures, tissue from each treated animal was processed adjacent to corresponding tissue from a matched vehicle animal on each slide that was run through the staining procedure. The first appearance of the lateral ventricle was used as marker to begin saving sections on slides and 16  $\mu$ m sections were then taken at fixed intervals. This minimized variability between the matched treated and vehicle sections on a given slide, with respect to rostrocaudal position within the striatum. In addition, the left or right position of the tissues from treated and vehicle animals were counterbalanced on the slide to avoid such position effects during staining. Therefore, the most appropriate comparison was the difference in staining intensity between the paired treated and vehicle tissues on each slide. This difference for each slide was averaged across slides for each pair of matched treated and vehicle mice. The non-parametric signed rank test was used to test for significant changes in immunolabeling intensity since changes in staining intensity within each slide were not normally distributed. As the name implies, this test ranks the absolute magnitude of differences between matched pairs and then, taking into account the sign of the differences along with the magnitude of their ranks, determines whether the distribution of differences is significantly far away from zero. The test is analogous to a paired t-test, except that the statistic is calculated based on the magnitude of ranks of the differences instead of on the actual magnitude of the differences themselves. This minimizes the effect of extreme values in non-normally distributed data. The test can assess whether a significant difference occurred between matched vehicle and treated mice within a dosage group, but does not provide information about differences across dosage groups. The statistical analysis of the data was conducted by Dan Ward, statistical consultant for the College of Veterinary Medicine.

**Results and discussion:** Figure 13 shows box and whisker plots of the distribution of mean differences in DAT immunolabeling between matched pairs of vehicle and treated mice, for each dose of PM. This type of plot provides a useful shorthand for depicting where the majority of data points are concentrated within a distribution. Unless an extreme value is present, indicated by a small triangle, the tips of the whiskers represent the respective minimum and maximum mean differences, while the length of the box represents the inter-quartile range of the mean differences. The line and the dot within the box respectively represent the median and mean of the distribution of difference scores. The number of matched pairs of mice examined at each dose is also shown in each box. As can be seen in the figure, the mean and median of the mean difference scores between vehicle and matched treated mice was negative for all doses of PM, indicating an overall trend toward a decrease in DAT immunolabeling in the treated mice. However, as indicated by the asterisk, only the 3.0 mg/kg dose was found to produce a significant decrease in DAT immunolabeling ( $S=18$ ,  $p<.0078$ ). It therefore appears that low doses of PM can alter not only the function of the DAT, as we have previously shown, but the amount of DAT protein within the striatum. The absence of a significant change in DAT immunolabeling in the high dose PM group indicates that a change in the amount of DAT protein does not underlie the decrease in dopamine uptake that we have previously demonstrated for this dose, and as mentioned above, a change in activity of the DAT or a different isoform of the DAT is being induced by PM.

Figure 14 shows similarly constructed box and whisker plots of the distribution of mean differences in TH immunolabeling between matched pairs of vehicle and insecticide-treated mice for a high dose of PM (200 mg/kg) and for a 50 mg/kg dose of CPF. No significant change in TH

immunolabeling was observed for either treatment, although the trend toward a decrease in TH immunolabeled protein in the PM-treated group was in the same direction as that observed for DAT immunolabeling at all doses. The absence of a significant change in immunolabeling of the TH enzyme in the high dose PM group suggests that the decrease in dopamine uptake that we have previously demonstrated at this dose is not a function of the degeneration of dopaminergic terminals within the striatum. However, our data cannot preclude the possibility that a lack of change in TH immunolabel is attributable to an upregulation of TH in striatal terminals that survive a wave of degeneration. The absence of a significant change in immunolabeling of the TH enzyme in the CPF-treated group is not surprising given our previous finding that dopamine concentration does not change following exposure to 100 mg/kg of CPF. In the coming year we are especially interested in evaluating combination treatments for their effect on TH staining.

**g. Explore toxicant effects on open field/rearing frequencies and pole climbing behaviors and search for correlations between behavioral impairment and neurochemical effects.**

**Methods:** Behavioral experiments focused on measurements of impairment of motor function. Alteration of motor behaviors was assessed using open field ambulation, rearing frequency, and a pole traction test similar to those described by Takahashi *et al.* (1989). Animals were tested at the end of the posttreatment period, prior to sacrifice for the neurochemical analyses. In the open field test, we assigned a movement unit whenever all 4 feet of the mouse entered a new square on the bottom of the arena. Movement units and the number of rearing behaviors produced were summed over a 3 min observation period. The number of rears was also summed for 3 min. The pole test apparatus consists of a 38 cm taped ring stand. Mice are placed at the top of the pole and allowed to hang by the forepaws. All mice are observed for up to 5 minutes. Times required for mice to turn (invert) and then climb down the pole are measured. Inversion times, climbing times, and the number of falls was determined.

**Results and Discussion:** By and large, effects on dopamine titers did not correlate with behavior changes in treated mice. CPF at 75 mg/kg had significant effects on movement and rearing behavior (Figs 15). Open field and rearing movements were unaffected by 20 mg/kg (Fig. 15) or 30 mg/kg (Fig. 16) MPTP, any of the PM treatments, or PM+MPTP. MPTP+CPF treated-mice showed less rearing, but the movement score was near control levels in one study (Fig. 15), but remained reduced in another (Fig. 16). In both cases, the triple treatment groups were not different from controls, perhaps reflecting different actions of these toxicants on overall levels of excitability of the nervous system.

The pole climbing data from two studies using 30 mg/kg MPTP was pooled and is shown in Figure 17. There is a definite trend towards a propensity to fall from the pole, however, with only one statistical degree of freedom, the treatment effects were not significant, even though the % falling is near four times the control value. Additional replicates will easily solve this problem.

PD results in tremors, bradykinesia, and incoordination (Bowman and Rand, 1980), which are reflected in the behavioral assays we have performed.. These behavioral studies have allowed us to observe some general correlations between behavior and neurochemical effects for insecticides, but not for MPTP alone, at least under the doses and dosing frequency we tested. However, bear in mind that it requires about 80% depletion of dopamine in humans before parkinsonian symptoms appear (Marsden, 1990), so some discordance between dopamine levels and behavior is expected. Literature reports of MPTP's attenuation of open field behavior in the mouse typically use multiple treatments and higher doses than we use here. For example, Sundstrom *et al.* (1990) used two 40 mg/kg treatments 16 hr apart and measured behavior and dopamine 4 weeks later. In these studies, they observed a 70-80% reduction in locomotion and rearing. Similarly, Ogawa *et al.* (1985) injected 30 mg/kg twice a day for 5 days and reported that both turning and down travel time in a pole test similar to the one we used were increased, although not much more than 2-fold.

injected 30 mg/kg twice a day for 5 days and reported that both turning and down travel time in a pole test similar to the one we used were increased, although not much more than 2-fold.

**h. Determine the extent of acetylcholinesterase inhibition following treatment with toxicants for comparison with other behavioral and neurochemical effects.**

**Methods:** We used the classical method of Ellman *et al.* (1961) to determine acetylcholinesterase activity in striatal synaptosomes from treated mice. The assay measures enzyme generation of yellow color by reaction of 5,5'-dithiobis-2-nitrobenzoic acid and thiocholine when acetylthiocholine is used as the enzyme substrate. Although  $V_{max}$  and  $K_m$  values for acetylcholinesterase activity were planned, standard measurement of cholinesterase involves a single substrate concentration at an incubation time in the linear range of activity. We used a substrate concentration of 400  $\mu$ M and an incubation time of 3 min.

**Results and Discussion:** Combination treatments were also evaluated for their effect on acetylcholinesterase activity in the striatum in a single experiment. CPF at 75 mg/kg gave about 70% inhibition of acetylcholinesterase (Fig. 18), and the behavioral effects of treatments containing CPF appear to be better correlated with cholinesterase inhibition than any effect on dopamine. PM and MPTP had little effect on cholinesterase, and combining the toxicants had little effect on enzyme activity compared to that observed with CPF alone. Certainly there was no indication of greater inhibition, and in fact, MPTP+CPF actually showed significantly less inhibition than CPF alone. None of the mice showed any signs of SLUD. Thus, although MPP<sup>+</sup>, the bioactivation product of MPTP, is an inhibitor of cholinesterase, with *in vitro*  $K_i = 0.197$   $\mu$ M (Zang and Misra, 1993), we did not observe any effect on enzyme activity in *ex vivo* striatal tissue. Apparently, the striatal concentration did not reach this level from a single treatment of 30 mg/kg MPTP, or any inhibited enzyme was replaced during the time between treatment and assay. Recall that this period of time for MPTP is about 2 weeks, but only 24 hr for the insecticides in our treatment regime (Figs. 1 and 2).

**i. Define any toxicant-induced changes in cholinergic receptor density or function with respect to agonist-induced dopamine release from striatal synaptosomes.**

**Methods:** This last objective of the research actually contains several separate neurochemical measurements. We have established methods for radioligand binding studies involving [<sup>3</sup>H]quinuclidinyl benzilate ([<sup>3</sup>H]QNB) and [<sup>3</sup>H]nicotine, with nicotine binding adapted from the procedures of Marks *et al.* (1986). In addition, two methods for measuring functional cholinergic modulation of dopamine release are still under development. The first will measure loss of label by repeated application of buffer with a pipettor. Alternatively, the labeled synaptosomes will have agonists superfused over them with a peristaltic pump and loss of label will be quantified in this manner. We have not yet performed any studies on the ability of cholinergic agonists to alter release of dopamine in striatal synaptosomes from insecticide-treated mice.

**Results and Discussion:**

In last year's report, we provided evidence that exposing mice to PM caused an apparent upregulation of muscarinic receptors, as evidenced by an increase in the  $B_{max}$  for [<sup>3</sup>H]QNB binding (Fig. 19), and have expanded these results to include findings at 25 mg/kg PM. Compared to controls, QNB binding was increased 32% at 25 mg/kg, 86% at 50 mg/kg, 131% at 100 mg/kg, and 111% at 200 mg/kg PM (Table with Fig. 19). ANOVA of the  $B_{max}$  values showed that all of these values were significantly different from control ( $P < 0.05$ , with Student-Newman-Keuls post test). There were also changes in apparent  $K_d$ , but there was considerable overlap in the 95% confidence limits for these values (Table with Fig. 19). ANOVA confirmed that none of the  $K_d$  values for PM-treated mice were significantly different from controls. In previous studies, systemic injection of pyrethroids (*ca.* 1 mg/kg) in young mice slightly down-regulated cortical expression of muscarinic

receptors (5-10%), but apparently not in the striatum (Eriksson and Fredriksson, 1991). These results however, may be suspect, since Eriksson and Fredriksson (1991) report a  $K_d$  value in the low micromolar range of what they call high affinity QNB binding. These data are several orders of magnitude different from the  $K_d$  we report for controls (13 pM), as well as that found by others: Goodwin *et al.* (1995) for heart ( $K_d = 63$  pM) and Niemeyer *et al.* (1995) for cat retina ( $K_d = 270$  pM). More detailed discussion of these issues is given in the cholinergic paper in the Appendices.

We have also performed studies of striatal QNB binding in mice treated with CPF (Fig. 20). Little effect in QNB binding was found for a dose of 50 mg/kg CPF. However,  $B_{max}$  was reduced 47% at a dose of 100 mg/kg (table in Fig. 20). This level of binding was significantly different from control (t-test,  $p < 0.05$ ). These results are similar to those in rats given a maximally tolerated dose of CPF (279 mg/kg, sc), where muscarinic receptor density as estimated from QNB binding was reduced as much as 55% (Chaudhuri *et al.*, 1993). This down-regulation of muscarinic receptors by CPF is opposite that of PM. This difference is interesting, since both insecticides are expected to increase synaptic levels of acetylcholine, but differ in how this action affects receptor regulation. The mechanisms underlying this difference deserve further investigation.

Due to lack of personnel, no experiments on [ $^3$ H]nicotine binding or cholinergic effects on dopamine release were performed in year 2 of the project.

## KEY RESEARCH ACCOMPLISHMENTS

Observed synergistic interactions in combination treatments of the insecticides PM and CPF and found that insecticides enhance the dopamine-depleting action of MPTP.

Confirmed that small, but statistically significant reductions in mitochondrial activity occurred in PM- and CPF-treated mice, and the extent of reduction was increased when insecticides were combined with MPTP.

Replicated studies showing dopamine transport up-regulation at extremely low doses of technical PM (1.5 mg/kg) and down-regulation of transport at doses  $\geq 12$  mg/kg.

Extended the dose-response range for PM up-regulation of muscarinic receptor density in the striatum of treated mice, and confirmed that CPF down-regulates these receptors.

## REPORTABLE OUTCOMES

### Meeting Presentations

The presenter is underlined in each citation, where an asterisk indicates the sponsoring author. Copies of the corresponding abstracts are appended

W. Li, P. Harp, D. Karen, B. Klein, and J. Bloomquist, Striatal Dopaminergic Pathways as Target for the Insecticides Permethrin and Chlorpyrifos, fall 2000, National Meeting of the Society for Neuroscience, New Orleans, Louisiana.

J. Bloomquist, P. Harp, D. Karen, and W. Li, Insecticide Action on Behavior and Striatal Cholinergic Biomarkers, fall 2000, National Meeting of the Society for Neuroscience, New Orleans, Louisiana.

D. Karen, P. Harp, W. Li, and J. Bloomquist, Effects of Multiple Exposures of Chlorpyrifos or Permethrin on Murine Behavior and Striatal Cholinergic Biomarkers, fall 2000, National Meeting of the Society for Environmental Toxicology and Chemistry, Nashville, Tennessee.

D. Karen, W. Li, P. Harp, J. Gillette, B. Klein, and J. Bloomquist, Striatal Dopaminergic Pathways as Targets of Chlorpyrifos or Permethrin Exposures: Comparison with the Parkinsonian Neurotoxin MPTP, spring 2001, National Meeting of the Society of Toxicology, San Francisco, California.

D. Karen, P. Harp, W. Li, J. Gillette, and J. Bloomquist, Effects of Subchronic Exposures of Chlorpyrifos or Permethrin on Behavior and Striatal Cholinergic Biomarkers in C57BL/6 Mice, spring 2001, National Meeting of the Society of Toxicology, San Francisco, California.

## Publications

Two publications were prepared along the lines of the two types of posters (cholinergic and dopaminergic) given in the past year. The dopamine work will be submitted to *NeuroToxicology* and the cholinergic research to *Toxicology and Applied Pharmacology*. Copies of these manuscripts are given in the Appendices.

## CONCLUSIONS

A number of major conclusions are derived from the second year of this project.

First, enhancement of the dopamine-depleting effect of MPTP was observed with insecticides, albeit at high doses. This synergism provides a facile way to visualize how insecticides might accelerate or intensify idiopathic disease processes. Although the up-regulation of dopamine transport occurring at low doses of PM (1.5 mg/kg) provides a ready mechanism for synergism with pyridinium toxins, such as MPP<sup>+</sup>, we have so far only demonstrated synergism at high doses of insecticides. Investigating this synergism is a major goal of the third year of the project, in the context of dose-response relationship for the synergistic effect.

Second, the loss of dopamine transport at higher doses of PM is probably related to other toxic effects, such as a reduction in mitochondrial activity. Even though the magnitude of the effect is small, any reduction in mitochondrial activity caused by PM and CPF may be significant over the long term. This year's GBR binding studies to estimate transporter density in striata from treated mice confirm our initial immunocytochemical studies suggesting no deficit in DAT levels in mice treated with 200 mg/kg PM. However, we did not observe a loss of striatal dopamine after 100 mg/kg CPF or 200 mg/kg PM, which would have been expected if significant cytotoxicity had occurred.

Third, the strong up-regulation of muscarinic receptors by PM was unexpected, and may play a role in reducing motor activity, since muscarinic agonists such as oxotremorine cause bradykinesia and tremor. The mild up-regulation of 25 mg/kg PM indicates it is near the NOEL for this effect. The down-regulation of muscarinic receptors caused by CPF is similar to that observed in previous studies.

The significance of these studies for the neurotoxicology of insecticides is applicable beyond the scope of Parkinson's disease, especially the upregulation of transport at low doses of PM. We have previously observed that the organochlorine heptachlor (Bloomquist *et al.*, 1998) and the pyrethroid deltamethrin increase dopamine transport (Kirby *et al.*, 1999). However, the latter studies did not include a dose-response analysis for this effect. Now, we have extended this observation to permethrin and shown that this action occurs at doses at least two orders of magnitude below the LD<sub>50</sub>. Thus, up-regulated DAT is a sensitive index of CNS exposure to insecticides and may be

generalized to include other classes of neurotoxins as well. Studies on mitochondrial impairment have also provided additional significant findings, since mitochondrial dysfunction is implicated in a number of neurodegenerative diseases besides PD (Beal *et al.*, 1993). Thus, our observation of compromised mitochondrial function following insecticide exposure may broaden the possible roles of insecticide exposure in other neurological conditions.

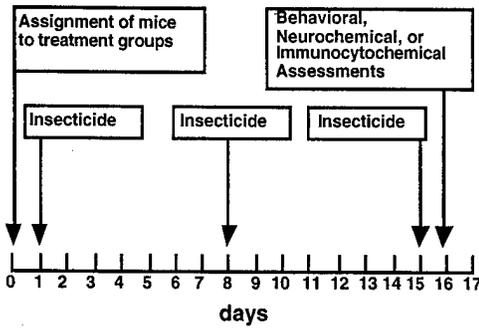


Figure 1. Treatment regime for studies with insecticides alone (PM, CPF) or in combination (PM+CPF).

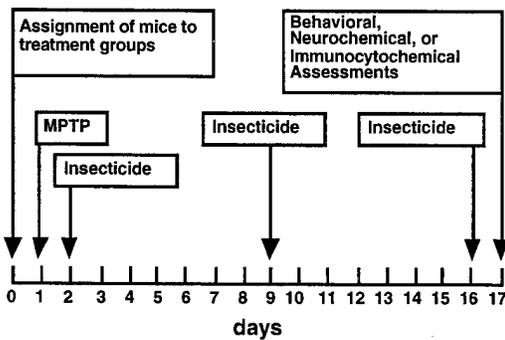


Figure 2. Treatment regime for studies of insecticides in combination with MPTP (MPTP, MPTP +PM, MPTP + CPF, MPTP+PM+CPF). Animals receiving MPTP alone were also given insecticide vehicle.

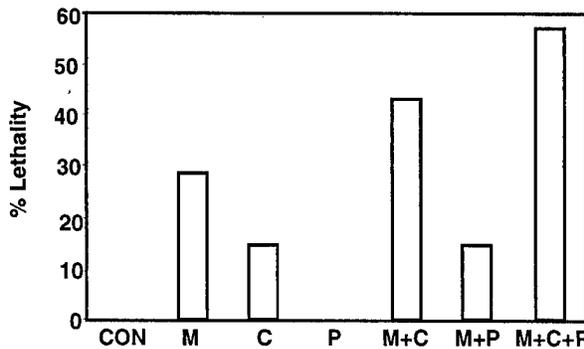


Figure 3. Lethality in combination toxicant treatments. Con = control, M = MPTP, C = chlorpyrifos, and P = permethrin. Mice were treated by injection with 30 mg/kg ip MPTP (M) in saline, 75 mg/kg subcutaneous chlorpyrifos (C) in corn oil, or 200 mg/kg permethrin (P) in methoxytriglycol. Unless otherwise indicated, these are the treatments used in all subsequent figures. Multiple control groups getting appropriate vehicle were also done. No vehicle effects were typically observed and controls getting all 3 vehicle treatments are typically shown.

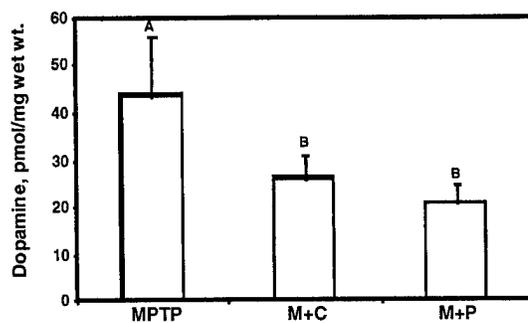


Figure 4. Dopamine titers in mouse striatum following injection with toxicants (M = 30 mg/kg, C = 75 mg/kg, and P = 200 mg/kg). Bars represent means  $\pm$  standard errors. Letters indicate results of ANOVA followed by Student-Newman-Keuls post test ( $p < 0.05$ ). Bars labeled by different letters are significantly different.

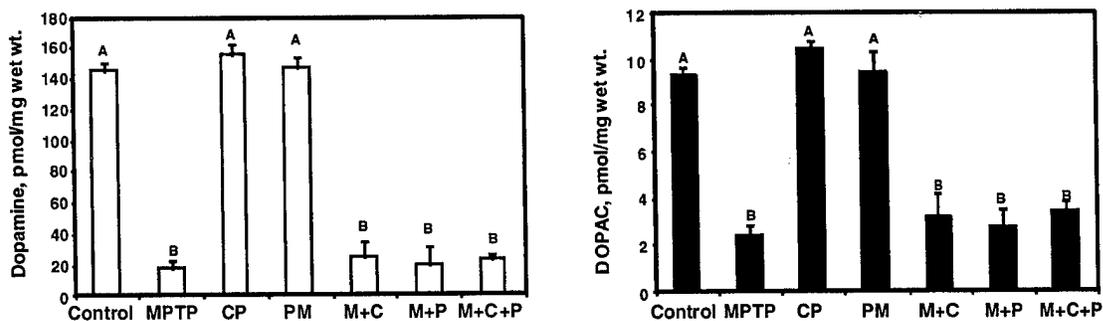


Figure 5. Dopamine (left) and DOPAC (right) titers in mouse striatum following injection with toxicants, abbreviations and doses as given in Fig. 4. Bars represent means  $\pm$  standard errors. Letters indicate results of ANOVA followed by Student-Newman-Keuls post test ( $p < 0.05$ ). Bars labeled by different letters are significantly different.

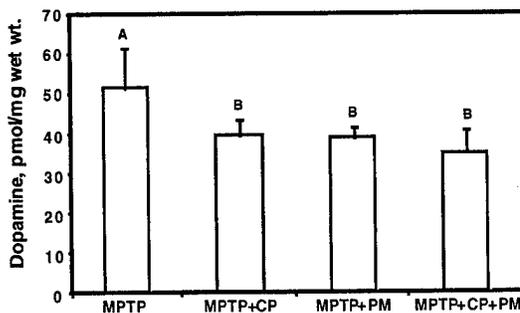


Figure 6. Dopamine titers in mouse striatum following injection with toxicants, abbreviations and doses as given in Fig. 4. Bars represent means  $\pm$  standard errors. Letters indicate results of ANOVA followed by Student-Newman-Keuls post test ( $p < 0.05$ ). Bars labeled by different letters are significantly different.

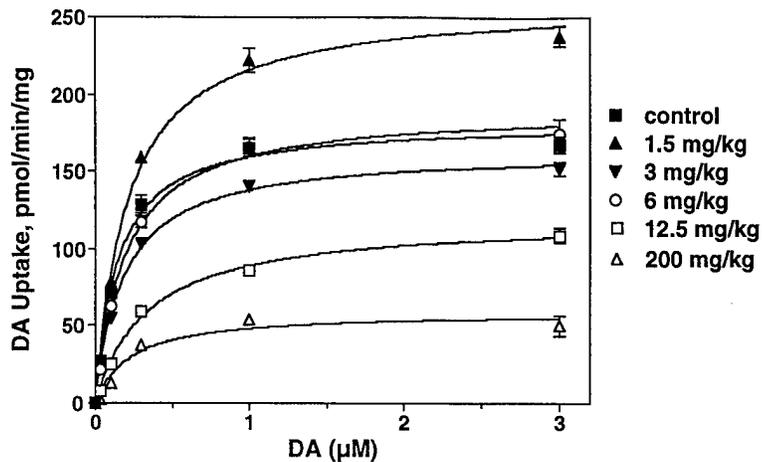


Figure 7. Representative isotherm plots of dopamine uptake in *ex vivo* striatal synaptosomes isolated from mice given the indicated doses of PM. Symbols represent means of three determinations from the same membrane preparation and bars the SEM. Most of the kinetic data was presented in last year's report and is not repeated here.

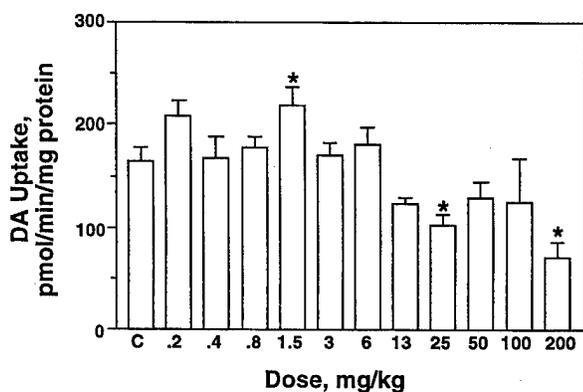
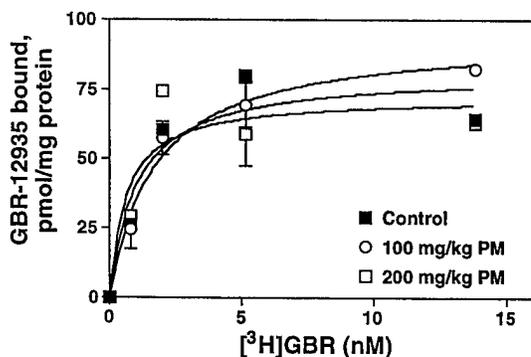
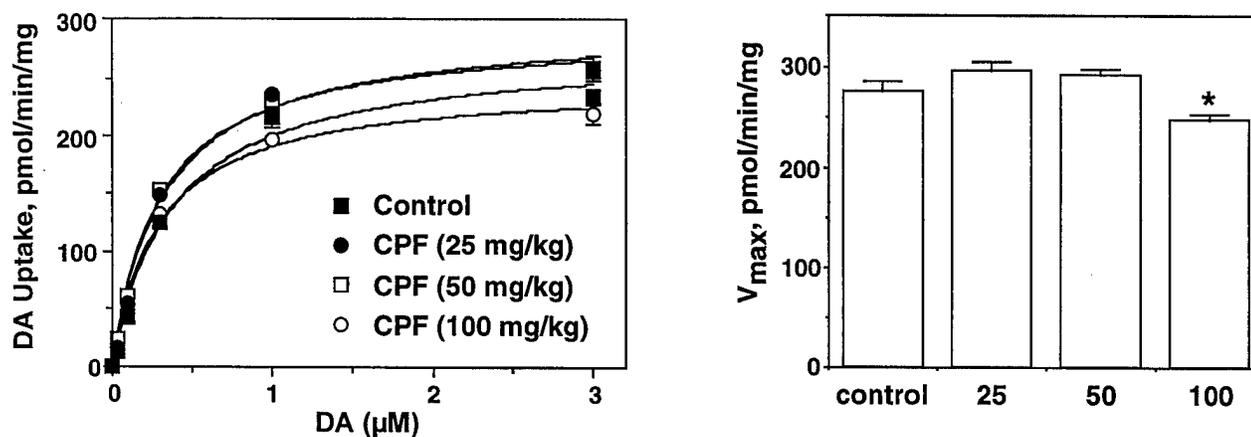


Figure 8. Changes in maximal dopamine uptake ( $V_{max}$ ) following treatment with PM. Asterisks indicate effects significantly different from control (t-test,  $p < 0.05$ ).



Equation 1	control	100 mg/kg PM	200 mg/kg PM
<b>Variables</b>			
BMAX	81.25	94.30	72.46
KD	1.046	1.734	0.6490
<b>Std. Error</b>			
BMAX	8.803	8.660	11.56
KD	0.4557	0.4787	0.4654
<b>95% Confd. Intervals</b>			
BMAX	60.95 to 101.5	73.82 to 114.8	45.12 to 99.79
KD	-0.005 to 2.096	0.602 to 2.866	-0.452 to 1.750
<b>Goodness of Fit</b>			
Degrees of Freedom	8	7	7
R squared	0.8858	0.9553	0.8107
Abs. Sum Squares	983.8	354.8	1355
Sy.x	11.09	7.120	13.91

Figure 9. GBR12935 binding to *ex vivo* striatal synaptosomes prepared from PM-treated mice. Left, isotherm binding plots; Right, kinetic and statistical parameters for the data.



Equation 1	Control	CPF (25)	CPF (50)	CPF (100)
<b>Variables</b>				
BMAX	275.1	295.9	291.0	246.9
KD	0.3737	0.3181	0.2966	0.2907
<b>Std. Error</b>				
BMAX	9.654	8.985	7.359	6.164
KD	0.04353	0.03327	0.02624	0.02550
<b>95% Confidence Intervals</b>				
BMAX	254.7 to 295.6	276.8 to 314.9	275.4 to 306.6	233.8 to 259.9
KD	0.28 to 0.46	0.24 to 0.39	0.24 to 0.35	0.23 to 0.34
<b>Goodness of Fit</b>				
Degrees of Freedom	16	16	16	16
R squared	0.9846	0.9871	0.9903	0.9904
Absolute Sum of Squares	2434	2457	1754	1253
Sy.x	12.34	12.39	10.47	8.848
<b>Data</b>				
Number of X values	6	6	6	6
Number of Y replicates	3	3	3	3
Total number of values	18	18	18	18
Number of missing values	0	0	0	0

Figure 10. Effect of CPF treatment on dopamine uptake in *ex vivo* striatal synaptosomes. Isotherm plots are shown in the upper left of the figure, along with a bar graph of Vmax values in the upper right. In the bar graph, the asterisk indicates an effect significantly different from control (t-test,  $p < 0.05$ ). Kinetic and statistical analysis of this data are given in the accompanying table.

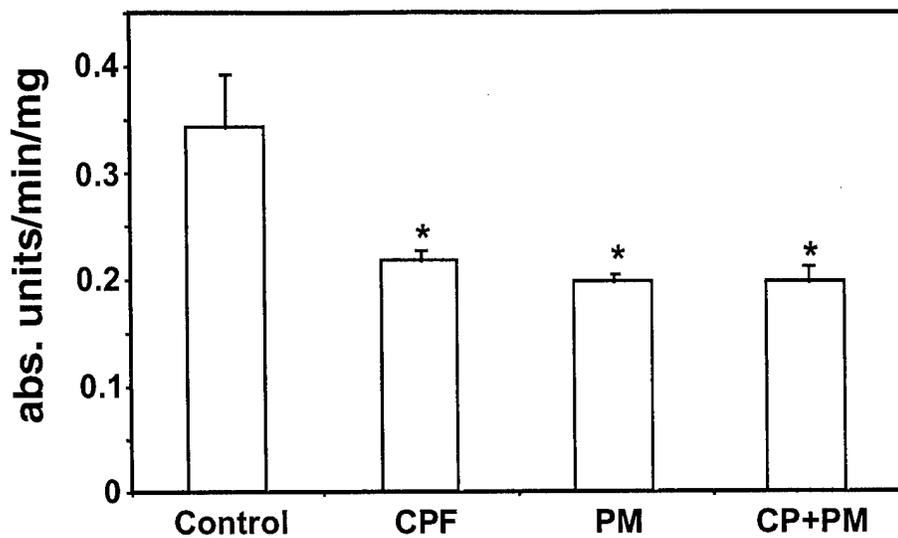


Figure 11. MTT reduction in striatal synaptosomes following insecticide treatment. Asterisk indicates that the difference is significantly different from control (t-tests,  $p < 0.01$ ).

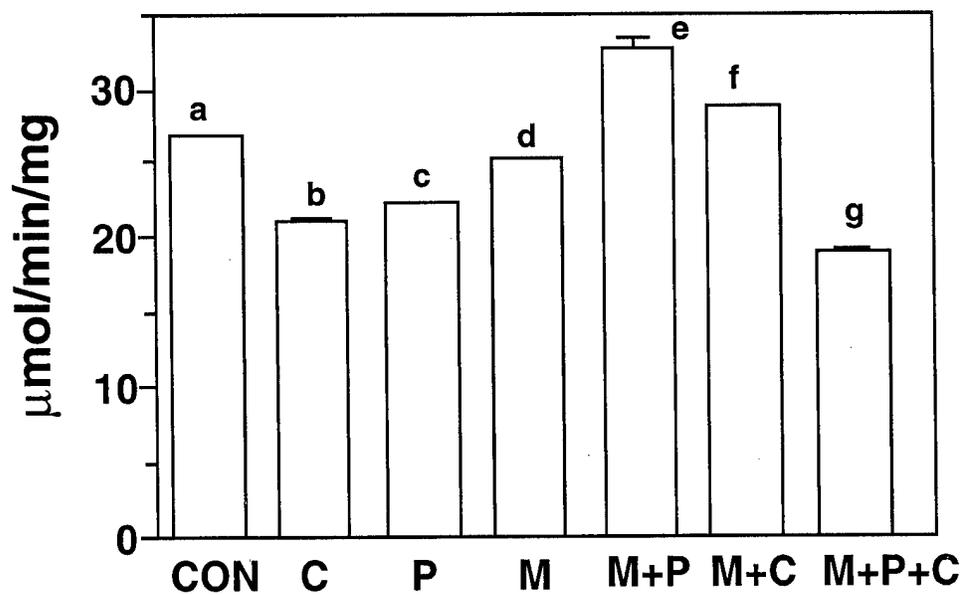


Figure 12. MTT reduction in striatal synaptosomes following toxicant treatment. Abbreviations as defined previously. Bars labeled by different letters are significantly different (ANOVA with Student-Newman-Keuls post test,  $p < 0.05$ ).

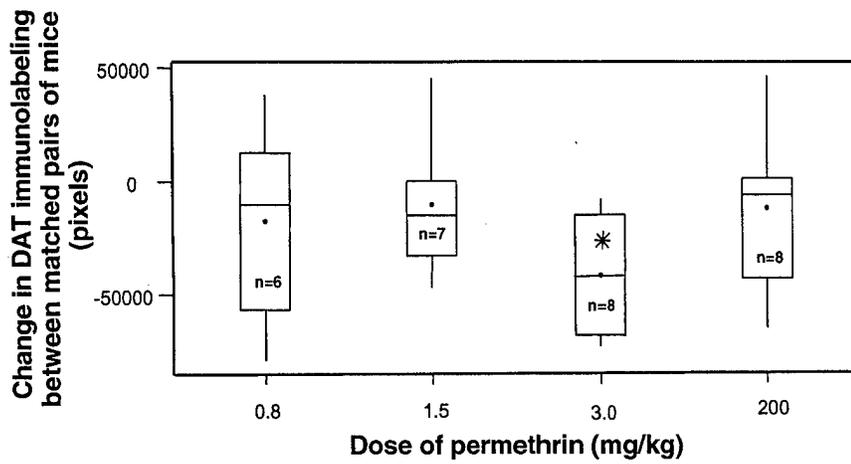


Figure 13. Change in immunolabeling of the DAT following treatment with PM. See text for explanation.

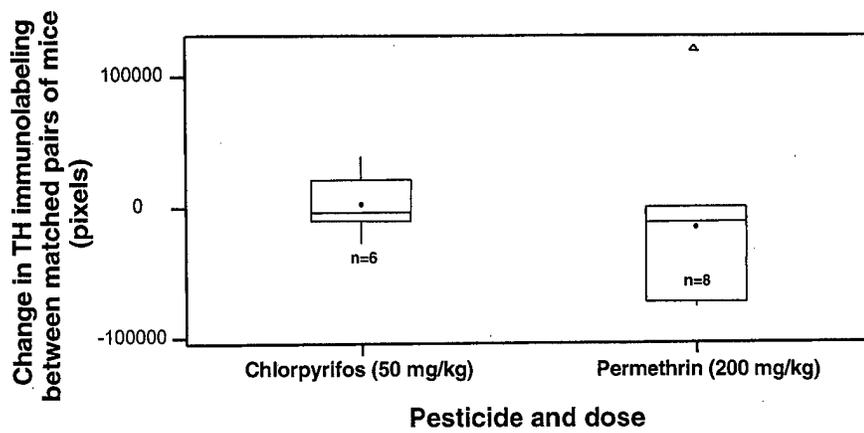


Figure 14. Effect of PM and CPF treatment on striatal TH labeling. See text for explanation.

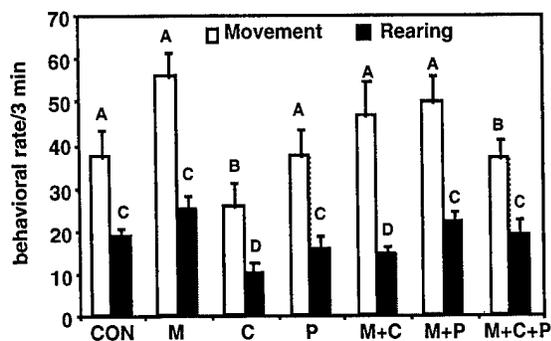


Figure 15. Movement and rearing following toxicant treatment. Abbreviations as defined previously and MPTP at 20 mg/kg. For movement or rearing measurements, bars labeled by different letters are significantly different (ANOVA with Student-Newman-Keuls post test,  $p < 0.05$ ).

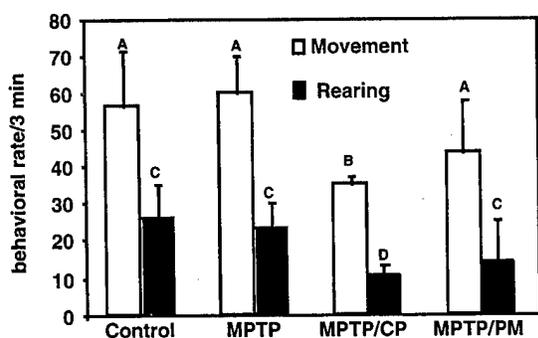


Figure 16. Movement and rearing following toxicant treatment. Abbreviations as defined previously and MPTP at 30 mg/kg. For movement or rearing measurements, bars labeled by different letters are significantly different (ANOVA with Student-Newman-Keuls post test,  $p < 0.05$ ).

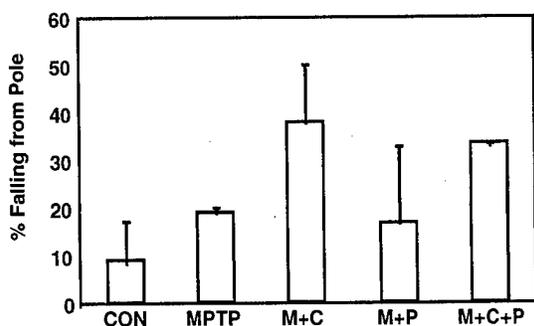


Figure 17. Falling behavior following toxicant treatment. Abbreviations as defined previously and MPTP at 30 mg/kg. Bars are the mean (with SEM) percentage falling, replicated twice.

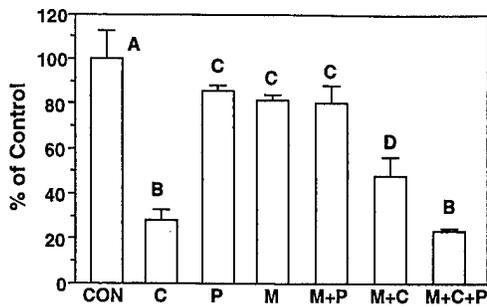
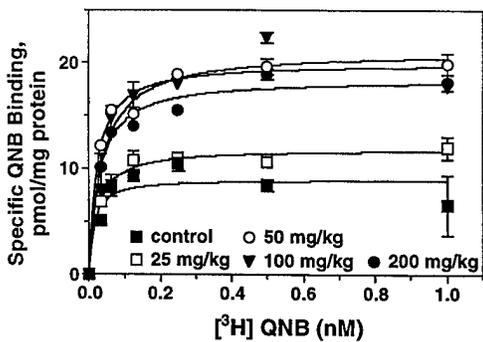
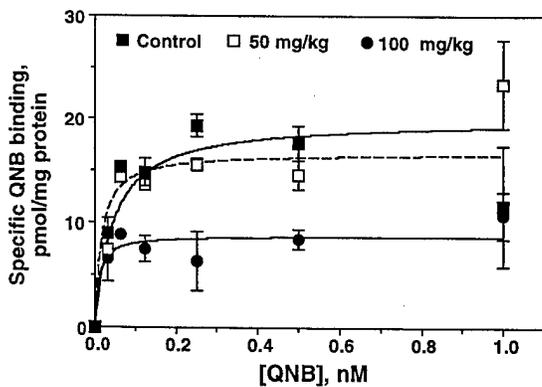


Figure 18. Striatal cholinesterase activity following toxicant treatment. Abbreviations as defined previously and MPTP at 30 mg/kg. Bars labeled by different letters are significantly different (ANOVA with Student-Newman-Keuls post test,  $p < 0.05$ ).



Equation 1	control	50 mg/kg	100 mg/kg	200 mg/kg	25 mg/kg
<b>Variables</b>					
BMAX	8.976	20.01	21.13	18.47	11.89
KD	0.01292	0.02184	0.03642	0.02771	0.02317
<b>Std. Error</b>					
BMAX	0.8692	0.5318	1.308	0.5293	0.4368
KD	0.01018	0.003506	0.01082	0.004279	0.004991
<b>95% Conf. Intervals</b>					
BMAX	7.082 to 10.87	18.85 to 21.17	18.28 to 23.98	17.32 to 19.63	10.94 to 12.84
KD	-0.009 to 0.035	0.014 to 0.029	0.013 to 0.06	0.018 to 0.037	0.012 to 0.034
<b>Goodness of Fit</b>					
Degrees of Freedom	12	12	12	12	12
R squared	0.7355	0.9759	0.9031	0.9741	0.9561
Abs. Sum Squares	43.59	14.09	69.03	12.77	9.308
Sy.x	1.906	1.083	2.398	1.031	0.8807

Figure 19. Enhancement of QNB binding following PM treatment. Left, typical isotherm plots; Right, kinetic and statistical analysis values.



Equation 1	Control	50 mg/kg	100 mg/kg
<b>Variables</b>			
BMAX	16.73	19.96	8.821
KD	0.01565	0.04490	0.009417
<b>Std. Error</b>			
BMAX	2.018	2.417	0.9086
KD	0.01367	0.02408	0.009733
<b>95% Confidence Intervals</b>			
BMAX	11.54 to 21.92	13.75 to 26.18	6.485 to 11.16
KD	-0.0195 to 0.0508	-0.0170 to 0.1068	-0.0156 to 0.0344
<b>Goodness of Fit</b>			
Degrees of Freedom	5	5	5
R squared	0.9084	0.9309	0.9239
Absolute Sum of Squares	23.36	22.00	5.287
Sy.x	2.162	2.098	1.028

Figure 20. Reduction of QNB binding following CPF treatment. Left, typical isotherm plots with the control curve given as a dashed line; Right, kinetic and statistical analysis values.

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## APPENDICES

(see following pages)

## Meeting Abstracts (formatted as per different society guidelines)

### Abstracts for Society for Neuroscience Meeting, Fall, 2000:

#### STRIATAL DOPAMINERGIC PATHWAYS AS TARGET FOR THE INSECTICIDES PERMETHRIN AND CHLORPYRIFOS.

W. Li\*, P. Harp, D. Karen, B. Klein, and J. Bloomquist. Lab. of Neurotoxicology, Department of Entomology, Virginia Polytechnic Institute and State University, Blacksburg, VA, 24061. To study the neurotoxic actions of insecticides permethrin (PM) and chlorpyrifos (CPF) as they related to the striatal dopaminergic pathways, C57BL/6 mice were exposed subchronically to PM (3 intraperitoneal doses at 0.2-200 mg/kg) and CPF (3 subcutaneous doses at 1.5-100 mg/kg), respectively during a two week period. The exposed mice were then analyzed by using behavioral, neurochemical, and immunocytochemical parameters. It was found that PM has effects on [<sup>3</sup>H]dopamine uptake in striatal synaptosomes of treated mice, displaying a bell-shaped curve with a peak increase of about 134% of control at doses of 1.5-6mg/kg. At higher doses of PM ( $\geq 25$  mg/kg), dopamine uptake declined to a level significantly below that of control (50% of control at 200 mg/kg,  $P < 0.01$ ). Since immunocytochemical labeling of the striatum showed that the level of transporter staining was near control, and we confirmed that there was no difference in [<sup>3</sup>H]GBR 12,935 binding between control and 200 mg/kg PM treatment groups, toxic effects may have been involved. We also found that CPF has no effects on [<sup>3</sup>H]dopamine uptake. Even though cytotoxicity was not reflected in decreased levels of striatal dopamine (either in 200 mg/kg PM or 100 mg/kg CPF treatment group) by high pressure liquid chromatograph, an increase in dopamine turnover at 100 mg/kg CPF, as indicated by a significant increase in titers of the dopamine metabolite, 3,4-dihydroxyphenylacetic acid, was observed. In *in vitro* dopamine release studies, we found that CPF at 10  $\mu$ M concentration stimulates about 80% dopamine release with an  $IC_{50}$  of 3  $\mu$ M. These findings suggest that dopaminergic neurotransmission may be affected by exposure to the PM and CPF and may contribute to the overall spectrum of toxicity targeted by these insecticides. Supported by the U.S. Army, contract DAMD17-98-1-8633.

#### INSECTICIDE ACTION ON BEHAVIOR AND STRIATAL CHOLINERGIC BIOMARKERS.

J. R. Bloomquist\*, P. Harp, D. J. Karen, and W. Li. Laboratory of Neurotoxicology, Department of Entomology, Virginia Polytechnic Institute and State University, Blacksburg, VA, 24061. A series of behavioral and neurochemical studies were undertaken to characterize the possible role of insecticide exposure in the etiology of Parkinson's disease as it may relate to Gulf War Syndrome. The insecticides under study were the organophosphorus compound chlorpyrifos (CP) and the pyrethroid, permethrin (PM), given three times over two weeks by injection (CP by sc; PM by ip). Chlorpyrifos at 25-100 mg/kg caused 15-84% inhibition of brain AchE, which correlated reasonably well with dose-dependent effects on open field, rearing, and pole climbing behaviors. Additionally, CP treatment increased striatal [<sup>3</sup>H]QNB binding ( $B_{max}$ ) at 25 mg/kg, but decreased binding at 100 mg/kg. While there was no consistent, dose-related effect of PM treatment on brain AchE activity, PM did cause a decline in open field behavior. This latter effect may have been related to a changes in striatal muscarinic receptor density. Permethrin treatment significantly increased striatal [<sup>3</sup>H]QNB binding at doses of 25-200 mg/kg. Increases in  $B_{max}$  over control ranged from 33% at 25 mg/kg to a 135% increase at 100 mg/kg. However,  $K_d$  values were not significantly altered by PM or CP treatments. These studies demonstrated significant effects on behavior and striatal cholinergic neurochemistry by these insecticides. Supported by the U.S. Army, contract DAMD-17-98-1-8633.

**Abstracts for Society of Environmental Toxicology and Chemistry meeting, Fall, 2000:**

**Murine Dopaminergic Pathways as Targets of Multiple Chlorpyrifos or Permethrin Exposures.** Li, W., Karen, D.J.\*, Harp, P., Klein, B., and Bloomquist, J.R., Virginia Polytechnic Institute and State University, Department of Entomology, Blacksburg, VA. C57BL/6 mice were exposed subchronically to permethrin (3 ip injections at 0.2-200 mg/kg) and chlorpyrifos (3 sc injections at 1.5-100 mg/kg) during a two week period to study neurotoxic actions as they related to striatal dopaminergic pathways. Chlorpyrifos had no effect on [<sup>3</sup>H]dopamine uptake. Cytotoxicity was not reflected in decreased levels of striatal dopamine in 200 mg/kg permethrin or 100 mg/kg chlorpyrifos. However, elevated dopamine turnover at 100 mg/kg chlorpyrifos, as indicated by significantly increased titers of the dopamine metabolite, 3,4-dihydroxyphenylacetic acid, was observed. During *in vitro* dopamine release studies, chlorpyrifos at 10  $\mu$ M concentration stimulated approximately 80% dopamine release with an IC<sub>50</sub> of 3  $\mu$ M. Permethrin (1.5 – 6 mg/kg) affected [<sup>3</sup>H]dopamine uptake in striatal synaptosomes of treated mice and displayed a bell-shaped curve peaking at 134% of control. At higher doses of permethrin ( $\geq$  25 mg/kg), dopamine uptake declined to a level significantly below the control (50% of control at 200 mg/kg,  $p < 0.01$ ). Immunocytochemical labeling of the striatum showed that the level of transporter staining was near control, and we confirmed that there was no difference in [<sup>3</sup>H]GBR 12,935 binding between control and 200 mg/kg permethrin treatment groups. Therefore, toxic effects may have been involved. These findings suggested that dopaminergic neurotransmission may be affected by exposure to permethrin and chlorpyrifos and may contribute to the overall spectrum of toxicity targeted by these insecticides.

**Effects of Multiple Exposures of Chlorpyrifos or Permethrin on Murine Behavior and Striatal Cholinergic Biomarkers.** Karen, D.J.\*, Harp, P., Li, W., and Bloomquist, J.R., Virginia Polytechnic Institute and State University, Department of Entomology, Blacksburg, VA. Several behavioral and neurochemical studies were performed to characterize the potential for insecticides to affect striatal cholinergic pathways. Chlorpyrifos and permethrin were given three times over two weeks by injection (chlorpyrifos by sc; permethrin by ip). While there was no consistent, dose-related effect of permethrin treatment on brain AChE activity, permethrin injections caused a decline in open field behavior. This latter effect may have been related to changes in striatal muscarinic receptor density, measured immunohistochemically. Permethrin treatment significantly increased striatal [<sup>3</sup>H]QNB binding at doses of 25-200 mg/kg. Increases in B<sub>max</sub> over control values ranged from 33% at 25 mg/kg to a 135% increase at 100 mg/kg. However, K<sub>d</sub> values were not significantly altered by permethrin or chlorpyrifos treatments. Chlorpyrifos caused up to 84% inhibition of brain AChE, which correlated reasonably well with dose-dependent effects on open field, rearing, and pole climbing behaviors. Chlorpyrifos treatment also increased striatal [<sup>3</sup>H]QNB binding at 25 mg/kg, but decreased binding at 100 mg/kg. These studies demonstrated significant effects on behavior and striatal cholinergic neurochemistry by these insecticides.

**Abstracts for Society of Toxicology meeting, Spring, 2001:**

**Striatal Dopaminergic Pathways as Targets of Chlorpyrifos or Permethrin Exposures: Comparison with the Parkinsonian Neurotoxin MPTP**

DJ Karen, W Li, P Harp, JS Gillette, B Klein and JR Bloomquist *Dept. of Entomology, Virginia Polytechnic Institute and State University, Blacksburg, VA*

To assess the effects of subchronic pesticide exposure on striatal dopaminergic pathways, male C57BL/6 mice from retired breeder stock were dosed for 2 weeks with permethrin (3 i.p. injections at 0.2-200 mg/kg) or chlorpyrifos (3 s.c. injections at 1.5-100 mg/kg). [<sup>3</sup>H]Dopamine uptake was significantly increased at lower chlorpyrifos doses, peaking at 108% of control, while higher doses (100 mg/kg) of chlorpyrifos significantly repressed [<sup>3</sup>H]dopamine uptake to 89% of controls. Dopamine uptake was also affected in a dose-dependent manner by permethrin treatment, resulting in a bell-shaped curve. At a dose of 1.5 mg/kg, dopamine uptake peaked at 125% of control, and declined at higher doses to 45% of control at 200 mg/kg. Immunocytochemical labeling of the striatum showed the level of transporter staining to be near that of controls. Further, there was no difference in [<sup>3</sup>H]GBR 12,935 binding between the control and 200 mg/kg permethrin treatment groups. Therefore, decreases in dopamine uptake at high doses of insecticide may be due to toxic effects. Permethrin at a dose of 200 mg/kg significantly decreases mitochondrial dehydrogenase activity compared to controls, as measured by the formation of MTT-formazan. Striatal dopamine levels were not affected by treatment with either 100 mg/kg chlorpyrifos or 200 mg/kg permethrin, however, a dose of 100 mg/kg chlorpyrifos significantly increased striatal titers of the dopamine metabolite 3,4-dihydroxyphenylacetic acid (DOPAC). Permethrin at a dose of 200 mg/kg did not change DOPAC levels. Additionally, permethrin or chlorpyrifos dosed in conjunction with the established dopaminergic neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) significantly decreased striatal dopamine levels as compared to controls and MPTP alone. These findings suggest that dopaminergic neurotransmission may be affected by exposure to permethrin and chlorpyrifos and may contribute to the spectrum of toxicity elicited by these insecticides, including parkinsonism.

**Effects of Subchronic Exposures of Chlorpyrifos or Permethrin on Behavior and Striatal Cholinergic Biomarkers in C57BL/6 Mice**

DJ Karen, P Harp, W Li, JS Gillette and JR Bloomquist *Dept. of Entomology, Virginia Polytechnic Institute and State University, Blacksburg, VA*

The C57BL/6 mouse given 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is an established animal model of Parkinson's disease (PD). Because cholinergic systems interact with the neural substrates of PD, behavioral and neurochemical studies were performed to characterize the effect of insecticides on striatal cholinergic pathways. Chlorpyrifos and permethrin were administered to retired male breeder C57BL/6 mice three times over two weeks by i.p. (permethrin) or s.c. (chlorpyrifos) injection. Brain AChE activity was little affected by permethrin treatment, however, permethrin treatment did cause a decline in open field behavior. This effect may have been due to changes in striatal muscarinic receptor density, since permethrin treatment significantly increased striatal [<sup>3</sup>H]QNB binding at doses of 25-200 mg/kg. Increases in  $B_{max}$  over control values ranged from 33% at 25 mg/kg to a 135% increase at 100 mg/kg.  $K_d$  values were not significantly altered by either chlorpyrifos or permethrin treatments. Chlorpyrifos (100 mg/kg) caused up to an 84% inhibition of brain AChE, which correlated reasonably well with dose-dependent effects on open field, rearing, and pole climbing behaviors. Chlorpyrifos treatment also increased striatal [<sup>3</sup>H]QNB binding at 25 mg/kg, but decreased binding at 100 mg/kg. These studies demonstrated significant effects on behavior and striatal cholinergic neurochemistry by these insecticides.







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## **PUBLICATIONS**

Gillette, J.S., Hansen, L.G., Rose, R.L., and Hodgson, E., Modulation of Testosterone and Estradiol Metabolism and In Vivo Estrogenicity of PCB 77 (3,3',4,4'-tetrachlorobiphenyl), PCB 110 (2,3,3',4',6-pentachlorobiphenyl), PCB 118 (2,3',4,4',5-pentachlorobiphenyl), and PCB 153 (2,2',4,4',5,5'-hexachlorobiphenyl). (2000) Submitted.

Gillette, J.S., Hansen, L.G., Rose, R.L., and Hodgson, E., Induction of Cytochrome P450 Isoforms and Modulation of Testosterone Metabolism in Male and Female CD-1 Mice by PCB 47 (2,2',4,4'-tetrachlorobiphenyl), PCB 110 (2,3,3',4',6-pentachlorobiphenyl), PCB 149 (2,2',3,4',5',6-hexachlorobiphenyl), and PCB 153 (2,2',4,4',5,5'-hexachlorobiphenyl). (2000) Submitted.

LeBlanc, G.A. and Gillette, J.S., Elevation of Serum Cholesterol Levels in Mice by the Antioxidant Butylated Hydroxyanisole. (1993) *Biochemical Pharmacology* 45: (2) 513-515.

## **PRESENTATIONS**

J.S. Gillette, R.L. Rose, and E. Hodgson. (1999). Modulation of Cytochrome P450 Isoforms and Steroid Hormone Metabolism in Juvenile CD-1 Mice by PCB 118 and PCB 153. Presented at the 9<sup>th</sup> North American Meeting of the International Society for the Study of Xenobiotics. Nashville, TN, Oct. 24-28 1999.

J.S. Gillette, R.L. Rose, and E. Hodgson. (1999). Alterations of Steroid Hormone Metabolism in Juvenile CD-1 Mice by PCB 153. *The Toxicologist*, 48(1-S), 1315.

## **PROFESSIONAL AFFILIATIONS**

International Society for the Study of Xenobiotics 1996-Present

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**STRIATAL DOPAMINERGIC PATHWAYS AS A TARGET FOR THE INSECTICIDES  
PERMETHRIN AND CHLORPYRIFOS**

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**Key words: Parkinson's disease, dopamine transport, pyrethroid, organophosphate**

## ABSTRACT

The neurotoxic actions of the insecticides permethrin and chlorpyrifos to striatal dopaminergic pathways were investigated. C57BL/6 mice were exposed to permethrin (3 intraperitoneal doses at 0.2-200 mg/kg) or chlorpyrifos (3 subcutaneous doses at 25-100 mg/kg) during a two week period. The exposed mice were then analyzed using behavioral and dopamine neurochemical parameters. It was found that permethrin altered the maximal [<sup>3</sup>H]dopamine uptake in striatal synaptosomes of treated mice, with changes in  $V_{max}$  displaying a bell-shaped curve. Uptake was increased to about 134% of control at a dose of 1.5 mg/kg. At higher doses of PM ( $\geq 25$  mg/kg), dopamine uptake declined to a level significantly below that of control (50% of control at 200 mg/kg,  $p < 0.01$ ). We also found a small, but statistically significant effect of chlorpyrifos on [<sup>3</sup>H]dopamine uptake, when given at a dose of 100 mg/kg. There was no significant effect on the  $K_m$  for dopamine transport in any of the insecticide-treated mice. No change in dopamine transporter density was observed in [<sup>3</sup>H]GBR 12935 binding in striatal membranes from mice given 100 and 200 mg/kg permethrin, so other effects may have been involved in the reduced uptake at these doses. Evidence of cell stress was observed in measures of mitochondrial function, which were reduced in mice given high-end doses of chlorpyrifos and permethrin. Although cytotoxicity was not reflected in decreased levels of striatal dopamine in either in 200 mg/kg PM or 100 mg/kg CPF treatment groups by high pressure liquid chromatography, an increase in dopamine turnover at 100 mg/kg CPF was indicated by a

significant increase in titers of the dopamine metabolite, 3,4-dihydroxyphenylacetic acid. Both permethrin and chlorpyrifos caused a decrease in open field behavior at the highest doses tested. These findings confirm that dopaminergic neurotransmission may be affected by exposure to pyrethroid and organophosphorus insecticides, and may contribute to the overall spectrum of toxicity targeted by these compounds.

## INTRODUCTION

Insecticides are widely used by the military (Corporate author, 1991), and pose a potential hazard to military personnel. In the military, insecticides are commonly used for disease vector control (Mount, 1996) and as termiticides in military buildings (Corporate Author, 1982; Gebhart, 1983). The pyrethroid and organophosphorus (OP) insecticides are members of two chemical classes of heavily used insecticides, and exposure data indicate that intoxication of individuals by OP insecticides is one of the most common forms of chemical poisoning (D'Mello, 1993). A hazard from exposure to insecticides exists from spraying, long-term storage, or from contact of personnel with insecticide-contaminated areas. Furthermore, the neurological health problems which comprise Gulf War Syndrome are thought to be due, at least in part, to exposure of personnel to various chemicals, including the insecticides permethrin (PM), chlorpyrifos (CPF), and the repellent N,N-diethyl-*m*-toulamide (DEET) (Abou-Donia *et al.*, 1996). Over 30,000 Gulf War veterans have reported having Gulf War Syndrome. In previous studies, we have documented effects of the pyrethroid deltamethrin (Kirby *et al.*, 1999) and the

organochlorine heptachlor (Bloomquist *et al.*, 1999; Kirby *et al.*, 2001) on dopaminergic nerve pathways, which might be a contributory factor in the etiology of environmentally-induced Parkinson's Disease (PD). The present study assessed effects on dopamine pathways following exposure to PM and CPF insecticides to ascertain whether damage to dopaminergic pathways and attendant parkinsonism might be a long term consequence of Gulf War chemical exposures.

## MATERIALS AND METHODS

### Chemicals.

Analytical grade chlorpyrifos was obtained from ChemService, Inc (West Chester, PA). PM (a mixture of *cis* and *trans* isomers) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma Chemical Co., St. Louis, MO. [<sup>3</sup>H]Dopamine (20.3 Ci/mmol) and [<sup>3</sup>H]GBR 12935 (53.5 Ci/mmol) were purchased from NEN Life Science Products, Inc., Boston, MA. Unlabeled GBR 12909, choline-Cl, KCl, MgCl<sub>2</sub>, CaCl<sub>2</sub>, and ascorbate were obtained from Sigma Chemical Co. Pargyline, D-glucose, sucrose, and HEPES were obtained from Fisher Scientific Co., Pittsburgh, PA.

### Animals and Treatments.

Male C57BL/6 retired breeder mice were utilized for all experiments. Mice were purchased from Harlan Sprague-Dawley, Dublin, VA, and were aged 7-9 months (28-40 g live weight) at the time of the experiments. Mice were assigned randomly to treatment groups, which contained a minimum of 6 mice, so that the mean weight of all treatment groups was

approximately equal. CPF carried in corn oil vehicle or PM carried in methoxytriglycol (MTG) vehicle were administered to the mice at multiple doses three times over a two week period according to the method of Bloomquist *et al.* (1999). CPF administration was by subcutaneous injection, while PM was administered by intraperitoneal injection. Control mice received 50 $\mu$ l corn oil or 10  $\mu$ l of MTG vehicle alone. On the day following the last treatment day, mice were killed by cervical dislocation, and striatal tissues were collected at this time.

### **Dopamine Uptake Studies.**

Labeled dopamine uptake studies were performed according to the method outlined in Kirby *et al.*, (1999). Briefly, synaptosomes were prepared from fresh striatal tissue dissected from treated mice, and incubated with [<sup>3</sup>H]dopamine at various concentrations for 2 min. Transport of dopamine was determined after washing and vacuum filtration, followed by liquid scintillation counting. Uptake rates were determined by the method of Krueger (1990) in incubations with and without sodium ions (equimolar choline chloride substitution) in order to correct for low affinity transport. Uptake parameters ( $V_{max}$  and  $K_m$ ) were determined by nonlinear regression to isotherm plots (Prism™, GraphPad Software, San Diego, CA). Aliquots of each synaptosomal preparation were frozen at -70° C for membrane protein determinations, which was according to the method of Bradford (1976).

### **MTT Cytotoxicity Assay**

This assay was run on synaptosomes by adapting the cultured cell methods of Carmichael *et al.* (1987). Striatal synaptosomes were prepared as described in Kirby *et al.*, (1999) and incubated with MTT dissolved in Krebs-Henseleit buffer containing (mM): NaCl (140), KCl (5.0), MgSO<sub>4</sub> (1.3), NaHCO<sub>3</sub> (5.0), Na<sub>2</sub>HPO<sub>4</sub> (1.0), HEPES (10), Glucose (10), and CaCl<sub>2</sub> (1.2), pH = 7.4. After 30 minutes at 37°C, the tubes were centrifuged for 5 minutes at 10,000 x g. The pellets were resuspended in DMSO to solubilize the formazan reduction product, and centrifuged again at 10,000 x g for 1 minute. Background absorbance of MTT (650 nm) was subtracted from test absorbance (580 nm) for the blue formazan product, both determined by a 96-well plate reader (Dynex Technologies, Inc. Chantilly, VA).

### **Dopamine and DOPAC Content.**

The methods employed were similar to those of Hall *et al.* (1992). Striata from individual mice were homogenized in 5% TCA containing 10 ng DHBA/mg tissue wet weight as an internal standard and frozen at -70°C until analysis. Prior to analysis, samples were thawed and centrifuged at 10,000xG to pellet tissues. Dopamine metabolites were separated by HPLC using an ODS 3 µm Phase 2 column (3.2x 100 mm). Mobile phase consisted of 170 mM NaH<sub>2</sub>PO<sub>4</sub>, 1.5 mM octanesulfonic acid, 5.5 % methanol, 1.5 % acetonitrile, and 93 % H<sub>2</sub>O, at a flow rate of 0.6 ml/min. Dopamine, DOPAC and DHBA standards were prepared to quantitate the amounts of dopamine and DOPAC in the samples.

## **Behavioral Assessments.**

On the last day of the study, behavioral effects were assessed by means of monitoring the number of open field movements and rearing in an arena during a three minute period. The open field was the floor of a 10 gallon aquarium, divided into six equally sized squares. A movement was counted when the animal's front paws crossed any grid line. A rear was counted when the mouse raised onto its rear paws, lifting the front paws from the floor of the field. The mice were also placed on the top of a pole, and time to descend the pole or a fall from the pole, was noted. Mice were timed for a maximum of three minutes while on the pole.

## **Statistical Analysis.**

Statistical significance was determined using one-way ANOVA and Student-Newman-Keuls means separation where applicable. Other statistical comparisons were by t-test. Calculations were performed using InStat™ (GraphPad Software).

## **RESULTS**

Maximal dopamine uptake in 9-month-old C57BL/6 mice treated with PM varied with the dose, increasing at a lower dose of PM, and at higher doses uptake declined until it was less than the control level (Fig. 1). PM treatment did not have any significant effect on the apparent  $K_m$  of the dopamine transporter in any of the treatment groups. A bar graph (Fig. 2) of PM-induced  $V_{max}$  values took the form of a bell-shaped curve, in which the maximal rate of dopamine uptake,  $V_{max}$ , peaked at a dose of 1.5 mg/kg. At this dose, dopamine uptake was significantly

greater (33%) than that of the control value, and this dose was replicated in four different groups of mice. All other doses were replicated at least twice. At higher doses of PM (> 25 mg/kg),  $V_{max}$  declined to a level significantly below that of control (50% of control at 200 mg/kg) (Fig.2). This effect of PM on dopamine uptake was also found to be dependent on the age of the C57BL/6 mouse, since 11-month-old mice given the same doses of PM did not exhibit any significant changes in striatal dopamine uptake (data not shown). Treatment with CPF also caused a reduction in dopamine uptake  $V_{max}$  at the highest dose administered (100 mg/kg) (Fig.3). However, doses of CPF below 25 mg/kg were not tested in these experiments.

Striatal MTT dehydrogenase activity, an assay of mitochondrial integrity, was performed on pooled membranes from treated mice. Production of reduced formazan was reduced by 100 mg/kg, but not 50 mg/kg of CPF, which was actually higher than control (Fig.4). The reduction caused by 100 mg/kg CPF was present at all concentrations of thiazolyl blue (MTT) tested, although only data at 0.55 mM is shown. PM also reduced MTT dehydrogenase activity, but at lower doses than CPF. At doses of 12.5, 25 and 50 mg/kg PM, MTT dehydrogenase activity was depressed 9, 12, and 14% respectively. Similarly, there was a statistically significant 9% decrease in mitochondrial activity in a separate group of mice given 200 mg/kg PM.

CPF (100 mg/kg), but not PM (200 mg/kg) increased striatal dopamine turnover, as indicated by significantly elevated titers of the dopamine metabolite, DOPAC (Fig. 5). The

effect of CPF at this dose was an increase of 14% above control. Neither CPF nor PM at these doses had any effect on striatal dopamine levels (data not shown).

CPF and PM both had similar effects on mouse behavior, according to movement and rearing tests. Statistically significant effects on rearing and movement were observed only at the highest doses of both compounds. Both movement and rearing frequency were decreased by treatment with 50 and 100 mg/kg CPF; however, the decrease at 50 mg/kg was not statistically significant (Fig. 6). High doses of PM decreased both frequency of open field movement and rearing frequency (Fig.7). This effect was only significant at the 50 and 200 mg/kg doses, and not the 100 mg/kg dose, however.

## DISCUSSION

The effect of 1.5 mg/kg, PM for increasing the maximal transport of dopamine uptake is a potent action of this compound, *in vivo*. This dose is about 3 orders of magnitude below the rat oral PM LD<sub>50</sub> (Budavari *et al.*, 1996), and we never observed any lethality at the highest dose (200 mg/kg) used in this study. Moreover, technical permethrin is a mixture of four (1*R,S cis* and 1*R,S, trans*) isomers, only one of which (1*R, cis*) has lethal effects in mammals (Casida *et al.*, 1983). If the 1*R, cis* isomer is responsible for the up-regulation, it is only 25% of the applied dose, and was actually given at about 0.4 mg/kg. We assume that the observed increase in dopamine uptake was compensatory for permethrin-dependent increases in dopamine release, *in vivo*. We have recently shown that the related pyrethroid deltamethrin releases a variety of

neurotransmitters from preloaded synaptosomes, with the effect on dopamine 2.4- and 8.6-fold more potent than serotonin or glutamate release, respectively (Kirby *et al.*, 1999).

As the dose of PM increased, maximal transport of dopamine decreased to a level about 50% below that of controls, most likely from an inability of the synaptosomes to retain dopamine, rather than a true effect on dopamine transport. This conclusion is based on the fact that [<sup>3</sup>H]GBR 12935 binding was not changed. We would expect toxicity of the nerve terminals to be reflected in loss of striatal dopamine, which was not observed. However, there was evidence of cell stress in mice treated with doses  $\geq 12.5$  mg/kg PM in the MTT assay, which is a measure of mitochondrial function (Carmichael *et al.*, 1987). In future studies, we do expect to observe an up-regulation of dopamine transporter (DAT) expression via increased GBR binding at doses near 1.5 mg/kg of PM. Previous work in our laboratory has shown that the organochlorine insecticide heptachlor increases dopamine transport in male C57BL/6 mice about 2-fold at a dose of 6 mg/kg and this increase in uptake was accompanied by an increase in DAT protein labeling in western blots of striatal membranes (Miller *et al.*, 1999). Moreover, the dose-response curve for heptachlor has a shape similar to that reported here for PM (Kirby *et al.*, 2001). We have also demonstrated this effect for the pyrethroid insecticide deltamethrin, which increased dopamine uptake by 70 % at a 6 mg/kg dose (Kirby *et al.*, 1999).

In contrast to PM, striatal dopamine uptake is not up-regulated by lower doses of CPF, however, in these experiments doses under 25 mg/kg CPF were not tested. At higher doses of

CPF (100 mg/kg), dopamine transport  $V_{max}$  is significantly decreased, as is the case with PM. Similarly, at a dose of 100 mg/kg CPF, MTT dehydrogenase activity is significantly depressed compared to controls.

CPF and PM failed to have an effect on striatal dopamine titers at the relatively high doses administered (data not shown). However, incipient effects on dopamine could be occurring that are masked when measured as total amount of striatal dopamine by HPLC. The effect may be similar to that seen in aged mice, in which 68% of the dopaminergic neurons are lost naturally, but there is a 103% increase in dopamine synthesis by the remaining neurons as a compensatory effect (Tatton *et al.*, 1991). DOPAC levels were increased by treatment with a high dose of CPF, but not PM. Loss of dopamine and DOPAC is a cardinal sign of PD (Hornykiewicz and Kish, 1987) and can reflect changes in both cellular levels of dopamine and cell death in the striatum. Elevated levels of DOPAC indicate greater turnover of dopamine in response to toxicant-induced processes (Hudson *et al.*, 1985). We assumed that CPF increased turnover through neuronal hyperexcitation caused by inhibition of acetylcholinesterase, although interaction with other targets cannot be ruled out. The related compound methyl parathion, given at low doses (0.1 mg/kg a day for 15 days) to neonatal rats had little or no effect on dopamine content (Kumar and Desiraju, 1992). Soman induced an increase in DOPAC levels, consistent with an increase in dopamine turnover, but no change in dopamine levels (el-Etri *et al.*, 1992; Fosbraey *et al.*, 1990). We were somewhat surprised by the lack of any effect of PM on

DOPAC, given that increased levels of striatal DOPAC had been demonstrated with this compound previously (Doherty et al., 1988). However the dose Doherty *et al.* used (1200 mg/kg oral) probably gave a greater effective brain concentration than the treatment we used in this study (200 mg/kg ip).

Movement, rearing and pole traction behaviors observed after CPF treatment are most likely due to inhibition of acetylcholinesterase activity, which is a hallmark of organophosphate exposure (Bowman and Rand, 1984). At doses above 25 mg/kg, there is a strong correlation between dose and impairment of movement and rearing. PM has a less clear dose-dependent effect on behavior than CPF. However, at doses above 50 mg/kg, PM decreases both movement and rearing frequencies. This action is consistent with results reported by Spinoso *et al.*, (1999), in which movement and rearing frequencies were reduced by 10 and 30 mg/kg of the pyrethroid fenvalerate.

We have shown that up-regulated dopamine transport and mitochondrial integrity assays are sensitive biomarkers of exposure to certain insecticides. However, we do not know whether the neurochemical effects observed are persistent, or only temporary changes occurring after the last insecticide treatment. The next phase of this work will be to determine if the extent of reversibility of PM and CPF effects on striatal neurochemistry.

## ACKNOWLEDGEMENTS

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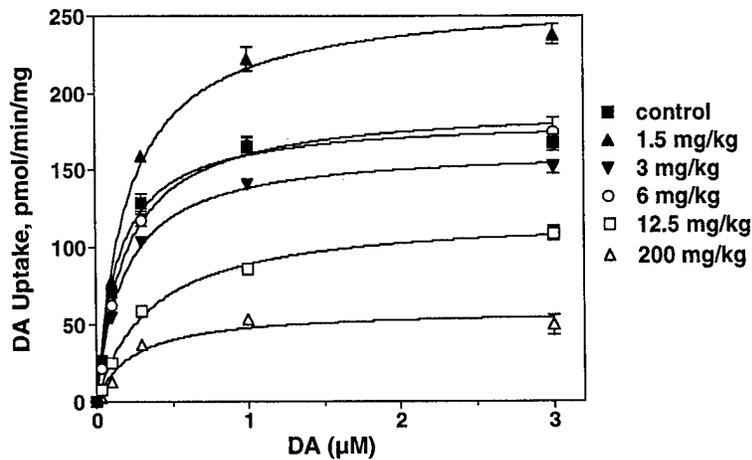


Figure 1. Representative isotherm plots of dopamine uptake in *ex vivo* striatal synaptosomes isolated from mice given the indicated doses of PM. Symbols represent means of three determinations from the same membrane preparation and bars the SEM.

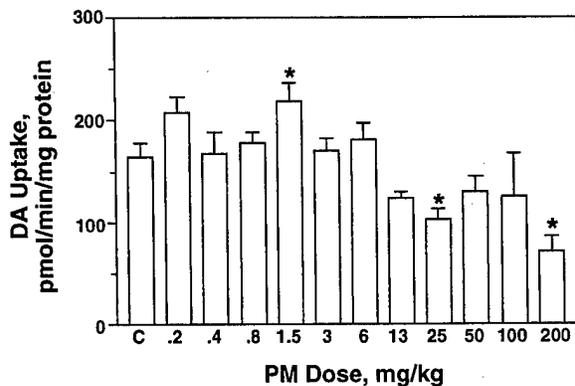


Figure 2. Changes in maximal dopamine uptake ( $V_{max}$ ) following treatment with PM. Asterisks indicate effects significantly different from control (t-test,  $p < 0.05$ ).

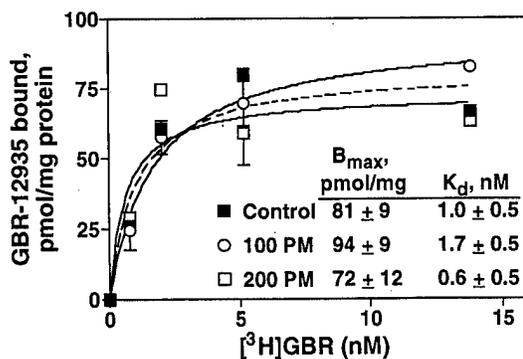


Figure 3. GBR12935 binding to *ex vivo* striatal synaptosomes prepared from PM-treated mice at 100 and 200 mg/kg. The dashed line is the curve for the controls. Symbols are means of two determinations and accompanying bars are the SEM.

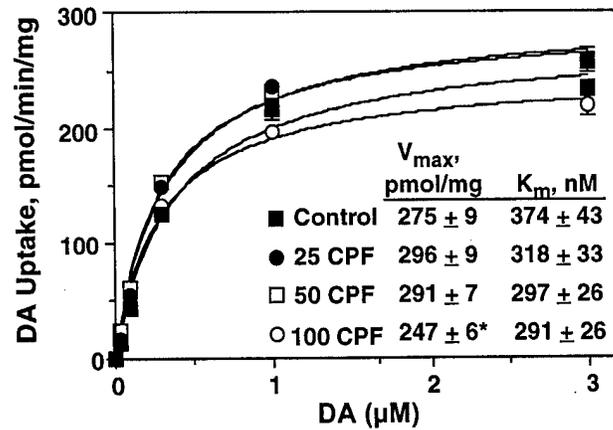


Figure 4. Effect of CPF treatment (25, 50, or 100 mg/kg) on dopamine uptake in *ex vivo* striatal synaptosomes. Kinetic and statistical analysis of this data are given in the inset table. In the table, the asterisk indicates an effect significantly different from control (t-test,  $p < 0.05$ ).

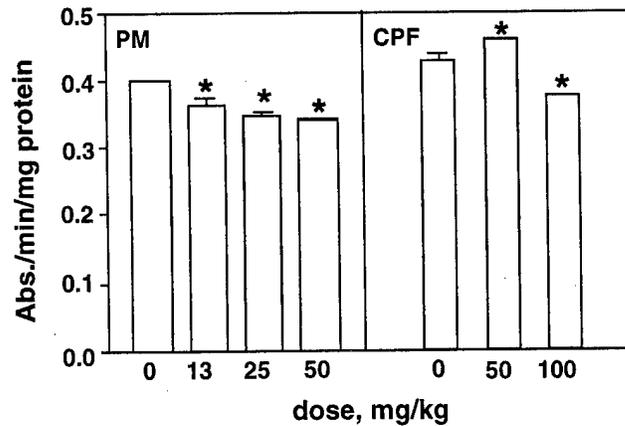


Figure 5. Effect of PM (left) and CPF (right) treatment on mitochondrial activity (MTT reduction) in *ex vivo* striatal synaptosomes. MTT was tested at a single concentration of 0.55 mM. In the bar graphs, the asterisk indicates an effect significantly different from control (ANOVA,  $p < 0.05$ ).

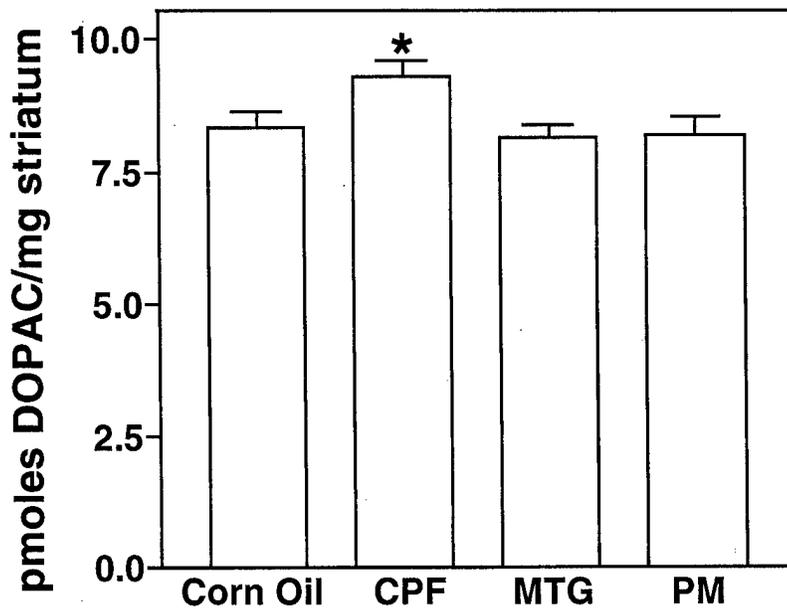


Figure 5. Changes in DOPAC titers following treatment with vehicle (Corn oil; MTG) or insecticide (100 mg/kg CPF; 200 mg/kg PM). Asterisk indicates effect significantly different from control (t-test,  $p < 0.05$ ).

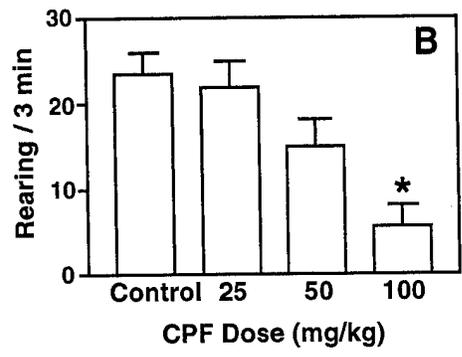
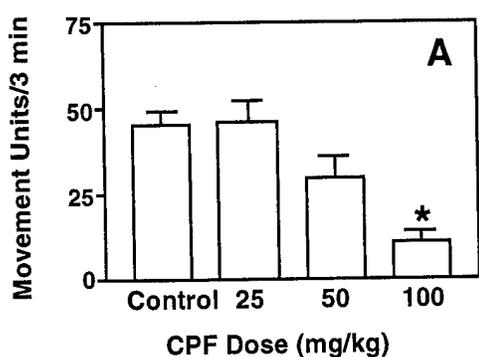


Figure 6. Changes in open field (A) and rearing frequency (B) at the indicated doses of chlorpyrifos. Asterisk indicates an effect significantly different from control (t-test,  $p < 0.05$ ).

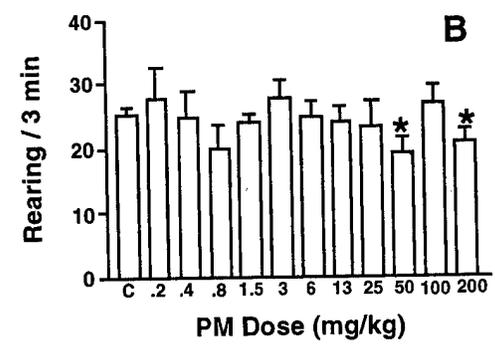
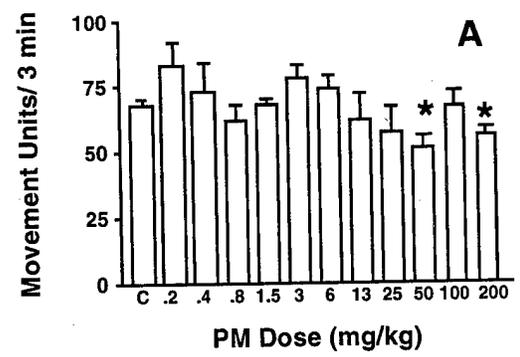


Figure 7. Changes in open field (A) and rearing frequency (B) at the indicated doses of permethrin. Asterisk indicates an effect significantly different from control (t-test,  $p < 0.05$ ).

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**Effects of Multiple Exposures of Chlorpyrifos or Permethrin on  
Murine Behavior and Striatal Cholinergic Biomarkers**

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## ABSTRACT

Several behavioral and neurochemical studies were performed to characterize the potential for insecticides to affect striatal cholinergic pathways. Chlorpyrifos and permethrin were given three times over two weeks by injection (chlorpyrifos by sc; permethrin by ip). While there was no consistent, dose-related effect of permethrin treatment on brain AChE activity, permethrin injections caused a decline in open field behavior. This latter effect may have been related to changes in striatal muscarinic receptor density, since permethrin treatment significantly increased striatal [<sup>3</sup>H]QNB binding at doses of 25-200 mg/kg. Increases in B<sub>max</sub> over control values ranged from 33% at 25 mg/kg to a 135% increase at 100 mg/kg. However, K<sub>d</sub> values were not significantly altered by permethrin or chlorpyrifos treatments. Chlorpyrifos caused 15, 54, and 84% inhibition of brain acetylcholinesterase at a doses of 25, 50, and 100 mg/kg, respectively, which correlated reasonably well with dose-dependent effects on open field, rearing, and pole climbing behaviors. Chlorpyrifos treatment had no effect on striatal [<sup>3</sup>H]QNB binding at 50 mg/kg, but decreased maximal binding at 100 mg/kg. These studies demonstrated significant effects on behavior and striatal cholinergic neurochemistry induced by these insecticides.

## INTRODUCTION

Insecticides are unique among pesticides in that most are designed to be neurotoxic and are deliberately placed in the environment for insect control. The insecticides selected for this

study were the pyrethroid, permethrin (PM) and the organophosphorus (OP) compound, chlorpyrifos (CP). PM was selected for study because it was impregnated into uniforms of U.S. service personnel for control of disease carrying insects (Abou-Donia *et al.*, 1996a). Thus, Gulf War veterans were exposed to PM, and CPF, and exposure to toxicant mixtures containing CP and PM has been implicated as a possible cause of the neurological problems associated with Gulf War Syndrome (Abou-Donia *et al.*, 1996a; Abou-Donia *et al.*, 1996b).

The anticholinesterase activity of OP insecticides causes a variety of signs consistent with poisoning of the parasympathetic system (Saunders and Harper, 1994), along with cognitive and psychiatric disturbances, schizophrenia, and depression (Gershon and Shaw, 1961; Metcalf and Holmes, 1969; Biskind and Mobbs, 1972). The striatum is a brain region particularly rich in cholinergic innervation (Bowman and Rand, 1980) that is strongly affected by OP insecticides, such as CP (Chakraborti *et al.*, 1993). Interestingly, there are often indications of excess parasympathetic activity in PD patients and atropine-like drugs have been used to treat PD (Bowman and Rand, 1980). A recent study specifically implicated exposure to organochlorine and organophosphorus insecticides in the etiology of PD (Seidler *et al.*, 1996). OP-induced delayed neuropathy (OPIDN) is a phenomenon associated with exposure to some OP compounds, and results in ataxia and paralysis via sciatic nerve demyelination (Bowman and Rand, 1980), as well as axonal and nerve terminal degeneration of ascending spinal tracts, brainstem nuclei, and the cerebellum (Tanaka *et al.*, 1989). In our view, the established neurotoxicity and neurodegenerative ability of OP insecticides, coupled with the demonstrated

sensitivity of the striatum to these compounds and parasympathetic involvement in PD, make a compelling argument that the actions OPs on the nigrostriatal pathway and their possible role in Gulf War Syndrome and PD need to be investigated further.

The objectives of this research were to (1) measure striatal acetylcholinesterase inhibition in CP and PM treated mice, (2) assess the effects of CP or PM treatment on striatal [<sup>3</sup>H] QNB binding, and (3) attempt to correlate insecticide effects on striatal cholinergic pathways with alterations in behavior by measuring open field, rearing, and pole climbing behaviors.

## MATERIALS AND METHODS

**Chemicals.** Analytical grade CP was obtained from ChemService, Inc (West Chester, PA). PM (a mixture of *cis* and *trans* isomers) was obtained from Sigma Chemical Co., St. Louis, MO. [<sup>3</sup>H]QNB (42.0 mCi/μmol) was purchased from NEN Life Science Products, Inc., Boston, MA. NaH<sub>2</sub>PO<sub>4</sub>, Na<sub>2</sub>HPO<sub>4</sub>, CaCl<sub>2</sub>, and MgSO<sub>4</sub> were purchased from Sigma Chemical Co., St. Louis, MO. Sucrose, HEPES, NaCl, and KCl were purchased from Fisher Scientific, Pittsburgh, PA. Acetylthiocholine iodide was obtained from Nutritional Biochemicals Corp., Cleveland, OH.

**Animals and Treatments.** Male C57BL/6 retired breeder mice were utilized for all experiments. Mice were purchased from Harlan Sprague-Dawley, Dublin, VA, and were aged 7-9 months (28-40 g live weight) at the time of the experiments. Mice were assigned randomly to treatment groups, which contained a minimum of 6 mice, so that the mean weight of all treatment groups

was approximately equal. CP carried in corn oil vehicle or PM carried in methoxytriglycol (MTG) vehicle were administered to the mice at multiple doses three times over a two week period according to the method of Bloomquist *et al.*, (1999). CP administration was by subcutaneous injection, while PM was administered by intraperitoneal injection. Control mice received 50µl corn oil or 10 µl of MTG vehicle alone. On the day following the last treatment day, mice were killed by cervical dislocation, and striatal tissues were collected at this time.

**Behavior Assessments.** On the last day of the study, behavioral effects were assessed by means of monitoring number of movements and rearing in an open field during a three minute period. The open field was the floor of a 10 gallon aquarium, divided into six equally sized squares. A movement was counted when the animal's front paws crossed any grid line. A rear was counted when the mouse raised onto its rear paws, lifting the front paws from the floor of the field. The mice were also placed on the top of a pole, and time to descend the pole or a fall from the pole, was noted. Mice were timed for a maximum of three minutes while on the pole.

**Acetylcholinesterase Assay.** Acetylcholinesterase activity was determined according to a modification of the method of Ellman *et al.* (1961). Mice were killed by cervical dislocation, striatal tissue was removed, homogenized, and then centrifuged at 10,000 x g for 5 min. at 4°C, and the resulting pellet was resuspended in 90 µl of 0.1 M NaPO<sub>4</sub> buffer. Tissue dilutions (1:40)

were prepared by combining tissue homogenates (1:10) with 3 parts 0.1 M NaPO<sub>4</sub> buffer (pH=7.8). Reactions were run in triplicate in a 96 well microplate; each well containing 20  $\mu$ l tissue homogenate, 175  $\mu$ l buffer (pH=7.8), 10  $\mu$ l DTNB (6.8 mM), and 5 $\mu$ l acetylthiocholine (42.4 mM). Absorbance was measured at 405 nm, eight times over a two-minute period, to calculate kinetic data from which activity was estimated. The molar extinction coefficient (Ellman *et al.*, 1961) was used to calculate activity and data were normalized to protein content ( $\mu$ mol/min/mg).

**QNB Binding Assay.** Mice were killed by cervical dislocation, striata were removed and homogenized in cold Kreb's-Ringer's HEPES (KRH) buffer (118 mM NaCl, 4.8 mM KCl, 1.2 mM MgSO<sub>4</sub>, 20 mM HEPES, 2.5 mM CaCl<sub>2</sub>) at a ratio of two striata per ml of buffer. Homogenates were centrifuged at 100xG for 15 min. at 4°C. The supernatant was retained and centrifuged again at 10,000xG for 15 min at 4°C. The resulting pellets were resuspended and homogenized in cold KRH buffer at a ratio of 435  $\mu$ l buffer per striatum. For analysis, samples were run in duplicate in the absence and presence of 0.2 mM unlabelled atropine. To measure total binding, 925  $\mu$ l of KRH buffer, 25  $\mu$ l of [<sup>3</sup>H]QNB (1, 0.50, 0.25, 0.125, 0.0625, and 0.03125 nM), and 50  $\mu$ l of tissue homogenate were mixed and incubated for 60 minutes at 37°C. The same procedure was followed to measure non-specific binding; however, 875  $\mu$ l KRH buffer and 50  $\mu$ l of a 0.2 mM unlabeled atropine solution were substituted for 925  $\mu$ l of KRH buffer. To

stop both reactions, 3 ml cold KRH buffer was added to each tube and the contents were filtered at 10 psi using 25 mm Whatman GF/B filter disks pre-wet with KRH buffer. Filters were each washed 3 times with 3 ml cold KRH buffer to remove any unbound radiolabel. Filters soaked overnight in 5 ml Scintiverse E before total counts were measured (~53% efficiency for [<sup>3</sup>H]). 25 ul of each [<sup>3</sup>H]QNB solution (1, 0.50, 0.25, 0.125, 0.0625, and 0.03125 nM) were mixed with 5 ml Scintiverse E cocktail to calculate the exact [<sup>3</sup>H]QNB concentrations in each reaction mixture. Nonlinear regression was used to determine  $V_{max}$  and  $K_m$ .

**Protein determination.** All data were normalized to protein content of the striatum. A standard curve spanning the assay's linear range (0.005 – 0.04 ug/ul) was used to quantify protein content in stored (-70° C) samples. Several volumes of a working dye stock solution, consisting of 50 ml Coomassie brilliant blue (1 g / 500 ml ethanol), and 100 ml H<sub>4</sub>PO<sub>3</sub> brought to 1 L with deionized water, were combined with volumes of a 1 mg/ml BSA solution to generate protein standards. Absorbance of BSA/dye and BSA/sample solutions was measured in a Dynex microplate reader at 595 nm. Sample concentrations were calculated from regressed standards.

**Statistical Analysis.** Statistical significance was determined using one-way ANOVA and Student-Newman-Keuls means separation where applicable. Calculations were performed using InStat (GraphPad Software)

## RESULTS

Significant striatal acetylcholinesterase (AChE) inhibition was observed following CP treatment (Fig. 1). Compared to the control, injections of 25, 50, and 100 mg/kg CP significantly reduced AChE activity in a dose-dependent manner. Moreover, there was about 15% mortality observed in the mice at 50 mg/kg and about 20% mortality at 100 mg/kg, although the animals showed no signs of SLUD. PM treatment did not inhibit AChE, and in fact several treatments were significantly elevated compared to the control (Fig. 2). Specifically, 0.4, 1.5, 6, and 25 mg/kg PM significantly elevated AChE activity, compared to the control. However, the magnitude of elevation in AChE activity was not clearly dose-dependent.

Striatal muscarinic binding, as determined by [<sup>3</sup>H]QNB isotherms, was also significantly affected by both CP (Figure 3) and PM (Figure 5), with the major effect expressed in changes in maximal binding,  $B_{max}$ . Bar chart representations of the maximal binding data generated from the QNB isotherms reveals that for CP,  $B_{max}$  is significantly depressed 47% following a dose of 100 mg/kg, but not 50 mg/kg (Fig. 4). PM was found to have the opposite effect of CP on QNB binding. Compared to controls, QNB binding was increased 32% at 25 mg/kg, 86% at 50 mg/kg, 131% at 100 mg/kg, and 111% at 200 mg/kg PM (Fig. 6). The bar graph of  $B_{max}$  values after PM treatment show a dose dependent up-regulation of  $B_{max}$  at doses of 25 and 50 mg/kg versus that of control mice. The effect saturated at doses above 50 mg/kg (Fig. 6). Changes in  $K_d$ , for both CP and PM exposed mice followed the same trend as  $B_{max}$ ; however, there was considerable overlap in the 95% confidence limits, and the difference was not statistically significant.

Open field, rearing, and pole climbing behaviors were measured at the end of each experiment and prior to neurochemical assessments. Performance in all three parameters was affected in a dose dependent manner by CP (Fig. 7A-C). At doses of 50 and 100 mg/kg CPF, movement and rearing frequency were both decreased as compared to the control; however, the decrease was statistically significant only at the 100 mg/kg dose (Figure 3). At doses of 25 mg/kg and above, a dose-dependent trend in the number of mice falling from the pole was also observed. This trend was significant at all doses, since no control mice fell from the pole. Likewise, PM also had an effect on both movement and rearing frequencies at high doses (Figure 8). At doses of 50 mg/kg and 200 mg/kg PM, movement and rearing frequencies were both decreased compared to the control mice. At the 100 mg/kg PM dose, movement and rearing frequencies were not significantly different than that of controls. Ability to descend the pole was not affected by treatment with PM at any dose, and no mice fell from the pole, even at the highest doses of PM (data not shown).

## DISCUSSION

The maximal level of cholinesterase inhibition observed at 100 mg/kg CPF (Figure 1) was similar to that reported for rat striatum (82-96% inhibition) treated with CPF or parathion (Liu and Pope, 1998). The extent of acetylcholinesterase inhibition by CPF typically did not correlate with effects on behavior (Nostrandt *et al.*, 1997), possibly due to compensatory changes in muscarinic receptors (Nostrandt *et al.*, 1997) and high affinity choline uptake (Liu *et al.*,

1995). In contrast, the present study showed a good correlation between enzyme inhibition and behavioral effects (movement, rearing, and falling; Figure 2).

We also observed an effect of PM treatment on acetylcholinesterase activity. In this case, there was an increase in enzyme activity in treated mice (Figure 7). The relationship was not clearly dose-dependent, so the biological relevance is somewhat questionable. It is interesting to note that exposure to the pyrethroid deltamethrin also caused a small but significant increase in acetylcholinesterase activity in rat brain (Husain *et al.*, 1994). Perhaps this effect is an adaptive response to high levels of synaptic acetylcholine caused by pyrethroid exposure, since pyrethroids are known to release a variety of neurotransmitters through effects on nerve terminals (Kirby *et al.*, 1999). In the present study, there was not a clear correlation between PM-elevated acetylcholinesterase activity and frequency of movement and rearing or time to descend the pole. Both movement and rearing frequencies were decreased by both 50 and 200 mg/kg of PM; however it is not clear why movement and rearing frequencies were not affected by 100 mg/kg PM. Other workers have shown that fenvalerate, a type II pyrethroid, caused a decrease in open field movement and rearing behaviors in rats dosed with 10 or 30 mg/kg of fenvalerate (Spinosa *et al.*, 1999).

In mouse brain striatal synaptosomes from controls, we observed [<sup>3</sup>H]QNB binding characteristics of  $K_d$  values in the picomolar range (13 pM) and 11 pmol/mg protein for  $B_{max}$ . This compares reasonably well with the values reported for heart membranes by Goodwin *et al.* (1995) of  $K_d = 60$  pM and  $B_{max} = 401$  fmol/mg protein. In addition, there is a good match

between our  $B_{max}$  value and that reported by Nostrandt *et al.* (1997), which was about 2.8 pmol/mg protein in rat striatum.

Exposing mice to PM or CP caused differential regulation of muscarinic receptors, as evidenced by changes in the  $B_{max}$  for [ $^3$ H]QNB binding. CP has been shown to down-regulate muscarinic receptors in the rat striatum (Chaudhuri *et al.*, 1993) and we have confirmed this effect in the C57 mouse. PM exposure caused an opposite effect to that of CPF (Figures 9 and 10). This difference is interesting, since both pesticides are expected to increase synaptic levels of acetylcholine, but differ in how this action affects receptor regulation. Systemic injection of pyrethroids (ca. 1 mg/kg) in young mice slightly down-regulated cortical expression of muscarinic receptors (5-10%), but apparently not in the striatum (Eriksson and Fredriksson, 1991). In subsequent studies by the same research group, oral doses of bioallethrin up-regulated muscarinic receptors about 6% (Talts *et al.*, 1998a), and mRNA analysis showed that it was specifically the M4 subtype whose expression was increased (Talts *et al.*, 1998b). These results however, may be suspect, since the  $K_d$  values reported in all these studies are in the low micromolar range, even what they define as high affinity QNB binding. This data is several orders of magnitude different from the  $K_d$  we report (13 pM), as well as that found by others: Goodwin *et al.* (1995) for heart ( $K_d = 63$  pM) and Niemeyer *et al.* (1995) for cat retina ( $K_d = 270$  pM). The mechanisms underlying the differential regulation of muscarinic receptors by OPs and pyrethroids merits further investigation.

## ACKNOWLEDGEMENTS

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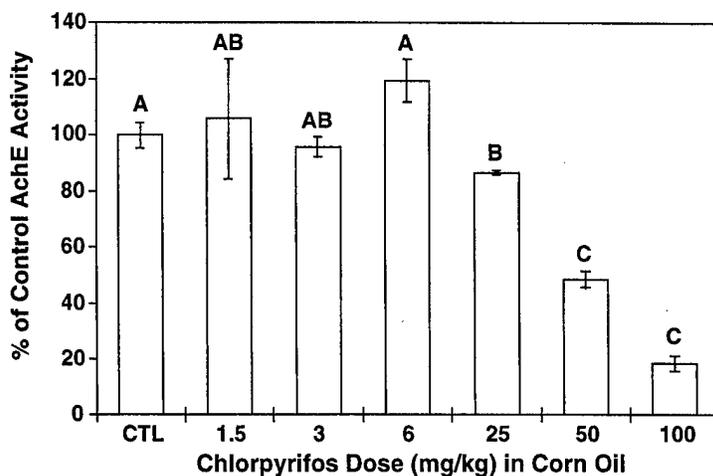
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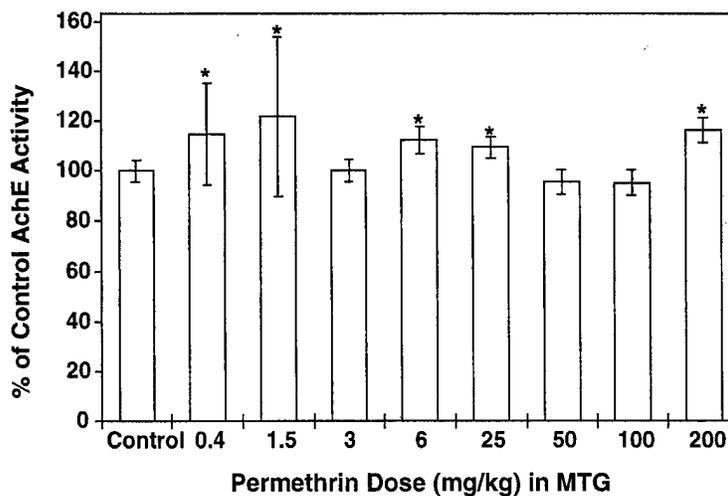
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**Figure 1.** Striatal acetylcholinesterase activity (as % control) following three sc injections of CP.

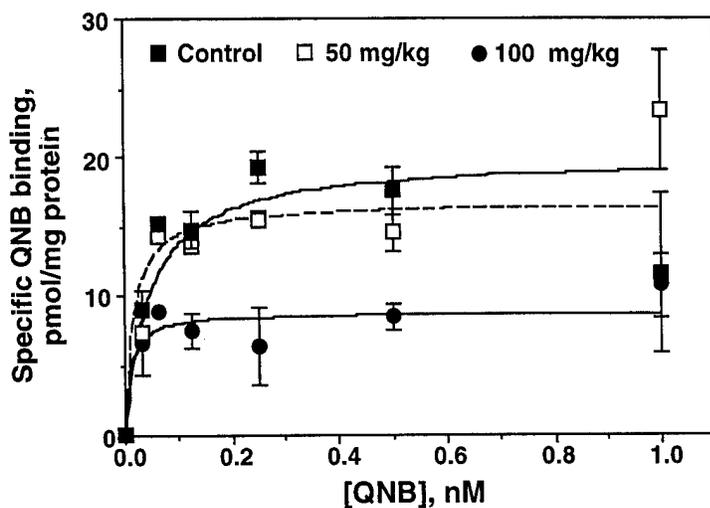
Data are presented as means with confidence intervals. Bars labeled by different letters are significantly different (ANOVA, followed by Student-Newman-Keuls post test,  $p < 0.05$ ).



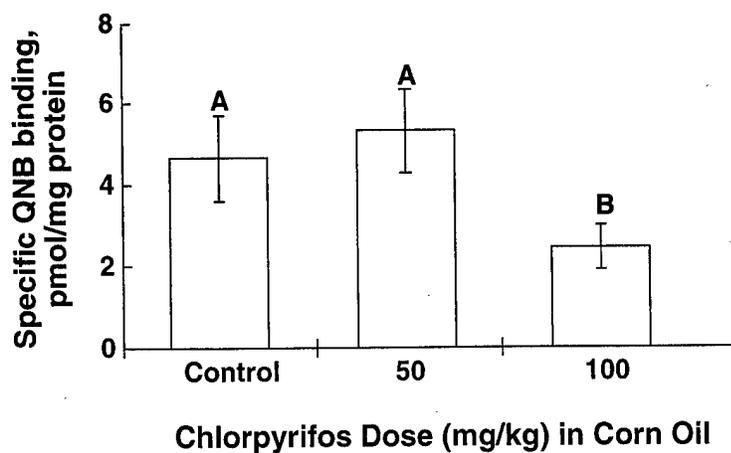
**Figure 2.** Striatal acetylcholinesterase activity (means  $\pm$  CIs), as percent of control, following three ip injections of PM carried in MTG. Asterisks denote treatment means that are significantly higher than the control mean.



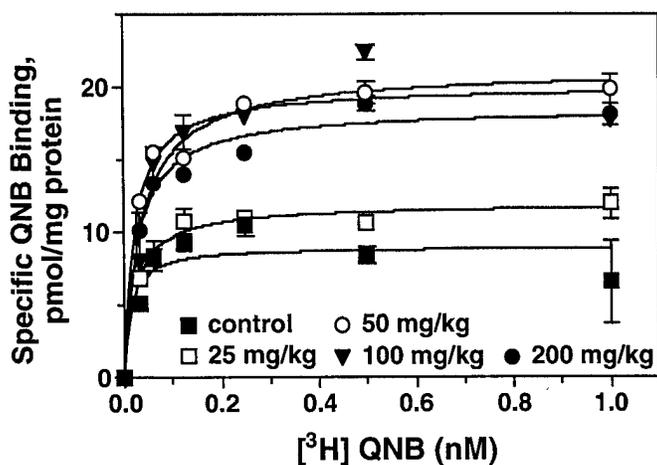
**Figure 3.** Isotherms of [<sup>3</sup>H]QNB binding following three sc injections of CP. Nonlinear regression was used to determine  $K_d$  and  $B_{max}$ . Symbols are presented as means with SEM.



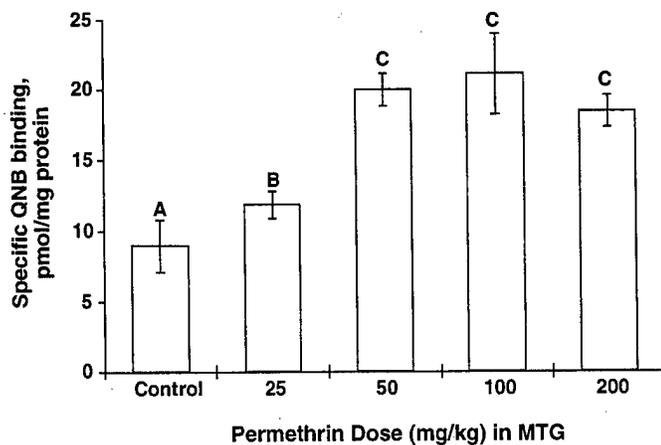
**Figure 4.** Maximal binding of [<sup>3</sup>H]QNB following three sc injections of CP. Data are presented as means with confidence intervals. Bars labeled by different letters are significantly different (ANOVA, followed by Student-Newman-Keuls post test,  $p < 0.05$ ).



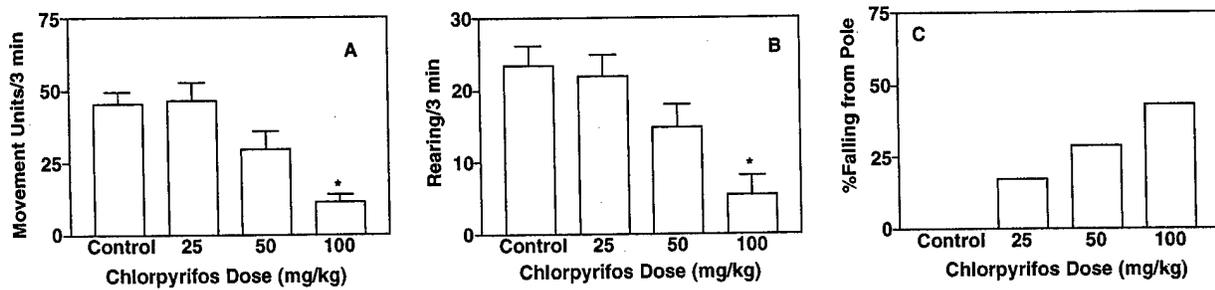
**Figure 5.** Isotherms of [<sup>3</sup>H]QNB binding (pmol/min/mg) following three ip injections of PM. Nonlinear regression was used to determine  $K_d$  and  $B_{max}$ . Data are presented as means with confidence intervals.



**Figure 6.** Maximal binding of [<sup>3</sup>H]QNB following three ip injections of PM. Data are presented as means with confidence intervals. Bars labeled by different letters are significantly different (ANOVA, followed by Student-Newman-Keuls post test,  $p < 0.05$ ).



**Figure 7.** Open field (A), rearing (B), and pole-climbing behaviors (C) measured following three sc injections of CP. Observations were made for three minutes. Open field and rearing behaviors are presented as total counts. Pole climbing behavior is presented as the percent that fell while attempting to descend the pole. Asterisks denote effects that are different from controls (t-test,  $p < 0.05$ ).



**Figure 8.** Open field and rearing behaviors measured following three ip injections of PM. Observations were made for three minutes. Open field and rearing behaviors are presented as total counts. Pole climbing behavior is presented as descent time (in seconds). Asterisks show treatment means that are significantly lower than the control mean (for movement).

