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14. ABSTRACT To study the etiology of Parkinson's disease as it may relate to Gulf War Syndrome, the insecticides chlorpyrifos (CPF) and/or permethrin (PM) were given 3 times over a two week period with or without a single dose of the Parkinsonian neurotoxin, MPTP. Biomarkers were then analyzed at t = 1, 14, and 28 days post-treatment. Potent up-regulation of dopamine transport biomarkers was observed with PM, but not CPF, at t = 1 day. Both insecticides reversibly altered the expression of nicotinic and muscarinic cholinergic receptors at high doses, with greater overall effects in the striatum compared to cortex. High doses of both insecticides also increased the toxicity of MPTP at t = 28 days post-treatment. Immunocytochemical analysis showed increased glial fibrillary acidic protein (GFAP) after MPTP or PM treatment, but not a significantly enhanced effect when applied together (T=1 day). Measurements of behavior at t = 1 day showed reduced locomotion at high doses of PM and CPF, as well as a trend for decreased movement in combined toxicant treatment groups as well (t = 28 days). Taken together, these results suggest that insecticide exposure at high doses may exacerbate idiopathic disease processes. Isomer studies showed that both the cis and trans isomers of PM contribute to the increase in expression of α -synuclein. Finally, 3 and 6 month weekly injections with low doses of PM found little or no toxic effect, with or without MPTP. Overall, these results suggest little Parkinsonian hazard from the proper use of these insecticides.						
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Table of Contents

Cover	1
SF 298	2
Introduction	4
Body	4
Key Research Accomplishments	19
Reportable Outcomes	19
Conclusions	22
References	23
Appendices	25

INTRODUCTION

This study was 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 (Bowman and Rand, 1980). We assessed the ability of each compound alone, or in combination, to directly induce neurochemical or neuropathological hallmarks of PD. In addition, tests with different isomers of PM attempted to identify those involved in its neurochemical effects on striatal dopaminergic systems. Since PD is hypothesized to have a multifactorial etiology (Siderowf, 2001), these insecticides were also 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 assessed the ability of the insecticides to accelerate or intensify idiopathic disease processes. Experiments were performed on the C57BL6 black mouse, which when given MPTP is a valid rodent model for the development of PD (Heikkila and Sonsalla, 1992). For each treatment group, effects consistent with metabolic insult and changes in cholinergic and dopaminergic neurotoxicity in the striatum were measured. Cell stress in striatal nerve terminals was evaluated by measurements of mitochondrial function. Other neurochemical studies measured 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 measured acetylcholinesterase activity, and the density/function of muscarinic and nicotinic receptors following insecticide treatment. Neuropathology studies focused on any gross changes in immunocytochemical markers for glial fibrillary acid protein (GFAP). Other antibody labeling studies assessed effects specific to the dopaminergic system, including antibody labeling of tyrosine hydroxylase and the dopamine transporter (DAT). These studies represent a unique combination of research approaches and provided a comprehensive and integrated evaluation of the possible Parkinsonian effects of these insecticides.

The project also included two supplements to extend the work. The first examined the isomeric dependence of PM effects on DAT expression, in order to identify whether any particular isomer was responsible for the enhancement we observed. This experiment addressed a potentially important issue, since only the 1*R*-cis isomer has any acute toxicity to mammals, whereas the 1*R*-trans isomer is toxic to insects and not mammals (Soderlund *et al.* 2002). Thus, if only the 1*R*-cis isomer enhanced DAT expression in mice, switching to PM formulations containing only the 1*R*-trans isomer would circumvent this effect while still providing good insect control. The second and final supplement examined whether prolonged exposures to low doses of PM had any cumulative dopaminergic neurotoxicity, either alone or in combination with MPTP. This study was undertaken because we observed enhancement of toxicity in co-treatments of high dose PM + MPTP (2003 report), and it was decided to ascertain whether this effect was mimicked by prolonged exposure to low doses of PM. The results of these just-completed experiments are included for the first time here.

BODY

The original Statement of Work (SOW) for the project had three main objectives: (1) to establish the no observable adverse effect level (NOAEL) for insecticide exposures using the

biomarkers listed below (SOW, year 1A,B,C; year 2A); (2) to assess any synergistic interactions between the insecticides and the Parkinsonian neurotoxin MPTP (SOW year 2B,C; year 3A); and finally, (3) evaluate the reversibility of effects by assessing biomarkers at various times post-treatment (SOW year 3 B,C). These objectives were pursued in retired breeder male (7-9 months old) C57 mice using the treatment regime shown in Fig. 1, with only a subset of selected doses and biomarkers proposed for objectives 2 and 3. Additional objectives pertain to supplemental work, including (4) define which isomer of PM is responsible for the up-regulation of the dopamine transporter; and (5) determine effects of long-term, low-dose exposures of PM on dopaminergic biomarkers with and without MPTP, as described above.

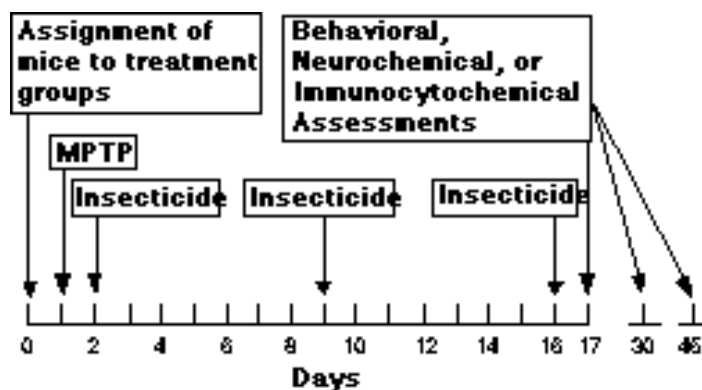


Figure 1. Treatment regime for studies of insecticides alone or in combination with MPTP, including studies of reversibility of effects. NOAELs of biomarker effects were determined 1 day after the last treatment (day 17 overall). Most reversibility experiments were evaluated at 28 days post-treatment (day 45 overall), but some were run at 14 days post-treatment (day 30 overall). Treatment groups were: Controls, PM, CPF, MPTP, MPTP + PM, MPTP + CPF, PM + CPF, and MPTP + PM + CPF. In synergism studies, animals receiving MPTP alone were also given insecticide vehicles; methoxytriglycol (PM) and corn oil (CPF).

Original Statement of Work

I. Year 1

A. Months 1-3 (Objective 1)

1. Initiate range-finding studies with permethrin.
 - a. Test 60 mg/kg permethrin to ascertain its usefulness as a benchmark dose.
 - b. If there is lethality, reduce the dose.
 - c. If no effects on PD biomarkers are documented, the dose will be increased.

B. Months 4-9 (Objective 1)

2. Replicate dosing studies with permethrin (controls & 3 different doses/experiment).
 - a. Change the dose in 2-fold steps to define Parkinsonian effects from doses that are just sublethal down to the NOAEL.

C. Months 10-12 (Objective 1)

1. Initiate range-finding studies with chlorpyrifos.
 - a. Test 40 mg/kg chlorpyrifos to ascertain its usefulness as a benchmark dose.
 - b. If there is lethality, reduce the dose.
 - c. If no effects on PD biomarkers are documented, the dose will be increased.

II. Year 2

A. Months 1-6 (Objective 1)

1. Replicate the dosing process with chlorpyrifos (controls & 3 different doses/experiment).
 - a. Change the dose in 2-fold steps to define Parkinsonian effects from doses that are just sublethal down to the NOAEL.

B. Months 7-9 (Objective 2)

1. Initiate insecticide synergism studies.
 - a. Select 1 or 2 doses of each insecticide for synergism studies.
 - b. Evaluate effects of permethrin and chlorpyrifos in combination

C. Months 10-12 (Objective 2)

2. Evaluate synergism of permethrin or chlorpyrifos in combination with MPTP at single doses (controls, insecticide, MPTP, and MPTP + insecticide).

III. Year 3

A. Months 1-3 (Objective 2)

1. Select and use single doses to evaluate synergism of permethrin and chlorpyrifos in combination with MPTP (controls, permethrin, chlorpyrifos, MPTP, and MPTP + permethrin + chlorpyrifos).

B. Months 7-9 (Objective 3)

1. Dose animals with permethrin or chlorpyrifos (single effective dose) for biomarker reversibility studies and analyze at various times following treatment until effects return to baseline.

C. Months 10-12 (Objective 3)

1. Dose animals with permethrin and chlorpyrifos for biomarker reversibility studies and analyze at various times following treatment until effects return to baseline.

Results for the entire grant period are organized by main objective (as given above), with an alphabetical listing of the biomarker assessments performed for each objective, as given in the amended grant proposal and in the annual reports for previous years. Again, only a subset of selected doses and biomarkers was proposed for objectives 2 and 3. This final report will rely heavily on published papers to summarize methods and results, as well as progress reports from past years.

Objective 1: Establish the No Observable Adverse Effect Level (NOAEL) for Insecticide Exposures Using the Biomarkers Listed Below (SOW, year 1A,B,C; year 2A)

- 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 for measuring dopamine and DOPAC content of striata are given in Karen et al. (2001, see appendix). We observed no effect of high insecticide doses on dopamine levels, whereas a Parkinsonian effect would be expressed by dopamine depletion. There was a small increase (ca. 10%) in DOPAC levels by 100 mg/kg chlorpyrifos (Karen *et al.*, 2001), that undoubtedly would disappear at 50 mg/kg.

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

GBR binding, as well as Western blot analysis of DAT, TH, α -synuclein, and synaptophysin was approached by the methods described in Bloomquist et al. (2002), Gillette and Bloomquist (2003), and Kou et al. (2006), all of which can be found in the appendix. Bloomquist et al. (2002) shows that PM-dependent up-regulation of GBR binding and DAT immunoblots are well correlated. We then focused on the up-regulation of the DAT because it could provide a molecular gateway for Parkinsonian toxins to access dopaminergic systems (e.g., MPP⁺). At a dose of 1.5 mg/kg, we observed an up-regulation of DAT function (dopamine transport) 24 hr after the last treatment (Karen *et al.*, 2001) that was correlated with DAT immunoblots and declined to statistical insignificance at about 0.1 mg/kg (Gillette and Bloomquist, 2003).

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

Methods for this standard cytotoxicity assay are given in Karen et al. (2001), and it measures mitochondrial dehydrogenase activity. In previous years, we found that PM and CPF caused dose-dependent, statistically significant reductions of about 15% in mitochondrial function (Karen et al., 2001), with little effect below 50 mg/kg for CPF and 12 mg/kg for PM. However, this assay proved to be less reproducible than Western blotting in our hands, and was not used much in subsequent studies.

- 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 general neurotoxicity within specific dopaminergic components of the nigro-striatal system using immunocytochemical staining for the catecholamine-synthesizing enzyme tyrosine hydroxylase (TH).*

- f. *Confirm whether functional changes in dopamine transport are due to fluctuating levels of dopamine transporter (DAT) protein using immunocytochemical identification.*

The methodology employed in these studies can be found in the appended publication of a portion of our previous data (Pittman et al., 2003, in the appendix), and presentation of these biomarkers are combined, since they were performed together as immunocytochemical studies conducted by Dr. Klein. In these studies, the major findings were a significant decrease in DAT expression at 3 mg/kg PM (no effect at 0.8 and 1.5 mg/kg), and an increase in GFAP at 200 mg/kg PM (no effect on TH or DAT at this dose). All these studies were done 24 hr after the last treatment, and are generally consistent with most of our western blot studies. We speculate that the lack of effect on DAT expression may be a difference in tissue sub-sampling, which is discussed at length in Pittman et al. (2003).

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

Methods for determining rearing and locomotion are given in Karen et al. (2001), where we saw a dose-dependent decrease in moving and rearing frequency after treatment with CP or PM. These results only occurred at high exposures, and disappeared at doses of 25-50 mg/kg (Karen et al. 2001). Effects on pole climbing were also observed with CPF, manifested as falling from the pole, and in increased hang time with PM (report for 2000). The unreliability of the pole climbing behavioral assay led us to de-emphasize its use in subsequent studies.

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

We used the classical method of Ellman et al. (1961) to measure acetylcholinesterase activity. In previous studies, we observed approximately 15, 50, and 80% inhibition of cholinesterase activity at CPF doses of 25, 50, and 100 mg/kg, respectively, and the extent of inhibition was nearly identical (within a few percent) in striatal and cortical tissue (t = 1 day, report for 1999). Thus, the NOAEL is about 13 mg/kg. The extent of inhibition of acetylcholinesterase by CPF correlated with reductions in open field and rearing frequencies (report for 1999).

We also observed that PM treatment increased acetylcholinesterase activity (Fig. 21, Report for 1999). The effect was small, typically an increase of 10-20%, and was not clearly dose-dependent, so the biological relevance is somewhat questionable. It is interesting to note that exposure to 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 the pyrethroids.

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

These two categories of cholinergic biomarkers are related, and will be dealt with together for simplicity. There are four biomarkers under this umbrella area, all related to cholinergic effects of insecticide treatments. These are: muscarinic receptor density as defined by quinuclidinyl benzilate (³H]QNB) binding, nicotinic receptor density as defined by [³H]epibatidine binding, and functional effects on muscarinic and nicotinic receptors on dopamine secretion in synaptosomal release assays. The binding studies used the standard techniques similar to those of Chaudhuri et al. (1993) for QNB binding and the procedures of Houghtling et al. (1995) for epibatidine binding. Binding isotherms were analyzed by nonlinear regression using Prism™ (GraphPad software, San Diego, CA). Single concentration data from binding assays or enzyme activity assays were analyzed by ANOVA/Student-Newmann-Keuls post test or T-test, as appropriate. An important component of this work is comparison of cortical with striatal effects, in order to assess the tissue selectivity expected in PD.

We are still deciding whether to prepare one or two papers on cholinergic marker effects and have some new data from previously frozen tissue analyzed during the last work period. In our original studies on QNB binding at t = 1 day post-treatment, we found little or no effect of any

insecticide treatment on the K_d value for QNB. Exposing mice to PM (50 mg/kg) caused an up-regulation of muscarinic receptors in both cortex and striatum, as evidenced by an increase in the B_{max} for QNB binding. There was little up-regulatory effect at doses below 25 mg/kg (the NOAEL, report for 2001). Eriksson and Fredriksson (1991) found that daily systemic injection of pyrethroids (*ca.* 1 mg/kg) in young mice slightly down-regulated cortical expression of muscarinic receptors (5-10%), but this effect was not observed in the striatum. We also observed that the up-regulation of QNB binding by PM tended to decline at doses above 50 mg/kg (2001 report). This finding is consistent with the evidence of cytotoxicity (attenuated MTT reductase activity) at high doses of PM observed by Karen et al. (2001). There was a significant down-regulation of QNB binding by CPF (75 mg/kg) in the striatum 24 hr after the last treatment, but no effect in cortex and no significant effect at lower doses (report for 2000). Reduction in QNB binding at high doses of CPF has been previously reported (*e.g.*, Chaudhuri et al. 1993, our report for 2000), and a study by Nostrandt et al. (1997) also found a decrease of QNB binding sites by 100 mg/kg chlorpyrifos in striatum, but not pons/medulla. Thus, we have confirmed specificity of effect on the striatum in our study.

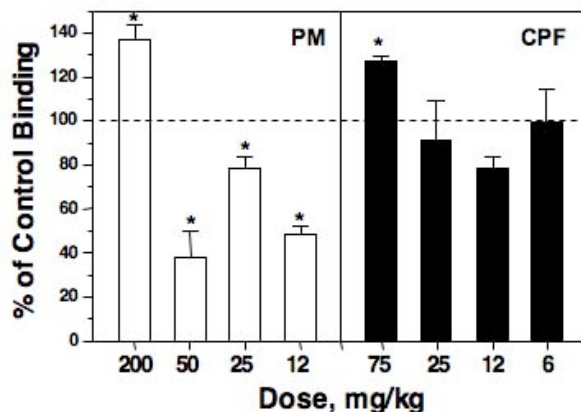


Figure 2. EB binding NOAEL experiments with PM and CPF at $t = 1$ day post-treatment. Bars are means \pm SEM and an asterisk indicates a value significantly different from its matched control (T-test, $p < 0.05$). The dashed line indicates 100% of control value for binding.

Houghtling et al. (1995) showed that the nicotinic receptor ligand [3 H]epibatidine (EB) binds to high and low affinity sites having K_d values of in rat forebrain membranes of 16 and 391 pM, respectively, with about equal densities of both sites. In 2001, we confirmed similar K_d values of 6 and 225 pM in mouse synaptosomal membranes isolated from striatum. Based on these affinities, and the fact that we observed only B_{max} effects on QNB binding, we estimated B_{max} using a single concentrations of ligand at 2 nM, with nonspecific binding defined by 300 μ M nicotine (Houghtling et al. 1995). In terms of the 1-day NOAEL, treatment with 200 mg/kg PM increased striatal binding site density 37% (Fig. 2), which is consistent with the 10-20% enhancement of [3 H]nicotine binding observed after treatment with deltamethrin to young mice (Eriksson and Nordberg, 1990). Unexpectedly, at lower doses of PM a decrease in binding was observed, which we are still trying to sort out, even if it requires running additional studies at our own expense. There was no effect of high dose PM or CPF treatment on cortical EB receptor density (Fig. 6). At 75 mg/kg, CPF increased EB binding site density in the striatum 27% (Fig.

2), with no statistically significant effect at lower doses (Fig. 2). This latter result is in opposition to that of Zheng et al. (2000), who found that 14 daily oral doses of 7.5 mg/kg CPF reduced low affinity (1 nM) epibatidine binding sites 32% in adult rats. In our hands, the neuroexcitatory toxicants studied here up-regulated presynaptic nicotinic receptors at high doses.

We did not perform any studies on the ability of cholinergic compounds (nicotine or muscarine) to alter release of dopamine in striatal synaptosomes from insecticide-treated mice. These studies turned out to be beyond the scope of what we could achieve in this project, mostly due to turnover in the postdoc positions. Only 1 of 4 postdocs on this project worked over 9 months, the others secured jobs very quickly. Thus, we were constantly re-training new people, and this hampered our efforts.

Objective 2: Assess any Synergistic Interactions Between the Insecticides and the Parkinsonian Neurotoxin MPTP (SOW year 2B,C; year 3A)

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.

In previous studies, we found at best small synergistic effects ($t = 1$ day post-treatment) of insecticides with MPTP in measures of striatal dopamine content (see conclusions from reports for 2000 and 2001), and only at the highest dose of MPTP we used (30 mg/kg). Reductions in dopamine titers at 10 and 20 mg/kg MPTP were not enhanced by insecticide co-exposure at this time point.

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

Additional studies used DAT protein expression as a biomarker in combination treatments assessed at 1 and 28 days post-treatment (Kou et al., 2006, see appendix). This approach was used because western blot analysis is essentially equivalent to dopamine titers for assessing striatal neurotoxicity (Tillerson et al. 2002), and can be performed on smaller treatment groups of mice than dopamine analysis by HPLC. In Kou et al. (2006) we reported that at 28 days post-treatment, but not at 1 day post-treatment, there was an enhancement of MPTP-induced reductions of DAT and TH proteins when it was given in combination with CPF (75 mg/kg) and PM (200 mg/kg). The lack of effect on synaptophysin expression suggested that the toxicity was specific for dopaminergic terminals, and not a general neurotoxic action. Due to the high doses required to see the effect, further immunocytochemical or reversibility studies were not performed.

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

An insecticide-dependent decrease in MTT activity (Karen et al. 2001) was confirmed in subsequent studies, but no greater effect was evident on this biomarker in combination treatments (report for 2000). As noted above, this assay provided uneven results and we emphasized other markers for combination treatments.

- 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 general neurotoxicity within specific dopaminergic components of the nigro-striatal system using immunocytochemical staining for the catecholamine-synthesizing enzyme tyrosine hydroxylase (TH).*
- f. *Confirm whether functional changes in dopamine transport are due to fluctuating levels of dopamine transporter (DAT) protein using immunocytochemical identification.*

Three experiments were performed to assess possible synergistic effects of 1) permethrin, 2) chlorpyrifos and 3) permethrin combined with chlorpyrifos upon MPTP neurotoxicity in immunocytochemical studies conducted by Dr. Klein's laboratory. These immunohistochemical MPTP synergy experiments were fairly consistent in demonstrating an expected decrease in TH immunoreactivity and an increase in GFAP immunoreactivity in the striatum in MPTP treated groups (2003 report). This result would be expected from the degeneration of nigrostriatal terminal neuropil. However, there was no evidence that permethrin and chlorpyrifos, alone or together, could alter the effects upon TH or GFAP immunoreactivity produced by MPTP (2003 report). This difference is most likely related to the fact that the immunocytochemical analyses were performed at t = 1 day, whereas the western blots showed the greatest effect at t = 28 days.

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

Methods for measuring rearing and movement frequencies in treated mice can be found in Karen et al. (2001), and we previously summarized our preliminary results of behavioral studies with combination treatments (reports for 2000 and 2001). In the 2003 report, we noted a clear trend for decreasing movement across all three time points in the combination treatments, especially the triple treatment group (MPTP+PM+CPF) at t = 28 days. However, there were few statistically significant effects when the data were subjected to a single large analysis set. In Parkinsonism, a loss of 80% of nigro-striatal dopamine occurs before clinical symptoms appear (Marsden, 1990), so neurochemical effects are more sensitive than behavioral analysis. Thus, a lack of statistical significance for the behavior is not too surprising. Accordingly, we left this data out of the manuscript by Kou et al. (2006)

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

Combination treatments were also evaluated for their effect on acetylcholinesterase activity in the striatum in a single experiment at t = 1 day (report for 2000). CPF at 75 mg/kg gave about 70% inhibition of acetylcholinesterase, while PM and MPTP treatment caused a small decrease in cholinesterase activity. Combining the toxicants showed little increase in effect on enzyme activity compared to that observed with CPF alone, with no indication of greater inhibition, and in fact, M+C actually showed significantly less inhibition than CPF alone (report for 2000).

Reasons for this remain unclear, and the overall paucity of biologically interesting effects in the combination treatments convinced us that there was little reason to study them further.

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

A limited series of experiments were run in mice treated with combinations of neurotoxicants that were assessed at $t = 1$ day post-treatment (see report for 2002). Complete comparisons of QNB and epibatidine binding for combination treatments are now reported (Figs. 3-6). The data are presented with $t = 1$ day and $t = 28$ day post-treatment times shown together on the same graph. The $t = 1$ day effects will be discussed here and the reversibility data at $t = 28$ days (Objective 3) in the next section of this report. A significant up-regulation of QNB binding after MPTP treatment was observed (Fig. 3). This up-regulation is consistent with the previous findings of Mizukawa et al. (1987), using autoradiographic analyses in MPTP-treated mice. PM alone had little effect on QNB binding at this dose (same as found in report for 2001), and while CPF showed the expected down-regulation, it was not quite statistically significant. When combined with doses of CPF or PM expected to down-regulate or up-regulate binding, respectively, the net effect was a reduction of the MPTP-induced up-regulation (Fig. 3). In the case of CPF, the expected down-regulation would appear to be an additive effect. For PM, the underlying cause of the reduced binding for M+P is less clear, but could be related to an overall increase in general toxicity. However, no greater effect was seen in the triple treatment group. A similar pattern of effects occurs in cortex at $t = 1$ day (Fig. 4).

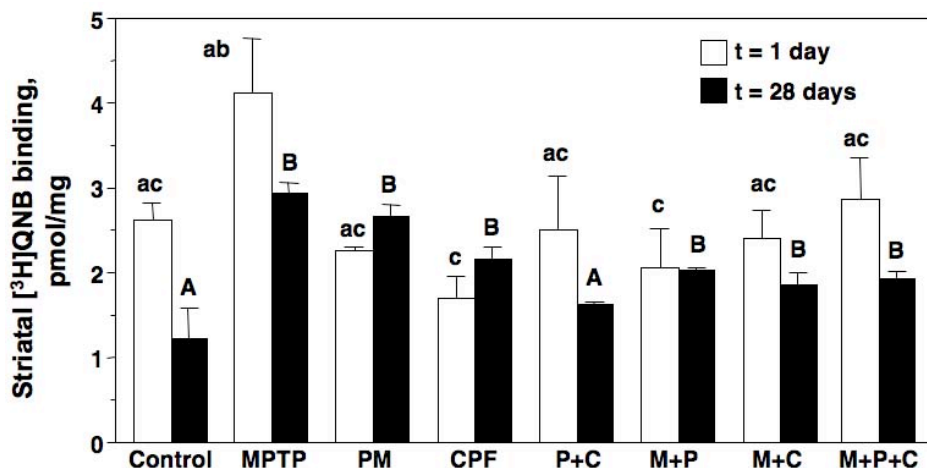


Figure 3. Effects of combination treatments on expression of QNB binding in striatal membranes at two post-treatment times. Bars are means with SEM and treatment abbreviations for combinations use the first letter of the toxicant (MPTP = M at 20 mg/kg; PM = P at 200 mg/kg; CPF = C at 75 mg/kg). Mean responses to toxicants within each post-treatment time were subjected to ANOVA, followed by Student-Newmann-Keuls post test. Within each post-treatment time, bars not labeled by the same letter are significantly different ($p < 0.05$). For $t = 1$ day, lower case letters are used and for $t = 28$ days, upper case letters are used.

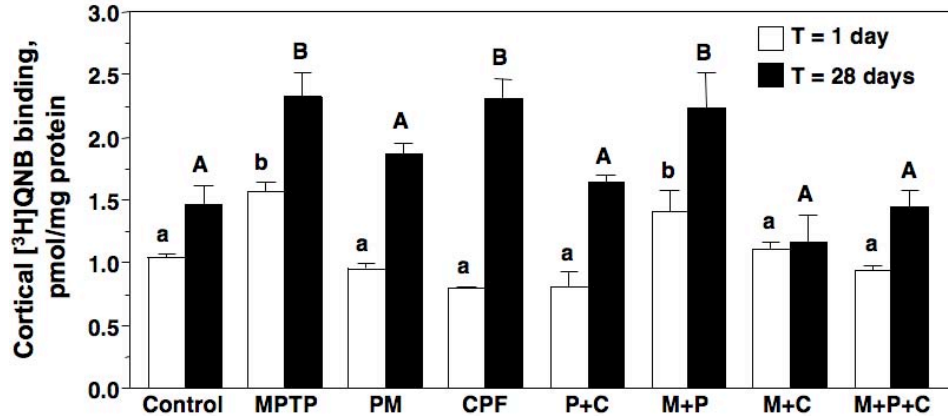


Figure 4. Effects of combination treatments on expression of QNB binding in cortical membranes at two post-treatment times. Bars are means with SEM and treatment abbreviations/doses are the same as given in Fig. 3. Mean responses to toxicants within each post-treatment time were subjected to ANOVA, followed by Student-Newmann-Keuls post test. Within each post-treatment time, bars not labeled by the same letter are significantly different ($p < 0.05$). For $t = 1$ day, lower case letters are used and for $t = 28$ days, upper case letters are used.

EB binding was increased in striatal membranes by both PM and CPF, with a decrease caused by MPTP, that was not quite statistically significant (Fig. 5). A previous study showed that MPTP treatment reduced nicotinic receptor expression (EB binding) in mice about 22% (Quik et al., 2003), so our results are similar. Effects of combination treatments tended to be additive in nature with two exceptions. P+C showed less than additive up-regulation, and overall binding in the triple treatment group was reduced, consistent with greater overall toxicity (Fig. 5).

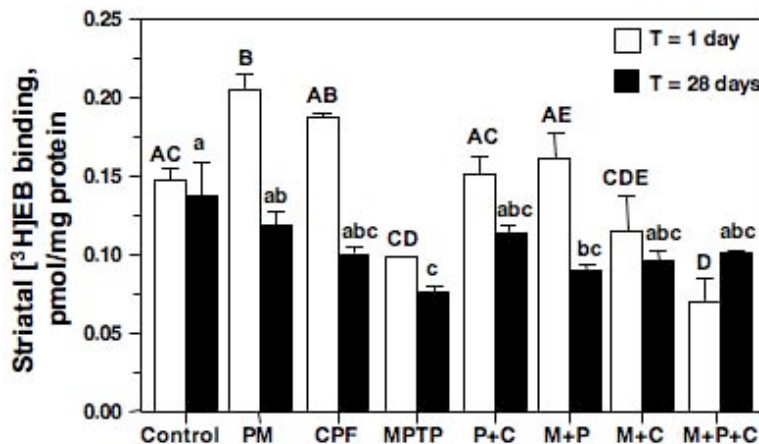


Figure 5. Effects of combination treatments on expression of epibatidine (EB) binding in striatal membranes at two post-treatment times. Bars are means with SEM and treatment abbreviations/doses are the same as given in Fig. 3. Mean responses to toxicants within each post-treatment time were subjected to ANOVA, followed by Student-Newmann-Keuls post

test. Within each post-treatment time, bars not labeled by the same letter are significantly different ($p < 0.05$). For $t = 1$ day, upper case letters are used and for $t = 28$ days, lower case letters are used.

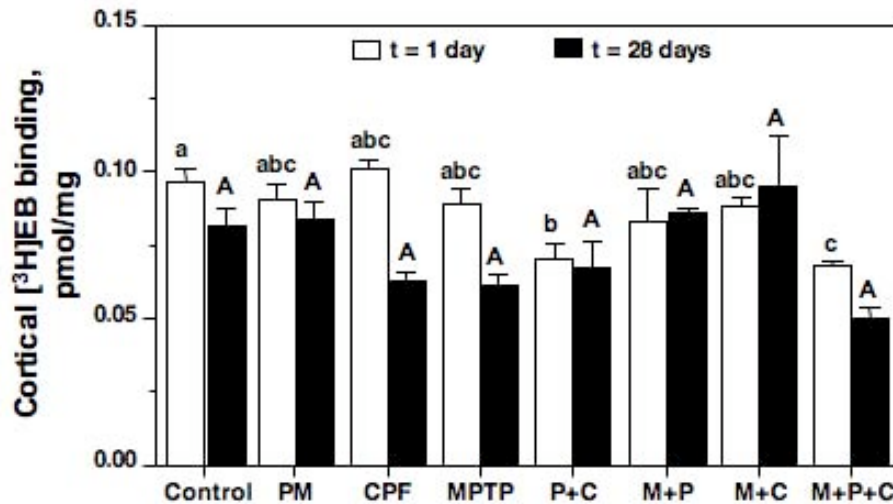


Figure 6. Effects of combination treatments on expression of epibatidine (EB) binding in cortical membranes at two post-treatment times. Bars are means with SEM and treatment abbreviations/doses are the same as given in Fig. 3. Mean responses to toxicants within each post-treatment time were subjected to ANOVA, followed by Student-Newmann-Keuls post test. Within each post-treatment time, bars not labeled by the same letter are significantly different ($p < 0.05$). For $t = 1$ day, lower case letters are used and for $t = 28$ days, upper case letters are used.

There was little effect of the individual toxicants on EB binding in the cortex at $t = 1$ day (Fig. 6), confirming a greater effect in the striatum. In fact, the only significant effects at $t = 1$ day was a reduction in binding in the P+C and triple treatment groups, consistent with a greater general toxicity. Quik et al. (2003) also observed that the reduction in EB binding by MPTP was specific for the striatum, and was not observed in cortex.

Objective 3: Evaluate the Reversibility of Effects by Assessing Biomarkers at Various Times Post-Treatment

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.

Because we observed only a small increase (ca. 10%) in DOPAC levels by a high dose of chlorpyrifos (Karen *et al.*, 2001), reversibility of this effect was not considered worthy of additional investigation.

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

As mentioned in the section on Objective 1, at a dose of 1.5 mg/kg, we observed an up-regulation of DAT expression 24 hr after the last treatment (Karen *et al.*, 2001; Gillette and Bloomquist, 2003). This up-regulation peaked at 28 days post-treatment, and declined thereafter (report for 2003, Fig. 2). These results demonstrate that this effect of PM can be slowly developing, and is reversible.

c.-g. Biomarkers

As mentioned in the previous sections, no reversibility studies of these effects were performed because of their small magnitude or poor reproducibility.

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

We determined the cortical acetylcholinesterase activity after CPF treatment at additional time points (1, 14, or 28 days), for correlation with measures of QNB binding and behavior (report for 2002). In these studies, 100 mg/kg CPF showed initially about 60% inhibition of enzyme activity (1 day), and it was stable for 2 weeks following the last dose. By $t = 28$ days, the activity had substantially recovered, and the enzyme was 18% inhibited, although this level of activity was not quite significantly different from the control ($p = 0.0583$).

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

In the 2002 report, we expanded the QNB studies to determine the extent of reversibility of the primary insecticide effects (up-regulation by 50 mg/kg PM and down-regulation by 100 mg/kg CPF) in both cortex and striatum. The down-regulation by CPF in striatum had rebounded by $t = 28$ days to levels significantly above that of controls, and the PM effect had reverted to control levels (report for 2002). We can find no precedent for this rebound effect in the literature, and observed it in both PM (200 mg/kg) and CPF (75 mg/kg) treated mice in recent $t = 28$ day studies (Fig. 3). Moreover, at $t = 28$ days QNB binding remained elevated by MPTP. With the exception of the C+P group, all other combination treatments showed elevated QNB binding compared to controls, but no more than that caused by the individual chemicals themselves ($t = 28$ days, Fig. 3). In the cortex, there was a pattern of effects at $t = 28$ days that was similar to striatum (Fig. 4). Although there is no effect of CPF on cortical QNB binding at $t = 1$ day (Fig. 4), the up-regulation of binding occurred in this tissue at $t = 28$ days. MPTP also maintained its effect on QNB. Of the combination treatments at $t = 28$ days, only M+P was changed (elevated) compared to control (Fig. 4).

The up-regulation of EB binding in striatum caused by PM and CPF at $t = 1$ day had subsided by $t = 28$ days (Fig. 5). Thus, the up-regulation is transitory. In contrast, at $t = 28$ days, MPTP caused a significant reduction in EB binding, while the M+P group was the only combination treatment significantly different from control (Fig. 6). There were no statistically significant effects on EB binding in cortical membranes by any of the treatments at $t = 28$ days, indicating that the reduction in binding by the P+C and triple treatment groups was a reversible effect.

Objective 4: Define Which Isomer of PM is Responsible for the Up-regulation of the Dopamine Transporter/ α -Synuclein.

PM has two chiral carbons in the acid moiety, and exists as four diastereomers (Fig. 1, of Kou *et al.*, 2006, in press). We used the treatment regime of Fig. 1 for these studies (assessments 1 day post-treatment) and highly purified samples of *cis*-PM and *trans*-PM for this work. Jinghong Kou's initial attempts to use DAT expression for these studies was not fruitful (see report for 2003), so we used α -synuclein expression as a biomarker instead. Technical PM (200 mg/kg) significantly increased α -synuclein expression by 19% (less than what we observed before, Gillette and Bloomquist, 2003), with the *cis* and *trans* isomers showing increases of 11% and 13%, respectively, for a roughly additive effect (Fig. 6 of Kou *et al.*, 2006, in press). Thus, both *cis* and *trans* isomers contributed to this response, whereas we expected 100% of the response to be from the *cis* isomer. Recall that only the 1*R*-*cis* isomer of PM (Fig. 1) has significant lethal activity in mice, principally via an effect on voltage-sensitive sodium channels of nerve membrane (Soderlund *et al.*, 2002). The corresponding 1*S*-*cis* and both *trans* isomers of PM are without lethal effect in mammals and do not appreciably modify sodium channel function [33]. Nonetheless, the overall effect of technical PM on α -synuclein expression was roughly additive for the *cis* and *trans* isomers, suggesting other targets are involved, such as voltage-sensitive calcium channels, or various phosphorylation signaling pathways (Soderlund *et al.*, 2002). In contrast, both the *cis* and *trans* isomers of PM are toxic to insects (Soderlund *et al.*, 2002), so if the *trans* isomer did not up-regulate α -synuclein, it would have been possible to eliminate this effect by using a PM formulation of pure *trans* isomer. Unfortunately, this is not the case.

Objective 5: Determine Effects of Long-Term, Low-Dose Exposures of PM on Dopaminergic Biomarkers With and Without MPTP.

During the last period of work, supplemental funding for a graduate student (Jinghong Kou) was provided by the sponsor to support studies on long-term, low-dose treatments with PM, alone or with MPTP (Fig. 7) to see if prolonged exposures showed enhanced toxicity. Biomarkers at the end of the exposures were levels of DAT, TH, and α -synuclein proteins as measured by Western blotting. These studies are incorporated into the attached manuscript for *Toxicology Letters* (Kou *et al.* see appendix).

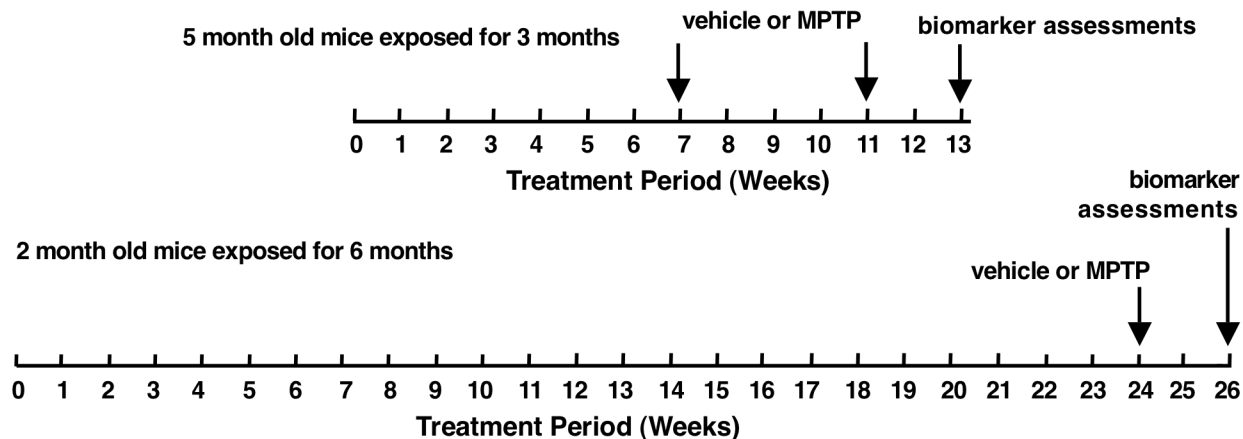


Figure 7. Experimental protocol for 3 month (12 treatments) and 6 month (24 treatments) exposure to PM using weekly ip injections. Vehicle or MPTP were given at either of 2 post-treatment times as indicated by the arrows. For the 13 week exposures, MPTP was given on week 7 (6 weeks before the last PM treatment) or week 11 (2 weeks before the last PM treatment). For the 6 month exposure, MPTP was given 2 weeks before the last PM treatment, only. Mice were sacrificed 24 hr after the last PM treatment, when 8 months of age.

Long-term exposure to low doses of PM did not cause a significant increase of general toxicity. During the 3-month or 6-month treatment period, no more than 2 mice died in any treatment group, and there was no significantly enhanced mortality in the PM and/or MPTP treatment groups. There was also no significant loss of body weight in the groups treated with PM and/or MPTP compared with controls (data not shown).

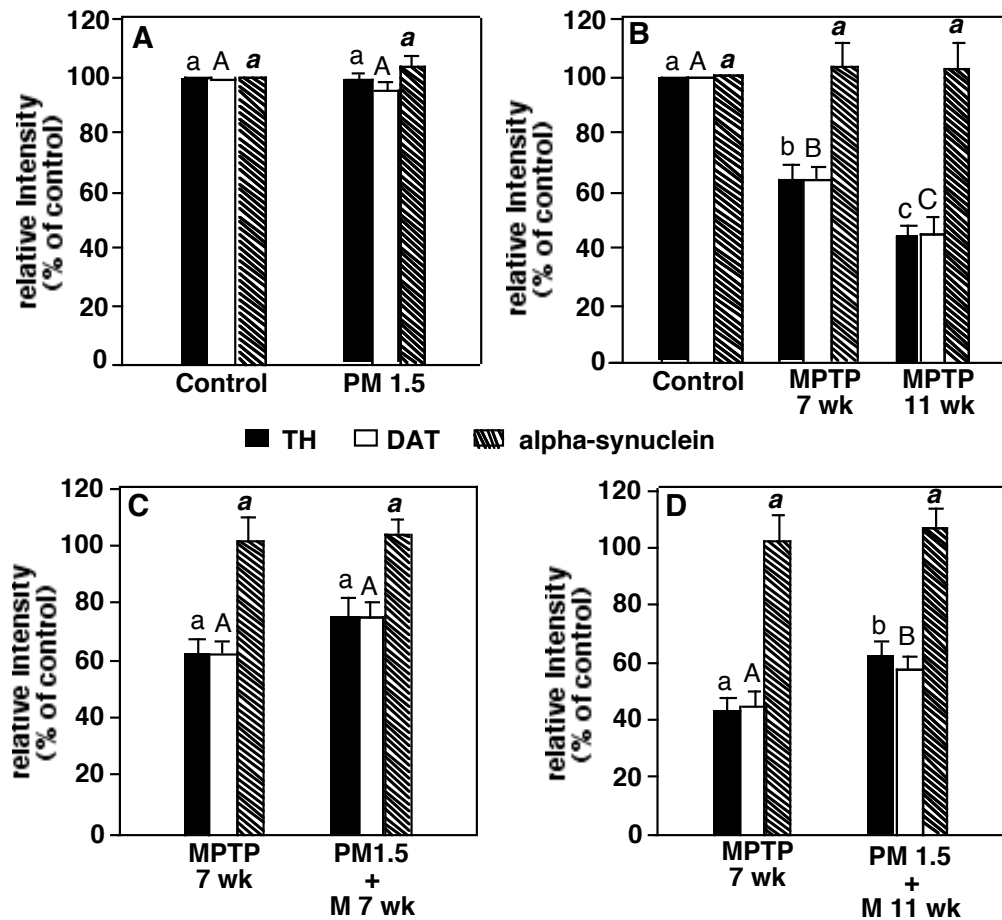


Figure 8. Western blot analysis of TH, DAT and α -synuclein protein of striatal tissue taken from C57BL/6 mice treated with PM (1.5 mg/kg) for 3 months, with or without a single injection with MPTP (M) (20 mg/kg) at the 7th week or the 11th week. Western blot data were expressed as the percentage of control. Panels A-D are arranged to best illustrate appropriate statistical comparisons. Bars labeled by different letters are significantly different (p<0.05). Statistical significance among treatment groups are designated for each

biomarker, with TH, DAT, and α -synuclein labeled by lower case, capital, and bold italic letters, respectively.

Representative western blots of DAT and TH proteins following low-term exposure to low doses of PM, either with or without MPTP, is shown in Figure 8. Western blot data revealed that 3-month exposure to PM (1.5 mg/kg) had no effect on the expression of TH and DAT protein in striatal dopaminergic terminals (Fig. 8A). A single treatment with 20 mg/kg MPTP persistently reduced the expression of both TH and DAT until 6 weeks after the MPTP injection, with a partial recovery over time (Fig. 8B). Long-term treatment with PM (1.5 mg/kg) for 3 months did not enhance the neurotoxicity of MPTP on the striatal dopaminergic system (Fig. 8C, D). Instead, 3-month exposure to 1.5 mg/kg PM significantly antagonized the down-regulation effect of MPTP on TH protein when MPTP was given at the 11th week ($p < 0.05$), but this effect was not quite significant when MPTP was given at the 7th week ($p = 0.07$) (Fig. 8C, D). The expression of α -synuclein protein was also quantified by western blot assay, but there was no significant change in α -synuclein expression in any of the treatment groups (Fig. 8A-D).

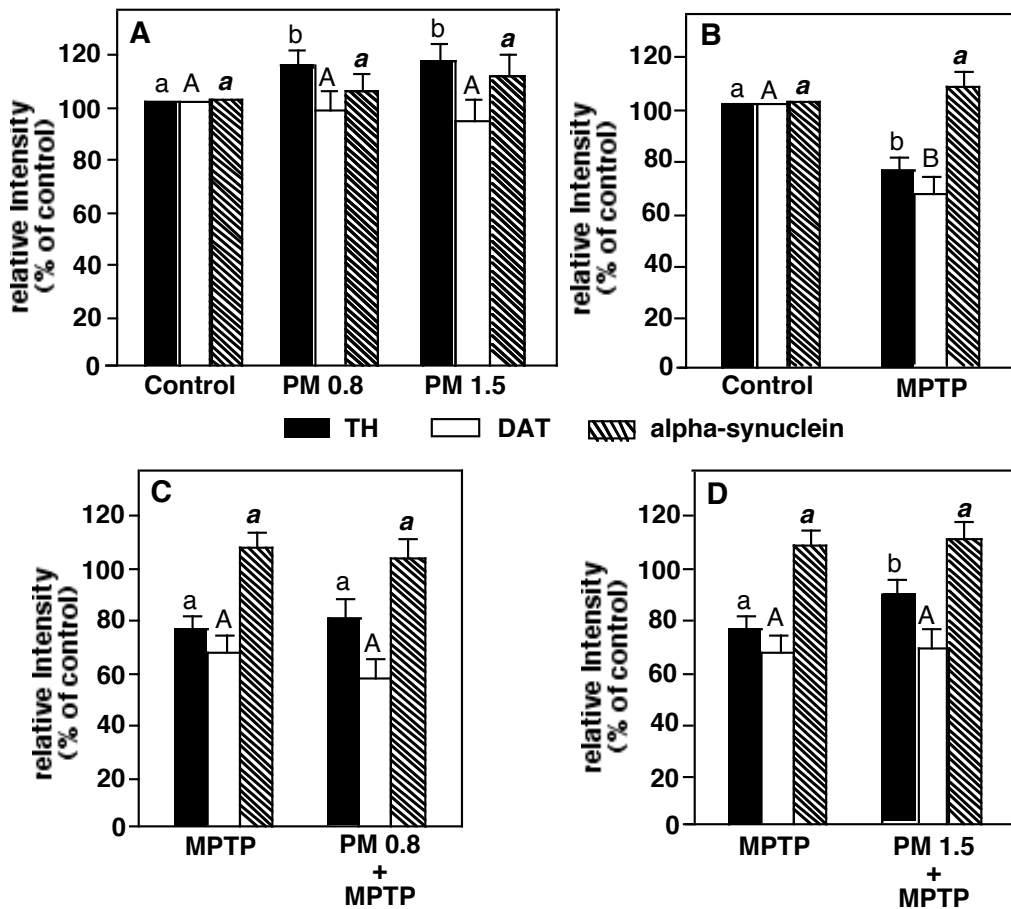


Figure 9. Western blot analysis of TH, DAT and α -synuclein protein of striatal tissue taken from C57BL/6 mice treated with PM (0.8/1.5 mg/kg) for 6 months, with or without a single injection with MPTP (20 mg/kg) given at 2 weeks before the end of the treatment period. Western blot data were expressed as the percentage of control. Bars labeled by different

letters are significantly different ($p < 0.05$). Panels A-D are arranged to best illustrate appropriate statistical comparisons. Statistical significance among treatment groups are designated for each biomarker, with TH, DAT, and α -synuclein labeled by lower case, capital, and bold italic letters, respectively.

Because the only statistically significant effects of PM on MPTP-induced neurotoxicity were observed at week 11, 6-month exposures were done at 2 doses, but only one MPTP treatment time (2 weeks prior to the end of the study). Six-month exposure to either 0.8 mg/kg PM or 1.5 mg/kg PM up-regulated TH expression about 17% compared to control, but did not alter the expression of DAT (Fig. 9A). A single dose of 20 mg/kg MPTP reduced the expression of both TH and DAT to 80% of control and to 75% of control, respectively (Fig. 9B). Treatment with PM (0.8 and 1.5 mg/kg) for 6 months did not enhance the neurotoxicity of MPTP on the striatal dopaminergic system either (Fig. 9C, D). Again, pre-exposure to 1.5 mg/kg of PM attenuated the effect of MPTP on TH reduction (Fig. 9D). The expression of α -synuclein protein was not changed in any of the treatment groups (Fig. 9A-D).

Thus, there was no evidence of PM-induced neurotoxicity in the 3 and 6 month exposures, and it did not exacerbate the effects of MPTP. In fact, PM generally decreased the effects of MPTP toxicity (Figs. 8D and 9D), in contrast to the increase in toxicity observed at high doses of PM and CPF (Kou et al., 2006). The mechanisms behind this effect remain unclear. There was an increase in TH expression by PM in the 6 month exposure, possibly a response to chronic, low level release of dopamine via effects on voltage-sensitive sodium channels (Soderlund *et al.* 2002). Additional Discussion of these results can be found in the accompanying paper draft for *Toxicology Letters* by Kou, Klorig, and Bloomquist (see appendix).

A final issue concerns the possible exposure of soldiers wearing PM-impregnated uniforms. A study in rabbits exposed them to cloth treated with [^{14}C]-labeled PM (0.125 mg/cm², the rate used for military uniforms) for 7 days (Snodgrass, 1992). At the end of the 7 day exposure, 3.2% of the available PM had reached the skin and about 2% was absorbed, as indicated by its presence in excreta, giving a daily uptake rate of 6×10^{-4} mg/kg/day (Snodgrass, 1992). This exposure is more than an order of magnitude less than any dose we found to have a statistically significant effect in our studies.

KEY RESEARCH ACCOMPLISHMENTS

Research accomplishments throughout the lifetime of the project were described under each of the objective/biomarker sections given above.

REPORTABLE OUTCOMES

Meeting Presentations (speaker underlined)

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.

W. Li, D. Karen, P. Harp, B. Klein, and J. Bloomquist, Murine Dopaminergic Pathways as Targets of Multiple Chlorpyrifos or Permethrin Exposures, fall 2000, National Meeting of the Society for Environmental Toxicology and Chemistry, Nashville, Tennessee.

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.

J. R. Bloomquist, Pesticides and Parkinsonism: Military Insecticide Exposures and Its Relevance to the General Public. Spring 2001, Invited Speaker, Capital Chapter of the National Parkinson Foundation, Parkinson's Community Support Group, Fairfax, Virginia.

J. R. Bloomquist, Pesticides and Parkinsonism in Gulf War Syndrome. Spring 2001, Invited Speaker, Department of Toxicology, North Carolina State University, Raleigh, North Carolina.

J. R. Bloomquist, Insecticide Exposure, Dopamine Neurotoxicity, and Parkinson's Disease. Spring 2001, Invited Speaker, Department of Entomology, University of California, Davis, California.

J. R. Bloomquist, Insecticide Exposure, Dopamine Neurotoxicity, and Parkinson's Disease. Summer 2001, Invited Speaker, Department of Entomology, University of California, Riverside, California.

J.T. Pittman, C.A. Dodd and B.G. Klein, Effects of the pyrethroid insecticide permethrin upon tyrosine hydroxylase and dopamine transporter immunoreactivity in the mouse striatum. Fall 2001, National Meeting of the Society for Neuroscience, .

J. R. Bloomquist, Impact of Organochlorine, Pyrethroid, and Organophosphate Insecticides on Striatal Neurochemistry, Summer 2001, Nineteenth International Neurotoxicology Conference: Parkinson's Disease, Environment and Genes, Colorado Springs, Colorado.

J. S. Gillette and J. R. Bloomquist, Modulation of Murine Striatal Dopamine Transporter Expression by the Pyrethroid Insecticide Permethrin, spring 2002, National Meeting of the Society of Toxicology, Nashville, Tennessee.

J. R. Bloomquist, Low Dose Effects of Insecticides to Dopaminergic Pathways Involved in Parkinsonism. Spring 2003, National Meeting of the American Chemical Society, New Orleans, Louisiana.

J. R. Bloomquist, Actions of Organophosphorus and Pyrethroid Insecticides on Dopaminergic Pathways Involved in Parkinsonism. Spring 2003, School of Pharmacy, Dept. of Pharmaceutical Science, Texas Tech University Health Sciences Center, Amarillo, Texas.

J. R. Bloomquist, Dopaminergic Neurotoxicity of Insecticides. Spring 2003, Interdisciplinary Neuroscience Seminar Series, Virginia Tech.

J. R. Bloomquist, Insecticide exposure in the MPTP-treated C57 mouse model of Parkinson's disease. Fall 2004, National Meeting of the American Chemical Society, Division of Agrochemicals, Philadelphia, Pennsylvania.

J. Kou, and J. R. Bloomquist. Interactions on striatal dopaminergic pathways following co-application of permethrin, chlorpyrifos, and MPTP. 2004, Poster, National Meeting of the Society for Neuroscience, San Diego, California.

J. R. Bloomquist. Impact of Insecticide exposure in the MPTP-treated C57 mouse model of Parkinson's disease. Spring 2005, University of Massachusetts, Department of Veterinary and Animal Science, Biomedicine and Biotechnology Program, Amherst, Massachusetts.

Publications

D. Karen, W. Li, P. Harp, J. Gillette, and J. Bloomquist, Striatal Dopaminergic Pathways as a Target for the Insecticides Chlorpyrifos and Permethrin. *NeuroToxicology* 22, 811-817 (2001).

J. Bloomquist, R. Barlow, J. Gillette, W. Li, and M. Kirby, Selective Effects of Insecticides on Nigrostriatal Dopaminergic Nerve Pathways. *NeuroToxicology* 23, 537-44 (2002).

J. S. Gillette and J. R. Bloomquist, Differential Up-Regulation of Striatal Dopamine Transporter and α -Synuclein by the Pyrethroid Insecticide Permethrin. *Toxicol. Appl. Pharmacol.* 192, 287-293 (2003).

J. Pittman, C. Dodd, and B. Klein, Immunohistochemical Changes in the Mouse Striatum Induced by the Pyrethroid Insecticide Permethrin. *Intl. J. Toxicol.* 22, 359-370 (2003). Figure 1 from this paper was selected as cover art for this issue of the Journal (see appendix).

J. Kou, J. G. Gillette, and J. R. Bloomquist, Neurotoxicity in Striatal Dopaminergic Pathways Following Co-application of Permethrin, Chlorpyrifos, and MPTP. *Pestic. Biochem. Physiol.* 85, 68-75 (2006).

J. Kou, D. Klorig, and J. R. Bloomquist, Alteration of Murine Striatal Dopaminergic Biomarkers Following Long-Term Application of Low Doses of Permethrin. (for submission to *Toxicol. Lett.* See appendix)

D. J. Karen, P. R. Harp, J. S. Gillette, J. Kou, and J. R. Bloomquist, Effects of Chlorpyrifos, Permethrin, and MPTP on Murine Behavior and Striatal Cholinergic Biomarkers. (in preparation)

OVERALL CONCLUSIONS FROM THIS PROJECT

Up-regulation of dopamine transport occurred at low doses of PM (NOAEL = 0.2 mg/kg) and by itself provides a mechanism for possible synergism with pyridinium toxins, such as MPP⁺, which use the DAT to gain entry into the neuron. The up-regulation of the DAT is potent, and occurs at doses at least two orders of magnitude below the LD₅₀ of PM. However, no enhancement of MPTP effects were observed, *in vivo*, perhaps due to pharmacokinetic interactions.

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.

The significance of these studies for the neurotoxicology of insecticides is applicable beyond the scope of Parkinson's disease, especially the up-regulation of dopamine transporter observed at low doses of PM. We have previously shown that the organochlorine heptachlor (Miller *et al.*, 1999) and the pyrethroid deltamethrin increase dopamine transport (Kirby *et al.*, 1999). 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.

GFAP expression seems to be a more sensitive indicator of toxic insult than TH levels in immunocytochemical studies, and although each was effective alone, there was no significant enhancement of effect when combining high doses of PM with MPTP. Differences in results between western blot and immunocytochemical studies may be due to sampling effects: the Westerns take essentially all of the striatum, whereas the immunocytochemical studies looked specifically at those subregions that decline in PD (see Pittman *et al.*, appendix).

The enhanced effect of PM, CPF, and MPTP in 28 day analyses of DAT, TH, and synaptophysin expression suggests that insecticide exposure could induce or exacerbate idiopathic disease processes, albeit at high doses.

Down-regulation of muscarinic receptors (QNB binding) as a response to cholinesterase inhibition shows a good correlation between these two biomarkers of cholinergic neurochemistry, as shown in previous studies by other investigators.

Insecticide-induced up-regulation in nicotinic receptors (EB binding) is transient, but its functional significance is unclear at the present time.

The primary conclusion of this work is that there appears to be no undue Parkinsonian hazard from any insecticide exposures likely to occur from wearing clothing impregnated with PM and proper use of CPF.

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PERSONNEL RECEIVING PAY FROM THIS PROJECT

Working with Dr. Bloomquist:

Dr. Paul Harp

Dr. Wen Li

Dr. Dan Karen

Dr. Jeff Gillette

Jinghong Kou, who finished her Ph.D. degree with funding from the end of this project.

Working with Dr. Klein:

Mr. Julian Pittman, who finished his M.S. degree with funding from this project.

Ms. Celia Dodd

Ms. Sarah Koss

APPENDICES

Meeting Abstracts (presenter*) Many of the presentations did not have abstracts.

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 [³H]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 (≥ 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 [³H]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 [³H]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 μ M concentration stimulates about 80% dopamine release with an IC_{50} of 3 μ 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.

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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 [³H]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 [³H]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 [3 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 μ M concentration stimulated approximately 80% dopamine release with an IC_{50} of 3 μ M. Permethrin (1.5 – 6 mg/kg) affected [3 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 [3 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.

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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 [3 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. However, K_d 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 [3 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.

Abstract for Nineteenth International Neurotoxicology Conference: Parkinson's Disease, Environment and Genes, Colorado Springs, Colorado, summer 2001.

IMPACT OF ORGANOCHLORINE, PYRETHROID, AND ORGANOPHOSPHATE INSECTICIDES ON STRIATAL NEUROCHEMISTRY. *JR Bloomquist. Department of Entomology, Virginia Polytechnic Institute and State University, Blacksburg, VA, 24061.

Neurochemical changes in C57BL/6 mice were investigated following three doses of the pyrethroids permethrin or deltamethrin, the organochlorine heptachlor, or the organophosphate chlorpyrifos, given over a two week period. All the insecticides altered [³H]dopamine uptake in striatal synaptosomes prepared from treated mice, which was observed by low doses of permethrin (1.5 mg/kg) and heptachlor (6 mg/kg), but only at high doses of chlorpyrifos (100 mg/kg). Upregulation of dopamine transporter protein by permethrin and heptachlor was confirmed by antibody labeling of synaptosomal protein in Western blots.

This upregulation may reflect toxicant-induced changes in synaptic levels of dopamine. Neurotransmitter release studies with striatal synaptosome preparations *in vitro* found that deltamethrin and heptachlor potentiated release of preloaded dopamine from synaptosomes with greater potency than other types of neurotransmitters. This neurotransmitter release effect correlated to a surprising degree with previous measurements of [³H]tyramine displacement from synaptic vesicles, although the relationship between this effect and release is unclear.

Although the insecticides had little effect on striatal dopamine content in this treatment paradigm, striatal DOPAC (dihydroxyphenylacetic acid, a dopamine metabolite) was increased 37% by deltamethrin and 14% by chlorpyrifos, further suggesting an increase in dopamine turnover *in vivo*. High doses of the insecticides were also found to disrupt nerve terminal respiration. While the findings do not show a discrete Parkinsonian effect of these insecticide classes, they demonstrate insecticide-induced neurochemical changes that might interact with other factors in idiopathic Parkinson's Disease.

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). [³H]Dopamine uptake was significantly increased at lower chlorpyrifos doses, peaking at 108% of control, while higher doses (100 mg/kg) of chlorpyrifos significantly repressed [³H]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 [³H]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 [³H]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 [³H]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.

Abstract for Society for Neuroscience, 2001

EFFECTS OF THE PYRETHROID INSECTICIDE PERMETHRIN UPON TYROSINE HYDROXYLASE AND DOPAMINE TRANSPORTER IMMUNOREACTIVITY IN THE MOUSE STRIATUM

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Low doses (1.5-6 mg/kg) of the insecticide permethrin (PM) increase dopamine (DA) uptake in striatal synaptosomes of C57BL/6 mice, and high doses (25-200 mg/kg) produce a decrease. To explore the underlying mechanism, we examined DA transporter (DAT) immunoreactivity (IR) in striata of black mice at low doses (0.8, 1.5 & 3.0 mg/kg) of PM, and both DAT and tyrosine hydroxylase (TH) IR at a high dose (200 mg/kg) of PM. Groups received 3 IP injections over 2 wks. Each PM mouse was paired with a vehicle control, comprising a case. Pixel counts of IR neuropil in dorsolateral striatum were made from 4 pre-selected fields per section. Counts were compared between matched sections, processed on the same slide, from a PM mouse and its vehicle control. A mean difference score, across slides, for each case was determined. All PM groups showed decreased DAT IR, but only the 3.0 mg/kg group showed a significant reduction ($p < .0078$). The 200 mg/kg group showed a trend toward reduced TH IR that was not significant. These data suggest that increased DA uptake, from low doses of PM, is not due to increased DAT protein in striatum and the reduction in DA uptake with high doses is not due to degeneration of DA terminals in striatum. Our low-dose decrease in DAT IR, in light of increased DA uptake, may indicate a very high affinity of DAT for DA, despite reduced DAT protein. Alternatively, synaptosomal data was sampled from whole striatum, while we sampled small regions of dorsolateral striatum. Regional differences may exist in the response of striatal afferents to PM exposure.

Abstract for Society of Toxicology, 2002:

MODULATION OF STRIATAL DOPAMINE TRANSPORTER EXPRESSION IN THE C57BL/6 MOUSE BY THE PYRETHROID INSECTICIDE PERMETHRIN.

*JS Gillette and JR Bloomquist. Dept. of Entomology, Virginia Polytechnic and State University, Blacksburg, VA, USA.

The pyrethroid insecticide permethrin may play a role in the development of Parkinson's disease as a consequence of Gulf War Syndrome. Previous work in our laboratory has shown that an i.p. dose of 1.5 mg/kg permethrin in the C57BL/6 mouse causes a significant increase in striatal dopamine uptake to 125% of control values. Recently, we have dosed C57 mice with either 0.8 or 1.5 mg/kg permethrin (3 i.p. injections over 2 weeks). To assess reversibility of effects on striatal dopamine transporter (DAT), a portion of the mice were sacrificed on the day following the last dose ($t=0$), while a second group was sacrificed 2 weeks post last treatment ($t=2$), and a third group 4 weeks post last treatment ($t=4$). At $t=0$, DAT protein as assayed by Western blotting was increased 119% and 107% in the 0.8 and 1.5 mg/kg group, respectively, over that of control mice. DAT binding was analyzed by incubating the ligand [3 H]GBR 12935 with striatal synaptosomes from control and treated mice. [3 H]GBR 12935 binding was increased 156% and 118% in mice treated with 0.8 and 1.5 mg/kg permethrin, respectively. Preliminary evidence suggests that this effect is persistent, since DAT protein was likewise elevated in the $t=2$ group of mice. Due to its effects on mouse DAT, the ability of permethrin to synergize the effects of the neurotoxin MPTP was ascertained. MPTP (30 mg/kg) was administered to C57BL/6 mice on day 1 of the treatments, followed the next day by administration of various doses of permethrin from 0.8 mg/kg to 200 mg/kg. Striatal dopamine and its metabolite dihydroxyphenylacetic acid (DOPAC) were determined by HPLC-ECD. Only the highest dose of permethrin (200 mg/kg) in combination with MPTP was able to deplete dopamine significantly greater than MPTP alone. However, DOPAC titers were significantly

elevated at doses above 50 mg/kg permethrin, suggesting an increase in dopamine turnover. Taken together, these data suggest that insecticides such as permethrin may have subtle effects on the dopaminergic system, and may be involved in the etiology of Parkinson's disease.

Dr. Jeffrey Gillette was selected as the second place winner in the Neurotoxicology Specialty Section poster competition held at this Society of Toxicology meeting in Nashville, spring 2002. The award consisted of a check in the amount of \$300 and an award certificate.

Abstract for Society for Neuroscience, 2004:

INTERACTIONS ON STRIATAL DOPAMINERGIC PATHWAYS FOLLOWING CO-APPLICATION OF PERMETHRIN, CHLORPYRIFOS, AND MPTP.

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Permethrin and chlorpyrifos exposure have been suggested to be contributory factors for both Gulf War illness and Parkinson disease, and the neurotoxic action of these compounds on striatal dopaminergic pathways was investigated in C57BL6 mice. Permethrin (200 mg/kg) and chlorpyrifos (75 mg/kg) were administered three times over a two-week period, with MPTP (20 mg/kg) given on the first day of the treatment period. The treated mice were sacrificed at twenty-four hours and four weeks after the last treatment. The alteration of dopamine transporter (DAT), tyrosine hydroxylase (TH), alpha-synuclein, and synaptophysin expression were analyzed as biomarkers. At twenty-four hours post-treatment, western blot assay showed that MPTP alone, or MPTP in conjunction with insecticides, decreased the expression of DAT, TH, and alpha-synuclein. Permethrin enhanced alpha-synuclein expression 20% by itself. At four weeks post-treatment, a significant reduction of DAT and TH were still observed in the MPTP-treated mice in conjunction with permethrin and/or chlorpyrifos, with greater effect in the triple treatment group. These findings demonstrate that the co-application of pyrethroid or organophosphorus insecticides enhanced the neurotoxicity of MPTP in C57BL6 mice.

Bibliography of Published Papers

See following attachments

Striatal Dopaminergic Pathways as a Target for the Insecticides Permethrin and Chlorpyrifos

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Abstract

Because insecticide exposure has been linked to both Parkinsons disease and Gulf War illness, the neurotoxic actions of pyrethroid and organophosphate insecticides on behavior and striatal dopaminergic pathways were investigated in C57BL/6 mice treated with permethrin (three i.p. doses at 0.2–200 mg/kg) or chlorpyrifos (three s.c. doses at 25–100 mg/kg) over a 2-week period. Permethrin altered maximal [3 H]dopamine uptake in striatal synaptosomes from treated mice, with changes in V_{max} displaying a bell-shaped curve. Uptake was increased to 134% of control at a dose of 1.5 mg/kg. At higher doses of PM (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 observed a small, but statistically significant decrease in [3 H]dopamine uptake by chlorpyrifos, when given at a dose of 100 mg/kg. There was no significant effect on the K_m for dopamine transport. 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 200 mg/kg PM or 100 mg/kg CPF treatment groups, 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. Although frank Parkinsonism was not observed, these findings confirm that dopaminergic neurotransmission is affected by exposure to pyrethroid and organophosphorus insecticides, and may contribute to the overall spectrum of neurotoxicity caused by these compounds. © 2001 Elsevier Science Inc. All rights reserved.

Keywords: Parkinson's disease; Dopamine transport; Pyrethroid; Organophosphate; Gulf War illness

INTRODUCTION

The pyrethroid and organophosphorus (OP) insecticides are members of two chemical classes of heavily used compounds, and hazards from exposure to insecticides exist from the manufacture, storage, spraying, and contact of personnel with insecticide-contaminated food or areas. Among the possible hazards is a consistent epidemiological linkage between insecticide exposure and the incidence of Parkinson's disease (Semchuk et al., 1992; Butterfield et al., 1993; Gorell et al., 1998). Further, the neurological health problems

which comprise Gulf War illness, reported by over 30,000 veterans, may be due to exposure of personnel to various chemicals. Specific compounds implicated include pyridostigmine bromide, the insecticides permethrin (PM) and chlorpyrifos (CPF), and the repellent *N,N*-diethyl-*m*-toluamide (DEET) (Abou-Donia et al., 1996; Abou-Donia et al., 2001). 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 to ascertain whether damage to dopaminergic pathways and attendant Parkinsonism

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might be a consequence of Gulf War chemical exposures.

MATERIALS AND METHODS

Chemicals

Analytical grade CPF was obtained from ChemService Inc. (West Chester, PA). PM (a mixture of four *R,S-cis* and *R,S-trans* isomers) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma Chemical Co., St. Louis, MO, USA. [³H]Dopamine (20.3 Ci/mmol) was purchased from NEN Life Science Products Inc., Boston, MA, USA. Choline-Cl, KCl, MgCl₂, CaCl₂, and ascorbate were obtained from Sigma Chemical Co., Pargyline, D-glucose, sucrose, and HEPES were obtained from Fisher Scientific Co., Pittsburgh, PA, USA.

Animals and Treatments

Male C57BL/6 retired breeder mice were utilized for all experiments. Mice were purchased from Harlan Sprague–Dawley, Dublin, VA, USA 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 six 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 2-week period according to the method of Bloomquist et al. (1999). CPF administration was by s.c. injection, while PM was administered by i.p. injection. Control mice received 50 μ l corn oil or 10 μ 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, crude synaptosomes were prepared from fresh striatal tissue dissected from treated mice, and incubated with [³H]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 (PrismTM, GraphPad Software, San Diego, CA, USA). Aliquots of each synaptosomal preparation were frozen at -20°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) to synaptosomes. 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), MgSO₄ (1.3), NaHCO₃ (5.0), Na₂HPO₄ (1.0), HEPES (10), glucose (10), and CaCl₂ (1.2), pH 7.4. After 30 min at 37°C, the tubes were centrifuged for 5 min at 10,000 \times g. The pellets were resuspended in DMSO to solubilize the formazan reduction product, and centrifuged again at 10,000 \times g for 1 min. 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, USA).

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 dihydroxybenzylamine (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 10,000 \times g to pellet tissues. Dopamine and its metabolite 3,4-dihydroxyphenylacetic acid (DOPAC) were separated by HPLC using an ODS 3 μ m Phase 2 column (3.2 mm \times 100 mm). Mobile phase consisted of 170 mM NaH₂PO₄, 1.5 mM octanesulfonic acid, 5.5% methanol, 1.5% acetonitrile, and 93% H₂O, 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 and prior to neurochemical analyses, behavioral effects were assessed by means of monitoring the number of open field movements and rearing frequency in an arena over a 3-min

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.

Statistical Analysis

Statistical significance was determined using one-way ANOVA and Student–Newman–Keuls means separation if a statistically significant main effect of treatment was observed. Other statistical comparisons were by *t*-test calculations performed using InStat™ (GraphPad Software).

RESULTS

Dopamine uptake rate in striatal synaptosomes displayed the expected saturation with increasing concentration of substrate (Fig. 1A). All curves showed a good fit to a Michaelis–Menten model, and typically had correlation coefficient (r^2) values ≥ 0.98 . PM treatment did not have any significant effect on the apparent K_m of the dopamine transporter in any of the treatment groups. The control values for K_m averaged 233 ± 28 nM (mean \pm S.E.M.). Maximal dopamine uptake in 9-month-old C57BL/6 mice treated with PM did vary with the dose, increasing at lower doses of PM, while at higher doses maximal uptake declined until it was less than the control level (Fig. 1A). Thus, a bar graph (Fig. 1B) of PM-induced V_{max} values from a broader range of doses 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

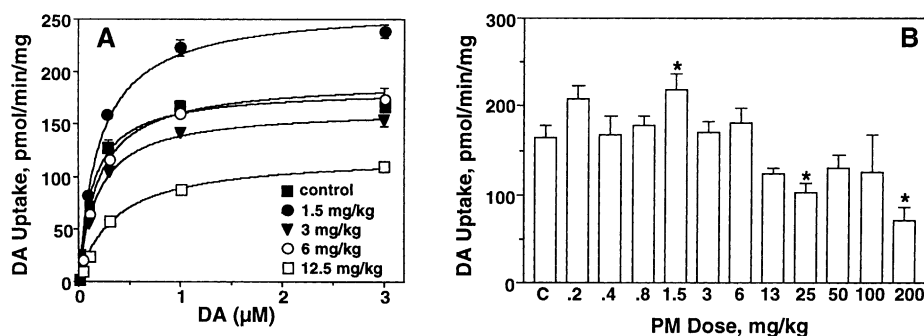


Fig. 1. Representative isotherm plots (A), and a bar graph (B) of the effects of PM on dopamine uptake. (A) Symbols represent means of three determinations with bars equal to the S.E.M. Absence of bars means that the S.E.M. was less than the size of the symbol. (B) Percentage changes in maximal dopamine uptake (V_{max}) following PM treatment at the indicated doses. Asterisks indicate effects significantly different from control (*t*-test, $P < 0.05$).

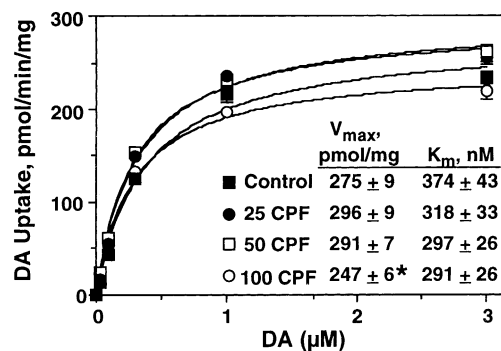


Fig. 2. Effect of CPF treatment on dopamine uptake in ex vivo striatal synaptosomes. Symbols represent means of three determinations with bars equal to the S.E.M. Absence of bars means that the S.E.M. was less than the size of the symbol. Kinetic and statistical analysis is given in the inset table. Asterisks indicate an effect significantly different from control (*t*-test, $P < 0.05$).

significantly greater (34%) 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. 1B). Treatment of mice with CPF also caused a reduction in dopamine uptake V_{max} at the highest dose administered (100 mg/kg) (Fig. 2). 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. 3). 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

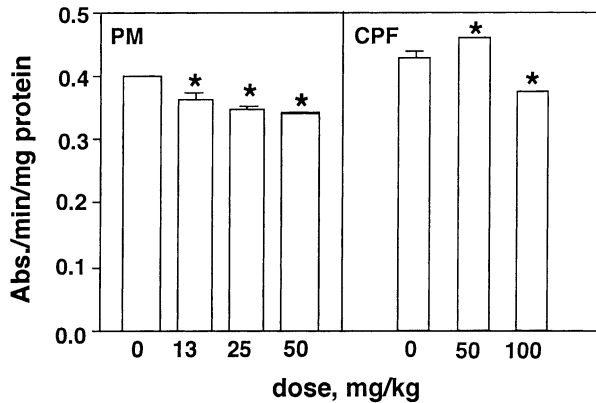


Fig. 3. Effect of PM (left) and CPF (right) treatment on mitochondrial activity (MTT reduction) in ex vivo striatal synaptosomes. MTT reduction activity is shown at a single concentration of 0.55 mM. In the bar graphs, the asterisk indicates an effect significantly different from control (ANOVA, $P < 0.05$).

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) increased striatal dopamine turnover, as indicated by significantly elevated titers of the dopamine metabolite, DOPAC (Fig. 4). The effect of CPF at this dose was an increase of 14% above control. In contrast, a high dose of PM (200 mg/kg) did not increase DOPAC titers. Neither CPF nor PM at these doses had any effect on striatal dopamine levels (data not shown).

CPF and PM both had similar dose-dependent 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. 5A and B). Similarly, high doses of PM decreased both frequency of open field movement and rearing frequency (Fig. 6A and B). The

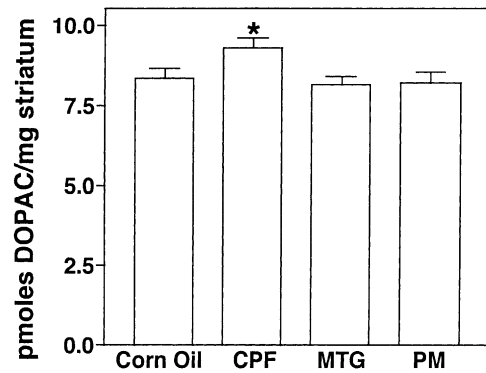


Fig. 4. Changes in DOPAC titers following treatment with vehicle (corn oil; MTG) or insecticide (100 mg/kg CPF; 200 mg/kg PM). Asterisks indicate effects significantly different from control (t -test, $P < 0.05$).

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 three orders of magnitude below the rat oral LD_{50} for PM (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 EC_{50} for dopamine release (48 nM) being 2.4- and

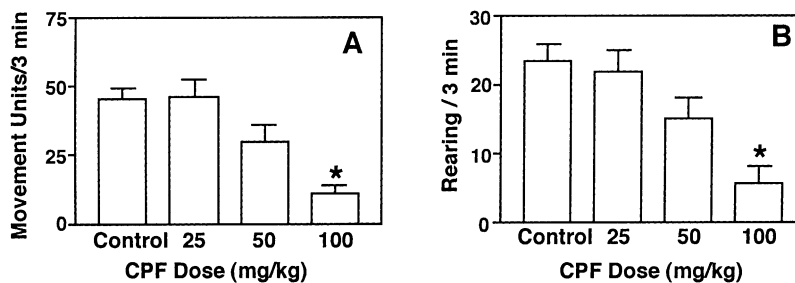


Fig. 5. Changes in open field (A), and rearing frequency (B) at the indicated doses of CPF. Asterisk indicates an effect significantly different from control (t -test, $P < 0.05$).

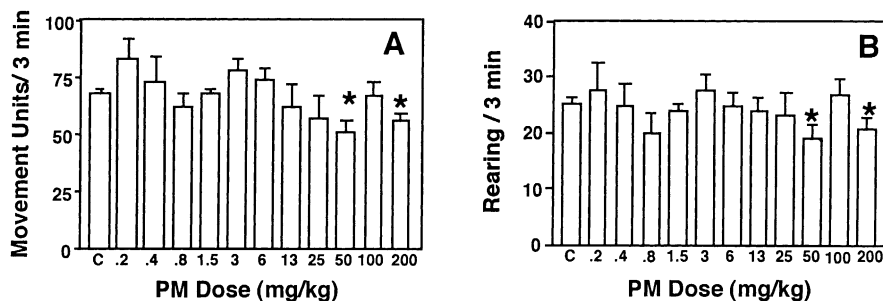


Fig. 6. Changes in open field (A), and rearing frequency (B) at the indicated doses of permethrin. Asterisk indicates an effect significantly different from control (*t*-test, $P < 0.05$).

8.6-fold more potent than serotonin or glutamate release, respectively (Kirby et al., 1999). Thus, increased dopamine outflow was presumably balanced by increased uptake to maintain normal dopaminergic neurotransmission.

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. We would expect degeneration 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 ≥ 12.5 mg/kg PM in the MTT assay, which is a measure of mitochondrial function (Carmichael et al., 1987). In future studies, we expect to observe an up-regulation of dopamine transporter (DAT) protein in western blots at doses near 1.5 mg/kg of PM. Our previous work has shown that the organochlorine insecticide heptachlor increases dopamine transport in male C57BL/6 mice about two-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% following three doses of 6 mg/kg (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 was the case for high doses of PM. Similarly, at a dose of 100 mg/kg CPF, MTT dehydrogenase activity is significantly depressed compared to controls, suggesting chemically-induced nerve terminal stress.

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 per 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, p.o.) probably gave a greater effective brain concentration than the treatment we used in this study (200 mg/kg, i.p.).

Changes in movement and rearing behaviors observed after CPF treatment are most likely due to inhibition of acetylcholinesterase activity, which is a hallmark of organophosphate exposure (Bowman and

Rand, 1980). At doses above 25 mg/kg, there is a good 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 Spinosa et al. (1999), in which movement and rearing frequencies were reduced by 10 and 30 mg/kg doses of the pyrethroid fenvalerate, which has greater mammalian toxicity than PM (Budavari et al., 1996). Although bradykinesia is a hallmark of PD (Bowman and Rand, 1980), it was not accompanied by a reduction in striatal dopamine in PM-treated mice, so another mechanism is probably responsible.

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 lack of any effect on dopamine titers does not support a rapidly developing Parkinsonism following short-term exposures to these compounds. Long-term exposure studies should be undertaken. A recent study (Lee et al., 2001) observed an interaction between the DAT and α -synuclein in cultured neurons, which led to increased dopamine uptake and dopamine-induced cellular apoptosis. Such a scenario would provide a mechanism whereby increased DAT expression by insecticides could play role in the development of Parkinsonism.

Future studies will determine the extent of synergism in the effects of these insecticides on the nigrostriatal dopaminergic pathway, since synergistic neurotoxicity has been observed with DEET, pyridostigmine bromide, chlorpyrifos, and permethrin in various combinations (Abou-Donia et al., 1996; Abou-Donia et al., 2001). Other studies will address the extent of reversibility of the observed PM and CPF effects on striatal neurochemistry.

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Selective Effects of Insecticides on Nigrostriatal Dopaminergic Nerve Pathways

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Abstract

A degeneration of the nigrostriatal pathway is a primary component of Parkinson's disease (PD), and we have investigated the actions of insecticides on this pathway. For *in vivo* exposures, C57BL/6 mice were treated three times over a 2-week period with heptachlor, the pyrethroids deltamethrin and permethrin, or chlorpyrifos. One day after the last treatment, we observed that heptachlor and the pyrethroids increased maximal [³H]dopamine uptake in striatal synaptosomes from treated mice, with dose-dependent changes in V_{max} displaying a bell-shaped curve. Western blot analysis confirmed increased levels of dopamine transporter (DAT) protein in the striatum of mice treated with heptachlor and permethrin. In contrast, we observed a small, but statistically significant decrease in dopamine uptake by 100 mg/kg chlorpyrifos. For heptachlor, doses that upregulated DAT expression had little or no effect on serotonin transport. Permethrin did cause an upregulation of serotonin transport, but required a 30-fold greater dose than that effective on dopamine uptake. Other evidence of specificity was found in transmitter release assays, where heptachlor and deltamethrin released dopamine from striatal terminals with greater potency than other transmitter types. These findings confirm that insecticides possess specificity for effects on striatal dopaminergic neurotransmission.

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Keywords: Parkinson's disease; Permethrin; Deltamethrin; Heptachlor; Chlorpyrifos; Dopamine transporter; Dopamine release

INTRODUCTION

Parkinson's disease (PD) results mainly from a degeneration of dopaminergic fibers of the nigrostriatal pathway (Bowman and Rand, 1980). Among the possible causes of PD, there is a consistent epidemiological linkage with pesticide exposure (Semchuk et al., 1992; Butterfield et al., 1993; Gorell et al., 1998).

Hazards from exposure to heavily used insecticides, such as the pyrethroids and organophosphates, exist from the manufacture, storage, spraying, and contact of personnel with insecticide-contaminated food or areas. Similarly, human exposure to organochlorine insecticides continues to be an important issue in human health, since the environmental persistence of these compounds has raised concerns regarding their documented ability for bioaccumulation (Matsumura, 1985).

Several studies have observed effects of organochlorines consistent with Parkinsonism. Early studies showed that rats fed 50 ppm dieldrin for 10 weeks had small reductions in whole brain dopamine after 4 weeks, but no change in the amount of striatal dopamine, even though striatal serotonin and norepinephrine levels were depressed (Wagner and Greene, 1974). Similarly, Mallard ducks (Sharma, 1973) and ring

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doves (Heinz et al., 1980) fed dieldrin also displayed significant reductions in brain dopamine. Chemical analysis of Parkinsonian human brain found that it contained significantly greater levels of organochlorines, especially dieldrin, than healthy brain (Fleming et al., 1994; Corrigan et al., 1998). In studies with mesencephalic neuron cultures, dieldrin application caused cytotoxicity in dopaminergic neurons more so than GABAergic neurons, suggesting some selectivity of action (Sanchez-Ramos et al., 1998).

We observed that the organochlorine insecticide heptachlor upregulated dopamine transport in striatal synaptosomes from treated mice at relatively low doses (Kirby and Bloomquist, 1996). Subsequent work expanded these findings, and showed that organochlorine and pyrethroid insecticides affect dopamine transporter (DAT) expression and dopamine release in the striatum (Kirby et al., 1999, 2001; Karen et al., 2001). The present paper will review our published work on these insecticides, and provide additional data confirming and extending our observations of the selective actions of insecticides on dopaminergic pathways.

MATERIALS AND METHODS

Chemicals

Analytical grade chlorpyrifos ($\geq 99\%$) was obtained from Chem Service Inc. (West Chester, PA). Permethrin (a mixture of four *R*, *S*-*cis* and *R*, *S*-*trans* isomers) was obtained from Sigma (St. Louis, MO), and deltamethrin (a single, *1R*-*cis*, α -*S* isomer) was supplied by Crescent Chemical Co. (Hauppauge, NY). Analytical grade ($\geq 99\%$ purity) heptachlor was purchased from Chem Service (West Chester, PA). [^3H]Dopamine (20.3 Ci/mmol) and [^3H]GABA (100 Ci/mmol) were purchased from New England Nuclear (Wilmington, DE). [^3H]Glutamate (56.0 Ci/mmol) and [^3H]serotonin (17.9 Ci/mmol) were purchased from Amersham (Buckinghamshire, UK). Buffer constituents were obtained from Sigma or Fisher Scientific Co. (Pittsburgh, PA).

Animals and Treatments

For experiments on insecticide-dependent changes in transporter regulation, we used male C57BL/6 retired breeder mice, purchased from Harlan Sprague-Dawley (Dublin, VA) that were 7–9 months old (28–40 g live weight). Treatments were administered three times over a 2-week period according to the method of Kirby et al. (1999). All insecticides were

delivered in methoxytriglycol (MTG) vehicle and injected i.p., except CPF, which was carried in corn oil vehicle and given s.c. Control mice received 50 μl corn oil or 10 μl of MTG vehicle alone. On the day following the last treatment, mice were killed by cervical dislocation and tissues were collected for neurochemical analysis.

Dopamine and Serotonin Uptake Studies

Neurotransmitter uptake studies were performed according to the methods of Kirby et al. (1999) for dopamine and Kirby et al. (2001) for serotonin. Briefly, crude synaptosomes were prepared from fresh striatal (for dopamine) or cortical tissue (for serotonin) dissected from treated mice. Synaptosomes were incubated with various concentrations of [^3H]dopamine or [^3H]serotonin for 2 min. Uptake was stopped by addition of ice cold buffer, followed by vacuum filtration, washing, and liquid scintillation counting. Aliquots of each synaptosomal preparation were frozen at -20°C for membrane protein determination, which was performed according to the method of Bradford (1976). Uptake rates were calculated by the method of Krueger (1990), using incubations with and without sodium ions (equimolar choline chloride substitution) to correct for nonspecific uptake. Uptake parameters (V_{max} and K_{m}) were determined by nonlinear regression to isotherm plots using PrismTM (GraphPad Software, San Diego, CA).

Western Blot Analysis

Western blots were used to quantify the amount of DAT protein present in samples of brain striatal tissue from treated mice. Crude synaptosomal membranes were prepared as previously described (Kirby et al., 2001) and then homogenized, heat denatured, and the proteins separated by SDS–PAGE electrophoresis (Laemmli, 1970). Separation was accomplished on a 10% SDS–PAGE gel (10 μg protein per lane) and transferred electrophoretically to a nitrocellulose membrane for 1 h at 100 V, according to the method of Towbin et al., (1979). The nitrocellulose membranes are stained with Ponceau S dye to confirm transfer of the proteins to the membrane, and it serves the added purpose of ensuring that equal amounts of protein have been loaded onto each lane. Blots were then incubated in 4% dry milk for 1 h, followed by a 1 h incubation in anti-DAT antibody (rat, Chemicon, Temecula, CA) diluted 1:1000, and 1 h with peroxidase-linked secondary antibodies. The blots were visualized using the ECL

Chemiluminescence detection kit (Amersham, Buckinghamshire, England), with exposure to the ECL reagent for 1 min, followed by autoradiography for various lengths of time, up to 2 min. Analysis of the blots was performed by digital image analysis using a Kodak Digital Camera and EDAS 290 System (Eastman Kodak Scientific Imaging Systems, Rochester, NY).

Neurotransmitter Release

Conventional neurotransmitter release assays in striatal or cortical synaptosomes were performed essentially as described in Kirby et al. (1999). Crude synaptosomes were prepared from the two tissue sources and the final pellets resuspended in incubation buffer containing either 100 nM [³H]dopamine, 115 nM [³H]serotonin, 40 nM [³H]GABA or 90 nM [³H]glutamate (5 min, 37 °C). Cortical tissue was used as a synaptosome source for assays with [³H]serotonin, due to the relatively low density of serotonergic terminals in the striatum. After loading, the membranes were centrifuged and the labeled pellets resuspended in buffer and incubated with toxicants for 10 min at 37 °C. Lipophilic toxicants were dissolved in DMSO and final DMSO concentrations in incubations did not exceed 0.1%, with controls receiving 0.1% DMSO alone. Synaptosomes were diluted with 3 ml of wash buffer (37 °C), vacuum-filtered, and then washed three times with 3 ml of 37 °C wash buffer. Radioactivity on the filters was determined as described before.

Statistical Analysis

For uptake studies, kinetic parameters (K_m and V_{max}) were determined by nonlinear regression to isotherm plots (GraphPad Software, San Diego, CA). For release studies, data were analyzed by nonlinear regression to a four-parameter logistic equation using either Prism™ 2.0 (GraphPad Software, San Diego, CA) or MacCurveFit 1.3 (Kevin Raner Software, Vic., Australia), which gave similar results. EC_{50} values for release of different neurotransmitters or dopamine uptake parameters following insecticide treatment were compared by *T*-test or by one-way ANOVA with Student–Newman–Keuls means separation test (InStat 2.03, GraphPad Software, San Diego, CA).

RESULTS

Dopamine uptake in striatal synaptosomes from treated mice was saturable and showed a good fit to

a Michaelis–Menten model (r^2 values ≥ 0.98). Permethrin treatment did not have any significant effect on the apparent K_m of the DAT (range: 375–545 nM) in any of the treatment groups. Maximal dopamine uptake in C57BL/6 mice treated with permethrin varied with the dose (Fig. 1A), and peaked at a dose of 1.5 mg/kg. At this dose, dopamine uptake was significantly greater (34%) than that of the control value, while at higher doses maximal uptake declined. A similar dose-response profile was observed for heptachlor. Increases in V_{max} had a threshold dose of 3 mg/kg and uptake was maximally enhanced at 6 mg/kg (Fig. 1B). Maximal induction of V_{max} for striatal dopamine transport was 217% of control at this dose. Again, at higher doses, transport declined. Changes in apparent K_m did not match in a dose-dependent way the increases in V_{max} and displayed a variable response to heptachlor treatment (Kirby et al., 2001). In contrast, treatment of mice with chlorpyrifos caused a significant 10% reduction in dopamine uptake V_{max} at 100 mg/kg, the highest dose administered. No significant effect on V_{max} was observed at lower doses of chlorpyrifos.

Upregulation of the DAT was confirmed in striatal tissue taken from treated mice. We observed that two major protein bands were labeled by DAT antibody (Fig. 2, top). Scanning densitometry found clear evidence of greater antibody labeling, especially at the 0.8 mg/kg dose of permethrin. In Fig. 2 (bottom), both bands were upregulated, with the total effect of about a 30% increase in labeling of each band. In addition, we have observed that permethrin-dependent upregulation

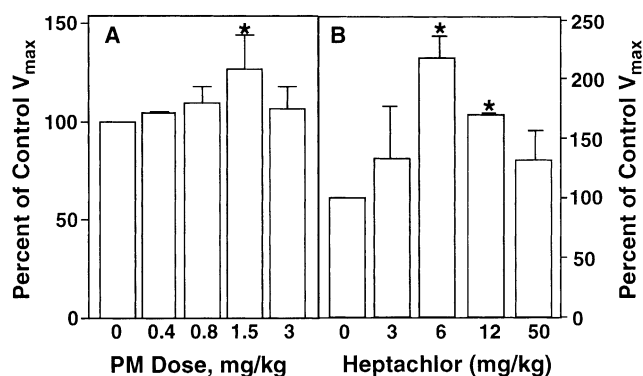


Fig. 1. Representative bar graphs of dopamine transport after permethrin (A) or heptachlor treatment (B). Bars represent mean (with S.E.M.) changes in V_{max} values, expressed as percent of control and replicated across different cohorts of treated mice. Asterisks indicate effects significantly different from control using the untransformed data (*T*-test, $P < 0.05$). The permethrin plot (A) is redrawn from Karen et al. (2001) and the heptachlor plot (B) is redrawn from Kirby et al. (2001).

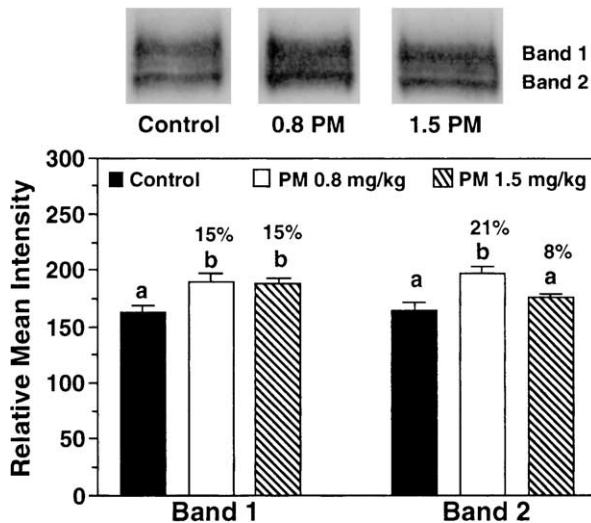


Fig. 2. Western blot of striatal membranes taken from mice treated with the indicated doses of permethrin (PM) three times over a 2-week period. Top: each lane shows two major protein bands, and is from a pooled striatal homogenate isolated from treated mice. Bottom: scanning densitometry of the bands shows an increase in DAT protein, with the percentage increase from matched controls given above the bars for the PM treated groups. Bars labeled by different letters are significantly different from controls (*T*-test, $P < 0.05$).

of the DAT is still present 4 weeks after the last 0.8 mg/kg treatment (data not shown).

Levels of cortical serotonin uptake following heptachlor treatment did not reflect the increase in maximal uptake observed for dopamine (Fig. 3). These experiments used 6 and 12 mg/kg heptachlor; the doses that in previous experiments produced the greatest

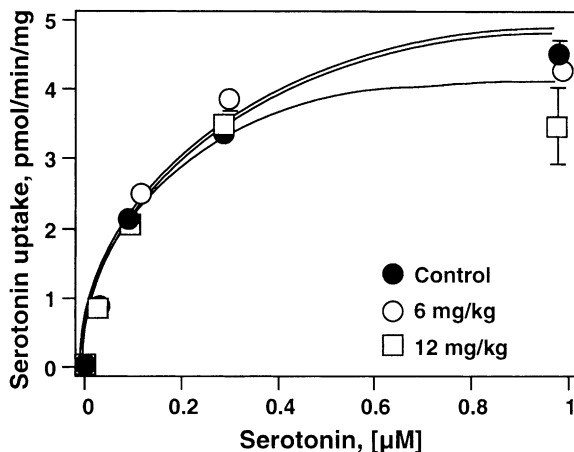


Fig. 3. Serotonin uptake following treatment at doses of heptachlor known to upregulate dopamine transport. Symbols indicate means, along with the S.E.M. Lack of error bars indicates that the S.E.M. was smaller than the size of the symbol. Redrawn from Kirby et al. (2001).

increases in V_{max} for dopamine uptake. In these studies, there was no significant change in V_{max} from control (5.1 ± 0.1 pmol/min/mg) in mice treated with 6 mg/kg heptachlor (5.0 ± 0.2 pmol/min/mg). There was a statistically significant, 21% reduction in calculated maximal rates of serotonin uptake for 12 mg/kg heptachlor-treated mice (4.1 ± 0.3 pmol/min/mg). However, this reduction was essentially attributable to a more variable decrease in uptake at 1 μ M serotonin only, since the other points on the curve (Fig. 3) overlapped closely those of the other treatment groups. No statistically significant change in K_m values was measured for serotonin uptake (range: 90–140 nM) in any of the heptachlor-treated mice.

Reduced sensitivity of cortical serotonergic pathways was also observed with permethrin treatment. Ex vivo cortical synaptosomes showed a dose-dependent upregulation of serotonin transport (Fig. 4), but at doses at least 30-fold greater than that required to upregulate dopamine uptake. Upregulation was essentially complete at 100–200 mg/kg permethrin, and virtually disappeared as the dose was lowered to 25 mg/kg (Fig. 4). In addition, the maximal extent of upregulation of serotonin transport (34%), was identical to that observed for dopamine. There was no statistically significant effect on the K_m values for serotonin transport (range: 69–289 nM).

Differential sensitivity of nerve terminals to pyrethroid- or heptachlor-evoked release of neurotransmitter was observed in synaptosome preparations from striatum and cortex of ICR mice (Table 1). The pyrethroid deltamethrin released a variety of neurotransmitters

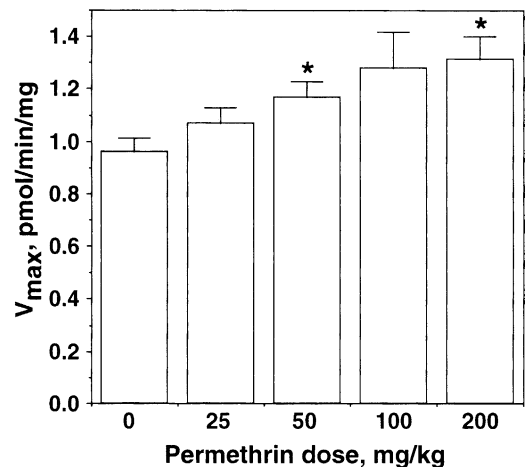


Fig. 4. Serotonin uptake in cortical synaptosomes following treatment with permethrin. The results are taken from pooled membranes of single cohorts of mice having typically five to six animals. Asterisks indicate serotonin transport significantly different from control (*T*-test, $P < 0.05$).

Table 1
Potency of insecticides for releasing different neurotransmitters from synaptosomes

Insecticide	Brain region	Transmitter	EC ₅₀ ± S.E.M. ^a
Deltamethrin ^b	Striatum	Dopamine	48 ^a ± 25 nM
	Cortex	Serotonin	117 ^b ± 37 nM
	Cortex	Glutamate	412 ^b ± 76 nM
Heptachlor	Striatum	Dopamine	1.1 ^a ± 1.1 μM
	Striatum	GABA	7.3 ^b ± 1.2 μM
	Striatum	Glutamate	13.7 ^c ± 1.2 μM
	Cortex	Serotonin	25.9 ^d ± 1.3 μM

^a EC₅₀ values labeled by different letters are significantly different from each other, as found by one-way ANOVA and Student–Neumann–Keuls means separation test ($P < 0.05$).

^b Data taken from Kirby et al. (1999).

from preloaded synaptosomes at nanomolar concentrations, with the EC₅₀ for dopamine release being 2.4- and 8.6-fold more potent than serotonin or glutamate release, respectively. The EC₅₀ for heptachlor-evoked release of [³H]dopamine from striatal synaptosomes (low micromolar range) was about 23-fold less potent than that of deltamethrin. Compared to dopamine, the EC₅₀ for heptachlor-induced release of GABA and glutamate from striatal synaptosomes were significantly greater (ca. 7- and 13-fold, respectively). Serotonergic terminals were the least sensitive of the nerve terminal types tested for heptachlor-evoked release and were, approximately 23-fold less sensitive than striatal dopaminergic terminals (Table 1).

An effect on dopamine release was also observed, in vitro, with chlorpyrifos (Fig. 5). Synaptosomes preloaded with dopamine were induced to release label

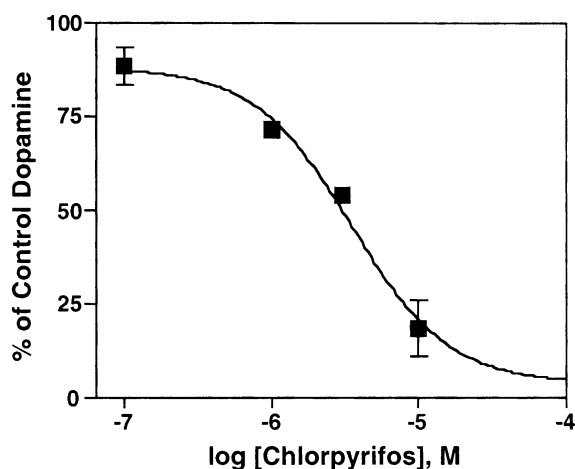


Fig. 5. Ability of chlorpyrifos to cause dopamine release from striatal synaptosomes. Symbols represent means of three determinations with bars equal to the S.E.M. Absence of bars means that the S.E.M. was less than the size of the symbol.

at micromolar concentrations of chlorpyrifos, and the EC₅₀ for this compound was calculated to be 3.8 ± 1.3 mM. The release was also complete, and showed an excellent fit to a sigmoidal model ($r^2 = 0.998$). No experiments with chlorpyrifos were attempted using other neurotransmitters.

DISCUSSION

The ability of insecticide exposure to increase dopamine transport reflects a potent action, in vivo. We demonstrated this effect previously for the pyrethroid insecticide deltamethrin, which increased dopamine uptake by 70% following three doses of 6 mg/kg (Kirby et al., 1999). Similarly, permethrin at 1.5 mg/kg increased significantly the maximal transport of dopamine, and this dose is about three orders of magnitude below the mouse i.p. LD₅₀ for this compound (Gray and Soderlund, 1985). 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*) causes lethality in mammals (Casida et al., 1983). If the 1*R*-*cis* isomer alone is responsible for the upregulation, it comprises only 25% of the applied dose, and it was therefore present at about 0.4 mg/kg. A potent enhancement of uptake also occurs following treatment with heptachlor, where 6 mg/kg represents about 4% of the LD₅₀ dose (145 mg/kg by i.p. injection; Cole and Casida, 1986). We assume that the observed increase in dopamine uptake was compensatory for increased levels of free synaptic dopamine, in vivo, and that balanced neurotransmission was maintained by increased expression of the transporter.

A greater abundance of DAT protein was confirmed in Western blots at doses of 0.8 and 1.5 mg/kg of permethrin. The apparent greater expression at 0.8 mg/kg, where transport was not significantly upregulated, can be ascribed to different responses of different cohorts of mice, and the neurochemical effects observed vary from group to group with respect to dose. Previous work has shown that heptachlor-dependent increase in dopamine transport was also accompanied by an increase in DAT protein labeling in Western blots of striatal membranes (Miller et al., 1999). Interestingly, we observed in the present study two labeled bands, instead of only one, which is more typical (Miller et al., 1999). These two closely spaced bands may represent different glycosylated forms of the DAT, since it is known to possess four consensus glycosylation sites (Hitri et al., 1994). Alternatively, they may be an artifact of tissue or blot processing.

Because persistent effects on DAT expression occur at low doses, it appears to be a sensitive index of sub-clinical toxicant insult and should be investigated further as a biomarker of environmental toxicant exposure. In addition, a recent study by Lee et al. (2001) observed an interaction between the DAT and α -synuclein in cultured neurons. This interaction led to increased dopamine uptake and dopamine-induced cellular apoptosis, and would provide a mechanism whereby increased DAT expression by insecticides could play role in the development of PD.

For both permethrin and heptachlor, transport declined at doses greater than those causing a maximal induction of dopamine uptake. This decline most likely occurred from the inability of the synaptosomes to retain dopamine, rather than a down regulation of the DAT. For both compounds, there is evidence of cell stress occurring at higher doses. In mice treated with doses ≥ 12.5 mg/kg permethrin, less activity was present in a synaptosomal MTT dehydrogenase assay (Karen et al., 2001), which is a measure of mitochondrial function (Carmichael et al., 1987). Similarly, polarographic measurements of mitochondrial respiration were reduced in striatal synaptosomes from mice treated with ≥ 25 mg/kg heptachlor (Kirby et al., 2001). In contrast to permethrin and heptachlor, striatal dopamine uptake was not upregulated at any dose of chlorpyrifos tested. In fact, at the highest dose (100 mg/kg), dopamine transport V_{\max} was slightly decreased. As was the case for elevated doses of permethrin, treatment with 100 mg/kg chlorpyrifos significantly depressed MTT dehydrogenase activity in ex vivo synaptosomes, suggesting chemically-induced nerve terminal stress (Kirby et al., 2001).

Whereas heptachlor increased dopamine uptake in the striatum, no change in maximal rates of serotonin uptake was detected in the cortex. There was an upregulation of serotonin transport following permethrin treatment, but only at doses at least 30-fold greater than that required to upregulate the DAT. The lack of comparable effect on serotonin uptake by insecticides suggests less disruption of cortical serotonergic pathways in vivo, since compounds that either block uptake (fluoxetine; Hrdina and Vu, 1993) or inhibit synthesis of serotonin (*p*-chlorphenylalanine; Rattray et al., 1996) will affect the expression of serotonin transporter in rat cortex.

Specificity in neurotransmitter release assays is evident in the greater sensitivity of nigrostriatal dopaminergic nerve terminals to insecticide-evoked release than those of either glutamatergic or GABAergic projections to the striatum, or serotonergic terminals

in the cortex. Pyrethroids and organochlorines may affect release through an action on sodium channels (Kirby et al., 1999) and calcium ion flux/homeostasis (Yamaguchi et al., 1979, 1980), respectively. The greater potency of insecticide-induced dopamine release, in vitro, is mirrored in the preferential ability of permethrin or heptachlor treatment to upregulate dopamine transport in ex vivo synaptosomes from C57 mice.

The mechanism of dopamine release by chlorpyrifos is not obviously related to its well-described action as an anticholinesterase, and we did not test its efficacy against other transmitters. However, Dam et al. (1999) found that this compound released about 20% of labeled norepinephrine from whole rat brain synaptosomes at 50 μ g/ml (142 μ M). Species, tissue, or transmitter selectivity could contribute to this difference. The release of norepinephrine was not antagonized by blockers of muscarinic (atropine) or nicotinic receptors (mecamylamine), so the mechanism remains unknown. The lack of DAT upregulation by chlorpyrifos treatment suggests that the release effect of this compound was not sufficient to induce transporter expression at the doses tested, or perhaps was interfered with by other poisoning processes.

Selective effects on dopaminergic nigrostriatal pathways is also observed with the mitochondrial poison, rotenone (Ferrante et al., 1997; Betarbet et al., 2000), and have been ascribed to a constitutive metabolic deficiency of nigral neurons (Marey-Semper et al., 1993). This characteristic might play some role in the greater effect of different insecticide classes having different modes of action on striatal dopamine release, although other differences in the structure or function of the release machinery for different transmitter types might also be involved.

A role for excessive release of dopamine as a neurotoxic mechanism in PD is supported by other experimental evidence. The toxic nature of elevated dopamine levels has been shown following in vivo injection (Filloux and Townsend, 1993) and through exposure to cultured neurons, in vitro (Ziv et al., 1994). Similarly, the compounds reserpine and tetrabenazine cause release of neurotransmitter from nerve terminals (Mahata et al., 1996), deplete dopamine and other monoamine levels in brain (Bowman and Rand, 1980), and Parkinsonism is a common side effect of their use as drugs in humans (Montastruc et al., 1994). Insecticides may manifest similar effects.

Although these studies have shown specific effects of insecticides on the nigrostriatal pathway, we have failed to demonstrate a reduction in dopamine titers

following heptachlor, chlorpyrifos, or permethrin treatment (Kirby et al., 2001; Karen et al., 2001). Loss of dopamine is a cardinal sign of PD (Hornykiewicz and Kish, 1987) and can reflect changes in both cellular levels of dopamine and cell loss in the nigrostriatal pathway. In previous studies, we measured the total amount of striatal dopamine by HPLC (Bloomquist et al., 1999; Karen et al., 2001). This technique may have missed incipient effects of insecticides on dopamine levels, because natural loss of dopaminergic neurons in aging is countered by an increase in dopamine synthesis by the remaining neurons (Tatton et al., 1991). In our studies, the lack of significant effect on dopamine titers by insecticides does not support a rapidly developing Parkinsonism following short term exposures to these compounds. Longer term studies, especially those at environmentally relevant exposures and in combination with other susceptibility factors (e.g. mitochondrial compromise), should be undertaken to reveal any effects of insecticides on striatal dopamine levels under these conditions.

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Differential up-regulation of striatal dopamine transporter and α -synuclein by the pyrethroid insecticide permethrin

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Abstract

The effects of permethrin on striatal dopaminergic biomarkers were assessed in this study. Retired breeder male C57 B1/6 mice were given an ip dose of permethrin (0.1–200 mg/kg) at 7-day intervals, over a 2-week period (Days 0, 7, and 14). Animals were then sacrificed 1 day ($t = 1$), 14 days ($t = 14$), or 28 days after the last treatment ($t = 28$). Dopamine transporter (DAT) protein as assayed by Western blotting was increased to 115% in the 0.8 mg/kg group over that of control mice at $t = 1$ ($P < 0.05$). At $t = 14$, this value increased to 140% of control, and declined slightly to 133% of control at $t = 28$. The mice given the 1.5 mg/kg dose displayed a significant increase in DAT protein only at $t = 28$, to 145% of controls. Thus, upregulation of the DAT at low doses of PM is variable 24 h after treatment, and seems to stabilize by $t = 28$. The threshold dose for increasing DAT expression in Western blots by $t = 28$ was 0.2 mg/kg permethrin. [3 H]GBR 12935, used to assay DAT binding, followed the same trend as that for the Western blotting data for 0.8 and 1.5 mg/kg doses of permethrin over the 4 weeks posttreatment. At 200 mg/kg permethrin, DAT protein was unchanged vs controls ($t = 1$), but had significantly increased by $t = 14$ and continued to increase at $t = 28$, suggesting that the reduced dopamine transport at this dose was due to nerve terminal stress and that recovery had occurred. The protein α -synuclein was also significantly induced at the 1.5 mg/kg dose at $t = 1$; however, unlike DAT up-regulation, this effect had declined to control values by $t = 14$. Maximal induction of α -synuclein protein occurred at a dose of 50 mg/kg permethrin. These data provide evidence that the pyrethroid class of insecticides can modulate the dopaminergic system at low doses, in a persistent manner, which may render neurons more vulnerable to toxicant injury.

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Keywords: Parkinson's disease; Striatum; Neurotoxicity; Protein regulation

Introduction

Parkinson's disease (PD), a chronic neurodegenerative disease of unknown etiology, is characterized by a loss of dopaminergic neurons in the substantia nigra and depletion of striatal dopamine (DA) (Fearnley and Lees, 1991). In humans, an 80% striatal DA loss is required before overt clinical symptoms appear, after which significant correlation exists between severity of PD symptoms and subsequent DA loss (Hornykiewicz and Kish, 1986).

The etiology of idiopathic PD is thought to be multifactorial (Paganini-Hill, 2001; Siderowf, 2001; Zhang et al., 2000), and it has long been known that there is an epide-

miological link between PD and persons who are associated with rural living and agricultural work. In particular, persons exposed to various herbicides and insecticides in an agricultural setting show an increased risk of developing PD (Semchuck et al., 1992, 1993; Gorell et al., 1998). Other work linked exposure to organochlorine insecticides such as dieldrin to PD (Fleming et al., 1994). The discovery that 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is able to produce parkinsonism in animals and humans via its oxidative metabolite MPP⁺ (Tipton and Singer, 1993) provided the original evidence for a putative environmental component in the etiology of PD (Jenner et al., 1992).

The so-called Gulf War Syndrome, which is manifest as various neurological maladies reported by veterans of the Persian Gulf War, may be linked to pesticide exposure. These compounds include pyridostigmine bromide (PB), an

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anticholinesterase, the insect repellent DEET, and the pyrethroid insecticide permethrin (PM), which was impregnated into the uniforms of soldiers who served in the war (Hoy et al., 2000). Personnel who served in the first Gulf War report a variety of symptoms of neurological dysfunction, with no mortality increases, which have been confirmed by epidemiological studies (Steele, 2001). Further, these symptoms may be related to a spectrum of generalized neurologic injury to the central, peripheral, and autonomic nervous systems (Haley et al., 1997a, 1997b).

The dopamine transporter (DAT) is a membrane-bound carrier molecule that mediates the action of DA in the nerve synapse via the reuptake of DA into the dopaminergic neuron, and the DAT is also capable of neuronal DA release (Horn, 1990). In addition, MPP⁺ is transported into the neuron via the DAT, where it blocks mitochondrial complex I respiration and subsequently causes cell death (Tipton and Singer, 1993). Thus, the DAT is a putative molecular gateway for exogenous and endogenous dopaminergic toxicants.

Using three ip doses over a 2-week period (Days 0, 7, and 14), our laboratory found that the organochlorine insecticide heptachlor has significant effects on DA transport and causes DAT up-regulation in C57B1/6 mice at relatively low doses (Miller et al., 1999; Kirby et al., 2000). We also showed that the pyrethroid insecticides deltamethrin and PM significantly increase DA uptake in treated mice (Kirby et al., 1999; Karen et al., 2001). For PM, DA uptake was increased 33% above controls ($P < 0.05$) at a dose of 1.5 mg/kg, but reduced 56% below control values ($P < 0.05$) at 200 mg/kg (Karen et al., 2001). The objective of this research was to further characterize the time and dose dependence of PM's effects on transporter ligand binding and DAT protein expression. Moreover, because mutations in the α -synuclein gene are associated with some forms of familial PD (Langston et al., 1998; Mizuno et al., 2001), and the herbicide paraquat causes up-regulation and increased aggregation of α -synuclein in C57B1/6 mice (Manning-Bog et al., 2001), we have expanded our studies to include Western blot analysis of α -synuclein expression in PM-treated mice. In this way, we hope to gain insight into the mechanisms by which compounds alter the dopaminergic system and render the neuron more susceptible to toxicant injury.

Materials and methods

Chemicals. Technical PM (mixture of 1-*R,S*-*cis* and 1-*R,S*-*trans* isomers) was purchased from Sigma-Aldrich GmbH. Sucrose, Hepes, KCl, and methanol were purchased from Fisher Scientific (Pittsburgh, PA). Methoxytriglycol (MTG), NaCl, CaCl₂, MgSO₄, and Ponceau-S stain were purchased from Sigma Chemical Co. (St. Louis, MO). GBR 12909 was obtained from Research Biochemicals International (Natick, MA), and [³H]GBR 12935 was obtained from NEN Life

Science Products, Inc. (Boston, MA). Acrylamide, bisacrylamide, ammonium persulfate, *N,N,N,N'*-tetramethylethylenediamine (TEMED), and Tris/glycine/SDS buffer were purchased from Bio-Rad (Hercules, CA). ECL Western blotting detection system RPN 2108 and ECL Hyperfilm were purchased from Amersham-Pharmacia Biotech, (Buckinghamshire, UK).

Animals and treatments. Retired breeder male C57B1/6 mice (Charles River Labs, Raleigh, NC) were weighed and segregated into randomized dosing groups ($n = 6$ mice per dose group) prior to ip treatment with PM at doses ranging from 0.1 to 200 mg/kg. Mouse weights ranged from 35 to 40 g, with an average weight of 38 g. Different cohorts of mice were used for the low (0.2–1.5 mg/kg) vs high (100 and 200 mg/kg) PM dose groups. Further, the time course experiments were repeated using different cohorts of mice. For dose-response studies, a dose of PM dissolved in MTG was given three times over a 2-week period (Days 0, 7, and 14) and sacrificed 1 day ($t = 1$) after the last dose as previously outlined in Kirby et al. (1999). This exposure paradigm is based on the results of Bloomquist et al. (1999), where a single treatment of MPTP caused significant depletion of striatal DA 2 weeks later. Control mice received MTG vehicle alone. For the time course studies, groups of mice were held for either 14 days ($t = 14$) or 28 days ($t = 28$) post last dosing, at which time they were sacrificed. At the time of necropsy, brain striatal tissues were dissected from the mice and prepared for assay as described below. All procedures were approved by the VPI&SU Animal Care and Use Committee.

[³H]GBR 12935-binding assay. Pooled striatal tissues from all mice within a treatment group were homogenized in ice-cold Krebs-Ringer's-Hepes (KRH) buffer (118 mM NaCl, 4.8 mM KCl, 2.5 mM CaCl₂, 1.2 mM MgSO₄, 20 mM Hepes, pH 7.5). Striata were then centrifuged at 18,000g and resuspended for 1 h in distilled water. The tissues were again centrifuged at 18,000g, resuspended in cold KRH buffer, and stored at -70°C until use. For the assay, samples were thawed and run in duplicate in the absence and presence of 0.5 μM GBR 12909. To measure total binding, [³H]GBR 12935 (100, 50, 25, 12.5, and 6.25 nM) was incubated with KRH buffer (20 mM Hepes, 118 mM NaCl, 4.8 mM KCl, 2.5 mM CaCl₂, 1.2 mM MgSO₄; pH 7.5) and tissue for 2 h at 4°C. The same procedure was used to measure nonspecific binding with the exception that excess cold GBR 12909 was added to the reaction mixture. The reactions were stopped by the addition of cold KRH buffer, and the contents of each reaction tube were filtered at 10 psi using 25 mm Whatman GF/B filters presoaked in 0.1% BSA. Filters were then washed 3 times with cold KRH buffer to remove unbound radioactivity. The filters were soaked overnight in Scintiverse E, and total counts were measured on a Beckman LS 6500 liquid scintillation counter. Aliquots of [³H]GBR 12935 solutions were added

to Scintiverse E cocktail to calculate the exact [^3H]GBR 12935 concentrations in each reaction mixture. Following protein determination by the method of Bradford (1976), nonlinear regression to isotherm plots was used to determine B_{max} , K_d , and their standard errors using Prism (GraphPad Software, San Diego, CA).

Western blot analysis. Pooled striatal synaptosomes from C57B1/6 mice were homogenized in cold isolation buffer and centrifuged at 1000g for 15 min. The supernatant was removed and centrifuged at 10,000g for 15 min. The resulting pellets were resuspended in a small volume of KRH buffer and stored at -70°C until use. At the time of assay, the tissues were thawed, mixed with sample buffer (60 mM Tris, pH 6.8, 2% SDS, 100 mM DTT, 0.001% bromphenol blue), and heated at 80°C for 5 min. Samples were run on either a 10% SDS gel (DAT, synaptophysin) or a 12% SDS gel (α -synuclein) and transferred to a nitrocellulose membrane according to the method of Towbin et al., (1979). For the DAT, α -synuclein, and synaptophysin blots, SDS gels were loaded with 10, 5, and 2 μl of protein, respectively. After transfer was complete, the membrane proteins were stained with Ponceau S dye to verify transfer. The membrane was incubated overnight in 5% dry milk at 5°C , followed by an overnight incubation at 5°C in either a mouse monoclonal anti- α -synuclein (Bioscience International, Saco, ME), a rat monoclonal anti-DAT, or a rat monoclonal anti-synaptophysin primary antibody (Chemicon Int'l, Temecula, CA). The blot was washed with TBST buffer and incubated in an appropriate peroxidase-linked secondary antibody (Sigma Chemical Co.) for 1 h at room temperature. After a second washing with TBST buffer, the blot was developed using the ECL Amersham chemiluminescence kit (Amersham-Pharmacia Corp.), and exposed to ECL Hyperfilm (Amersham-Pharmacia Corp.) for varying lengths of time. Protein bands on the film were quantitated using the Kodak EDAS 290 system (Eastman Kodak Co.). Protein content was determined by the method of Bradford (1976). Gels were run with 3 protein samples (determinations) from the pooled tissue of each treatment group, and were quantitated by densitometry and then averaged. All blots contained matched controls, to account for any differences in staining or photographic development.

Statistical analysis. Statistical significance was determined using one-way ANOVA and Student-Newman-Keuls means separation if a statistically significant effect of treatment was observed. Other statistical comparisons were by t test calculations. Analysis was performed using InStat (GraphPad Software).

Results

Signs of pyrethroid toxicity were not observed in any of the treatment groups, even at the highest dose of 200 mg/kg.

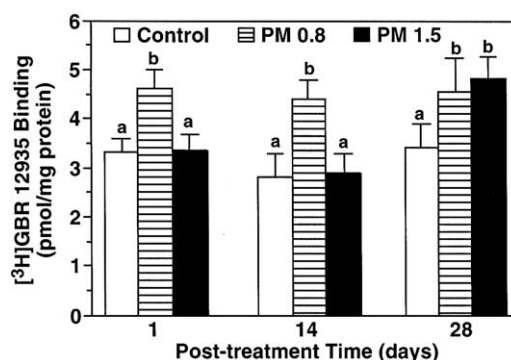


Fig. 1. Time course experiments of [^3H]GBR 12935 binding in C57B1/6 mice treated with 0.8 or 1.5 mg/kg PM. Bars represent means with SEM in this and all subsequent figures. Letters denote results of ANOVA with Student-Newman-Keuls means separation test, where treatments within a particular time period (1, 14, or 28 days) are significantly different ($P < 0.05$) when labeled by different letters.

Mouse weights were also not significantly altered by PM treatment in any of the dose groups (data not shown).

Since maximal DA uptake occurred at 1.5 mg/kg PM in our earlier DA uptake experiments (Karen et al., 2001), it was decided to select the PM doses of 0.8 and 1.5 mg/kg for the initial [^3H]GBR 12935-binding assays and Western blotting studies. At $t = 1$ day posttreatment, the B_{max} value for [^3H]GBR 12935 was significantly elevated in the 0.8 mg/kg dose group to 137% of control, but was unchanged in the 1.5 mg/kg dose group (Fig. 1). At $t = 1$ day, K_d values (nM, $\pm\text{SE}$) for the controls and 0.8 and 1.5 mg/kg groups were 2.7 ± 0.7 , 3.9 ± 0.9 , and 3.4 ± 0.6 , respectively. Other groups of mice from the same experimental cohort were assessed either 14 or 28 days beyond the last dose, with no further treatments given. At $t = 14$ days, GBR 12935 B_{max} increased to 157% of control in the 0.8 mg/kg dose group, with the 1.5 mg/kg dose group remaining unchanged. At $t = 14$ days, K_d values (nM, $\pm\text{SE}$) for the controls and 0.8 and 1.5 mg/kg groups were 3.4 ± 1.9 , 4.3 ± 1.2 , and 6.0 ± 1.8 , respectively. At $t = 28$ days, both the 0.8 and 1.5 mg/kg groups demonstrated a significantly increased [^3H]GBR 12935 B_{max} ($\sim 130\%$ for both groups) over that of controls (Fig. 1). At $t = 28$ days, K_d values (nM, $\pm\text{SE}$) for the controls and 0.8 and 1.5 mg/kg groups were 5.2 ± 1.9 , 4.0 ± 1.7 , and 3.5 ± 1.4 , respectively. [^3H]GBR 12935 B_{max} at $t = 1$ was not significantly different from that of controls at PM doses of 100 and 200 mg/kg (Fig. 2). At $t = 1$ day, K_d values (nM, $\pm\text{SE}$) for the controls and 100 and 200 mg/kg groups were 6.2 ± 2.2 , 5.2 ± 2.7 , and 6.2 ± 2.4 , respectively. The K_d of [^3H]GBR 12935 was not significantly different from that of controls for any dose or time groups ($P > 0.05$, ANOVA).

Western blotting of DAT protein largely reflected the above [^3H]GBR 12935-binding results for each time period tested. At $t = 1, 14,$ and 28 days posttreatment, DAT protein was significantly elevated to 115, 140, and 133% of controls, respectively, in the 0.8 mg/kg treatment group (Fig. 3).

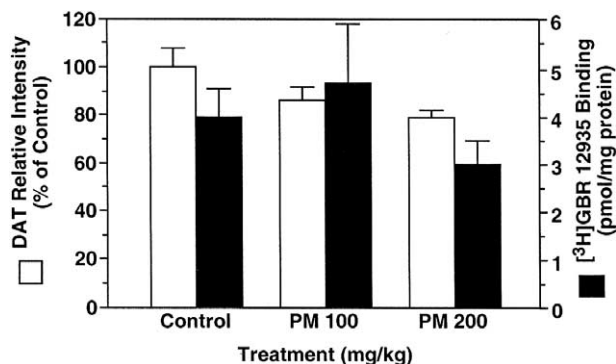


Fig. 2. DAT Western blot densitometry and [³H]GBR 12935 binding in C57B1/6 mice sacrificed 1 day after the final treatment with 100 or 200 mg/kg PM. No statistically significant differences were observed (*t* test, *P* > 0.05).

The 1.5 mg/kg treatment group did not show a significant induction of DAT protein until the *t* = 28 time period, where it was enhanced to 145% of control (Fig. 3). A similar effect on DAT was observed in mice treated with 200 mg/kg PM (Fig. 4). As before, DAT protein was not significantly different at 24 h posttreatment; however at *t* = 14 and *t* = 28, DAT protein was significantly upregulated to 135 and 147% of control, respectively, by PM (Fig. 4).

In an effort to determine the threshold dose of PM for up-regulating DAT protein expression, doses of 0.1, 0.2, or 0.4 mg/kg PM were administered according to the standard dosing scheme and sacrificed at *t* = 1, 14, or 28 days posttreatment. At *t* = 1, there was a significant increase in DAT protein at doses of 0.2 and 0.4 mg/kg, but not at 0.1 mg/kg. This effect on DAT protein was persistent for both doses at both 2 and 4 weeks. Again, in the highest dose group, there was a significant increase in DAT protein from *t* = 1 to *t* = 14, which then remained constant until *t* = 28 days. Thus, the threshold dose for significant up-regulation of DAT protein by technical PM was 0.2 mg/kg (Fig. 5) under the dosing regime used in this study.

α -Synuclein protein was also quantified by Western blot-

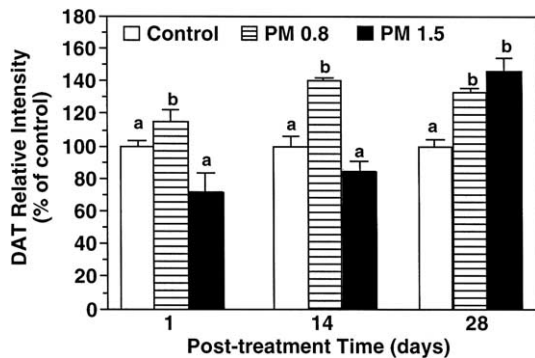


Fig. 3. Time course experiments of DAT Western blot densitometry in C57B1/6 mice treated with 0.8 or 1.5 mg/kg PM. ANOVA was performed on the raw data before calculation as percentage of control. ANOVA and means separation as described for Fig. 1.

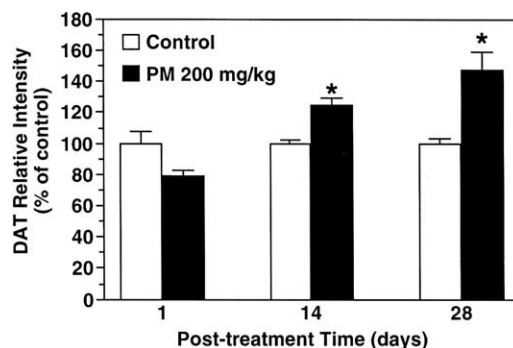


Fig. 4. Time course studies using densitometry of DAT Western blots of striatal synaptosomes from mice treated with 200 mg/kg PM. Raw data were analyzed by *t* test before calculation as percentage of control, and bars labeled by an asterisk for a particular time point (1, 14, or 28 days) are significantly different (*P* < 0.05).

ting in the same 0.8 and 1.5 mg/kg dose groups used to assay the DAT (Fig. 6). At a PM dose of 1.5 mg/kg, α -synuclein was up-regulated to 180% of control in the mice sacrificed 1 day after the last PM dose. However, unlike DAT expression, this effect was not persistent, as evidenced by the lack of any increase in either the 14- and 28-day mice (Fig. 6). At *t* = 1, α -synuclein protein expression demonstrated a bell-shaped curve over a wide range of PM doses from 25 to 200 mg/kg, with a peak increase at 50 mg/kg to 244% of control. α -Synuclein protein was also significantly up-regulated at doses of 25 and 100 mg/kg PM (145 and 137% of controls, respectively), but was not significantly changed at the 200 mg/kg PM dose (Fig. 7).

An antibody to synaptophysin, a neuronal vesicle-bound protein, was used in Western blotting techniques to verify uniform loading of protein in Western blots. Synaptophysin protein remained unchanged across treatment groups in all tissues tested, as opposed to the up-regulation observed for DAT and α -synuclein (Fig. 8). Since staining across all DAT bands was increased, all were included in the quantitation. Uniform protein loading was also ensured by semi-quantitatively measuring the Ponceau-S staining of each Western blot lane, which again remained constant across all treatment groups and time points (data not shown).

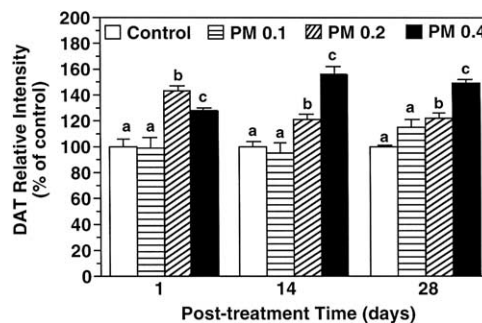


Fig. 5. Time course studies using densitometry of striatal synaptosomes on DAT Western blots from mice treated with either 0.1, 0.2, or 0.4 mg/kg PM. ANOVA and means separation as described for Fig. 3.

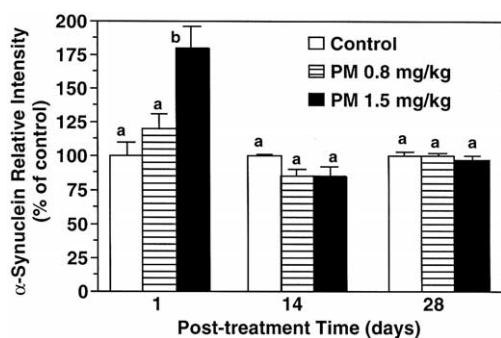


Fig. 6. Densitometry of α -synuclein in Western blots of striatal synaptosomes from mice treated with either 0.8 or 1.5 mg/kg PM at 1, 14, and 28 days posttreatment. ANOVA and means separation as described for Fig. 3.

Discussion

The present study confirms and extends the results of Karen et al. (2001), and establishes a potent, slowly developing up-regulation of the DAT by the pyrethroid, PM. In general, pyrethroids are quickly metabolized and excreted from the body, although some pyrethroid metabolites may be persistent (Casida et al., 1983). In light of this rapid metabolism, it was surprising to us that at doses as low as 0.2 mg/kg PM, DAT protein was significantly induced at 4 weeks posttreatment. Thus, DAT expression can occur at doses about an order of magnitude below those previously reported to have this effect (Karen et al., 2001). The initial doses of PM selected for this study, 0.8 and 1.5 mg/kg given 3 times over 2 weeks, are consistent with a reported dermal dose of 0.13 mg/kg/day to humans exposed during the first Gulf War (Abou-Donia et al., 2001). It is also noteworthy that of the four PM stereoisomers, only one of them (1*R*-*cis* configuration across the cyclopropane ring) has significant acute toxicity to mammals (Casida et al., 1983). If this isomer is also solely responsible for the observed effects on

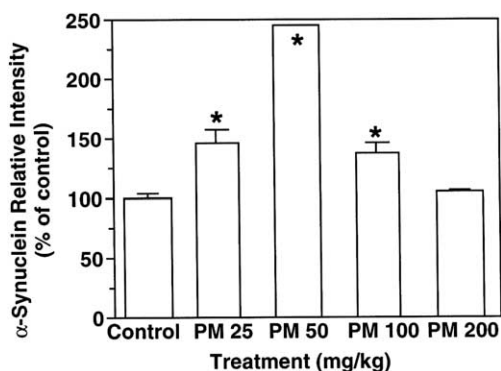


Fig. 7. Densitometry of α -synuclein in Western blots of striatal synaptosomes from mice treated with increasing doses of PM. Letters denote results of a *t* test, where bars labeled by an asterisk for a particular time point (1, 14, or 28 days) are significantly different from controls ($P < 0.05$). The SEM of the 50 mg/kg group was too small to be seen at the y-axis scaling of the graph.

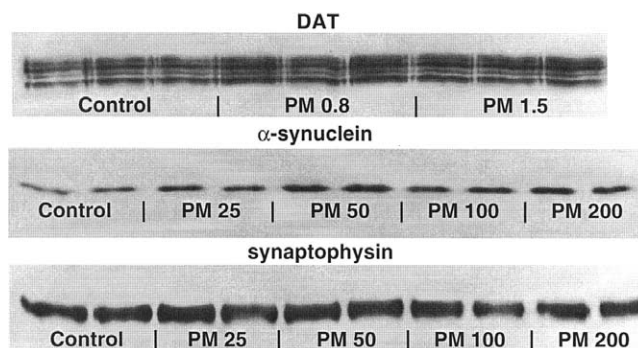


Fig. 8. Examples of representative Western blots of DAT, α -synuclein, and synaptophysin. Treatments are labeled below stained bands, and are mg/kg PM.

the DAT, then the actual doses of PM responsible for this effect are 4-fold lower than those used in this study. Support for this conclusion is found in published studies with the related pyrethroid, deltamethrin. This compound is composed of a single 1*R*,*cis*, α S isomer that is toxic to mammals (murine ip LD50 = 10 mg/kg, Casida et al., 1983) and it up-regulates DA transport in ex vivo synaptosomes (Kirby et al., 1999).

It is compelling that our published DA uptake data, in which transport is increased at low PM doses (Karen et al., 2001), are mirrored by the [3 H]GBR 12935-binding studies and quantitation of DAT protein by Western blotting of this study. However, there has been some debate over the use of [3 H]GBR 12935 as a DAT-specific ligand. Pristupa et al. (1994) found that binding of [3 H]GBR 12935 in human COS-7 cells transfected with human DAT was not saturable up to 22 nM. It has also been shown that [3 H]GBR 12935 is able to bind to dopamine-insensitive “piperazine acceptor sites,” one of which has been identified as human CYP2D6 (Allard et al., 1994; Hiroi et al., 1997). In our experiments, [3 H]GBR 12935 binding was specific and saturable, showed excellent correlation with other biomarkers as noted above, and yielded a B_{\max} value for C57B1/6 control mice of about 3 pmol/mg protein, similar to values reported by Horn (1990) for rat striatum (5.5 pmol/mg) or human caudate tissue (2.7 pmol/mg).

The lack of a statistically significant response at the 1.5 mg/kg dose until $t = 28$ in the binding and Western blot studies was surprising, since the maximal increase in DA uptake in other cohorts of mice occurred at this dose 1 day posttreatment. It is likely that there are cohort-related differences in the effectiveness of the 1.5 mg/kg PM dose. This possibility is supported by our previous data in which up-regulation of DAT protein did occur at the 1.5 mg/kg dose at 1 day posttreatment (Bloomquist et al., 2002). A slowly developing effect on DAT was observed across all doses tested, since DAT protein was induced to a greater extent in the 14- and 28-day groups vs the 1-day posttreatment groups given 0.2–0.4 mg/kg (Fig. 5) and 200 mg/kg PM (Fig. 4). Thus, induction of DAT up-regulation is variable or

delayed until 28 days posttreatment, when it apparently stabilizes. At high doses of PM (e.g., 200 mg/kg), the delayed response in DAT up-regulation, as well as reduced DA uptake, may be due to nerve terminal toxicity, since this dose causes a reduction in mitochondrial function in previous studies (Karen et al., 2001). After a recovery period, DAT up-regulation again becomes evident. Persistent DAT up-regulation was also noted in Sprague-Dawley rats exposed to heptachlor in the gestational, perinatal, and adolescent stages of development. In this case, DAT binding to [³H]mazindol remained significantly increased into adulthood (Purkerson-Parker et al., 2001).

Unlike DAT, the up-regulation of α -synuclein protein was not a persistent effect, and it had returned to normal levels in the 14- and 28-day treatment groups. Mutations in α -synuclein are believed to cause the fibrillar aggregates that are the major components of Lewy bodies, a pathological hallmark of PD (Murray et al., 2001). Mutations in the α -synuclein gene have also been associated with some forms of familial PD (Langston et al., 1998; Mizuno et al., 2001). Manning-Bog et al. (2001) found the herbicide paraquat (10 mg/kg) to cause up-regulation and increased aggregation of α -synuclein in C57B1/6 mice. This up-regulation was also not persistent, with protein levels returning to control values by Posttreatment Day 7.

It has been reported that human α -synuclein can complex with human DAT, causing acceleration of cellular DA uptake and DA-induced cellular apoptosis (Lee et al., 2001). DA itself can be metabolized to toxic free radical species that have neurotoxic effects, including inhibition of mitochondrial respiration (Ben-Shachar et al., 1995). Thus, the combined increase in DAT and α -synuclein by pesticides such as PM may exacerbate this potential for cellular toxicity, especially if sufficiently prolonged. Our observed up-regulation of α -synuclein is likely a compensatory mechanism, since it has been reported that α -synuclein is able to inactivate c-Jun N-terminal kinase (JNK), thus protecting cells against oxidative stress (Hashimoto et al., 2002). Although the signaling pathway for protein up-regulation by pyrethroids is unknown, these compounds have a high-affinity interaction with the β -subunit of GTP-binding proteins (Rossignol, 1991a, 1991b), and are reported to increase protein phosphorylation levels by enhancing the effects of both protein kinase C (Enan and Matsumura, 1992) and protein kinase A (Matsumura et al., 1989). The bell-shaped dose-response relationships observed for α -synuclein suggest that the signaling pathways are modified at different threshold doses, and that the pathway(s) apparently shuts down at higher doses, at least at $t = 1$.

To date, studies of pyrethroid effects on dopaminergic pathways have emphasized doses that result in acute intoxication. Electroencephalographic recordings found that pyrethroid-induced intoxication in the rat seemed to originate in neurons of the caudate nucleus and the dopaminergic neurons of the substantia nigra before spreading to other brain regions (Ray and Cremer, 1979; Ray, 1980). Thus,

acutely toxic doses of pyrethroids affect regions of the brain involved in PD, and because they are the first affected, these areas would seem to display an exceptional sensitivity to the action of pyrethroids. Acute treatments of pyrethroids at high doses caused a slight decrease in striatal DA levels (Husain et al., 1991), but a significant increase of 23–37% in levels of DOPAC (Doherty et al., 1988), suggesting an increase in DA turnover. At 200 mg/kg, PM had no effect on striatal DA or DOPAC levels in the treatment paradigm we used (Karen et al., 2001). The low dose effects we show here are more likely to mirror body burdens found in humans, and it remains to be determined whether sustained exposures to low doses can result in loss of striatal DA content, a cardinal sign of PD (Hornykiewicz and Kish, 1986).

Acknowledgments

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Immunohistochemical Changes in the Mouse Striatum Induced by the Pyrethroid Insecticide Permethrin

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Epidemiological studies have linked insecticide exposure and Parkinson's disease. In addition, some insecticides produce damage or physiological disruption within the dopaminergic nigrostriatal pathway of non-humans. This study employed immunohistochemical analysis in striatum of the C57BL/6 mouse to clarify tissue changes suggested by previous pharmacological studies of the pyrethroid insecticide permethrin. Dopamine transporter, tyrosine hydroxylase, and glial fibrillary acidic protein immunoreactivities were examined in caudate-putamen to distinguish changes in amount of dopamine transporter immunoreactive protein from degeneration or other damage to dopaminergic neuropil. Weight-matched pairs of pesticide-treated and vehicle-control mice were dosed and sacrificed on the same days. Permethrin at 0.8, 1.5 and 3.0 mg/kg were the low doses and at 200 mg/kg the high dose. Brains from matched pairs of mice were processed on the same slides using the avidin-biotin technique. Four fields were morphometrically located in each of the serial sections of caudate-putamen, digitally photographed, and immunopositive image pixels were counted and compared between members of matched pairs of permethrin-treated and vehicle-control mice. For low doses, only 3.0 mg/kg produced a significant decrease in dopamine transporter immunostaining. The high dose of permethrin did not produce a significant change in dopamine transporter or tyrosine hydroxylase immunostaining, but resulted in a significant increase in glial fibrillary acidic protein immunostaining. These data suggest that a low dose of permethrin can reduce the amount of dopamine transporter immunoreactive protein in the caudate-putamen. They also suggest that previously reported reductions in dopamine uptake of striatal synaptosomes of high-dose mice may be due to nondegenerative tissue damage within this region as opposed to reductions of dopamine transporter protein or death of nigrostriatal terminals. These data provide further evidence that insecticides can affect the primary neurodegenerative substrate of Parkinson's disease.

Keywords Dopamine Transporter, Glial Fibrillary Acidic Protein, Parkinson's Disease, Permethrin, Striatum, Tyrosine Hydroxylase

Despite a wealth of investigation into Parkinson's disease (PD), the etiology of the disorder remains elusive. Although there is agreement on a significant genetic contribution in some forms of early onset Parkinson's-like disorders, the role of genetic inheritance in the most common form of the disease, adult- or late-onset PD, remains equivocal (Olanow and Tatton 1999; Tanner et al. 1999; Sveinbjornsdottir et al. 2000; Mouradian 2002). This, in addition to the fact that the xenobiotic compound 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is used to produce one of the principal mammalian models for Parkinson's disease (Royland and Langston 1998; Schmidt and Ferger 2001), has prompted a great deal of work on potential environmental causes or triggers for PD.

Insecticides are a widely used class of environmental chemicals that induce their toxic effects by acting on the nervous system. A number of epidemiological studies support a link between insecticide exposure and Parkinson's disease in humans (Semchuck, Love, and Lee 1992; Butterfield et al. 1993; Gorell et al. 1998; Corrigan et al. 2000; Zorzon et al. 2002). Furthermore, a number of recent experimental studies have shown that some insecticides can produce damage or physiological disruption within the dopaminergic nigrostriatal pathway (Bloomquist et al. 1999; Kirby, Castagnoli, and Bloomquist 1999; Miller et al. 1999; Betarbet et al. 2000; Karen et al. 2001; Purkerson-Parker, McDaniel, and Moser 2001; Kirby, Barlow, and Bloomquist 2001). This pathway is the principal focus of degeneration in idiopathic PD (DiMonte and Langston 1995; Poewe and Wenning 1998).

The synthetic pyrethroid insecticides are derivatives of the natural insecticidal pyrethrins found in the chrysanthemum. The principal target of the pyrethroids, in both insects and mammals, is the voltage-gated sodium channel (Ghiasuddin and Soderlund 1985; Ray 2001). The opened state of the channel is prolonged, which produces a hyperexcitability within the target tissue. There is some evidence that other sites in excitable tissues may be

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affected as well, such as voltage-gated calcium channels (Hagiwara, Irisawa, and Kameyama 1988; Duce et al. 1999), γ -aminobutyric acid receptors (Lawrence, Gee, and Yamamura 1985; Bloomquist, Adams, and Soderlund 1986) and neurotransmitter membrane transporters (Kirby, Castagnoli, and Bloomquist 1999; Karen et al. 2001; Bloomquist et al. 2002).

Pyrethroids have been reported to affect the function of components of the dopaminergic nigrostriatal pathway, sometimes in a selective fashion. For example, in rats, decamethrin produced effects on electroencephalographic recordings in striatum and substantia nigra prior to affecting other brain regions (Ray 1980). In addition, deltamethrin-induced increases in blood flow were greater in caudate nucleus compared with cerebral cortex (Ray 1982). In rabbit brain slices, fenvalerate was capable of inducing the release of dopamine and acetylcholine from striatum, but did not cause release of norepinephrine or acetylcholine from hippocampus (Eels and Dubocovich 1988). In neurotransmitter release studies using preloaded synaptosomes, it has been shown that deltamethrin induces dopamine release from striatal synaptosomes at EC_{50} values 2.4- to 8.6-fold more potent than cortical synaptosomes containing serotonin or glutamate, respectively (Kirby, Castagnoli, and Bloomquist 1999; Bloomquist et al. 2002). Pyrethroids have also been shown to increase the concentration of the dopamine metabolite 3,4-dihydroxyphenylacetic acid (DOPAC) in the striatum (Doherty et al. 1988). In prenatally exposed rat pups examined as adults, a fenvalerate-induced increase in DOPAC and DOPAC/dopamine ratio suggested an enhanced activity of the dopaminergic nigrostriatal pathway that was not seen for noradrenergic or serotonergic systems (Lazarini et al. 2001). The fairly limited number of studies on any given pyrethroid, in addition to the variety of dependent variables employed, suggest it would be premature to consider a specific pyrethroid as a reliable, positive experimental control for pyrethroid-induced insult to the nigrostriatal pathway. Nevertheless, there appears to be sufficient evidence to warrant further exploration of the role of pyrethroids in such damage.

Pyrethroid insecticides have also been shown to modulate neurotransmitter uptake (Kirby, Castagnoli, and Bloomquist 1999; Karen et al. 2001; Bloomquist et al. 2002). A recent study has demonstrated that the type I pyrethroid permethrin can alter maximal dopamine uptake (V_{max}) by mouse striatal synaptosomes (Karen et al. 2001). At low doses V_{max} was increased, whereas at higher doses V_{max} was decreased, as was open field behavior, compared to vehicle controls. The lack of an accompanying change in K_M suggested that these pharmacological changes reflected an alteration in the amount of dopamine transporter (DAT) present within the synaptosomal tissue sample, rather than a change in the efficiency of the transporter. Furthermore, the reversal of increased maximal dopamine uptake seen at the high doses of permethrin could reflect damage or degeneration of dopaminergic neuropil within the striatum. In the present study, immunohistochemical techniques were employed in the mouse striatum to clarify the tissue changes suggested by the aforementioned kinetic studies. This technique permitted the

evaluation of changes in a topographically defined region of the striatum: that which contains the highest density of nigrostriatal dopaminergic afferents (Heimer, Zahm, and Alheid 1995) and the region most likely to be affected in PD (Kish, Shannak, and Hornykiewicz 1988; Graybiel, Hirsch, and Agid 1990; Miller et al. 1999). DAT, tyrosine hydroxylase (TH), and glial fibrillary acidic protein (GFAP) immunoreactivities were examined in an attempt to distinguish changes in the amount of the DAT from degeneration or other damage to dopaminergic striatal neuropil.

MATERIALS AND METHODS

Animals and Treatments

Male C57BL/6 retired breeder mice were used because this study attempted to assess the effects of permethrin on the nigrostriatal pathway and the C57BL/6 mouse is the most common, and sensitive, rodent model used in studies of chemically induced parkinsonism (Heikkila, Hess, and Duvoisin 1984; Royland and Langston 1998). Mice were 7 to 9 months old at the time of the experiment and were obtained from Harlan Sprague-Dawley, Dublin, VA, USA. They were assigned to groups by weight, such that every mouse designated for insecticide treatment had a paired, weight-matched control. These weight-matched pairs were randomly assigned to different dosage groups. Insecticide-treated mice were given intraperitoneal (IP) injections of permethrin (Sigma Chemical, St. Louis, MO, USA) in a methoxytriglycol vehicle, whereas control mice received vehicle alone. The permethrin compound was 96.6% pure and was comprised of 71.8% *trans*-isomer and 24.8% *cis*-isomer. Regardless of dose, all mice received three injections over a 2-week period according to the methods of Bloomquist et al. (1999) and Karen et al. (2001), with day 1 being the first injection, followed by injections on days 8 and 15 and sacrifice on day 16. For every permethrin-treated mouse that was sacrificed on a given day, its weight-matched, vehicle-control partner was also sacrificed. Mice receiving 0.8, 1.5, or 3.0 mg/kg of permethrin were considered the "low-dose" group and those receiving 200 mg/kg were considered the "high-dose" group. These doses were selected because permethrin treatments of similar magnitude were previously shown to produce the greatest changes in dopamine uptake in the mouse striatal synaptosome preparation (see Karen et al. 2001). In addition, the extent of the dosing spectrum was limited to these values in order to maximize resources and minimize the number of mice used in the experiment.

Fixation, Tissue Sectioning, and Histochemistry

On the day of sacrifice, the permethrin-treated and matched vehicle-control mice were deeply anesthetized with sodium pentobarbital and perfused transcardially with a phosphate-buffered saline (PBS) rinse (0.05 M, pH 7.4), followed by 4% paraformaldehyde fixative solutions (in 0.1 M phosphate buffer) at respective pH values of 7.0 and 10.5. Brains were then removed and post-fixed in the pH 10.5 fixative for 4 hours, rinsed three

times with PBS, and cryoprotected overnight in 10% sucrose at 4°C.

Using a cryostat, 16- μm sections through the striatum were cut onto slides beginning at the rostral-most appearance of the lateral ventricle. For the high-dose mice, three consecutive sections were cut to provide exposure to TH antibody (Protos Biotech, New York, NY, USA, formerly Eugene Tech, catalogue no. CA-101), cresyl violet staining, and either DAT antibody or GFAP antibody (DAKO, Glostrup, Denmark, catalogue no. Z0334). For the low-dose mice, two consecutive 16- μm sections were cut, respectively, for exposure to DAT antibody (Chemicon International, Temecula, CA, USA, catalogue no. AB1591P) or cresyl violet staining. Sections for TH and GFAP staining were not prepared for this condition because previous work provided no reason to suspect death or damage of striatal neuropil following the low dose regimen (see Karen et al. 2001). The sectioning sequence was repeated at 320- μm intervals, through the striatum, until 12 sets of multiple sections were cut for primary antibody staining. During cutting of the fifth set of slides, a supplemental slide set was prepared (set 5A) from tissue immediately adjacent (within 16 μm) to the sections cut for antibody staining. This supplemental set was used as an omission control for primary antibody staining.

Given the operational definition of the cutting procedure described above, sectioning began at approximately 1.94 mm rostral to bregma and ended at approximately 1.68 mm caudal to bregma. The caudate-putamen extends from 1.94 mm rostral to bregma through 2.30 mm caudal to bregma (Franklin and Paxinos 1997). After completing the sectioning of a given permethrin-treated brain, corresponding sections from the matched-vehicle brain were placed on the same slide, which insured identical histochemical treatment for both sections. The two sectioned brains comprised a single case. The slide positions of the treated and vehicle-control brains were counterbalanced across cases, as well as the order in which the brains were cut.

Because the two brains were cut in series, as opposed to parallel within the same block, it was possible that there were minor variations in section thickness that could have affected the subsequent quantification of stained neuropil described below. However, the counterbalancing of the order in which brains were cut would help to distribute such error randomly. Furthermore, variation in section thickness is likely to occur across serial sections within a given brain and not just across different brains, further ensuring a random distribution of this variability.

Immunohistochemistry was performed directly on the slides according to a modification of methods previously outlined by this laboratory (Klein and Blaker 1990; Klein et al. 1992; Misra and Klein 1995). Each antiserum used in this study was commercially available and has been used in a multitude of studies on the nervous system, many of which involve analysis of striatal tissue (e.g., Lew et al. 1992; Pu et al. 1994; Freed et al. 1995; Kordower et al. 1996; Vaughan et al. 1996; Wirth, Rufer, and Unsicker 1996; Betarbet et al. 1997; Zimmer, Ennis, and Shipley 1997; Sortwell, Collier, and Sladek 1998; Sugama et al. 2003). Speci-

ficity of these antibodies has been previously verified by others using a variety of techniques including Western blot, enzyme-linked immunosorbent assay (ELISA), immunoelectrophoresis, and immunohistochemistry. Briefly, sections were rinsed with PBS, incubated with 3% hydrogen peroxide to quench endogenous peroxidase, and rinsed again. The slides were then incubated sequentially in (1) 10% normal goat blocking serum containing 0.15% Trinton X-100 and (2) polyclonal rabbit antisera to either DAT (1:600), TH (1:400), or GFAP (1:6400), overnight, at room temperature. Tissue was then processed by the avidin-biotin complex method using a Vectastain Elite kit (Vector Labs, Burlingame, CA, USA, catalogue no. PK6101) and 0.05% diaminobenzidine in 0.01% hydrogen peroxide as the chromogen. Sections were then dehydrated in alcohols, cleared in xylene, and coverslipped. Alternate sections were stained with 0.3% cresyl violet in 50% ethanol. Diluent for primary and secondary (biotinylated goat anti-rabbit, included in the kit) antibody solutions was 1% goat serum in PBS containing 0.15% Trinton X-100. Tissue incubations were performed in a humidity chamber. Immunostaining produced with the three primary antibodies can be seen in Figures 1 and 2. These photographs are simply intended to show examples of the general quality of tissue staining obtained in the study. However, they also serve as evidence of positive and/or negative controls for primary antibody staining. The region of the caudate-putamen, a region known to be rich in dopaminergic afferents, exhibits dense immunoreactivity for both the DAT and TH antibodies in Figures 1 and 2. Furthermore, there is a drastic reduction of this staining at the medial and lateral borders of the caudate-putamen, in the respective regions of the lateral globus pallidus and the external capsule. This can be seen for DAT in Figures 1 and 2, and the reduction in TH at the external capsule can be seen in Figure 2. This lateral portion of the globus pallidus and the external capsule are regions where dopaminergic input is known to be limited (Parent and Smith 1987). Immunopositive staining in the above-noted primary antibody omission-control sections was virtually absent.

Morphometric Location of Fields for Image Analysis

An individual data point in this study was defined as the difference in the amount of immunoreactive neuropil (DAT, TH, or GFAP) between a brain section from a permethrin-treated mouse and the corresponding section from its matched vehicle control, located on the same slide. The amount of immunoreactive neuropil in a given brain section was a mean, calculated from four 3070- μm^2 fields, distributed within the dorsolateral portion of the striatum (Figure 1B). This region is the primary striatal target of dopaminergic neurons originating from the rodent substantia nigra pars compacta (Heimer, Zahm, and Alheid 1995). In order to insure consistent sampling between sections from treated and matched vehicle-control sections, a morphometric procedure was used to define the location of the four fields within each brain section. Consistent identification of the caudate-putamen, across sections, was aided by the fact that (1) throughout most of its

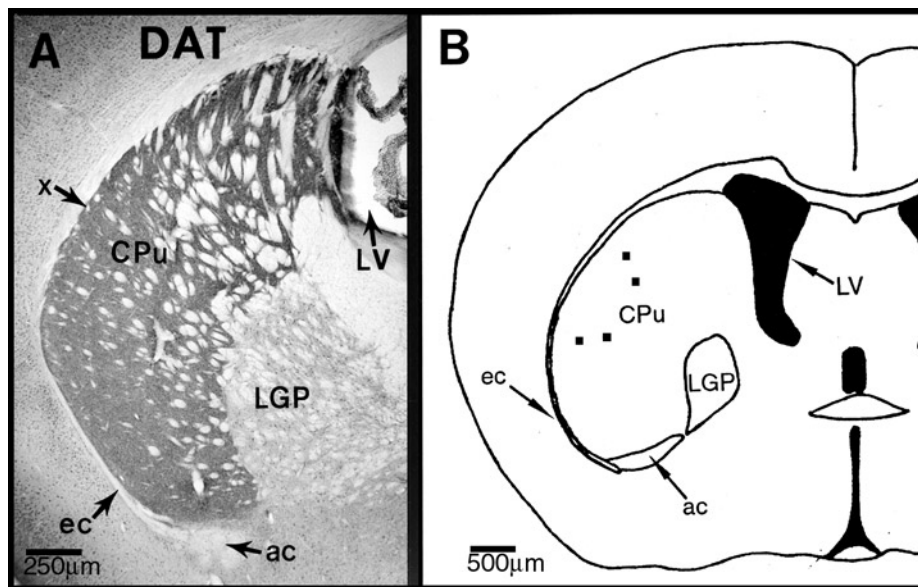


FIGURE 1

(A) Low-magnification image of a coronal section through the striatum showing immunostaining for DAT. The region indicated by the "x" is shown at higher magnification in Figure 2. (B) Schematic diagram of a similar section, modified from a mouse brain atlas (Franklin and Paxinos 1997), showing the location and size ($3070 \mu\text{m}^2$) of each of the four analysis fields in the dorsal lateral portion of the striatum. Both images also illustrate prominent anatomical landmarks in the region. CPu = caudate-putamen; ec = external capsule; LV = lateral ventricle; ac = anterior commissure; LGP = lateral globus pallidus.

rostrocaudal course, the caudate-putamen is bordered dorsally, ventrally, and laterally by the external capsule, a well-defined band of white matter; (2) the dorsomedial border of the nucleus is adjacent to and can be easily distinguished from the cavity of the lateral ventricle; and (3) the ventromedial border of the nucleus can be histologically distinguished from the less densely staining globus pallidus (see Figure 1A) in both immunostained and cresyl violet-stained sections (Franklin and Paxinos 1997). In the rostral third of the nucleus, where the external capsule does not extend to the ventral border, the lateral striatal stripe and the anterior commissure were used to locate the ventral border of the caudate-putamen and distinguish it from the nucleus accumbens.

Morphometric location of the four analysis fields was done on camera-lucida tracings of brain sections. Initially, a central point of the caudate-putamen was operationally defined as follows: The dorsal and ventral tips of the nucleus were located with the aid of the external capsule. At the dorsoventral midpoint of the nucleus, a horizontal line was drawn extending from the external capsule laterally, to the pallidal border medially. The midpoint of this line was operationally defined as the center point of the caudate putamen.

After operationally locating the center point of the caudate-putamen, the outer circumference of the nucleus was traced from the ventral-most tip to the dorsal border with the lateral ventricle. Along this arc, marks were made at $1/3$ and $2/3$ of the total distance. Lines were then drawn from these marks to the center

point of the nucleus. Along each of these two radii, marks were made at $1/4$ and $1/2$ the distance between the perimeter of the nucleus and the center point. This yielded four marks, designating the centers of the four fields that were to be digitally photographed for image analysis (Figure 1B). Around these fields, numerous tissue landmarks were traced, such as unstained fiber bundles or imperfections, to aid in relocating these fields for photography. The fields identified by the procedure described above were consistently distributed within the dorsolateral quadrant of the caudate-putamen and permitted measurements from similar regions within permethrin-treated and matched vehicle-control brains.

As alluded to above, and as can be seen in Figures 1 and 2, coronal sections through the striatum are characterized by fairly numerous, unstained fiber bundles of passage, the pattern of which varies among individual animals. In order to minimize the effect of these fibers of passage upon the analysis, when such a bundle fell within the measurement field designated for photography, the position of the field was adjusted to incorporate the closest adjacent field of homogenous immunolabeling. Thus, the fields such as those depicted in Figure 1 could vary by as much as $50 \mu\text{m}$ in any direction, but all still remained within the dorsolateral quadrant of the caudate-putamen.

After morphometrically locating and photographing the four analysis fields from the caudate-putamen, the convergence of the mediolateral and dorsoventral midpoints of the corpus callosum was visually located and photographed in each brain section.

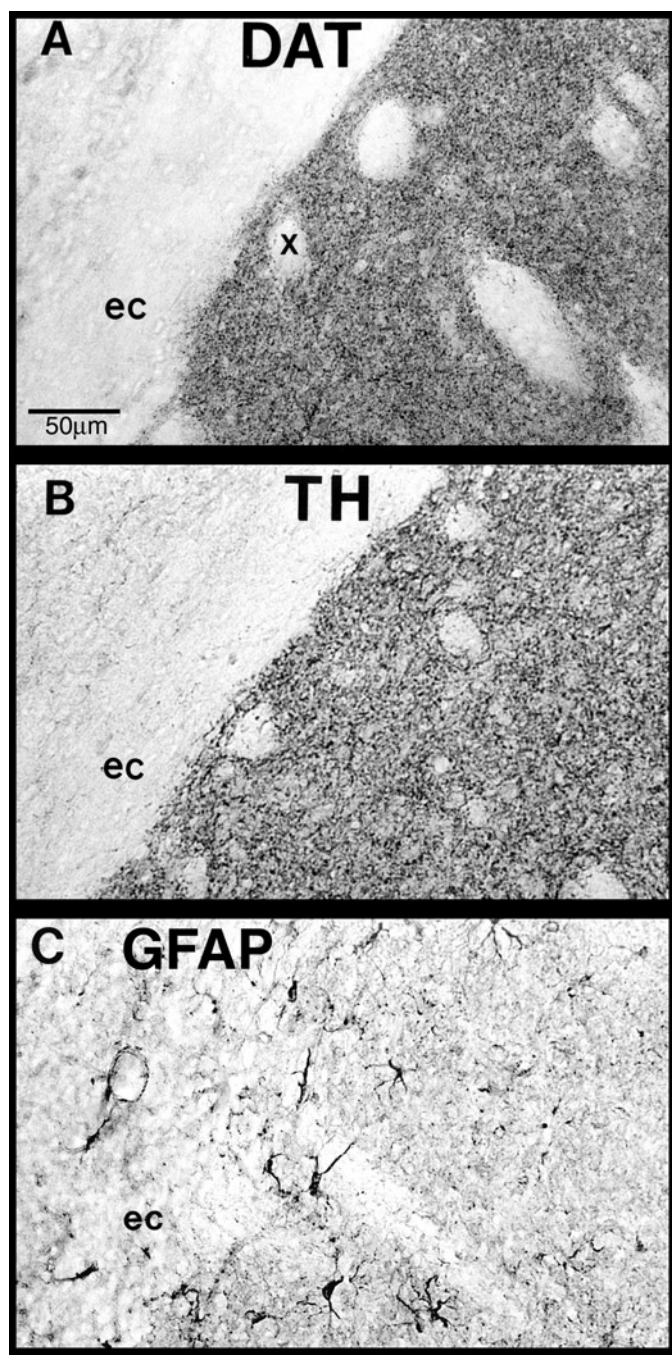


FIGURE 2

(A) Higher-magnification image of a portion of the section shown in Figure 1A. The “x” in both figures indicates the same tissue location. (B and C) Similar locations within the caudate-putamen, relative to A, showing staining for TH and GFAP, the other two antibodies used in the study. The anatomical landmark of the external capsule is indicated in all three photomicrographs. DAT = dopamine transporter; TH = tyrosine hydroxylase; GFAP = glial fibrillary acid protein; ec = external capsule.

This allowed assessment of a possible change in GFAP immunolabeling in a nonstriatal region and addressed whether changes in GFAP immunoreactivity were a generalized response to insecticide exposure. The mediolateral midpoint was located by following an imagined, vertically oriented line, continuing from the dorsal median fissure (the fissure that separates the two cerebral hemispheres) through the corpus callosum. At this midline location, the dorsal and ventral borders of the callosum could be easily recognized and the midpoint between these borders was visually located. A digital photograph was then taken that was centered on this point.

Image Analysis

The amount of immunostained neuropil in photographs of each of the four fields within a section was quantified using the “Histogram” feature of Adobe Photoshop 5.0 (Adobe Systems, San Jose, CA, USA). Digital images of a given size are comprised of a fixed array of loci called pixels. For any selected area of pixels from a digital photograph, the “Histogram” function can determine the mean grayscale value (0 to 255, where 0 is black and 255 is white) and the number of pixels that are darker than a specified grayscale value.

A threshold value for immunostained neuropil was determined by sampling the mean grayscale value of varicosities, which were visually judged to be unambiguously immunopositive, within every brain section of the vehicle-control mice. The grayscale value for two such varicosities was averaged for each field and the mean across the four fields was taken as the average value for the section. The mean across all sections from each vehicle-control mouse was determined, and the mean grayscale value across all vehicle-control mice was used as the threshold value for identifying immunostained neuropil in both vehicle-control mice and permethrin-treated mice. Image pixels as dark, or darker than this value, were then considered as immunopositive labeling, irrespective of how much darker they were. Pixels lighter than this threshold value were not counted. Using the above-noted threshold value, the number of pixels darker than threshold was counted for each photograph of the four measurement fields from each striatal section. This value was averaged across the four measurement fields from each section and was considered the amount of immunostained neuropil in that section. These immunopositive pixel counts were then compared between vehicle and insecticide-treated mice. The determination of immunopositive labeling and statistical analysis for the callosal field was performed in the same fashion.

The typical distribution of grayscale values for the pixels from a photograph of a given measurement field was bell-shaped. A random sample of 50 photographs from among all vehicle-treated mice in the low-dose condition revealed that, on average, the darkest 22% of DAT-stained pixels were being counted. Although this probably reduced the chance of detecting real differences between matched vehicle- and insecticide-treated mice, it

likely minimized the probability that any differences were attributable to variation in nonspecific background staining, even though such staining appeared minimal in omission controls.

Statistical Analysis

As alluded to above, on each microscope slide, a brain section from a permethrin-treated animal was paired with a section from a matched vehicle-control animal. These mice were injected on the same days, sacrificed on the same day, and the sections were taken from similar rostrocaudal positions within the striatum. In addition, the paired sections on a slide shared identical tissue processing conditions. Therefore, the individual data points subjected to statistical analysis in this study were the differences in immunopositive staining (pixel counts), as described above, between the paired permethrin-treated and matched vehicle-control sections on a microscope slide.

Initially, scatterplots were used to assess the effects of microscope slide section position and date of tissue processing on differences in immunostained neuropil between paired sections. Based on this analysis, an analysis of variance (ANOVA) model, fitted using the GLM procedure of SAS (SAS Institute,

Cary, NC, USA), was used to correct for the effect of processing date on each data point. The corrected differences in immunostaining were then consistent with the assumptions for analysis by a paired *t* test. For a pair of permethrin-treated and matched vehicle-control brains, the difference in immunostained neuropil was averaged across microscope slides. Then, for each dose concentration group, the grand mean of these corrected mean differences was tested for its difference from zero using an alpha level of 0.05. Inflation of the type I error rate was avoided by treating each dose concentration group as a separate experiment.

RESULTS

Figure 3 is a box and whisker plot of the distribution of mean differences in DAT immunostaining, between matched pairs of permethrin-treated and vehicle-control mice, within each of the "low-dose" concentration groups. The tips of the whiskers respectively represent the minimum and maximum mean differences, whereas the length of the box represents the interquartile range. The line and solid square within each box respectively represent the median and grand mean of the distribution

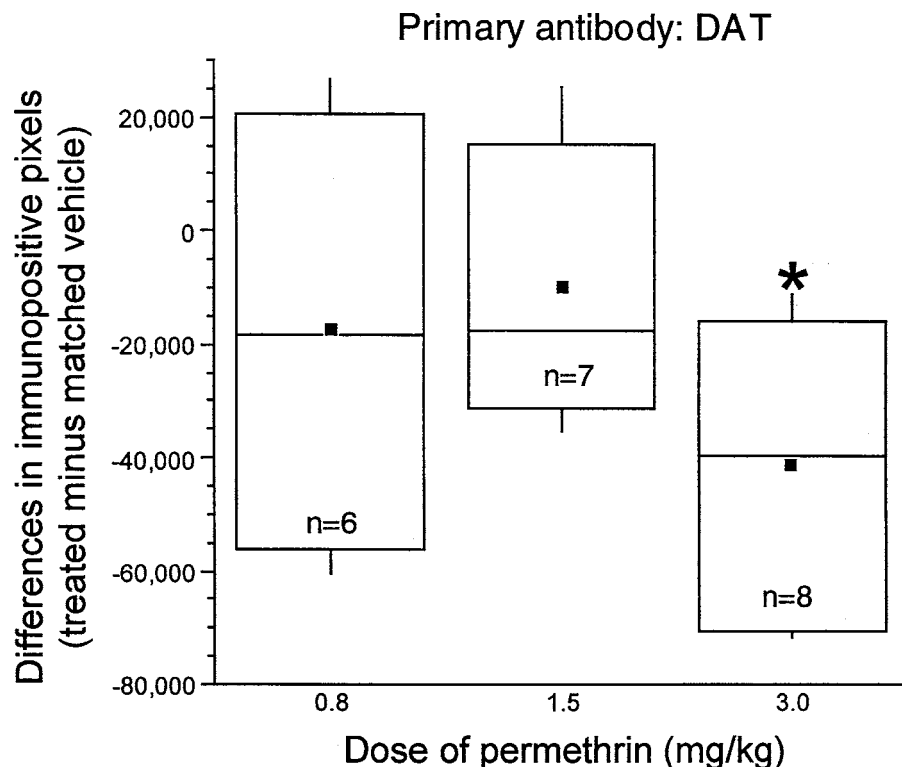


FIGURE 3

Distributions of differences in immunopositive staining for DAT, between pairs of pesticide-treated and matched vehicle-control mice, for each of the low doses of permethrin. For each box and whisker plot, whisker tips respectively represent minimum and maximum differences whereas box length shows the interquartile range of the differences. The line and black square respectively indicate the median and mean of the difference scores. The asterisk indicates a mean of difference scores significantly different from zero. *n* = number of matched pairs of permethrin-treated and vehicle-control mice.

of mean differences. The number of matched pairs of treated and vehicle-control mice is also indicated for each dose. A grand mean of zero represents no change in immunostaining between matched pairs of mice. As can be seen in Figure 3, only the 3.0-mg/kg dose of permethrin produced a significant decrease in DAT immunostaining within the caudate-putamen, compared with matched vehicle-control mice ($df = 7, p = .007$), although all “low-dose” permethrin-treated mice showed a trend toward decreased labeling. The mean decrease of 41,713 pixels in the 3.0-mg/kg group was 48.6% of the mean number of pixels in the matched vehicle control group. An example of the change in DAT immunopositive staining, that reflects this mean decrease, can be seen in Figures 5C and 5D. Although this percentage provides some idea of the magnitude of change, the reader should note that it is based upon a corrected mean across vehicle-control mice that was not used in the formal statistical analysis of the results. As noted in the Materials and Methods, that analysis was based on *differences* between individual brain sections from treated mice and their matched-vehicle controls that were processed under the same conditions.

Figure 4 is a box and whisker plot of mean differences in DAT, TH, and GFAP immunostaining, between matched pairs of treated and vehicle-control mice, in the “high-dose” concentration group (200 mg/kg permethrin). As seen in the figure, the “high dose” of permethrin did not produce a significant change in DAT or TH immunostaining within the caudate-putamen, compared with matched-vehicle controls, although there was a trend toward a decrease for both types of labeling. However, as in-

dicated by the asterisk, the “high dose” of permethrin resulted in a significant increase in striatal GFAP immunostaining, compared to matched vehicle controls ($df = 7, p = .048$). The mean increase of 4524 pixels was 879% of the mean number of pixels in the matched vehicle control group. Again, the value of this percent change should be interpreted in light of the caveat noted above. An example of the change in GFAP immunopositive staining, which reflects this mean increase, can be seen in Figures 5A and 5B.

Comparison of the callosal field between matched pairs of treated and vehicle-control mice in the “high-dose” concentration group (200 mg/kg of permethrin) revealed a mean difference of -10.4 pixels ($N = 8$; median = 51.3; range = 781.8; interquartile range = 200.3). This change, which represented only a 5.3% decrease relative to the mean number of pixels in the matched vehicle-control group, was not significantly different compared with a mean difference of zero.

DISCUSSION

The results of this experiment provide further support for the notion that environmental chemicals, in this case insecticides, can produce changes in components of the dopaminergic nigrostriatal pathway; a pathway that is the primary neurodegenerative substrate of Parkinson’s disease. Taken together with a previous report on mouse striatal synaptosomes, these data indicate that a low dose (1.5 to 3.0 mg/kg) of the pyrethroid insecticide permethrin cannot only alter the kinetics of dopamine uptake

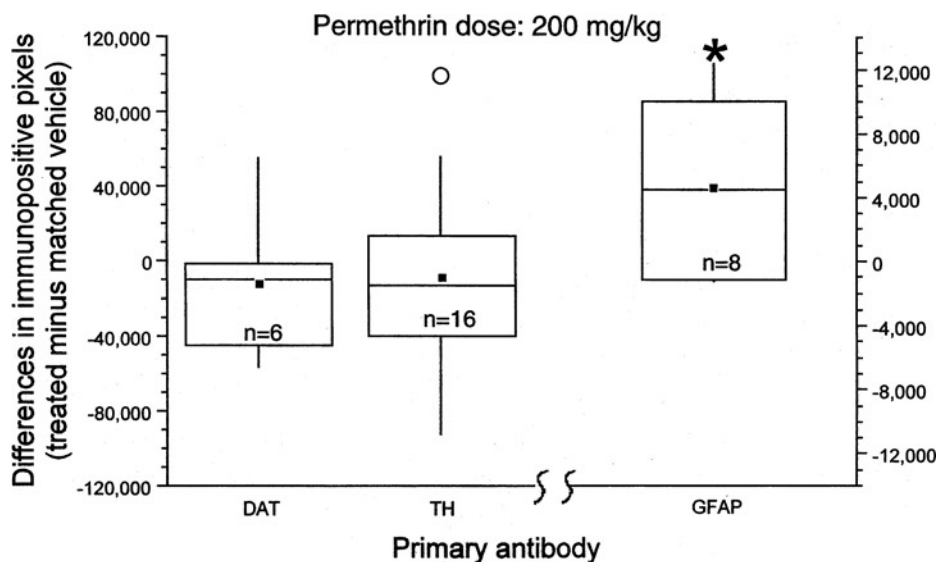


FIGURE 4

Box and whisker plots of differences in immunopositive staining for DAT, TH, and GFAP, between matched pairs of pesticide-treated and vehicle-control mice, for the high dose (200 mg/kg) of permethrin. The *open circle* is a difference score that is more than 1.5 interquartile ranges above the median. The *asterisk* indicates a mean of difference scores significantly different from zero. Note that the y-axis for the GFAP data is drawn to a different scale because the amount of GFAP immunopositive material is normally an order of magnitude more sparse compared with that for DAT and TH.

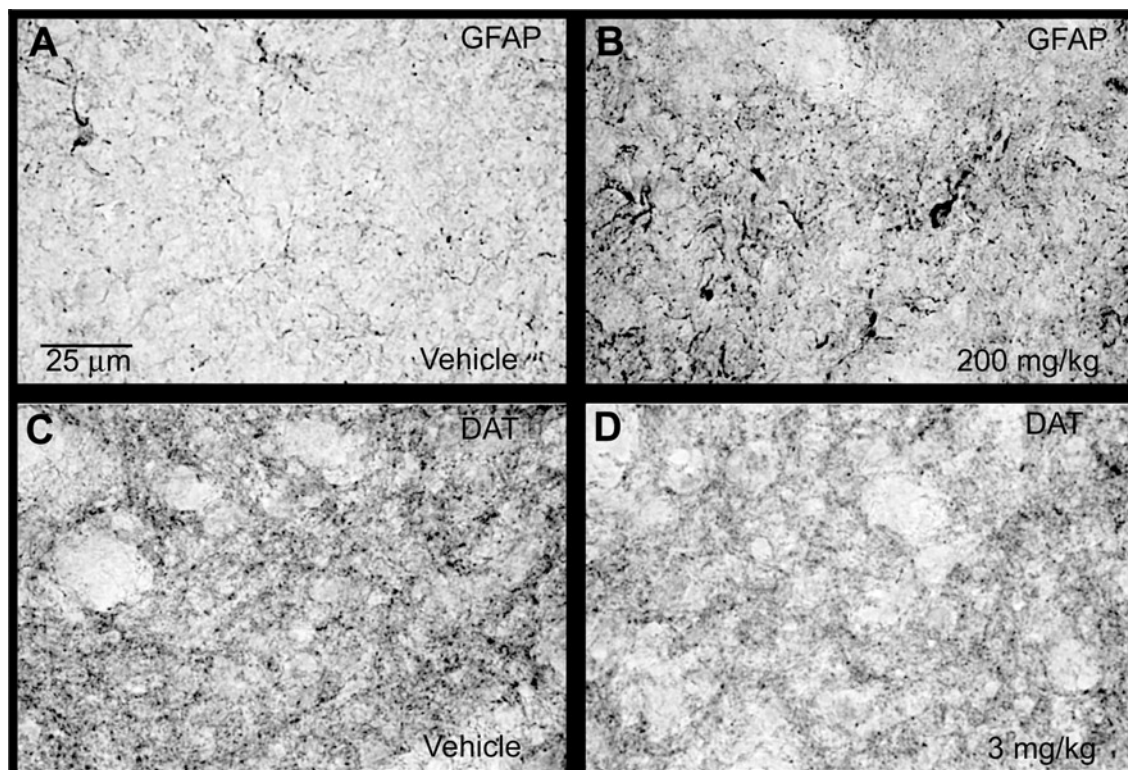


FIGURE 5

High magnification images illustrating the two significant changes in immunoreactivity reported in this experiment. The increase in GFAP immunoreactivity observed at the 200-mg/kg dose of permethrin can be seen by comparing *A* and *B*, whereas the decrease in DAT immunoreactivity is apparent by comparing *C* and *D*. All photographs were made from the dorsolateral sample field, at the same magnification and level of illumination. The quantitative differences in immunoreactivity between the photographs of matched sections from treated and vehicle controls are of the same magnitude as the mean changes in immunoreactivity reported in the text.

(Karen et al. 2001), but can change the amount of DAT immunoreactive protein within the caudate-putamen. Furthermore, high doses of permethrin, which also modify the kinetics of dopamine uptake, can induce glial responses in the caudate-putamen, which have been associated with neuronal tissue damage. The lowest doses at which such changes are observed are more than two orders of magnitude lower than the LD_{50} reported for IP administration of the commercial formulation of permethrin known as Ambush (Williamson et al. 1989).

Although low doses of permethrin can affect both dopamine uptake and the amount of DAT immunoreactive protein in components of the dopaminergic nigrostriatal pathway, the effects are in different directions. A 1.5-mg/kg dose of permethrin has been reported to produce an increase in maximal dopamine uptake in striatal synaptosomes (Karen et al. 2001), whereas in the present experiment, 3.0 mg/kg produced a decrease in DAT-immunoreactive neuropil within the caudate-putamen. These two findings would be concordant if an increase in the efficiency of DAT transport overcompensated for a decrease in the amount of DAT protein. However, Karen et al. (2001) failed to find a

change in the K_M for synaptosomal dopamine uptake, arguing against increased DAT efficiency.

Alternative explanations for the difference in the direction of the two findings noted above relate to differences in the nature of the tissue samples analyzed. For example, in the present immunohistochemical study, anatomical landmarks within transverse forebrain sections were used to select analysis fields within the caudate-putamen, the region of the striatum containing the vast majority of dopaminergic afferents from the substantia nigra (Heimer, Zahm, and Alheid 1995). Furthermore, each of these analysis fields was only on the order of $3070 \mu m^2$. The kinetic data of Karen et al. (2001) was gathered in a synaptosomal preparation made from the entire dissected striatum. This sample may have included varying amounts of tissue from striatal regions outside the caudate-putamen, such as the nucleus accumbens, or nonstriatal regions, such as the globus pallidus, both of which are physically contiguous with the caudate-putamen (Franklin and Paxinos 1997). Therefore, the present study analyzed changes in a smaller sample of tissue, from a more topographically restricted region of the striatum; the

region that is most closely associated with degenerative changes in PD (Kish, Shannak, and Hornykiewicz 1988; Graybiel, Hirsch, and Agid 1990; Miller et al. 1997). This suggests the possibility that different topographic subregions within the striatum may be differentially affected by permethrin exposure. Indeed, it has been shown that portions of the dopaminergic input to the ventral and medial striatum are more resistant to the neurodegenerative effects of MPTP and rotenone compared with the dopaminergic input to the more dorsolateral striatal region containing the caudate-putamen (Gerhardt et al. 1985; Mizukawa, Sora, and Ogawa 1990; Betarbet et al. 2000). In addition, using a whole striatal preparation of mouse synaptosomal membranes, it has recently been reported that low systemic doses of permethrin increased the amount of DAT protein as revealed by Western blot analysis (Bloomquist et al. 2002).

Although the sampling within the present experiment was more restricted with regard to striatal topography, the immunohistochemical analysis most likely surveyed a more extensive portion of individual nigrostriatal neuronal morphology compared with analysis of synaptosomes. Synaptosomal preparations are designed to isolate the synaptic bouton and sometimes contain a portion of the immediate postsynaptic membrane (Webster 2001). Alternatively, any DAT-containing portion of a neuron that lies near the surface of the striatal tissue section is available for labeling by the immunohistochemical procedure. Electron microscopic immunohistochemical studies of the dorsolateral striatum and substantia nigra pars compacta have reported that DAT-immunoreactive protein can be found not only at the plasma membranes of synaptic boutons, but at the membranes of axonal segments lying between the boutons, as well as at somal and dendritic membranes of dopaminergic neurons (Nirenberg et al. 1996; Hersch et al. 1997). Because interbouton axonal segments course through the caudate-putamen, immunohistochemical procedures should label axon-associated DAT, in addition to bouton-associated DAT. Synaptosomal preparations would be expected to primarily sample bouton-associated DAT, because these structures do not contain axonal membrane, and any portion derived from the membrane of a postsynaptic striatal neuron would be almost exclusively non-dopaminergic. Therefore, the regional intraneuronal population of DATs examined in the present study may have differed from that examined in studies using synaptosomal preparations from whole striatum (Karen et al. 2001; Bloomquist et al. 2002). In some dopaminergic neurons, there is a functional differentiation between DATs located in different regions of the cell. For example, dendritic DATs in the substantia nigra are capable of releasing dopamine, through reverse transport, under normal physiological conditions (Falkenburger, Barstow, and Mintz 2001). This potential for functional differentiation between DATs in different parts of the neuron, combined with a probable difference in intraneuronal populations of DATs examined, could also account for the difference in direction of the kinetic data of Karen et al. (2001) and the present immunohistochemical study.

As noted earlier in this article, a decrease in maximal dopamine uptake (V_{max}) has been reported for striatal synaptosomes following higher doses of permethrin (25 or 200 mg/kg) (Karen et al. 2001). This suggested that these doses may induce significant death of dopaminergic striatal terminals. The dopamine synthesizing enzyme TH is commonly used as an immunohistochemical marker to identify the presence of dopaminergic neuropil within the striatum (e.g., Gerhardt et al. 1985; Nirenberg et al. 1996, 1997; Ho and Blum 1998; Brooks et al. 1999; Canudas et al. 2000; Betarbet et al. 2000). Although this enzyme is also critical for synthesis of norepinephrine and epinephrine, there is an insignificant presence of neurons that contain these transmitters within the striatum (Aston-Jones, Shipley, and Grzanna 1995; Saper 2000). Therefore, in the "high-dose" portion of the present work, TH immunoreactivity was used as an additional marker for dopaminergic terminal death within the striatum because the digital image analysis, by itself, cannot distinguish between a reduction in DAT-immunoreactive protein alone and the disappearance of DAT-containing terminals within the striatum. It was possible that the DAT protein could be down-regulated without accompanying degeneration of dopaminergic striatal afferents. The lack of change in the amount of DAT- and TH-immunoreactive neuropils, at doses of 200 mg/kg of permethrin, fails to support the notion that dopaminergic terminal death, within the caudate-putamen, is a substrate for the previously reported decrease in synaptosomal V_{max} . The absence of a decrease in DAT and TH immunoreactivities is consistent with a previous report that this dose did not change striatal levels of dopamine (Karen et al. 2001), which also argues against terminal death being responsible for the decrease in V_{max} .

Increased GFAP, an intermediate filament protein of astrocytes, has been shown to be a marker of the onset, degree, and locus of neuropathology (Norton et al. 1992; O'Callaghan 1993; O'Callaghan, Jensen, and Miller 1995; Eng, Ghimikar, and Lee 2000). Such increases correspond not only to sites where there is easily identifiable loss of neuropil, such as the striatum following MPTP or 1-methyl-4-phenylpyridinium (MPP⁺) exposure (Schneider and Denaro 1988; Francis et al. 1995; Canudas et al. 2000; Akari et al. 2001), but where there is histological evidence of tissue damage that is more subtle than frank degeneration (O'Callaghan, Jensen, and Miller 1995). Although the lack of change in TH and DAT immunoreactivities within the caudate-putamen argue against the death of dopaminergic nigrostriatal terminals in the "high-dose" condition, the corresponding increase in GFAP immunoreactivity suggests the possibility of axon-terminal damage that has not advanced to frank degeneration. Such damage may be sufficient to render the DAT inoperative, which would be consistent with the decrease in maximal dopamine uptake reported by Karen et al. (2001). Furthermore, nonfatal damage to the lipid bilayer of striatal synaptic boutons could permit non-transporter-mediated leakage of dopamine from nigrostriatal terminals. This could also account for a decrease in synaptosomal V_{max} , while leaving DAT and TH

immunoreactivities unchanged. Again, such comparisons with the aforementioned synaptosomal study should be tempered by the possibility of a topographic difference in the striatal tissue samples examined.

It should be noted that the observed increases in GFAP immunoreactivity could also represent damage to glial cells, because such increases have been reported following damage to oligodendrocytes and in astrocytes that survive direct astrocytic insult (Smith, Somera, and Eng 1983; Takada, Li, and Hattori 1990). Although the increase in GFAP immunoreactivity may not be solely attributable to neuronal insult, it does appear to be regionally restricted as opposed to a generalized response to introduction of permethrin into the body. This is suggested by the absence of a significant change in GFAP immunoreactivity within the corpus callosum. Finally, there is evidence suggesting that MPTP-induced damage to the dopaminergic nigrostriatal pathway is capable of inducing an up-regulation of TH protein and mRNA (Greenwood et al. 1991; Bezard et al. 2000; Rothblat, Schroeder, and Schneider 2001), as well as sprouting (Song and Haber 2000), in surviving nigrostriatal neurons, across a variable time frame. If a high dose of permethrin were capable of a similar effect, a reduction in TH immunoreactivity, due to the death of nigrostriatal terminals, could be masked, while still inducing an increase in GFAP. This scenario cannot be ruled out. However, such compensatory effects within the nigrostriatal pathway may be dependent on the type of damage since lesions of this pathway, induced with 6-hydroxydopamine, produced a down-regulation of TH mRNA in surviving nigral neurons (Sherman and Moody 1995).

As noted in Materials and Methods, brain sections were not prepared for TH and GFAP staining in the "low-dose" condition because previous work provided no reason to suspect death or damage of striatal neuropil following the low-dose regimen (see Karen et al. 2001). Therefore, degeneration of dopaminergic terminals within the striatum, and hence, a reduction in their numbers, could not be definitively ruled out as a possible substrate for the decrease in DAT immunoreactive protein observed at the 3.0-mg/kg dose of permethrin. As noted earlier, a decrease in DAT was not seen at the next higher dose of permethrin used in this experiment (200 mg/kg). In light of this, it should be noted that analyses of dopamine uptake into striatal synaptosomes have revealed significant changes at a very restricted range of low doses, without changes in the same direction for several higher doses. This can be seen for exposure to both permethrin and the organochlorine heptachlor (Karen et al. 2001; Bloomquist et al. 2002).

The present immunohistochemical data, combined with previous kinetic data, suggest that pyrethroid insecticides are capable of altering the normal functional status of the nigrostriatal pathway. The tissue damage suggested by increased GFAP within the caudate-putamen has obvious implications for the putative connection between pesticide exposure and PD. However, alterations of DAT protein within this region may also represent an important substrate modulating the onset and severity

of Parkinson's disease, because this transporter is the means by which Parkinson's disease-like inducing chemicals (e.g. MPP⁺) can enter dopaminergic neurons (Mayer, Kindt, and Heikkila 1986; Gainetdinov et al. 1997; Bezard et al. 1999). Such changes in the integrity of the DAT may be important for future investigations of the role of synergistic interactions between environmental chemicals in the development of PD.

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Neurotoxicity in murine striatal dopaminergic pathways following co-application of permethrin, chlorpyrifos, and MPTP

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Abstract

The neurotoxic action of permethrin and chlorpyrifos on striatal dopaminergic pathways was investigated in C57BL/6 mice. Technical permethrin (50/50 ratio of *cis* and *trans* isomers, 200 mg/kg) and/or chlorpyrifos (75 mg/kg) were administered three times over a two-week period, with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP, 20 mg/kg) given on day one. Alterations in expression of α -synuclein, dopamine transporter (DAT), and tyrosine hydroxylase (TH) were analyzed at 1 or 28 days post-treatment. MPTP alone produced a long-lasting lesion in striatal dopaminergic pathways, with a depression of TH and DAT protein at both post-treatment times. Chlorpyrifos or permethrin alone had no effect on TH or DAT expression levels. No greater effect on protein expression was observed in mice treated with both MPTP and insecticides at 1 day post-treatment. However, by day 28 a significant reduction ($p < 0.05$) of TH and DAT was observed in the mice treated with MPTP, permethrin, and chlorpyrifos, compared with the mice given MPTP alone. Significant alteration ($p < 0.05$) of α -synuclein expression by MPTP (45% decrease) and permethrin (20% increase) occurred at 1 day post-treatment, but reverted to control levels by day 28. Parallel experiments with pure *cis* or *trans* isomers of permethrin (100 mg/kg), showed that each isomer caused about half the up-regulation of α -synuclein. These findings demonstrate that the co-application of pyrethroid or organophosphorus insecticides enhance the neurotoxicity of MPTP in C57BL/6 mice, and that a slowly developing neurotoxicity may occur after termination of high-dose exposure.

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Keywords: Parkinson's disease; Insecticide; Striatum; Dopamine transporter; Tyrosine hydroxylase; α -Synuclein; Synaptophysin

1. Introduction

PM and CPF² are two widely used insecticides in the pyrethroid and organophosphorus compound classes. PM and CPF exposure may be contributory to Gulf War syndrome, since approximately 30,000 veterans complained of neurological symptoms after exposure to multiple chemicals, in

particular PB, DEET, PM, and CPF [1–4]. Animal experiments indicated that relatively high doses of PB, DEET, PM, or CPF alone caused minimal neurotoxicity, while co-exposure to the same doses of these compounds significantly increased the severity of motor deficits [1]. Thus, mixed exposures to neurotoxicants may have unforeseen effects.

Epidemiological studies [5] have implicated organophosphorus insecticide exposure as a possible contributory factor in PD. Moreover, previous studies revealed that PM and CPF affect the nigro-striatal system, the primary brain pathway lesioned in PD [6]. PM enhanced dopamine uptake [7] and increased DAT expression [8] at the dose of 1.5 mg/kg to C57BL/6 mice. Similar effects of other pyrethroid insecticides on striatal dopaminergic neurochemistry, such as deltamethrin, has also been documented [9,10]. At high doses, both PM (200 mg/kg) and CPF (100 mg/kg) significantly reduce maximal [³H]dopamine uptake and caused a decrease

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² Abbreviations used: CPF, chlorpyrifos; DAT, dopamine transporter; DEET, *N,N*-diethyltoluamide; DOPAC, dihydroxyphenylacetic acid; DTT, dithiothreitol; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; MTG, methoxytriglycol; PB, pyridostigmine bromide; PD, Parkinson's disease; PM, permethrin; TEMED, *N,N,N,N'*-tetramethylethylenediamine.

in locomotor activity [7]. In addition, PM treatment up-regulated α -synuclein [8], an important component of Lewy bodies, proteinaceous tangles found in PD [11]. Taken together, these findings suggest that dopaminergic neurotransmission is significantly affected by exposure to pyrethroid and organophosphorus insecticides.

The present study is focused on the neurotoxic actions of PM (200 mg/kg) and CPF (75 mg/kg) alone or in combination, and their interactions with the well-established Parkinsonian neurotoxin, MPTP. This approach provides an opportunity to assess the ability of insecticides to intensify the development of PD, as modeled by exposure to MPTP. Western blot analyses were conducted to evaluate the alteration of biomarkers specific to dopaminergic pathways in striatum, including the expression of DAT and TH proteins. Reduction of DAT and TH indicates dopaminergic nerve terminal injury, because DAT and TH are mainly expressed in dopaminergic terminals and great reductions in DAT and TH expression occurs in the brains of MPTP-lesioned mice [12,13] or human Parkinsonian patients [14]. Moreover, previous studies (e.g., Tillerson et al. [13]) showed that Western blot measures of DAT and TH expression are appropriate and selective markers for striatal dopaminergic neurotoxicity. We also evaluated expression of α -synuclein, as well as synaptophysin; the latter an abundant synaptic protein widely expressed in neurons [15], as a check for specificity of effect.

2. Materials and methods

2.1. Chemicals

Technical PM (a 50/50 mixture of 1-*R,S-cis* and 1-*R,S-trans* isomers, Fig. 1) was purchased from Sigma–Aldrich

GMBH. CPF (99%) and the resolved *cis* (99%) and *trans* (94%) isomers of PM were obtained from ChemService (West Chester, PA). The MPTP used originated from Research Biochemicals International, Natick, MA. Buffer components were purchased from Fisher Chemicals, Fair Lawn, NJ. Bio-Rad (Hercules, CA) was the commercial source for 30% acrylamide, ammonium persulfate, SDS, and TEMED. Rat monoclonal anti-DAT, mouse monoclonal anti-TH, and anti-synaptophysin primary antibodies were purchased from Chemicon International (Temecula, CA). Mouse monoclonal anti- α -synuclein primary antibody was obtained from Biodesign International (Saco, ME). The secondary peroxidase-linked antibody was from Sigma Chemical (St. Louis, MO). ECL Western blotting detection system RPN 2108 and ECL Hyperfilm were purchased from Amersham-Pharmacia Biotech (Buckinghamshire, UK).

2.2. Animals and treatments

C57BL/6 retired breeder male mice (Harlan–Sprague–Dawley, Dublin, VA) were used for all the experiments, which were approved by the Virginia Tech Animal Care and Use Committee. Mice aged 7–9 months, weighing from 36 to 42 g were used because this age range gives a consistent dopamine depletion following MPTP treatment [16]. Mice were randomly assigned to treatment groups according to weight, with typically five mice in each group. The mean weight of all treatment groups was not significantly different. High, but sublethal doses of PM (200 mg/kg) and CPF (75 mg/kg) were selected in the present study based on our previous studies of these two insecticides in C57BL/6 mice [7]. A single dose of 20 mg/kg MPTP was chosen because this dose caused approximately

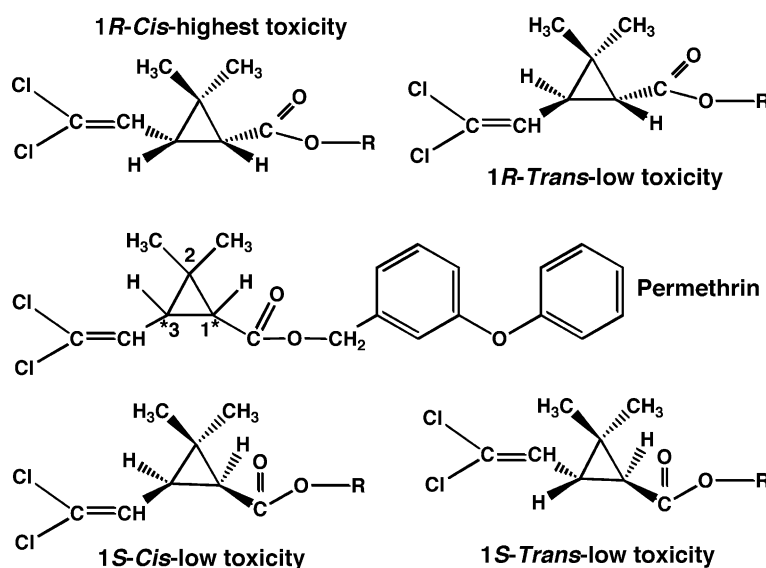


Fig. 1. Chemical structures of the PM isomers discussed in the text. In the middle generic structure, chiral carbon atoms 1 and 3 in the cyclopropane ring are indicated by asterisks. Consensus pyrethroid nomenclature defines the *R* or *S* stereochemistry at carbon 1, with *cis* or *trans* designations defined by projections relative to the cyclopropane ring, so as to consistently define biologically active conformations, as opposed to formal stereoisomeric nomenclature, as described by [34]. Relative mammalian toxicities for PM isomers, are as given by Soderlund et al. [33].

30% dopamine depletion under a similar exposure regimen [16]. PM carried in MTG or CPF carried in corn oil was administered three times over a 2-week period, with MPTP dissolved in saline given once on the first day of the treatment period (Fig. 2). The single dose of MPTP was given before the first insecticide treatment to ensure that insecticide was not interfering with the bioactivation of MPTP, since MPTP is completely metabolized to MPP⁺, the actual neurotoxin, within 2 h after ip injection in C57BL/6 mice [17]. MPTP-treated mice (Fig. 2) also received treatment with equal volumes of vehicle used with the insecticides. Both MPTP and PM were administered by ip injection, while CPF was given by sc injection. Different injection routes were used in order to avoid multiple ip injections on the same day, as much as possible. Control mice received the same amount of vehicle. Mice were sacrificed at 24 h or 28 days after the last treatment, and brain striatal tissue was collected for immunoblotting assays.

2.3. Western blot analysis

Striatal synaptosomes were prepared according to the methods described by Wright et al. [18]. In brief, striatal tissue was homogenized in 4 ml ice-cold sucrose buffer (0.32 M sucrose, 4.2 mM Hepes; pH 7.4). The homogenates were centrifuged at 1000g for 15 min at 4 °C. The supernatants were collected and re-centrifuged at 10,000g for 15 min at 4 °C. The resulting pellets were resuspended in KRH buffer (NaCl, 118 mM; KCl, 4.8 mM; CaCl₂, 2.5 mM; MgSO₄, 1.2 mM; Hepes, 20 mM, pH 7.5), stored at –70 °C. Prior to Western blot assay, the frozen tissue was thawed, and the protein concentration of the tissue samples was determined by Bradford assay [19]. The tissue samples were then mixed with 25% of sample buffer (Tris, 50 mM; SDS, 2%; DTT, 100 mM; bromphenol blue, 0.001%, pH 6.8) and heated at 90 °C for 5 min. A 10% SDS gel was used for the analysis of DAT, TH, and synaptophysin, with a 15% SDS gel for α -synuclein. A sample of 10 μ g protein was loaded for DAT, 5 μ g protein was loaded for TH and α -synuclein, and 2 μ g protein was loaded for synaptophysin. Uniform protein loading was ensured by

adjusting tissue samples to the same protein concentration before loading. SDS gels were run according to the method of Towbin et al. [20]. The resulting protein bands were transferred to a nitrocellulose membrane, and equal loading was re-confirmed by Ponceau-S staining of each Western blot lane on the membrane. The Ponceau-S was then removed by washing with TBST buffer (Tris, 50 mM; NaCl, 138 mM; KCl, 2.7 mM; Tween 20, 0.05%, pH 8.0). Membranes were incubated with nonfat milk (5%) at room temperature for 1 h, followed by incubation with the appropriate monoclonal primary antibody at 4 °C. After an overnight incubation, the membrane was washed with TBST buffer, followed by one-hour incubation with peroxidase-linked secondary antibody at room temperature. After three 5-min washes with TBST buffer, the membrane was developed using the ECL Amersham Chemiluminescence kit and exposed to ECL Hyperfilm for an appropriate time. After washing with tap water and air drying, the protein content on the film was quantitated using a Kodak EDAS 290 image analysis system.

2.4. Permethrin isomer study

The isomeric specificity of permethrin in its effects on α -synuclein expression was tested in C57BL/6 mice (7–9 months). Technical PM (a 50/50 mixture of *cis/trans* isomers at 200 mg/kg), or 100 mg/kg of pure *cis* or *trans* isomer was given to mice under standard conditions as previously described (Fig. 2). α -Synuclein levels in striata were determined by Western blot at 1 day post-treatment, as described above.

2.5. Statistical analysis

Data were analyzed using InStat (Graph-Pad Software, San Diego, CA), and in consultation with the Virginia Tech Statistical Consulting Center. Statistical analysis was focused on treatment effect within each post-treatment time point. Significance was determined by one-way ANOVA, followed by Student–Newman–Keuls post hoc test if significance ($p < 0.05$) was observed.

3. Results

Densitometry analysis of TH showed that a single dose of 20 mg/kg MPTP decreased TH expression in striatum by 46%, as shown in Fig. 3A. Neither CPF (75 mg/kg) nor PM (200 mg/kg) changed the expression of TH by itself, but the CPF + PM group did display a small, but statistically significant reduction in TH staining at 1 day post-treatment (Fig. 3A). Co-exposure to MPTP with CPF or PM caused a reduction in TH expression of about the same magnitude as that of MPTP by itself, but less depression of TH in the triple treatment group at 1 day post-treatment compared to MPTP alone or MPTP given with PM/CPF (Fig. 3A). By 28 days post-treatment, the following pattern of effects had emerged. There was still significant TH reduction in

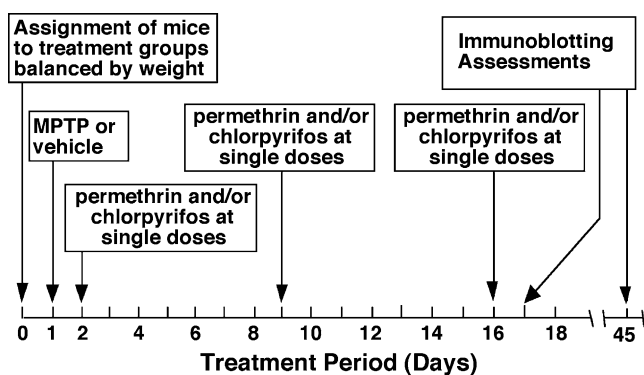


Fig. 2. Timeline for treatment of insecticides with and without MPTP, followed by biomarker assessments at 1 and 28 days post-treatment.

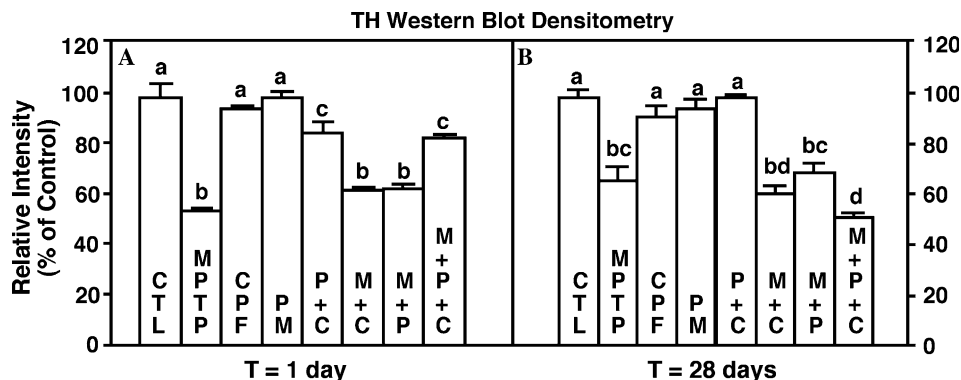


Fig. 3. Densitometry analysis of antibody-labeled TH in Western blots of striatal tissue taken from C57BL/6 mice at 1 day (A) and 28 days (B) post-treatment. Treatment abbreviations are CTL (control, vehicle), MPTP (MPTP, 20 mg/kg), CPF (CPF, 75 mg/kg), PM (PM, 200 mg/kg), P + C (PM + CPF), M + C (MPTP + CPF), M + P (MPTP + PM), and M + P + C (MPTP + PM + PPF). For each post-treatment time, letters indicate results of ANOVA followed by Student–Newman–Keuls post hoc test, where bars not labeled by the same letter are significantly different ($p < 0.05$).

the MPTP group (66% of control), but little effect of either insecticide alone or any enhanced effect by combination treatment, except for the triple treatment group (Fig. 3B). In this latter case, the extent of TH depletion was enhanced to 51% of control. Representative Western blots of the effects of MPTP, and MPTP + insecticides on TH expression are shown in Fig. 4.

Densitometry analysis of Western blots showed effects on DAT expression parallel to those of TH. A single dose of 20 mg/kg MPTP decreased DAT expression in striatum 24%, as shown in Fig. 5A. Neither insecticide alone nor in combination changed the expression of the DAT, relative to that of MPTP, 1 day post-treatment. Western blot analysis at 28 days post-treatment (Fig. 5B) showed that DAT expression remained at a slightly reduced level in MPTP

(87% of control) or MPTP + PM/CPF treatment groups, but a significantly greater reduction in staining was observed in the triple treatment group (69% of control). There was no effect on DAT expression by the insecticides alone, or in combination (CPF + PM), 28 days post-treatment (Fig. 5B). Representative Western blots of the effects of MPTP, and MPTP + insecticides on DAT expression are shown in Fig. 4.

At 24 h post-treatment, Western blot analysis showed that a single dose of 20 mg/kg MPTP to mice decreased subsequent α -synuclein expression by 45% compared to control (Fig. 6A). CPF had no influence on α -synuclein expression by itself, whereas PM alone induced an elevation of α -synuclein, about 20% above control. Combining PM and CPF caused a 28% depression of α -synuclein pro-

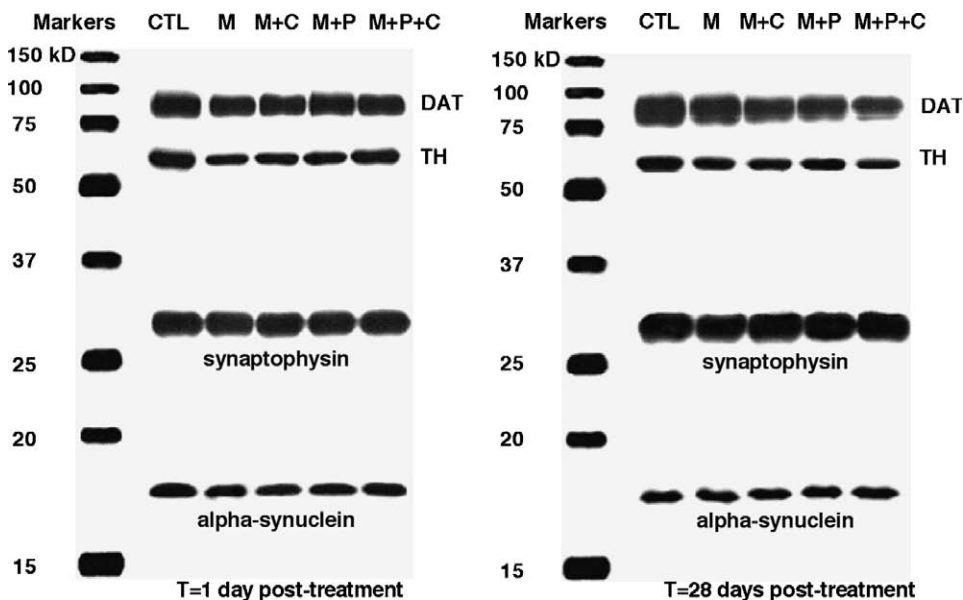


Fig. 4. Representative Western blots of DAT (80 kDa), TH (60 kDa), synaptophysin (34 kDa), and α -synuclein (19 kDa). Monoclonal antibodies were used to identify appropriate molecular weight bands in each blot. The molecular weight for the band of α -synuclein was also identified by using pure α -synuclein (19 kDa) as positive control (not shown). The figure is composed of several scanned Western blots films, with background subtracted using Adobe Photoshop (Adobe Systems, San Jose, CA). Treatments are labeled above the bands (CTL, control; M, MPTP; C, CPF; P, PM). The Western blots of striatal tissue were taken from C57BL/6 mice at 1 day (left) and 28 days (right) post-treatment.

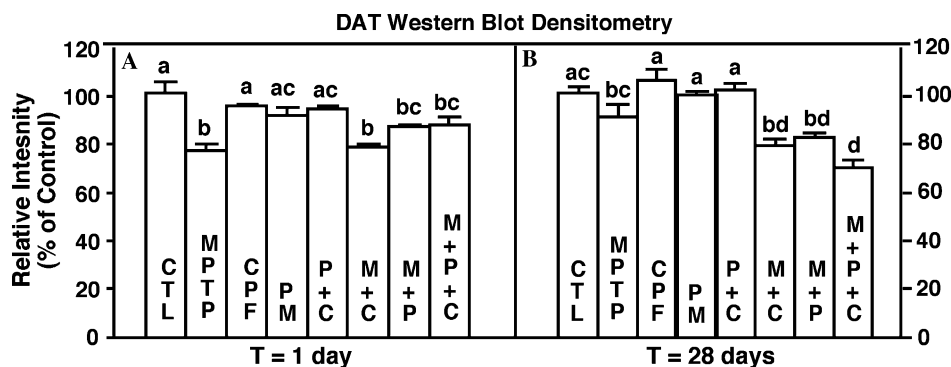


Fig. 5. Densitometry analysis of antibody-labeled DAT in Western blots of striatal tissue taken from C57BL/6 mice at 1 day (A) and 28 days (B) post-treatment, with doses and abbreviations as defined in Fig. 3. For each post-treatment time, letters indicate results of ANOVA followed by Student–Newman–Keuls post hoc test, where bars not labeled by the same letter are significantly different ($p < 0.05$).

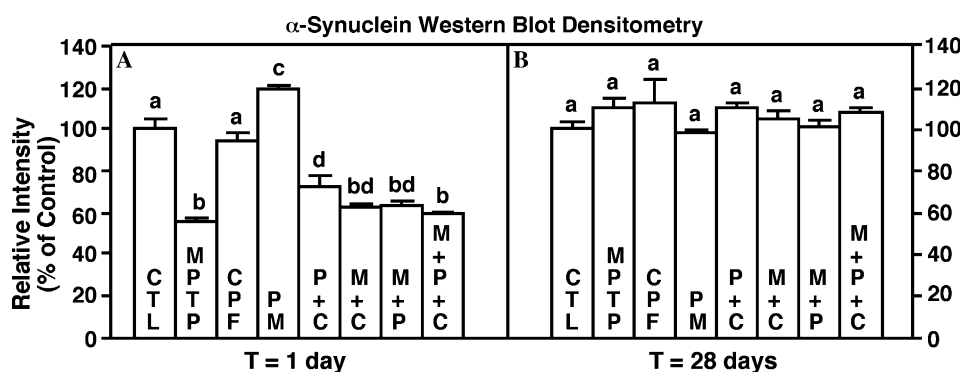


Fig. 6. Densitometry analysis of antibody-labeled α -synuclein in Western blots of striatal tissue taken from C57BL/6 mice at 1 day (A) and 28 days (B) post-treatment, with doses and abbreviations as defined in Fig. 3. For each post-treatment time, letters indicate results of ANOVA followed by Student–Newman–Keuls post hoc test, where bars not labeled by the same letter are significantly different ($p < 0.05$).

tein. Co-exposure to MPTP with CPF or/and PM changed α -synuclein expression in a pattern and magnitude similar to that of MPTP itself (Fig. 6A). The alteration of α -synuclein biomarker is temporary, since α -synuclein expression returned to control levels for all treatments 28 days later (Fig. 6B). Representative Western blots of the effects of MPTP, and MPTP + insecticides on α -synuclein expression are shown in Fig. 4.

Data on the stereospecificity of PM isomers were collected 24 h after the last treatment. These studies showed that technical PM significantly increased α -synuclein expression by 19% (Fig. 7), similar to that observed in other experiments (Fig. 6A). Treatment with the purified *cis* and *trans* isomers showed increases of 11 and 13%, respectively, for a roughly additive effect (Fig. 7, bottom).

There was no statistically significant change in synaptophysin expression across any of the treatment groups or post-treatment times, as evident in Western blots (Fig. 4) or the resulting densitometry analysis (Figs. 8A and B).

4. Discussion

The MPTP-treated C57BL/6 mouse is a well-established animal model of PD, showing a selective destruction of nigrostriatal dopamine neurons, and associated neuro-

chemical and neuropathological changes [21,22]. The levels of both DAT and TH protein were significantly reduced by a single dose of 20 mg/kg MPTP at 1 day after the termination of treatments, and this reduction remained at a similar level 28 days later. This conclusion agrees with previous reports, which suggested that older C57BL/6 mice, such as those used here, do not recover from the effects of MPTP because of an age-related decline in the potential for recovery [23–25]. CPF or PM alone did not change the expression of DAT and TH protein in striatum 1 day post-treatment, which agrees with our previous finding that high doses of CPF (100 mg/kg) and PM (200 mg/kg) did not decrease dopamine content in striata [7]. A combined treatment with MPTP and CPF/PM did not lead to further reduction of TH or DAT protein compared with MPTP-treated mice. Surprisingly, there was increased expression of TH protein in the triple treatment group at 1 day post-treatment, which may reflect a stimulatory effect in the remaining dopaminergic neural terminals as a response to the presence of the insecticides.

In the present study, neither CPF nor PM synergized the toxicity of MPTP in binary treatments at either post-treatment analysis time. A previous study did observe enhancement of MPTP-induced neurotoxicity when diisopropylfluorophosphate was given the day before MPTP

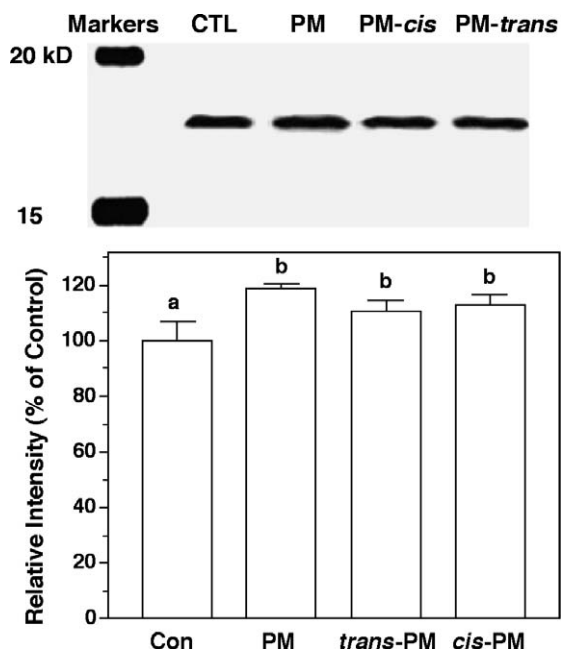


Fig. 7. Isomeric dependence of increased α -synuclein expression by PM. (Top) Representative Western blots of α -synuclein. (Bottom) Densitometry analysis of antibody-labeled α -synuclein was performed on Western blots of striatal tissue taken from C57BL/6 mice treated with 200 mg/kg of a 50/50 mixture of *cis/trans* technical permethrin (PM), or 100 mg/kg doses of pure *cis* (*cis*-PM) or *trans* (*trans*-PM) isomer. Letters indicate results of a paired ANOVA followed by Student–Newman–Keuls post hoc test, where bars not labeled by the same letter are significantly different ($p < 0.05$).

treatment, and biomarkers (loss of TH activity, dopamine, and DOPAC) were assessed 30 days post-treatment [26]. The lack of enhancing effect by CPF in the present study could be due to differences in the timing of the treatments, dose, etc.

A small, but significant enhancement of MPTP toxicity was shown in the triple treatment group at 28 days post-treatment. This phenomenon indicates a delayed interaction between MPTP with CPF and PM, consistent with greater dopaminergic neurotoxicity. Apparently, any abili-

ty of insecticides to stimulate TH expression, as hypothesized above, is lost by 28 days post-treatment. Synaptophysin expression was used to test whether the apparent dopaminergic toxicity suggested by the reduction in TH and DAT expression was specific, or represented a more general neurotoxic insult. The lack of changes in synaptophysin expression indicated that widespread synaptic injury was not responsible for the observed effects. In addition, synaptophysin served as an internal control for nervous tissue labeling, and the absence of synaptophysin alteration confirmed the uniform loading of Western blots across the treatment groups.

The fact that high doses of PM or CPF reduce mitochondrial integrity might be a contributory factor to the effects observed with MPTP. Our previous work showed that there was a statistically significant (ca. 13%) decrease in mitochondrial dehydrogenase activity in the mice given 100 mg/kg CPF or ≥ 13 mg/kg PM [7]. Along these lines, Gassner et al. [27] reported that micromolar concentrations of permethrin inhibited mitochondrial complex I activity in isolated rat liver mitochondria. Given this mitochondrial-directed action of PM, toxic effects reminiscent of MPTP treatment might be expected. We can only surmise that PM did not attain sufficient levels *in vivo* over the required period of time in order to cause neurotoxicity via this mechanism, even though it was tested at a relatively high dose (200 mg/kg) and given three times. However, even a small effect on mitochondrial integrity, either direct or indirect, could increase MPTP toxicity.

The underlying mechanism behind the enhanced toxicity we observed is unclear, but pharmacokinetic interactions seem an unlikely source. Increased bioactivation of MPTP is obviated by our treatment regime, although reduced clearance of MPP⁺ from the brain by insecticides remains to be investigated. Reduced metabolism of PM or CPF through interference with carboxylesterase or P450-mediated detoxication remains a possibility, but in the present study, no indication of greater toxicity was observed following the combination of these two insecticides, except a slight reduction of TH at 1 day post-treatment.

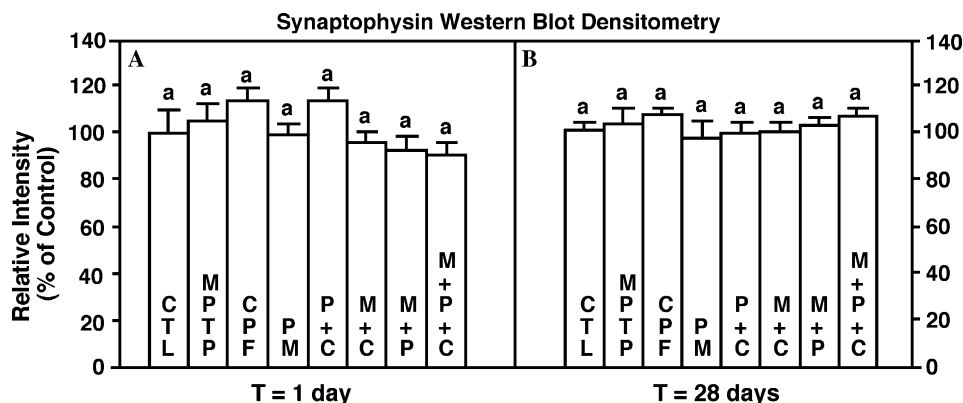


Fig. 8. Densitometry analysis of antibody-labeled synaptophysin Western blots of striatal tissue taken from C57BL/6 mice at 1 day (A) and 28 days (B) post-treatment, with doses and abbreviations as defined in Fig. 3. For each post-treatment time, letters indicate results of ANOVA followed by Student–Newman–Keuls post hoc test, where bars not labeled by the same letter are significantly different ($p < 0.05$).

Western blot analysis revealed a significant decrease in α -synuclein expression two weeks after a single MPTP injection. This finding is consistent with recent work showing that repeated 20 mg/kg doses of MPTP to C57BL/6 mice resulted in a reduction in α -synuclein mRNA expression 24 h after the last treatment [28]. However, other investigations reported an up-regulation of α -synuclein in substantia nigra dopaminergic neurons [29] or an absence of α -synuclein alteration [30], in striatum following MPTP administration. These discrepancies may be due to differences in treatment regime or analysis parameters. The reduction of α -synuclein after MPTP injection observed in the present study correlates with the reductions in TH and DAT expression, which are usually interpreted as neurotoxicity. However, the recovery of α -synuclein expression to control levels at 28 days post-treatment is not consistent with such an interpretation. A temporary up-regulation of α -synuclein was found in PM-treated mice at 1 day post-treatment, which confirmed our previous finding that PM administration in this exposure paradigm (Fig. 2) led to a significant, but reversible augmentation of α -synuclein expression, albeit at lower doses [8]. A similar reversible increase in α -synuclein expression was observed in C57BL/6 mice treated with paraquat, that also resulted in formation of protein aggregates [31]. While we have not yet looked for such aggregates in PM-treated mice, immunohistochemical analysis of mice treated with 200 mg/kg PM in the same exposure paradigm we used here found no change in TH or DAT staining, but an increase in glial fibrillary acidic protein, a marker for incipient neuropathology, at 1 day post-treatment [32].

Studies on the isomer-dependence of α -synuclein expression found that both *cis* and *trans* isomers contributed to this response, whereas we expected 100% of the response to be from the *cis* isomer. Recall that only the 1 *R-cis* isomer of PM (Fig. 1) has significant lethal activity in mice, principally via an effect on voltage-sensitive sodium channels of nerve membrane [33]. The corresponding 1 *S-cis* and both *trans* isomers of PM are without lethal effect in mammals and do not appreciably modify sodium channel function [33]. Nonetheless, the overall effect of technical PM on α -synuclein expression was roughly additive for the *cis* and *trans* isomers (Fig. 7), suggesting other targets are involved, such as voltage-sensitive calcium channels, or various phosphorylation signaling pathways [33]. In contrast, both the *cis* and *trans* isomers of PM are toxic to insects [33], so if the *trans* isomer did not up-regulate α -synuclein, it would have been possible to eliminate this human exposure hazard by using a PM formulation of pure *trans* isomer. Unfortunately, this is not the case.

The overall findings demonstrated that short-term, high-dose exposure to pyrethroid or organophosphorus insecticides slightly enhanced the neurotoxicity of MPTP in C57BL/6 mice, even though they had no significant effects on striatal dopaminergic pathways when given alone. It remains to be determined whether long-term, low-dose exposures will have similar effects.

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Neurotoxicity in Murine Striatal Dopaminergic Pathways Following Long-Term Application of Low Doses of Permethrin and MPTP

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ABSTRACT

Our previous work showed that short-term, high-dose exposure to pyrethroid and organophosphorus insecticides enhanced the neurotoxicity of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) in C57BL/6 mice at 4 weeks post-treatment. In the present study, the long-term effects of low doses of permethrin (PM) and its interaction with MPTP, on striatal dopaminergic pathways was investigated in C57BL/6 mice. In a 3-month exposure study, technical PM (1.5 mg/kg) was administered once per week, with MPTP (20 mg/kg) given once on either the 7th week or the 11th week. In a 6-month exposure study, technical PM (0.8 or 1.5 mg/kg) was administered once per week for 26 weeks, with MPTP (20 mg/kg) given once, 2 weeks before the last PM injection. Alterations in the expression of tyrosine hydroxylase (TH), dopamine transporter (DAT), and α -synuclein proteins were analyzed 1 day after the last PM treatment using western blot assay. Low doses of PM had no significant neurotoxic effect on striatal dopaminergic pathway by itself, whereas MPTP alone significantly reduced the expression of TH and DAT protein in striatal synaptosomes. No greater effect on protein expression was observed in mice treated with both MPTP and PM, at either 3-month or 6-month exposure studies. Instead, treatment with 1.5 mg/kg PM significantly antagonized the toxic effect of MPTP on TH and DAT expression ($p < 0.05$). There was no significant alteration of α -synuclein expression following any exposure to PM and/or MPTP. [³H]Tetrabenazine binding assay for expression of vesicular monoamine transporter (VMAT) in the striatum showed no effect by PM, but the reduction in this protein caused by MPTP was attenuated by PM, consistent with other dopaminergic biomarkers. The possible mechanisms underlying the antagonism of MPTP-induced neurotoxicity by low doses of PM is discussed.

Key words: Parkinson's disease, insecticide, striatum, dopamine transporter, vesicular monoamine transporter, tyrosine hydroxylase, α -synuclein

INTRODUCTION

Parkinson's disease (PD) is a neurodegenerative condition characterized by severe damage to dopaminergic neurons of the nigrostriatal system (Sundstrom *et al.*, 1990). The etiology of idiopathic PD is still unknown, but epidemiological studies suggest that exposure to various classes of insecticide contributes to the increased risk of PD development (Hertzman *et al.*, 1990; Jimenez-Jimenez *et al.*, 1992; Rajput *et al.*, 1986). For example, an organophosphorus insecticide exposure was identified as a possible contributory factor in PD (Seidler *et al.* 1996). Postmortem brain analysis found elevated levels of the organochlorine dieldrin in PD patients (Fleming *et al.*, 1994). P

Pyrethroid insecticides are heavily used in agriculture and household for pest control, and PM is impregnated into uniforms of U.S. military service personnel for control of disease carrying insects (Abou-Donia *et al.*, 1996a). Thus, Gulf War veterans may have been exposed to PM, and toxicant mixtures containing PM have 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).

Recent studies have reported effects of pyrethroids on dopamine neurochemistry to evaluate any Parkinsonian hazard from pyrethroid exposure. PM enhanced dopamine uptake (Karen *et al.*, 2001) and increased DAT expression (Gillette and Bloomquist, 2003) at a dose of 1.5 mg/kg (3 injections over 2 weeks) to C57BL/6 mice. Similar effects of PM were observed by Elwan *et al.* (2005). Other pyrethroid insecticides, such as deltamethrin (Doherty *et al.* 1988; Kirby *et al.* 1999), have also been reported to affect striatal dopaminergic neurochemistry. At high doses, PM (200 mg/kg) significantly reduced maximal [³H]dopamine uptake and caused a decrease in locomotor activity (Karen *et al.* 2001). In addition, it was documented that three injections of PM (1.5 mg/kg) over a 2-week period up-regulated α -synuclein protein (Gillette and Bloomquist 2003), an important component of Lewy bodies, the proteinaceous tangles found in PD (Spillantini *et al.* 1997). Short-term exposure (3 injections over 2 weeks) to high doses of PM (200 mg/kg) or CPF (75 mg/kg) significantly enhanced the neurotoxicity of the well-established Parkinsonian neurotoxin MPTP in C57BL/6 mice evaluated 28 days post-treatment, even though they had no significant effects on the striatal dopaminergic pathway when given alone (Kou *et al.*, 2006).

In the present study, the neurotoxic effect of long-term exposure (once per week for 3 months or 6 months) to low doses of PM (0.8 and 1.5 mg/kg), and their interactions with MPTP was investigated in C57BL/6 mice. Western blot analyses were conducted to evaluate the alteration of biomarkers specific to dopaminergic pathways in the striatum, including the expression of DAT and TH proteins. Reduction of DAT and TH indicates dopaminergic nerve terminal injury, because DAT and TH are mainly expressed in dopaminergic terminals and great reductions in DAT and TH expression occur in the brains of MPTP-lesioned mice (Sundstrom *et al.* 1990; Tillerson *et al.* 2002) or human Parkinsonian patients (Innis *et al.* 1993). We also evaluated the expression of α -synuclein, because the up-regulation of α -synuclein was reported in C57BL/6 mice given MPTP (Vila *et al.* 2000) or PM (Gillette and Bloomquist, 2003; Kou *et al.*, 2006).

MATERIALS AND METHODS

Chemicals

Technical PM was purchased from Sigma-Aldrich GMBH. The MPTP used originated from Research Biochemicals International (Natick, MA). Buffer components were purchased from Fisher Chemicals, Fair Lawn, NJ. Bio-Rad (Hercules, CA) was the commercial source for 30% acrylamide, ammonium persulfate, sodium dodecyl sulfate (SDS), and N,N,N,N'-tetra-methyl-ethylenediamine (TEMED). Rat monoclonal anti-DAT and mouse monoclonal anti-TH primary antibodies were purchased from Chemicon International (Temecula, CA). Mouse monoclonal anti- α -synuclein primary antibody was obtained from Biodesign International (Saco, ME). The secondary peroxidase-linked antibody was from Sigma Chemical Co. (St. Louis, MO). ECLTM western blotting detection system and ECLTM hyperfilm were purchased from Amersham-Pharmacia Biotech (Buckinghamshire, UK). [³H]TBZ was obtained from American Radiolabeled Chemicals (St. Louis, MO).

Animals and treatments

C57BL/6 mice (Harlan-Sprague-Dawley, Dublin, VA) were used for all experiments, and were used in accordance with NIH guidelines. In the 3 month exposure study, mice (5 months old) were randomly assigned to the treatment groups according to their body weight, with 7 mice in each group. The mean weight of the mice in all treatment groups was not significantly different. PM (1.5 mg/kg) carried in MTG was administered once/week for 13 weeks, with MPTP (20

mg/kg) dissolved in saline given once at either the 7th week or the 11th week (Fig. 1). In the six-month exposure study, mice (2 months old) were randomly assigned to the treatment groups in the same way described above, with 10 mice in each group. PM (0.8 or 1.5 mg/kg) was administered once/week for 26 weeks, with MPTP (20 mg/kg) given once at the 24th week (Fig. 1). Both MPTP and PM were administered by i.p. injection. Control mice received the same amount of vehicle. The body weight of each mouse was measured before each injection to ensure the same dose given as their body weight increased during the treatment period. Weekly treatments with PM were given because it is cleared from the body within 7 days after exposure (Tomalik-Scharte et al., 2005). MPTP was given 2 weeks or 4 weeks before the end of the PM treatments, consistent with our previous studies (Kou et al., 2006). Mice were sacrificed by cervical dislocation 24 hours after the last treatment, and the brain striatal tissue was collected for immunoblotting assays.

Western blot analysis

Western blot analysis was used to measure the change of the protein biomarkers. Striatal synaptosomes were prepared according to the methods described by Wright *et al.* (1998). In brief, striatal tissue was homogenized in 1 ml ice-cold sucrose buffer (0.32 M sucrose, 4.2 mM HEPES; pH 7.4). The homogenates were centrifuged at 1,000 x g for 15 min at 4 °C. The supernatants were collected and re-centrifuged at 10,000 x g for 15 min at 4 °C. The resulting pellets were resuspended in KRH buffer (NaCl: 118 mM, KCl: 4.8 mM, CaCl₂: 2.5 mM, MgSO₄: 1.2 mM, HEPES: 20 mM; pH: 7.5), and stored at -70 °C. Prior to western blot assay, the frozen tissue was thawed, and the protein concentration of the tissue samples was determined by a protein dye-binding assay (Bradford 1976). The tissue samples were then mixed with 25% of sample buffer (Tris-HCl: 60 mM, SDS: 2%, glycerol: 10%, bromphenol blue: 0.001%; pH: 6.8) and heated at 90 °C for 5 minutes. A 10% SDS gel was used for the analysis of DAT and TH, with a 15% SDS gel for α -synuclein. A sample of 10 μ g protein was loaded for DAT, 5 μ g protein was loaded for TH, and 3 μ g protein was loaded for α -synuclein. Uniform protein loading was ensured by adjusting tissue samples to the same protein concentration before loading. SDS gels were run according to the method of Towbin *et al.* (1979). The resulting protein bands were transferred to a nitrocellulose membrane, and equal loading was re-confirmed by Ponceau-S staining of each western blot lane on the membrane. The Ponceau-S was then removed by washing with TBST buffer (Tris: 50 mM, NaCl: 138 mM, KCl: 2.7 mM, Tween 20:

0.05%; pH: 8.0). Membranes were incubated with non-fat milk (5%) at room temperature for 1 hour, followed by incubation with the appropriate monoclonal primary antibody at 4°C at the following dilutions: DAT, 1:3000; TH, 1:12,000; and synaptophysin: 1:12,000. After an overnight incubation, the membrane was washed with TBST buffer, followed by one-hour incubation with peroxidase-linked secondary antibody at room temperature. After three, 5-minute washes with TBST buffer, the membrane was developed using the ECL Amersham Chemiluminescence kit and exposed to ECL hyperfilm for an appropriate time. After washing with tap water and air drying, the protein content on the film was quantitated using a Kodak EDAS 290 image analysis system.

[³H]TBZ binding assay

Frozen striatal synaptosomal tissue was resuspended in incubation buffer (NaCl: 10 mM, KCl: 130 mM, MgCl₂: 5 mM, HEPES: 20 mM; pH: 7.4). To measure the total binding of TBZ to VMAT, [³H]TBZ (2 nM) was incubated with synaptosomal tissue for 1 hour at room temperature, with 100 μM of reserpine used to define the nonspecific binding. After incubation, the suspension was diluted by the addition of 3 ml of incubation buffer, and vacuum-filtered through Whatman GF/C filters. The filters were rinsed 3 times with 3 ml of incubation buffer to remove any unbound radioactivity. Filters were air-dried, placed in scintillation vials with cocktail (Scintiverse E, Fisher Scientific) and radioactivity was counted by liquid scintillation spectrometry after standing overnight. Protein concentration was measured using the protein dye-binding assay (Bradford 1976), and the binding data were expressed as pmol [³H]TBZ /μg protein.

Statistical analysis

The raw densitometry western blot data were used for statistical analysis. Data were analyzed in the method of ANOVA using SAS (SAS Institute, Cary NC) and done in consultation with the Virginia Tech Statistical Consulting Center. [³H]TBZ binding data were analyzed using InStat (Graph-Pad Software, San Diego, CA), with differences across the treatment groups determined by one-way ANOVA, followed by Student-Newmann-Keuls post hoc test if significance ($p < 0.05$) was observed.

RESULTS

Long-term exposure to low doses of PM did not cause a significant increase of general toxicity. During the 3-month or 6-month treatment period, no more than 2 mice died in any treatment group, and there was no significantly enhanced mortality in the PM and/or MPTP treatment groups. There was also no significant loss of body weight in the groups treated with PM and/or MPTP compared with controls (data not shown).

Representative western blots of DAT and TH proteins following low-term exposure to the low doses of PM, either with or without MPTP, is shown in figure 2. Western blot data revealed that 3-month exposure to PM (1.5 mg/kg) had no effect on the expression of TH and DAT protein in striatal dopaminergic terminals (Fig. 3A). A single treatment with 20 mg/kg MPTP persistently reduced the expression of both TH and DAT until 6 weeks after the MPTP injection, with a partial recovery over time (Fig. 3B). Long-term treatment with PM (1.5 mg/kg) for 3 months did not enhance the neurotoxicity of MPTP on the striatal dopaminergic system (Fig. 3C, D). Instead, 3-month exposure to 1.5 mg/kg PM significantly antagonized the down-regulation effect of MPTP on TH protein when MPTP was given at the 11th week ($p < 0.05$), but this effect was not quite significant when MPTP was given at the 7th week ($p = 0.07$) (Fig. 3C, D). The expression of α -synuclein protein was also quantified by western blot assay, but there was no significant change in α -synuclein expression in any of the treatment groups (Fig. 3A-D).

Six-month exposure to either 0.8 mg/kg PM or 1.5 mg/kg PM up-regulated TH expression about 17% compared to control, but did not alter the expression of DAT (Fig. 4A). A single dose of 20 mg/kg MPTP reduced the expression of both TH and DAT to 80% of control and to 75% of control, respectively (Fig. 4B). Treatment with PM (0.8 or 1.5 mg/kg) for 6 months did not enhance the neurotoxicity of MPTP on the striatal dopaminergic system (Fig. 4C, D). Again, pre-exposure to 1.5 mg/kg of PM attenuated the effect of MPTP on TH reduction (Fig. 3D). The expression of α -synuclein protein was not changed in any of the treatment groups (Fig. 4A-D).

[³H]TBZ binding experiments showed that neither 3-month nor 6-month exposure to low doses of PM had significant effect on the [³H]TBZ binding to VMAT (Fig. 5A, B), even though 6-month treatment with 0.8 mg/kg of PM tends to reduce the [³H]TBZ binding compared to the control (Fig. 5B). In the 3-month treatment study, one single dose of 20 mg/kg MPTP, given at the 11th week during the treatment period, significantly reduced the [³H]TBZ binding to 56% of

the control level (Fig. 5A). The data taken from the mice injected with MPTP at the 7th week demonstrated that the reduction of [³H]TBZ by MPTP was persistent, but with partial recovery over the time, to 75% of the control value (Fig. 5A). Pretreatment with 1.5 mg/kg of PM antagonized the reduction of [³H]TBZ binding by MPTP when MPTP was given at 11th week, but not at the 7th week over the 13-week treatment period (Fig. 5A). In the 6-month treatment experiment, one single injection of 20 mg/kg MPTP again remarkably reduced the [³H]TBZ binding, but the [³H]TBZ binding result in the groups treated with either dose of PM + MPTP were not significantly different from the mice treated with MPTP alone (Fig. 5B).

DISCUSSION

In general, long-term, low-dose exposure to PM did not cause significant neurotoxicity to the striatal dopaminergic system. The elevation of TH protein after 6-month exposure to 0.8 or 1.5 mg/kg PM suggests that an enhanced requirement for dopamine synthesis. The underlying mechanisms are unclear, but could be due to increased dopamine release from hyperexcitatory nerve firing via modification of sodium channel function (Soderlund *et al.* 2002). No effects of PM on DAT and α -synuclein proteins were seen in either the 3-month or the 6-month treatment study. Our previous data showed that PM up-regulated DAT and α -synuclein proteins at doses of 0.8 and 1.5 mg/kg, after 3 injections over a 2-week period (Gillette and Bloomquist 2003). This pyrethroid-induced up-regulation of DAT at low doses was also documented by Elwan *et al.* (2005) under the same treatment regimen. In contrast to the lack of PM-induced toxicity to striatal neurons observed here, Abdel-Rahman *et al.* (2001) found that a daily dermal dose of PM (0.13 mg/kg for 60 days) to rats degraded or reduced neurons in the motor cerebral cortex, dentate gyrus, CA1/CA3 subfields of the hippocampus, and cerebellum. The longer exposures and different brain regions examined would account for the different results observed.

Long-term exposure to low doses of PM for either 3 or 6 months did not enhance the toxicity of MPTP, expressed as the reduction of TH or DAT protein. Instead, pre-treatment with 1.5 mg/kg of PM for 3 months significantly reduced the effect of MPTP on DAT and TH protein when MPTP was given 2 weeks before the end of the last PM injection. However, exposure to 1.5 mg/kg PM for 6 months attenuated the reduction of TH caused by MPTP, but had less of an effect on expression of DAT protein. This time dependence is similar to our previous studies (Kou *et al.*, 2006), where 3 doses of PM (200 mg/kg) and CPF (75 mg/kg) were given over 2

weeks, and a single dose of MPTP (20 mg/kg) was given on day 1. When assayed at 1 day post-treatment, MPTP-induced decreases in TH labeling were antagonized by co-injection of PM + CPF, with less of an effect on DAT expression. By 4 weeks post-treatment with all three toxicants, both TH and DAT levels had significantly declined below the levels caused by MPTP alone (Kou et al. 2006).

The underlying mechanism whereby PM reduced the toxicity of MPTP is unclear, but one possibility would be PM-induced increase in the expression of VMAT. Numerous evidence indicates that MPP⁺ is sequestered within synaptic vesicles by acting as a substrate for VMAT, therefore preventing the interaction of MPP⁺ with mitochondria to execute its cytotoxic effect (Del Zompo, *et al.*, 1991; Peter *et al.*, 1994). [³H]TBZ binding assay revealed that the binding capacity of VMAT for [³H]TBZ was not changed by PM alone, but was significantly reduced by one single injection of MPTP. This latter finding is consistent with recent work showing that doses of MPTP to C57BL/6 mice resulted in reduced gene expression of VMAT 24 hr following MPTP administration (Tillerson *et al.*, 2002; Xu *et al.*, 2005). However, the reduction of [³H]TBZ binding by MPTP was attenuated by weekly pre-treatment with 1.5 mg/kg PM for 3-months when MPTP was administered 2 weeks before the end of the study. Thus, increased VMAT expression by PM did not underlie its antagonism of MPTP-induced neurotoxicity, but changes in this biomarker were consistent with the changes in expression levels of DAT and TH proteins.

Pharmacokinetic interaction between PM and MPTP is another possible mechanism behind the observed antagonism. PM treatment may inhibit or down-regulate the MAO activity in brain. Therefore, reduced bioactivation of MPTP to its toxic metabolite MPP⁺ would provide protection from MPTP-induced toxicity. *In vitro* experiments on inhibition of MAO-A by PM, found it competitively inhibits MAO-A in rat brain preparations, *in vitro* (Rao and Rao 1993). It is difficult to envision how this effect would be expressed, *in vivo*, at the low doses used here, but not at the high doses used in our previous studies (Kou et al., 2006), where 200 mg/kg PM did not antagonize the effects of MPTP, but required the additional presence of CPF. Thus, we conclude that inhibitory effects on MAO are unlikely to explain the antagonism of MPTP neurotoxicity we observe. Another possible factor that might contribute to the attenuated toxicity of MPTP after long-term PM treatment may be due to the ability of PM to increase cytochrome P₄₅₀ mono-oxygenase activity, since P450 monooxygenases are known to metabolize

MPTP to nor-MPTP (Upadhyaya et al. 2001). Along these lines, it was reported that administration of PM (100 mg/kg) daily for 20 days resulted in a slight induction of P₄₅₀ in rat hepatocytes (Krechniak and Wrzesniowska 1991). Considering the approximately 100-fold lower doses used in the current long-term PM treatment study, this mechanism also seems unlikely.

The overall findings demonstrate that long-term, low-dose exposure to PM alone did not cause signs of neurotoxicity to striatal dopaminergic neural terminals, or enhance the effects of MPTP. A study in rabbits exposed them to cloth treated with [¹⁴C]-labeled PM (0.125 mg/cm², the rate used for military uniforms) for 7 days (Snodgrass, 1992). At the end of the 7 day exposure, 3.2% of the available PM had reached the skin and about 2% was absorbed, as indicated by its presence in excreta, giving a daily uptake rate of 6 x 10⁻⁴ mg/kg/day (Snodgrass, 1992). Thus, use of PM as an insecticide would seem to pose little Parkinsonian hazard under typical use conditions, including when impregnated into cloth to control biting insects.

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Figure 1:

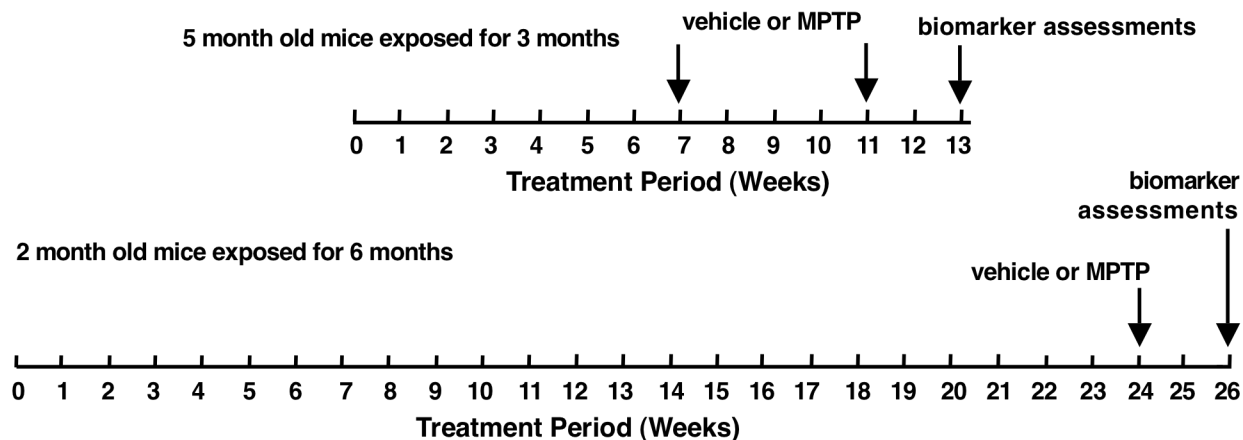


Figure 1. Experimental protocol for 3 month (12 treatments) and 6 month (24 treatments) exposure to PM using weekly ip injections. Vehicle or MPTP were given at either of 2 post-treatment times as indicated by the arrows. For the 13 week exposures, MPTP was given on week 7 (6 weeks before the last PM treatment) or week 11 (2 weeks before the last PM treatment). For the 6 month exposure, MPTP was given 2 weeks before the last PM treatment, only. Mice were sacrificed 24 hr after the last PM treatment, when 8 months of age.

Figure 2:

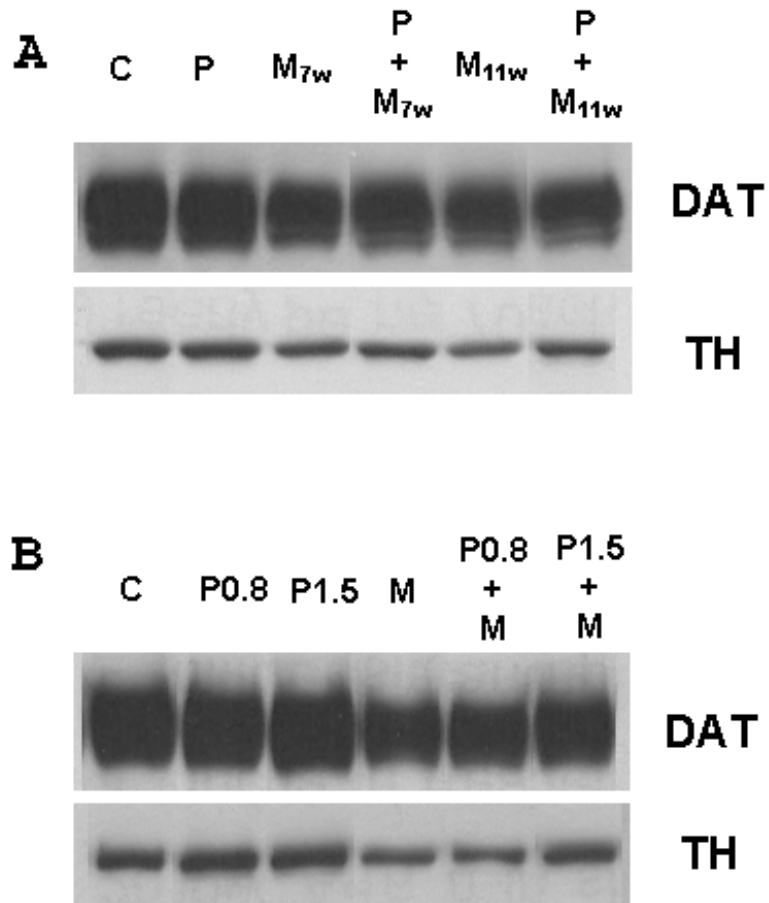


Figure 2: Representative western blots of DAT and TH following toxicant treatment. Monoclonal antibodies were used to identify appropriate bands in each blot, confirmed by molecular weight markers (not shown). Treatments are labeled above the bands (CTL: control; M: MPTP; M₇: MPTP given at the 7th week; M₁₁: MPTP given at the 11th week; P: PM).

Figure 3:

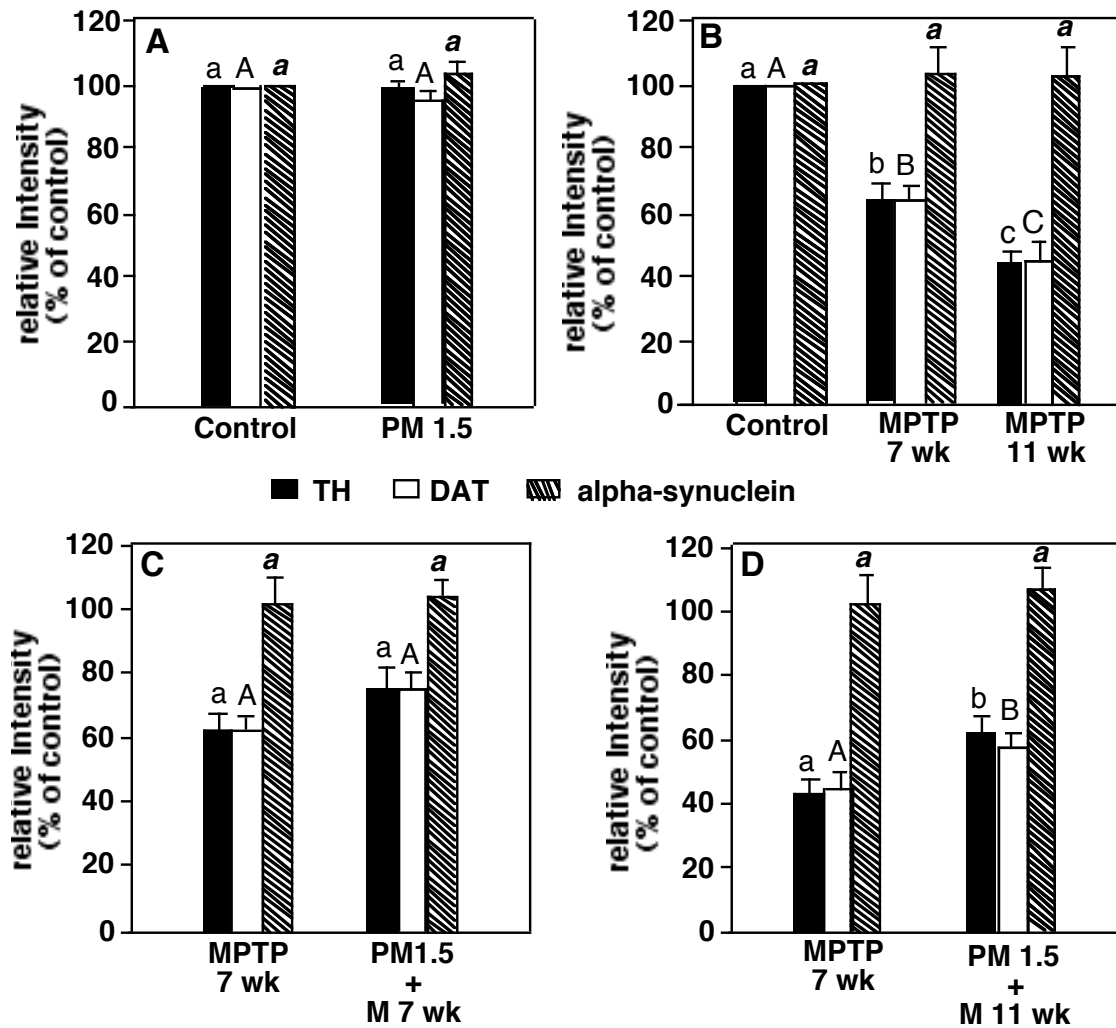


Figure 3: Western blot analysis of TH, DAT and α -synuclein protein of striatal tissue taken from C57BL/6 mice treated with PM (1.5 mg/kg) for 3 months, with or without a single injection with MPTP (M) (20 mg/kg) at the 7th week or the 11th week. Western blot data were expressed as the percentage of control. Panels A-D are arranged to best illustrate appropriate statistical comparisons. Bars labeled by different letters are significantly different (p<0.05). Statistical significance among treatment groups are designated for each biomarker, with TH, DAT, and α -synuclein labeled by lower case, capital, and italic letters, respectively.

Figure 4:

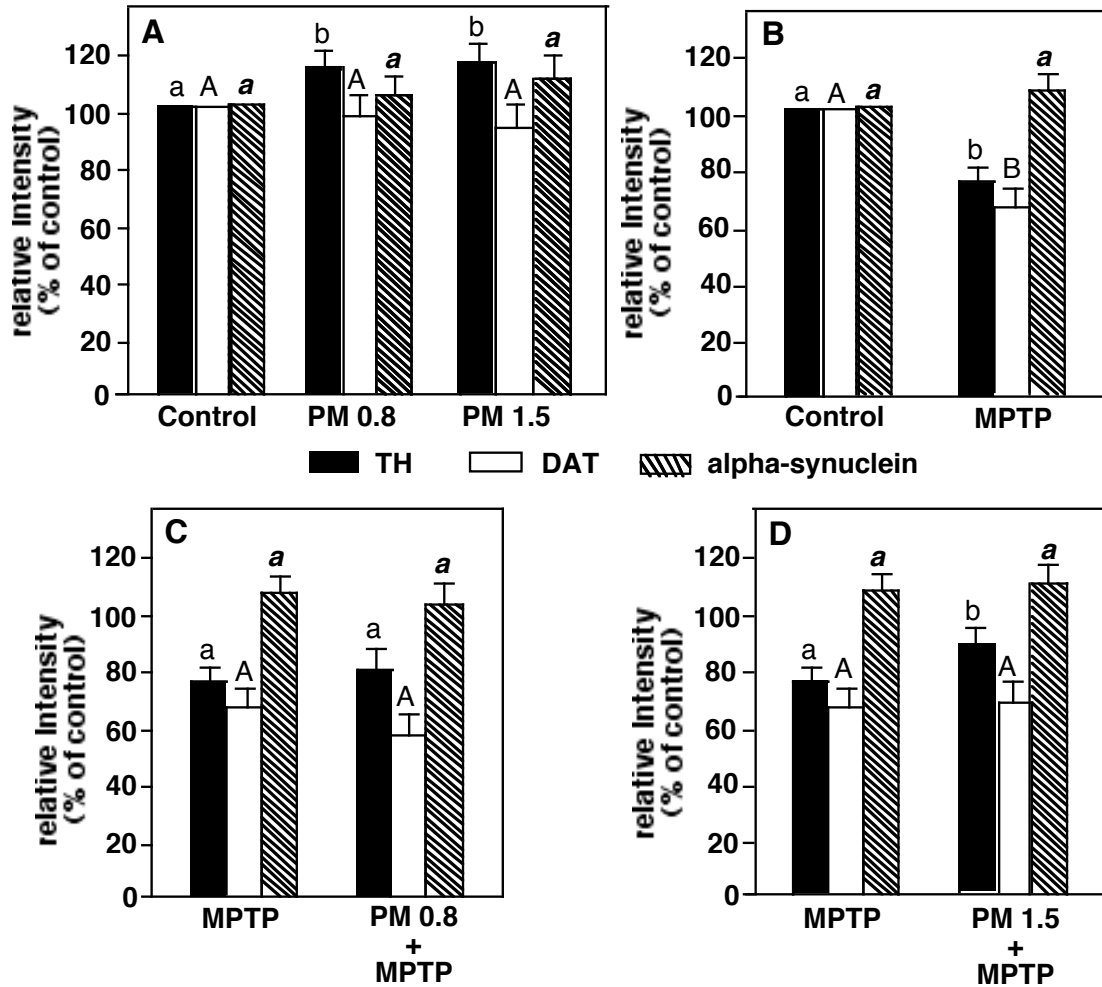


Figure 4: Western blot analysis of TH, DAT and α -synuclein protein of striatal tissue taken from C57BL/6 mice treated with PM (0.8/1.5 mg/kg) for 6 months, with or without a single injection with MPTP (20 mg/kg) given at 2 weeks before the end of the treatment period. Western blot data were expressed as the percentage of control. Bars labeled by different letters are significantly different ($p < 0.05$). Panels A-D are arranged to best illustrate appropriate statistical comparisons. Statistical significance among treatment groups are designated for each biomarker, with TH, DAT, and α -synuclein labeled by lower case, capital, and italic letters, respectively.

Figure 5:

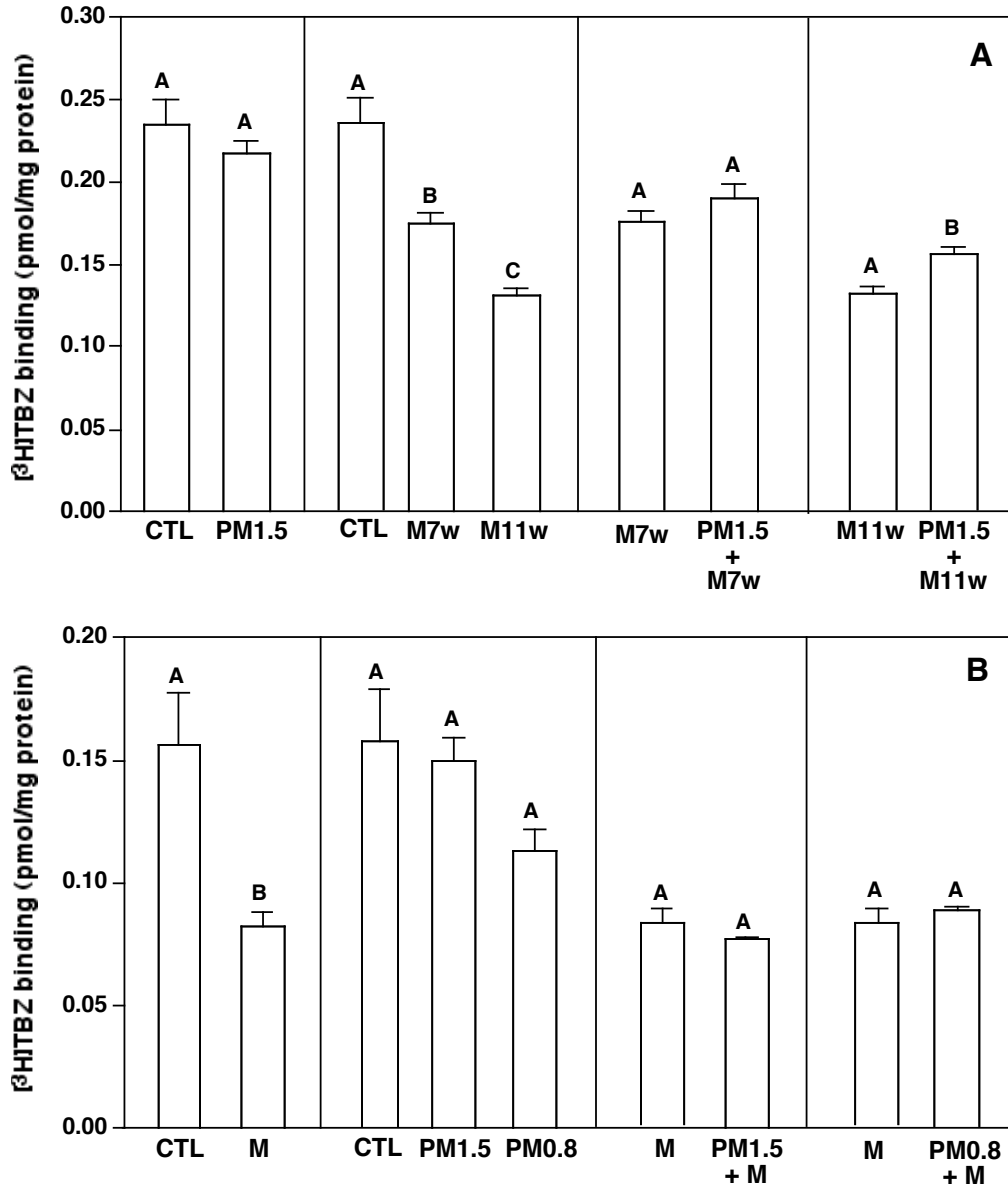


Figure 5: ^3H TBZ binding data taken from the C57BL/6 mice treated with PM (1.5 mg/kg) for 3 months (A), or the mice treated with PM (0.8 or 1.5 mg/kg) for 6 months (B), either with or without MPTP (M). Control (CTL) = vehicle alone. In the 3 month study (A), MPTP was given on week 7 (M7w) or week 11 (M11w). In the 6 month study (B), MPTP was only given 2 weeks before the end of the experiment. Bars represent means with SEM, and the bars labeled by different letters are significantly different (p < 0.05) within each matched experimental grouping, as defined by the plot frames.