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Table of Contents

Cover	1
SF 298	2
Table of Contents	3
Introduction	4
Body	5
Key Research Accomplishments	11
Reportable Outcomes	12
Conclusions	13
References	14
Appendices	15

INTRODUCTION

The subject of this research is to understand the role of genes, aging, and neurotoxicants on the etiology of Parkinson's disease (PD). One specific gene, the human α -synuclein gene, was selected for study because of its role in a familial form of Parkinson's disease. Recent evidence also implicates α -synuclein in idiopathic or other forms of Parkinson's disease. The presence of the α -synuclein protein in Lewy bodies and dystrophic neurites has been found. In addition, polymorphisms or unique haplotypes exist in some patients with sporadic Parkinson's disease suggesting that dysregulation of this gene and its wild-type protein may play a role in idiopathic Parkinson's disease. The functional role of both wild-type and mutated α -synuclein remains unclear. The use of transgenic animals with functional human α -synuclein in the nigrostriatal system will assist in understanding these functions.

The other major risk factor for Parkinson's disease is environmental exposure to potential neurotoxicants. MPTP is a known cause of a form of Parkinsonism. It has been speculated, based on epidemiologic studies and animal studies, that other environmental agents may predispose to sporadic forms of Parkinson's disease. Recent studies in our lab and other labs have suggested that paraquat may be selective dopaminergic neurotoxicant. In addition, the environmental dithiocarbamate pesticides have been found to augment toxicity of both MPTP and paraquat. We have investigated these pesticides in wild-type mice and our transgenic mice to understand their actions. We have also explored their mechanisms *in vitro* and *in vivo*.

The purpose of this research is to develop appropriate models, to study the individual effects and interactions of α -synuclein, neurotoxicants, and age. These models will demonstrate whether dysfunction and death occur in different mouse models and how it may be prevented. Our primary method of investigation is the use of transgenic mice, expressing either the wild type human α -synuclein or a doubly mutated form of α -synuclein. These mice are studied in the context of normal aging and following exposure to neurotoxicants. We also explore the actions and mechanisms of environmentally relevant neurotoxicants.

BODY

Our application stated that environmental neurotoxicants or genetic mutations alone might produce forms of Parkinson's disease. We also hypothesized that sporadic cases of Parkinson's disease may be the result of interactions between three factors: genetic predisposition, aging, and either life long exposures to neurotoxicants or acute intermittent exposure to neurotoxicants. We hypothesized that: (1) a mutated form of human α -synuclein would be toxic to substantia nigra neurons via a toxic gain of function when over expressed in the mouse and (2) that over expression of either the mutated α -synuclein or the wild type α -synuclein gene would contribute to increased vulnerability to environmental neurotoxicants in the mouse substantia nigra. We finally hypothesized that aging would play an important role in the interaction of human α -synuclein and neurotoxicants in contributing to Parkinson's disease.

The fourth year of this grant has been a successful year in terms of abstracts and manuscripts. We continue to demonstrate data that support our hypotheses. We published our first manuscript on our transgenic mice which was well received and differed from the results of others in several important ways. The successful model we have generated will be extensively used by us and others. Two groups have already requested these mice for their studies and we intend to honor requests, provided they do not overlap with other requests or our studies. Michael Schwarzschild at Harvard University and Ann Graybiel from MIT will begin using our mice this year for their studies. Dr. Schwarzschild will study the role human α -synuclein in mesolimbic function related to drug preference and its possible role in PD. Dr. Graybiel will study nigrostriatal function and plasticity related to PD.

We will summarize our work in relationship to our most recent SOW. We will also include data on other related projects we are involved in that relate to models of PD.

Years 1 to 3. Data from Years 1 to 3 have been presented in the previous annual reports. Specific work completed in Years 1 to 3 include the following:

Ia. Transgenic mouse lines over expressing wild type or double mutated human α -synuclein under control of TH promotor have been completed.

Iib. Reagents and characterization of both constructs at the DNA, RNA and protein level has been completed.

Ic. Assessment of these constructs for functionality and response to neurotoxicants using behavioral, neurochemical and stereological measures have been completed. This work will continue with more characterization and study. Some of this data is presented in our paper entitled "Behavioral and neurochemical effects of wild-type and mutated human α -synuclein in transgenic mice" included in the appendix.

Id. Create a universal somatic mosaic vector for the production of somatic mosaic lines and test all functional DNA elements. This has been completed.

IIa. Two α -synuclein XAT constructs will be made. *In vitro* testing of the constructs using transfected cells. We have cloned the α -synuclein gene into our pUSMTV along with the human prion promotor. All clones to date were in the wrong orientation. We have redesigned our PCR primers to ensure correct orientation, but have yet to complete the testing. We have delayed this aim because of the success

and importance of using our current transgenic model for our studies. As we demonstrate, this model has been successful and is capable of answering all the biological questions we and others are interested in. The use of a somatic mosaic transgenic carries less importance as the data from our current approach mounts.

Iic. Generation of somatic mosaic transgenic mice. This task has not yet been completed, due to efforts spent in Year 4 on other studies to be described using the lines already created. Most of the hypotheses we made can be tested using standard transgenic models as we have done and will continue to do. We hope to finish sequencing of the new somatic mosaic constructs so that they can be sent to our transgenic core for generation of mice.

Iid. Begin testing for aging and neurotoxicant effects using the transgenic lines. This work has been accomplished and will be discussed in detail in Year 4 summary.

Ic. We have completed studies on the effects of amphetamine in two lines from each construct and that data are presented in our first manuscript. These data demonstrate that both constructs are functional in the nigrostriatal system. The demonstration of functional activity is the key issue for Drs. Schwarzschild and Graybiel to pursue the use of these mice. Their studies will further explore the functional roles of human α -synuclein in normal and Parkinsonian mice.

Year 4

Iid, IIIb, IIIc, and IVb. As mice age they have a decline in locomotor activity, similar to humans. When severe, it may be due to PD. We have completed a two year prospective, longitudinal study on the motor activity in both genders from all three lines of mice, nontransgenic littermates and lines $hw\alpha$ -SYN-5 and $hm^2\alpha$ -SYN-39. This long, but necessary and valuable study, in conjunction with age-related stereological counts of SNpc neurons, will be very valuable in understanding the behavioral, neurochemical, and morphological relationships in mouse models of the PDP. This data will also be useful for planning interventional studies. The behavioral and stereological data are shown below (**Fig. 1**). Our conclusions from these data include the following. Line $hm^2\alpha$ -SYN-39 show considerable decline in locomotor activity with age that correlates with loss of TH+ neurons in the SNpc. The relationship is not straightforward and the decline in locomotor activity seems to be less dramatic than the loss of TH+ neurons suggesting compensation by the surviving neurons. Locomotor activity in males of all the lines declines more than in females consistent with epidemiology of PD in which men are more commonly affected than women. Nontransgenic males have a significant decline in locomotor activity over 20 months, but this was not reflected in loss of TH+ neurons, suggesting other factors are important in their decline. Line $hw\alpha$ -SYN-5 had the second greatest degree of locomotor decline over time. They also lost a significant number of TH+ neurons with aging, although they also started with an increased number. These data suggest that the wild-type human α -synuclein may affect neuron number at birth when overexpressed and may adversely affect TH+ neuron survival. SNpc TH+ neurons declined much more slowly in line $hw\alpha$ -SYN-5 compared to line $hm^2\alpha$ -SYN-39. This would support, perhaps, a role for the wild-type form in the development of PD, but the contribution is much less than the mutated form.

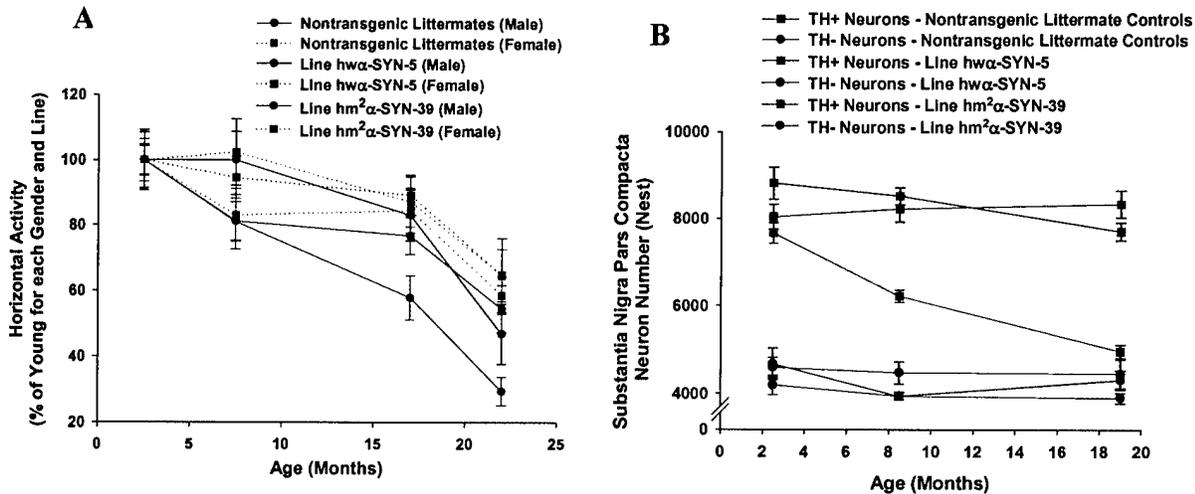


Fig. 1. Locomotor behavior and SNpc neuron counts in the h α -SYN model over time. **A.** We measured locomotor behavior longitudinally in both genders over time in nontransgenic littermate controls (● male, ■ female), a line expressing the human wild-type α -synuclein, line h ω α -SYN-5 (● male, ■ female), and line h m^2 α -SYN-39 (● male, ■ female). Each animal served as its own control and data were normalized to activity levels at the first test. Males had a greater decline in locomotor activity than females in their respective group. Line h m^2 α -SYN-39 males declined to greater extent than the males in other lines. **B.** Stereologic counting of TH+ and TH- neurons in the SNpc was performed in males only at different ages in nontransgenic littermate controls (■ TH+, ● TH-), line h ω α -SYN-5 (■ TH+, ● TH-), and line h m^2 α -SYN-39 (■ TH+, ● TH-). Line h m^2 α -SYN-39 had a clear age-related loss in TH+ neurons without change in TH- neurons. Line h ω α -SYN-5 had a significantly greater number of neurons at a young age, but also lost a significant number of neurons with aging, albeit at a much reduced rate compared to line h m^2 α -SYN-39. No significant age-related changes were found in TH- neurons or TH+ neurons in the nontransgenic littermate controls.

As part of this study, we also determined whether the loss of SNpc neurons and striatal dopamine could be reversed by dopamine agonists. We completed testing of reversal of motor declines using apomorphine. We used two doses of apomorphine, a low dose known to act presynaptically on nigrostriatal terminals and a higher dose felt to act postsynaptically on striatal neurons. We found a reduced presynaptic response in line h m^2 α -SYN-39 compared to the other two groups. This is consistent with their abnormal nigrostriatal system and abnormal response to amphetamine. Using the higher dose, we found reversal of the locomotor deficit only in line h m^2 α -SYN-39 consistent with denervation supersensitivity. We conclude that all of the longitudinal and pharmacological data are consistent with an aging-dependent model of the PDP seen in line h m^2 α -SYN-39.

We are writing a second manuscript documenting these age-related declines in locomotor activity, neuron counts and the reversal of the functional decline with dopaminergic agonists. We feel this aging study completes that aim and now allows us to study a variety of consequences and mechanisms related to this neuronal loss. To that end, we are now looking for evidence of aggregation and morphological changes in SNpc neurons in all lines. We feel this model may be an ideal age-related model of parkinsonism in mice.

We have begun performing electron microscopy of our lines to look for evidence of aggregation or other evidence of dysfunction in line h m^2 α -SYN-39 compared to the other lines (**Fig. 2**). We have examined only a few mice, but differences are present and require further work. The most obvious finding

is the absence of aggregates and inclusions as seen in most, but not all other models. The reason for the absence of inclusions in these mice, despite the clear loss of neurons and other features remains unknown, but requires additional EM and other studies to confirm.

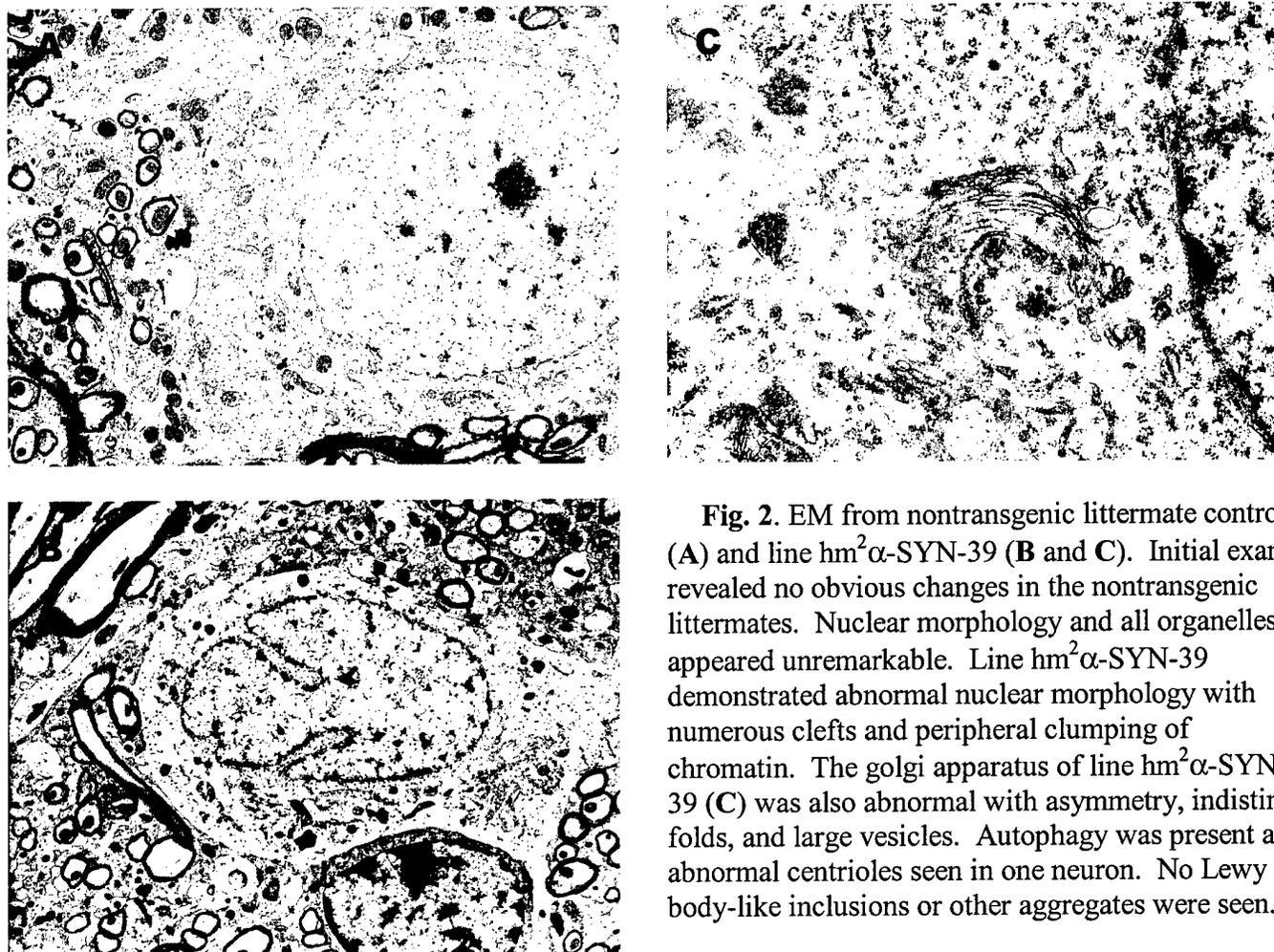


Fig. 2. EM from nontransgenic littermate controls (A) and line $hm^2\alpha$ -SYN-39 (B and C). Initial exam revealed no obvious changes in the nontransgenic littermates. Nuclear morphology and all organelles appeared unremarkable. Line $hm^2\alpha$ -SYN-39 demonstrated abnormal nuclear morphology with numerous clefts and peripheral clumping of chromatin. The golgi apparatus of line $hm^2\alpha$ -SYN-39 (C) was also abnormal with asymmetry, indistinct folds, and large vesicles. Autophagy was present and abnormal centrioles seen in one neuron. No Lewy body-like inclusions or other aggregates were seen.

We are also performing staining of paraffin embedded tissue to look for inclusions and other morphological changes. To date, staining for ubiquitin, human α -synuclein, and tyrosine hydroxylase has not demonstrated inclusions. Increased lipofuchsin as a sign of dysfunction may be present and needs confirmation.

Ic, IId, IIIb, and IVb. We have made considerable progress in demonstrating an interaction between mutated α -synuclein and neurotoxicants. We have completed the behavioral, neurochemical, and morphological aspects of this study, but are working on identifying evidence for oxidative stress as a key mediator. We feel this additional mechanistic data will be important for linking the actions of the mutation and the neurotoxicants. We presented this data in last years report. Briefly, we treated non-transgenic controls, lines $hw\alpha$ -SYN-5 and $hm^2\alpha$ -SYN-39 with chronic maneb and paraquat. We performed behavioral measures, neurochemical measures, and stereologic measures, which are illustrated below (**Fig. 3** and **4** and **Table**). In this study we used half the normal doses of maneb and paraquat that we normally use in non-transgenic littermates because line $hm^2\alpha$ -SYN-39 was too sensitive. Despite this lower dose, line hm^2 -39 was affected with highly significant losses in locomotor activity (**Fig 3**), associated with significant losses in dopamine and dopamine metabolites (**Fig 4**) and significant losses in TH positive neurons in the substantia

nigra pars compacta (Table). This data will be published once we finish measuring several markers of oxidative stress. Increased oxidative stress in the transgenic mice, the neurotoxicant treated mice, or the combined group will be informative data.

Fig 3. Behavior was assessed immediately after dosing, 24 hrs later, or 10 days after the last dose to assess acute effects, immediate recovery and permanent effects. This figure shows final locomotor behavior 10 days following the last dose of saline or combined PQ+MB. *PQ+MB resulted in a permanent and significant decline ($p < 0.0001$) in locomotor activity **only** in line $hm^2\alpha$ -SYN-39.

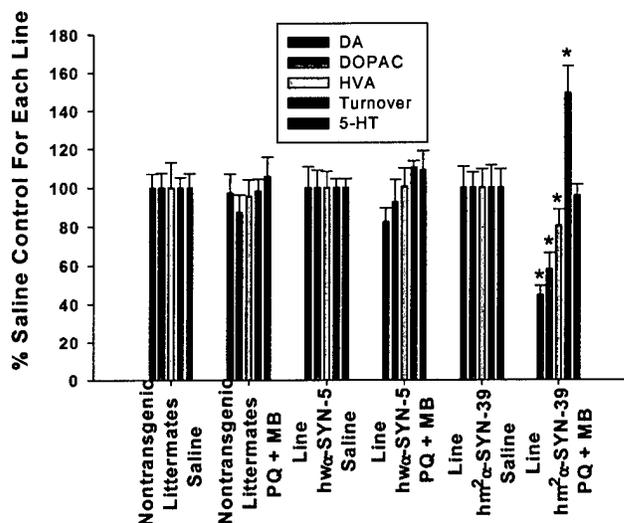
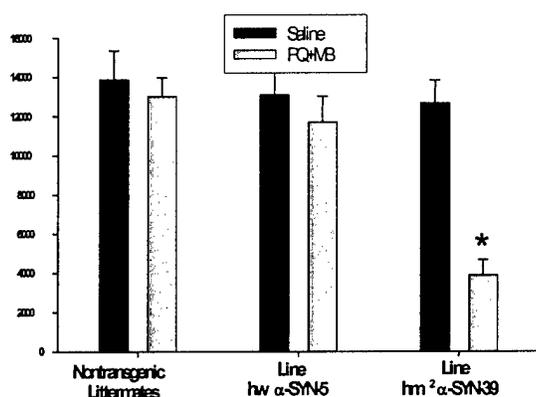


Fig 4. DA and metabolite values were normalized to the saline control for each line. This allows the data to be visualized on one plot at the same scale. Values for the nontransgenic littermates treated with saline are as follows (ng/mg protein \pm SEM): DA (106.6 ± 7.6), DOPAC (10.1 ± 0.8), HVA (13.4 ± 1.9), DA turnover (16.0 ± 0.9), 5-HT (5.8 ± 0.4). Values indicated by * for PQ+MB are significantly ($p < 0.005$) different from their saline controls.

Mouse line	Treatment	n	TH+ Nest	% saline	TH- Nest	Total Neuron (calculated)
Nontransgenic Littermates	Saline	3	$8,213 \pm 308$		$4,467 \pm 254$	$12,680 \pm 533$
Nontransgenic Littermates	PQ+MB	3	$6,507 \pm 278^a$	79%	$4,493 \pm 53$	$11,000 \pm 323$
Line $hw\alpha$ -SYN-5	Saline	3	$8,507 \pm 278$		$4,860 \pm 89$	$13,346 \pm 213$
Line $hw\alpha$ -SYN-5	PQ+MB	3	$7,066 \pm 116^a$	83%	$4,461 \pm 94$	$11,666 \pm 232$
Line $hm^2\alpha$ -SYN-39	Saline	3	$6,213 \pm 141$		$3,933 \pm 71$	$10,146 \pm 187$
Line $hm^2\alpha$ -SYN-39	PQ+MB	3	$2,991 \pm 70^a$	48% ^b	$4,116 \pm 607$	$7,107 \pm 616$

Table. Stereologic counts of mouse SNpc neurons in the three lines following saline or PQ+MB. Values marked by ^a indicate significant difference following PQ+MB versus saline ($p < 0.0001$) and by ^b indicate significant difference ($p < 0.0001$) from nontransgenic littermates and line $hw\alpha$ -SYN-5. Note that combined PQ+MB produced equal and significant neuronal loss of neurons in nontransgenic littermates and line $hw\alpha$ -SYN-5 without changes in behavior, DA, or DA metabolites. This is consistent with our previous work. Note also that line $hm^2\alpha$ -SYN-39 had significantly fewer neurons at middle age than the other two groups. Whether this represents a loss at this age will not be known until we complete our neuronal counts at the younger age.

We have also worked on several other projects related to neurotoxicants and their role in the Parkinson's disease phenotype (See reportable outcomes and appendix). Given the importance of neurotoxicants in our hypotheses, these studies are important for understanding their mechanisms prior to implementation with the transgenic mice. We have studied the role of paraquat and maneb at different ages and for different survival times (See appendix). We show that age is an important variable for neurotoxicity and that the effects of these neurotoxicants are permanent and progressive. We are now in the position to test these agents in the transgenic mice to determine the interaction between human α -synuclein, age and the neurotoxicants. We also have demonstrated that these agents may result in toxic effects when administered in utero and followed by treatment as an adult (See appendix). Prenatal maneb on its own did not result in a neurotoxic effect as demonstrated by locomotor behavior or neurochemistry as an adult in either gender. However, rechallenge with paraquat in males as an adult resulted in a significant permanent decline in locomotor behavior and striatal dopamine. These data will be presented at the Soc. for Neuroscience in November. We are completing stereological counts of SNpc neurons which should help clarify the significant and nonsignificant effects. These data, however, already clearly demonstrate that prenatal exposures to one neurotoxicant result in a permanent change in SNpc neurons that predispose them to a later insult that will lead to a PDP.

We are also submitting a manuscript (see appendix) that demonstrates several important findings that relate to the neurotoxicology of the two environmentally relevant pesticides we use. We first finding demonstrates that paraquat is not transported via the dopamine transporter as often presumed based on its chemical similarity to MPP⁺. We also show that several selective dithiocarbamates act to increase the concentration of dopamine in synaptosomes *in vitro* and paraquat *in vivo*. Although several mechanisms are possible, we believe this effect is due to inhibition of an efflux transporter, possibly an organic cation transporter. The DTCs having these effects are also the ones that increase the neurotoxic effect of both MPTP and paraquat in mice. This correlation between the toxicokinetic effects *in vitro* and *in vivo* and the toxicodynamic effects suggest the kinetics of dopamine, paraquat, and MPP⁺ may be a central factor in their adverse mechanisms and are related to the area under curve for these agents.

KEY RESEARCH ACCOMPLISHMENTS IN YEAR 4

1. Publication of “Behavioral and neurochemical effects of wild-type and mutated human α -synuclein in transgenic mice” (see Appendix).
2. Publication of “Reporter gene transfer induces apoptosis in primary cortical neurons. ” (see Appendix).
3. Publication of “Developmental paraquat and maneb exposure and Parkinsonism ” (see Appendix).
4. Submission of “Age-related and irreversible nigrostriatal dopaminergic neurotoxicity following exposure to the pesticides paraquat and maneb” (see Appendix).
5. Submission of “Selective dithiocarbamates increase synaptosomal dopamine content, brain concentration of paraquat and correlate with potentiation of MPTP and paraquat toxicity” (see Appendix).
6. Completion of longitudinal aging study. Manuscript in preparation.
7. Completion behavioral, neurochemical, and stereological data of neurotoxicant-gene interaction. Manuscript in preparation.
8. Initiation of morphological studies to determine if aggregation or inclusions play a role in the transgenic mice.
9. Initiation of oxidative stress studies to link a mechanism to the neurobiological outcomes in our different models.
10. Identification of “silent” toxicity related to prenatal exposure to maneb.

REPORTABLE OUTCOMES

Manuscripts Accepted

Detrait, E.R., Bower, B., Halterman, M., Guiliano, R., Bennice, L., Federoff, H.J., and Richfield, E.K. (2002) Reporter gene transfer induces apoptosis in primary cortical neurons. *Molecular Therapy* 5(6):723-730.

Richfield, E.K., Cory-Slechta, D.A., Thiruchelvan, M.J., Wuertzer, C.A., Gainetdinov, R., Caron, M., Di Monte, D.A., and Federoff, H.J. (2002) Behavioral and neurochemical effects of wild-type and mutated human α -synuclein in transgenic mice *Exp. Neurol.* 175:35-48.

Thiruchelvam, M., Richfield, E.K., Goodman, B.M., Baggs, R., Di Monte, D.A., and Cory-Slechta, D.A. (2002) Developmental paraquat and maneb exposure and Parkinsonism: Permanent dopaminergic neurotoxicity and enhanced vulnerability to subsequent exposures. *Neurotoxicology* (in press).

Manuscripts Submitted

Thiruchelvam, M., Richfield, E.K., McCormack, A., Baggs, R., Tank, A.W., Di Monte, D.A., and Cory-Slechta, D.A. Age-related and irreversible nigrostriatal dopaminergic neurotoxicity following exposure to the pesticides paraquat and maneb (submitted).

Barlow, B.B., Bennice, L., Thiruchelvam, M.J., Cory-Slechta, D.A., Ballatori, N., and Richfield, E.K. Selective dithiocarbamates increase synaptosomal dopamine content, brain concentration of paraquat and correlate with potentiation of MPTP and paraquat toxicity (submitted).

Manuscripts in Preparation

Thiruchelvam, M., Federoff, H.J., Cory-Slechta, D.A., and Richfield, E.K. Adverse effect of age and environmental neurotoxicants in a mouse model of Parkinsonism (in preparation).

Abstracts

Richfield, E.K., Barlow, B.K., Thiruchelvam, M., and Cory-Slechta, D.A. Selective Dithiocarbamates increase synaptosomal dopamine content, brain concentration of paraquat and correlate with potentiation of MPTP and paraquat toxicity. *Society for Neuroscience Abst.*, (2002).

Barlow, B.K., Thiruchelvam, M., Richfield, E.K., and Cory-Slechta, D.A. Prenatal exposure to maneb increases adult vulnerability to the neurotoxic effects of paraquat: Implications for Parkinson's Disease. *Society for Neuroscience Abst.*, (2002).

Thiruchelvam, M., Cory-Slechta, D.A., Federoff, H.J. and Richfield E.K. Age-related changes associated with overexpression of wild-type and mutated human α -synuclein in mice. *Society for Neuroscience Abst.*, (2002).

CONCLUSIONS

We have made considerable progress in the fourth year of this award. We have demonstrated significant effects of both the wild-type and double mutant form *in vivo* in transgenic mice. It is important to emphasize that this is one of the few papers in which a transgenic human protein has been demonstrated to have a functional effect related to its likely normal action in man and mouse. This model is the only model to show neuronal loss in the SNpc that is age-related. We also show that the double-mutant line responds incompletely to a presynaptic dose of apomorphine and excessively to a postsynaptic dose indicative of denervation supersensitivity. We also demonstrate the importance of gender and age to neurotoxicant exposures. We demonstrate a potential mechanism for the augmentation of neurotoxicity by selective dithiocarbamates.

REFERENCES

None.

APPENDICES

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Reporter Gene Transfer Induces Apoptosis in Primary Cortical Neurons

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Modern cell biologists typically use reporter genes either alone or co-expressed with a protein of interest to facilitate the localization or quantification of protein expression. Our work demonstrates that reporter genes should be used cautiously, as several common reporter gene products are toxic to primary cortical neuronal cultures. We used the herpes simplex virus-based viral amplicon vector to transduce cortical neurons with three different reporter genes and assessed whether any reporter gene products were toxic over time, by monitoring neurite disintegration and apoptosis. Toxicity varied as a function of the reporter gene, the gene product localization, and the level of reporter gene expression. Transduction of enhanced green fluorescent protein or nuclear-localized β -galactosidase was more toxic than non-nuclear localized β -galactosidase. This work underscores the need for careful design of gene expression constructs. Moreover, in studies where cell injury or toxicity is being evaluated, their use should be carefully considered.

Key Words: reporter gene, apoptosis, transgene toxicity

INTRODUCTION

Cell and molecular biologists have been using reporter genes for many years, both in mammalian and non-mammalian cell culture and *in vivo* [1,2]. They share a common feature: their gene products are easily visualized either directly or by a simple colorimetric assay. Genes used as reporters include those encoding fluorescent molecules like the green fluorescent protein (GFP) or encoding an enzyme such as firefly luciferase or *Escherichia coli* β -galactosidase (encoded by *lacZ*) [1,2]. Due to the convenient detection of gene products, these genes have been expressed alone, included in DNA constructs to make fusion proteins, or used as surrogate markers to monitor the expression of another gene that is not readily detectable. Reporter genes have also been incorporated into transient and stably transfected cell lines to serve as markers of successful transfection.

Despite the extensive use of reporter genes, few studies have focused on the potential toxicity of foreign protein overexpression. Indeed, some reporter proteins have been suspected or shown to induce toxicity. For example, the efficiency of establishing stable cell lines expressing GFP is typically low and the reason for this is unknown,

but may be attributed to GFP-mediated toxicity [3]. GFP has been reported to induce cell death by production of free radicals when intensely excited for extended periods [4]. Felts and colleagues have reported eGFP toxicity in stably transfected HeLa cell lines [5]. These human cancer cell lines lost nearly all the expression of eGFP within 21 days and after only four passages. GFP toxicity was also investigated on a variety of non-neuronal cell lines transfected with a GFP or LacZ-expressing construct [3]. These studies showed that GFP expression, but not that of LacZ, was toxic, inducing apoptosis within 48 hours post-transfection. These observations have led to the development of less cytotoxic variants of GFP [5].

Given the growing number of applications of reporter molecules both *in vivo* and *in vitro*, a systematic study of toxicity appears warranted. Overexpression of any native or exogenous protein may disrupt cellular processes such as intracellular trafficking or signaling, and may ultimately disrupt critical biochemical processes that lead to dysfunction and/or cell death.

Our study examined neuronal toxicity of two reporter molecules usually believed to be innocuous: eGFP and β -galactosidase. We used the herpes simplex virus (HSV) amplicon viral vector system to transduce these genes into

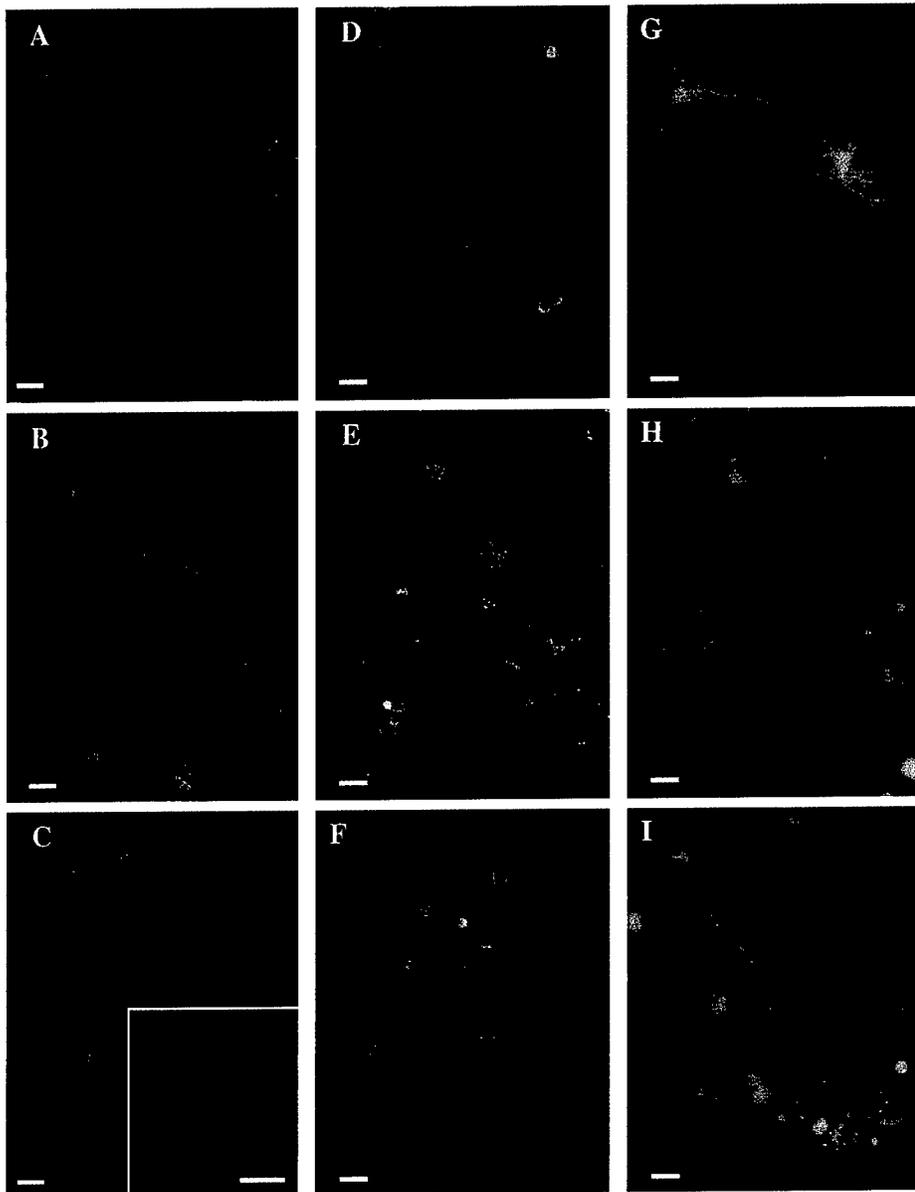


FIG. 1. Loss of neuronal processes over time. We transduced primary cortical neurons with helper virus-packaged HSVlac (A–C) or HSVeGFP (D–F) or with helper virus-free HSVlac (G–I). At 1 (A, D, G), 3 (B, E, H), and 5 (C, F, I) days after transduction, we processed cultures for ICC using a primary antibody against the appropriate reporter gene product and a secondary antibody labeled with TRITC (A–F) or FITC (G–I). Neuronal processes disintegrated over time, and cell bodies rounded up. This process occurred faster in neurons expressing eGFP than with LacZ. Scale bar, 20 μ m.

cultured primary cortical neurons. We quantified the toxicity elicited by each transgene, and we related resultant toxicities to the level and localization of transgene product. We show that eGFP expression induced apoptotic cell death more rapidly than vector-transduced β -galactosidase, but that both reporter molecules were ultimately toxic, inducing apoptosis when expressed at high levels.

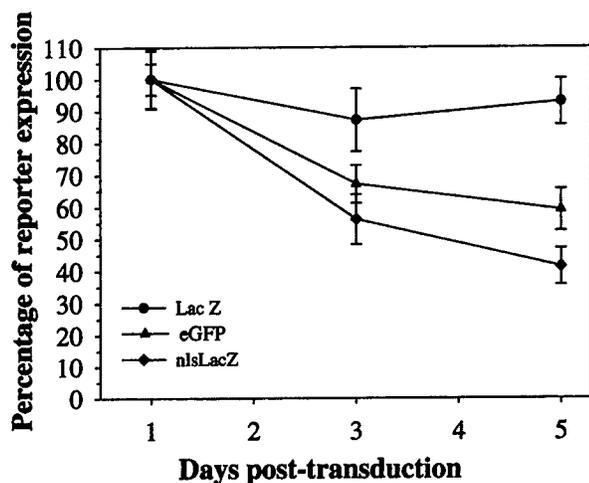
RESULTS

Morphology of Cortical Neurons Expressing a Reporter Gene

We transduced primary cortical neurons with helper or helper-free HSV packaged amplicon virus stocks expressing

one of the following reporter genes under the control of the IE4/5 promoter: *eGFP* (HSVeGFP), *lacZ* (HSVlac), or *nlslacZ* (HSVnlsLac). We examined the morphology of transduced cells 1, 3, and 5 days after transduction. Neurons transduced with HSVnlsLac were indistinguishable from eGFP-expressing neurons. Morphological changes of cells transduced with either the helper virus containing (Figs. 1A–1C) or helper virus-free (Figs. 1G–1I) vectors appeared comparable. This suggested the packaging system was not in itself the cause of toxicity. After 24 hours of transduction, most transduced cells expressing reporter gene products manifested long processes filled with the reporter molecule (Figs. 1A, 1D, and 1G). After 3 days, we observed the morphological changes typical of dying cells [6] in a subset of cells transduced with either HSVeGFP or HSVnlsLac. Processes appeared to disintegrate

and cells rounded up at this time point (Fig. 1E). In contrast, after 3 days, the cells transduced with HSVlac exhibited morphology similar to 1 day after transduction (Figs. 1B and 1H). At 5 days post-transduction, nearly all neurons expressing *eGFP* or *nlslacZ* were devoid of processes. Many cell bodies were rounded (Fig. 1F) and the very few remaining processes were fragmented. At this same time point, the HSVlac-transduced neurons did not exhibit such drastic morphological changes. Although most cells lost their processes and their somata displayed a rounded appearance (Figs. 1C and 1I), some transduced neurons still displayed long, healthy processes (Fig. 1C, inset). These results illustrate that cortical neurons transduced with amplicons expressing reporter genes lost processes over time, with



HSVeGFP and HSVnlsLac inducing this loss more rapidly than HSVLac. The viral packaging system used for transduction, whether helper virus-based or helper virus-free, did not modify these observations.

Rate of Cell Loss

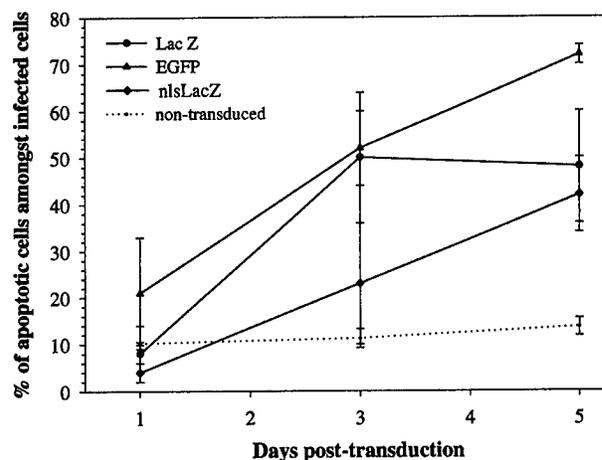
To assess the impact of reporter gene expression on neuronal viability, we transduced cortical neurons with HSVLac, HSVeGFP, or HSVnlsLac using the helper virus packaging system and we enumerated gene-product-positive cells at 1, 3, and 5 days post-transduction (Fig. 2). The number of cells expressing *eGFP* and *nlsLacZ* decreased significantly over time (ANOVA main effect of time $F(2,27) = 45$, $P < 0.0001$). The number of cells expressing these reporter proteins fell to 59% and 41% at 5 days respectively, which was significantly fewer than the number of cells expressing these reporters at day 1 or 3 post-transduction ($P < 0.0001$ for day 5 versus day 3 or day 1 and for day 3 versus day 1 for both reporter molecules). In contrast, the number of cells expressing *lacZ* did not decrease significantly over time (92% remaining after 5 days; both $P > 0.05$ for day 5 compared with day 1 and day 3). ANOVA also identified a significant effect of reporter molecule type on the number of

FIG. 3. Increased percentage of apoptotic neurons transduced with a reporter gene-expressing amplicon vector. We transduced cortical neurons with helper virus-packaged HSVLac, HSVeGFP, or HSVnlsLac. We transduced neurons 7 days after plating and processed cultures at 1, 3, and 5 days post-transduction for ICC using primary antibody against the appropriate reporter gene product. We stained nuclei with DAPI and counted labeled neurons with an apoptotic nucleus at the three time points. We used two-way factorial ANOVA to analyze the effect of time post-transduction and reporter type on the percentage of apoptotic cells. The number of apoptosis was significantly greater at day 3 and day 5 post-transduction compared with day 1 ($P < 0.001$ for HSVLac day 5 versus day 1, $P < 0.01$ for HSVeGFP and HSVnlsLac day 5 versus day 1, $P < 0.01$ comparing HSVLac and HSVeGFP day 3 versus day 1, $P < 0.05$ comparing HSVnlsLac day 3 versus day 1, $P < 0.05$ comparing non-transduced cells day 5 versus day 1). Error bars correspond to SD.

FIG. 2. Loss of reporter expression over time in cortical neuron cultures transduced with helper virus-packaged HSVnlsLac or HSVeGFP, but not in cells transduced with HSVLac. We transduced neurons 7 days after plating, and processed cultures at 1, 3, and 5 days post-transduction for ICC using primary antibody against the appropriate reporter gene product. We enumerated labeled cells at the three time points and calculated percentages taking the number of infected neurons at day 1 as 100%. Two-way factorial ANOVA analyzed the effect of time post-transduction and reporter type on the percentage of reporter expression. The number of neurons transduced with HSVeGFP or HSVnlsLac was significantly lower at days 3 and 5 post-transduction compared with day 1 ($P < 0.0001$); this number was also significantly lower at day 5 compared with day 3 in HSVnlsLac-transduced neurons ($P < 0.01$). Error bars correspond to SD.

immunolabeled cells ($F(2,27) = 106$, $P < 0.0001$). The number of cells expressing β -galactosidase was significantly higher than the number of cells expressing *eGFP* or *nlsLacZ* genes at 3 and 5 days post-transduction ($P < 0.0001$ at both time points for both reporter). These results suggest that the expressions of different reporter molecules vary in either their loss of expression levels or the rate of cell loss in culture. This effect was greatest for HSVeGFP, followed by HSVnlsLac and HSVLac.

To assess whether the cortical neurons expressing a particular reporter gene were dying, we identified by immunocytochemistry (ICC) neurons expressing a reporter gene and quantified the number of apoptotic cells at the three time points. We identified apoptotic cells by the morphology of the nucleus labeled with DAPI. Cells with a fragmented or condensed nucleus were defined as being apoptotic. Immunolabeled cells with an absent or "ghost nucleus" were classified as being dead. The percentage of apoptotic neurons expressing a reporter molecule increased over time with all three reporters (Fig. 3; ANOVA main effect of time $F(2,24) = 49$, $P < 0.0001$). Five days after transduction, the percentage of apoptotic cells reached 42, 48, and 72% of cells expressing *nlsLacZ*, *lacZ*, and *eGFP*, respectively, and was significantly greater than at 1 day post-transduction ($P < 0.01$ for *lacZ* and *nlsLacZ*, $P < 0.001$



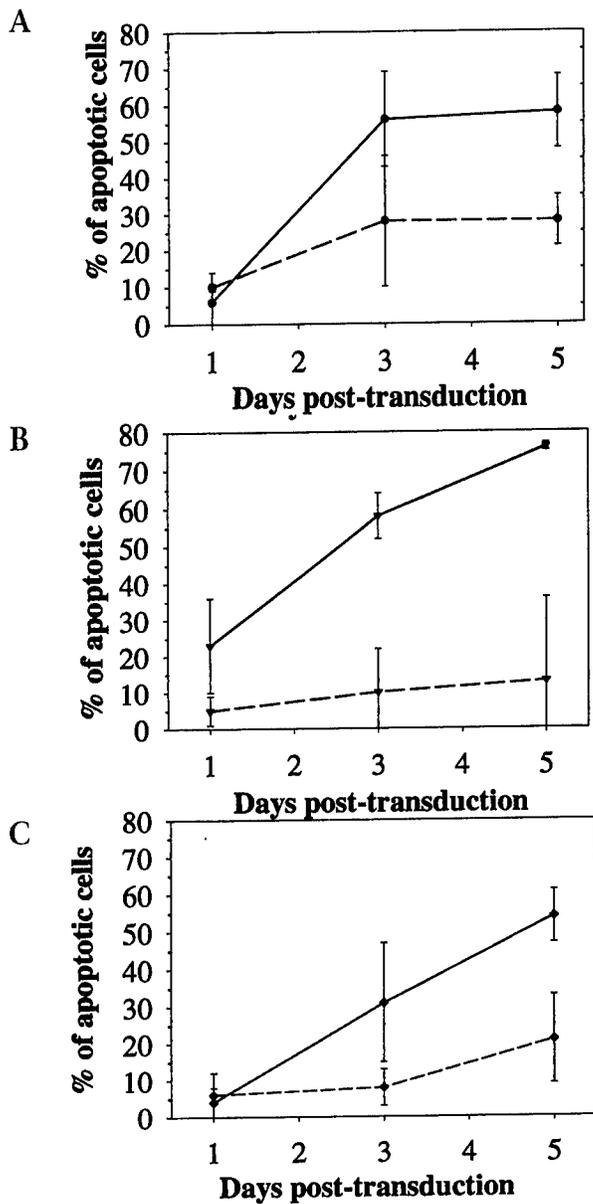


FIG. 4. The rate of apoptosis is higher in neurons expressing a high level of reporter than in neurons expressing a low level of reporter. We transduced cortical neurons with helper virus-packaged HSVlac (A), HSVeGFP (B), or HSVnlsLac (C). We processed cultures at 1, 3, and 5 days post-transduction for ICC using primary antibody against the appropriate reporter gene product, and we stained nuclei with DAPI. We counted labeled neurons with an apoptotic nucleus at the three time points. We defined a "low expressing" cell (dotted lines) as a cell for which the capture time was $4\times$ longer than the "high expressing" cells (full line). We used two-way factorial ANOVA to analyze the effect time post-transduction and level of expression of each reporter on the percentage of apoptotic cells. The number of apoptosis in neurons expressing low levels of reporter protein was significantly smaller than in neurons expressing high levels of reporters ($P < 0.001$ for HSVeGFP at day 3 and 5, $P < 0.01$ for HSVlac and HSVnlsLac at day 5, $P < 0.05$ for HSVlac and HSVnlsLac at day 3). The number of apoptotic cells significantly increased over time in neurons expressing high levels of reporter proteins ($P < 0.001$ for HSVlac and HSVeGFP comparing day 5 versus day 1, $P < 0.01$ for HSVlac and HSVeGFP comparing day 3 versus day 1 and for HSVnlsLac comparing day 5 versus day 1, $P < 0.05$ for HSVeGFP comparing day 5 versus day 3 and for HSVnlsLac comparing day 5 versus day 3 and days 3 versus day 1). In contrast, the number of apoptosis in neurons expressing low levels of reporter proteins did not significantly increase over time with any of the reporters ($P > 0.05$).

over time. We confirmed these results using helper virus-free amplicon stocks and TUNEL staining to assess apoptosis (data not shown).

The Rate of Cell Death Is Dependent on the Level of Expression of the Gene Product

To assess whether the toxicity of a given reporter gene product correlated with the level of expression of the gene, we quantified the percentage of cells displaying an apoptotic phenotype among the cells expressing a reporter molecule at a high and a low level (Fig. 4). The differentiation between high and low expression was based on the intensity of the immunolabeling. At a low level of reporter expression, the percentage of apoptotic cells varied from 10% to 20% depending on the reporter and did not significantly increase over time (main effect of time: $F(2,6) = 2.8$ for *lacZ*, $F(2,6) = 0.2$ for *eGFP*, $F(2,6) = 2.7$ for *nlsLacZ*, $P > 0.05$ with all three reporter molecules). However, at a high level of expression, the percentage of apoptotic cells increased significantly to 55–75% at day 5 post-transduction. The overall effect of time post-transduction on the number of apoptotic cells expressing a high level of reporter molecules was significant: *lacZ*, $F(2,6) = 23$, $P = 0.0014$; *eGFP*, $F(2,6) = 31$, $P = 0.0006$; *nlsLacZ*, $F(2,6) = 17$, $P = 0.0034$. The number of apoptotic cells was always significantly higher at day 5 versus day 1 ($P < 0.001$ for *lacZ* and *eGFP*; $P < 0.01$ *nlsLacZ*, Fig. 4). The percentage of apoptotic cells was significantly higher in high expressing cells than in low expressing cells (overall effect of the expression level for *lacZ*, $F(1,12) = 13$, $P < 0.01$; *eGFP*, $F(1,12) = 54$, $P < 0.0001$; *nlsLacZ*, $F(1,12) = 15$, $P < 0.01$). The level of apoptosis was significantly greater after 3 days ($P < 0.05$) and 5 days ($P < 0.05$) compared with day 1 for all three reporter proteins.

for *eGFP*). By contrast, the percentage of apoptosis among neurons that did not express a reporter gene product remained fairly constant over time (around 10%), although it was slightly higher after 5 days ($P < 0.05$ day 5 versus day 1). The ANOVA comparing reporter genes showed a significant effect of reporter molecule ($F(3,24) = 31$, $P < 0.0001$); the number of apoptotic cells was higher in transduced cells than in non-transduced cells at day 5 post-transduction ($P < 0.01$ for all three reporters). These results illustrate that the neurons transduced with HSVlac initiated an apoptotic process, although the number of β -galactosidase-positive cells did not significantly decrease

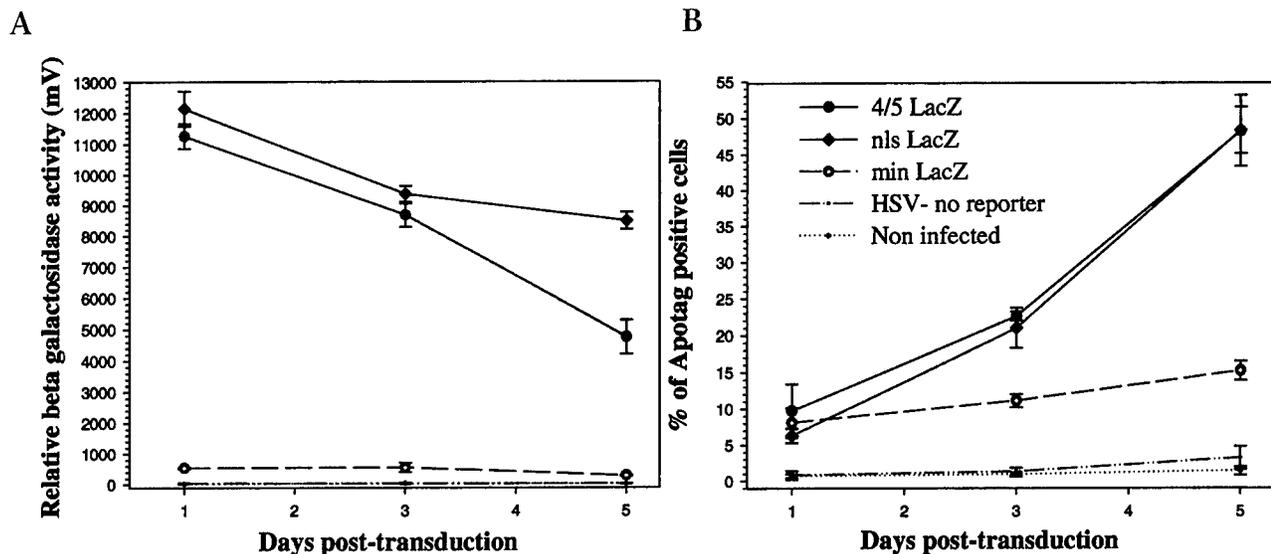


FIG. 5. The percentage of apoptotic neurons is related to the level of reporter expression. We transduced cortical neurons with helper virus-free packaged HSVLac, HSVnlsLac, HSVminPrLac, or with control vector HSVPrPuc. (A) We assessed the relative β -galactosidase expression by Galacto-Lite assay. (B) We quantified the percentage of apoptosis among transduced cells by TUNEL staining in corresponding cultures, and we used two-way factorial ANOVA to analyze the effect time post-transduction and the type of amplicon on the percentage of apoptotic cells. The percentage of apoptotic neurons was significantly lower when a low expressing promoter drove the reporter gene ($P < 0.001$ comparing HSVLac or nlsLac with HSVminLac at day 3 or 5). The number of apoptotic cells was also lower in neurons transduced with HSV amplicon or non-transduced than in neurons expressing a reporter protein ($P < 0.001$ at day 3 and 5 with any reporter construct and $P < 0.05$ at day 1). The number of apoptotic neurons significantly increased over time in neurons expressing a reporter ($P < 0.001$ for HSVLac and HSVnlsLac comparing day 5 versus day 1, $P < 0.01$ for HSVLac and HSVnlsLac comparing day 3 versus day 1 and for HSVminLac comparing day 5 versus day 1, $P < 0.05$ for HSVminLac comparing day 3 versus day 1).

To further examine the relationship of reporter gene product level to induction of apoptosis, we used amplicons that harbored different promoters to drive the expression of the *lacZ* transgenes: a low *lacZ*-expressing amplicon (HSVminPrLac) driving the expression of *lacZ* and the high expressing IE4/5 promoter driving the expression of *lacZ* or *nslacZ* (HSSLac and HSVnlsLac, respectively). Neurons transduced with the HSV amplicon without a reporter (HSVPrPuc) and non-transduced neurons served as controls. We quantified both the relative β -galactosidase activity by Galacto-Lite assay (Fig. 5A) and the degree of apoptosis by TUNEL staining (Fig. 5B). The level of β -galactosidase activity was greater when the *lacZ* transgene was driven by IE4/5 promoter (HSSLac) than by the minimal promoter (HSVminPrLac; Fig. 5A). The percentage of apoptotic neurons transduced with HSSLac and HSVnlsLac, both driven by the strong IE 4/5 promoter, increased significantly with time post-transduction (F(2,30) = 250, $P < 0.0001$; day 1 versus day 3, $P < 0.01$, and day 1 versus day 5, $P < 0.001$, with both reporter molecules). The number of apoptotic cells was significantly greater at days 3 and 5 in cultures transduced with HSSLac and HSVnlsLac than when transduced with HSVminPrLac or HSVPrPuc, or non-transduced (main effect of reporter: F(4,30) = 310, $P < 0.0001$, HSSLac or HSVnlsLac versus any of the other constructs $P < 0.001$ at both time points).

Apoptotic cell percentages reached 48% of cells at 5 days; no significant difference was found between HSSLac and HSVnlsLac amplicons (Fig. 5B). In contrast, the extent of apoptosis in HSVminPrLac-transduced cultures increased modestly to 15% after 5 days ($P < 0.01$ at day 5 versus day 1 post-transduction). The number of apoptotic neurons was still, however, significantly greater than cultures transduced with HSVPrPuc or non-transduced control cultures ($P < 0.001$ both at day 3 and 5). The percentages of apoptotic cells in the non-transduced cultures or in cultures transduced with HSV amplicon devoid of a reporter gene remained low (less than 5% after 5 days).

DISCUSSION

This study investigated the toxicity of three reporter molecules expressed in primary cortical neuron cultures using HSV amplicon vectors. We delivered the three reporter genes (*lacZ*, *nslacZ*, and *eGFP*) using two different HSV viral vector packaging systems: helper-based and helper virus-free. We obtained similar results with both viral packaging systems, ruling out the possibility that the toxicity was linked to the presence of helper virus.

Our data show that the expression of these reporter genes is toxic to cortical neurons in culture as a function of time. The number of cells transduced with HSVeGFP or

HSVnlsLac decreased rapidly over time with concomitant loss of processes. In contrast, HSVlac-transduced neurons did not exhibit such drastic morphological changes, and transgene expression did not decrease as rapidly as observed with the two other test vectors. This suggests that, although these three reporters are toxic to cortical neurons, there is a gradation of their toxicity, with *lacZ* being least toxic as *nlslacZ* and *eGFP*. Toxicity associated with β -galactosidase is likely delayed in time as compared with the other two reporters because evidence for apoptosis was clearly present in HSVlac-transduced cultures at 5 days post-transduction.

Transgene-mediated cell death occurred via a mechanism consistent with apoptosis. The percentage of apoptotic neurons among transduced neurons increased as a function of time. *eGFP* expression resulted in the highest percentage of cell death, but the two *lacZ*-expressing constructs also induced significant levels of apoptosis. It is therefore likely that the gradation in toxicity observed by the decrease in reporter-positive neurons reflects a difference in the kinetics of the induction of apoptosis, with *eGFP* inducing apoptosis more quickly than either *nlslacZ* or *lacZ*.

The *in vitro* toxicity of reporter genes has not been previously reported in primary cortical neurons. However, *eGFP*, but not *lacZ* expression, has been linked to the induction of apoptosis in several non-neuronal cell lines [3]. The lack of data concerning such toxicity may be due to experimental design. Numerous studies using reporter molecules focus on very short-term expression (from hours to a few days) and do not necessarily investigate the occurrence of apoptosis [7]. In such experimental conditions, this study clearly indicates that *lacZ*-mediated toxicity may be easily missed, as the number of neurons expressing β -galactosidase did not decrease after 5 days and cell morphology was only significantly altered at day 5 post-transduction. In addition, potential β -galactosidase-mediated toxicity is not always highlighted because the cells transduced with this gene are often considered as a control group in experiments focused on other genes of interest.

In vivo, long-term expression of reporter genes, such as in the case of transgenic animals, is well established, suggesting a lack of *in vivo* toxicity [8,9]. The apparent contradiction between *in vivo* and *in vitro* studies might be explained by several reasons. First, the *in vivo* cellular environment is much more complex than the *in vitro* situation. Therefore, one can expect protective mechanisms and molecules to exist in a transgenic animal expressing a reporter gene to be absent *in vitro*. Second, the type of promoter driving expression of a given reporter gene *in vivo* may explain the lack of toxicity observed in this setting. Indeed, transgenic animals often harbor a cellular promoter to drive the transgene of interest in a cell-type-specific manner. The use of such a promoter may be more tightly regulated than a viral foreign promoter (that is, HSV IE4/5), which may not be subject to similar cellular

regulatory mechanisms. Jin and colleagues previously reported the importance of the type of promoter for the successful long-term transgene expression in neurons [8]. They suggested that in neuronal cells the HSV IE4/5 promoter was less tightly regulated than 9-kb tyrosine hydroxylase promoter, which in the nigrostriatal pathway was able to drive longer-term gene expression. Although the authors did not suggest any promoter effect on potential toxicity of the transgene, one can expect that a lack of regulation of transgene expression might induce toxicity in some cases. Third, the lack of reports of reporter gene-mediated toxicity *in vivo* might be a consequence of compensatory changes that occur in transgenic animals during development, cellular alterations that may be invisible to the investigator. For example, transgenes expressed early during embryonic development may induce toxicity in a subset of cell types. If the animals are to be viable, a compensatory change must occur at the cellular level to adapt to the insult. This may come in the form of dampening transgene expression levels or the activation of protective cellular mechanisms that may never be evident (or expected) later in the adult animal. In any case, further examination of transgenic animals at early embryonic stages may sort out this very important question and garnering such knowledge may help to define the conditions in which reporter molecules may be safely used *in vivo* via gene transfer vectors.

Our data show that the expression of a reporter gene via an HSV amplicon vector in neuronal cells is toxic *in vitro* and induces apoptosis as a function of timing and level of gene product expression. These results emphasize that the use of reporter genes should be evaluated carefully before incorporating such foreign genes into vectors and into cells. In particular, great caution needs to be exercised in interpreting experimental results in which these molecules are used as controls. Similarly, we would recommend avoiding the use of these reporters as control genes for any toxicity study. We suggest using alternative techniques to visualize the expression of the transgenes. When antibodies are available, immunocytochemistry is a method of choice to monitor transgene expression, which simultaneously allows for the assessment of transduction efficiency and gene product subcellular localization. When antibodies are not available and the use of a reporter is required, it may be desirable to use a low-expressing promoter to drive the expression of the reporter gene, which should decrease reporter gene product-induced toxicity.

MATERIAL AND METHODS

Primary cortical neuron cultures. We cultured embryonic day (E) 14.5 cortical neurons as described [10,11]. We dissected mouse cortices at E14.5 and plated cells on polylysine-coated glass coverslips (30 μ g/ml overnight) at a density of 200,000 cells per well (24-well plates). Plating medium consisted of Neurobasal medium with added B27 supplement, 0.8% fetal bovine serum (FBS), glutamic acid (3.7 μ g/ml), and glutamine (0.5 mM).

After 5 days in culture, we replaced 50% of the medium with feed medium (plating medium without FBS and glutamic acid). These culture conditions produced a nearly pure neuronal culture after 10 days (less than 2% astrocytes) as assessed by counting GFAP-positive cells versus the total number of cells in culture (data not shown).

Amplicon vector construction, packaging, and transduction. We used the following constructs based on the pHSVPrPuc amplicon: HSVPrPuc-4/5 promoter-Lac [12], -eGFP, or -nuclear localization sequence β -galactosidase (nlsLac). Three of the amplicon vectors that we studied contained transgenes under the transcriptional control of HSV-derived immediate-early 4/5 promoter. To construct pHSVeGFP, the parent amplicon vector, pHSVPrPuc [13], was digested with *EcoRI*, filled in with T4 polymerase and subsequently digested with *XbaI*. We subcloned the enhanced eGFP insert from the vector pEGFP-C2 (Clontech, Inc., Palo Alto, CA) into the prepared pHSVPrPuc as a *NheI/DraI* fragment. The pSVLac was described [12]. We subcloned the nlsLac encoding sequence from the PM48 plasmid into pHSVPrPuc amplicon at the *BamHI* site to create pHSVnlsLac. In addition to constructs with the immediate early 4/5 promoter (IE4/5), we used a construct possessing a basal promoter (minPr) driving LacZ expression to investigate whether the level of expression of a transcript was linked to gene product-induced toxicity. We constructed the minimal promoter-containing amplicon plasmid, pHSVminPrLac, by cloning a 0.7-kb fragment from pGRES-2 [14] into the pHSVminOriSlac amplicon vector. The cloned fragment contains the adenovirus 2 major late promoter (Ad2-MLP), followed by a segment of the rabbit β -globin exon 2-intron 2-partial exon 3 driving the expression of LacZ.

We packaged these constructs by two methods, one using a helper virus-based system as reported [15,16], and the second method using helper virus-free amplicon packaging technology prepared as described [17]. We titered Amplicon stocks as described [18], whereas we enumerated the helper virus component of helper virus-prepared stocks by plaque assay [19]. We transduced primary neuronal cultures 7 days after plating. We incubated cells for 3 hours in feed medium in the presence of virus (MOI 0.2), and we washed cultures twice with warm MEM and re-fed with fresh medium without virus.

Toxicity and apoptosis. The toxicity of reporter genes was assessed 1, 3, and 5 days after transduction, using several methods: 1) studying cellular morphology in neurons expressing a reporter; 2) counting the number of cells expressing a reporter gene product over time; and 3) counting the number of apoptotic neurons over time identified either by nuclear morphology (fragmented and condensed nucleus) or by TUNEL staining. We performed all experiments in triplicate.

At the three time points, we fixed transduced cultures for 1 hour at room temperature with freshly prepared paraformaldehyde (4%) in phosphate buffered saline (PBS). We briefly washed cultures three times in PBS, and two times for 5 minutes with Tris buffered saline (TBS, 0.1 M). We permeabilized cells for 10 minutes with TBS (0.1 M)-Triton X-100 (0.1%). We blocked nonspecific antibody binding by a 1-hour incubation at room temperature in blocking solution (0.1 M TBS, 1% Triton, 2% BSA, and 10% goat serum). Primary incubation was done overnight at 4°C with one of the following antibodies diluted in dilution buffer (TBS (0.1 M), 0.1% Triton, 2% goat serum): rabbit polyclonal anti-GFP (Chemicon, Temecula, Ca, 1:1000), mouse monoclonal anti- β -galactosidase (Chemicon, 1:1000), and rabbit polyclonal anti-glial fibrillary acidic protein (GFAP; Chemicon, 1:1000). The primary incubation was followed by five washes of 5 minutes in TBS (0.1 M), 10 minutes incubation in dilution buffer, and 2 hours of secondary incubation at room temperature with a Molecular Probe Alexa 488 goat anti-mouse or anti-rabbit secondary antibody (1:500 in dilution buffer). We subsequently washed cultures twice with TBS, incubated for 20 minutes with 4,6-diamidino-2-phenylindole (DAPI 1:10,000), and finally washed twice with distilled water before mounting.

TUNEL assay was performed after the permeabilization step and before the blocking of ICC using a modified version of the protocol provided with the Apotag Kit (Intergen, Purchase, NY). We washed permeabilized cultures twice with distilled water and then processed for tunnel staining following manufacturer's instructions until the end of the washing step with the Intergen Stop/Wash buffer. Cultures were then blocked as described above, and processed for primary antibody incubation (overnight

at 4°C) with a mixture of primary antibodies diluted in Intergen dilution buffer. The mixture of antibodies contained a primary antibody directed against one of the reporters and the Intergen sheep anti-digoxigenin secondary antibody labeled with rhodamine. Subsequent steps were identical to the ICC protocol described above. However, we performed the secondary incubation using a mixture of an Alexa 488 goat anti-mouse or anti-rabbit secondary antibody, and an Alexa 568 donkey anti-sheep antibody (Molecular Probe, Eugene, OR), which increased and prolonged the fluorescence red labeling of the TUNNEL assay.

For cell counting, we used a Provis Olympus microscope equipped with a 20 \times objective, fluorescence illumination, and filter set for FITC, TRITC, and DAPI fluorescence. We performed all counting on triplicates of each experimental condition.

Estimation of the level of expression of a reporter. We estimated the level of expression of the reporter by two different techniques. In a first series of experiments, we used a qualitative technique and an Optronix video camera with different image capture times. We defined a "low expressing" cell as a cell for which the capture time was 4 \times longer than for a "high expressing" cell. This technique allowed assigning a qualitative level of expression (high or low) of the reporter in a neuron that we subsequently analyzed for the occurrence of apoptosis.

The second method used to determine the expression level of reporter protein involved the assessment of β -galactosidase enzymatic activity of reporter gene using the Galacto-lite assay. We performed this assay in accordance with manufacturer's instructions (Applied Biosystems, Foster City, CA). We subsequently normalized the resultant β -galactosidase activity to the number of viral genomes detected using quantitative real-time PCR (QRT-PCR) analysis on viral and cellular DNA isolated from Galacto-Lite lysate. We performed QRT-PCR on duplicate samples using primers corresponding to the β -galactosidase gene present in the amplicon plasmid as published [18]. This second technique has the advantage of providing a quantified level of expression per viral genome, but the quantification of the level of expression and the quantification of apoptosis had to be performed in parallel cultures simultaneously transduced.

Statistical analysis. We performed two-way factorial ANOVA using the Statview 5 statistical package (Abacus Concepts, Inc., Cary, NC) to analyze the overall effect of time post-transduction and of the type of expressed reporter molecule. Fisher's post hoc tests were used to determine differences between time points or between different reporter molecules if we first identified an overall significant effect using ANOVA. Significance level was set at $P < 0.05$.

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Behavioral and Neurochemical Effects of Wild-Type and Mutated Human α -Synuclein in Transgenic Mice

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Human α -synuclein (h α -SYN) is implicated in the Parkinson's disease phenotype (PDP) based on a variety of studies in man, animal models, and *in vitro* studies. The normal function of h α -SYN and the mechanism by which it contributes to the PDP remains unclear. We created transgenic mice expressing either wild-type (hw α -SYN) or a doubly mutated (hm² α -SYN) form of h α -SYN under control of the 9-kb rat tyrosine hydroxylase promoter. These mice expressed h α -SYN in cell bodies, axons, and terminals of the nigrostriatal system. The expression of h α -SYN in nigrostriatal terminals produced effects in both constructs resulting in increased density of the dopamine transporter and enhanced toxicity to the neurotoxin MPTP. Expression of hm² α -SYN reduced locomotor responses to repeated doses of amphetamine and blocked the development of sensitization. Adult hw α -SYN-5 transgenic mice had unremarkable dopaminergic axons and terminals, normal age-related measures on two motor coordination screens, and normal age-related measures of dopamine (DA) and its metabolites. Adult hm² α -SYN-39 transgenic mice had abnormal axons and terminals, age-related impairments in motor coordination, and age-related reductions in DA and its metabolites. Expression of hm² α -SYN adversely affects the integrity of dopaminergic terminals and leads to age-related declines in motor coordination and dopaminergic markers. © 2002 Elsevier Science (USA)

Key Words: α -synuclein; transgenic mice; Parkinson's disease; nigrostriatal system; substantia nigra; dopamine; MPTP.

INTRODUCTION

Parkinson's disease (PD) is characterized by neuronal loss, inclusions, and gliosis in the substantia nigra (SN) resulting in dopamine (DA) deficiency in projection regions and clinical symptoms when the loss is extensive (20). The Parkinson's disease phenotype

(PDP) results from different mechanisms including neurotoxin exposure (25) or mutations including those in the α -synuclein (α -SYN) gene (23, 41). The causes of the PDP are poorly understood, but reflect risks associated with genetic background, environmental exposures, and aging (5). The human α -synuclein (h α -SYN) gene is implicated based on two known mutations (A53T and A30P; 23, 41) associated with autosomal dominant forms of the PDP, the presence of α -SYN in aggregates (2, 45), and possibly the presence of a polymorphism in the 5'-untranslated region of the h α -SYN gene associated with increased risk (21, 24). The function of α -SYN is unclear, but a role in presynaptic function has been suggested (1, 7). Overexpression of α -SYN in cell culture (38), transgenic mice (29), and transgenic *Drosophila* (12) suggests adverse effects and the generation of aggregates.

The first reported transgenic mice expressing wild-type h α -SYN gene demonstrated inclusions and motor dysfunction with reduced tyrosine hydroxylase (TH) at an older age (29). Other transgenic models identified abnormalities in neurons and processes (27, 50), but did not identify differences between wild-type and mutated h α -SYN. Other transgenic mice have failed to demonstrate a phenotype (30). We created transgenic mice using the 9-kb rat TH promoter to express either wild-type (hw α -SYN) or a doubly-mutated (hm² α -SYN) form of h α -SYN in dopaminergic neurons. In these mice h α -SYN is functionally active in DA terminals and its effects may be mediated in part through the dopamine transporter (DAT). Expression of hm² α -SYN leads to altered DA terminals with reduced response to repeated administration of amphetamine and DA terminal failure with aging resulting in progressive motor impairment. We propose that mutations in h α -SYN that result in the PDP may do so either by a gain of a toxic action or by interfering with the activity of hw α -SYN acting in DA terminals.

EXPERIMENTAL PROCEDURES

Generation and screening of transgenic mice. A clone (ID No. 48811) containing the full length wild-type h α -SYN cDNA was obtained from the IMAGE consortium (Research Genetics Inc., Huntsville, AL). A 469-bp fragment of the coding sequence was obtained using the PCR with forward (hSYNU1, 5'-caggtaccgacagcttggtgtaaggaat) and reverse (hSYNLI1, 5'-gatagctataaggcttcaggttcgtagctct) primers and subcloned into pGEMT (Promega Corp., Madison, WI). This vector was subjected to two rounds of *in vitro* mutagenesis (QuikChange site-directed mutagenesis kit, Stratagene Cloning Systems, La Jolla, CA.) with mutagenesis primers hSYNmut1U (ggagtggtgcatggtgtgacacagctggctgagaagacc) and hSYNmut1L (ggttctcagccactggttcacaccatgcaccactcc) to introduce the G209A mutation and primers hSYNmut2U (cagggtgtggcagaagcaccaggaagacaaaag) and hSYNmut2L (ctttgtcttctctggtgctcttccacacacctg) to introduce the G88C mutation. All constructs were sequenced. The h α -SYN and hm² α -SYN genes were subcloned into pUTHTV. pUTHTV is a cloning vector constructed from pBlue-script II KS (pBSKS, Stratagene Cloning Systems) by first introducing nine additional rare restriction endonuclease sites in the multiple cloning site to create pEKR.3. The 9-kb rat tyrosine hydroxylase promoter (34, 47), a splice donor/intron/splice acceptor (18), and a poly(A) site from the human growth hormone gene (46) were subcloned from 5' to 3' into pEKR.3 to create pUTHTV. Subcloning of h α -SYN into pUTHTV resulted in pUTHTV/h α -SYN and subcloning of hm² α -SYN into pUTHTV resulted in pUTHTV/hm² α -SYN. The transgenes were removed by a *PacI* digest, purified, and injected into oocytes obtained from C57/BL6 females. Founders and offspring were screened using the original PCR primers. The two constructs can be identified by restriction digests using *MwoI* following the PCR.

Mice. Mice were group or individually housed in microisolation cages, given free access to food and water, kept on a 12-h light/dark cycle, and cared for as approved by the University of Rochester Committee on Animal Resources. Mice were sacrificed by rapid cervical dislocation followed by rapid dissection of regions or whole brain removal followed by freezing and storage at -20°C. Other mice were anesthetized with Nembutal and perfused transcardially with 4% fresh paraformaldehyde (PFA). Perfused brains were postfixed for an additional 2 h in 4% PFA, processed through a graded series of sucrose, and stored at 4°C.

Unless otherwise stated, young mice (age 2–3 months) were used for all characterization studies. We used middle-aged (7–9 months) and old-aged (13–23 months) mice for selected behavioral and neurochemical studies.

Southern analysis. Tail genomic DNA was digested using *NcoI* and resulted in a fragment of 443 bp that was identified using a ³²P-labeled probe to the PCR product made using primers described earlier (hSYNU1 and hSYNLI1).

Reverse transcriptase polymerase chain reaction (RT-PCR). RT-PCR was performed on mRNA from brain, eyes, and adrenal gland. mRNA was obtained using the MicroPoly(A)Pure kit (Ambion, Austin, TX). mRNA was reverse transcribed using the RETROScript kit (Ambion) with 1–2 μ l of product used with PCR primers (SDU3, ttctggagccggaggg and 3'UTRL1, cgatgcccaggaatgctct). These primers cross the SD/SA region and will detect a product of size 1136 bp in genomic DNA and 846 bp in a mRNA/cDNA product that has been appropriately spliced.

Real-time quantitative reverse transcriptase polymerase chain reaction (QRT-PCR). QRT-PCR was performed after obtaining mRNA, which was reverse transcribed as described earlier and 1–2 μ l of product were used along with PCR primers (3'UTRFwd, ggcagctagaagccacagct and 3'UTRRev, tggacaaggtcgagacattcc) directed to the 3'-UTR region of the transgene and a FAM-labeled probe (FAM-cggcactgcacgatgcccga-TAMRA).

In situ hybridization histochemistry (ISHH). ISHH was performed using a 48-mer DNA oligonucleotide probe (aatgtctcgacctgttcctatgtccttctctgaagcagtagagcagcccc) directed to the 3'-UTR region using methods previously described (44) with a hybridization temperature of 35°C and a wash temperature of 39°C. No hybridization was seen in nontransgenic littermates.

Immunohistochemistry (IHC). Brains were sectioned at 40 μ m using a sliding freezing microtome, and sections were collected in cryoprotectant and stored at -20°C. Single- and double-label free-floating IHC was performed using a rabbit polyclonal anti-human α -SYN Ab (1:7,500, Affiniti Research, Exeter, UK). This Ab is specific for h α -SYN protein as mouse α -SYN protein was not detected under the conditions used for IHC nor following Western blotting. The Alexa Fluor 594 goat anti-rabbit IgG fluorescent secondary Ab (1:750, Molecular Probes, Eugene, OR) was used. To identify mouse TH, a mouse monoclonal anti-TH Ab (Chemicon Int., Temecula, CA) was used (1:250) along with the Alexa Fluor 488 goat anti-mouse fluorescent secondary Ab (1:500, Molecular Probes). All sections were also incubated with 4',6-diamidino-2-phenylindole (DAPI, 1:10,000, Sigma, St. Louis, MO) to identify nuclear DNA. All sections were coverslipped with Mowiol 4-88 (Polysciences, Inc., Warrington, PA). Slides were observed using either an Olympus Provis microscope or an Olympus Fluoview Personal Confocal microscope system (v1.2).

The integrated optical density of different antibody staining was measured in the dorsal striatum, ventral striatum, and olfactory tubercle of different lines of

mice. Each section served as its own control as only the ratio between different regions on the same section was measured, eliminating difficulties in controlling for labeling intensity across animals, antibodies, and experiments. The ratio of integrated optical density was calculated comparing optical densities between ventral striatum/dorsal striatum and olfactory tubercle/dorsal striatum using the rabbit polyclonal anti-human α -SYN Ab and the mouse monoclonal anti-TH Ab. Measurements were taken from 3–12 different mice. Values reported were the mean \pm SEM.

Two-dimensional (2-D) polyacrylamide gel electrophoresis (PAGE) and Western blotting. Protein from mouse striata was subjected to two-dimensional electrophoresis (36) by Kendrick Labs, Inc. (Madison, WI). Isoelectric focusing (IEF) was first carried out using 2% pH 4–8 ampholines (BDH ampholines, Gallard Schlesinger, Long Island, NY) for 9600 V-h. One microgram of an IEF standard, tropomyosin, was added and migrates as a doublet with the lower polypeptide spot of MW 33,000 and pI 5.2. After equilibration in buffer "0" (10% glycerol, 50 mM dithiothreitol, 2.3% SDS, and 0.0625 M Tris, pH 6.8) each tube gel was sealed to the top of a stacking gel on top of a 10% acrylamide slab gel. After slab gel electrophoresis, the gel was placed in transfer buffer (12.5 mM Tris, pH 8.8, 86 mM glycine, 10% methanol) and electrophoretically transferred onto PVDF paper overnight at 200 mA and approximately 100 V/two gels. The blot was stained with Coomassie brilliant blue.

The PVDF membranes were prewetted in 100% MeOH, transferred to Tris-buffered saline containing Tween (TBS-T, 20 mM Tris, pH 7.6, 137 mM NaCl, 0.1% Tween 20), blocked in TBS-T plus 5% milk, and incubated for 3 h in TBS-T plus 5% milk with an anti- α -SYN Ab (1:5,000, Chemicon Inc.) that recognizes both the mouse and human forms of α -SYN. Membranes were rinsed in TBS-T, incubated with an HRP-conjugated goat anti-rabbit secondary (1:5000, Chemicon Inc.) for 1 h, and then sequentially rinsed in TBS-T, TBS, and finally Tris-HCl (0.1 M, pH 8.6). Membranes were transferred to freshly prepared enhanced chemiluminescence solution containing 5.5 mg luminol, 0.28 mg *p*-coumaric acid, 7.7 μ l of H₂O₂ (30%) in 25 ml Tris-HCl (0.1 M, pH 8.6), and gently agitated for 90 s. Membranes exposed to Kodak Biomax MR film.

Films were digitized using a CCD camera and analyzed using the 2-D gel analysis module of the MCID image analysis system (Imaging Research, Inc., St. Catharines, Ontario, Canada). The spot volume was determined as the area times the optical density.

DAT quantitative autoradiography (QAR). DAT autoradiography was performed and analyzed as previously described (43). Mouse sections were incubated with [³H]GBR 12935 (0.25 nM) and Zn²⁺ (50 μ M). Films were exposed for 10 days.

High-performance liquid chromatography (HPLC). HPLC was performed by either of two methods in two different labs depending on the age group of the animals. HPLC was always performed comparing transgenic mice with age-matched nontransgenic littermates. Values were normalized to concurrent nontransgenic age-matched littermate controls. Dissected striata of adult mice were homogenized in 0.1 M HClO₄ containing 100 ng/ml 3,4-dihydroxybenzylamine (DHBA) as an internal standard. Homogenates were centrifuged, and supernatants were filtered and analyzed for levels of DA, 3,4-dihydroxyphenylacetic acid (DOPAC), and homovanillic acid (HVA) using high-performance liquid chromatography with electrochemical detection (51). DA and metabolites were separated on a microbore reverse-phase column (C-18, 5 μ m, 1 \times 150 mm, Unijet, BAS) with a mobile phase consisting of 0.03 M citrate-phosphate buffer with 2.1 mM octyl sodium sulfate, 0.1 mM EDTA, 10 mM NaCl, and 17% methanol (pH 3.6) at a flow rate of 90 μ l/min and detected by a 6-mm glass carbon electrode (Unijet, BAS) set at +0.8 V.

Alternatively, striatal tissues were processed as previously described (49). Briefly, tissue was dissected and placed in 0.1 M perchloric acid. The tissues were sonicated and centrifuged. The supernatants were stored at -80°C until analyzed for the concentrations of DA, DOPAC, and HVA by HPLC-EC. The pellets were later digested in 1 ml of 0.5 N NaOH for measurements of protein concentration using Bio-Rad assay reagents. The concentrations of the neurotransmitters were expressed in terms of ng/mg protein. DA turnover was expressed as the ratio DOPAC/DA.

Striatal MPP⁺ determinations. Striatal MPP⁺ levels were determined at 90 and 240 min after a single ip injection of 15 mg/kg MPTP. These two time points were chosen since previous work has shown that, under these experimental conditions, maximum levels of striatal MPP⁺ are reached at 90 min, whereas approximately 50% of MPP⁺ is eliminated by 240 min (4). MPP⁺ was assayed in acid-extracted samples by HPLC using a Selectosil 5 SCX column (Phenomenex, Torrance, CA). The mobile phase was delivered at a flow rate of 1.0 ml/min and consisted of a 90% mixture of 0.1 M acetic acid and 0.15 M triethylamine hydrochloride (adjusted to pH 2.3 with formic acid) and 10% acetonitrile. Detection wavelength was set at 295 nm and retention time was 11.5 min.

Behavioral assessments. Locomotor activity was quantified using automated locomotor activity chambers (Opto-Varimex Minor, Columbus Instruments International Corporation, Columbus, OH) as previously described (3, 49). Each chamber was equipped with infrared photobeams. Total photobeam breaks were recorded each minute for 45 min for horizontal, vertical, and ambulatory movements. Mice were initially

habituated to the locomotor activity chambers in three 45-min sessions (baseline activity) occurring on consecutive days, with all mice receiving vehicle ip injections prior to the session. Activity is presented as total counts.

After the third habituation session, some mice (non-transgenic littermate controls, lines $hw\alpha$ -SYN-5, and $hm^2\alpha$ -SYN-39) were administered 15 mg/kg MPTP ip and motor activity assessed 1 h, 24 h, and 5 days after each injection. MPTP injections were separated by 1 week. Two doses of MPTP were used. Animals were sacrificed 6 days after the second MPTP treatment.

After the third habituation session other mice (non-transgenic littermate controls, line $hw\alpha$ -SYN-5, and line $hm^2\alpha$ -SYN-39) were administered 0.375 mg/kg amphetamine ip and the effects on motor activity assessed immediately afterward (first treatment). Amphetamine was repeated 7 days later and the effects assessed immediately afterward (second treatment). In a separate experiment, mice (nontransgenic littermate controls, line $hw\alpha$ -SYN-88, and line $hm^2\alpha$ -SYN-10) were treated with biweekly injections of amphetamine (1 mg/kg) and motor activity assessed immediately afterward. Mice received six biweekly injections and then received a rechallenge dose 1 week later.

The motor coordination task was an inverted wire screen hanging test. Mice were placed individually on top of a square wire screen (13 × 13 cm of No. 4 mesh) mounted horizontally on a metal rod. The rod was then rotated 180°. The time taken to climb to the top of the screen was recorded with 2 min recorded if mice fell or remained clinging to underside of the screen. This test has demonstrated sensitivity to drugs such as haloperidol and results in fewer failures (e.g., falling or clinging) and less opportunity for incompatible interfering behavior than techniques such as the Rotarod (8, 31). Each mouse was tested on three separate trials at each session and the mean value of the last two trials was recorded.

Statistical analyses. Analysis of DA and metabolites were carried out with repeated measures analyses of variance (RMANOVA), with transgene status as a between-groups factor and neurotransmitter/metabolite as a within factor since these levels were derived from the same brain. Similarly, DAT densities were analyzed using RMANOVA with brain region as a within group variable. This analytical approach provided conservative estimates. Motor activity level and inverted screen time across age were analyzed using RMANOVA with block (time) as a within-group factor and transgene status as a between groups factor. Total activity counts were compared with one-factor ANOVA using transgene status and age. Locomotor activity changes following MPTP treatment were also analyzed by RMANOVA across conditions (baseline-MPTP-recovery) and DAT density by RMANOVA with brain region as a within-factor. Significant effects were de-

termined as $P \leq 0.05$. Significant effects determined by RMANOVA were followed by one-factor ANOVAs or Fisher's protected least significant differences test as appropriate.

RESULTS

Description of mice. We created multiple lines of transgenic mice expressing either the wild-type $h\alpha$ -SYN ($hw\alpha$ -SYN) gene or a doubly mutated ($hm^2\alpha$ -SYN) form of the $h\alpha$ -SYN gene containing both reported human mutations associated with the PDP (Fig. 1A). Five founders from each construct were generated. Lines transmitting the transgene to offspring were characterized by Southern analysis, RT-PCR, QRT-PCR, ISHH, IHC, and 2-D PAGE followed by Western blotting. Digestion of genomic DNA using *Nco*I demonstrated a transgene specific fragment and mRNA expression was detected in the midbrain, eye, and adrenal gland using RT-PCR in all lines (data not shown). Lines $hw\alpha$ -SYN-5 and $hm^2\alpha$ -SYN-39 had higher expression of mRNA in the midbrain using QRT-PCR (data not shown) and ISHH (Fig. 1B) compared to lines $hw\alpha$ -SYN-88 and $hm^2\alpha$ -SYN-10. High levels of $h\alpha$ -SYN protein were detected in the cell bodies of dopaminergic neurons in the midbrain (Fig. 1C) and its main projection region the striatum (Fig. 1D) in all lines. Non-transgenic littermate controls did not express transgene $h\alpha$ -SYN mRNA nor protein as assessed using IHC and a $h\alpha$ -SYN specific Ab (Fig. 1E) nor by 2-D PAGE and Western blotting using a non-species-specific α -SYN Ab (Fig. 1F). In the brain of transgenic mice, catecholaminergic nuclei expressed both $h\alpha$ -SYN mRNA and protein. Labeling of $h\alpha$ -SYN protein was highest in the cell bodies and dendrites of SN and locus ceruleus (LC), their axons and terminal axon fields. The distribution of cells expressing the transgene and protein was consistent with others using this TH promoter (34, 47). The quantity of $h\alpha$ -SYN protein was measured in two lines from each construct using 2-D PAGE and Western blotting. The endogenous $m\alpha$ -SYN protein migrated as two spots with one larger isoform compared to a second very small isoform migrating at a slightly more basic pI and a slightly smaller mass and may represent a dephosphorylation product (Fig. 1F; 35). Both the $hw\alpha$ -SYN (Fig. 1H) and $hm^2\alpha$ -SYN (Fig. 1G) protein migrated as single spots having slightly more acidic pIs than the endogenous $m\alpha$ -SYN protein and similar apparent masses. The amount of $h\alpha$ -SYN protein was always less than the amount of endogenous $m\alpha$ -SYN in the striatum. The percentage of $h\alpha$ -SYN compared to $m\alpha$ -SYN (100% control) varied between the different transgenic lines ($33.1 \pm 8.9\%$ in $hw\alpha$ -SYN-5, $41.3 \pm 32.3\%$ in $hw\alpha$ -SYN-88, $19.4 \pm 10.2\%$ in $hm^2\alpha$ -SYN-10, and $48.8 \pm 5.0\%$ in $hm^2\alpha$ -SYN-39). The fraction of $h\alpha$ -SYN to the total amount of α -SYN ($h\alpha$ -SYN + $m\alpha$ -SYN) in the striatum of these

constructs and lines was calculated ($24.7 \pm 5.1\%$ in hw α -SYN-5, $27.3 \pm 16.6\%$ in hw α -SYN-88, $15.7 \pm 7.5\%$ in hm $^2\alpha$ -SYN-10, and $32.8 \pm 2.3\%$ in hm $^2\alpha$ -SYN-39; mean \pm SD, $n = 2-4$).

One line of each construct (hw α -SYN-5 and hm $^2\alpha$ -SYN-39) was selected for further detailed study based on their high and comparable levels of h α -SYN mRNA expression in the SN, h α -SYN protein expression in the SN and striatum determined by IHC, and h α -SYN protein levels in the striatum determined by 2-D PAGE and Western blotting. Two additional lines (hw α -SYN-88 and hm $^2\alpha$ -SYN-10) were examined to confirm and extend selected findings.

Immunohistochemistry. The localization of h α -SYN protein was compared to TH using double-label IHC (Fig. 2) in both lines of transgenic mice and in nontransgenic littermate controls. Both h α -SYN and TH (Figs. 2A–2C) were colocalized in cell bodies (Figs. 2D–2F) and dendrites of the SN, VTA, and LC, their axons (Figs. 2G–2I), and in their terminal projection regions, the dorsal striatum (DS), nucleus accumbens (Acb), olfactory tubercle (OT), and cerebral cortex. The amount of h α -SYN protein varied among cells of the SNpc and VTA and was present in most cells that also expressed TH protein. The number of cells expressing the h α -SYN protein or the amount of protein per cell was observed to be greater in the VTA than in the SN. This corresponded to the amount of h α -SYN protein in projection regions which were observed to be greater in the Acb and OT than in the DS as visualized using IHC (Fig. 2B). The ratios of optical density for anti-TH labeling in nontransgenic littermate controls, line hw α -SYN-5, and line hm $^2\alpha$ -SYN-39 in dorsal striatum/ventral striatum (0.99 ± 0.03 , 1.01 ± 0.01 , and 1.03 ± 0.00 , respectively) and olfactory tubercle/dorsal striatum (0.99 ± 0.03 , 1.03 ± 0.02 , and 0.97 ± 0.04 , respectively) were all similar (all P s > 0.05 comparing transgenic lines to nontransgenic littermate controls). The ratios of optical density for anti-h α -SYN labeling in lines hw α -SYN-5 and hm $^2\alpha$ -SYN-39 in dorsal striatum/ventral striatum (1.13 ± 0.02 , $p = 0.02$ and 1.22 ± 0.03 , $P = 0.006$, respectively) and olfactory tubercle/dorsal striatum (1.01 ± 0.03 , $P > 0.05$; and 1.15 ± 0.04 , $P = 0.02$, respectively) were different. Whereas TH was strictly cytoplasmic in dopaminergic neurons (Figs. 2D and 2F), h α -SYN was cytoplasmic and nuclear (Figs. 2E and 2F) as originally described by others (28). Nuclear localized h α -SYN protein was absent in the nucleolus. In line hm $^2\alpha$ -SYN-39, the abnormal axons visualized in the median forebrain bundle (Fig. 2L) were more dilated and beaded in appearance compared to those seen in nontransgenic littermate controls (Figs. 2J). The terminals in the Acb of mice from line hm $^2\alpha$ -SYN-39 also appeared abnormal (Fig. 2M) with the smaller caliber processes and terminals often dilated or enlarged compared to those seen in nontransgenic

littermate controls (Fig. 2K). Neither compact round cytoplasmic inclusions nor intranuclear inclusions were identified in either transgenic line at 4 months of age. The cytoplasmic staining of TH and h α -SYN proteins was heterogeneous.

Dopamine transporter autoradiography. The density of the DAT was determined in young mice using QAR in three regions (DS, Acb, and OT) receiving dopaminergic input (Fig. 3A). The DAT density was increased in different subregions of the striatum in line hw α -SYN-5 (11–21%) and in line hm $^2\alpha$ -SYN-39 (12–23%) compared to nontransgenic littermates, although the small sample size might have limited the degree of significance (RMANOVA main effect of transgene status $F(2,9) = 3.395$, $P = 0.08$, $n = 4$ per group). Post hoc analysis of the effect of specific transgenes suggested again that the density of the DAT was greater in each transgenic line compared to nontransgenic littermate controls (Fisher's PLSD comparing line hw α -SYN-5 to nontransgenic littermates, $P = 0.08$ and line hm $^2\alpha$ -SYN-39 to nontransgenic littermates, $P = 0.04$). The elevated DAT density in the transgenic lines was greatest in the Acb, with a 23% increase in line hm $^2\alpha$ -SYN-39 and a 21% increase in line hw α -SYN-5 consistent with the region expressing the greatest amount of h α -SYN protein visualized using IHC.

Effect of amphetamine. We next sought to determine if expression of h α -SYN altered the response to systemic amphetamine (Fig. 3B). We habituated young mice to a locomotor chamber following ip injections of saline. We then injected mice with either saline or a low dose of amphetamine (0.375 mg/kg) ip for two treatments separated by 1 week. RMANOVA revealed a significant interaction between lines of mice, treatment, and drug ($F(5,29) = 17.16$, $P < 0.0001$). There was no significant effect of amphetamine compared to saline after the first treatment (Fig. 3B, left). However, a significant effect of amphetamine after the second treatment occurred revealing a significant interaction between line of mice and drug ($F(5,29) = 16.7$, $P < 0.0001$, Fig. 3B, right). A post hoc analysis showed significant increases in horizontal activity in both transgenic lines and nontransgenic littermate controls compared to saline (all P s < 0.05). The effect of amphetamine was significantly different in both transgenic lines compared to nontransgenic littermates, with line hw α -SYN-5 having a significantly greater response ($P < 0.02$) and line hm $^2\alpha$ -SYN-39 having a significantly reduced response ($P < 0.003$) compared to nontransgenic littermate controls.

We then determined if the reduced sensitization-like response to amphetamine seen in line hm $^2\alpha$ -SYN-39 was present in line hm $^2\alpha$ -SYN-10 using a repeated dosage paradigm. We treated male nontransgenic littermate controls and lines hw α -SYN-88 and hm $^2\alpha$ -SYN-10 with biweekly doses of amphetamine (1 mg/

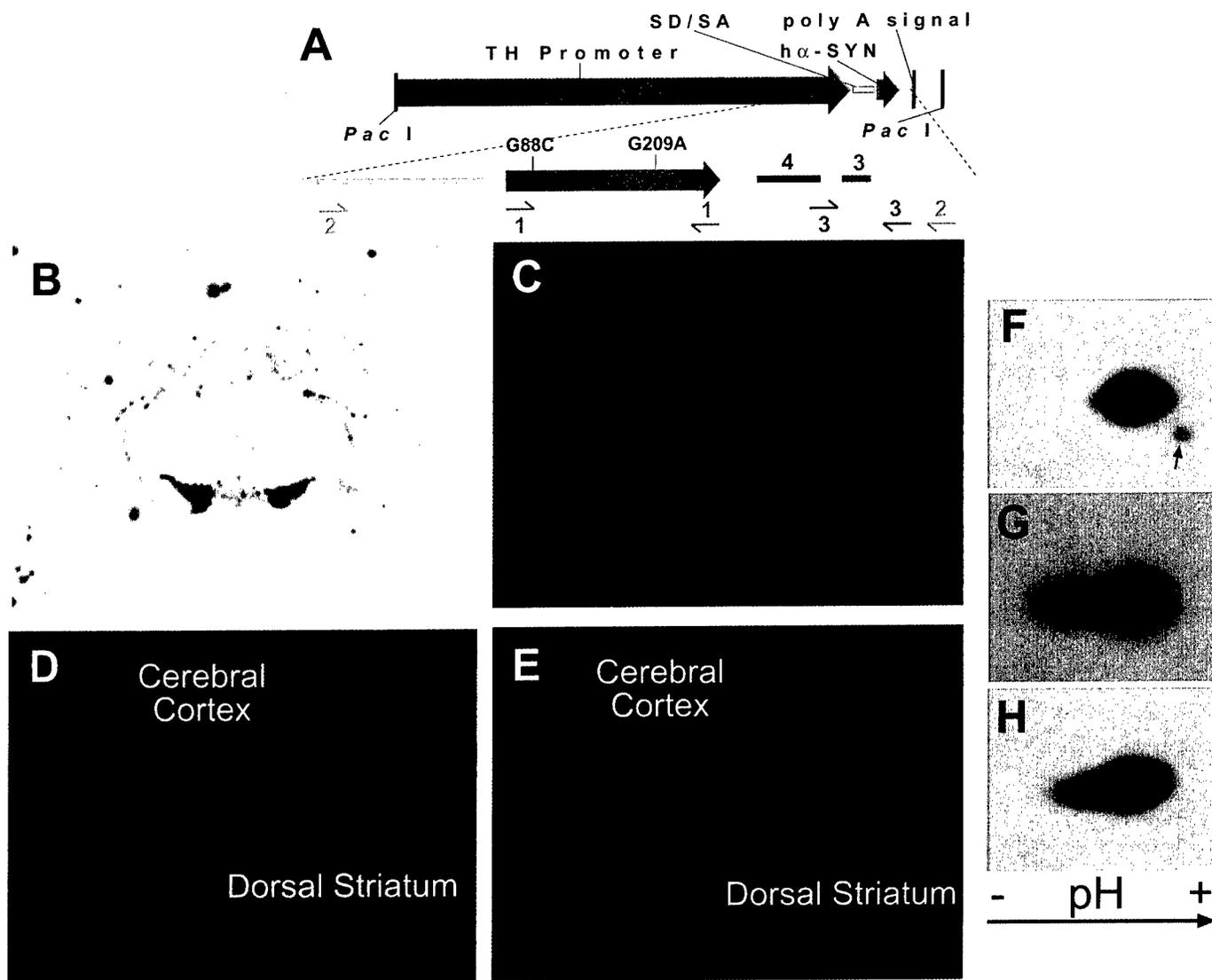


FIG. 1. Generation and characterization of $h\alpha$ -SYN transgenic mice. (A) Map of the pUTHTV/ $hm^2\alpha$ -SYN construct. Sites for PCR primers used to screen genomic DNA and amplify the original $h\alpha$ -SYN cDNA (1), sites for PCR primers used for RT-PCR to identify mRNA/cDNA and appropriate splicing of the construct (2), real-time QRT-PCR primers (3) and probe (3) for detecting and quantifying mRNA, and the site of the 48-mer oligonucleotide probe (4) used for ISHH. (B) Image of ISHH from line $hm^2\alpha$ -SYN-39 at the level of the SN. (C-E) IHC using a human specific polyclonal Ab to $h\alpha$ -SYN in line $hm^2\alpha$ -SYN-39 (C, D) or a nontransgenic littermate control (E). This Ab detected only $h\alpha$ -SYN protein (C, D), but not $m\alpha$ -SYN (E). Human α -SYN protein was most highly expressed in the cell bodies and dendrites in the SN and VTA, their axons, and in the striatum (D). Striatal protein was subjected to 2-D PAGE followed by Western blotting using a polyclonal Ab to α -SYN that recognizes both mouse ($m\alpha$ -SYN) and human ($h\alpha$ -SYN) α -SYN. (F) Nontransgenic littermate controls demonstrated a larger single spot migrating at approximately 15 kDa and a much smaller spot (arrow) migrating at a slightly smaller MW and at a slightly more basic pI suggestive of a dephosphorylated form of $m\alpha$ -SYN. Lines $hm^2\alpha$ -SYN-39 (G) and $hw\alpha$ -SYN-5 (H) demonstrated unique spots migrating at a similar MW to $m\alpha$ -SYN, but at different and slightly more acidic pIs.

kg). The seventh dose of amphetamine (rechallenge dose) was given after a 1 week drug-free interval. There was a significant interaction between line of mice, treatment, and drug ($F(35,574) = 1.695$, $P < 0.009$). Post hoc testing demonstrated that line $hm^2\alpha$ -SYN-10 did not sensitize to amphetamine ($P > 0.05$) at the seventh dose in contrast to both nontransgenic littermate controls and line $hw\alpha$ -SYN-88 which did sensitize (both P s < 0.05).

Effect of MPTP. We tested if vulnerability to MPTP neurotoxicity was altered in the transgenic lines. Young mice were habituated to an automated locomotor apparatus and then received two low doses of MPTP (15 mg/kg) ip separated by 1 week with locomotor testing 1 h, 1 day, and 5 days after each treatment. Both $h\alpha$ -SYN transgenic lines demonstrated enhanced behavioral sensitivity to MPTP (Fig. 4A) and altered postmortem densities of the DAT (Fig. 4B) following

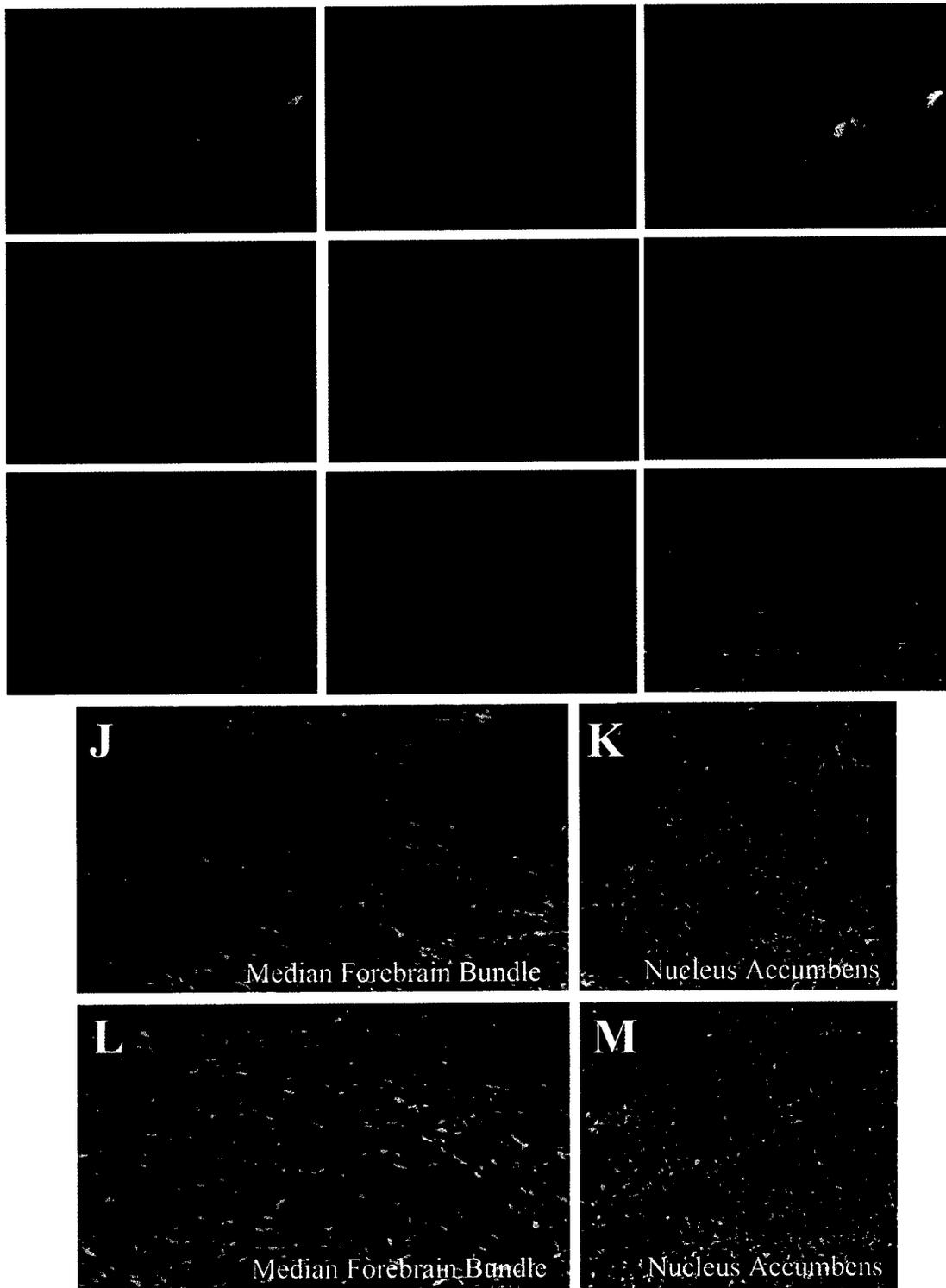


FIG. 2. Colocalization of $h\alpha$ -SYN protein and abnormal axons and neurites in young $h\alpha$ -SYN transgenic mice. Double-label fluorescent IHC for TH (A, D, G, green), $h\alpha$ -SYN (B, E, H, red), both $h\alpha$ -SYN and TH (C, F, I) in a sagittal section from line $hm^2\alpha$ -SYN-39 (A–C), a line $hw\alpha$ -SYN-5 SN neuron (D–F), or from a line $hm^2\alpha$ -SYN-39 mouse median forebrain bundle (MFB, G–I). Confocal microscopy was used to image sections at $0.4\ \mu\text{m}$ thickness using either the red or green filter (D–I) or routine fluorescent microscopy at $1.25\times$ (A–C). The $h\alpha$ -SYN protein was present in dopaminergic cell bodies and dendrites (B and E) and nigrostriatal axons and terminals (B and H) in a distribution that matched that of TH (A, D, and G). The $h\alpha$ -SYN protein was present in both the cytoplasm and the nucleus of neurons (E). Abnormal neurites were present in dendrites of SN and VTA neurons and axons to the striatum (G–I) and elsewhere. Fluorescent IHC for TH was performed in a nontransgenic littermate control mouse (J and K) or a mouse from line $hm^2\alpha$ -SYN-39 (L and M) in the MFB (J and L) or the nucleus accumbens (K and M). Confocal microscopy using a $100\times$ objective at $0.5\text{-}\mu\text{m}$ steps was performed and a total of 10 sections stacked to make the final image. TH is normally present in MFB axons of control mice in a discontinuous manner with the majority of axons having a uniform diameter with occasional modest dilations (J). In $hm^2\alpha$ -SYN-39 transgenic mice TH axons were more beaded and dilated in appearance with more discontinuities (L). In the Acb, TH was present in smaller caliber processes and terminals of control mice (K). In $hm^2\alpha$ -SYN-39 transgenic mice the smaller caliber processes and terminals were more dilated and punctate (M).

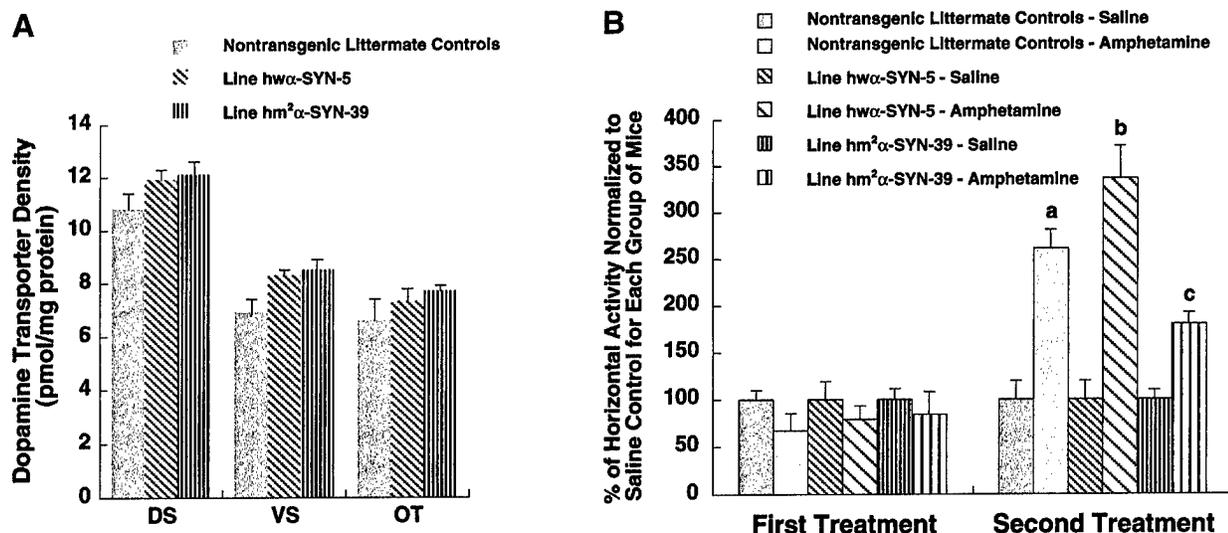


FIG. 3. Density of the dopamine transporter (DAT) and locomotor response to amphetamine in young $h\alpha$ -SYN transgenic mice. (A) Both transgenic lines ($hw\alpha$ -SYN-5 and $hm^2\alpha$ -SYN-39) demonstrated modest, but significant, increases (10–20%) in the density of the DAT that varied by line and region. (B) Mice were given either saline or amphetamine (0.375 mg/kg) ip and assessed for locomotor activity over a 45-min test session (first treatment). The treatment was repeated seven days later (second treatment). RMANOVA revealed a significant interaction between lines of mice, treatment, and drug ($F(5,29) = 17.16$, $P < 0.0001$). Post hoc testing revealed no significant differences between lines of mice or drug during the first treatment ($F(5,29) = 0.55$). Post hoc testing revealed significant interactions between line of mice and drug on the second treatment ($F(5,29) = 16.7$, $P < 0.0001$). ^{a,b,c}Significant effect of amphetamine compared to saline were found in all three lines on the second treatment (all P values < 0.05). Both lines ^a $hm^2\alpha$ -SYN-39 ($P < 0.03$) and ^b $hw\alpha$ -SYN-5 ($P < 0.02$) were significantly different from nontransgenic littermate controls and from each other ($P < 0.0001$).

the second treatment with MPTP. Significantly greater decreases in locomotor activity (1 hour after injection) compared to baseline were seen following both doses of MPTP in line $hw\alpha$ -SYN-5 (81% decrease from baseline, $P < 0.001$) and line $hm^2\alpha$ -SYN-39 (78% decrease from baseline, $P < 0.0001$) compared to nontransgenic littermates (34% decrease). Both lines $hw\alpha$ -SYN-5 line (11% decrease from baseline) and $hm^2\alpha$ -SYN-39 (21%

decrease from baseline) exhibited an incomplete recovery in locomotor activity 24 h following the first dose of MPTP. The density of the DAT (Fig. 5B) measured 6 days after the last dose of MPTP was reduced to a significantly greater extent following the two MPTP treatments in both transgenic $h\alpha$ -SYN lines (main effect of transgenic status $F(2,30) = 4.76$, $P = 0.016$) compared to nontransgenic littermate control mice.

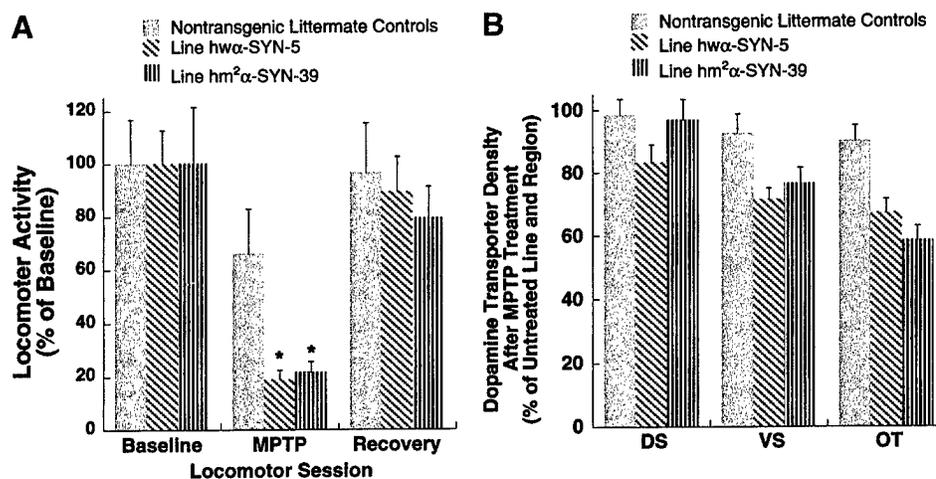


FIG. 4. Effects of MPTP on young $h\alpha$ -SYN transgenic mice. (A) Locomotor activity as percentage of score in mice prior to MPTP (baseline), 1 h (MPTP), and 5 days after MPTP (recovery). Both transgenic lines demonstrated significantly greater reduction in locomotor activity 1 h after MPTP compared to nontransgenic littermate controls ($*P < 0.001$). (B) The density of the DAT (reported as the % of the untreated value in each line for each region) decreased in both lines of transgenic mice to a greater extent than in nontransgenic littermate controls after the two low doses of MPTP (ANOVA main effect of transgenic status $F(2,30) = 4.76$, $P = 0.016$). The decreases varied by region and line.

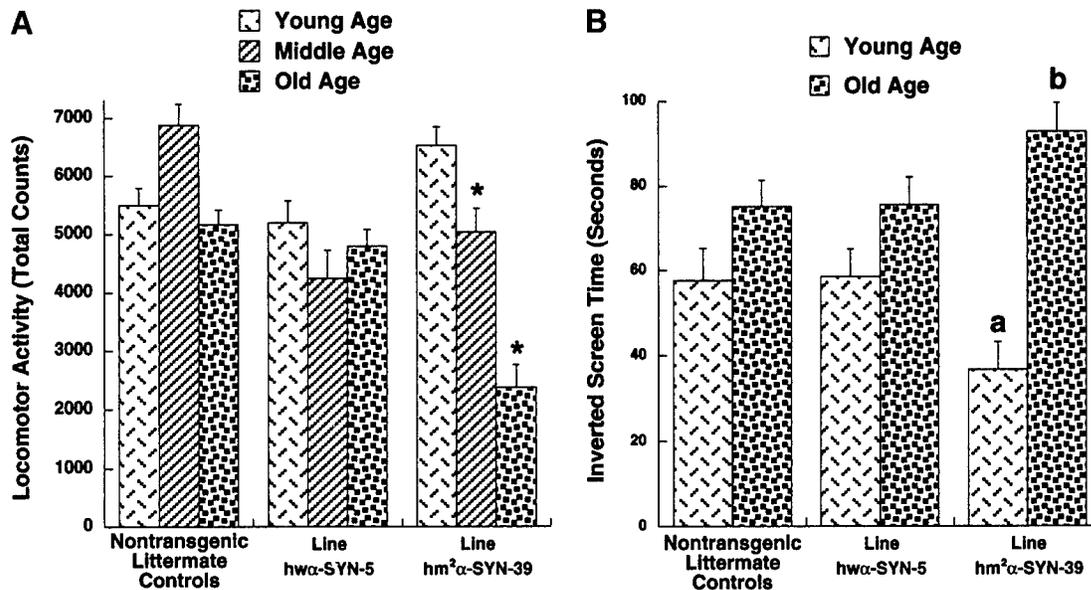


FIG. 5. Age-related behavioral measures. (A) Horizontal activity was measured for each group at three ages, young age (2–3 months), middle age (7–9 months), and old age (13–23 months). RMANOVA demonstrated a significant effect of transgenic status ($F(2,286) = 11.0$, $P < 0.0001$), age ($F(2,286) = 17.3$, $P < 0.0001$) and an interaction between group and age ($F(4,286) = 11.5$, $P < 0.0001$). ^aIndividual post hoc analyses demonstrated that at each age, line h $m^2\alpha$ -SYN-39 was significantly different from nontransgenic controls and line h ω α -SYN-5 (all P values < 0.05). ^{*}Line h $m^2\alpha$ -SYN-39 demonstrated a progressive age-related decline with both the middle and old age groups significantly different from the young age group (both P values < 0.0001). (B) Time to right from an inverted screen was measured for each group at two ages, young age (2–3 months) and old age (13–23 months). RMANOVA demonstrated a significant interaction of transgenic status and age ($F(2,181) = 4.6$, $P = 0.01$). ^aIndividual post hoc analyses demonstrated that line h $m^2\alpha$ -SYN-39 at a young age was significantly different from both nontransgenic littermate controls and line h ω α -SYN-5 (both P s < 0.03). ^bIndividual post hoc analyses demonstrated that line h $m^2\alpha$ -SYN-39 at an old age was significantly different compared to the younger age ($P < 0.0001$).

The decline in DAT density was greatest in the Acb and OT compared to the DS.

We next tested the possibility that increased sensitivity to MPTP may be due increased tissue levels of MPP⁺, the metabolite resulting in toxicity of MPTP. We measured striatal MPP⁺ levels after a single ip dose of 15 mg/kg of MPTP (same dose used in the prior experiment) at two time points, 90 and 240 min after injection. These times represent the peak and ~50% elimination times in mice (4). Values at 90 and 240 min were 19.0 ± 2.3 and 10.5 ± 2.9 (nontransgenic littermates, mean \pm SEM), 14.9 ± 2.6 and 5.2 ± 0.4 (line h ω α -SYN-5), and 16.6 ± 3.1 and 8.8 ± 1.8 (line h $m^2\alpha$ -SYN-39), respectively. There was a significant decline at 240 min compared to 90 min for each of the three lines examined (nontransgenic littermates ~45% decline, line h ω α -SYN-5 ~65% decline, and line h $m^2\alpha$ -SYN-39 ~47% decline, ANOVA $F(1,17) = 21.4$, $P = 0.0002$, all post hoc P s < 0.05 comparing 90 to 240 min for each line). Although both transgenic lines had less MPP⁺ in the striatum at both time points compared to nontransgenic littermates, the differences between lines were not statistically significant ($F(2,17) = 2.23$, $P = 0.13$). There was no significant transgenic status versus time interaction ($F(2,17) = 1.9$, $P = 0.91$). These data suggest that the behavioral and DAT density differences after MPTP seen in both transgenic lines

(h ω α -SYN-5 and h $m^2\alpha$ -SYN-39) compared to nontransgenic littermates were not due to differences in striatal MPP⁺ levels or elimination rate.

Effect of aging. Finally, we examined age-dependent and gender effects of h α -SYN expression on two measures of motor behavior, spontaneous locomotor activity (Fig. 5A) and the ability to right from an inverted position (Fig. 5B). Neither transgenic line had differences in weight compared to nontransgenic littermates at any age. Analyses by gender did not demonstrate a significant gender effect in any group at any age, so genders were combined for both behavioral measures. Horizontal locomotor activity was measured at three ages, young age (2–3 months), middle age (7–9 months), and old age (13–23 months) and the inverted screen test was performed at two ages, young age (2–3 months) and old age (13–23 months). Both transgenic lines and nontransgenic littermates were always age-matched for both behavioral measures. In both behavioral assays, there was a significant effect of age, of transgene status, and an interaction between transgene and age (all P values < 0.05). At the young age, line h $m^2\alpha$ -SYN-39 was more active and had a shorter time to right than either line h ω α -SYN-5 or nontransgenic littermate controls (all P values < 0.05). Line h $m^2\alpha$ -SYN-39 had a progressive and significant decline

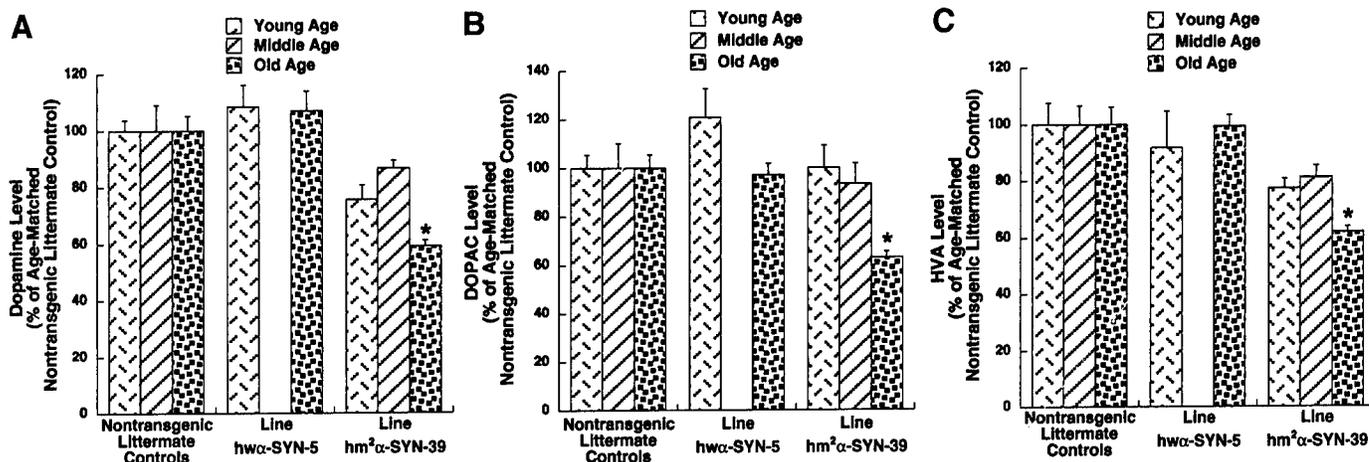


FIG. 6. Age-related striatal neurotransmitter levels. HPLC data for DA (A), DOPAC (B), and HVA (C) from striatum were obtained at three ages: young age (2–3 months), middle age (7–9 months), and old age (16–18 months) except for line $hw\alpha$ -SYN-5 in which values were obtained only at the young and old ages. The HPLC data were collected in two different labs using different procedures and equipment. However, nontransgenic littermate control mice were always included in each assay. All values were normalized to the mean of the nontransgenic littermates at the appropriate age (error bars indicate SEM). The absolute values (mean \pm SEM in units of ng/mg wet tissue weight or in units of ng/mg protein) for the nontransgenic littermate group from the two labs: DA (7.59 ± 0.29 ; 121.5 ± 6.63), DOPAC (1.04 ± 0.06 ; 8.92 ± 0.56), and HVA (1.01 ± 0.08 ; 8.18 ± 0.51). Levels of DA and DA metabolites in line $hw\alpha$ -SYN-5 were similar to those of nontransgenic littermates at the two ages examined. RMANOVA demonstrated a significant effect of transgene, age, and an interaction for each measure (all P values < 0.05). *Post hoc analyses revealed a significant decline in all three measures in line $hm^2\alpha$ -SYN-39 at the old age compared to nontransgenic littermates and line $hw\alpha$ -SYN-5 (all P values < 0.0002).

in locomotor activity (Fig. 5A) at the middle ($P < 0.05$) and old ($P < 0.05$) ages compared to the young age which was matched by a significant increase in the inverted screen time in the old age group ($P < 0.0001$, Fig. 5B) compared to the young age group. Both nontransgenic littermate controls and line $hw\alpha$ -SYN-5 had an age-related increase in the inverted screen time, but neither was significantly different from their young age group.

DA, HVA, and DOPAC were measured in the striatum at three ages, young age (2–3 months), middle age (7–9 months), and old age (13–23 months) using HPLC (Figs. 6A–6C). No gender-related differences were seen in any line for any measure. DA, DOPAC, and HVA levels were altered by age, transgenic status, and by an interaction ($F(7,108) = 6.7$, $P < 0.0001$; $F(7,108) = 4.9$, $P < 0.0001$; $F(7,108) = 4.7$, $P < 0.0001$, for DA, DOPAC, and HVA, respectively). Post hoc analyses revealed the primary change to occur in line $hm^2\alpha$ -SYN-39, where an age-related decline was seen primarily in the old age group for all three measures (all P s < 0.001) compared to nontransgenic littermates and line $hw\alpha$ -SYN-5 (Figs. 6A–6C). The locomotor changes seen in line $hm^2\alpha$ -SYN-39 at a young age were associated with a decrease in DA levels and a normal level of DOPAC producing a significantly increased ratio of DOPAC/DA ($P = 0.02$). At the old age the ratio of DOPAC/DA was not altered due to similar levels of decline.

DA, HVA, and DOPAC were measured in the striatum of lines $hw\alpha$ -SYN-88 and $hm^2\alpha$ -SYN-10 at 7–9

months of age. Compared to both nontransgenic littermate controls and line $hw\alpha$ -SYN-88, line $hm^2\alpha$ -SYN-10 had 27–31% lower levels of DA (both P s < 0.05) and HVA (both P s < 0.05). Levels of DOPAC were preserved in line $hm^2\alpha$ -SYN-10 at this age. These changes are similar to those seen in line $hm^2\alpha$ -SYN-39 at this age. Line $hw\alpha$ -SYN-88 did not differ in any measure from nontransgenic littermates similar to line $hw\alpha$ -SYN-5 examined at both young and old ages.

DISCUSSION

The $hw\alpha$ -SYN protein expressed in the transgenic mice reported here is biologically active in terminals of the dopaminergic nigrostriatal pathway as demonstrated by an increased density of the DAT, altered locomotor activity responses to amphetamine, and increased sensitivity to MPTP. The biological effect of $hw\alpha$ -SYN protein differed between the $hw\alpha$ -SYN and $hm^2\alpha$ -SYN forms with the $hm^2\alpha$ -SYN displaying adverse effects including altered morphology of DA processes, reduced locomotor response to amphetamine, lack of amphetamine-induced sensitization, increased motor activity at a young age, progressive motor impairments with increasing age, and age-related alterations in DA and metabolite levels. These findings have significant implications for understanding the normal role of α -SYN in DA terminals and the role of $hm^2\alpha$ -SYN in contributing to the PDP.

Functional effects of $hw\alpha$ -SYN expression. The specific function(s) of α -SYN remains to be determined.

The cellular localization in terminals and other studies suggest a role in presynaptic function (7, 35). α -SYN has been shown to interact with a variety of proteins including synphilin-1 and TH (10, 40) and a functional interaction with the DAT has been demonstrated (26). We demonstrate that expression of h α -SYN in transgenic mice resulted in increased density of the DAT. The greater amount of h α -SYN protein in the Acb and OT compared to the DS as visualized by IHC might have contributed to the greater increase in density of the DAT in the Acb and OT than in the DS. Finally, the toxicity of MPTP, as determined by the magnitude of the decrease in regional DAT levels, was greater in the Acb and OT than in the DS in transgenic mice. These findings can be experimentally verified by further examination of other lines of h α -SYN transgenic mice expressing either reduced or increased amounts of h α -SYN or lines with different levels of regional expression. A dose-response effect may be important to relate to humans where the concentration or activity of h α -SYN protein may be a predisposing factor in the PDP.

Expression of h α -SYN in these transgenic mice resulted in a variety of behavioral effects, which might relate to interactions with the DAT or other synaptic proteins. h α -SYN expression in transgenic mice resulted in an increased sensitization-like response to a low dose of amphetamine on a second administration (line h α -SYN-5). The low dose used was reported to not have an effect following the first administration, but resulted in sensitization on further injections (32). Sensitization occurred after repeated doses of amphetamine in line h α -SYN-88. These results are consistent with the increased response to amphetamine seen in rats transduced with a h α -SYN expressing virus and the decreased response to amphetamine seen in α -SYN knockout mice (1, 22).

Adverse effects of hm² α -SYN. Expression of hm² α -SYN demonstrated several differences from h α -SYN suggesting it may be toxic. Differences included morphologic abnormalities in processes and age-related alterations in behavior likely reflecting changes in DA and metabolites. The behavioral differences between h α -SYN and hm² α -SYN lines were dramatic. At the youngest age examined, line hm² α -SYN-39 demonstrated significantly increased locomotor activity and a shorter time to right compared to line h α -SYN-5 and nontransgenic littermates. These behavioral effects seen in line hm² α -SYN-39 were associated with reduced DA levels and increased DA turnover, suggesting that these behavioral effects might be related to abnormal DA homeostasis in terminals. These changes are suggestive of increased or abnormal DA release resulting in increased metabolism or turnover. Locomotor dysfunction in line hm² α -SYN-39 was observed with aging, leading to a parkinsonian phenotype of bradykinesia and hypokinesia. This phenotype was as-

sociated with further reductions in DA levels and significant reductions in DOPAC and HVA.

Expression of hm² α -SYN also impaired locomotor responses to amphetamine. Locomotor response to a second low dose of amphetamine was significantly reduced in line hm² α -SYN-39 and sensitization to repeated higher doses of amphetamine was blocked in line hm² α -SYN-10. These findings suggest an important role for h α -SYN in regulating responses to repeated doses of amphetamine and are consistent with the location of α -SYN in terminals. This effect may be related to the DAT or other proteins interacting with α -SYN in DA terminals.

Both intracytoplasmic (12, 29) and intranuclear (29) inclusions have been identified in other transgenic models expressing α -SYN. We did not identify either type of inclusion in our mice. This may relate to strong cytoplasmic staining of h α -SYN obscuring the presence of inclusions, the young age we have examined so far, the limited number of neurons examined, or the fact that we have expressed h α -SYN in cells also expressing m α -SYN which may act to reduce the tendency for h α -SYN to spontaneously aggregate. This was demonstrated by *in vitro* aggregation studies combining m α -SYN and h α -SYN (45). High levels of h α -SYN in cells not expressing α -SYN may be more likely to aggregate. This is a testable hypothesis *in vivo*. Alternatively, it may be that posttranslational modification of h α -SYN is required for aggregation (16, 39) and does not occur in these transgenic mice.

Others have reported adverse properties of h α -SYN in a variety of model systems ranging from cell culture (38) to transgenic flies (12) and mice (29, 50). Both the wild-type and mutated form of h α -SYN appear to be toxic in these studies, with some studies suggesting greater toxicity with mutant forms (38). Although the mutant forms of h α -SYN are toxic in humans because of their association with inherited forms of the PDP; the role of wild-type α -SYN in causing neurodegeneration has been less clear. The ability of α -SYN to spontaneously aggregate *in vitro* and *in vivo* under physiologic and altered environments suggests this may be a leading factor in the detrimental effects of overexpressed α -SYN (13). The widespread ability of this protein to be toxic in a variety of cells and conditions suggests some cells may not tolerate this gene product. The restricted location of α -SYN expression in the CNS suggests that transgenic or ectopic expression in other neuronal groups may be adverse. Data from our study suggest that h α -SYN was not directly toxic to SN neurons, at least up to 18 months of age in the absence of exogenous neurotoxins and did not form aggregates as described by others. There are a variety of explanations for the discrepancy between our transgenic mice and other studies demonstrating either a toxic role for h α -SYN or the absence of a toxic role with mutated forms of h α -SYN (30, 42). One may relate to the

amount of protein expression. This is particularly important for a protein with a tendency to aggregate in a concentration-dependent manner such as α -SYN. The stoichiometric relationship between transgene product and endogenous gene product may be most relevant in the *in vivo* setting. In the transgenic lines reported here, the level of h α -SYN accounted for ~25 to 33% of the total amount of α -SYN (h α -SYN plus m α -SYN) present in the mouse striatum. This fraction is useful for comparing differences between lines and perhaps other transgenic models where a similar value can be derived. We presume that at this stoichiometric ratio h α -SYN is insufficient to produce a pathologic phenotype. Another difference between studies is the transcriptional control of the transgene. In our mice the TH promoter was used to direct expression to catecholaminergic neurons. Others have employed promoters yielding more widespread expression. This is in contrast to the normally restricted regional expression in rodents (19). This raises the possibility that forced expression of h α -SYN in cells not normally expressing it may result in toxicity. A third difference between models is the strain background. Considerable data have shown marked effects of strain background on a variety of genetically engineered genotypes and following neurotoxic exposure (6, 11, 17). Another difference is our use of a doubly mutant form of h α -SYN which may have unique properties compared to the two single mutations. Irrespective of which of these considerations may apply it is clear that TH promoter driven h $m^2\alpha$ -SYN, but not h α -SYN elicits adverse effects in the nigrostriatal system in these lines. Future studies will aid in sorting out these differences among models and hopefully shed light on the human condition.

Model and mechanisms. A model for the role of h α -SYN in neurodegeneration may be emerging. The subcellular location of h α -SYN suggests it may have more than one function, but it clearly plays a role in presynaptic terminals. Alterations in the amount or function of the DAT may have significant effects on dopaminergic neurons, via dopamine (26), its metabolites, or neurotoxicants. Modest increases in mouse striatal DAT (20–30%) may increase significantly the response to MPTP (>50% loss of neurons; 9). Likewise, reductions in the DAT reduce the toxic response to MPTP (14). Alternatively, dysfunction of h α -SYN due either to a mutation, the amount of protein, aging, or an interaction with environmental toxins may lead to altered function either as aggregates or as a toxic interaction with another protein. This may result in impaired axonal transport and/or terminal dysfunction recognized by dystrophic neurites or impaired DA terminal metabolism. Affected DA terminals would be predicted to be more susceptible to oxidative injury. The observed increases in the DAT may over time

contribute to presynaptic dysfunction through accelerated and prolonged energy-consuming reuptake of DA.

The mechanism for nigral neurodegeneration resulting from mutations in h α -SYN is most consistent with a gain of toxic function. The behavioral, anatomical, and neurochemical observations in the h α -SYN and h $m^2\alpha$ -SYN lines suggest a possible pathogenic sequence. In young adult mice expression of h α -SYN is sufficient to elicit presynaptic DAT upregulation without perturbing DA metabolism. Expression of the h $m^2\alpha$ -SYN at the same age results not only in presynaptic DAT upregulation, but also adversely affects DA metabolism and responses to amphetamine. We speculate that nigrostriatal presynaptic dysfunction is achieved in part by a h α -SYN-mediated increase in DAT activity and attendant increased presynaptic metabolic activity associated with neurochemical dysregulation. Others have provided evidence linking an association between the levels of the DAT and the vesicular monoamine transporter (VMAT2) in the pathogenesis of toxin-induced and idiopathic PDP (33) suggesting an important role for the DAT. However, other proteins interacting with α -SYN must still be considered.

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DEVELOPMENTAL EXPOSURE TO THE PESTICIDES PARAQUAT AND MANEB AND THE PARKINSON'S DISEASE PHENOTYPE

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ABSTRACT

Idiopathic Parkinson's disease (PD) is associated with advanced age, but it is still unclear whether dopaminergic neuronal death results from events initiated during development, adulthood or represents a cumulative effect across the span of life. This study hypothesized that paraquat (PQ) and maneb (MB) exposure during critical periods of development could permanently change the nigrostriatal dopamine (DA) system and enhance its vulnerability to subsequent neurotoxicant challenges. C57BL/6 mice were treated daily with saline, 0.3 mg/kg PQ, 1 mg/kg MB or PQ+MB from postnatal days 5-19. At 6 weeks, a 20% decrease in activity was evident only in the PQ+MB group, with a further decline (40%) observed at 6 months. A subset of mice were re-challenged as adults with saline, 10 mg/kg PQ, 30 mg/kg MB, or PQ+MB 2x a week for 3 weeks. Mice exposed developmentally to PQ+MB and re-challenged as adults were the most affected, showing a 70% reduction in motor activity two weeks following the last re-challenge dose. Striatal DA levels were reduced by 37% following developmental exposure to PQ+MB only, but following adult re-challenge levels were reduced by 62%. A similar pattern of nigral dopaminergic cell loss was observed, with the PQ+MB treated group exhibiting the greatest reduction, with this loss being amplified by adult re-challenge. Developmental exposure to PQ or MB alone produced minimal changes. However, following adult re-challenge, significant decreases in DA and nigral cell counts were observed, suggesting that exposure to either neurotoxicant alone produced a state of silent toxicity that was unmasked following adult re-exposure. Taken together, these findings indicate that exposure to pesticides during the postnatal period can produce permanent and progressive lesions of the nigrostriatal DA system, and enhanced adult susceptibility to these pesticides, suggesting that developmental exposure to neurotoxicants may be involved in the induction of neurodegenerative disorders and/or alter the normal aging process.

Key Words: Parkinson's disease, development, substantia nigra, pesticides, striatum.

INTRODUCTION

Parkinson's Disease (PD) is typically considered an aging-related neurodegenerative disorder given its typical onset after 60 years of age and subsequent progression. This pattern of manifestation of signs and symptoms later in life is not, of course, necessarily indicative of the timing of etiological factors. In fact, one possibility that can be posited is that PD could arise from events that occur early in development that have long-term but delayed adverse consequences for the nigrostriatal dopamine (DA) system. Possible developmental events could include exposures to environmental neurotoxicants. Under such scenarios, it is conceivable that the population of nigrostriatal DA cell bodies is reduced early in life, and that with normal aging-related loss, the DA system will eventually reach levels associated with PD. Alternatively, or in conjunction with such a model, is the possibility that nigrostriatal DA system damage early in development, whether or not it is associated with cell body loss, nevertheless renders the system more vulnerable to subsequent environmental risk factors associated with PD, effectively increasing their potency, resulting in PD that might not otherwise have occurred.

Environmental risk factors have, in fact, long been implicated in the etiology of Parkinson's disease (PD). Several epidemiological studies report an increased incidence of PD in association with pesticide exposures and associated conditions that include well water drinking, farming, and rural living (Gorell, et al., 1998; Semchuk, et al., 1992; Tanner, 1989; Tanner, et al., 1987). The increased prevalence of PD in industrialized countries and its geographic heterogeneity have also been suggested to be the result of the greater use of environmental chemicals (Li, et al., 1985; Morens, et al., 1996; Schoenberg, et al., 1985; Schoenberg, et al., 1988). The potential for environmental risk factors to contribute to PD gained particular attention following the report of a study of 19000 pairs of twins, the largest of its kind, by Tanner and

colleagues (Tanner, et al., 1999) citing no difference in PD rates between monozygotic and dizygotic twins with onset of PD after age 60.

Our laboratory recently established a model of environmental Parkinsonism in adult mice that resulted from repeated combined exposure to the herbicide/dessicant paraquat (PQ) with the ethylenebisdithiocarbamate fungicide maneb (MB), both of which are known to adversely impact DA systems. These exposures produced selective nigrostriatal DA system neurotoxicity, including loss of striatal DA and of cell bodies of DA neurons in the substantia nigra pars compacta (Thiruchelvam, et al., 2000a; Thiruchelvam, et al., 2000b). Compared to the commonly used neurotoxicant model MPTP, combined PQ+MB produces permanent effects. While the mechanism of toxicity of MPTP is relatively well understood, those underlying the selective nigrostriatal DA neurotoxicity of PQ+MB remain to be determined. PQ is a free radical generator, due to its ability to redox cycle, and is commonly used experimentally as an oxidative stressor (Dey, et al., 1990; Woolley, et al., 1989). While previous studies report somewhat equivocal effects of systemically-administered PQ on the nigrostriatal system, new findings show that PQ alone, even at very low doses, can produce DA cell loss in the substantia nigra and increase expression of alpha-synuclein (Manning-Bog, et al., 2002; Thiruchelvam, et al., In preparation). Less is known about the mechanism of neurotoxicity of MB, but it has been reported to inhibit glutamate transport and disrupt DA uptake and release (Vaccari, et al., 1998; Vaccari, et al., 1999; Vaccari, et al., 1996).

The susceptibility of the developing nervous system to degeneration following exposure to environmental toxicants is well recognized. Exposure to neurotoxicants such as 1,1,1-trichloro-2,2-bis(p-chlorophenyl)ethane (DDT), polychlorinated biphenyls (PCBs), and pyrethroids produce permanent behavioral changes as well as neurochemical changes in the

cholinergic system in the CNS of adult animals when administered even at low doses during critical periods of development (Ahlbom, et al., 1994; Ahlbom, et al., 1995; Eriksson, et al., 1992; Eriksson, et al., 1991a; Fredriksson, et al., 1993a). Developmental exposure to PQ or MPTP also produce permanent changes in striatal DA and behavior in the adult animal (Fredriksson, et al., 1993b; Ochi, et al., 1991; Perez-Otano, et al., 1992; Weissman, et al., 1989). Effects of developmental exposure to MB have been studied, but its effects on the DA system per se have not been examined (Bancroft, et al., 1973; Chernoff, et al., 1979; Sobotka, et al., 1972).

The dopaminergic system develops both pre- and postnatally, with receptor development and the brain growth spurt occurring predominantly in the postnatal period (Giorgi, et al., 1987; Voorn, et al., 1988). The hypotheses posed here were that developmental exposure to either PQ or MB alone, or in combination, would result in permanent nigrostriatal DA system neurotoxicity, and, secondly, would render the nigrostriatal DA system more susceptible to environmental chemical challenges later in life.

EXPERIMENTAL PROCEDURES

Animals and Drug Administration

C57BL/6 male mice were injected i.p. with either vehicle (saline), paraquat dichloride hydrate (PQ, Sigma, St. Louis, MO) at a dose of 0.3 mg/kg, maneb (MB, manganese bisethylenedithiocarbamate; Dow Chemical) at a dose of 1 mg/kg, or with the combination from postnatal days 5-19. Both PQ and MB were dissolved in saline. For combined injections, two separate injections were administered. At 6 1/2 months of age, a subset of these animals were re-challenged with the original treatment using saline, 10 mg/kg PQ, 30 mg/kg MB or the combination of PQ + MB. A separate group of mice were treated only as adults to these same doses, yielding three exposure groups of mice; a) postnatal only, b) postnatal + adult, c) adult only. Postnatal exposure was carried out daily from days 5 to 19 (15 total doses), and subsequent adult re-exposure occurred twice a week for 3 1/2 weeks (total of 7 treatments). **Figure 1** shows the experimental time line. Mice were housed in a room maintained under constant temperature (72 – 74 °F) and humidity conditions with a 12:12 light-dark cycle. During post-natal exposure, mice were housed with dams and littermates. Following weaning, mice to be used for behavioral studies were housed one per cage; all other mice were housed four per cage. To prevent litter-specific effects, a total of 30 litters were generated for each treatment group, with one mouse derived from each litter.

Food and water were available *ad libitum*. Body weights were obtained periodically over the course of the experiment. Animals were cared for and treated in accord with NIH and the University of Rochester Animal Care and Use Committee guidelines.

Chemicals

Solvents for high performance liquid chromatography with electrochemical detection (HPLC-EC) were purchased from Sigma (St Louis, MO). All other chemicals, if not specified, were at least analytical grade and were purchased from Sigma (St. Louis, MO).

Locomotor Activity

Automated locomotor activity chambers equipped with infrared photobeams (Opto-Varimex Minor, Columbus Instruments International Corporation, Columbus, OH) were used to quantify locomotor activity. Photobeam breaks were recorded each minute for 45 minutes for horizontal, vertical, and ambulatory movements. Following postnatal exposure, motor activity was assessed at 6 weeks and again at 6 months of age. To assess the effects of developmental exposure to these toxicants at 6 weeks of age, all mice were run on three consecutive days and the data from the third locomotor session is reported here. At 6 months of age, mice were also habituated to the locomotor activity chambers in three 45-minute sessions occurring on consecutive days, with all mice receiving i.p. vehicle injections prior to the session. After the third habituation session, treatments began either as re-challenge (postnatal + adult) or first challenge (adult only) or to vehicle only (postnatal), and effects on motor activity were assessed immediately and 24 hours after each injection in 45 minute test sessions with activity counts totaled in 3 min blocks across the session. Activity was also determined again 2 weeks after the final treatment to determine whether there were persistent effects of adult re-exposure. This behavioral session was preceded by a saline injection.

Dopamine and Metabolite Analyses by HPLC

Neurotransmitter concentrations were measured two weeks following the last injection of the assigned treatment. All groups (postnatal only, postnatal + adult, adult only exposure) were sacrificed at the same time point. Following rapid decapitation, striatal blocks were dissected and placed in 0.1N perchloric acid. The tissues were sonicated and centrifuged for 15 min at 1,000 X g. The supernatants were stored at -80°C until analyzed for the concentrations of DA, dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA) and serotonin (5HT) by HPLC - EC. The pellets were digested in 1 ml of 0.5N NaOH for measurements of protein concentration using the Bio-Rad assay. HPLC analysis was carried out as described previously (Thiruchelvam et al., 2000a). The concentrations of the neurotransmitters were expressed in units of ng/mg protein. DA turnover was expressed as the ratio (DOPAC + HVA)/DA.

Immunohistochemistry (IHC) for Tyrosine Hydroxylase and Cell Counting

Tissue Preparation Paraformaldehyde (PFA) post-fixed brains were used for immunolabeling studies. Fixed brains were cut into 30µm sections and collected in cryoprotectant. Sections were washed with 0.1 M phosphate buffer (PB), blocked for non-specific binding, and incubated with a 1° antibody (Ab) to TH (Chemicon International, Temecula, CA) for 48 hours at a dilution of 1:4000. Sections were subsequently incubated with a 2° biotinylated anti-rabbit Ab (Vector Laboratories, Burlingame) at a dilution of 1:200. Sections were subsequently washed and incubated with avidin-biotin solution using the Vectastain Elite kit (Vector Laboratories, Burlingame) for 1 hour at room temperature. Sections were developed in 3-3'-diaminobenzidine tetrachloride (DAB) for 2-3 minutes. Following several rinses, sections were mounted, counterstained with cresyl violet and cover-slipped.

Stereological Analysis After delineation of the substantia nigra pars compacta (SNpc) at low magnification (4X objective), every fourth section from the entire region was sampled at higher magnification (100X objective) using the stereology module of the MCID imaging program (Imaging Research, St. Catherines, ON) with an Olympus Provis microscope. The optical fractionator method was used to count TH+ and TH- cells. The entire depth of field was sampled ignoring the upper and lower 1.5 μm to avoid counting cells that might be missing nuclei. The thickness of each section was measured. The total number of TH positive (TH+ and cresyl violet positive neurons) and TH negative (cresyl violet positive only) neurons in the substantia nigra was estimated using the optical fractionator method.

Peripheral Organ Histopathology

Representative sections of lung, heart, kidney, and liver (n=6 per treatment group) were prepared by formalin fixation, paraffin embedding, sectioning at 4 microns, and staining with hematoxylin and eosin. Sections were examined without knowledge of treatment group for evidence of alterations in microscopic pathology.

Statistical Analysis

Overall effects of treatment on horizontal locomotor activity were first analyzed with repeated measure analyses of variance (RMANOVA) using treatment and developmental group (i.e., postnatal, postnatal + adult, or adult only exposure) as between-group factors and injections as a within-group factor. This was followed by individual ANOVAs using treatment and developmental group as between-group factors for each injection and subsequent Fisher's post-hoc tests to compare treatment groups. To assess treatment-related changes within an activity

session, RMANOVAs with treatment and developmental group as between group factors and time block as a within group factor were utilized; significant main effects of treatment or interactions were followed by ANOVAs at each time point. Changes in DA, DOPAC and turnover were first evaluated using treatment and developmental group as between group factors for ANOVA. Effects of all other endpoints were analyzed using one factor ANOVA with treatment and development group as the between group factors, followed by Fisher's tests in the event of significant main effects of treatment.

RESULTS

Body Weight and Pathology

No treatment-related changes in body weights were observed in any of the groups at any time point in the experiments. Lungs were graded for signs of alveolitis, bronchiolitis, bronchitis, lymphoid aggregation, bronchiectasis, and fibrosis and found to be histologically normal. Similarly, no pathological changes were observed in heart, kidney or liver.

Locomotor Activity

Locomotor activity was evaluated at 6 weeks of age and again at 6 months of age after postnatal (PN) exposure to saline, 0.3 mg/kg PQ, 1 mg/kg MB or the combination of the two (Figure 2). At 6 weeks of age, only the group that received combined PQ+MB showed a significant decrease (23%) in horizontal activity, as confirmed by a main effect of treatment in the statistical analysis ($F(3,34) = 3.456, p = 0.027$) with subsequent post-hoc tests indicating lower activity levels than groups treated with saline and with MB alone (both p 's < 0.05). The PQ

only treated group showed a marginal decrease in activity levels at this early time-point (14%). By 6 months of age, the PQ+MB treated group showed a further reduction in locomotor activity (38%) such that activity levels were significantly lower than those of the other three groups (all p 's<0.05), confirmed by a significant main effect of treatment ($F(3,34) = 3.376, p = 0.03$) in the RMANOVA. This represented a further decrease of approximately 15% in the PQ+MB group between 6 weeks and 6 months (**Figure 2**), demonstrating progressive effects of PQ+MB (p <0.0001).

Challenges with saline, 10 mg/kg PQ, 30 mg/kg MB or the combination of the two were carried out in a subset of mice after the 6 month motor activity assessment. Additional naive mice were treated only as adults with the same doses and PN-only exposed group was treated with saline. Locomotor activity was evaluated immediately after these treatments and again 24 hours later followed by an additional assessment 2 weeks later preceded by saline only injections. The corresponding locomotor activity levels are depicted in **Figure 3**. As it indicates, mice that received PQ+MB postnatally and were subsequently re-challenged with PQ+MB as adults exhibited a marked decrease (70%) in locomotor activity. A significant main effect of treatment ($F(3,106) = 6.36, p = 0.0005$) and group (PN, PN + adult, and adult only; $F(2,106) = 3.00, p = 0.05$) were confirmed in the statistical analysis. The levels of activity in the PN + adult PQ+MB group were lower than those of the PN only and adult only groups exposed to PQ+MB (both p 's<0.01) and were also lower than levels in the PN + adult groups that received either PQ or MB alone (both p 's<0.01). MB alone mice exposed both postnatally and as adults exhibited a slightly greater reduction in activity than the groups that were exposed either postnatally only or as adults only, although these decreases were not statistically significant.

Striatal Dopamine, Metabolites, Turnover and Serotonin

Striatal levels of DA, DOPAC, HVA, 5HT and DA turnover were evaluated 2 weeks after the last adult exposure, immediately after last locomotor session (**Figure 4**). All groups of mice were sacrificed at the same time. Striatal DA levels (**Figure 4A**) were significantly affected by treatment ($F(3,105) = 24.8, p < 0.0001$), and differed by group as well (PN, PN + adult, and adult only: $F(2,106) = 20.3, p < 0.0001$; treatment by group interaction: $F(6,105) = 3.8, p = 0.0018$). PN only exposure to PQ+MB significantly decreased DA levels by about 36% relative to saline, PQ or MB alone ($p < 0.05$), while PQ alone marginally decreased DA. Adult only exposure to PQ+MB decreased DA levels by approximately 15% ($p < 0.05$), findings consistent with our previous observations (Thiruchelvam, et al., 2000).

With PN + adult exposure, PQ alone decreased DA levels (**Figure 4A**) by 36%, MB alone by 30%, and PQ+MB by 62% as compared to the corresponding saline treated group. This stands in contrast to the lack of effect on DA levels in response to PQ or MB alone following adult only exposure. Similarly, PQ+MB treated mice in the PN + adult exposed group showed enhanced reductions in DA levels compared to those exhibited by the corresponding PN only or adult only groups ($p < 0.001$).

Changes in DOPAC levels paralleled those seen in DA (**Figure 4B**), with a significant effect of treatment ($F(3,105) = 11.7, p < 0.0001$), group ($F(2,105) = 9.8, p < 0.0001$), and an interaction between treatment and group ($F(6,105) = 2.31, p = 0.03$). For all three treatment protocols, exposure to PQ alone, or to PQ + MB decreased DOPAC levels, with this reduction being greatest in the PN + adult group ($p < 0.01$). In fact, the decrease in DOPAC levels in the PN + adult exposed PQ+MB appears additive of the effects observed in the PN only and adult only exposure groups. While MB alone treatment did not alter DOPAC following exposure either

during development or in adulthood, PN + adult exposure did result in significant and notable decreases in DOPAC ($p < 0.05$). Effects on HVA levels (**Figure 4C**) mirrored those seen with DOPAC, but were of smaller magnitude, with significant effects of treatment ($F(3,105)=2.9$, $p=0.037$) and group ($F(2,105)=2.88$, $p=0.05$) in the statistical analysis, but not an interaction between the two.

DA turnover (**Figure 4D**), changes reflected the alterations in DA and metabolite levels (main effect of treatment ($F(3,105)=4.6$, $p = 0.004$) and group ($F(2,105)=0.002$). Specifically, PQ+MB treatment during PN days 5 – 19 significantly increased turnover by 20% compared to effects produced by either compound alone or by saline treatment. Adult only exposure to PQ+MB did not alter DA turnover in any of the groups. Exposure to PQ+MB both PN and as adults significantly increased DA turnover (50%) compared to the corresponding saline group. This increase was also significantly higher than that of the PN only treated PQ+MB group. Although not statistically significant, both PQ and MB alone increased DA turnover (16% and 10%, respectively) in the PN + adult group. No significant changes in 5HT levels were observed in any of the treatment groups (data not shown), indicating the selectivity of these treatments for the dopaminergic system.

Nigral Dopaminergic Cell Counts

The number of dopaminergic cells in the substantia nigra pars compacta (SNpc) was determined 2 weeks after the last adult treatment (**Figure 5**), with all mice sacrificed at the same time point. TH⁺ neurons (**Figure 5A**) actually represent TH⁺ and cresyl violet positive neurons indicating that the changes observed are a true loss of dopaminergic neurons rather than just a down regulation of the enzyme, with no change in TH⁻ neurons. PN only exposure to any of the

treatments (PQ or MB alone or combined PQ+MB) all decreased the number of TH+ neurons as compared to the corresponding saline-treated group significantly (all comparisons, $p < 0.0001$), with the PQ+MB group exhibiting the largest reduction, and differing from PQ and MB alone. Adult only exposure to PQ alone and to PQ+MB also reduced the number of dopaminergic cells (both comparisons, $p < 0.0001$). The PN + adult exposure regimen decreased numbers of TH+ cells most dramatically ($p < 0.0001$), again with the PQ+MB group showing the largest decrease (67% decrease) relative to saline ($p < 0.0001$). PQ+MB also reduced TH+ neurons to a significantly greater extent than PQ alone or MB alone. The decreases in the PN + adult groups were all potentiated relative to corresponding losses in either the PN or adult only groups for all the treatments. These effects were confirmed in the statistical analysis by a significant main effect of treatment ($F(3,36) = 275$, $p < 0.0001$), group ($F(2,36) = 110$, $p < 0.0001$), and an interaction of treatment and group ($F(6,36) = 31.1$, $p < 0.0001$). In contrast, there were no differences in TH- neurons under any conditions (**Figure 5B**), suggesting that these neurotoxicant treatments destroyed only dopaminergic neurons of the substantia nigra pars compacta.

DISCUSSION

Although PD is considered a neurodegenerative disorder, the possibility that it results from damage to the nigrostriatal system incurred developmentally but expressed only as normal aging processes unfold, while speculated, has yet to be examined. The aims of this study were to examine the hypothesis that targeting the nigrostriatal dopaminergic system developmentally could result in permanent neurotoxicity to the DA system and, further increase its vulnerability to subsequent neurotoxic challenges occurring later in life. In concert with those assertions, developmental exposures to PQ or MB alone as well as their combination impart sustained alterations to the nigrostriatal dopamine system that are still evident at 6 months of age, i.e., 5 1/2 months after the last exposure, suggesting permanent DA system alterations. This was demonstrated by locomotor activity, especially in the PQ+MB treated group, where activity levels were reduced at 6 weeks of age but had fallen even further by 6 months of age, consistent with progressive neurotoxicity. The increased dopamine turnover in the PN treated PQ+MB group evident even 6 months after exposure suggests a continuous alteration of DA metabolism, an effect that could signal the sustained formation of free radicals as a mechanism for the progressive degeneration that appears to take place. Determining the increases in free radical generation and its by-products will allow us to examine this possibility.

In addition to demonstrating permanent and progressive effects from developmental only exposures, two other major findings were notable. First, developmental exposures markedly enhanced vulnerability to subsequent pesticide treatments, and, secondly, developmental only exposures were associated with “silent neurotoxicity” that was only unmasked by later challenges to the DA system. The effects of adult re-challenge following developmental exposure to either PQ or MB alone or PQ+MB produced markedly greater reductions in

locomotor activity, striatal DA and metabolites as well as nigral dopaminergic neurons compared to either PN only or adult only exposures to these neurotoxicants. With respect to the second finding, the effects of MB alone during development were found to be modest and without substantial behavioral impairments. However, following adult re-exposure to MB, a marked decrease in nigrostriatal dopaminergic function was observed. Developmental exposure to MB alone therefore produced a “silent toxicity” such that the system was already vulnerable, and re-exposure thereafter to the neurotoxicant produces dramatic effects.

Calne and Langston (Calne, et al., 1983) posited a decline in dopaminergic function with age that could be accelerated by an environmental insult in early or middle life that eventually reduces dopamine function below that necessary to maintain normal function as a model for PD (Figure 6 – solid black line (A & B)). Alternatives or additions to this hypothesis are posed in Figure 6 (blue (6D) and red (6C) lines). The progressive decline in locomotor activity with age following developmental exposure may be an indication of progressive loss of dopaminergic function across the life span. To document this, of course, would require a longer, time-course experiment. However, if one makes that assumption, several hypotheses can be explored. As depicted in Figure 6, developmental insult might result in a loss of dopaminergic neurons at the onset of exposure, thus depleting the total pool of neurons, and this process may continue with age resulting in the disease phenotype appearing earlier in time compared to what might be predicted with normal aging alone (Fig 6D – solid blue line). In another proposed scheme, neuronal number may not be altered with developmental exposure at the beginning of life, but rather the developmental insult could enhance the rate of cell death across the life span, again, leading to an earlier onset of the disease phenotype (Fig 6C – solid red line). Under both scenarios, a subsequent environmental insult during adulthood can shift these curves even further

downward, as schematized (Figure 6E). This study clearly shows that developmental exposures to these toxicants can render a system more vulnerable to subsequent exposures. Furthermore, it raises the possibility that individuals that may not have evidenced susceptibility through genetic background alone could, by virtue of early damage to the system, become vulnerable to the PD phenotype. It is important to point out however, that to date, only behavioral data has been presented to support the possibility of progressive effects following developmental exposure alone, making it premature to conclude that developmental exposure alone results in progressive damage to the nigrostriatal system. More experiments looking at the time course of these effects will have to be carried out to ascertain this possibility.

Other neurotoxicants have been shown to have a greater effect on the developing central nervous system compared to the adult brain (Ahlbom et al., 1994; Eriksson et al., 1992; Eriksson, et al., 1991b; Moser, et al., 1998; Moser, et al., 2001; Spyker, et al., 1977). Developmental exposure to MPTP, the commonly used neurotoxicant model of PD, produces permanent effects on the nigrostriatal system both in mice and non-human primates following developmental treatment but the changes observed are unlike those following adult exposure (Ali, et al., 1993; Ochi et al., 1991; Perez-Otano, et al., 1995; Perez-Otano et al., 1992; Weissman et al., 1989). A previous study reported that exposure to PQ on PND 10-11 produced permanent changes in striatal dopamine as well as behavioral changes (Fredriksson et al., 1993b). These findings are not completely consistent with those reported here, which could reflect differences in route of exposure (oral vs i.p. in our studies) and/or the timing of the exposure. Exposure to the insecticide DDT (1,1,1-trichloro-2,2-bis(p-chlorophenyl)ethane) during PND 10-11 enhanced the effects produced by adult exposure to bioallethrin (Eriksson, et al., 2000), as assessed using behavioral endpoints and cholinergic receptor pharmacology. However, the current study is, to

the best of our knowledge, the first demonstration of a developmental effect on the nigrostriatal system that is progressive, and also leads to enhanced nigrostriatal system vulnerability to subsequent exposures, having obvious implications for the etiology of PD.

The mechanism(s) by which developmental exposure to PQ and MB alone or in combination enhances the toxic impact of subsequent exposures requires further investigation. PQ itself has been repeatedly shown to adversely affect the nigrostriatal dopamine system (Barbeau, et al., 1985; Brooks, et al., 1999). While the extent to which PQ crosses the blood brain barrier (BBB) has been questioned, it can indeed enter the brain and actually appears to accumulate differentially in certain regions following systemic injections of ¹⁴C-PQ (Bageetta, et al., 1992; Corasaniti, et al., 1993; Corasaniti, et al., 1990; Corasaniti, et al., 1992; Lindquist, et al., 1988; Rose, et al., 1976). Furthermore, systemically administered PQ uptake in brain depends upon the age of the animal, with higher brain concentrations achieved in very young and older animals (Corasaniti, et al., 1991; Widdowson, et al., 1996).

Based on the neurochemical and behavioral changes, and it's apparently selective disruption of the nigrostriatal system, it is assumed that MB is able to cross the BBB. The development of the BBB proceeds from late gestation and continues through the postnatal period and may be a time of increased permeability. Thus, exposure to an environmental toxicant during this period could be associated with increased uptake and also disruption of the normal development and maturation of this crucial barrier (Kneisel, et al., 1996; Rodier, 1994; Rodier, 1995; Saunders, et al., 1991). An incomplete or immature BBB in the developing central nervous system could result in greater uptake of neurotoxic compounds into the central nervous system, which then exert greater effects on the dopaminergic system. Such a possibility could account for the more pronounced effects associated with developmental only as compared to adult only

exposures in these experiments. The postnatal period is often one of greater vulnerability to toxic insults as compared to the adult stage of the life cycle because excretion processes are functionally inefficient at this point and many drug metabolizing systems have not yet completely developed (Gange, et al., 1972). This would potentially result in a protracted time course of toxicant effects. While the basis for the potentiated neurotoxicity of combined PQ and MB has yet to be resolved, preliminary findings from our laboratory suggest that MB may increase the accumulation of PQ in the brain as compared to PQ administration alone (unpublished observation).

Thus, exposure to PQ and MB during critical periods of development may be disrupting the normal development of the BBB. Developmental exposure to several pesticides has been shown to alter BBB development and its functionality in the adult (Banks, et al., 1996; Gupta, et al., 1999; Srinivas, et al., 1993). Consequently, a “leaky” BBB as a result of incomplete or aberrant maturation could increase subsequent permeability to toxicants, a scenario that might underlie the heightened susceptibility of adult animals treated postnatally and re-challenged as adults. Similar impacts could be envisaged if defense mechanisms, xenobiotic metabolizing enzyme systems, and/or neurotrophic factor networks were incompletely or incorrectly developed due to disruption during the critical periods by the effects of these exposures. Developmental expression of neurotrophic factors is important for numerous processes and its expression is critically dependent on timing, duration and also context (Barone, 1999). Furthermore, perturbation of any of these systems may also have profound effects on downstream signaling processes that play an important role in the maintenance of cellular homeostasis.

Apoptotic cell death is also an important developmental process and occurs during both pre- and postnatal development. Exposure to toxicants during critical periods may perturb this

process by altering the systems that regulate apoptotic signals, resulting in undesirable increases in apoptosis and consequent decreases in cell numbers. This altered cell number may lead to neurological dysfunction as seen in several neurological disorders including PD (Burke, et al., 1998).

The findings described here using this environmental exposure model have clear implications for the risk assessment processes used to evaluate pesticide safety for human populations. Numerous studies have now begun to document the placental transfer of various pesticides to the fetus (Siddiqui, et al., 1981; Waliszewski, et al., 2001). The environmental reality, of course, is that exposures occur to mixtures of chemicals rather than to single agents, as might be predicted from the fact that environmental pesticides are widely used in overlapping geographical locations in areas in the US and some even on the same crops (USGS, 1998). Recently published studies now beginning to examine human exposures to pesticides, primarily organophosphates, confirm the presence of metabolites of multiple pesticides in urine of children. In some cases these levels are not seasonal as might have been expected, and are present in higher concentrations than predicted by recent non-probability based samples (Adgate, et al., 2001; Lu, et al., 2001). Clearly, a further understanding of the mixtures of pesticides to which humans are exposed and the levels of these exposures is needed to permit a precise determination of the risks of such exposures both for various developmental disabilities as well as their potential contributions as risk factors in the etiology of neurodegenerative diseases.

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FIGURE LEGENDS

FIGURE 1

Experimental time-line indicating postnatal exposure and adult re-challenge paradigms.

FIGURE 2

Total horizontal locomotor activity 6 weeks and 6 months following postnatal exposure to saline, 0.3 mg/kg paraquat, 1 mg/kg maneb or the combination of the two. Data is shown as group mean \pm S.E. (n = 10 per treatment group). Post-hoc analysis revealed significance from: * saline; + MB alone; # PQ alone; ~ 6 week old mice treated with PQ+MB.

FIGURE 3

Horizontal locomotor activity 2 weeks after last adult re-challenge. Data is shown as group mean \pm S.E. (n = 10 per treatment group), normalized to respective saline controls. The three groups, postnatal only (PN), postnatal + adult exposure (PN + adult) as well as adult only (adult) exposure are all shown as percent of representative saline treated group. Adult re-challenge with either saline, 10 mg/kg PQ, 30 mg/kg MB or the combination of the two was carried out at 6 1/2 months of age. Postnatal only treated mice were injected with saline prior to being placed in activity chambers. Post-hoc analysis showed significant difference from; * saline; + MB alone; # PQ alone; ~ PQ+MB postnatal only treated mice; ^ PQ+MB adult only treated mice.

FIGURE 4

Striatal DA (A), DOPAC (B), HVA (C), and DA turnover (DOPAC+HVA)/DA (D) levels two weeks after the last injection of saline or 10 mg/kg PQ, 30 mg/kg MB or the combination of the two (n = 10 for each treatment group). Post-natal exposure was carried out from postnatal day 5 to 19 (PND 5 – 19), with adult exposure occurring at 6.5 months of age. Data are shown as group mean \pm S.E (percent of corresponding saline treated group). Fisher's post-hoc confirmed significant difference from; * saline, # from adult only exposure, + PN only exposure, ~ MB alone, ^ PQ alone.

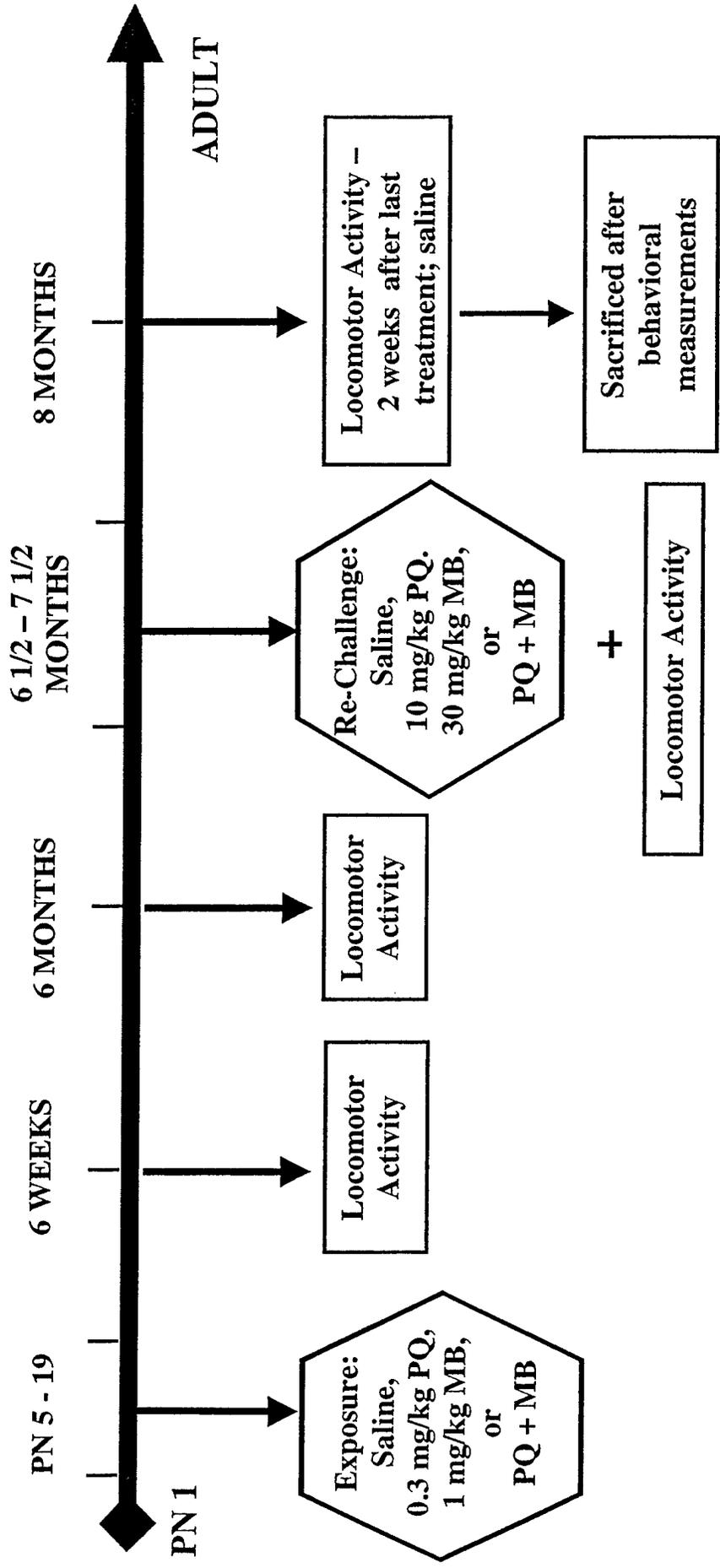
FIGURE 5

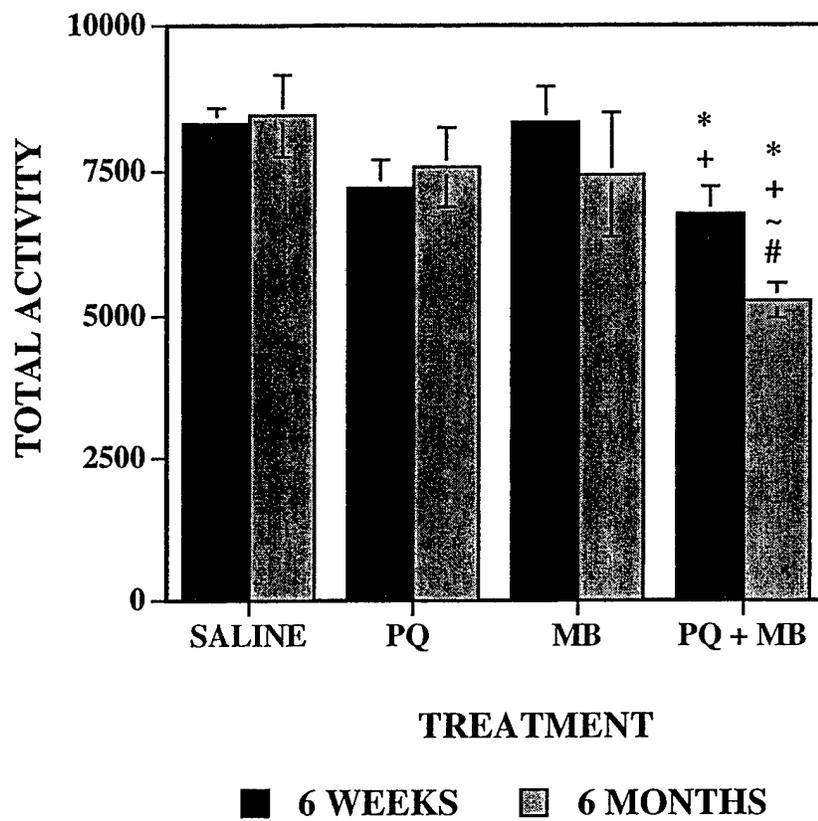
Total number of TH+ (A) and TH- (B) neurons in the substantia nigra pars compacta two weeks after the last treatment of either saline or 10 mg/kg PQ, 30 mg/kg MB or the combination of the two (n = 4 for each treatment group). Postnatal exposure from days 5 to 19 (PN 5 – 19) were followed by adult re-challenge with corresponding compounds twice a week for a total of 7 injections, with mice being sacrificed 2 weeks after the last dose. Data represents total number of TH+ / cresyl violet positive (A) or cresyl violet only positive (B) neurons and is represented as group mean \pm S.E. Post-hoc analysis further revealed significance from: * saline, # from adult only exposure, + PN only exposure, ~ MB alone, ^ PQ alone.

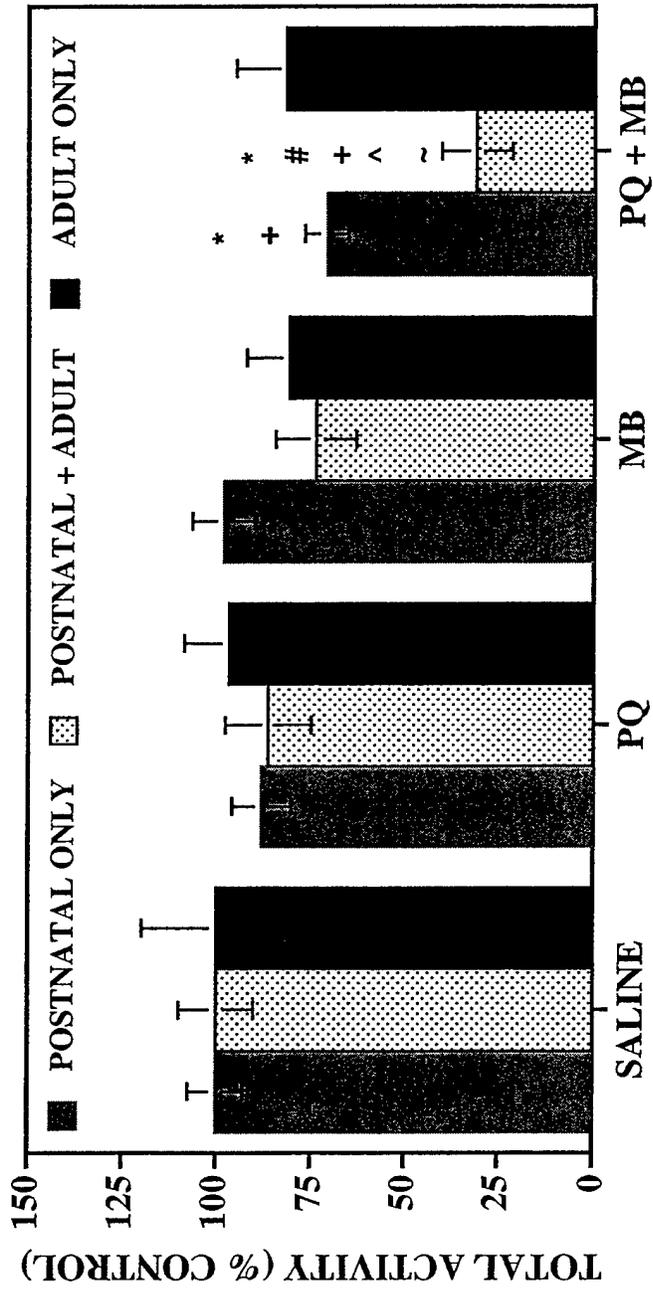
FIGURE 6

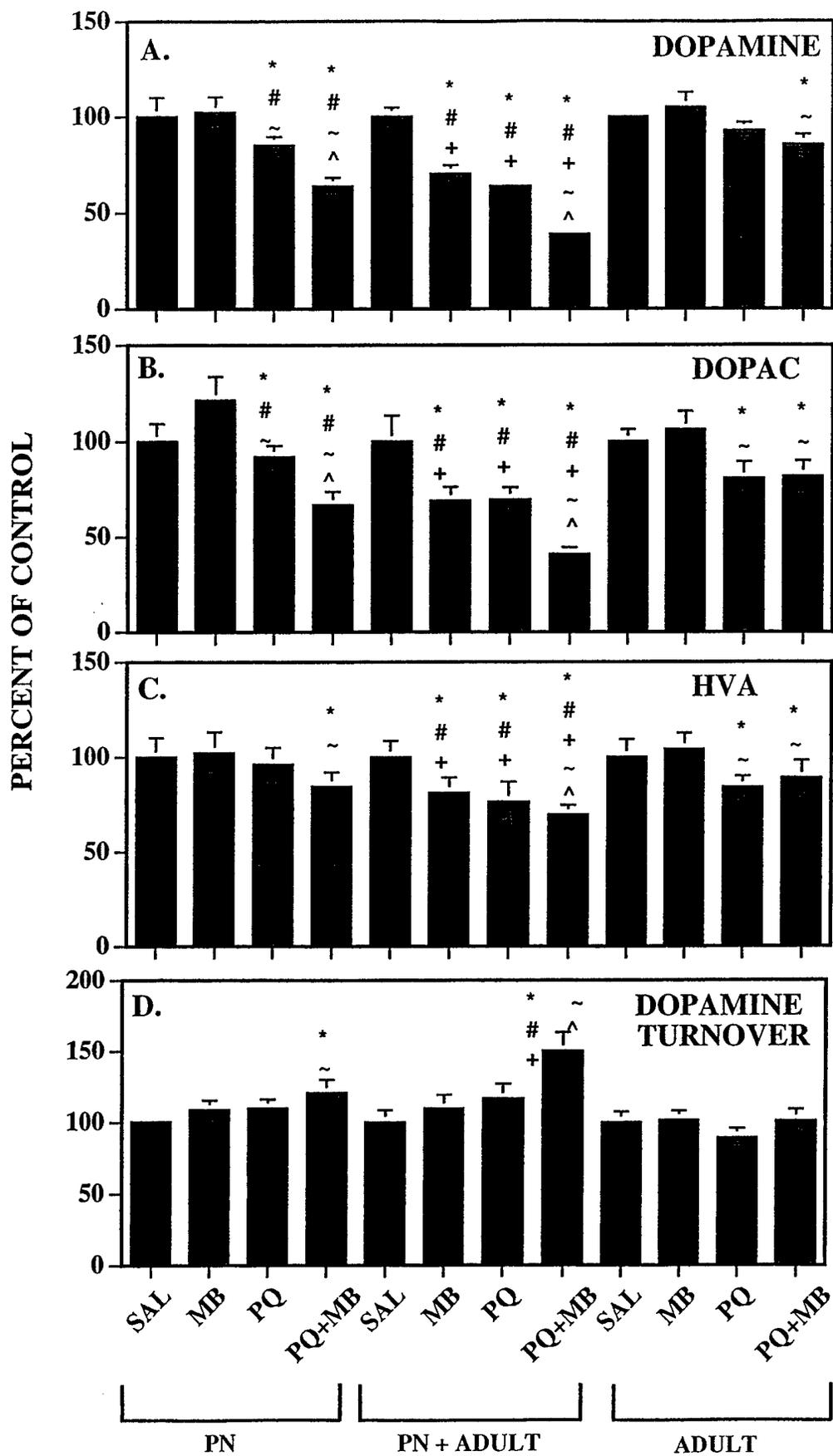
Schematic representation of three different scenarios leading to the Parkinson's disease phenotype, with the developmental exposure hypothesis relatively unexplored. The first (solid black line - A) shows how an environmental insult somewhere in the 3rd or 4th decade of life

(dotted black - B) might produce parkinsonism around the age of 60, assuming that dopaminergic (DA) function continues to decline with aging and an 80% or greater depletion of dopaminergic function is required for symptoms to develop. Contrary, exposure to DA neurotoxicants during critical periods of development can result in loss of DA function at the intercept of exposure (blue line - D) or accelerate the age-related decline of dopaminergic function (red line - C), both leading to the disease phenotype sooner. However, an adult re-exposure to such an insult (dotted black - E) could lead to a parkinsonian phenotype in the 5th or 6th decade of life.

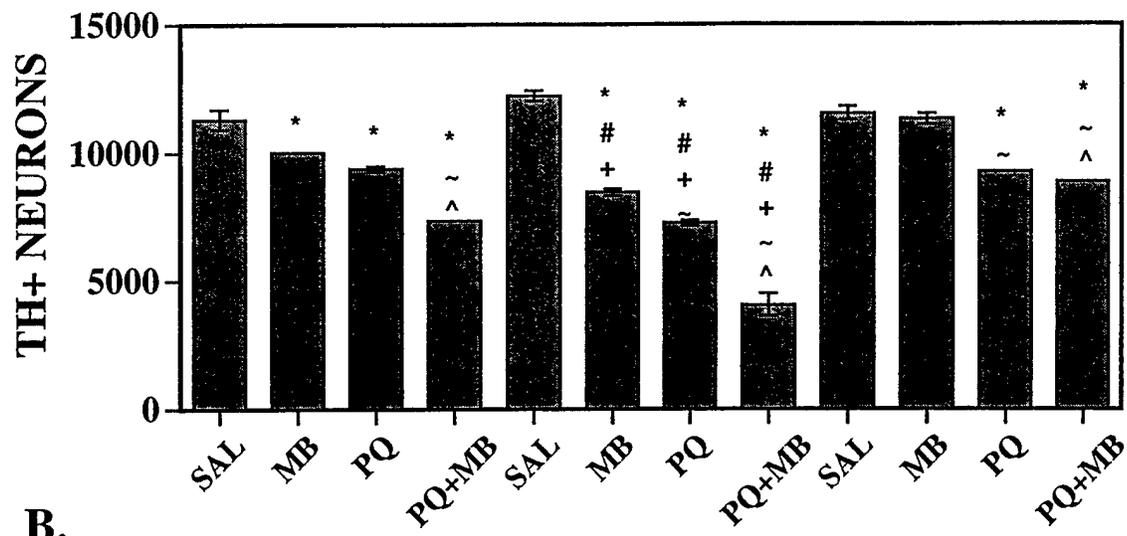




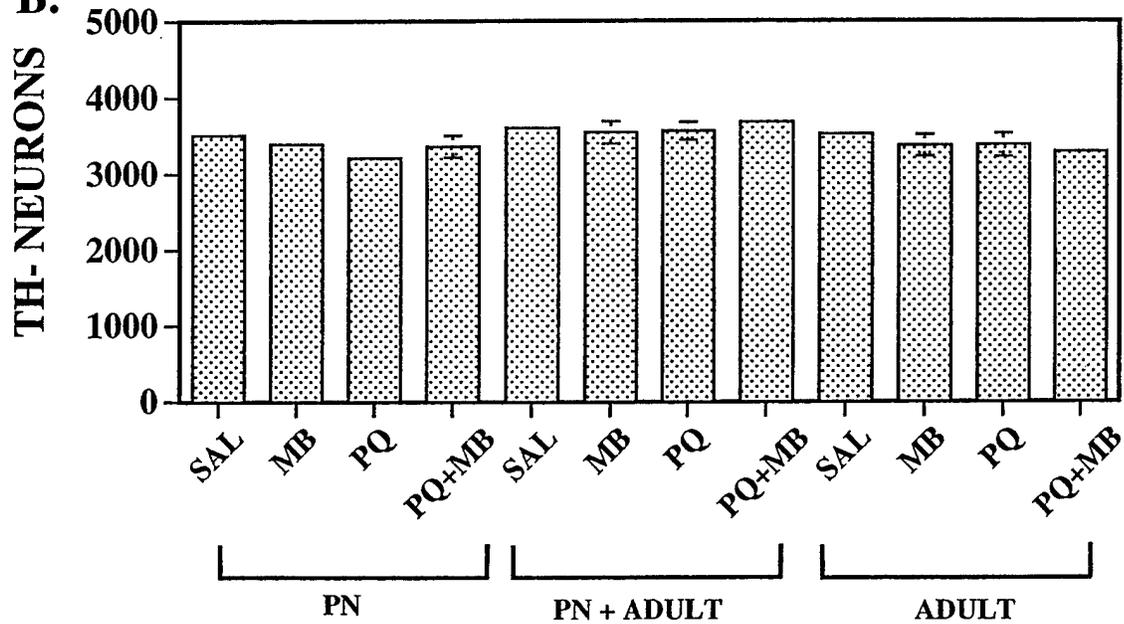


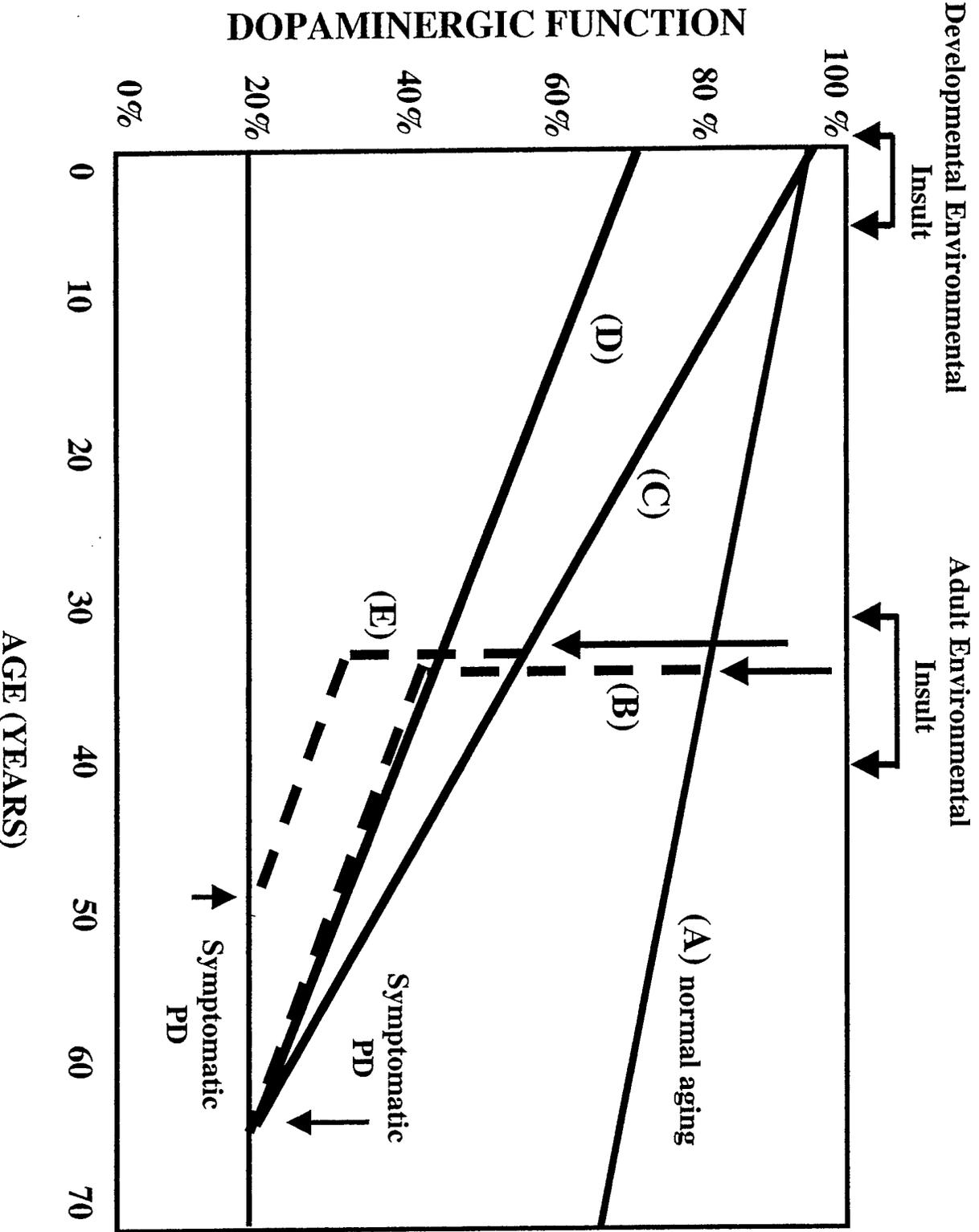


A.



B.





Section: Behavioral/System Neuroscience

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**Age-Related and Irreversible Nigrostriatal Dopaminergic Neurotoxicity
Following Exposure to the Pesticides Paraquat and Maneb**

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ABSTRACT

While advancing age is the only unequivocally accepted risk factor for idiopathic Parkinson's disease (PD), it has been postulated that exposure to environmental toxicants combined with aging could increase the risk for developing PD. The current study tested this hypothesis by exposing C57BL/6 mice that were 6 weeks, 5-months or 18-months of age to the herbicide paraquat (PQ) and/or the fungicide maneb (MB). Mice were injected 2X/week for 3 weeks with saline, 10 mg/kg PQ, 30 mg/kg MB or PQ+MB. PQ+MB-induced decreases in locomotor activity and motor coordination were age-dependent, with 18-month mice most affected and also showing the most pronounced failure to recover 24 hr post-injection. Levels of dopamine (DA), DA metabolites and DA turnover were also reduced to the greatest extent in 18 month PQ+MB mice as assessed 10 days post-treatment. Measurement of tyrosine hydroxylase (TH) enzyme activity indicated that 6 week and 5-month, but not 18-month old mice, compensated for striatal TH protein and/or DA loss following exposure with increased TH activity. Stereological assessment of nigrostriatal dopaminergic neurons demonstrated decreases at all ages following PQ alone and PQ+MB exposure. Three months post-treatment, only the 5 and 18 month PQ+MB groups still evidenced reductions of locomotor activity and deficits in motor coordination. Furthermore, striatal TH protein levels of both these groups remained 40% below control values. Collectively, these data demonstrate an enhanced sensitivity of the nigrostriatal pathway to these pesticides, particularly PQ+MB during advancing age with effects of these exposures being irreversible and progressive.

Keywords: dopamine, aging, striatum, substantia nigra, tyrosine hydroxylase, locomotor activity, stereology, irreversible

Although the etiology of idiopathic Parkinson's disease (PD) remains unknown, several risk factors have been attributed to the disease phenotype, including genetic background, environmental toxicants, and the aging process (Tanner, 1989; Koller et al., 1990; Polymeropoulos et al., 1997). Aging is perhaps the best-established of the putative risk factors for the sporadic form of PD. The disease rarely begins before the fourth decade of life and increases in prevalence through the seventh decade. Thus, any consideration of the etiology of PD must ultimately take into account the age-related nature of this disorder.

Several hypotheses have linked environmental neurotoxicant exposure, particularly pesticides, to aging in the induction of PD. Calne and Langston (Calne and Langston, 1983) suggested the possibility that exposure to a neurotoxicant could induce a discrete but limited insult to the nigrostriatal system in early or middle life, but compensatory mechanisms existed to preserve the normal dopaminergic function until later in life. This hypothesis assumes that aging is accompanied by a decline in the nigrostriatal dopaminergic system, and when added to the earlier lesion, will accelerate the natural decline of the system to below 80% of controls. An alternative hypothesis was put forward by Barbeau (Barbeau, 1984), suggesting that an insult in early or middle life would make the dopaminergic system respond by increasing dopamine production in the remaining terminals. This compensatory response would add to the stress of the system, leading to additional neuronal death. A third, more simplistic hypothesis merely postulates that there is age-dependent susceptibility to the effects of neurotoxicants, with younger individuals being more resistant.

The effects of the most commonly used neurotoxicant model of PD, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) have been well established, and confirm an enhanced susceptibility of older animals (Jossan et al., 1989; Walsh and Wagner, 1989; Date et al., 1990;

Irwin et al., 1992; Tatton et al., 1992; Irwin et al., 1993). MPTP, however, is not a common environmental agent. While the herbicide/dessicant paraquat has repeatedly been implicated in PD because of its structural similarity to MPP⁺, the active metabolite of MPTP, paraquat's effects have been equivocal following systemic exposures in rodents (Corasaniti et al., 1992; Widdowson et al., 1996a).

The environmental reality, however, is that exposure occurs to mixtures of chemicals rather than to single agents. In light of this, we developed a model of combined exposures to two toxicants i.e., the herbicide/dessicant paraquat (PQ) and the fungicide maneb (MB), based on the known dopaminergic neurotoxicity of each when administered separately (Takahashi et al., 1989; Corasaniti et al., 1992). Combined PQ+MB exposures produce potentiated and selective loss of nigrostriatal dopaminergic cell bodies and of striatal dopamine in young mice (Thiruchelvam et al., 2000a; Thiruchelvam et al., 2000b), with no effects of each when administered individually. To examine the hypothesis that an environmental toxicant could act in conjunction with aging to enhance the Parkinson's phenotype, the effects of PQ and MB, alone and in combination, were evaluated in mice that were either 6 weeks, 5 or 18 months of age at the beginning of treatment. The resulting data clearly demonstrate an age-related enhancement of sensitivity to combined PQ+MB. These changes were both permanent and progressive, with effects still evident 3 months post-exposure. Combined PQ+MB exposure may be a more relevant model than the routinely used MPTP model, since like PD, it shows selective age-related and irreversible nigrostriatal dopaminergic system neurotoxicity.

EXPERIMENTAL PROCEDURES

Animals

Male C57BL/6 mice 6 weeks, 5 months and 18 months of age from the National Institute of Aging (NIA) aging rodent colony (Harlan Sprague Dawley Inc.) were housed in a room maintained under constant temperature (72 - 74° F) and humidity conditions with a 12:12 light-dark cycle. Those used for behavioral studies were housed one per cage; all other mice were housed four per cage. Food and water were available *ad libitum*. Mice were habituated to the vivarium for at least one week prior to commencement of experiments. Body weights were obtained daily over the course of the exposures. Animals were cared for and treated in accord with NIH and the University of Rochester Animal Care and Use Committee guidelines.

Chemicals

Solvents for high performance liquid chromatography with electrochemical detection (HPLC-EC) were purchased from Sigma (St Louis, MO). All other chemicals, if not specified, were at least analytical grade and were purchased from Sigma (St. Louis, MO).

Drug Administration

Mice were injected i.p. with either saline (vehicle), 10 mg/kg paraquat (1,1'-dimethyl-4,4'-bipyridinium) dichloride hydrate (Sigma, St. Louis, MO), 30 mg/kg maneb (manganese bisethylenedithiocarbamate; gift from DuPont Agricultural Products) or PQ+MB. Both PQ and MB were dissolved in saline. Mice were injected twice a week for 3 weeks for a total of 6 injections. For combined injections, two separate injections were administered. Animals were sacrificed either 2 weeks or 3 months following the last treatment.

Locomotor Activity and Motor Function

Automated locomotor activity chambers equipped with infrared photobeams (Opto-Varimex Minor, Columbus Instruments International Corporation, Columbus, OH) were used to quantify locomotor activity. Photobeam breaks were recorded each minute for 45 minutes for horizontal, vertical, and ambulatory movements. Mice were initially habituated to the locomotor activity chambers in three 45-minute sessions occurring on consecutive days, with all mice receiving i.p. vehicle injections prior to the session. After the third habituation session, treatments began, and effects on motor activity were assessed immediately and 24 hours after each injection (sessions 1 and 2, respectively) in 45 minute test sessions with activity counts totaled in 3 min blocks across the session. The final locomotor assessment was carried out either 2 weeks or 3 months after the last treatment.

Motor Screen

The motor screen used consisted of two tasks: 1) an inverted wire screen hanging test and 2) a horizontal beam test. For the first task, mice were placed individually on top of a square wire screen 13 x 13 cm of #4 mesh mounted horizontally on a metal rod. The rod was then rotated 180°. The time taken to climb to the top of the screen was recorded, with 2 min recorded if mice fell to the table below or remained clinging to the screen. This is a test with demonstrated sensitivity to drugs such as haloperidol and results in fewer failures (e.g., falling or clinging) than techniques such as the rotarod (Coughenour et al., 1977; McDermott et al., 1994). For the horizontal beam test, mice were placed at one end of a 20 inch horizontal beam and were required to walk across the beam to a platform at the other end. The beam was placed 3 feet above the ground. For both tasks, each mouse was tested on three separate trials at each session

and the mean value of the last two trials were recorded.

Dopamine and Metabolite Analyses by HPLC

Neurotransmitter concentrations were measured 2 weeks or 3 months following the 6th injection of either saline, PQ, MB, or PQ + MB. Following rapid decapitation, striatal pieces were dissected and placed in 0.1N perchloric acid. The tissue was sonicated and centrifuged for 8 min at 1,000 X g. The supernatants were stored at -80°C until analyzed for the concentrations of DA, dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA) and serotonin by HPLC - EC. The pellets were digested in 1 ml of 0.5N NaOH for measurements of protein concentration using Bio-Rad assay reagents. HPLC analysis was carried out as described (Thiruchelvam et al., 2000a). The concentrations of the neurotransmitters were expressed in units of ng/mg protein. DA turnover was expressed as the ratio DOPAC/DA.

Immunohistochemistry (IHC) for Tyrosine Hydroxylase and Cell Counting

Paraformaldehyde fixed brains were cut into 40µm sections, collected in cryoprotectant and were later used for immunolabeling studies. Sections were rinsed in PBS at room temperature before immunostaining. Immunostaining was carried out as previously described (Chan et al., 1997) and incubated with a rabbit polyclonal anti-TH 1° AB (1:1000; Pel Freez Biologicals, Rogers, Arkansas). The total number of TH positive and Nissl-stained neurons in the substantia nigra pars compacta were counted using the optical fractionator method and previously described counting criteria (West et al., 1993; Chan et al., 1997). After delineation of the region at low magnification (4X objective), every sixth section from the entire substantia nigra was sampled at higher magnification (100X objective) using the Castgrid system

(Olympus).

Tyrosine Hydroxylase and Protein Levels Using Western Blot Analysis

Western blot analysis for TH protein was carried out as previously described (Osterhout et al., 1997). Briefly, following rapid decapitation, the striata were removed and stored at -80°C until analysis. Protein concentrations were determined using the Bio-Rad assay reagents. Each treatment group had a sample size of 8. For each sample, three different amounts of protein (5, 10, and 20 ug) were loaded onto separate lanes on a SDS polyacrylamide gel. The samples were subjected to electrophoresis, transferred to nitrocellulose, and immunoblotted with a rabbit anti-serum 1° Ab specific for TH. Detection was carried out with the ECL kit using autoradiography. The autoradiograms were scanned and the autoradiographic bands were quantitated using NIH image software to calculate the density. Only density values that were within the linear range of the autoradiographic film were utilized. The density for each TH protein band was normalized to the amount of protein loaded onto the gel for that particular sample and then divided by the density of standards of TH protein loaded onto that gel. TH protein values were expressed as ug of TH protein/mg of protein loaded.

Tyrosine Hydroxylase Enzyme Activity

Striata were dissected and rapidly frozen on dry ice and stored at -80°C. Striata were homogenized in 30mM potassium phosphate (pH 6.8), 10mM NaF, and 0.1mM EDTA. A 50ul aliquot was used for the assay and was carried out in duplicate. TH activity was assayed using the coupled decarboxylation assay (Fossom et al., 1991) and 6-methyl-5,6,7,8-tetrahydropterine as cofactor and expressed as nanomoles of $^{14}\text{CO}_2$ formed per minute per milligram of protein.

Protein was measured using the Bio-Rad assay.

Lung Histopathology

Since PQ is a known toxicant of the lung, representative sections of lung (n=6 for each treatment group) were prepared by formalin fixation, paraffin embedding, sectioning at 4 microns, and staining with hematoxylin and eosin. Sections were examined without knowledge of treatment group for evidence of alterations in alveoli, respiratory ducts, bronchioles and bronchi.

Statistical Analysis

Overall effects of treatment and age on horizontal locomotor activity were first analyzed with repeated measure analyses of variance (RMANOVA) using treatment and age as between-group factors and injections as a within-group factor. This was followed by individual ANOVAs using treatment and age as between-group factors for each injection and subsequent post hoc Bonferroni/Dunn tests to compare treatment groups. Bonferroni/Dunn tests control for numbers of comparisons and thus provide a conservative estimate of significance. To assess treatment-related changes within an activity session, RMANOVAs with treatment and age as between group factors and time block as a within group factor were utilized; significant main effects of treatment or interactions were followed by ANOVAs at each time point. Changes in DA, DOPAC and turnover were first evaluated using treatment, age and time point (2 weeks and 3 months) as between-groups factors for ANOVA. This was followed by separate ANOVAs for each time point for each measure. Effects of all other endpoints were analyzed using ANOVA with treatment and age as the between group factors, followed by Bonferroni/Dunn tests in the

event of significant main effects or interactions.

RESULTS

Body Weight and Pathology

No treatment-related changes in body weights were observed at any time point in the experiments, either when: 1) body weights prior to each motor activity session were compared across the entire experiment, or 2) when body weights from the final habituation session prior to treatment were compared to: a) body weights on the 6 injection days, b) body weights on the day following each injection, or c) body weights on the last day of the experiment. Lungs were graded for signs of alveolitis, bronchiolitis, bronchitis, lymphoid aggregation, bronchiectasis, and fibrosis and all were found to be histologically normal.

Locomotor Activity and Motor Screen

Locomotor activity was evaluated immediately and 24 hours after each treatment and then again at 2 weeks and 3 months after the last treatment. Figure 1 shows total ambulatory activity immediately, 24 hours and 3 months after the last treatment (6th treatment) for each of the age and treatment groups. *Immediately following exposure*, 5 and 18 month old groups exhibited significant reductions in locomotor activity ($p < 0.05$) following all treatments (PQ alone, MB alone and PQ+MB), whereas in 6 week old mice, only PQ+MB treatment produced significant reductions in activity (interaction of treatment by age: $F(5,101) = 3.523$, $p = < 0.0001$). PQ alone and MB alone each produced age-related decreases in locomotor activity, with 18 month old mice showing the lowest activity scores. Combined PQ+MB produced significantly lower activity levels in all age groups relative to either chemical administered alone as well as

compared to their age-matched controls ($p < 0.0001$). The 5 (12% of control) and 18 month (8 % of control) old mice were significantly less active compared to the 6 week (46% of control) old mice that received PQ+MB.

When measured 24 hours following the last exposure, significant main effects of treatment ($F(3,101) = 6.118$; $p = 0.007$) and age ($F(2,101) = 14.143$; $p < 0.0001$) were obtained, but not a significant interaction of the two factors. The 6 week old mice that received PQ alone, MB alone and the combination were no different than controls. The 5 month old mice that were treated with PQ alone or MB alone exhibited a decrease (20%) in activity that was not statistically different from age-matched saline treated mice. However the 5 month group that received PQ+MB showed a partial recovery in activity compared to levels measured immediately after dosing, but still significantly lower than corresponding age-matched saline control levels (61% of control, $p < 0.05$). The 18 month old mice that received either compound alone or the combination failed to show recovery 24 hours after the last dose, exhibiting significantly lower activity levels compared to their corresponding age-matched saline treated group ($p < 0.001$)

Three months after the last dose, 6 week old mice looked like corresponding saline-treated counterparts. Levels of activity in PQ alone and MB alone treated 5 month old mice had recovered to saline levels at this time point, even though they were 20% of controls at 24 hours after the 6th dose. However, 3 months after the last dose the PQ+MB treated 5 month old animals showed a persistent decrease in activity that were comparable to the levels observed 24 hours after the last dose. The 18 month old mice treated with PQ alone and MB alone also showed an irreversible decrease in activity, with activity levels at 76% of control values 3 months post-treatment. The PQ+MB treated 18 month old mice showed a further, progressive decrease in activity, with levels down to 24% of control 3 months post-treatment as compared to 62% when

measured 24 hours after the last dose.

The balance beam and inverted screen tasks were carried out 10 days and again 3 months after the last treatment day. The time taken to either cross a beam to a platform (beam walk) or to right from an inverted position (inverted screen) was determined and the outcomes are presented as percent of control values (Figure 2). Ten days after the last treatment, a significant interaction of treatment by age was obtained for both the beam walk and inverted screen tasks ($F(6,106) = 5.986, p < 0.0001$; $F(6,106) = 2.325, p = 0.038$, respectively). When tested 3 months after the last treatment, a significant interaction of treatment by age was obtained for the beam walk but not the inverted screen task ($F(6,106) = 7.199, p < 0.0001$; $F(6,106) = 1.185, p = 0.32$, respectively). The 6 week old animals showed no change in latency regardless of behavioral paradigm or treatment. Both 5 and 18 month old mice treated with MB alone showed no adverse effects in either paradigm. PQ alone treated 5 month old mice showed an increased latency on the beam walk task measured 10 days after treatment, but this effect was no longer evident 3 months post-treatment. The 18 month old mice treated with PQ alone showed a significant increase in latency on both tasks ($p < 0.0001$) as measured ten days after the last treatment, and this latency increase was sustained on the inverted screen task at 3 months post-treatment. Combined exposure to PQ+MB produced a significant age-related increase in latency in both 5 and 18 month old mice at 10 days ($p < 0.0001$) after treatment, with this increase sustained even 3 months after treatment ended for both tasks ($p < 0.0001$).

Striatal Dopamine, Metabolites, Turnover and Serotonin

Changes in striatal DA, DOPAC, HVA, DA turnover (DOPAC/DA) and 5HT levels were evaluated at 2 weeks and 3 months after the last treatment. **Two weeks** after the last treatment

(Figure 3), 18 month old mice treated with PQ+MB showed significant decreases in DA levels (21%) compared to the corresponding age-matched saline, PQ alone values and relative to 6 week old mice given PQ+MB ($p=0.03$, 0.03 , and 0.007 , respectively). Similarly, DOPAC levels were significantly lower in this age group after combined PQ+MB (40%). Although not statistically significant, the 6 week and 5 month combined PQ+MB mice also showed decreases in DOPAC levels (15-18%). DA turnover was decreased at this time point mainly for 6 week and 18 month old mice (21-25%, $p=0.005$ and 0.002 , respectively). All the PQ treated groups showed an approximate though non-significant 10% decrease in DA turnover.

Three months following the last treatment (Figure 4), modest but significant decreases in DA were still evident in the 5 (19%) and 18 month (25%) PQ+MB treated mice. Changes in DOPAC depended upon age and treatment at 3 months post-treatment (interaction effect: $F(6,105) = 2.185$, $p=0.05$). An age-related decrease in DOPAC was seen with PQ alone, with even more pronounced reductions in response to PQ+MB. DOPAC levels were reduced by 62% in the 18 month old PQ+MB group. Similarly, HVA levels were also significantly reduced by the combination of PQ+MB in both 5 and 18 month old mice. DA turnover was significantly decreased (interaction of treatment by age: $F(6,105)=3.420$, $p=0.004$), in 5 and 18 month old mice given PQ+MB ($p<0.0001$ for both groups). No changes in 5HT levels were observed with any of the treatment or age groups, indicating a selective vulnerability of the nigrostriatal system. When effects at 2 weeks are compared to those 3 months post-treatment, DOPAC levels, and consequently DA turnover showed progressive reductions in 5 month old mice given PQ+MB, falling an additional 38% and 32% respectively in DOPAC and turnover and an additional 16% in DOPAC in 18 month old mice given PQ alone and an additional 32% and 22% decrease in DOPAC and turnover ($p<0.001$) in those given PQ+MB.

Tyrosine Hydroxylase Enzyme Activity

Changes in TH enzyme activity in the striatum were determined 2 weeks after the last treatment (Figure 5) and revealed an age-related decrease in saline-treated mice (interaction of treatment by age: $F(6,77)=2.388$, $p=0.036$), with 18 month old mice having the lowest TH activity. The 6 week and 5 month old mice showed significant increases in TH activity relative to corresponding age-matched saline control levels following PQ alone (20-40%) and in response to PQ+MB treatment (25-38%). In contrast, 18 month old mice showed a 35% decrease in TH activity following combined PQ+MB compared to the age matched control group and TH activity in response to PQ alone that were significantly lower than the corresponding 6 week and 5 month old mice given PQ alone.

Nigral Dopaminergic Cell Counts

The number of dopaminergic cells in the substantia nigra pars compacta was determined 2 weeks after the last treatment and are depicted in Figure 6. Both PQ alone and PQ+MB produced significant age-dependent reductions in the number of TH positive neurons (interaction of treatment and age: $F(6,31)=6.432$, $p=0.0002$). PQ alone produced a 25-30% decrease across age groups, with the 18 month animals showing the greatest mean loss ($p<0.0001$). Combined PQ+MB decreased TH positive neurons by 25-37% ($p<0.0001$). The 5 ($p<0.0001$) and 18 month ($p=0.02$) PQ+MB treated mice had greater cell loss than the corresponding age-matched PQ alone treated mice. Saline and MB alone however, produced no age-related change in the number of TH positive neurons. A similar decrease was observed when all Nissl positive neurons (total number of neurons) were counted, indicating that these neurotoxicants destroyed only dopaminergic neurons of the substantia nigra pars compacta.

Striatal Tyrosine Hydroxylase Protein Levels

Striatal TH protein levels were determined 2 weeks and 3 months after the last treatment (Figure 7). The 3 month time-point should reflect permanent changes in the terminals of the nigrostriatal system. At the 2 week time-point, a significant age related decrease was observed following exposure to PQ and PQ+MB (significant treatment and age effect; $F(3,60) = 4.056$, $p=0.011$; $F(2,60) = 3.055$, $p=0.05$ respectively). Three months post-treatment, PQ alone decreased striatal TH protein levels only in 18 month old mice by 18%. PQ+MB reduced striatal TH protein concentrations in both 5 and 18 month groups (interaction of treatment by age: $F(6,57)=2.818$, $p=0.018$), with levels reduced by 18 and 23%, respectively. The decrease in TH protein after PQ+MB treatment was age-related, with 18 month mice having lower protein levels than either the 6 week ($p<0.0001$) or 5 month old mice ($p=0.017$). Similarly, the 5 month old mice treated with PQ+MB had protein levels that were significantly lower than those of the 6 week old mice ($p=0.05$). MB alone did not result in any change in TH protein levels.

DISCUSSION

The intent of this study was to determine whether the parkinsonian phenotype produced by exposure to the environmental neurotoxicants, PQ and MB, would be enhanced by aging. The most commonly used approach to study PD has been the MPTP model, the effects of which are enhanced by aging (Irwin et al., 1993; Irwin et al., 1994). However, in addition to the fact that MPTP is not an environmental neurotoxicant, its effects are often reversible (Saitoh et al., 1987; Date et al., 1990), limiting its ability to mimic a progressive disorder, such as PD. We have previously demonstrated that combined exposure to the herbicide/dessicant paraquat (PQ) and the fungicide maneb (MB) produce a selective and potentiated neurotoxicity to the nigrostriatal dopamine system in young mice that includes loss of terminal function as well as cell bodies (Thiruchelvam et al., 2000a; Thiruchelvam et al., 2000b). We therefore examined whether the nigrostriatal dopaminergic neurotoxicity produced by PQ+MB would be enhanced by aging and, importantly, whether any effects observed would be progressive and/or irreversible. Thus the outcomes of exposures to these pesticides alone and in combination were assessed in mice aged 6 weeks, 5 months or 18 months of age at 2 weeks and again 3 months post-termination of treatment. Consistent with the hypothesis described above, the effects of PQ and MB were clearly enhanced by aging, with older mice (both 5 and 18 month old, depending upon the outcome measure) more susceptible to the adverse effects of these toxicants alone as well as in combination compared to young mice (6 weeks). Moreover, some of the effects of PQ+MB were permanent and even progressive.

The first indication that aging enhanced vulnerability was the fact that the total number of treatments administered had to be abbreviated. In our previous studies using 6 week old mice, a total of 12 treatments were administered (Thiruchelvam et al., 2000a; Thiruchelvam et al.,

2000b). Our initial intention was to duplicate that treatment regimen with older animals. However, it was deemed necessary to stop the total number of injections at 6, since 18 month old PQ+MB treated mice began to exhibit a lack of recovery of locomotor activity 24 hr post-treatment. Since locomotor activity has been an accurate predictor of underlying dopaminergic changes, particularly cell loss, in our previous as well as unpublished studies, treatment was halted in the interest of ensuring survival.

Treatment-related reductions in locomotor activity were also age-dependent, with older mice exhibiting more pronounced reductions after treatments (Figure 1), especially combined PQ+MB, but also following PQ alone and MB alone. Furthermore, the residual reductions in locomotor activity 24 hours after treatment were age-related as well, with 5 and 18 month old mice showing lack of recovery, whereas 6 week old mice exhibited complete recovery. Reduced levels of locomotor activity were still observed 3 months post-treatment in 5 and 18 month old mice. Furthermore, these changes were both irreversible and progressive in 18 month old mice given PQ+MB, where partial recovery of locomotor activity was evident at 24 hr post-treatment (62% of control), but when activity was assessed again 3 months post-treatment, levels had declined further to only 24% of control. A similar pattern of age-related enhancement of neurotoxicity was apparent when levels of striatal DA and numbers of dopaminergic neurons in the nigra were examined (Figures 3-4 and 6), particularly for combined PQ+MB treatment. Progressive decreases in DOPAC levels occurred in 6 week old PQ+MB mice between the 2 week and 3 month post-treatment time points. Both 5 and 18 month old mice showed a decrease in DA 2 weeks after the last PQ+MB treatment that was still evident 3 months after the final dose. Even more notable was the progressive reductions in these groups in DOPAC and HVA levels (only 3 month data shown), and thus DA turnover, from the 2 week to 3 month post-

treatment time points. To further verify the rate and nature of this progressive neurotoxicity, a more complete time course study will have to be conducted.

The increases in striatal TH enzyme activity (Figure 5) in the groups that received PQ alone relative to corresponding age-matched controls, and in 6 week and 5 month old mice that received PQ+MB suggests that striatal toxicity was most likely masked by an apparent compensatory mechanism in remaining terminals to increase DA levels. However, 18 month old mice given PQ alone showed TH enzyme activity levels similar to those of saline-treated controls, and those given PQ+MB actually demonstrated a significant reduction in TH enzyme activity relative to all other conditions, suggesting the loss of this compensatory capability with age. In concert with that assertion and with progressive and permanent neurotoxicity is the observation of a further reduction in levels of DA and metabolites and DA turnover in the 18 month PQ+MB group between the 2 week and three month post-treatment time points.

Our findings that older mice are more vulnerable to the effects of PQ and MB are consistent with the fact that age represents a period of enhanced vulnerability to such neurotoxicants. Advancing age is accompanied by decreases in defense mechanisms, in the activity of xenobiotic metabolizing enzymes and in anti-oxidant levels (e.g., glutathione) (Slivka et al., 1987). Aging is also a period in which blood-brain-barrier function is compromised (Mooradian, 1988). Consistent with that, greater regional brain levels of ^{14}C paraquat were found in older (24 month old) mice or rats compared to their younger counterparts following systemic injections (Corasaniti et al., 1991; Widdowson et al., 1996b). Thus greater levels of exogenous neurotoxicants may cross the blood-brain-barrier and reach the central nervous system with age. If elimination is delayed, neurotoxicants can potentially exert deleterious toxic effects for a longer period of time. Our initial studies using this model showed a surge of DA immediately

following exposure to either of these compounds alone with this effect potentiated by the combined exposure (Thiruchelvam et al., 2000a; Thiruchelvam et al., 2000b). If these compounds are indeed being excreted at a slower rate in older mice, then the repeated surges in DA may prove to be the toxic mediator leading to terminal dysfunction as well as dopaminergic cell death. Furthermore, in this mouse strain, monoamine oxidase (MAO) A and B show increased activity with age (Jossan et al., 1989; Irwin et al., 1992; Irwin et al., 1997). Metabolism of DA by MAO generates H_2O_2 that can in turn participate in free radical generation and oxidative stress, leading to neuronal degeneration. PQ+MB might also be inhibiting the enzymes involved in the metabolism of DA or altering the kinetics of these enzymes. Another possibility is that upon reentry into the cell, DA becomes sequestered in vesicles at a more rapid rate, preventing its metabolism. The changes in DA and metabolites and TH protein levels, and the DA cell loss clearly indicate that these effects are permanent, as well as progressive. Further studies will obviously be required to ascertain the operative mechanisms.

For any model of parkinsonism, various factors must be considered. One of these is the extent to which changes related to age per se occur in the nigrostriatal system of mice, for which the evidence is certainly conflicting (McNeil et al., 1984; McNeil and Koek, 1984; Ricaurte et al., 1987; McNeil et al., 1988; McNeil and Koek, 1990; Greenwood et al., 1991; Tatton et al., 1991a; Tatton et al., 1991b). Recent studies, including this one, using more sensitive techniques to evaluate dopaminergic changes, suggest that there is no change in striatal DA levels overall (Osterburg et al., 1981; Saitoh et al., 1987; Date et al., 1990; Greenwood et al., 1991; Irwin et al., 1993) or any loss of nigral dopaminergic neurons in C57BL/6 mice with age (McNeil et al., 1988; McNeil and Koek, 1990). Contrary to other reports (Greenwood et al., 1991), however, our data do suggest a decrease in dopamine synthesis with age, as indicated by the reduction in TH

enzyme activity in the dorsal striatum (Figure 5). TH protein levels, as observed in our studies, as well as reported by others, and gene expression do not appear to change with age (Gupta et al., 1990). Collectively these findings suggest that the available enzyme has a lowered specific activity. But they do suggest that the hypothesis of Calne and Langston (Calne and Langston, 1983) that an age-accompanied loss of dopaminergic neurons in the substantia nigra increases vulnerability to the effects of neurotoxicants, or that an insult during middle life can accelerate this age-related decline in dopaminergic function to a level below that needed for normal function, cannot be addressed using this model. These findings stand in contrast, however, to the decline in nigrostriatal function with age that appears to occur in human and non-human primates (Hiral, 1968; Carlsson and Winblad, 1976; McGeer et al., 1977; Mann and Yates, 1983; McGeer et al., 1988; Fearnley and Lees, 1991; Irwin et al., 1994; Emborg et al., 1998; Kordower et al., 2000)

From the perspective of an environmental model, it is difficult to accurately determine the extent to which combined PQ+MB fully mimics human exposures, given the paucity of information on both the types and levels of pesticides to which humans are ultimately exposed. It is obvious though, given environmental uses, that mixtures of pesticides must ultimately be considered. Nevertheless, combined PQ+MB has distinct advantages as a model for PD. Unlike MPTP, its effects are progressive and irreversible, an important component of PD. Moreover, they are enhanced by advancing age. Whether this model results in the development of Lewy bodies, considered a hallmark of PD, is as yet determined. It may be unlikely with the current treatment regimen, since it produces what appears to be a relatively early parkinsonian phenotype, and that a more protracted treatment regimen would be necessary to detect such inclusions. The fact that PQ+MB provides a model of preclinical parkinsonism presents the

specific advantage of permitting assessment of some of the early operative mechanisms by which progressive nigrostriatal dopaminergic pathogenesis can occur and persist.

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FIGURE LEGENDS

Figure 1

Total horizontal locomotor activity. Group mean \pm S.E. (n=10) (plotted as percent of control group values) measured immediately, 24 hours and 3 months after the last dose (6th injection). Mice at three ages (6 weeks, 5 and 18 months) were exposed to either saline, 10 mg/kg paraquat (PQ), 30 mg/kg maneb (MB) or their combination (PQ + MB). Fisher's post-hoc tests for each treatment day indicate differences: * from age-matched control group; ~ from MB alone; ^ from PQ alone; + from 6 week old mice receiving identical treatment; # from 5 month old mice receiving identical treatment.

Figure 2

Age-related and long term effects on a motor coordination task, A) the beam walk and B) inverted screen in 6 week, 5 and 18 months old mice treated twice a week for three weeks with saline, 10mg/kg PQ, 30mg/kg MB or combined PQ+MB. Data indicates latency to traverse a beam (beam walk) or to get to a upright position (inverted screen). Group mean \pm S.E. (n=10) is represented as percent of appropriate age-matched controls. Behavioral measurements were carried out 10 days and 3 months after the last treatment day. Fisher's *post-hoc* confirmed significant differences from: *saline; ~ from MB alone; + 6 week old mice; @saline, MB alone & 6 week and 5 month old mice; #saline, MB alone, PQ alone and 6 week and 5 month old mice (all compared to age-matched groups).

Figure 3

Striatal levels of DA, DOPAC, and DA turnover (DOPAC/DA). Group mean \pm S.E. as a

percent of control levels of DA, DOPAC, and DA turnover assessed 2 weeks after the 6th i.p. injection of saline, 10 mg/kg paraquat (PQ 10), 30 mg/kg maneb (MB 30) or their combination (PQ + MB). Fisher's post-hoc analysis indicate significant difference: *saline; ~ from MB alone; ^ from PQ alone; + 6 week old mice; # 5 month old mice.

Figure 4

Striatal DA, DOPAC, HVA, DA turnover and 5HT three months after the 6th treatment of either saline (n=10), 10 mg/kg PQ (PQ 10, n=10), 30 mg/kg MB (MB 30, n=10) or the combination (PQ + MB, n=10). Data are shown as group mean \pm S.E (percent of age-matched controls). Fisher's *post-hoc* confirmed significant differences from: *saline; ~ from MB alone; ^ from PQ alone; + 6 week old mice; # 5 month old mice (all compared to age matched groups).

Figure 5

Tyrosine hydroxylase enzyme activity in the striatum. Enzyme activity was determined 2 weeks after the last treatment (6th injection). Data is represented as pmol of ¹⁴CO₂ formed per min per milligram protein (n=8 for each group). Post-hoc analysis showed significant differences from: *saline; ~ from MB alone; ^ from PQ alone; + 6 week old mice; # 5 month old mice.

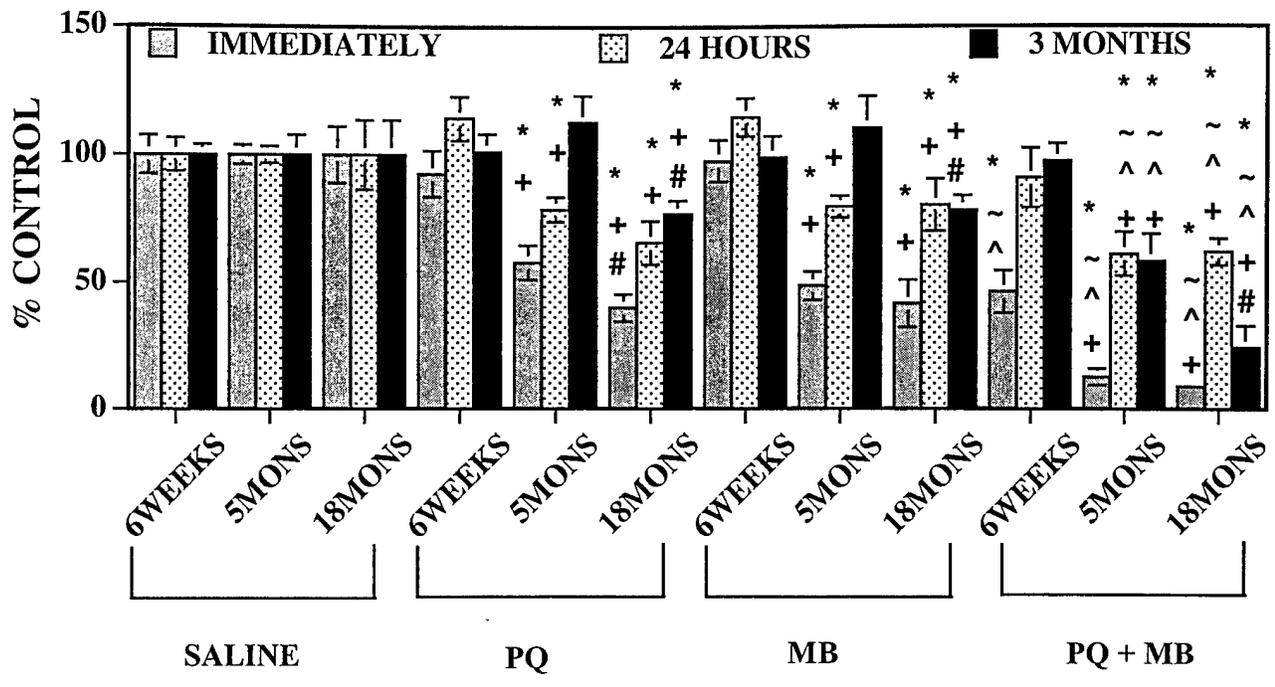
Figure 6

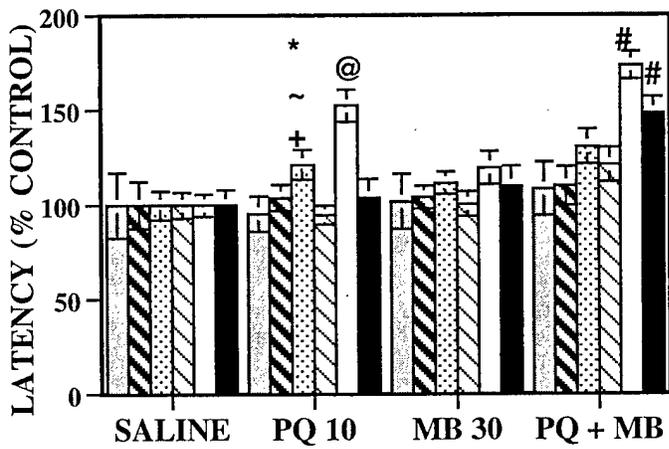
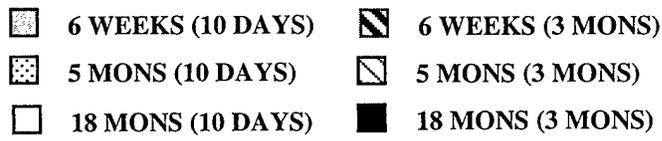
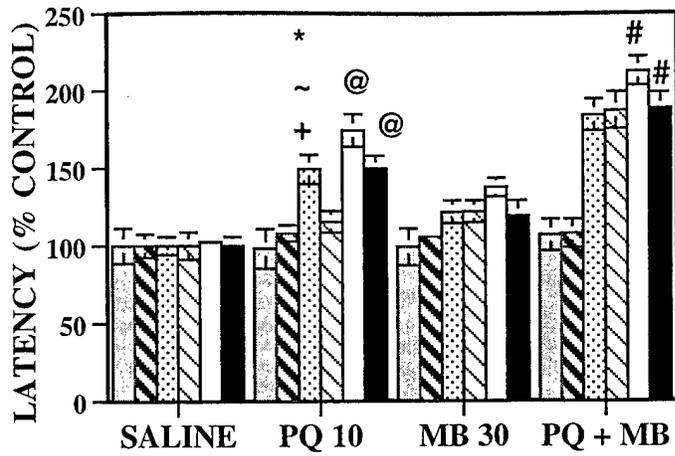
Total number of TH neurons in the substantia nigra pars compacta. Animals were sacrificed two weeks after the last treatment of either saline, 10 mg/kg PQ (PQ), 30 mg/kg MB (MB) or the combination of the two (PQ + MB). Data represents total number of TH positive and cresyl violet positive neurons (n=3-5 per treatment group). Similarly, total number of neurons in

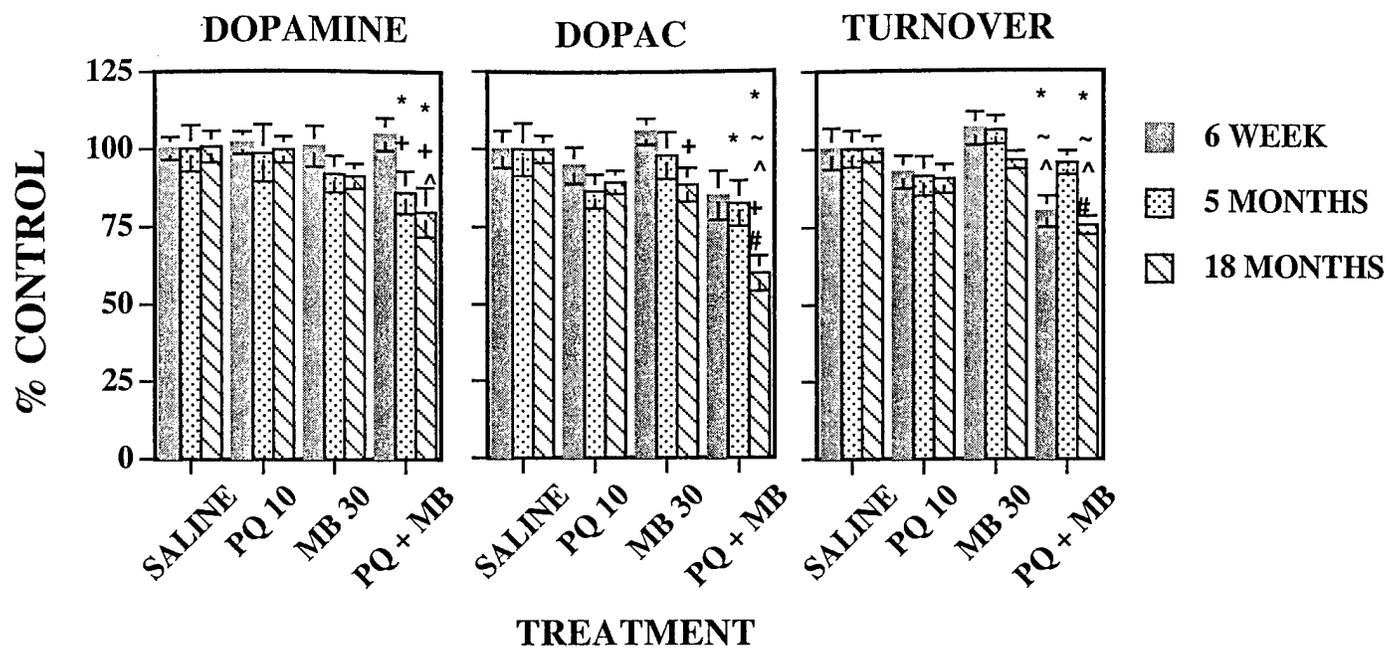
the substantia nigra decreased to the same extent (data not shown). Fisher post-hoc analysis revealed significance from: *saline; ~ from MB alone; ^ from PQ alone; + 6 week old mice; # 5 month old mice.

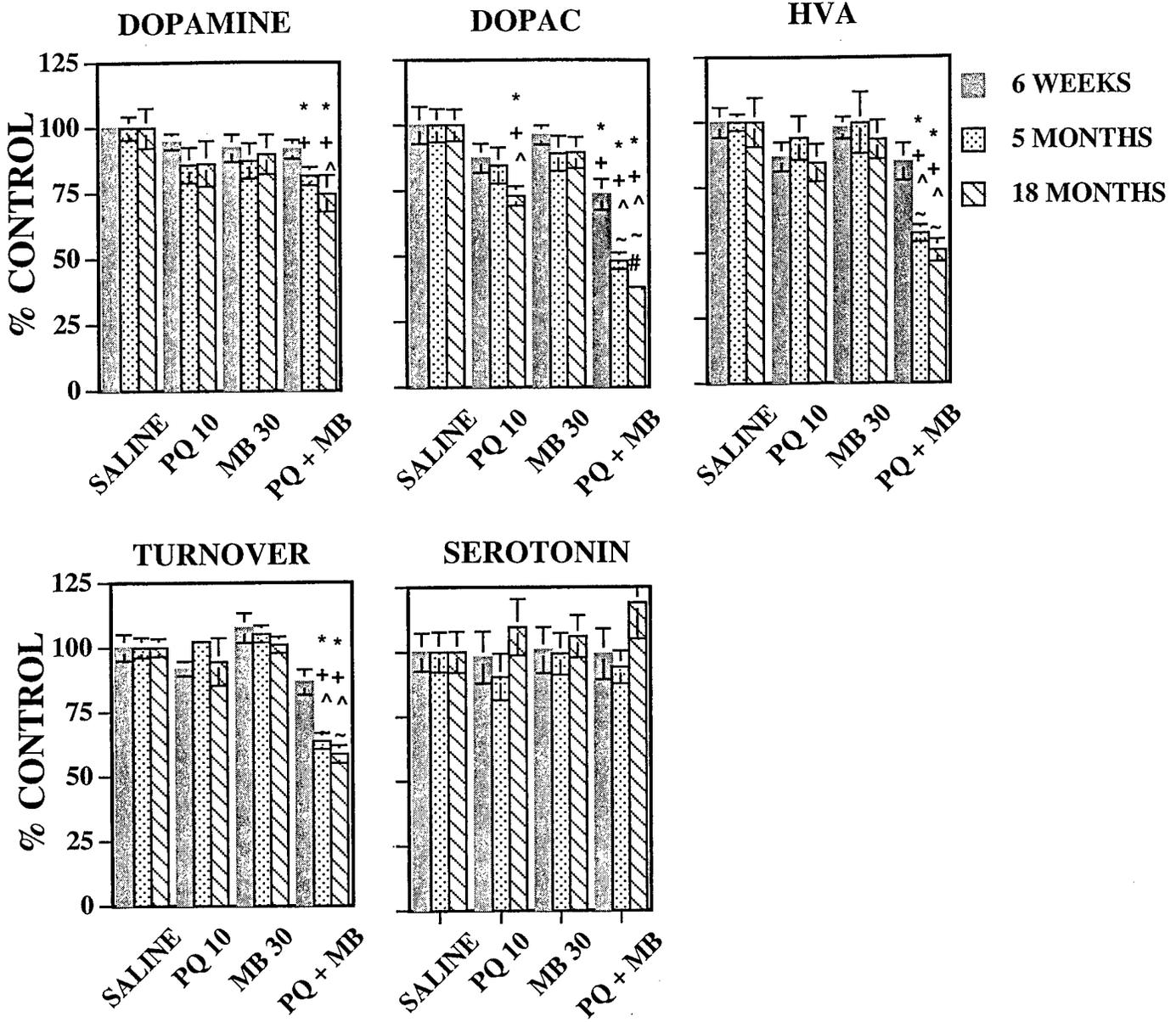
Figure 7

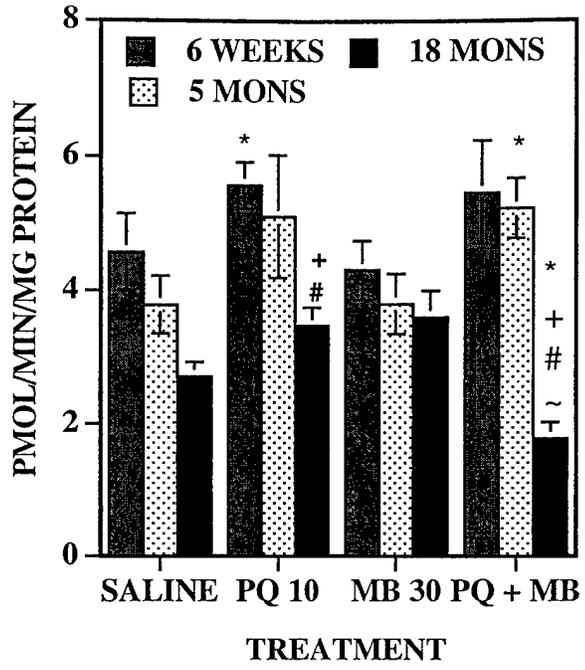
Western blot analysis of TH protein in mouse striatum. Group mean \pm S.E. levels of tyrosine hydroxylase protein levels (plotted as % of control group values) in the dorsal striatum 3 months after the 6th and last injection of saline (n=8), 10 mg/kg paraquat (PQ 10; n=8), 30 mg/kg maneb (MB 30; n=8) or their combination (PQ 10 + MB 30; n=8). Fisher's post-hoc analysis indicate significant difference: *saline; ~ from MB alone; ^ from PQ alone; + 6 week old mice; # 5 month old mice.

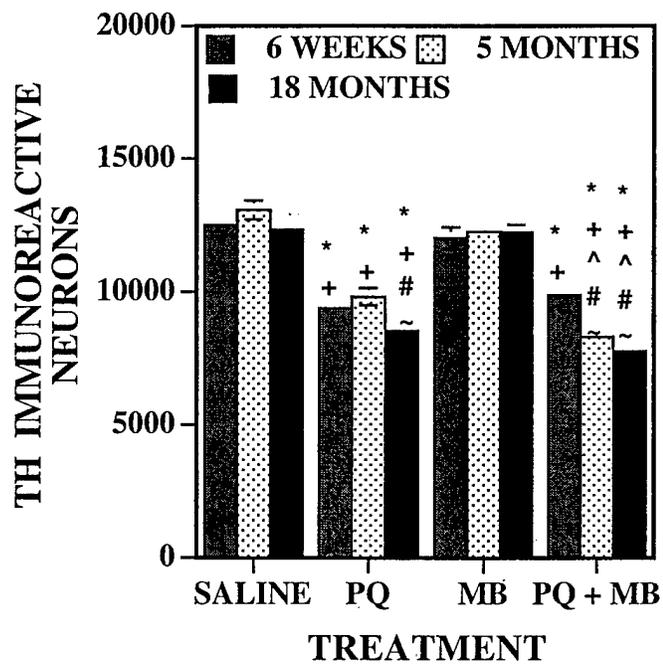


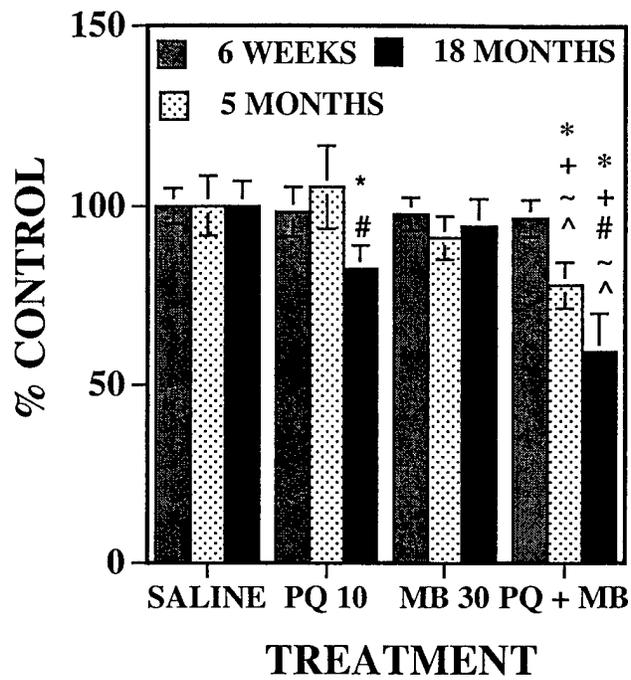












Selective Dithiocarbamates Increase Synaptosomal Dopamine Content, Brain Concentration of Paraquat, and Correlate with Potentiation of MPTP and Paraquat Neurotoxicity

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Abbreviations

AUC – area under the curve

DA – dopamine

DAT – dopamine transporter

DTCs – dithiocarbamates

PAT – polyamine transporter

PDP – Parkinson's disease phenotype

PQ – paraquat

SNpc – substantia nigra pars compacta

TD – toxicodynamic

TK – toxicokinetic

* These authors contributed equally to this paper.

Summary

Pesticides may be risk factors for Parkinson's disease based on epidemiologic data in humans, animal models, and *in vitro* studies. Different dithiocarbamate pesticides potentiate the toxicity of MPTP and/or paraquat in mouse models of Parkinsonism by an unknown mechanism. This study examined the effects of commercially used dithiocarbamates on [³H]-dopamine transport in striatal synaptosomes and on the concentration of [¹⁴C]-PQ *in vivo* in mice. Different ethylenebisdithiocarbamates and diethyldithiocarbamate increased dopamine accumulation in synaptosomes, whereas dimethyldithiocarbamates and methyldithiocarbamate did not. Increased dopamine accumulation in synaptosomes was dose-dependent. The increased accumulation of dopamine was due to the carbon backbone of these molecules. Dithiocarbamates increasing accumulation of dopamine did not alter the influx of dopamine, but delayed the efflux out of synaptosomes. Selective dithiocarbamates also increased tissue uptake and retention of [¹⁴C]-paraquat. Paraquat was not transported via the dopamine transporter. There was a consistent relationship between dithiocarbamates increasing synaptosomal accumulation of dopamine and tissue accumulation of paraquat with those previously demonstrated to enhance MPTP and/or paraquat toxicity *in vivo*. We hypothesize that certain dithiocarbamates potentiate the neurotoxicity of MPTP and paraquat by inhibiting an efflux transporter(s), effectively increasing and prolonging intracellular exposure to dopamine and/or the neurotoxicants.

Introduction

Pesticides (herbicides, insecticides, and fungicides) have been implicated in predisposing to the Parkinson's disease phenotype (PDP) based on epidemiological data in humans¹⁻⁵, animal models⁶⁻⁹, and biochemical studies *in vitro*¹⁰⁻¹¹. Identification of these pesticides, their mechanism(s) of action and their interaction with other risk factors for the PDP are under active study. The dithiocarbamates (DTCs) are a class of pesticides that have been used extensively world-wide for the last 40-50 years. Estimates of use range from 25-35,000 metric tons world-wide as of 1988¹². A variety of neurotoxicant effects related to DTCs have been documented including parkinsonism, although the mechanism(s) of neurotoxicity remain unclear^{1,13}. Originally studied as an inhibitor of superoxide dismutase, diethyldithiocarbamate (diethyl-DTC) was shown to potentiate the effect of the human PDP-inducing neurotoxicant MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) in mice¹⁴⁻²³. The mechanism for potentiation of MPTP toxicity of substantia nigra pars compacta neurons (SNpc) by DTCs has not been clearly defined. However, the mechanism must relate to basic concepts of chemical mixture toxicology²⁴. These include three basic concepts as originally defined by Bliss²⁵: (1) Simple similar action in which the different agents contribute to toxicity in proportion to their dose acting via a similar mechanism of toxicity; (2) Simple dissimilar action in which the agents do not affect each others' toxic effects. The mechanism and site of toxicity differ between the agents; (3) Interactions result if agents modify the magnitude or nature of the toxic effect of each other. These interactions may relate to toxicokinetics (uptake, distribution, metabolism, or excretion) or toxicodynamics (effect on specific organ or protein target). Understanding the mechanism of action of DTCs in neurotoxicant mouse models of the PDP will be important for determining if DTCs play a similar role in humans and what the mechanism of potentiation might be.

Paraquat (N,N'-dimethyl-4,4'-bipyridylium), another commonly used pesticide²⁶, has often been implicated as a human risk factor for the PDP based on epidemiologic studies²⁷⁻²⁹ because of its structural similarity to MPTP^{10,30}. Animal data on the effect of paraquat on the nigrostriatal system has been inconsistent with several studies failing to show a neurotoxic effect^{10,31-33}. However, recent data on the effects of paraquat alone or in combination with DTCs, using alternative outcome measurements at different ages, has been more supportive of a potential role for paraquat in the PDP in mice^{7-9,34-35}. Furthermore, because DTCs and PQ are used in geographically overlapping areas, and because these agrichemicals may persist in the environment and as food residues, it is important to understand how exposures to combinations of these chemicals may contribute to the development of the PDP in humans.

How DTCs might enhance the toxicity of both MPTP and paraquat in mice is not known. One protein proposed to link the effects of MPTP and paraquat is the dopamine transporter (DAT). The DAT has been proposed to be the primary route of access to nigrostriatal terminals for both MPP⁺ (1-methyl-4-phenylpyridium ion, the active metabolite of MPTP) and paraquat, although uptake of paraquat through the DAT has not been demonstrated. Another known route for brain uptake of PQ is via a polyamine transporter(s) (PAT) which has been demonstrated in lung and brain³⁶ to transport different polyamines and paraquat into cells in an energy-dependent and saturable manner³⁷⁻³⁹. We sought to determine if DTCs had an effect on the DAT as a common link between these two mouse PDP-inducing neurotoxicants and whether PQ might also be transported through the DAT.

Experimental Procedures

Tissue Procurement and Processing. Mice were acquired and cared for in accordance with guidelines published in the National Institutes of Health Guide for Care and Use of Laboratory Animals. Adult C57Bl/6J mice were sacrificed by cervical dislocation followed by harvesting of the brain. The dorsal striatum was rapidly dissected on ice, weighed, and immediately homogenized as previously described⁴⁰. Briefly, tissue was homogenized in 20 volumes of ice cold 0.32 M sucrose, 50 mM Tris-HEPES (pH 7.4), using a Teflon glass homogenizer (clearance, 0.15 mm), and using 14 up-and-down rotating strokes by hand. The homogenate was then centrifuged at 900 x g for 10 minutes. The supernatant was then centrifuged at 17,000 x g for 20 minutes. The pellet was resuspended in 20 volumes of ice cold incubation buffer and mixed by vortexing. The incubation buffer consisted of 10 mM Tris-HEPES, pH 7.4 (145 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂, 10 mM glucose, 0.25 mM ascorbate, and 15 μM pargyline).

DA Uptake. The final reaction volume was 0.5 ml. [³H]-DA was used at a concentration of 10 nM for all experiments. Other drugs and ions were prepared in incubation buffer (except as noted below) and kept on ice until added to the reaction mixture. The reaction mixture was kept on ice until a 5 minute prewarming step at 37 °C followed by incubation at 37 °C. Uptake studies were initiated by the addition of the tissue. Uptake was terminated by the addition of 3 ml ice cold normal saline and tubes were placed on ice until filtration (except for efflux studies). For efflux studies, synaptosomes were pre-filled with [³H]-DA for 12-14 minutes and then diluted 10 fold by the addition of warm incubation buffer containing no [³H]DA. After variable efflux times, the tubes were filtered. Efflux studies were done in the absence or presence of either Mn²⁺-ethylenebis-DTC or cocaine in the buffer. In these studies, synaptosomes were pre-filled in the absence or presence of

a compound and then placed in warm incubation either with or without the same compound. After variable efflux times, the tubes were filtered. In saturation experiments, the K_m and V_{max} for DA were calculated after incubating with a range of concentrations of unlabeled DA (1 nM to 100 nM) in the presence or absence of a single concentration of Mn^{2+} -ethylenebis-DTC (0.5 μM) for two durations of influx.

The synaptosomes were rapidly filtered (Whatman GF/C filter paper) using a 48 probe harvester (Brandel Incorporated, Gaithersburg, Maryland) and washed twice with 3 ml of cold saline. Filters were counted using a liquid scintillation counter and 5 ml of Ecoscint H (National Diagnostics) cocktail. Counting efficiency was corrected for each sample. Total uptake occurring at 37 °C was corrected by subtracting uptake occurring at 0 °C and the temperature specific uptake always exceeded 90%. Uptake was proportionate to tissue concentration and was time-dependent (data not shown). Protein was measured using the Bio-Rad protein assay (Bio-Rad, Richmond, CA) or the NanoOrange protein assay (Molecular Probes, Eugene, OR).

Nine different agrichemicals (**Fig. 1A**), two cations (Mn^{2+} and Zn^{2+}) and cocaine (a known DAT blocker) were used at final concentrations ranging from 0.1 to 100 μM . Triadimefon (a known DAT blocker) and thiram (1 mM) were dissolved in ethanol (10 and 25%, respectively) before incubation buffer was added to dilute them.

***In vivo* paraquat measurements.** Two sets of experiments were done. First, a time course comparing the uptake of [methyl- ^{14}C]-paraquat alone or with Mn^{2+} -ethylenebis-DTC at different time points was analyzed. Subsequently, the effect of different DTCs was examined only at the one hour time point. Male 2-3 month old C57BL/6J (B6) mice were injected ip with 100 μCi of [methyl- ^{14}C]-paraquat dissolved in saline. In other mice, Mn^{2+} -ethylenebis-DTC (30 mg/kg) dissolved in saline was administered in a separate ip injection immediately prior to injection of [methyl- ^{14}C]-

paraquat. For the time course study, mice (n = 4) were sacrificed at four different time points (0.5, 1, 6, and 12 hours) after injections. At each time point, blood sampling was obtained from orbital bleeds. Following this, mice were anesthetized with 100 mg/kg of pentobarbital, perfused with heparinized saline to remove blood from organs, and finally harvested of organs including brain, lung, liver, kidney, and heart. The brain was dissected and samples from four regions (striatum, midbrain, cerebellum, and frontal cortex) obtained and weighed. Samples from the other organs were blocked and weighed. All tissue samples were added to 1 ml of Biosol (National Diagnostics, Atlanta, GA) to dissolve tissue overnight or until clear at 50 °C in an oven followed by the addition of 10 ml of Bioscint (National Diagnostics, Atlanta, GA) for liquid scintillation counting. Values reported are the mean and SEM from 4 separate mice for each condition and time point. The effect of two additional DTCs, methyl-DTC (30 and 100 mg/kg) and diethyl-DTC (100 and 150 mg/kg) were examined in the same manner after 60 min.

Materials. [³H]-Dopamine (specific activity 28-31.6 Ci/mmol) was purchased from NEN Life Science Products, Inc (Boston, MA) and [methyl-¹⁴C]-paraquat dichloride (specific activity 110 mCi/mmol) was purchased from Amersham-Pharmacia Biotech (Arlington Heights, IL) or received as a gift from Syngenta (Cheshire, UK). Cocaine, DA, pargyline, diethyl-DTC, paraquat, all cations and buffers were obtained from Sigma (St. Louis, MO). The pesticides Mn²⁺-ethylenebis-DTC (maneb), Na⁺-ethylenebis-DTC (nabam), Zn²⁺-ethylenebis-DTC (zineb), dimethyl-DTC (thiram), Zn²⁺-dimethyl-DTC (ziram), methyl-DTC (vapam), and triadimefon were obtained from Chem Service (West Chester, PA).

Data and Statistical Analysis. For the DA uptake studies, data were averaged from 2-5 independent experiments. For *in vivo* [methyl-¹⁴C]-paraquat studies, 3-4 animals were used for each

condition. Unpaired Student's t-tests were used to compare the effects of a compound to the effect in the absence of any compounds. A two-factor ANOVA was used to analyze the *in vivo* time course study with treatment and time-point as between group factors. A one-factor ANOVA was used to analyze the effect of different DTCs on [methyl-¹⁴C]-paraquat uptake. Significant main effects of treatment or interactions were followed by selected post hoc analyses. The area under the curve was calculated in SigmaPlot[®] 2000 using a macro and the trapezoidal rule for equal or unequally spaced x values.

Results

***In vitro* DA uptake Studies.** Initial uptake studies were performed using a 10 nM concentration of [³H]-DA and a 6 minute incubation period. We tested a variety of DTC pesticides, two other pesticides (paraquat and triadimefon), several cations, and cocaine at varying concentrations (100 nM to 100 μM). The ethylenebis-DTCs, Mn²⁺-ethylenebis-DTC and Na⁺-ethylenebis-DTC demonstrated a similar increase in synaptosomal DA content of about 18% at 500 nM (**Fig. 1B**). Mn²⁺-ethylenebis-DTC resulted in a dose-dependent increase in synaptosomal content of DA, with a maximal increase of about 30% at a concentration of 5 μM (**Fig. 2**). Significant increases in DA content were seen at concentrations ranging from 500 nM to 50 μM. No effect was seen at lower concentrations and a reduction in DA content was seen at 100 μM. Similar dose-dependent effects were seen with Na⁺-ethylenebis-DTC and diethyl-DTC (data not shown). No significant increase was seen with incubation of the complexed cation Mn²⁺ at similar concentrations (**Fig. 2**).

Zn²⁺-ethylenebis-DTC did not produce a significant increase in DA content (**Fig. 1B**). However, the zinc cation alone resulted in a significant decrease (~15%) in DA content (as previously reported⁴⁰) and likely prevented an increase from the parent ethylenebis-DTC compound. Diethyldithiocarbamate (diethyl-DTC) also resulted in a significant increase in DA content (~24%). Two dimethyldithiocarbamates (dimethyl-DTC, and Zn²⁺-dimethyl-DTC) and the methyl-DTC did not significantly increase synaptosomal DA content at any concentration tested.

The pesticide PQ did not alter DA content at any concentration tested (0.1 to 50 μM, shown at 0.5 μM in **Fig. 1B**). The pesticide triadimefon, a known inhibitor of DA uptake⁴¹, produced a significant dose-dependent decrease in uptake, as did the classic DA uptake inhibitor cocaine.

We next performed more detailed kinetic studies of DA accumulation in the absence or presence of Mn²⁺-ethylenebis-DTC (1 μM, **Fig. 3A and B**). Uptake was time-dependent with or

without Mn^{2+} -ethylenebis-DTC (**Fig. 3A**). The initial rate of DA uptake was not affected by the presence of Mn^{2+} -ethylenebis-DTC. Uptake was rapid and reached equilibrium after 10 to 14 minutes. Accumulation over time was greater in the presence of Mn^{2+} -ethylenebis-DTC and reflected the net effect of influx and efflux. We then measured efflux of [3H]-DA from prefilled synaptosomes (**Fig. 3B**). [3H]-DA efflux was rapid in the absence of Mn^{2+} -ethylenebis-DTC. However, in the presence of Mn^{2+} -ethylenebis-DTC, [3H]-DA efflux was slower. In the absence of Mn^{2+} -ethylenebis-DTC, efflux of DA was nearly complete after 20 minutes. However, in the presence of Mn^{2+} -ethylenebis-DTC efflux was only about 50% complete after 20 minutes. The area under the curve (AUC) was increased in the presence of Mn^{2+} -ethylenebis-DTC (145% greater).

Efflux studies were also performed in the absence or presence of cocaine (0.5 μM) to determine if cocaine blockade of the DAT might alter DA efflux (**Fig. 4**). We preloaded synaptosomes with [3H]-DA for 13-14 min in the absence or presence of cocaine (0.5 μM) and then initiated efflux in solutions with or without cocaine (0.5 μM). The rate of DA efflux was similar in all conditions, suggesting that DA efflux is not modulated by cocaine at the DAT under these conditions.

We sought to determine if Mn^{2+} -ethylenebis-DTC would alter the K_m or V_{max} for [3H]-DA influx via the DAT after two durations of incubation (**Table 1**). After a short incubation (30 seconds, before efflux is likely to be significant), saturation studies in the absence or presence of Mn^{2+} -ethylenebis-DTC resulted in similar K_m s ($0.311 \pm 0.122 \mu M$ and $0.272 \pm 0.184 \mu M$, respectively, mean \pm SEM, $P > 0.05$) and V_{max} s (166.5 ± 108.9 pmol/min/mg protein and 161.7 ± 100.5 pmol/min/mg protein, respectively $p > 0.05$). After a longer incubation (6 min, when influx and efflux may both be operative), saturation studies in the absence or presence of Mn^{2+} -ethylenebis-DTC resulted in similar K_m s ($0.042 \pm 0.004 \mu M$ and $0.047 \pm 0.007 \mu M$, respectively, $P > 0.05$), but significantly different V_{max} s (7.7 ± 0.3 pmol/min/mg protein and 10.2 ± 0.7 pmol/min/mg protein,

respectively, $P < 0.05$). The higher V_{\max} in the presence of Mn^{2+} -ethylenebis-DTC is consistent with blockade of efflux without altering the affinity of the DAT for DA.

***In vivo* [^{14}C]-paraquat studies.** [^{14}C]-paraquat was rapidly transported from peritoneum into blood and then into organs with significantly greater uptake into peripheral organs compared to the brain (**Fig. 5A to D**). Levels generally peaked around 1 hour. The simultaneous administration of Mn^{2+} -ethylenebis-DTC resulted in significantly higher concentrations in all organs between 1 and 12 hours (**Figs. 5 and 6**). Mn^{2+} -ethylenebis-DTC also enhanced organ concentrations by 50 to 350% between 1 and 6 hours. The area under the curve between 0.5 and 12 hours (AUC) for each organ and brain region was increased in the presence of Mn^{2+} -ethylenebis-DTC (midbrain – 94%, cerebral cortex – 105%, striatum – 78%, cerebellum – 94%, serum – 127%, lung – 205%, kidney – 168%, heart – 143%, and liver 248%). Organ concentrations were still elevated at 12 hours post injection in the presence of Mn^{2+} -ethylenebis-DTC (**Fig. 6A**). By 12 hours, most organs had eliminated the [^{14}C]-paraquat, with brain and lung being the slowest to eliminate it in the presence of Mn^{2+} -ethylenebis-DTC (**Fig. 6B**). A similar significant increase in the presence of diethyl-DTC (150 mg/kg) was observed at one hour in all organs examined (**Fig. 7**). A lower dose of diethyl-DTC (100 mg/kg) produced a significant increase in some, but not all organs. Methyl-DTC (30 and 100 mg/kg) did not significantly augment [^{14}C]-paraquat concentration in any organ. The dimethyl-DTCs were toxic to mice at the lowest dose tested (30 mg/kg).

Discussion

Our data demonstrate novel information about distinct environmental agents, PQ and DTCs, implicated in the PDP. A potent effect of certain DTCs on the kinetics of [³H]-DA synaptosomal accumulation *in vitro* and tissue concentrations of systemic [¹⁴C]-PQ *in vivo* were identified. Several ethylenebis-DTCs and diethyl-DTC had this effect, whereas this effect was not seen with two other classes of DTCs having a very similar chemical structure, methyl-DTC and dimethyl-DTCs. DTCs having this kinetic effect also enhance MPTP and PQ neurotoxicity, whereas DTCs not having the studied effect either do not enhance neurotoxicity or have not been tested (**Table 2**). PQ was not transported via the DAT, but was likely transported via a widely expressed polyamine transporter (PAT). These findings have implications for these compounds in contributing to the PDP independently and in combinations.

Effect, Mechanism, and Implications for DTCs. The effect of the selective DTCs to increase synaptosomal [³H]-DA accumulation *in vitro* was seen at concentrations as low as 500 nM suggesting high sensitivity and occurred after a short 6 min incubation period suggesting a rapid onset. The effect lasted for at least 20 min *in vitro* and 12 hrs *in vivo* suggesting a prolonged effect. Because the K_m for DA uptake was similar for short and longer incubations, the effect did not relate to an alteration in the K_m of the DAT for DA. We found an apparent increase in the V_{max} of the DAT for DA, but only after a 6 min incubation. Based on subsequent kinetic experiments, this effect was likely due to the blockade of DA efflux. Thus, at longer incubations, synaptosomal DA content reflects the net effect of influx and efflux. If efflux is slowed, synaptosomal DA content increases to a new equilibrium. It does not appear that this effect of certain DTCs is via an action on the DAT. The rate of uptake of DA was not altered by any DTCs. Efflux of DA through the DAT was unlikely as cocaine did not alter efflux in this preparation as it did in other types of studies⁴². This effect was seen *in vitro* for DA, but also occurred *in vivo* for PQ and MPP⁺ suggesting that DTCs

may have effects on other endogenous and exogenous compounds, both in the brain and in other organs.

Certain DTCs have been shown to enhance the toxicity of both MPTP and PQ (**Table 2**). We identified a strong correlation between those DTCs increasing the content of synaptosomal DA *in vitro*, increasing and prolonging tissue concentrations of [¹⁴C]-PQ *in vivo* (toxicokinetic, TK), and potentiating the neurotoxicity (toxicodynamic, TD) of MPTP and PQ in the nigrostriatal system (**Table 2**). This enhanced toxicity may relate to a toxicokinetic (TK) effect the DTCs have on the neurotoxicant, a kinetic effect on DA, or both. Other mechanisms have been proposed for DTC potentiation, but none have demonstrated a clear correlation between DTCs potentiating MPTP and/or PQ neurotoxicity and those not doing so. The effect of DTCs on mitochondria was examined, but did not have the appropriate correlation with *in vivo* potentiation of MPTP¹⁴. The TD effect of a combined neurotoxicant and a selective DTC does not exclude other possible mechanisms for enhanced neurotoxicity of the combinations. Other noncommercial DTCs exist that either do (dipropyldithiocarbamate, diisopropyldithiocarbamate, and dicycloheylldithiocarbamate) or do not (ethylcyclohexyldithiocarbamate and piperidinyldithiocarbamate) potentiate systemic MPTP toxicity that could be tested to extend this correlation¹⁴. However, those DTCs were custom synthesized, are not readily available, and are not environmental risk factors. Also, there are numerous thiocarbamate pesticides with similar structures to the DTCs that have the potential for similar effects, but have not yet been examined for potentiation of MPTP or paraquat toxicity.

A TK mechanism of enhancement for DTCs is supported by the results of other agents that enhance the neurotoxicity of MPTP/MPP⁺ *in vivo* (**Table 2**)^{17, 43, 44}. Ethanol, acetaldehyde, and probenecid have all been shown to increase and prolong MPP⁺ tissue levels and potentiate neurotoxicity, with the augmentation lasting hours to days. Detailed quantitative and temporal data are not available for each agent due to the need for HPLC analysis of MPP⁺. Detecting changes in

the TK of [¹⁴C]-PQ by these agents is easier as PQ is not metabolized and requires only liquid scintillation counting. We have verified that probenecid (100 and 250 mg/kg) also enhances tissue concentrations of [¹⁴C]-PQ (Thiruchelvam, Cory-Slechta, and Richfield, unpublished observations).

If this effect of DTCs is mediated by inhibition of an efflux transporter(s), the transporter will have a number of properties. First, it *likely* exists in the brain and other tissues. We have demonstrated an effect in striatal synaptosomes *in vitro* and in several organs *in vivo*. Second, the transporter(s) may be used for the efflux of multiple compounds having similarities in structure. DA, MPP⁺, and PQ all share similar features of being small organic cations. However, alternative mechanisms remain.

Paraquat Implications. PQ has been implicated in the PDP via different lines of evidence including epidemiologic studies in man^{27,29}, animal models^{6,8,9,45}, and *in vitro* studies¹¹. It has long been speculated that based on the similar structure between PQ and MPP⁺ that PQ might enter SNpc neurons via the DAT^{10,30}. However, evidence from this study suggests that this is not the case. PQ did not compete with [³H]-DA for transport via the DAT *in vitro* at any concentration tested as shown by others for MPP⁺^{46,47}. Also, the concentration of [¹⁴C]-PQ was not increased in the striatum compared to other brain regions, at any time point between 0.5 and 12 hours after injection as would be expected if transport occurred only or preferentially via the DAT. In fact, the striatum had the lowest concentration with or without Mn²⁺-ethylenebis-DTC at every time point compared to the other regions examined.

How might PQ enter neurons? PQ, along with several other polyamines (spermine, spermidine, putrescine, and cadaverine) have been demonstrated to accumulate in brain slices and specifically alveolar type I and II epithelial cells of the lung via a polyamine transporter (PAT) that is energy-dependent and saturable^{11,38,39,48-50}. The specific transporter has yet to be identified. PATs are part of a large superfamily of transporters, the Amino acid/Polyamine/Organocation (APC)

superfamily (see⁵¹). At least 175 proteins make up this superfamily, with 10 smaller families and are present in prokaryotes, eukaryotes, or both having various specificities for different compounds. PATs have been identified in brain^{37, 52, 53}. Cellular location in brain for the PATs remains unclear, but PATs on glia have been identified *in vitro*⁵². If PQ is transported via a PAT into cells in the brain, our data suggest widespread brain uptake without selectivity for the SNpc. Yet, SNpc neurons have been demonstrated to be selectively vulnerable to PQ in mice^{8, 9, 45}. Selective SNpc vulnerability may relate to the presence of DA and the susceptibility of dopaminergic cells to oxidative stress and reactive oxygen species. PQ has the property of undergoing a single electron reduction from the cation to form a free radical that is stable in the absence of oxygen^{11, 54-58}. In the presence of oxygen, the free radical is immediately converted back to the cation with the reduction of oxygen to form a superoxide anion. This reaction is spontaneous, rapid, and unending if there is a supply of electrons (NADPH) and oxygen. Since PQ is not metabolized, the only way to terminate redox cycling is for a cell to remove PQ or suffer consequent damage.

Models of neurotoxicity. Our data suggest a possible mechanism for the potentiation of both MPTP and PQ by DTCs in models of the PDP. We hypothesize an unidentified efflux transporter as central in potentiating the toxicity of MPP⁺, PQ, and DA in leading to the PDP (**Fig 8A**). In this model, systemic MPTP is converted to MPP⁺ and taken up into nigrostriatal terminals by the DAT. Systemic PQ is presumably taken up into terminals or cells by a PAT. Effective DTCs block the efflux of those neurotoxicants and DA enhancing their toxicity by increasing the concentration of each and prolonging intracellular exposure yielding an increased cellular AUC. MPP⁺, PQ, and DA all result in the generation of ROS, which may ultimately reach a threshold for irreversible cellular damage (**Fig 8B**). Others have identified and discussed the importance of TK or biodisposition in the neurotoxicity of MPTP^{17, 59}. Species sensitivity to MPTP may be related to this issue with the primate being the most sensitive and having the highest brain AUC following a

single injection⁵⁹. We extend this concept to include PQ and compounds (DTCs, ethanol, acetaldehyde, and probenecid) altering their TK. In this model, nontoxic doses of a neurotoxicant (MPTP, PQ, and/or DA) do not result in neuronal death due to failure to achieve a threshold neurotoxicant level. This threshold relates to either a minimal peak concentration, duration, or AUC before cell death occurs. Sufficient data is not available yet to determine if peak dose, duration, or the AUC are independent factors or which factor might be most critical. Thresholds are likely to vary between organs, brain regions, and cell types, vary with age, and vary with other risk factors related to the PDP. However, SNpc neurons appear to be most sensitive. Effective DTCs or other transport inhibitors are capable of converting a nontoxic dose to a toxic dose^{9, 22, 34, 35}. Repeated doses will result in progressive neuronal loss with the rate of loss proportional to the magnitude above a threshold. Features of this model are testable.

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Figure Legends

Figure 1. A. Structure of selected dithiocarbamates. The structure of the four major classes of dithiocarbamates is illustrated. Molecules are color-coded by structural class and correspond to results in **B**. The dithiocarbamate backbone and complexed cations are also shown. **B.** Effect of agrichemicals, cations, and cocaine (all at 0.5 μM) on dopamine uptake. Values represent the percentage of DA uptake in the presence of added compounds (mean \pm SEM) compared to DA uptake in the absence of these compounds ($n = 2$ to 4 independent experiments). Values do not reflect the maximal effect or the lowest effective concentration of the different compounds, but demonstrate the relative effect of each compound at the same concentration (0.5 μM). All compounds were tested at higher and lower concentrations and the effects were consistent at similar concentrations (**Fig 2**). *Significantly different from control. Mn^{2+} -ethylenebis-DTC, Na^+ -ethylenebis-DTC, and diethyl-DTC all significantly increased DA synaptosomal content ($P < 0.05$). Zinc, triadimefon, and cocaine all significantly decreased DA synaptosomal content ($P < 0.05$). Manganese, Zn^{2+} -ethylenebis-DTC, Zn^{2+} -dimethyl-DTC, dimethyl-DTC, methyl-DTC, and paraquat showed no significant effect.

Figure 2. Effect of different concentrations of Mn^{2+} -ethylenebis-DTC (●) or Mn^{2+} (○) on synaptosomal DA content. Values represent the percentage of DA uptake in the presence of Mn^{2+} -ethylenebis-DTC or Mn^{2+} (mean \pm SEM) compared to DA uptake in the absence of the compound ($n = 3$ to 5 independent experiments). Mn^{2+} -ethylenebis-DTC showed a dose-dependent effect on synaptosomal DA content. Concentrations of Mn^{2+} -ethylenebis-DTC that showed significant increases ranged from 0.5 to 50 μM (all P s < 0.05). Mn^{2+} -ethylenebis-DTC significantly decreased DA content at 100 μM ($P < 0.05$). Mn^{2+} -ethylenebis-DTC showed no significant effect at the lowest

concentration (0.1 μM). Mn^{2+} showed no significant effect on DA uptake from 0.5 to 50 μM (all P s > 0.05), but did show an inhibitory effect at the lowest concentration ($P < 0.05$).

Figure 3. Influx and efflux of synaptosomal DA. **A and A'**. DA uptake was rapid in the absence (\circ) or presence (\bullet) of Mn^{2+} -ethylenebis-DTC (1 μM) and typically reached a steady state after 10-14 minutes. **B and B'**. In the absence of MB (\circ), DA efflux was rapid and exponential, falling to near zero after 20 min. In the presence of Mn^{2+} -ethylenebis-DTC (1 μM , \bullet), efflux was dramatically slowed and was only about 50% complete after 20 min. Values represent the mean \pm SEM from 3-5 independent experiments. **A'** and **B'** represent values normalized to a common 100% maximal value to allow comparison of the shape of the curves.

Figure 4. **A and A'**. Effect of cocaine on efflux of synaptosomal DA. Cocaine was included in various combinations during DA influx and efflux; both influx and efflux (\bullet), influx only (\circ), efflux only (\blacksquare), or neither (\square). Cocaine reduced DA influx as expected when included in the influx buffer ($\bullet\circ$). However, cocaine had no effect on efflux. **A'** represent values normalized to a common 100% maximal value to allow comparison of the shape of the curves.

Figure 5. Time course of [^{14}C]-paraquat in various organs and brain regions over time following ip injection. Organs and tissues were harvested at various times (0.5 to 12 hrs) after injection of [^{14}C]-paraquat (100 μCi) alone (filled symbols) or with Mn^{2+} -ethylenebis-DTC (30 mg/kg, open symbols). Concentrations (pmol/mg wet weight) in brain are much lower than those in other organs or serum. Values peaked at 30 min in serum or around 1 hour for other organs. Values in all organs and brain were increased in the presence of Mn^{2+} -ethylenebis-DTC between one and 12

hours. In brain and lung the augmentation by Mn^{2+} -ethylenebis-DTC was greater than in other organs, most noticeably at 6 and 12 hours.

Figure 6. Ratios of [^{14}C]-paraquat concentration in different organs or regions of brain. **A.** The change in [^{14}C]-paraquat concentration in the presence of Mn^{2+} -ethylenebis-DTC compared in its absence. After 30 min all organs displayed increased concentrations with the greatest effect between one and six hours. Increases due to Mn^{2+} -ethylenebis-DTC were still present after 12 hours. **B.** After 12 hours, the effect of Mn^{2+} -ethylenebis-DTC was similar in brain and lung and exceeded that in other organs when compared to values after one hour.

Figure 7. Effect of two additional DTCs on [^{14}C]-PQ concentration in the striatum. Mice (n=3 or 4) per condition were treated with [^{14}C]-PQ as described previously combined with methyl-DTC (30 and 100 mg/kg) or diethyl-DTC (100 and 150 mg/kg). Tissue was harvested after one hour. The dimethyl-DTCs were too toxic to use in combination. Effects in the striatum were similar in all other regions of brain and other organs. A dose-effect was seen with diethyl-DTC, but only the 150 mg/kg dose was significantly different from [^{14}C]-PQ alone (*, $P = 0.005$). Value obtained with Mn^{2+} -ethylenebis-DTC shown for comparison (*, $P = 0.002$).

Figure 8. Models of the potential mechanism of TD potentiation by certain DTCs related to the “Area Under the Curve” (AUC) hypothesis. **A.** Synaptosomal model based on data described in this report and published data on MPP⁺ and DA uptake^{46,47}. Dopaminergic terminals (synaptosomes) take up both DA and MPP⁺ via the DAT, whereas PQ is likely taken up via a polyamine transporter (PAT). Selective DTCs may block an additional unidentified cationic transporter used to export these compounds from terminals. The effect of this blockade will be to

increase the concentration and duration (Area Under the Curve, AUC) of intracellular exposure to DA and neurotoxic compounds both of which will contribute to adverse cellular effects. Cell vulnerability will depend on the combination of transporter expression (DAT, PAT, and the exporter) and tolerance to reactive oxygen species. **B.** Model of the effect of repeated exposure to neurotoxicants (MPTP or PQ) in the absence or presence of a TK potentiator (selective DTCs, probenecid, ethanol, acetaldehyde). In this model, a low dose of either dopaminergic neurotoxicant may not result in neuronal death. A dose insufficient to cause cell loss can be potentiated by the concomitant exposure to a TK potentiator resulting in either a higher peak concentration, a longer duration of exposure, or a combination of the two increasing the AUC and culminating in cell death.

Table 1. Parameters derived from saturation experiments performed at two different time points (30 seconds or 6 minutes) in the absence or presence of Mn²⁺-ethylenebis-DTC (0.5 or 5 μM). Mn²⁺-ethylenebis-DTC was used at a higher concentration for the 30 second incubation to maximize the likelihood of detecting an effect based on data demonstrated in **Fig. 3**. Varying concentrations of DA (1-100 nM) were incubated with synaptosomes for either 30 seconds or 6 minutes. * represents a significant difference in the V_{max} in the presence of Mn²⁺-ethylenebis-DTC compared to incubation in the absence of Mn²⁺-ethylenebis-DTC (*P* < 0.05). The effect of Mn²⁺-ethylenebis-DTC on the V_{max} at the longer incubation time reflects the net effect of influx and efflux.

Parameter (Units)	Mn ²⁺ -ethylenebis-DTC (μM)	Incubation Time (sec)	n	Mean ± SEM
K _m (nM)	0	30	3	311 ± 122
K _m (nM)	5	30	3	271 ± 184
V _{max} (pmol/mg protein/min)	0	30	3	166.5 ± 62.9
V _{max} (pmol/mg protein/min)	5	30	3	161.7 ± 100.5
K _m (nM)	0	360	5	42 ± 4
K _m (nM)	0.5	360	5	47 ± 7
V _{max} (pmol/mg protein/min)	0	360	5	7.7 ± 0.3
V _{max} (pmol/mg protein/min)	0.5	360	5	10.2 ± 0.7*

Table 2. Compounds enhancing neurotoxicant-induced PDP possibly via an alteration in toxicokinetics (TK). Interacting compounds have been shown to enhance the neurotoxicant (toxicodynamic, TD) effect of MPTP, paraquat or both as determined using different outcome measurements. These compounds have also been shown to alter the TK of MPP⁺, paraquat, or both in the brain or body. Fewer studies have addressed interactions with paraquat as it is a more recent model of the PDP. The DTCs having effects on MPTP and PQ neurotoxicity *in vivo* correlate with their effect on DA accumulation *in vitro* in synaptosomes (**Highlighted in Bold**). *In vivo* TK alterations of PQ have been studied for the four classes of DTCs (**Highlighted in Bold**) and also correlate with their effects using *in vitro* synaptosomal DA accumulation.

Neurotoxicant	Enhancer	Dose and Frequency	Neurotoxic Outcome Measurement	Toxicokinetic Effect	Effect on Synaptosomal DA Concentration	Reference
MPTP	ethanol	1 g/kg x 3	↓ striatal DA and SNpc neuron number	↑ striatal MPP ⁺ concentration and delayed excretion	not tested	44
MPTP	acetaldehyde	250 mg/kg x 3	↓ striatal DA and SNpc neuron number	↑ striatal MPP ⁺ concentration and delayed excretion	not tested	44
MPTP	probenecid	250 mg/kg x 10	↓ striatal DA and SNpc neuron number	↓ urinary excretion of MPTP metabolites	not tested	43, 60
MPTP	Mn ²⁺ -ethylenebis-DTC	various	↓ striatal DA, SNpc neuron number, and locomotor activity	not reported	increased	20
MPTP	ethylenebis-DTC	various	↓ striatal DA	not reported	increased	18
MPTP	diethyl-DTC	400 mg/kg x 1	↓ striatal DA and SNpc neuron number	↑ striatal MPP ⁺ concentration and delayed excretion	increased	14, 15, 17-19, 22
MPTP	methyl-DTC	various	no effect	not reported	no effect	18
MPTP	dimethyl-DTCs	not tested	not reported	not reported	no effect	not reported
PQ	Mn ²⁺ -ethylenebis-DTC	30 mg/kg	↓ striatal DA, SNpc neuron number, locomotor activity	↑ [¹⁴ C]-PQ AUC	increased	8, 9
PQ	diethyl-DTC	100 and 150 mg/kg	↓ locomotor behavior	↑ [¹⁴ C]-PQ concentration	increased	shown here
PQ	dimethyl-DTCs	30 mg/kg	high mortality	not tested, high mortality	no effect	shown here
PQ	methyl-DTC	30 and 100 mg/kg	no effect	no effect on [¹⁴C]-PQ concentration	no effect	shown here

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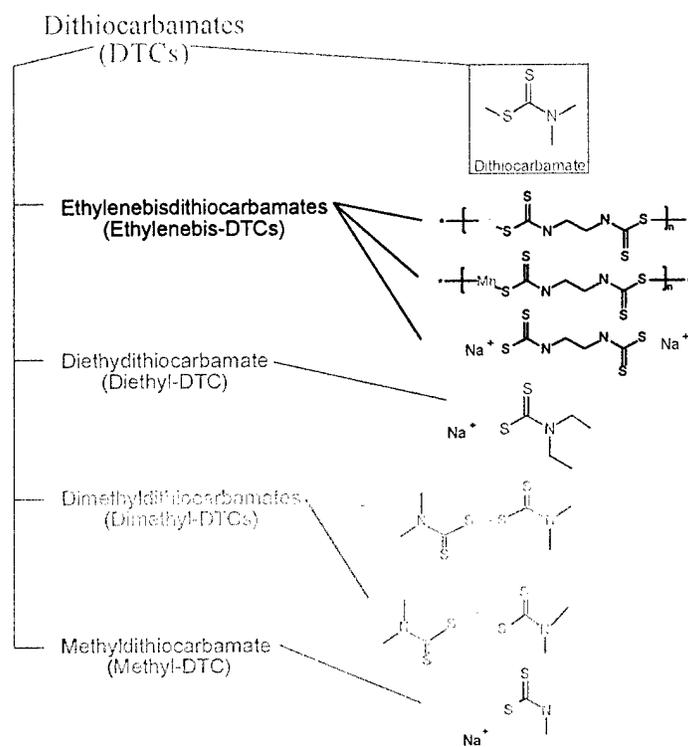


Figure 1A

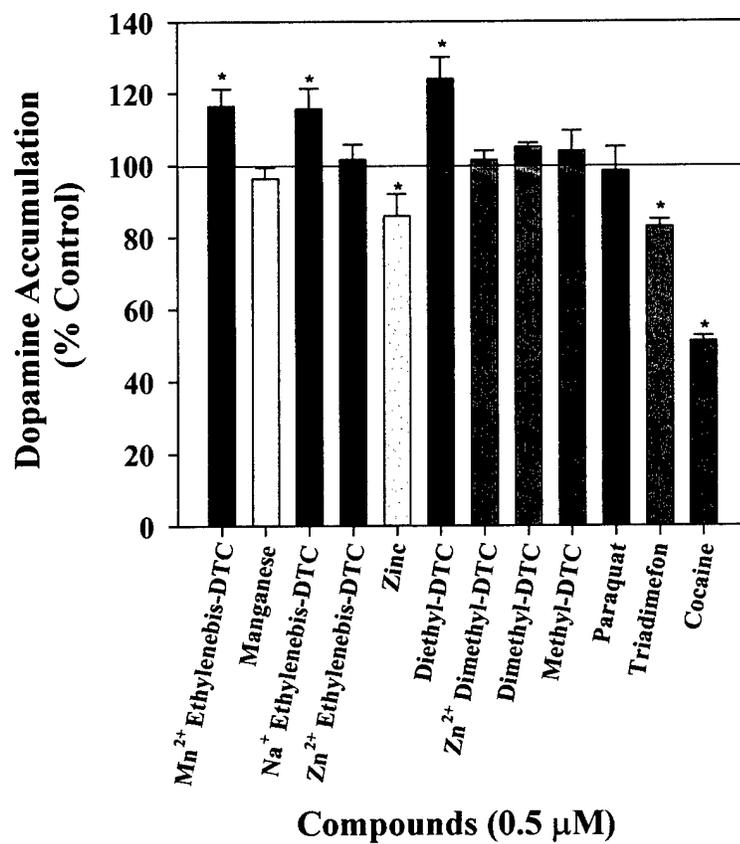


Figure 1B

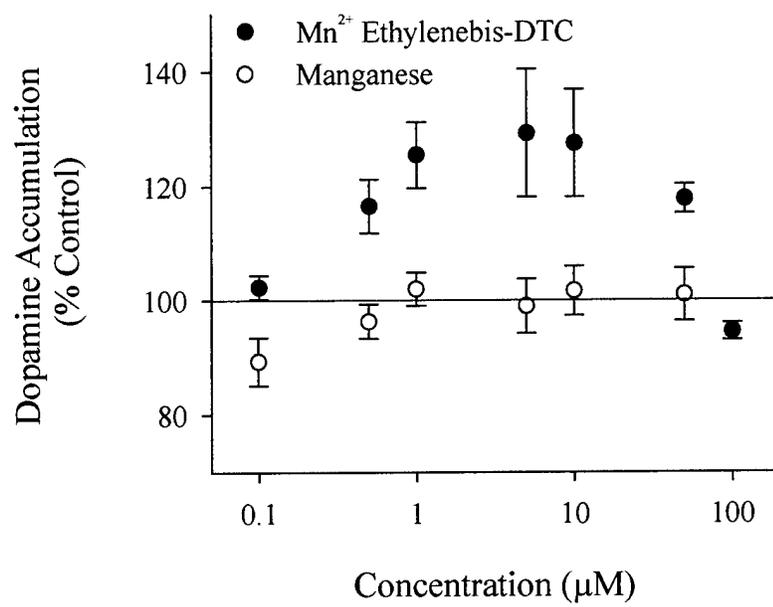


Figure 2

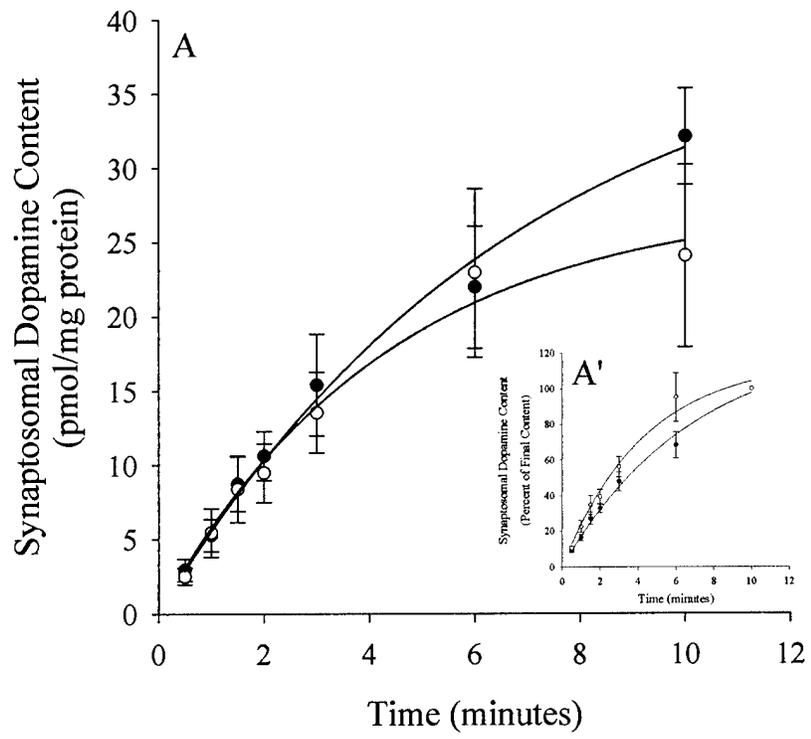


Figure 3A & 3A'

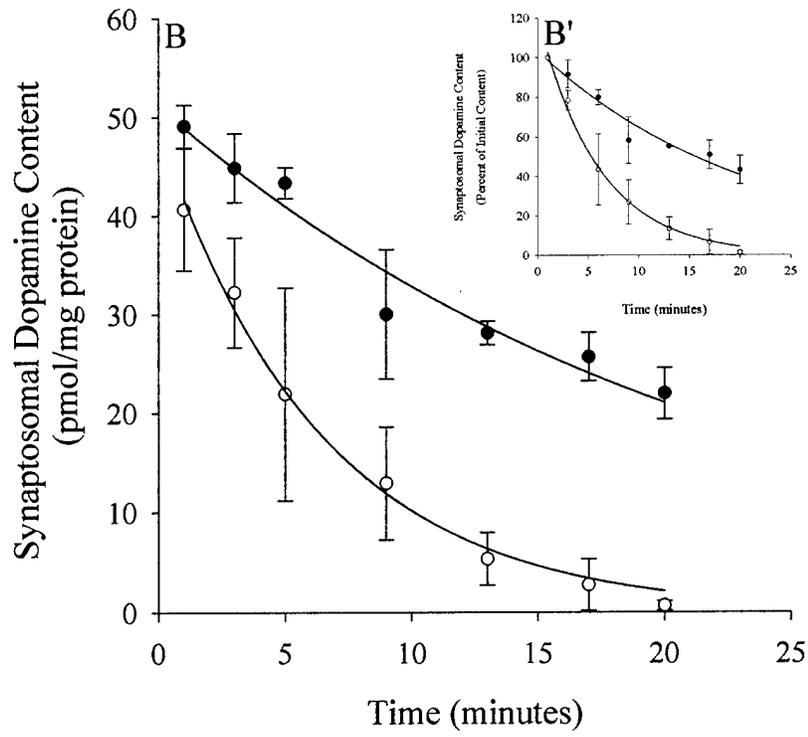


Figure 3B & 3B'

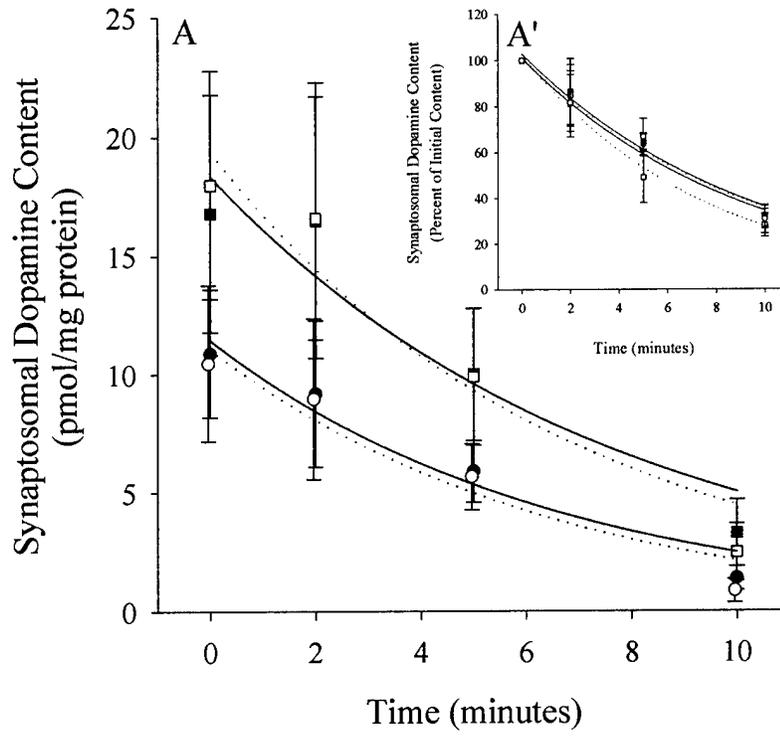


Figure 4

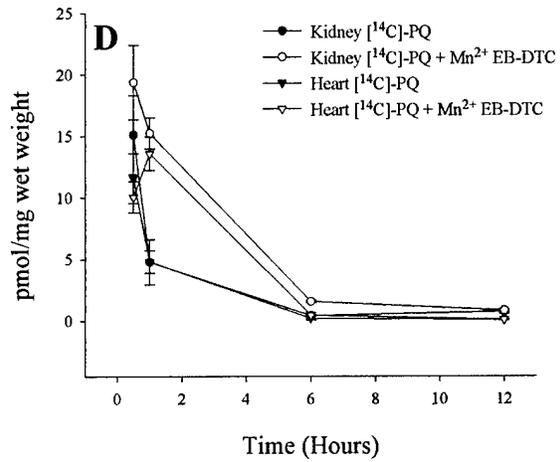
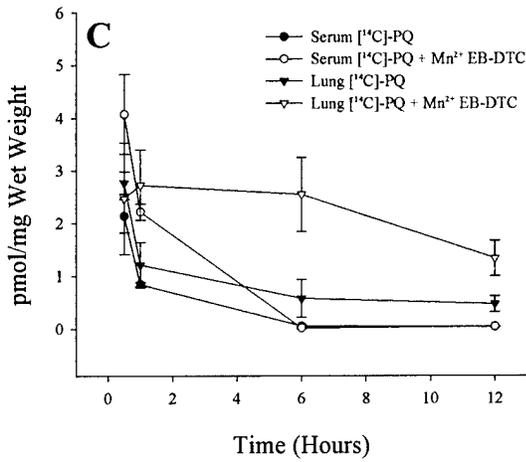
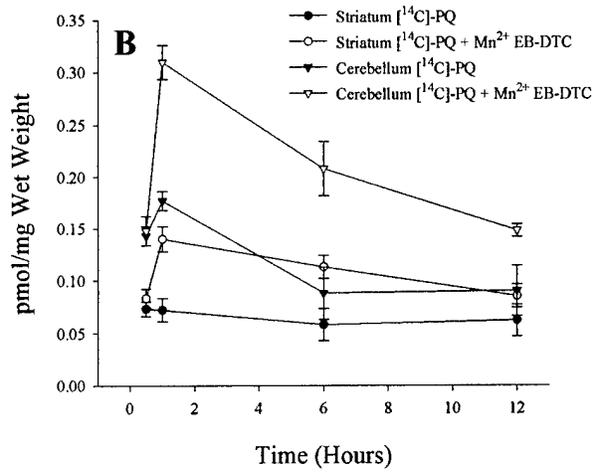
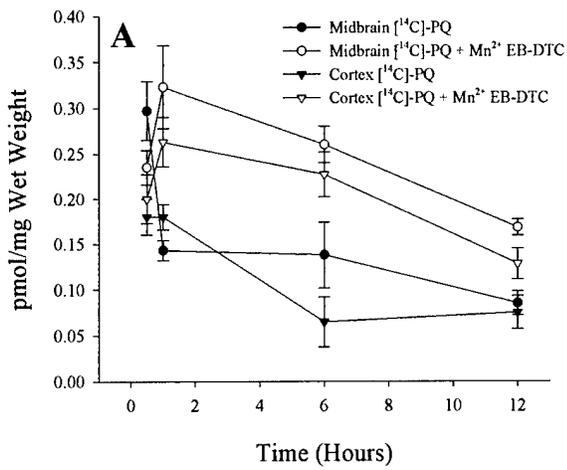


Figure 5A-D

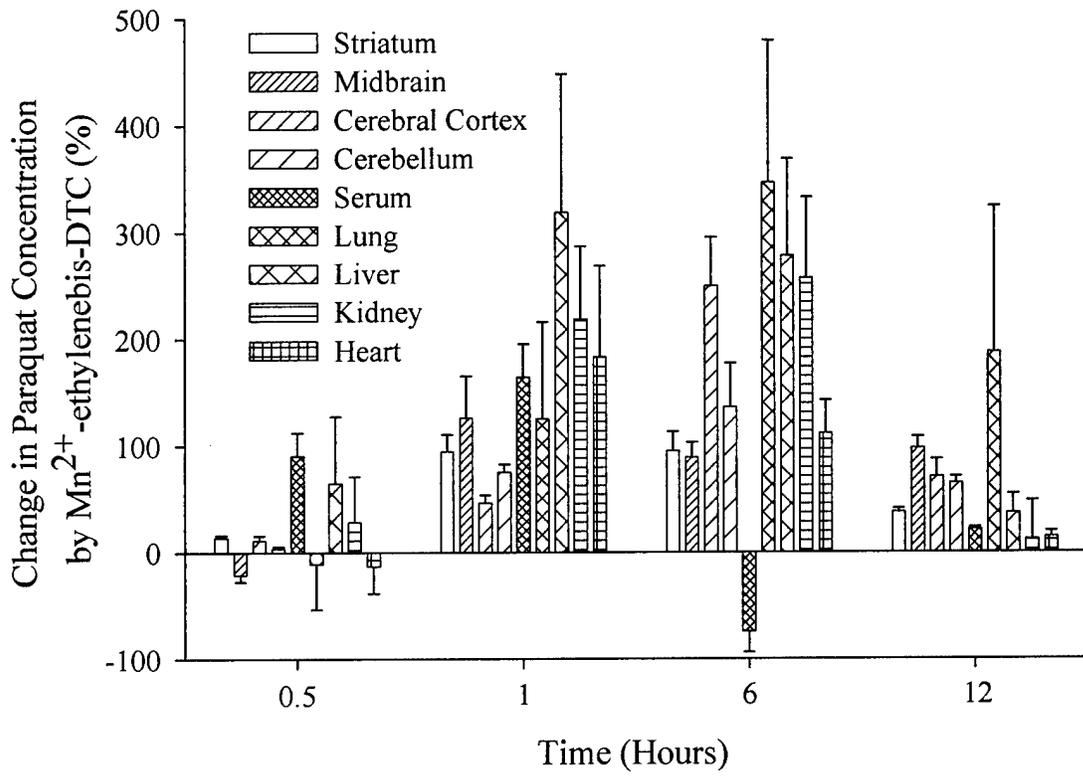


Figure 6A

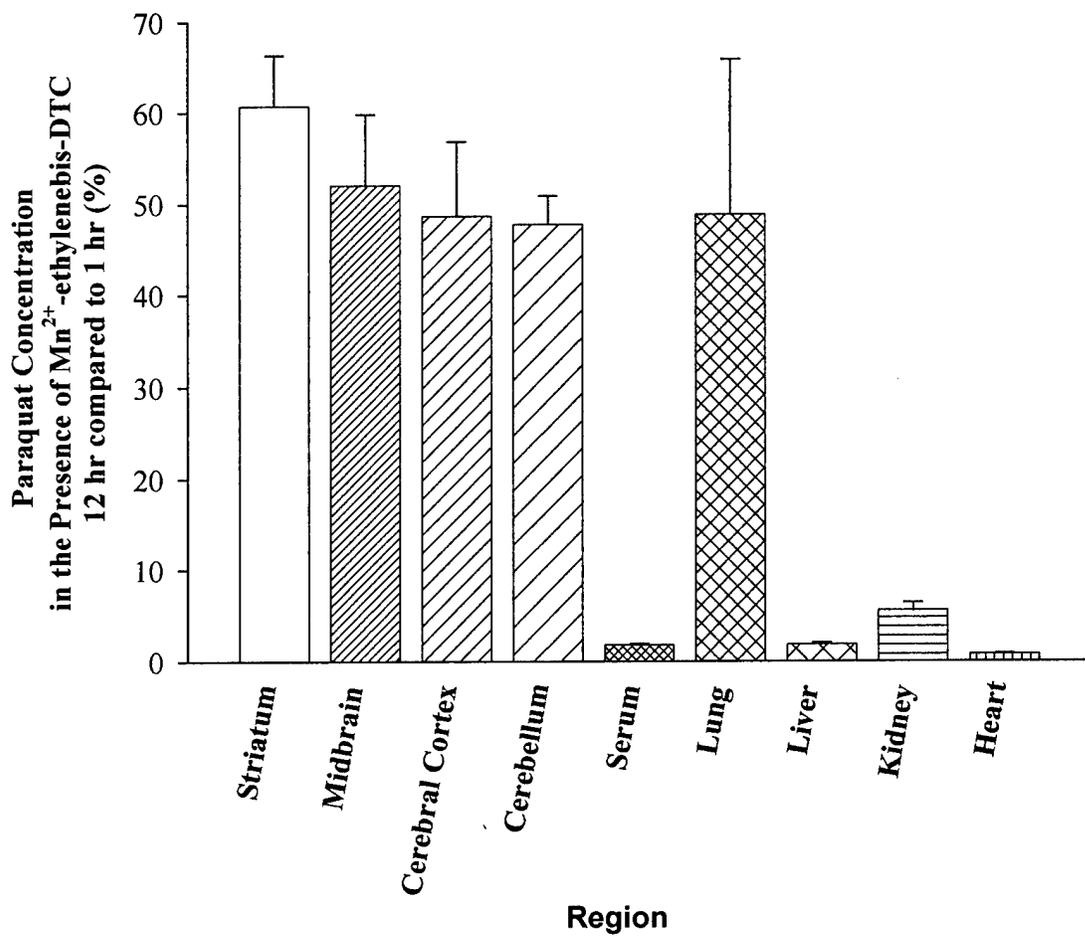


Figure 6B

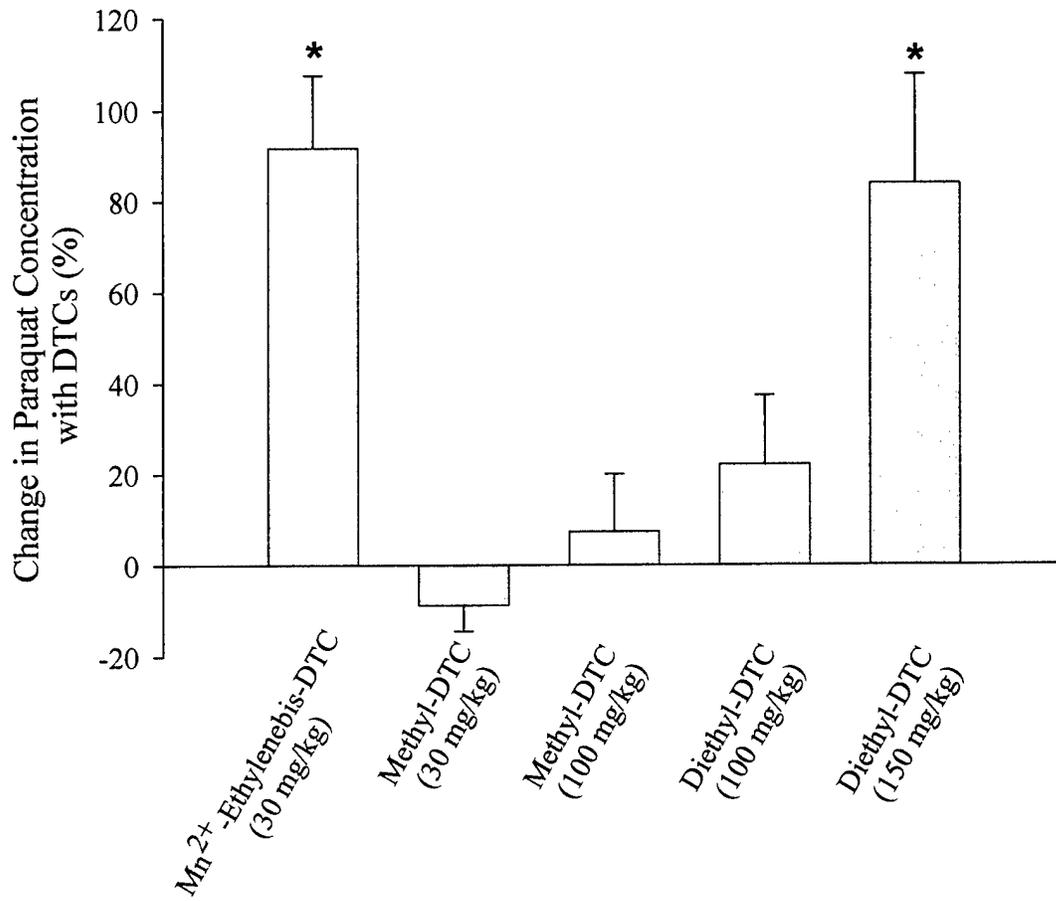


Figure 7

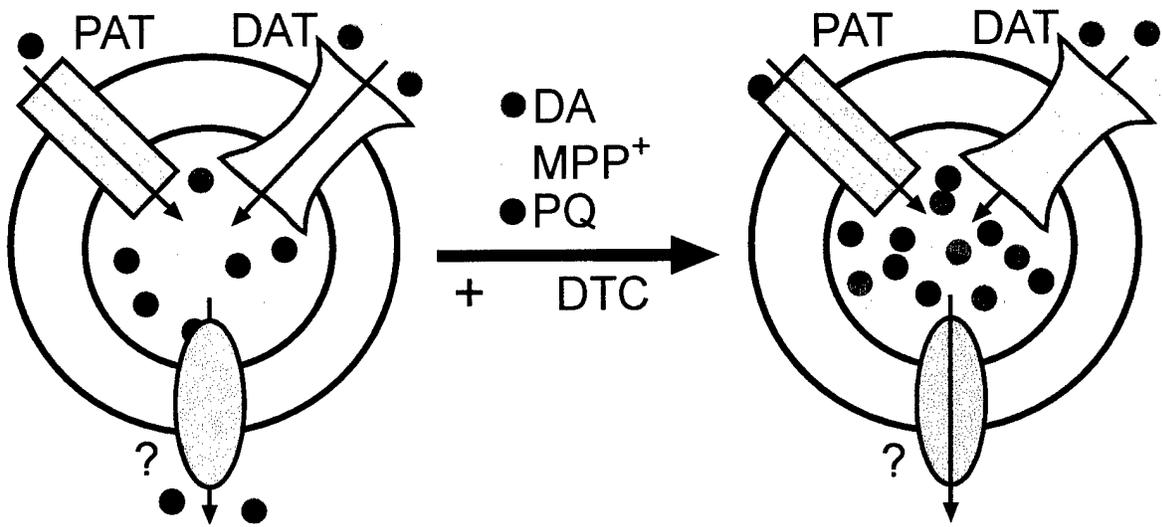


Figure 8A

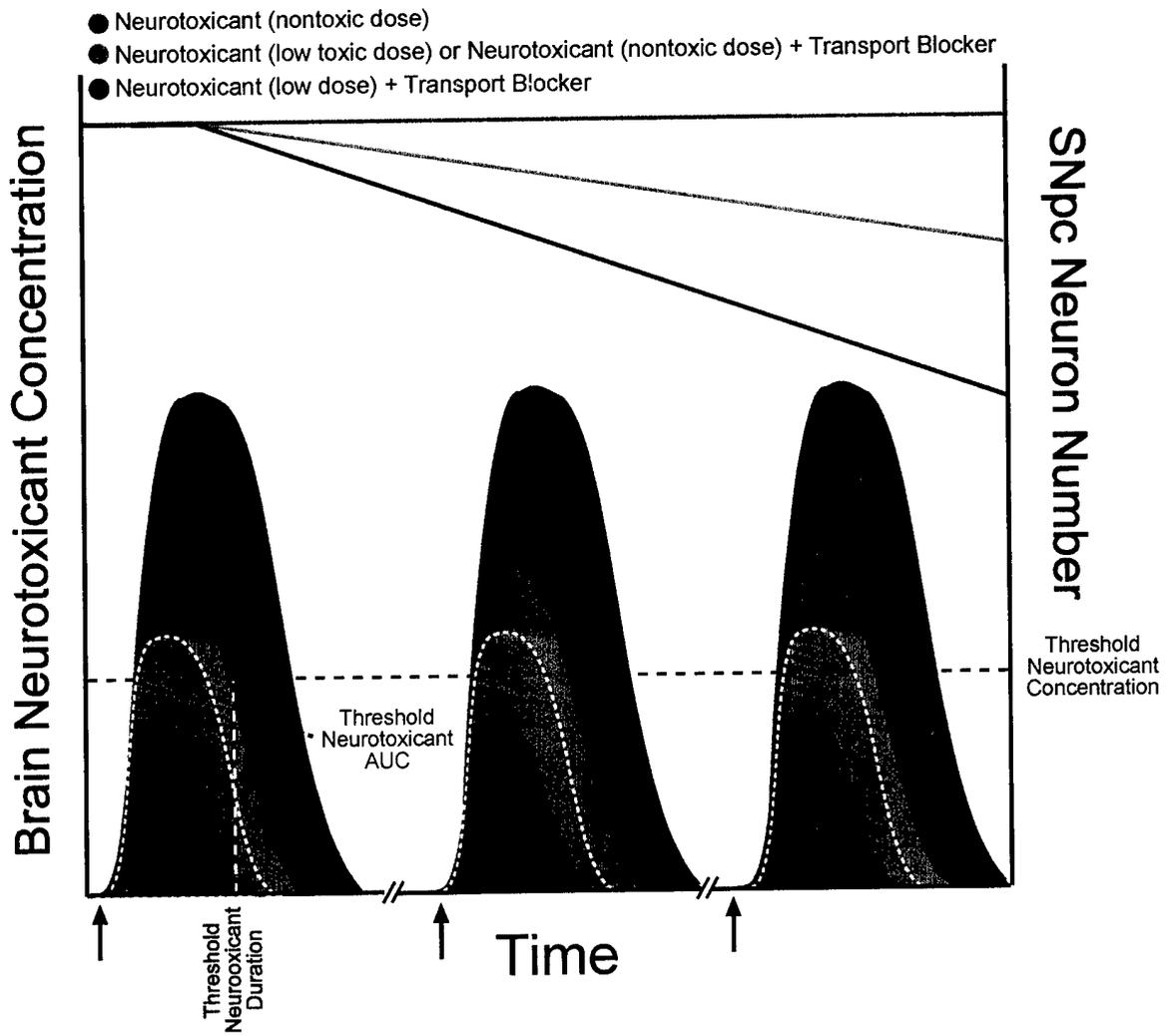


Figure 8B

Abstract View

SELECTIVE DITHIOCARBAMATES INCREASE SYNAPTOSOMAL DOPAMINE CONTENT, BRAIN CONCENTRATION OF PARAQUAT, AND CORRELATE WITH POTENTIATION OF MPTP AND PARAQUAT TOXICITY

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Pesticides have been implicated in Parkinson's disease based on epidemiologic data, animal models, and *in vitro* studies. Different dithiocarbamate pesticides (DTCs) increase the toxicity of MPTP and paraquat in mouse models. We examined certain classes of commercially used DTCs on [³H]-dopamine (DA) transport in synaptosomal vesicles from the striatum and the concentration of [¹⁴C]-paraquat *in vivo* in mice. Different ethylenebisdithiocarbamates (ethylenebis-DTCs) and diethyldithiocarbamate (diethyl-DTC) increased DA accumulation, whereas dimethyldithiocarbamates (dimethyl-DTCs) and methyldithiocarbamate (methyl-DTC) did not. Increased DA accumulation was dose-dependent. The increased accumulation was due to the carbon backbone and not the complexed cation. DTCs increasing accumulation of DA did not alter the influx of DA, but delayed the efflux out of synaptosomes. There was a consistent relationship between DTCs increasing synaptosomal accumulation of DA with those demonstrated to enhance MPTP and/or paraquat toxicity *in vivo*.

Selective DTCs also increased the tissue uptake and retention of [¹⁴C]-paraquat given ip. Paraquat was not transported via the DA transporter. We hypothesize that certain DTCs potentiate the neurotoxicity of MPTP and paraquat by inhibiting a transporter(s) and increasing intracellular exposure to DA and the neurotoxicants. These results suggest that certain DTCs may enhance the toxicity of a wide variety of endogenous and exogenous compounds.

Supported by: DAMD17-98-1-8628; NIH ES11839, ES01247, and ES06484

Close

Abstract View**PRENATAL EXPOSURE TO MANEB INCREASES ADULT VULNERABILITY TO THE NEUROTOXIC EFFECTS OF PARAQUAT: IMPLICATIONS FOR PARKINSON'S DISEASE.**

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Lack of strong evidence for a genetic heritability of idiopathic Parkinson's disease (PD) has focused attention on environmental toxins. While PD is associated with advanced age, it is unclear whether neuronal damage to the nigrostriatal pathway is due to an insult during development, adulthood, or a cumulative effect across the lifespan. This study hypothesized that prenatal exposure to maneb (MB) would disrupt the development of the nigrostriatal DA system and enhance its vulnerability to neurotoxicant exposures later in life. Pregnant C57BL/6 mice were treated on gestational days 10-17 with saline or MB (1 mg/kg). Locomotor activity in offspring, when evaluated at 6 weeks of age, showed no significant effect of prenatal MB exposure in either gender. Pups were then treated for 8 consecutive days with saline, MB (30 mg/kg), or PQ (5 mg/kg). Males exposed prenatally to MB and later to PQ showed a 95% reduction in locomotor activity, with no changes seen in the controls or females treated similarly. This behavioral effect was reflected in the neurochemistry of the MB-PQ males, with greater than 50% reductions in striatal DA and DOPAC and 40% increase in DA turnover compared to controls, with no change in 5HT levels. These results suggest that prenatal exposure to MB produces a state of silent toxicity, that is selective and permanent to the nigrostriatal system which enhances adult susceptibility to subsequent exposures. Thus, developmental exposure to neurotoxicants may be involved in the induction of neurodegenerative disorders such as PD, and/or alter the normal aging process.

Supported by: DAMD17-98-1-8628, ES10791, ES01247 and ES11839

Close

Age-Related Changes Associated With Overexpression of Wild-Type and Mutated Human α -Synuclein in Mice

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Numerous lines of evidence, including its abundance in Lewy bodies have implicated human α -synuclein, (α -SYN) in Parkinson's disease (PD). The precise role of this protein both in normal and diseased state, however, remains to be determined. To further elucidate the role of α -SYN in PD, transgenic mice expressing both known human mutations associated with PD ($hm^2\alpha$ -SYN) or the human wild-type α -SYN ($hw\alpha$ -SYN) were generated. These mice express approximately 30-40% of endogenous mouse α -SYN. Mice expressing $hm^2\alpha$ -SYN showed abnormal axons and terminals, and in adult mice potentiated decreases in locomotor activity and dopamine (DA) levels produced by combined paraquat + maneb treatment as compared to comparably treated non-transgenic (tg-) littermate controls or mice expressing the $hw\alpha$ -SYN. Between 2 and 23 months of age, $hm^2\alpha$ -SYN mice exhibited age-related impairments in locomotor activity and decreases in levels of striatal DA and metabolites. Additionally, age-related nigral DA neuronal loss of 40% occurred by 23 months of age. $hw\alpha$ -SYN mice showed only marginal decreases in locomotor activity and DA cell loss (12%) at this time. Furthermore, a presynaptic dose of apomorphine (0.2 mg/kg) decreased locomotor activity of both tg- and $hw\alpha$ -SYN mice, while no changes were found in $hm^2\alpha$ -SYN mice. A postsynaptic dose (1.5 mg/kg) decreased or failed to alter locomotor activity levels of tg- and $hw\alpha$ -SYN mice while increased activity occurred in $hm^2\alpha$ -SYN mice, consistent with a possible supersensitization of DA receptors following denervation. Adverse effects of α -SYN overexpression, moreover, were gender specific, with males more affected than females. Collectively, the effects of overexpression appear to be consistent with a gain of toxic function. The age-related changes associated with $hm^2\alpha$ -SYN mimic effects associated with PD and thus may serve as a valuable animal model to study the mechanism of cell death observed with this disorder. Supported by DAMD17-98-1-8628, ES10791 and ES01247.