Original Paper

Developmental Neuroscience

Dev Neurosci 2004;26:11–23
DOI: 10.1159/000080707

Received: December 12, 2003
Accepted: February 17, 2004

A Fetal Risk Factor for Parkinson's Disease

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Key Words
Maneb · Paraquat · Parkinson's disease

Abstract
A lack of strong evidence for genetic heritability of idiopathic Parkinson's disease (PD) has focused attention on environmental toxicants in the disease etiology, particularly agrichemicals. PD is associated with advanced age, but it is unclear whether specific neuronal damage could result from insults during development. This study hypothesized that prenatal exposure to pesticides would disrupt the development of the nigrostriatal dopamine (DA) system and enhance its vulnerability to dopaminergic neurotoxicant exposures later in life. Pregnant C57BL/6J mice were treated on gestational days 10–17 with saline or the pesticides maneb (MB, 1 mg/kg) or paraquat (PQ, 0.3 mg/kg). When offspring were evaluated in adulthood, there were no significant effects of prenatal MB or PQ exposure on locomotor activity. Subsequently, offspring were treated for 8 consecutive days with saline, MB (30 mg/kg), or PQ (5 mg/kg). One week after the last exposure, only males exposed to prenatal MB and adulthood PQ showed significant reductions in locomotor activity (95%) and changes in striatal neurochemistry. Stereological assessment of the substantia nigra pars compacta (SNpc) and ventral tegmental area correspondingly confirmed selective dopaminergic-neuron loss in SNpc. The lack of changes in other exposure groups suggests a specificity to the sequence of exposures as well as gender specificity. These results suggest that prenatal exposure to MB produces selective, permanent alterations of the nigrostriatal dopaminergic system and enhances adult susceptibility to PQ exposure. This study implicates a role for developmental neurotoxicant exposure in the induction of neurodegenerative disorders such as PD.

Introduction

Parkinson's disease (PD) is a neurodegenerative disorder resulting, in part, from the progressive loss of dopamine (DA) neurons in the substantia nigra pars compacta (SNpc). Environmental factors have been implicated in the etiology of idiopathic PD [Tanner et al., 1999; Di Monte et al., 2002], and exposure to agrichemicals has consistently been linked with the development of PD [Israeli et al., 1983; Tanner, 1989; Semchuck et al., 1992; Gorell et al., 1998; Priyadarshi et al., 2000]. Animal models, clinical case reports and epidemiological studies spe-

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cifically identify the widely used pesticides paraquat (1,1′-dimethyl-4,4′-bipyridinium, PQ) and maneb (manganese ethylene-bis-DTC, MB) as potential contributing factors for Parkinsonism [Ferraz et al., 1988; Meco et al., 1994; Liou et al., 1997; Brooks et al., 1999; Thiruchelvam et al., 2000a, 2000b].

Behavioral, neurochemical, and anatomical measures demonstrate that PQ can selectively damage DA neurons [Tawara et al., 1996; Brooks et al., 1999; McCormack et al., 2002]. The dithiocarbamate (DTC) family of pesticides, which includes MB, can also produce a broad range of neurotoxic effects [Miller, 1982; Mitchell et al., 1989; Morato et al., 1989; Vaccari et al., 1998; Vaccari et al., 1999] and augment the toxicity of the known dopaminergic neurotoxicant N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) [Corsini et al., 1985; Irwin et al., 1987; Takahashi et al., 1989; Yurek et al., 1989; Miller et al., 1991; Walters et al., 1999; McGrew et al., 2000]. Additionally, administration of PQ and MB in combination produces synergistic effects that specifically target the nigrostriatal system [Thiruchelvam et al., 2000a, 2000b, 2002]. These results highlight the 'multiple-hit' model of PD, which suggests that repeated exposures to combinations of environmental toxicants enhances reaction(s) to subsequent exposure(s), and eventually elicits a threshold loss of dopaminergic function [Walters et al., 1999; Thiruchelvam et al., 2002].

Although idiopathic PD typically affects humans beyond the fourth decade of life [Tanner, 1989], it is still unclear whether insults as early as prenatal development might enhance neurodegeneration or vulnerability [Ling et al., 2002; Riederer and Foley, 2002]. Catecholamine systems, including DA systems, are among the earliest neuronal populations to replicate and differentiate [Johnston, 1985] and are important for further brain development [De Vitry et al., 1991; Levitt et al., 1997]. Due to underdeveloped protective mechanisms and rigid schedules of interconnecting biochemical events, the embryonic nervous system is particularly sensitive to disruption, and may never fully recover after early insult [Rodier, 1994, 1995; Gupta et al., 1999; Sobotka et al., 1972]. While neonatal exposure to several kinds of agrichemicals can disrupt brain development and functioning and potentiate the reaction to later exposures, developmental exposures can also occur during prenatal periods, at the times when the brain growth spurt is sensitive to insult [Siddiqui et al., 1981; Eriksson, 1997]. While effects of prenatal exposure to MPTP are equivocal, MPTP can have adverse effects after crossing the placenta into the fetus in mice and non-human primates [Weissman et al., 1989; Melamed et al., 1990; Ochi, et al., 1991; Perez-Otano et al., 1992, 1995]. Likewise, PQ and DTCs are able to cross the placenta [Ingebrigsten et al., 1984; Larsson et al., 1976; Chernoff et al., 1979; Shukla et al., 2001] but effects of these compounds on the nigrostriatal DA system have not been reported.

Even in the absence of overt effects, however, there is a need to consider the subtle biological effects of environmental agents in the 'survivors' that do not show classic signs of teratogenesis [Beck, 1990]. These subtle effects include alterations in the development of the nervous system, where developmental exposure to neurotoxicants can induce a state of 'silent toxicity', primed for potentiated reaction to later-in-life exposure to the same and/or another kind of neurotoxicant, and thus accelerate neurodegeneration. This study sought to determine if prenatal exposure to MB or PQ would result in permanent deleterious changes to the nigrostriatal DA system, if this exposure would alter adult susceptibility to exposure to MB or PQ, and if these adult exposures would unmask any prenatal neurotoxicity and lead to potentiated changes in the nigrostriatal DA system.

Methods

Animals

Six-week-old male and female C57BL/6J mice were purchased from Jackson Laboratories. Animals were housed in a room maintained under constant temperature (72–74°F) and humidity conditions (40–70%) with a 12/12 hour light/dark cycle. Food (Purina Mills Rodent Chow 5001, LabDiet) and water were available ad libitum throughout the studies. Animals were habituated to the vivarium for at least 1 week before mating. For mating, 1 male was housed with 3 females for 5 days. Each day, females were examined for the presence of a seminal plug, and females who evidenced mating (day 0 of pregnancy) were removed and housed 1 per cage. Pups were housed with dams and littermates until day 25. Surviving pups were weaned and housed 1 per cage for the duration of the experiment. Animals were cared for and treated in accord with the National Institutes of Health and the University of Rochester Animal Care and Use Committee Guidelines.

Chemicals

Maneb was a gift from DuPont Chemicals (Wilmington, Del.). Paraquat dichloride and all solvents for high performance liquid chromatography with electrochemical detection (HPLC-EC) were purchased from Sigma (St. Louis, Mo., USA).

Drug Administration

The effects of prenatal exposure to either MB or PQ and to adult re-challenge with MB or PQ were studied in two independent but similar experiments, each with complete sets of corresponding control conditions. Dams were assigned to exposure groups and injected subcutaneously with saline (SAL), MB (1 mg/kg dissolved in saline,

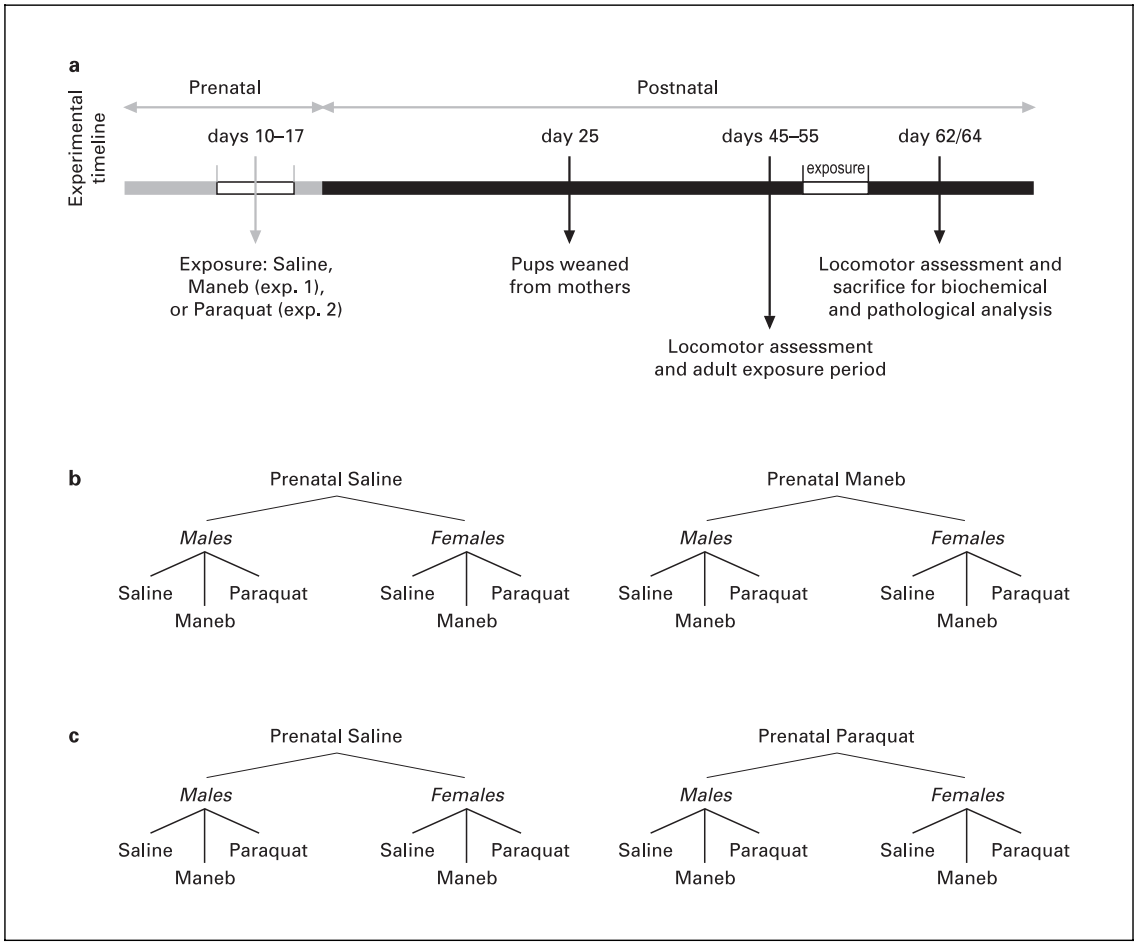

Fig. 1. Experimental design: Timeline and exposure groups. **a** For 8 days during gestation (days 10–17), pregnant females were injected with either saline, maneb (1 mg/kg), or paraquat (0.3 mg/kg). On postnatal day 25, pups were weaned. On postnatal days 45–47, animals were habituated to locomotor activity boxes. Animals were then exposed to either SAL, MB (30 mg/kg), or PQ (5 mg/kg) for 8 consecutive days, with locomotor assessment on days 1, 4, and 8 of this exposure period. One week following the last exposure (day 62), a final locomotor assessment was made, and animals were sacrificed. The study was conducted in two parts. In the first experiment, pregnant females were exposed to saline or to maneb (**b**); in the second, pregnant females were exposed to saline or to paraquat (**c**). Males and females were used in both experiments, and both experiments included complete sets of control conditions.

0.3% of LD_{50}) or PQ (0.3 mg/kg dissolved in saline, 1% of LD_{50}) daily on days 10–17 of gestation. This regimen was selected to correspond to the emergence of the nigrostriatal dopaminergic system [Golden, 1972], and doses were chosen based on mortality data from initial studies, other developmental and teratogenicity reports [Larsson et al., 1976; Chernoff et al., 1979; Thiruchelvam et al., 2002]. At weaning, pups were assigned to adult-exposure groups while balancing for potential litter effects (no more than 2 pups per litter for each treatment group), and both male and female pups were used for all the studies. An 8-day adult-exposure schedule was established for these pups with daily intraperitoneal injections of either SAL, MB (30 mg/kg) or PQ (5 mg/kg) on postnatal days 48–55 (adulthood); other work considering adulthood combined exposures to these chemicals has used similar doses of the compounds (30 mg/kg MB, 10 mg/kg PQ) but administered them concurrently and less frequently over a longer time period [Thiruchelvam et al., 2000b, 2002]. The present paradigm yielded six exposure groups for each gender (n = 7–11 for each group) for each experiment (see figure 1 for experimental design).

Locomotor Activity

Automated locomotor activity chambers equipped with infrared photobeams (Opto-Varimex Minor, Columbus Instruments International Corporation, Columbus, Ohio, USA) were used to quantify locomotor activity. Photobeam breaks were recorded every minute for 45 min for horizontal, vertical, and ambulatory movements. At 6 weeks of age, offspring were habituated to the locomotor activity chambers in three 45-min sessions on consecutive days, with all mice receiving i.p. saline injections prior to the session. After the third habituation session, adult exposures began, and effects on locomotor

activity were assessed immediately after each injection on days 1, 4, and 8 of the rechallenge paradigm. Activity was also measured 1 week following the last exposure to determine the persistence of effects of exposures.

DA and Metabolite Analyses by HPLC

Neurotransmitter concentrations in the striatum and the frontal cortex were measured 9 days following the eighth adulthood injection of SAL, MB, or PQ. Following cervical dislocation and rapid decapitation, frontal cortex and striatal blocks were dissected. HPLC-EC analysis was performed as previously described [Thiruchelvam et al., 2000a], and normalized to protein concentration as measured by the Bio-Rad DC protein assay (Bio-Rad, Richmond, Calif., USA). Concentrations of the neurotransmitters were expressed in terms of ng/mg protein. DA turnover in striatum was expressed as the ratio (DOPAC+HVA)/DA, and as the ratio DOPAC/DA in frontal cortex [Ricaurte et al., 1986; Felten et al., 1992].

Immunohistochemistry for Tyrosine Hydroxylase

Brains used for immunolabelling studies were post-fixed in 4% paraformaldehyde for 96 h and stored in 30% sucrose. Fixed brains were cut into 30-μm sections and collected in cryoprotectant. Sections were prepared as previously described [Thiruchelvam et al., 2002], using a primary antibody to tyrosine hydroxylase (TH, Chemicon, Temecula, Calif., USA) for 48 h at a dilution of 1:4,000, and with a secondary goat anti-rabbit antibody (Vector, Burlingame, Calif., USA) for 24 h at a dilution of 1:500. Sections were mounted on gelatin-coated slides, counterstained with cresyl violet for visualization of neuronal nuclei, and coverslipped for stereological analysis.

Stereological Analysis

After delineation of the SNpc and of the ventral tegmental area (VTA) at low magnification (×4 objective), one side of every eighth section from the entire region was sampled at higher magnification (×100 objective) using the stereology module of the Stereo Investigator imaging program (MicroBrightField, Inc., Williston, Vt., USA) with an Olympus Provis microscope. The optical fractionator method, an unbiased quantitative technique, was used for counting TH+ (TH-positive and cresyl-violet-positive neurons) and TH– (cresyl-violet-positive only) cells [West et al., 1993]. The mean thickness of each sample was determined by measuring two fields from 5 sections per sample, and the entire depth of field was sampled, ignoring the upper and lower 0.5 μm. The nucleator method was used to estimate mean cross-sectional area and cytoplasmic volume of TH+ neurons using a 5-point ray. All samples were evaluated by one experimenter without knowledge of mouse status.

Peripheral Organ Histopathology

Representative sections of lung, heart, kidney, and liver (n = 5 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 exposure and gender on horizontal locomotor activity were first analyzed with repeated-measures analysis of variance (RMANOVA) using exposure condition and gender as between-groups factors and time-point as a within-group factor. A p value ≤ 0.05 was considered to be statistically significant. This was followed by individual analysis of variance (ANOVA) using exposure condition and gender as between-groups factors for each time-point and followed by Fisher's post hoc test where appropriate to compare the exposure groups. Effects on neurochemistry and stereological cell counts were first evaluated using a two-factor ANOVA with exposure condition and gender as between-groups factors. A significant ANOVA was followed by Fisher's post hoc tests to compare genders and exposure groups. All values are expressed as group means ± SEM.

Results

Pregnancy, Litter Size, Body Weights, and Organ Pathology

Body weights were monitored throughout pregnancy. All females gained weight over time (both experiments, all p values <0.0001), and no exposure-related changes in weight gain throughout pregnancy were observed between SAL- and MB- or PQ-exposed females (n = 13–16, 17, and 13, respectively). Furthermore, there were no significant exposure-related effects on length of gestation, number of pups delivered, number of pups at weaning, or number of non-surviving pups. At weaning, animals exposed to prenatal MB had slightly (~10%) but significantly lower body weights than SAL controls, and this effect was still detectable at 6 weeks of age (all p values <0.05); animals exposed to prenatal PQ showed no weight difference from controls. Since the body weights of the prenatally exposed mice were lower, no further decline with age was observed, indicating that the mice were healthy otherwise. In both experiments, males weighed slightly more than females at weaning and throughout behavioral testing (all p values <0.05). In both experiments, RMANOVA including weights on the first day of behavioral testing, the last day of adult exposure, and the 1-week recovery day showed a main effect for weight change (all increases) over time [$F_{(2,198)} = 166.6$ and $F_{(2,202)} = 371.2$, respectively, for each experiment (both p values <0.0001)] but no interactions of gender and exposure over time. No pathological changes were observed in the lung, heart, kidney or liver.

Locomotor Activity

Before adulthood exposures began, total horizontal activity counts were analyzed from the third day of habituation (fig. 2). In both experiments, females showed slightly lower horizontal activity (both p values <0.05) but there was no significant effect of prenatal exposure to MB (fig. 2a) or PQ (fig. 2b) and no main effect of the interaction of gender and exposure. To examine effects of expo-

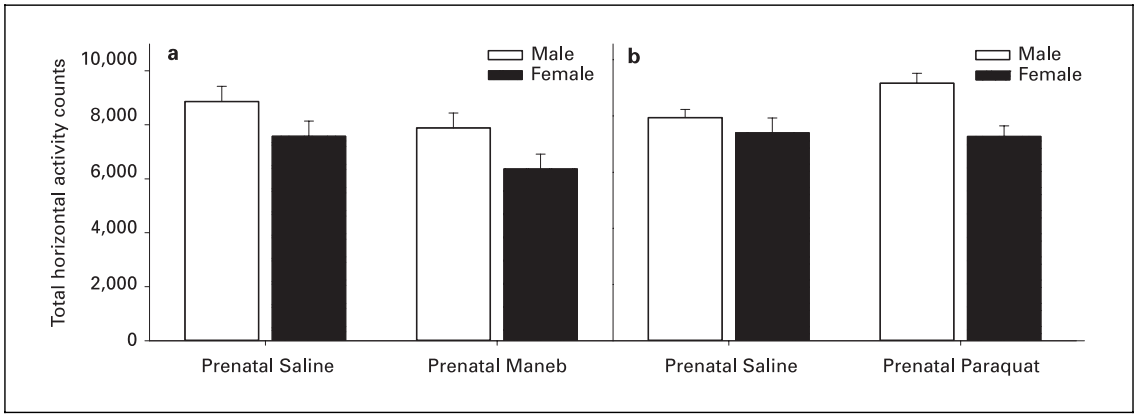

Fig. 2. Total horizontal activity on the third day of habituation. Activity (mean ± SEM, n = 24–30 per condition) was measured over a period of 45 min immediately following injection of saline. While females demonstrated slightly lower total activity than males ($p < 0.05$), there were no behavioral effects of prenatal exposure to maneb (**a**) or paraquat (**b**). Individuals in each prenatal exposure group were randomly assigned to one of three adult exposure groups (saline, maneb, or paraquat) and subsequent behavioral measures for each group were normalized to activity on the third habituation day.

sure over time and to compare behavior between groups, all subsequent behavioral analyses were performed after each group had been normalized to activity on this third day of habituation.

Total horizontal activity was examined over time to examine effects of adult challenge with MB or PQ following prenatal exposure to MB (fig. 3a, c). There was a significant effect of gender, of exposure, and of time (all $p < 0.05$), and interactions of gender and treatment [$F_{(5,198)} = 2.38$, $p < 0.05$], of gender and time [$F_{(2,198)} = 22.00$, $p < 0.0001$], of exposure and time [$F_{(10,198)} = 12.23$, $p < 0.0001$], and of gender and exposure over time [$F_{(10,198)} = 9.28$, $p < 0.0001$]. There were gender and exposure effects both on day 8 of exposure and 1 week after exposure ($p < 0.05$ for both time points), and a significant effect of the interaction of gender and exposure 1 week after exposure [$F_{(5,99)} = 13.58$, $p < 0.0001$].

On day 8 of exposure (fig. 3a), all animals exposed to MB in adulthood showed reductions in total horizontal activity but recovered normal activity levels 1 week after exposure (fig. 3c). Most male groups (with the notable exception of prenatal MB/adult PQ males) had similar activity levels 1 week after exposure compared to the third day of habituation; most female groups demonstrated increased behavior 1 week after exposure compared to the third day of habituation (all p values <0.05). The most dramatic change in activity was observed in males exposed prenatally to MB and to PQ in adulthood. While no differences in activity were seen in this group on day 8 of exposure (fig. 3a), this group showed a dramatic decrease in activity, exceeding 95%, 1 week after exposure (fig. 3c). Neither prenatal exposure to MB alone nor adult exposure to PQ alone caused any exposure-related changes in males' activity at any point, but the sequential combination resulted in profound effects on behavior compared to all other relevant male exposure groups and compared to similarly exposed females (all p values <0.05).

Total horizontal activity was likewise examined over time to examine effects of adult challenge with MB or PQ following prenatal exposure to PQ (fig. 3b, d). There were significant effects of exposure and time (both p values <0.05), and an interaction of exposure and time [$F_{(10,202)} = 6.35$, $p < 0.0001$]. There were significant effects of gender and exposure on day 8 of treatment (both p values <0.05) but not 1 week after exposure. Similar to results from the first experiment, all animals exposed to MB in adulthood showed reductions in total horizontal activity on day 8 of exposure (fig. 3b) but recovered normal activity levels 1 week after exposure (fig. 3d). No other significant effects were detected.

Neurochemistry

Striatal and frontal cortical levels of DA, DOPAC, HVA (striatum only), DA turnover, and 5HT were evaluated after the 1-week post-exposure locomotor activity assessment. In experiment 1, involving prenatal exposure to MB, striatal levels of DA and DOPAC were significant-

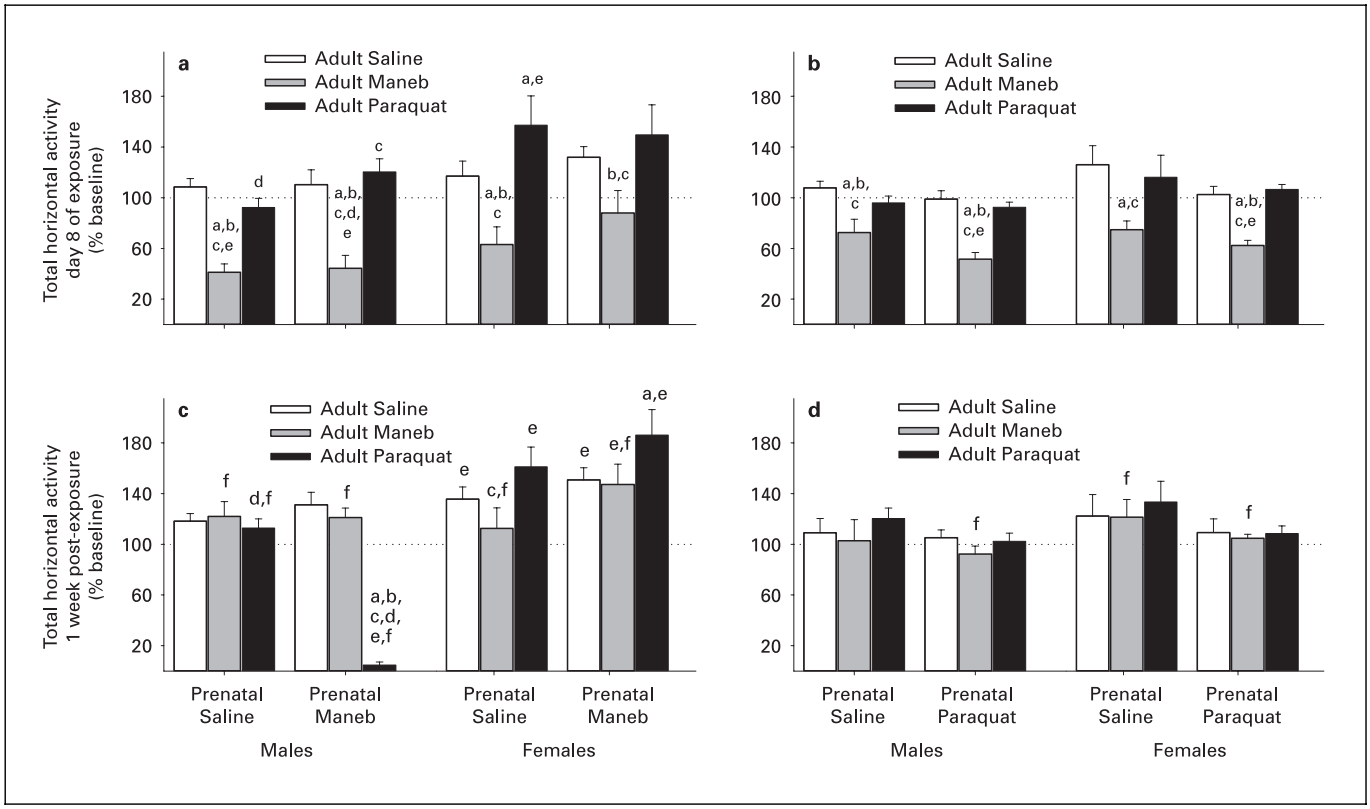

Fig. 3. Effect of gender and prenatal exposure to maneb or paraquat on locomotor activity in response to adulthood exposures. Total horizontal activity was normalized to group means from the third day of habituation to locomotor chambers (mean ± SEM, n = 7–11 per condition) and represents activity for 45 min immediately after the adult exposure injection (day 8 of consecutive exposures) or after injection of saline 1 week after exposure. Results from the first experiment, with prenatal exposure to MB (**a**, **c**), revealed a significant effect of gender and of exposure condition, and of the interaction of gender and exposure over time [$F_{(10,198)} = 9.28$, $p < 0.0001$]. On day 8 of exposure, all animals exposed to maneb in adulthood showed reductions in activity (**a**) but recovered to normal levels within 1 week of the last exposure (**c**). Most exposure groups in both genders showed increased activity between the final day of the exposure period and 1 week after exposure, with the notable exception of MB-PQ males who showed a dramatic (> 95%) reduction in activity (**c**) compared to other male exposure conditions. Furthermore, there was a striking gender differential in susceptibility to this combination of agrichemicals. Results from the second experiment, with prenatal exposure to paraquat (**b**, **d**), produced no significant main interaction of gender and exposure condition on activity over time. Fisher's post hoc analysis revealed significant differences ($p < 0.05$) from: [a] same gender SAL-SAL; [b] same gender MB-SAL (experiment 1) or PQ-SAL (experiment 2); [c] same gender SAL-PQ; [d] opposite gender same exposure condition (shown only on male bars); [e] activity during habituation, and [f] activity on day 8 of exposure.

ly affected by exposure ($p < 0.001$ and $p < 0.05$, respectively), expressed as percent SAL-SAL in figure 4a, b. There was an interaction of gender and exposure on striatal DA turnover [$F_{(5,98)} = 2.54$, $p < 0.05$]. Striatal levels of HVA and 5HT were unaffected. Neither prenatal exposure to MB alone nor adult exposure to PQ alone induced any significant change in dopaminergic neurochemistry in either gender. However, reflecting the pattern of results seen in assessment of locomotor activity, males exposed to the combination of prenatal MB and PQ in adulthood showed significant changes in neurochemical measures. In this group, DA levels were 50% lower, DOPAC levels were 35% lower, and DA turnover was 40% greater than in male control groups and 30% greater than similarly exposed females (all p values <0.05). In frontal cortex, DA, DOPAC and DA turnover were unaffected by gender and/or exposure.

In experiment 2, involving prenatal exposure to PQ, striatal levels of DA were affected by gender ($p < 0.05$) with females having slightly (<10%) higher DA levels, but no other significant effects of gender, exposure, or the interaction of gender and exposure were detected (ex-

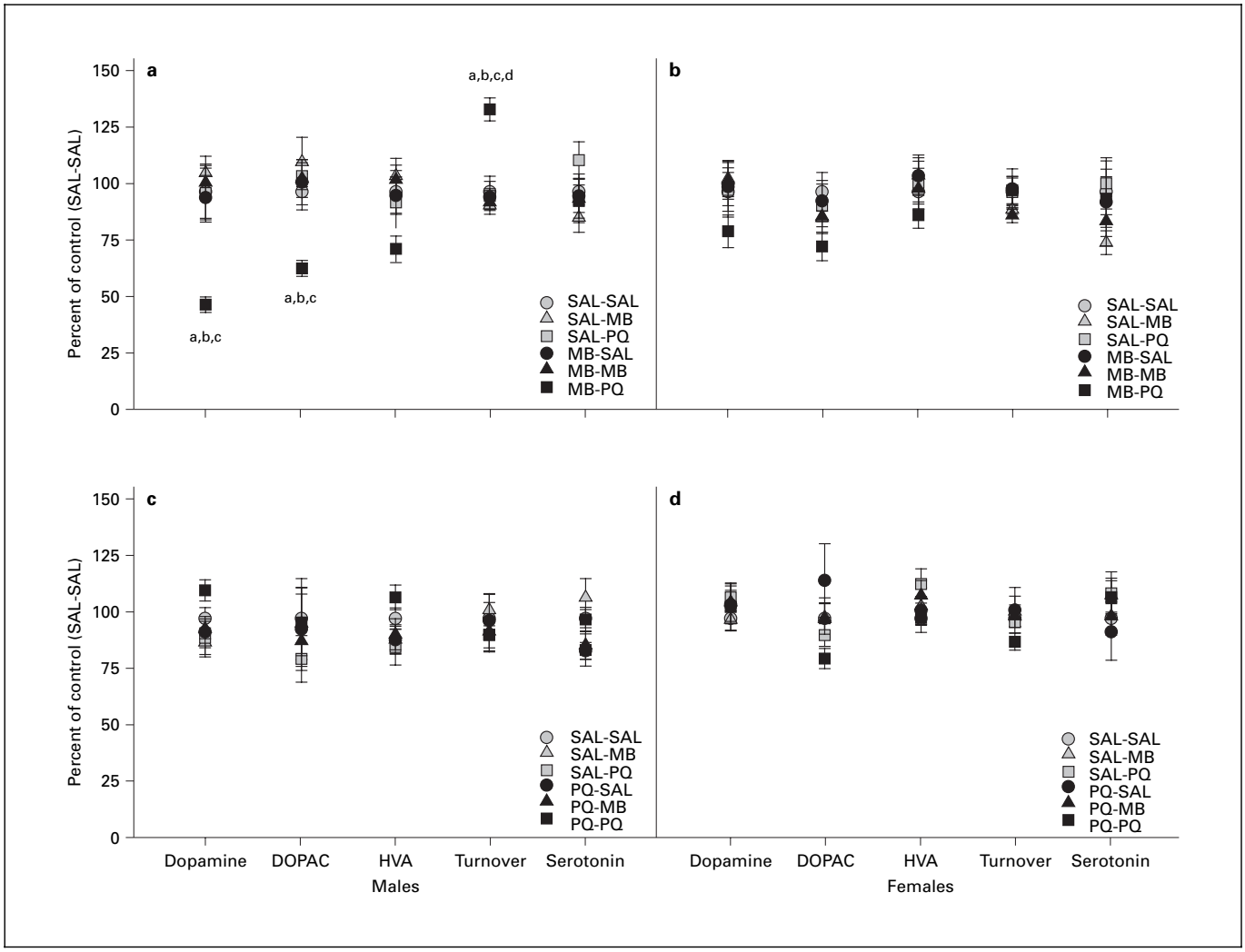

Fig. 4. Effect of gender and exposure condition on DA, DOPAC, HVA, DA turnover, and serotonin (5HT) levels in the striatum. Values represent the percent of SAL-SAL of each gender (**a**, **c**: males; **b**, **d**: females) for each experiment (**a**, **b**: experiment 1; **c**, **d**: experiment 2). Paralleling locomotor activity, only males exposed prenatally to maneb and to paraquat in adulthood showed significant differences from corresponding control groups, including 50% lower DA and 40% greater DA turnover than SAL controls, while displaying no change in 5HT levels (**a**). Similarly treated females showed no significant alterations in striatal neurochemistry (**b**). Prenatal exposure to paraquat did not impact catecholaminergic neurochemistry of the striatum, nor make these systems more vulnerable to damage by adulthood maneb or paraquat exposure (**c**, **d**). Control values (SAL-SAL, mean ± SEM) for DA (ng/mg protein), DOPAC (ng/mg protein), HVA (ng/mg protein), DA turnover, and 5HT (ng/mg protein) were as follows, respectively: **a** 150.6 ± 18.8, 10.0 ± 0.9, 18.6 ± 1.9, 0.21 ± 0.02, 7.9 ± 0.5; **b** 136.3 ± 12.5, 11.9 ± 1.1, 16.9 ± 1.4, 0.22 ± 0.01, 8.4 ± 0.9; **c** 191.5 ± 9.9, 14.1 ± 1.6, 27.2 ± 1.4, 0.22 ± 0.02, 4.0 ± 0.2; **d** 193.0 ± 10.6, 13.9 ± 1.0; 24.1 ± 0.8; 0.20 ± 0.01, 3.5 ± 0.3. Values for SAL-SAL groups were similar between males and females in both experiments. Fisher's post hoc analysis revealed significant differences ($p < 0.05$) from: [a] same gender SAL-SAL, [b] same gender MB-SAL, [c] same gender SAL-PQ, [d] opposite gender same exposure condition (shown only on male bars).

pressed as percent SAL-SAL in figure 4c, d). In frontal cortex, DA and DA turnover were affected by gender (male DA ~30% greater, female DA turnover ~30% greater; all p values <0.05); DOPAC and 5HT in frontal cortex were unaffected.

Stereological Assessment

The numbers of dopaminergic (TH+) cells in the SNpc and in the VTA were determined, along with the number of non-dopaminergic (TH–) neurons (fig. 5). Males exposed to prenatal MB combined with adult exposure to

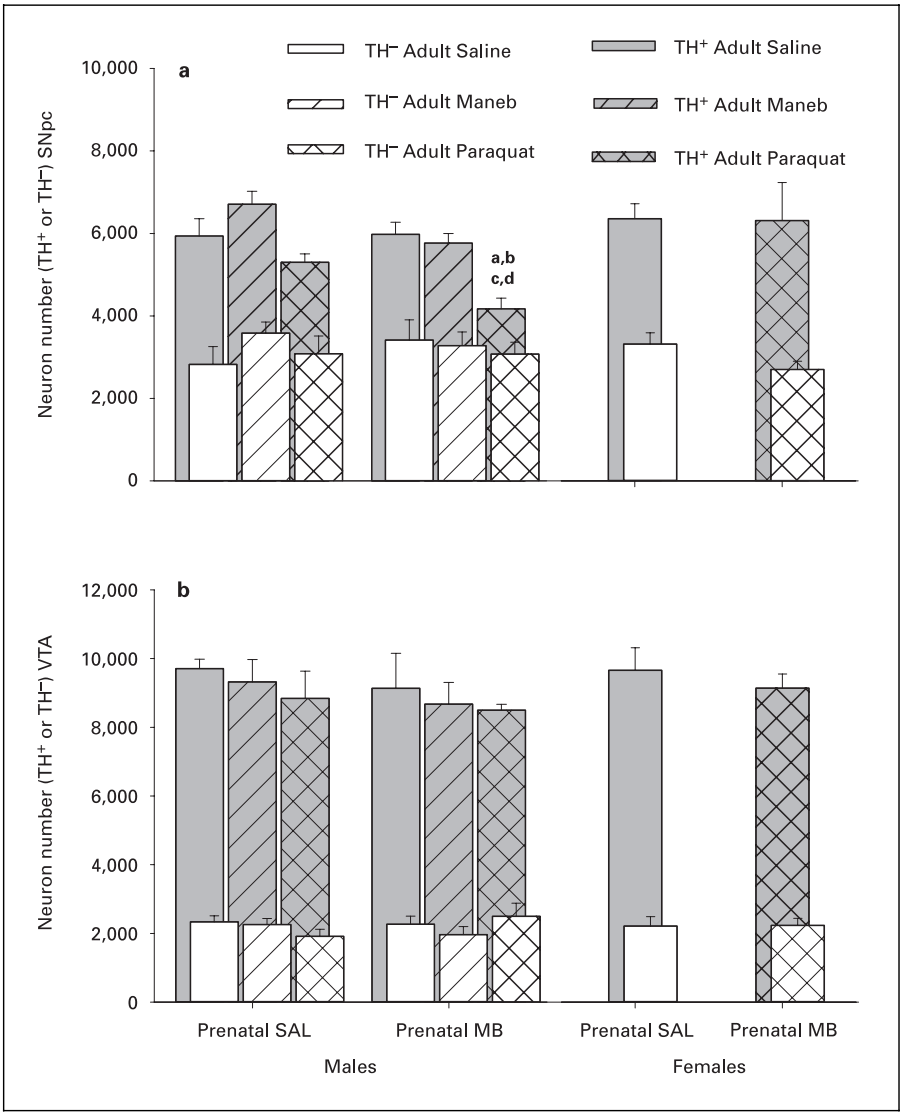

Fig. 5. Total number of TH+ and TH– neurons in the SNpc and VTA 1 week after the last adulthood exposure to saline, maneb, or paraquat (mean ± SEM, n = 4–5 per condition). **a** In the SNpc, only prenatal MB exposure combined with PQ exposure in adulthood led to a significant reduction in TH+ neurons when compared to all control groups and similarly exposed females. The loss of TH+ neurons was observed only in males of the MB-PQ combined exposure condition, implying that females exhibit protective mechanisms against the challenge presented by this combined sequence of exposures. The number of TH– neurons in SNpc was not significantly affected by any exposure condition, and was similar between genders. **b** In the VTA, the numbers of TH+ neurons as well as the number of TH– neurons were unaffected by exposure or gender. The loss of neurons observed in MB-PQ males was regionally selective for the TH+ neurons of the SNpc, as the number of TH+ neurons in VTA did not mirror the loss seen in the SNpc. Fisher's post hoc analysis revealed significant differences ($p < 0.05$) from: [a] same gender SAL-SAL, [b] same gender MB-SAL, [c] same gender SAL-PQ, [d] opposite gender same exposure condition.

PQ showed a loss of TH+ neurons when compared to SAL-SAL males (30% lower, $p < 0.001$), MB-SAL males (30% lower, $p < 0.001$), and SAL-PQ males (21% lower, $p < 0.05$). There were no significant effects of any exposure on the number of TH– neurons in SNpc. No change was observed in TH+ or TH– neurons in the VTA by any exposure or combination of exposures, suggesting a selective loss of TH+ neurons only in the SNpc. Importantly, prenatal exposure to MB alone caused no alteration in the number of TH+ neurons in the SNpc, suggesting that the susceptibility to PQ neurotoxicity observed in the sequential combined exposure group is not related to a decreased initial number of dopaminergic neurons. Because exposure condition caused no significant behavioral or neurochemical alterations in females, only SAL-SAL and MB-PQ females were included in stereological assessments to compare effects between genders (fig. 5). There were gender and exposure effects ($p < 0.05$ for both), and a significant interaction of gender and exposure [$F_{(1,15)} = 6.82$, $p < 0.05$] on the number of TH+ neurons in the SNpc, but no change in TH– cells in the SNpc or VTA was found. No gender-associated differences were observed in the SAL-SAL groups for either TH+ or TH– cells in either SNpc or VTA. Furthermore, unlike the dramatic effect of the MB-PQ combined exposure on the males' TH+ neuron number, TH+ and TH– cells of both the SNpc and the VTA were unaffected by this exposure in females. Males exposed to MB-PQ had significantly fewer TH+ neurons

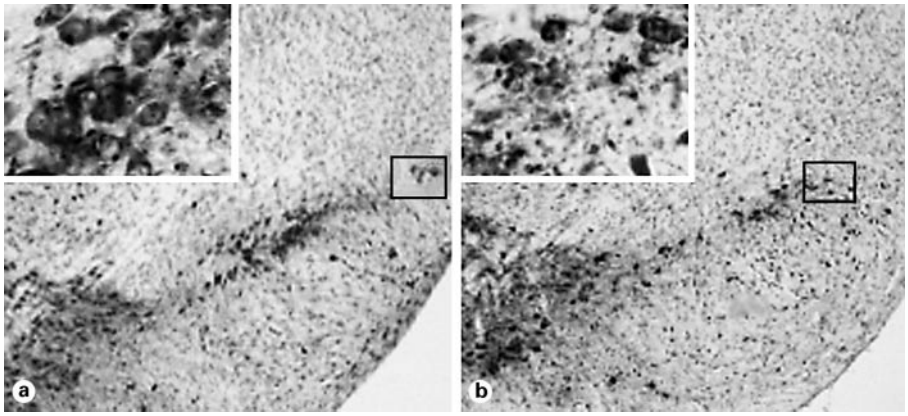

Fig. 6. Representative photomicrographs of TH staining in the SNpc at × 4 and × 40 magnifications. Sections from males of the SAL-SAL condition (**a**, ~20% greater TH+ neuron counts than group mean) and from the MB-PQ condition (**b**, ~5% lower TH+ neuron counts than group mean) are shown. × 40 magnifications (inset) were obtained from the dorsal lateral tip of the SNpc of each section. While stereological assessment is depicted in the previous figure, these representative sections display the substantial loss of TH+ neurons in the SNpc in the MB-PQ exposure condition, and show that the area and volume of remaining intact cells was unaffected by exposure.

in the SNpc than corresponding females (35%, $p < 0.01$), while TH– neurons of the SNpc and the neurons of VTA were unaffected by gender.

In all conditions examined, there was a greater number of TH+ neurons in the VTA than in the SNpc (t test, all p values <0.05), consistent with previous studies [Zaborszky & Vadasz, 2001]. In all conditions except SAL-PQ and MB-MB males, the regional volume of the VTA was significantly larger than the SNpc (t test, all p values <0.05).

Cross-sectional area and volumes of TH+ neurons in the SNpc and the VTA were determined only for SAL-SAL and MB-PQ males because of the extreme differences observed in behavioral and neurochemical measures. In the SNpc, there were no significant differences in cell area for SAL-SAL (244 ± 7 μm^2) compared to MB-PQ (240 ± 4 μm^2), or in cell volume for SAL-SAL (3,152 ± 140 μm^3) compared to MB-PQ (3,089 ± 85 μm^3) [n = 5 brains per condition, cells per brain = 124 ± 12 (SAL-SAL) and 104 ± 8 (MB-PQ)], and this can be seen in the representative photomicrographs in figure 6. Likewise in VTA, there were no significant differences in cell area for SAL-SAL (209 ± 2 μm^2) compared to MB-PQ (196 ± 5 μm^2), or in cell volume for SAL-SAL (2,526 ± 33 μm^3) compared to MB-PQ (3,089 ± 85 μm^3) [n = 5 brains per condition, cells per brain = 239 ± 9 (SAL-SAL) and 213 ± 4 (MB-PQ)]. All values expressed as group means ± SEM.

Discussion

Behavioral, neurochemical, and anatomical measures in this study demonstrated that low-level prenatal exposure to MB induced a state of silent toxicity that was only unmasked by adulthood exposure to PQ, and only in males. These effects were selective for the nigrostriatal DA system, delayed (did not emerge until 1 week after the exposure period ended) and persistent (neuronal loss). The nature of the 'static lesion' induced by MB is not known, but since prenatal MB exposure does not decrease the adulthood number of dopaminergic neurons in the SNpc, it seems to suggest altered neuronal homeostasis. In concordance with other research, these results show that developmental exposure to neurotoxicants can produce a system that is more vulnerable to environmental insult(s) later in life [Melamed et al., 1990; Eriksson et al., 1993; Eriksson, 1996; Gupta et al., 1999; Thiruchelvam et al., 2002]. Moreover, the effects observed were not due simply to the combination of pesticides but to the specific sequence of exposure: males developmentally exposed to MB had a potentiated reaction to adulthood exposure to PQ, whereas males in the reverse-sequence exposure condition (PQ-MB) displayed no discernible damage to the nigrostriatal DA system. While combined exposure to MB and PQ has been shown to induce behavioral and neurochemical changes associated with the PD phenotype

(PDP), previous studies have not examined sequential exposure to the individual compounds. Exposure to these agrichemicals, especially while the individual is developmentally sensitive, need not occur simultaneously but can be separated by several weeks to produce potentiated effects.

The development of the blood-brain barrier (BBB) can be disrupted by prenatal exposures to a range of environmental toxicants, and this protective system may never fully mature after early insult [Gupta et al., 1999]. PQ has been shown to cross the BBB [Corasaniti et al., 1991; Bagetta et al., 1992], it is a free-radical generator [Yang and Sun, 1998], and can interfere with mitochondrial functioning [Tawara et al., 1996]. If the BBB is weakened by developmental exposure to MB, then more PQ might enter the brain upon adulthood exposure, thus increasing the brain concentration of PQ and potentially leading to greater cell damage and cell loss. Alternatively, MB may itself indirectly or directly induce a persistent stress on the nigrostriatal dopaminergic neurons, such that they are already compromised and vulnerable when PQ exposure occurs. In vitro evidence suggests that an immediate effect MB is altered toxicokinetics of DA, such that efflux is impeded, thus increasing DA concentration [Barlow et al., 2003]. During development, DA has autocrine and paracrine functions [De Vitry et al., 1991; Levitt et al., 1997], but excess intracellular DA may be oxidized and interfere with mitochondrial function [Berman and Hastings, 1999]. Acutely, MB exposure increases striatal DA levels in adult mice and rats [Thiruchelvam et al., 2000b; Zhang et al., 2003], MB has also been shown to inhibit mitochondrial complex III [Zhang et al., 2003]. These actions of MB, while not resulting in fewer dopaminergic neurons, may induce biochemical changes and/or altered gene expression, which could lower the viability of these neurons and alter the rate of cell decline.

A proposed model of PD suggests that DA function declines progressively with normal aging, that environmental insult(s) in early or middle life could accelerate this loss and thus reduce nigrostriatal DA function below the threshold necessary to maintain a normal phenotype [Calne and Langston, 1983]. An extension of this proposal suggests that developmental exposure to neurotoxicants can reduce the integrity of the nigrostriatal DA system by either altering the initial number of neurons or the rate of neuronal loss such that the age at which damage falls below the threshold is an earlier one [Thiruchelvam et al., 2002]. Stereological assessment demonstrated that prenatal exposure to MB does not itself cause any reduction in the number of dopaminergic neurons at adulthood. MB may, however, help to establish a 'mutant steady state' (MSS) [Clarke et al., 2000] in which the homeostatic state of those cells is abnormal and confers an increased rate of cell death upon challenge with another neurotoxicant (PQ). While not examined in this study, other natural challenges associated with aging may lead to a greater number, proportion, or rate of cell death, and thus reach a threshold cell loss for the PDP at an earlier age. Taken together, the model raises the possibility that even in the absence of genetic risk factors, early damage caused by exogenous toxin exposure may induce a vulnerability to developing the PDP.

Additionally, like other studies on developmental dopaminergic challenges, this model includes differential gender effects on behavioral and neurochemical measures following developmental exposures and adult insult [Busidan and Dow-Edwards, 1999; Gomes-da-Silva et al., 2000]. While reports suggest that developmental sexual dimorphisms exist in phenotypic properties of dopaminergic neurons [Engele et al., 1989; Ovtscharoff et al., 1992], it is clear that male and female mice reach adulthood with similar numbers of dopaminergic neurons, whether exposed to SAL or MB prenatally, and that females, after prenatal MB exposure, do not demonstrate the potentiated reaction to adulthood exposure to PQ that males do. It is not clear, however, if this protection is due to a differential MB-induced MSS, differential protective mechanisms against PQ neurotoxicity, or differential rates of cell damage and loss between males and females. Epidemiological and clinical studies indicate that there is a higher prevalence of PD among men [Diamond et al., 1990; Baldereschi et al., 2000]. An estrogen-dependent protection of neural integrity has been demonstrated using the dopaminergic neurotoxicants MPTP and 6-OHDA, where males show greater susceptibility to damage by these compounds than females [Dluzen et al., 1996; Miller et al., 1996; Callier et al., 2002]. In primates, estrogen has been suggested to exert neuroprotective effects on nigrostriatal dopaminergic cells, allowing greater neuronal preservation over time, which may in turn slow the development of PD [Leranth et al., 2000]. The extent and mechanisms of estrogen-mediated neuroprotection have not been fully determined, but there are several possible actions within neurons, including antioxidant effects, alterations of gene expression, activation of metabolic pathways, and neurotransmitter receptor actions [Behl et al., 1997; Green and Simpkins, 2000]. Further understanding of gender differences in susceptibility to environmental neurotoxicants will help provide insight into the pathogenesis of PD.

The potential for human exposure to DTCs such as MB, alone or in combination with PQ, displays the importance of understanding their mechanism(s) of action. There is extensive geographical overlap in agricultural use of PQ and MB [USGS, 1998], and our results demonstrate that exposure does not need to be concurrent to yield deleterious effects. While human exposure is difficult to quantify, exposure to these pesticides is not limited to agricultural workers who apply it but can reach the general population through spills, drifts, and residues on food products [Newsome, 1976; Ames et al., 1993; McGrew et al., 2000]. Fungicidal DTCs have been used, increasingly, for over 50 years and are applied to a broad range of crops; they can persist in soil for 20–75 days, and have been detected on crops up to 3 weeks after application, and even after washing [Newsome, 1976, 1979; Brocker and Schlatter, 1979; Patsakos et al., 1992; Meco et al., 1994]. Families of agricultural workers have extensive opportunities for exposure to agrichemicals, making the effects of developmental and combined exposures critically relevant. Twenty-one percent of workers' homes are within 50 yards of pesticide mixing areas, 27% of workers store pesticides in their homes, and over half of wives and children do farm work that places them in direct contact with pesticides [Gladen et al., 1998]. There are potentially serious consequences of maternal and early childhood exposures for the developing system, especially since studies have shown that pesticides may be transferred to a fetus via the placenta, and that some pesticides are able to induce overt deleterious effects on progeny [Siddiqui et al., 1981; Nurminen, 1995; Garcia, 1998; Engel et al., 2000]. Such studies also suggest the inadequacy of current human health risk assessment strategies, since these are generally based on single adulthood exposures rather than developmental, sequential, and/or combined exposures.

Without overt teratogenesis or behavioral delays, acute prenatal exposure to low levels of MB can induce, in males, a state of silent toxicity that is primed for an enhanced reaction to later insult. While the mechanism(s) by which this exposure paradigm induces these potentiated effects has not yet been unraveled, this study clearly shows that developmental exposure to neurotoxicants may be involved in the induction of neurodegenerative disorders such as PD. Further studies on the time course of effects would provide insight into the possible progressive neurodegeneration of the DA system in response to environmental toxic insult, and this holds implications for risk assessment paradigms as well as preventative measures to slow the progression of the neurodegeneration seen in PD.

Acknowledgments

This work was supported by grants from the Department of Defense DAMD17-98-1-8628 (EKR), from the National Institutes of Health ES11839 (EKR), and ES10791, ES01247 (DCS). The authors would like to thank Becky M. Goodman for excellent technical assistance and Dr. Raymond B. Baggs for histopathological analysis.

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