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Developmental Heptachlor Exposure Increases Susceptibility of Dopamine Neurons to *N*-Methyl-4-Phenyl-1,2,3,6-Tetrahydropyridine (MPTP) in a Gender-Specific Manner

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Abstract

Parkinson's disease (PD) is primarily thought of as a disease of aging. However recent evidence points to the potential for exposure to xenobiotics during development to increase risk of PD. Here, we report that developmental exposure to the organochlorine pesticide heptachlor alters the dopamine system and increases neurotoxicity in an animal model of PD. Exposure of pregnant mice to heptachlor led to increased levels of the dopamine transporter (DAT) and vesicular monoamine transporter 2 (VMAT2) levels at both the protein and mRNA level in their offspring. Increased DAT and VMAT2 levels were accompanied by alterations of mRNA levels of nuclear transcription factors that control dopamine neuron development and regulate DAT and VMAT2 levels in adulthood. At 12 weeks of age, control and heptachlor-exposed offspring were administered a moderate dose (2×10 mg/kg) of the parkinsonism-inducing agent MPTP. Greater neurotoxicity as evidenced by a greater loss of striatal dopamine and potentiation of increased levels of glial fibrillary acidic protein and α -synuclein was observed in heptachlor-exposed offspring. The neurotoxicity observed was greater in the male offspring than the female offspring, suggesting that males are more susceptible to the long-term effects of developmental heptachlor exposure. These data suggest that developmental heptachlor exposure causes long-term alterations of the dopamine system thereby rendering it more susceptible to dopaminergic damage in adulthood.

Keywords

heptachlor; Parkinson's disease; pesticide; dopamine transporter; vesicular monoamine transporter 2; fetal basis of adult disease

Parkinson's disease (PD) is a progressive neurodegenerative disorder with full clinical manifestation typically observed in adults over 50 years of age (Van den Eeden et al., 2003).

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Although most cases of PD are observed later in life, there is evidence that the disease process begins long before the disease has progressed to the point at which it is diagnosed (Calne and Langston, 1983; Fearnley and Lees, 1991; Di Paola and Uitti, 1996). The presence of a lengthy preclinical phase and the linkage of environmental exposures to increased risk of PD has led to speculation that early life exposures to environmental toxicants may enhance dopaminergic neurodegeneration or increase vulnerability of the dopamine system in adulthood leading to an increased risk of PD (Carvey et al., 2003; Cory-Slechta et al., 2005; Richardson et al., 2006; Barlow et al., 2007).

Pesticide exposure has been one of the most studied environmental risk factors for PD (Semchuk et al., 1992; Tanner and Goldman, 1996; Le Couteur et al., 1999; Priyadarshi et al., 2000; Asherio et al., 2006). However, the possibility that developmental pesticide exposure contributes to PD has only recently begun to be explored. Early postnatal exposure of mice to the herbicide paraquat and the fungicide maneb has been demonstrated to damage the dopamine system and increase susceptibility to later challenges with the same compounds (Thiruchelvam et al., 2002). Similar effects have been observed in mice exposed during the same period to endosulfan and zineb (Jia and Misra, 2007). *In utero* exposure to the fungicide maneb has been demonstrated to cause increased susceptibility of the offspring to dopaminergic damage following paraquat challenge in adulthood (Barlow et al., 2004). We have recently reported that developmental exposure to the organochlorine pesticide dieldrin results in increased neurotoxicity of the parkinsonism-inducing neurotoxin *N*-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP; Richardson et al., 2006). Thus, developmental exposure to pesticides has been demonstrated to either cause direct damage to the dopamine system or alter it in a manner that renders it vulnerable to future insult.

Previous research from our laboratory has demonstrated that perinatal exposure of mice to the organochlorine pesticide heptachlor is associated with alterations of the dopamine system in male mice during adolescence (Caudle et al., 2005). Specifically, we observed alterations of the dopamine transporter (DAT) and vesicular monoamine transporter 2 (VMAT2) which led to an increased ratio of DAT:VMAT2. This finding may be of particular relevance to PD because the ratio of DAT:VMAT2 has been demonstrated to define regions of susceptibility for neurodegeneration in Parkinson's disease and increase susceptibility to the dopaminergic neurotoxin MPTP (PD; Miller et al., 1999; Richardson et al., 2006). In this study, we sought to test the hypothesis that the alterations of DAT and VMAT2 levels observed in mice developmentally exposed to heptachlor would lead to enhanced sensitivity to MPTP and whether there were gender differences in the effects of developmental heptachlor exposure on the dopamine system.

Materials and methods

Chemicals

Heptachlor (purity $\geq 98\%$) was obtained from Chem Service Inc. (West Chester, PA). Monoclonal anti-rat DAT and polyclonal anti-rabbit VMAT2 were purchased from Chemicon (Temecula, CA). Monoclonal anti-mouse α -tubulin and polyclonal glial fibrillary acidic protein (GFAP) was purchased from Sigma (St. Louis, MO). The monoclonal antibody (mAb) against α -synuclein was purchased from BD Transduction (Lexington, KY, USA). Secondary antibodies coupled to horseradish peroxidase were purchased from ICN (anti-rat; Costa Mesa, CA) and Bio-Rad (anti-rabbit and anti-mouse; Hercules, CA). SuperSignal West Dura Extended duration substrate and stripping buffer were obtained from Pierce (Rockford, IL). Monoamine standards for dopamine (DA), serotonin (5-HT), 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), and 5 hydroxy-indole acetic acid (5-HIAA) were obtained from Sigma (St. Louis, MO). Mobile phase was obtained from ESA Inc. (Chelmsford, MA).

Experimental Design

Eight week old female and male C57BL/6J mice purchased from Jackson Laboratory (Bar Harbor, ME) were used for developmental studies. Mice were maintained on a 12:12 light/dark cycle and food and water were available ad libitum. All procedures were conducted in accordance with the Guide for Care and Use of Laboratory Animals (National Institutes of Health) and previously approved by the Institutional Animal Care and Use Committee at Emory University.

Treatments were conducted as described previously (Caudle et al. 2005). Briefly, female mice were administered 3 mg/kg heptachlor dissolved in corn oil vehicle and mixed with peanut butter every 3 days for two weeks prior to introduction of male mice for breeding. Control mice received an equivalent amount of corn oil vehicle in peanut butter. Mice were monitored to ensure total consumption of the treatment, which generally occurred within 10 min. Oral exposures were chosen since the most likely route of exposure to organochlorine pesticides in the human population is through the ingestion of contaminated food. The dosage chosen is 20-fold less than the acute oral LD₅₀ in mice and is based on previous work from our laboratory and other studies (Moser et al., 2001; Caudle et al. 2005). Although the dose utilized is much higher than what would be found in typical human exposures, it is within the range of concentrations found in contaminated milk in Hawaii in the early 1980's (Smith, 1982). The use of peanut butter as a vehicle was chosen to reduce stress to the dam during gestation, since stress from repeated injections during gestation has been shown to alter GABA_A subunit development (Liu et al. 1997). Dosing continued on the same schedule throughout gestation and lactation ending upon weaning of the pups on postnatal day (PND) 22. Mice were then separated by litter and by sex into separate cages until 12 weeks of age.

MPTP Administration and Determination of MPP⁺ Levels

At 12 weeks of age, male and female offspring of control and heptachlor treated animals were administered 2 injections of saline or 10 mg/kg MPTP subcutaneously 10 hr apart (total dosage 20 mg/kg) and sacrificed 7 days after the second injection (Richardson et al. 2006, Tillerson et al. 2002). MPP⁺ levels in the striatum were determined 90 min following a single injection of MPTP (20 mg/kg i.p.) as described previously (Richardson et al., 2006). Briefly, striata were sonicated in 5% TCA and centrifuged for 10 min at 14,000g. MPP⁺ levels were determined in the supernatants by HPLC with UV detection at 290 nm using a reverse-phase Altima C18 column (Alltech Associates Inc., Deerfield, Illinois, USA) and a mobile phase consisting of 89% 50 mM KH₂PO₄ and 11% acetonitrile. MPP⁺ was identified and quantified by comparison of retention time with known standards.

Western Blot Analysis

Western blots were used to quantify the amount of DAT, VMAT2, TH, and AADC. For the MPTP experiment, GFAP and α -synuclein levels were quantified. α -tubulin levels were determined to ensure equal sample loading (Richardson and Miller, 2004; Richardson et al., 2006). Briefly, samples (5-10 μ g protein) were subjected to polyacrylamide gel electrophoresis on 10% precast NuPage gels (Invitrogen, Carlsbad, CA). Samples were electrophoretically transferred to a polyvinylidene difluoride membrane, and nonspecific sites were blocked in 7.5% nonfat dry milk in Tris-buffered saline (135 mM NaCl, 2.5 mM KCl, 50 mM Tris, and 0.1% Tween 20, pH 7.4). Membranes were then incubated overnight in a monoclonal antibody to the N-terminus of DAT (Miller et al. 1997). DAT antibody binding was detected using a goat anti-rat horseradish peroxidase secondary antibody and enhanced chemiluminescence. The luminescence signal was captured on an Alpha Innotech Fluorochem imaging system and stored as a digital image. Membranes were stripped for 15 minutes at room temperature with Pierce Stripping Buffer (Rockford, IL) and sequentially re-probed with polyclonal antibodies against VMAT2, and TH. For the MPTP samples, blots were probed for GFAP and α -synuclein.

α -Tubulin blots were used to ensure equal protein loading across samples. DAT immunoreactivity was detected as a single band between 80-85 kDA (Miller et al., 1997). VMAT2 immunoreactivity was detected as three bands at 75, 55, and 45 kDA, representing the glycosylated, native, and truncated form (Miller et al., 1999). For VMAT2 quantitation by densitometry, only the fully glycosylated 75 kDA band was quantified. AADC and TH were detected as single bands at 50 and 60 kDA, respectively. Densitometry of α -tubulin revealed a single band at 55 kDA.

HPLC-EC Determination of Dopamine

HPLC-EC analysis of neurochemistry was performed as previously described by Richardson and Miller (2004). Striata were sonicated in 0.1M perchloric acid containing 347 μ M sodium bisulfite and 134 μ M EDTA. Homogenates were centrifuged at 15,000 x g for 20 minutes at 4°C and the supernatant filtered through a 0.22 micron filter by centrifugation at 15,000 x g for 20 minutes. The supernatants were then analyzed for levels of dopamine, DOPAC, and HVA by HPLC with an eight-channel coulometric electrode array (ESA Coullarray, Chelmsford, MA). Quantification was made by reference to calibration curves made with individual standards for dopamine, DOPAC, and HVA.

Primer Sequences

Primers for mouse DAT, TH, VMAT2, NURR1, and Ptx3 were designed using the Primer Select software program (Lasergene) and are reported elsewhere (Richardson et al., 2006). All primers were submitted to the National Center for Biotechnological Information (NCBI) for nucleotide comparison by the basic local alignment search tool (BLASTn). Primers for the 18S ribosomal subunit were obtained from Ambion Inc. (Austin, TX).

Quantitative Reverse Transcriptase PCR (real-time PCR)

Real-time PCR was performed as described elsewhere (Richardson et al. 2006) using the ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Bedford, MA), to determine mRNA expression in midbrain or whole brain samples from mice. Briefly, reactions were performed in a total volume of 25 μ l using SyBr Green Master Mix reagent (Applied Biosystems); 2 μ l of cDNA/sample was used as template for the reaction, with 10 μ M forward and reverse primers. Both target and 18S (normalizing gene) amplifications were performed in duplicate. Thermal cycling conditions included 2 min at 50°C and 10 min at 95°C, followed by 40 cycles of 95°C for 15 s and 1 min at the appropriate annealing temperature for each primer set. Results are expressed as relative levels of mRNA normalized to 18S mRNA, referred to as control samples (the calibrator), chosen to represent 1x expression of the gene.

Determination of Brain Heptachlor and Heptachlor-Epoxy Levels by Gas Chromatography

The method for detection of heptachlor and heptachlor-epoxy levels were performed as described previously for the detection of dieldrin in mouse brain (Richardson et al. 2006; Hatcher et al., 2007). Briefly, frozen tissue samples were homogenized in a 1:1 mixture of hexane and acetone containing hexachlorobenzene as an internal standard. Following centrifugation for 10 minutes at 2,500 rpm, the extract solution was reduced by heating the contents of each tube to 60°C. The dry residue was weighed, dissolved in 1 mL of 1:1 hexane:acetone, and transferred to a 25 mL solid-phase extraction column (Alltech Assoc., Deerfield, IL), containing 5 g of Florisol™ and 1 g of anhydrous sodium sulfate that had been preconditioned with hexane. The Florisol™ column was eluted with hexane and the extract reduced to ~1.0 mL by heating to 60°C. The vials, centrifuge tubes, and autosampler vials weighed before and after each addition, extraction and drying sequence using an analytical balance (Mettler Toledo, Model no. AG104, Columbus, OH) to obtain the wet and dry tissue weights, water content, lipid content, and final extract volume.

The sample extracts were analyzed for heptachlor, heptachlor epoxide, and hexachlorobenzene using an Model 6890N gas chromatograph (Agilent Technologies, Palo Alto, CA) equipped with an autosampler, micro electron capture detector (μ ECD) and 30 m $\times$ 0.25 mm DB-5 column (J&W Scientific, Folsom, CA) with a 0.25 μ m internal film thickness. The injector was operated in splitless mode at 200°C, with helium as the carrier gas, and an injection volume of 1 μ L. The oven was operated at a flow rate of 1.5 mL/min, with an initial temperature of 60°C and programmed to increase at 10°C/min to a final temperature of 275°C, which was held for three minutes to yield a total run time of 24.5 minutes. The detector was operated at 270°C with nitrogen as the make up gas at a combined flow rate of 60 mL/min. At least five calibration standards were prepared in hexane over the relevant concentration range and analyzed at the beginning and end of each GC run. Chromatograph peaks were identified by comparison of retention times, with independent standards and confirmed by analysis of selected samples using a Varian STAR 3400 GC equipped with a Saturn 2000 mass spectrometer (hexachlorobenzene = 14.07 min., heptachlor = 16.0 min., heptachlor epoxide = 17.4 min.). The method level of detection (LOD) for heptachlor epoxide in the extract solution was determined to be 0.06 ppb. Recovery of spiked hexachlorobenzene from the samples was greater than 96% in all cases.

Statistical Analysis

Litter was considered the smallest unit of analysis, with each litter representing an independent replication (n = 4-6 litters per treatment group). Body weight gain for dams and pups were analyzed by repeated measures ANOVA. All other neurochemical data were analyzed using two-tailed Student's t-test, ANOVA, or 2-way ANOVA when gender comparisons within a given time point were assessed. Statistical significance is reported at the $p \leq 0.05$ level unless otherwise noted.

Results

Administration of heptachlor to pregnant mice throughout gestation and lactation was used to simulate a developmental exposure paradigm likely to occur in the human population. Similar to that observed in our previous study (Caudle et al., 2006), exposure to heptachlor did not result in overt toxicity to the pregnant mice or their offspring as evidenced by no significant alteration of weight gain and the absence of clinical signs such as tremors or convulsions. Likewise, there was no apparent alteration of litter size or sex distribution of litters from control or heptachlor exposed mice (data not shown).

Previously (Caudle et al., 2005), we found elevated levels of the DAT and VMAT2 in postnatal day 28 male striatum following developmental exposure to heptachlor. Here, we assessed DAT and VMAT2 protein and mRNA levels in the male and female offspring of mice exposed to heptachlor throughout gestation and lactation at 12 weeks of age. DAT protein levels were significantly increased by 82% and 28% ($p < 0.05$) in the striatum of male and female offspring (Fig. 1A). The increased protein levels were accompanied by a 54% increase ($p < 0.05$) in DAT mRNA in midbrain samples in males (Fig. 1B). The female offspring demonstrated a non-significant increase in DAT mRNA (20%). VMAT2 protein levels were increased by 36% and 59% ($p < 0.05$) in males and females, respectively (Fig. 2A), which was accompanied by increased VMAT2 mRNA levels of 75% and 135% ($p < 0.05$; Fig. 2B).

In our previous study exploring the basis of alterations in DAT levels by developmental dieldrin exposure (Richardson et al., 2006), we found increased expression of the transcription factors Nurr1 and Pitx3, which work in concert to regulate DAT levels during development (Martinat et al., 2006), were associated with increased DAT levels. In the offspring of mice exposed to heptachlor throughout gestation and lactation, Nurr1 mRNA levels were increased by 338% in the males and 58% in the females ($p < 0.05$; Fig. 3A). Pitx3 mRNA levels were also increased

in the females by 62% ($p < 0.05$), but decreased in the males by 54% ($p < 0.05$; Fig. 3B). To confirm the relationship between alterations in *Nurr1* and *Pitx3* mRNA expression as it relates to the increased DAT levels observed, we determined the protein and mRNA levels of aromatic amino acid decarboxylase (AADC), an enzyme involved in dopaminergic neurotransmission that is not regulated by *NURR1*. Developmental heptachlor treatment had no significant effect on striatal AADC protein or AADC mRNA expression in the midbrain (Fig. 4A,B), providing support that the alterations of *Nurr1* and *Pitx3* expression may be responsible for the elevated DAT levels observed here.

In the developmental paradigm employed in this study, exposure of developing mice to heptachlor occurred not only *in utero*, but also through lactation. Indeed, we found levels of heptachlor-epoxide, the primary metabolite of heptachlor, in the brains of the pups at 1 day, 4 weeks, and 12 weeks of age (Table 1). To gain insight into whether there existed a critical period of exposure for heptachlor's effects on the dopamine system, we analyzed mRNA levels of DAT, VMAT2, *Nurr1*, and *Pitx3* in 1 day old pups. We found no significant effects on mRNA levels of any of these targets at 1 day of age (Fig. 5A,B,C,D), suggesting that the exposure that occurs during the lactational period may be responsible for the neurochemical effects observed here. However, cross-fostering studies will be required to confirm this.

In animal models with genetic alteration of DAT and VMAT2, an increased ratio of DAT:VMAT2 has been demonstrated to increase susceptibility to the dopaminergic neurotoxin MPTP (Gainetdinov et al., 1997;1998;Donovan et al., 1999). Here, we found that the DAT:VMAT2 ratio was significantly increased by 34% in the male offspring of heptachlor-exposed mice ($p < 0.05$; Fig. 6A). However, there was no significant change observed in the female offspring. Administration of a moderate dose (2×10 mg/kg) of the dopaminergic neurotoxin MPTP decreased striatal dopamine by 63% in the male offspring of control mice ($p < 0.05$; Table 2) and this effect was significantly greater (77%) in the male offspring of heptachlor-exposed mice ($p < 0.05$). In the female offspring, there was no difference in dopamine depletion between the offspring of control (67% reduction) and heptachlor-exposed (71% reduction) mice. To further assess the enhanced neurotoxicity of MPTP in the male offspring of heptachlor-exposed mice, we measured the protein levels of glial fibrillary acidic protein (GFAP) and α -synuclein, both of which have been demonstrated to increase following MPTP administration. GFAP levels were increased by 31% in the male offspring of control mice and this increase was significantly greater (44%; $p < 0.05$) in the male offspring of heptachlor-exposed mice (Fig. 6B). Similar results were found with α -synuclein levels, with increases of 15% observed in control male offspring and 31% in heptachlor-exposed offspring ($p < 0.05$; Fig. 6C). The greater enhanced neurotoxicity of MPTP observed in the male offspring of heptachlor-exposed mice was not the result of altered MPTP metabolism since MPP⁺ levels were not significantly different between the two groups (Table 3).

Discussion

Epidemiological studies have repeatedly indicated that pesticide exposure is a significant risk for Parkinson's disease (PD) and there has been speculation that developmental pesticide exposure may be a risk factor for PD. Here, we report that developmental exposure to the cyclodiene insecticide heptachlor alters the dopamine system and increases susceptibility to the dopaminergic neurotoxin MPTP later in life. These effects had a distinct gender pattern, with the male offspring more affected than the female. These results suggest that developmental exposure to pesticides such as heptachlor induces a state of "silent toxicity" that renders the dopamine system vulnerable to future insult and provides further experimental support that early-life exposures that alter the dopamine system may increase risk for PD.

Heptachlor was widely used as an insecticide throughout the 1960s and 1970s in agricultural and household settings. Although its uses have been banned for over 25 years, there are still significant residues of heptachlor and its active metabolite, heptachlor-epoxide, present in the environment (Schmitt, 2002). Perhaps more importantly, there has been documented developmental exposure through heptachlor-contaminated milk in Hawaii in the early 1980s (Baker et al., 1991). Even though there did not appear to be a significant increase in teratogenic effects in children born during this exposure (Le Marchand et al., 1986), the present study and others (Moser et al., 2001; Smialowicz et al., 2001; Purkerson-Parker et al., 2002) suggest that there may be long term adverse effects that may not be clinically apparent until later in life.

As we observed previously in early adolescent male mice (Caudle et al., 2005), perinatal exposure to heptachlor increases dopamine transporter (DAT) and vesicular monoamine transporter 2 (VMAT2) protein levels. The effects on the DAT were more pronounced in the male mice, similar to that observed in the offspring of rats exposed to 4.2 mg/kg heptachlor from gestation day 10-21 (Purkerson-Parker et al., 2002). We have also observed a similar effect with mice exposed to dieldrin, another chlorinated cyclodiene pesticide (Richardson et al., 2006). The increased DAT levels were accompanied by increased DAT mRNA expression, suggesting that the increased DAT levels observed in this study and the ones mentioned above appear to be the result of enhanced transcription. In contrast to the gender differences observed with DAT levels in the heptachlor-exposed offspring, there were no significant differences observed with VMAT2 levels. As with the DAT, increased VMAT2 levels were associated with increased VMAT2 mRNA expression. Because we observed no increases in DAT or VMAT2 mRNA at one day of age in our mice, the effects we observed appear to have been primarily mediated through lactational exposure, as we have previously observed with dieldrin (Richardson et al., 2006). However, cross-fostering studies will be required to definitively determine this as the possibility of an increased duration of exposure could also explain these data.

Previously, we had hypothesized that increased neuronal activity resulting from inhibition of GABA_A receptors and subsequent activation of transcription factors that regulate DAT and VMAT2 levels could be a potential mechanism for the elevated levels of DAT and VMAT2 observed following perinatal exposure to heptachlor (Caudle et al., 2005). We focused on the nuclear transcription factors Nurr1 and Pitx3, which have been demonstrated to work in concert to regulate DAT and VMAT2 levels during development (Hermanson et al., 2003; Smidt et al., 2004; Martinat et al., 2006). Nurr1 expression has been demonstrated to be increased by neuronal activity (Brosenitsch and Katz, 2001) and increased expression of Nurr1 and Pitx3 have been demonstrated to be associated with increased DAT and VMAT2 levels in mice developmentally exposed to dieldrin, which is thought to act on GABA_A receptors in a similar manner as heptachlor-epoxide (Richardson et al., 2006). Nurr1 mRNA expression was significantly increased in both male and female offspring of mice exposed to heptachlor. However, this effect was much more pronounced in the male offspring. In contrast, Pitx3 mRNA expression was significantly increased in females and significantly decreased in males. This is in contrast to what we found in our previous study with perinatal exposure to dieldrin where Pitx3 expression was increased in both male and female offspring (Richardson et al., 2006). Because heptachlor and dieldrin are thought to act on a common target (Narahashi et al., 1992), it is not clear why we see these differences in gene expression. However, this observation may be the result of the continuing presence of higher levels of heptachlor-epoxide in the brains of the animals in this study (28.78 ng/g) compared to the levels of dieldrin (15.5 ng/g) observed in our previous study. Alternatively, it is possible that there are additional targets for heptachlorepoide that contribute to the effects observed here.

Ever since the first observations that heptachlor increased DAT levels in mice (Miller et al., 1999b; Kirby et al., 2001; Purkerson-Parker et al., 2002), there has been speculation that the

alteration of DAT:VMAT2 ratio observed following heptachlor exposure would result in increased susceptibility of dopamine neurons to endogenous neurotoxic dopamine metabolites or exogenous neurotoxins that use the DAT as a molecular gateway into dopamine neurons. In the case of the parkinsonism-inducing neurotoxin MPTP, genetic deletion of the DAT has been demonstrated to render mice resistant to MPTP neurotoxicity (Gainetdinov et al., 1997), while genetic overexpression of the DAT leads to enhanced MPTP neurotoxicity (Donovan et al., 1999). To test whether developmental heptachlor exposure resulted in increased susceptibility of the dopamine system, we administered the dopaminergic neurotoxin MPTP to the offspring of mice exposed to heptachlor throughout gestation and lactation. MPTP administration resulted in increased loss of dopamine and increased levels of glial fibrillary acidic protein and α -synuclein in the male offspring of heptachlor-exposed mice, providing evidence that the altered DAT:VMAT2 ratio observed in the male mice does indeed increase susceptibility of dopamine neurons. MPTP dopaminergic neurotoxicity was not increased in the female offspring of heptachlor-exposed mice because there was no change in DAT:VMAT2 ratio. Because heptachlor is a known inducer of P450 enzymes (den Tonkelaar et al., 1974), we considered the possibility that the differences that we observed in MPTP-induced neurotoxicity might be the result of altered metabolism of MPTP. However, we observed no significant differences in brain levels of MPP⁺, the active metabolite of MPTP, between control and heptachlor-exposed offspring, providing further support for the role of increased DAT:VMAT2 in the enhanced neurotoxicity observed here.

The gender differences we have observed in this study may have particular relevance to PD as well. Male gender has been demonstrated to be a risk factor for PD (Van den Eeden et al., 2003) and pesticide exposure has been found to be associated with increased risk of PD in men but not women (Baldereschi et al., 2003), although this is not the case in all studies (Asherio et al., 2006). However, the male gender preference of dopaminergic effects observed in experimental studies involving developmental exposures to pesticides (Thiruchelvam et al., 2002; Barlow et al., 2004; Caudle et al., 2005; Richardson et al., 2006) suggests that further research is warranted into the developmental susceptibility of males to pesticide exposure as it relates to dopaminergic function.

There is an ever emerging shift in the environmental health sciences towards the idea that early life exposures may lead to disease and dysfunction later in life (Heindel, 2007). However, the observation that environmental factors acting during critical periods of development lead to adult expression of disease (Barker et al., 1989) or a “silent state” of neurotoxicity that becomes manifest upon aging or neurotoxic challenge (Reuhl, 1991) has been around for some time. The idea of a potential role for early life exposures as a contributing factor in PD has recently started to gain traction in the PD field with several groups demonstrating that in utero exposures to various stressors or toxicants can result in outright dopaminergic neurotoxicity that could shorten the time to development of PD (Perez-Ontano et al., 1995; Ling et al., 2002; Thiruchelvam et al., 2002; Barlow et al., 2004; 2007; Ling et al., 2004; Lloyd et al., 2006; Richardson et al., 2006). This study and our previous work with dieldrin (Richardson et al., 2006) demonstrate that developmental exposure to these organochlorine pesticides are capable of sensitizing the dopamine system to future insult. Because we have observed similar effects with both dieldrin and heptachlor, these effects on the developing dopamine system may be a characteristic of this class of organochlorine pesticides which may suggest the need to alter current risk assessment strategies. Additionally, the findings that organochlorine pesticides have been found elevated in post-mortem PD brain samples (Fleming et al., 1994; Corrigan et al., 1998; 2000) and the persistence of these pesticides in the environment suggest that even though these pesticides have long been banned, they may still pose a danger to the human population. This may be particularly relevant to those developmentally exposed to heptachlor through contaminated milk in Hawaii in the early 1980s. Interestingly, two studies have found an association between dairy consumption and an increased risk for PD (Park et al., 2005;

Chen et al., 2007), with the study by Park and co-workers (2005) reporting there was a 2.3-fold excess of PD in men who consumed >16 oz of milk/day in the Honolulu-Asian Aging Study. Taken in concert, these findings suggest that developmental exposures to organochlorine pesticides may have implications for human health as individuals that have been exposed age or are exposed to other chemicals that target the dopamine system.

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Abbreviations

(PD), Parkinson's disease
 (MPTP), *N*-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
 (DAT), dopamine transporter
 (VMAT2), vesicular monoamine transporter 2
 (TH), tyrosine hydroxylase
 (MPP⁺), *N*-methyl-4-phenyl pyridinium
 (DA), dopamine
 (5-HT), serotonin
 (DOPAC), 3,4-dihydroxyphenylacetic acid
 (HVA), homovanillic acid
 (5-HIAA), and 5 hydroxy-indole acetic acid
 (GD), gestation day
 (PND), postnatal day
 (HPLC-EC), high performance liquid chromatography with electrochemical detection
 (GFAP), glial fibrillary acidic protein
 (GAT-1), Gamma amino butyric acid transporter
 (NET), norepinephrine transporter
 (SERT), serotonin transporter
 (AADC), aromatic amino acid decarboxylase

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Figure 1.

Developmental heptachlor exposure increases (A) dopamine transporter (DAT) protein levels in male and female offspring at 12 weeks of age as determined by western immunoblotting and (B) DAT mRNA determined by RT-PCR in male offspring of female mice exposed to 3 mg/kg heptachlor throughout gestation and lactation. Representative western blots of DAT and tubulin are presented below. Data represent mean $\pm$ SEM ($n=3-4$ animals per treatment group). * indicates groups are significantly different from control for each gender ($p \leq 0.05$) by 2-way ANOVA. # indicates significant differences between genders as a result of treatment. Control levels were not significantly different between males and females.

Figure 2.

Developmental heptachlor exposure increases (A) vesicular monoamine transporter 2 (VMAT2) protein levels at 12 weeks of age as determined by western immunoblotting and (B) VMAT2 mRNA determined by RT-PCR in male and female offspring of female mice exposed to 3 mg/kg heptachlor throughout gestation and lactation. Representative western blots of VMAT2, including the glycosylated, non-glycosylated, and truncated forms, and tubulin are presented below. Data represent mean $\pm$ SEM ($n=3-4$ animals per treatment group). * indicates groups are significantly different from control for each gender ($p \leq 0.05$) by 2-way ANOVA. Control levels were not significantly different between males and females.

Figure 3.

Developmental heptachlor exposure differentially affects (A) NURR1 and (B) Pitx3 mRNA at 12 weeks of age in male and female offspring of mice exposed to 3 mg/kg heptachlor throughout gestation and lactation as determined by RT-PCR. Data represent mean $\pm$ SEM (n=3-4 animals per treatment group). * indicates groups are significantly different from control for each gender ($p \leq 0.05$) by 2-way ANOVA. # indicates significant differences between genders as a result of treatment. Control levels were not significantly different between males and females.

Figure 4. Developmental heptachlor exposure has no significant effect on (A) aromatic amino acid decarboxylase (AADC) protein levels at 12 weeks of age as determined by western immunoblotting or (B) AADC mRNA determined by RTPCR in male and female offspring of female mice exposed to 3 mg/kg heptachlor throughout gestation and lactation. Representative western blots of AADC and tubulin are presented below. Data represent mean $\pm$ SEM (n=3-4 animals per treatment group).

Figure 5. Developmental heptachlor exposure has no significant effects on (A) DAT mRNA, (B) VMAT2 mRNA, (C) NURR1 mRNA, or (D) Pitx3 mRNA in 1 day old male and female offspring of mice exposed to 3 mg/kg heptachlor throughout gestation and lactation as determined by RT-PCR. Data represent mean $\pm$ SEM (n=3 animals per treatment group).

Figure 6.

Developmental heptachlor exposure increases the (A) DAT:VMAT2 ratio in male but not female offspring at 12 weeks of age and exacerbates the neurotoxicity of MPTP in male offspring as evidenced by (B) a significantly greater reduction of striatal dopamine levels and (C) a significantly greater increase of striatal glial fibrillary acidic protein (GFAP) and α -synuclein levels as determined by western immunoblotting. Data expressed as mean $\pm$ SEM ($n = 4-5$). Representative western blots of GFAP, α -synuclein, and tubulin are presented below each graph. For the DAT:VMAT2 ratio * indicates groups are significantly different from control for each gender ($p \leq 0.05$) by 2-way ANOVA. For graphs B and C, * indicates significantly different from Control-Saline group and # indicates significantly different from

the Control-MPTP group ($p \leq 0.05$) as determined by one-way ANOVA followed by means separation by the Student-Newman-Keuls method.

Table 1

Mean Brain Heptachlor-Epoxy Levels (ng/g tissue)

	1 Day	4 weeks	12 weeks
Male	718.33 ± 133	93.35 ± 23.35	28.78 ± 2.38
Female	NA	NA	21.78 ± 2.38

Data expressed as mean ± SEM (n = 2-4). Female mice were administered heptachlor (3 mg/kg) every three days throughout gestation and lactation. Whole brains were collected from 1 day old and 4 week old pups, while striatum was collected from 12 week pups for determination of heptachlor-epoxide levels by gas chromatography with electron capture detection. The parent compound, heptachlor, was not detected in any of the brains. NA (not assayed).

Table 2

Striatal MPP+ Levels (ng/mg tissue)

	Control	Heptachlor
Male	3.05 ± 0.33	3.64 ± 0.26
Female	2.67 ± 0.21	2.47 ± 0.07

Data expressed as mean ± SEM (n = 2-4). Female mice were administered corn oil (control), or 3 mg/kg heptachlor every three days throughout gestation and lactation. At 12 weeks of age, the offspring were administered a single dose of MPTP (20 mg/kg, i.p.) and sacrificed 90 min later. The striatum was dissected and MPP+ levels determined by HPLC with UV detection. MPP+ was identified by comparison of retention time with known standards and concentrations calculated from a standard curve of known concentrations of MPP+.