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Journal Title: Experimental Neurology
Volume: Volume 204, Number 2
Publisher: Elsevier: 12 months | 2007-04, Pages 619-630
Type of Work: Article | Post-print: After Peer Review
Publisher DOI: 10.1016/j.expneurol.2006.12.020
Permanent URL: http://pid.emory.edu/ark:/25593/fjd8w

Final published version: http://dx.doi.org/10.1016/j.expneurol.2006.12.020

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Accessed November 10, 2019 4:56 AM EST
Dieldrin exposure induces oxidative damage in the mouse nigrostriatal dopamine system

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Abstract

Numerous epidemiological studies have shown an association between pesticide exposure and an increased risk of developing Parkinson’s disease (PD). Here, we provide evidence that the insecticide dieldrin causes specific oxidative damage in the nigrostriatal dopamine (DA) system. We report that exposure of mice to low levels of dieldrin for 30 days resulted in alterations in dopamine-handling as evidenced by a decrease in dopamine metabolites, DOPAC (31.7% decrease) and HVA (29.2% decrease) and significantly increased cysteinyl-catechol levels in the striatum. Furthermore, dieldrin resulted in a 53% decrease in total glutathione, an increase in the redox potential of glutathione, and a 90% increase in protein carbonyls. α-Synuclein protein expression was also significantly increased in the striatum (25% increase). Finally, dieldrin caused a significant decrease in striatal expression of the dopamine transporter as measured by [3H]-WIN 35,428 binding and [3H]-dopamine uptake. These alterations occurred in the absence of dopamine neuron loss in the substantia nigra pars compacta. These effects represent the ability of low doses of dieldrin to increase the vulnerability of nigrostriatal dopamine neurons by inducing oxidative stress and suggest that pesticide exposure may act as a promoter of PD.

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Keywords
Parkinson’s disease; Pesticide; Dopamine; Environmental; cysteinyl-dopamine; cysteinyl adducts; quinone; insecticide

Parkinson’s disease (PD) is a chronic, progressive neurological disorder clinically characterized by resting tremor, bradykinesia, postural instability and rigidity (Marsden, 1984). Pathologically, PD presents as dopaminergic neuronal loss in the substantia nigra pars compacta (SNc), striatal depletion of the neurotransmitter dopamine (DA), the loss of neurochemical markers, such as the dopamine transporter (DAT), and the presence of Lewy bodies containing α-synuclein (Spillantini et al., 1997). Because only 5–10% of PD cases are thought to be primarily genetic in origin, investigation of the role of environmental factors in the pathogenesis of the disease is warranted (Dauer & Przedborski, 2003).

Numerous epidemiological studies have shown an association between pesticide exposure and an increased risk of developing PD (Kanthasamy et al., 2005; Le Couteur et al., 1999; Priyadarshi et al., 2000; Semchuk et al., 1991, 1992). In addition, a correlation between the presence of the organochlorine insecticide, dieldrin, in the brain and diagnosis of PD has been reported (Fleming et al., 1994). Similar observations have been reported in two small studies by Corrigan and coworkers, who observed elevated levels of dieldrin in post-mortem brains from PD patients living in the United Kingdom (Corrigan et al., 1998; Corrigan et al., 2000). However, the mechanism(s) by which pesticide exposure increases the risk of PD is still unknown.

Dieldrin is part of a class of synthetic organochlorine pesticides known as cyclodienes that were commonly used during the 1950s-70s. Due to their low volatility, chemical stability, and lipophilic properties, these compounds have a strong tendency to bioaccumulate and biomagnify and are persistent in the environment (Jorgenson, 2001). The Agency for Toxic Substances and Disease Registry (ATSDR) lists dieldrin as the most hazardous cyclodiene pesticide based on its toxicity, widespread distribution, and potential for human exposure (ATSDR, 2005). Collectively, these factors result in continued concern over the toxicity of these compounds decades after their use was banned in the U.S.

Despite the potential link between dieldrin and PD, there have been few studies to investigate the biological impact of dieldrin on the nigrostriatal dopaminergic system in animals. Thiffault and colleagues report no effect on striatal DA levels in mice following a single high dose (40 or 80 mg/kg) injection of dieldrin (2001), however they did not examine the overall state of the dopamine system. Our lab has previously reported that developmental exposure of mice to dieldrin leads to persistent alterations in the dopamine system and increases susceptibility to a dopaminergic toxin (Richardson et al., 2006). In this study, we hypothesized that exposure to low levels of dieldrin to adult mice over an extended period of time would lead to subtle alterations to the nigrostriatal dopamine system. We report dieldrin-induced cysteinyl-catechol formation, decreased striatal glutathione, and disruption of DA handling and DAT expression and function, all of which reflect an increase in oxidative stress. The effects reported here support the link between pesticide exposure and increased risk of developing PD.

METHODS
Materials
Analytical grade (purity ≥ 98%) dieldrin was obtained from ChemService Inc. (West Chester, PA). 3H-dopamine (58 Ci/mmol) and 3H-WIN 35,428 (85 Ci/mmol) were purchased from Perkin-Elmer Life Sciences (Boston, MA). The source of immunochemical reagents is as
follows: rat monoclonal antibody to DAT (cat #MAB369), rabbit polyclonal antibody to TH (cat #AB152), mouse monoclonal antibody to TH (cat #MAB318), rabbit polyclonal antibody to GAT-1 (cat #AB1570W), polyclonal guinea pig antibody to GLT (cat #AB783), and goat anti-guinea pig secondary antibody (cat #AP108P), Chemicon (Temecula, CA); monoclonal mouse antibody to α-tubulin (cat #T-9026), Sigma (St. Louis, MO); polyclonal mouse antibody to α-synuclein (cat #610787), BD Transduction (Bedford, MA); goat anti-rat (cat #112-065-143) and Cy3 goat anti-mouse (cat #115-165-166) secondary antibodies, Jackson ImmunoResearch (West Grove, PA); goat anti-rabbit (cat #170-6515) and goat anti-mouse (cat #170-5047) secondary antibodies, Bio-Rad (Hercules, CA). Super Signal West substrate and stripping buffer were obtained from Pierce (Rockford, IL). Acetone (Certified ACS, 99.6% purity), hexane (Certified ACS, 99.7% purity), and anhydrous sodium sulfate (Certified ACS, 99.4% purity, 10–60 mesh) were purchased from Fisher Chemicals (Fair Lawn, NJ). All other reagents were obtained from Sigma (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA).

Animals

Male C57BL/6J mice (8 weeks of age) were obtained from Jackson Laboratories (Bar Harbor, ME). Animals were group-housed (6 per cage) under a 12:12 light-dark cycle and acclimatized for 1 week prior to initiation of experiments. Standard rodent chow and tap 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. Control mice were injected intraperitoneally with vehicle (methoxytriglycol; n=12) and treated mice were injected with dieldrin (0.3, 1, or 3 mg/kg in methoxytriglycol; n=12 each) every 3 days for 30 days. Three days following the last treatment, striatal tissue was dissected out and prepared for assay as described below.

Pesticide Extraction/Analytical Methods

The tissue extraction procedure was based on procedures previously described (Corrigan et al., 2000; Grandjean et al., 2001). Approximately 150 mg of frozen cortical mouse tissue was added to 5 ml of a 1:1 mixture of hexane and acetone in an amber vial. The concentration of dieldrin from this brain region is representative because dieldrin is evenly distributed throughout the brain (Supplemental Fig. 2). The tissue was homogenized and the vials were placed in a sonication bath for 15 minutes, vortexed for an additional 2 minutes and then allowed to incubate at room temperature overnight. The supernatant was separated by centrifugation for 10 minutes at 1,250 × g and transferred to a 35 ml glass centrifuge tube. This extraction sequence was repeated an additional four times, to yield a total extract volume for each tissue sample of approximately 25 ml. The extract solution was then reduced by evaporating the contents of each tube at room temperature (RT) in an exhaust fume hood. The dry residue was weighed and dissolved in 2 ml of 1:1 hexane. The 2 ml volume, plus two 1 ml rinses, were transferred to a 25 ml solid-phase extraction column, containing 5 g of Florisil™ and 1 g of anhydrous sodium sulfate that had been preconditioned with 8 ml of hexane. The Florisil™ column was then eluted with 5 ml of methyl tertiary-butyl ether (MTBE), and the process was repeated four times to yield an effluent volume of approximately 25 ml. The hexane/MTBE extract was then evaporated. The dried samples were resuspended in 1.0 ml hexane volume, plus two 0.4 ml hexane rinses, and transferred to glass autosampler vials, which were immediately sealed with crimp top caps and stored at 4°C in the dark for 48 hours or less prior to analysis.

The sample extracts were analyzed for dieldrin using a Model 6890N gas chromatograph (GC; Agilent Technologies, Palo Alto, CA) equipped with micro electron capture detector (μECD). Separation was achieved using a 30 m × 0.25 mm DB-5 column (J&W Scientific, Folsom, CA) with an internal film thickness of 0.25 μm. The injector was operated in splitless mode at 200°
C, with helium as the carrier gas, and an injection volume of 1 μl. 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 (Supplemental Fig. 2). Chromatograph peaks were identified by comparison of retention time with independent standards and confirmed by analysis of selected samples using a Varian STAR 3400 GC equipped with a Saturn 2000 mass spectrometer (Berthouex PM, 2002). The minimum detection limit for dieldrin was 1.7 ng/g and 0.5 ng/ml in the tissue and the extract, respectively. Extraction efficiency of dieldrin was greater than 96%.

Organotypic culture
Organotypic culture was performed using the method first described by Stoppini and colleagues (Stoppini et al., 1991) and modified by Testa and colleagues (Testa et al., 2005). Briefly, post-natal day 7 rat pups with dams were obtained from Charles-River (Wilmington, MA). Pups were housed with dam and littermates under a 12:12 light-dark cycle and were allowed to acclimate for 1 week prior to initiation of experiments. Standard rodent chow and tap 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. A total of 12 pups were used for these experiments. On post-natal day 14, pups were decapitated and brains were rapidly removed and transferred into cold, sterile chopping buffer (in mM, 110 sucrose, 60 NaCl, 3 KCl, 1.25 NaH₂PO₄, 28 NaHCO₃, 5 D-glucose, 0.5 CaCl₂, 7 MgCl₂, 0.6 ascorbate) in a sterile hood. The brain was rapidly blocked, glued to the slicer chuck (OTS-4000 tissue slicer, Hatfield, PA) and transferred to the slicer bath filled with cold, oxygenated chopping buffer. Coronal midbrain slices were cut at 300 μm and transferred to a sterile petri dish containing cold dissection buffer (Gey’s balanced salt solution (Sigma, St. Louis, MO) with 0.5% glucose and 3 mM KCl). Slices were inspected, and three representative slices were bisected and each hemi-section was transferred onto a Millicell-CM membrane insert (Millipore, Bedford, MA) set in a 6-well plate in 1 ml of OPTI-MEM media containing 20% (v/v) Hank’s BSS, 20% (v/v) horse serum, and 0.4% glucose. Cultures were maintained in a humidified incubator at 37°C in 5% CO₂ with 87% relative humidity for 2–4 days before switching to, and maintaining cultures in, a serum-free Neurobasal-A (Gibco, Grand Island, NY) media containing serum-free B27 anti-oxidant-free supplement and 0.5% (v/v) L-glutamine which was changed three times a week. Seven days after plating, one hemisection of each brain slice was treated with dieldrin while the other hemisection served as the control and received ethanol alone. Dieldrin was dissolved in 100% ethanol and final concentrations in media were 1 and 10 μM. The final concentration of ethanol was ≤0.1% for all experiments and had no effect on any of the parameters studied (data not shown). Control experiments were performed in the presence of ethanol at the same concentration as that used in the dieldrin-treated slices.

High Performance Liquid Chromatography (HPLC)
Monoamine levels were determined in the striatum as previously described (Richardson & Miller, 2004). Briefly, dissected left striata were sonicated in 0.1 M perchloric acid containing 347 μM sodium bisulfite and 134 μM EDTA. Homogenates were centrifuged at 15,000 × g for 20 min at 4 °C, the supernatant removed, and filtered through a 0.22 μm filter by centrifugation at 15,000 × g for 20 min. The supernatants were then analyzed for levels of dopamine, DOPAC, and HVA using HPLC with an eight-channel coulometric electrode array (ESA Coularray, Chelmsford, MA) and expressed as μg/mg tissue. Quantification was made by reference to calibration curves made with individual monoamine standards.

Determination of the cysteinylation-catechols, cysteinyldopamine (cys-DA) and cysteinyldopamine (cys-DOPAC), was determined as described (Fornstedt-Wallin & Bergh, 1995) with...
modifications. Briefly, striatal samples were sonicated (1:10 dilution) in 0.1 M perchloric acid containing 347 μM sodium bisulfite and 134 μM EDTA. Homogenates were centrifuged at 12,000 × g for 10 min at 4°C, the supernatant removed, and filtered through a 0.22 μm filter by centrifugation at 12,000 × g for 10 min. The supernatants (50 μl) were then analyzed for levels of dopamine, DOPAC, HVA, cys-DA, and cys-DOPAC. All analyses were performed on a Waters Alliance HPLC (Milford, MA) equipped with a Waters 2465 electrochemical detector set at an oxidizing potential of +0.65 V. Analytes were separated with a Waters X Terra C18 column (5 μm; 150 × 4.6 mm) maintained at 32°C. The mobile phase was MD-TM mobile phase (ESA, Chelmsford, MA) containing 2 mM NaCl and adjusted to pH 2.1 with concentrated HCl. Quantification was made by reference to calibration curves constructed with pure (>98%) cys-DA and cys-DOPAC standards obtained from the NIMH Chemical Repository (Research Triangle Institute International, Research Triangle Park, NC). Retention times for cys-DA and cys-DOPAC were 4.993 and 6.040 min, respectively (Supplemental Fig. 3). Detection limits, as determined by Hubaux-Vos method (Hubaux and Vos, 1970), were found to be 0.0292 pmol and 0.0234 pmol for cys-DA and cys-DOPAC, respectively. As a positive control for cys-DA detection during method development, C57BL/6J mice were administered reserpine (5 mg/kg; i.p.) and sacrificed 16 hr later. Striata from these mice were isolated as described above and assayed for cys-DA. Reserpine treatment increased cys-DA concentrations in the striatum by 135% at this time point, similar to that observed by Forstedt and Carlsson (Forstedt & Carlsson, 1989).

Analysis of glutathione levels was performed by HPLC with fluorescence detection as previously described (Jones et al., 2004). Briefly, fresh striatal tissue was collected and immediately transferred to a microcentrifuge tube containing ice-cold 5% (w/v) PCA, 0.2M boric acid and 10μM γ-Glu-Glu as internal standard. Tissue was immediately homogenized and stored at −80°C (< 1 month) prior to further processing to form N-dansyl derivatives and analysis by HPLC with fluorescence detection. The stability of glutathione has been validated under these storage conditions. Glutathione was quantified by integration relative to the internal standard (Jones, 2002) and the total glutathione measurement was determined as the sum of glutathione and its disulfide forms. The redox potential of glutathione (Eh) was calculated according to the Nernst equation: Eh = E0 + (RT/nF)*ln([GSSG]/[GSH]2) (Watson et al., 2003).

Carbonyl Detection

Protein carbonyls were measured in tissue using the Oxyblot™ Protein Oxidation Detection Kit (Chemicon, Temecula, CA) according to the manufacturer’s protocol. Briefly, following treatment with pesticides, tissue was collected and protein extracted using TPER™ Tissue Protein Extraction Reagent (Pierce, Rockford, IL) lysis buffer supplemented with 1μl/ml leupeptin, aprotinin, and pepstatin and 1% β-mercaptoethanol (BME). The lysate was then centrifuged at 10,000 × g for 10 min at 4°C. The supernatant was collected as the soluble protein fraction. Protein levels were determined by using the BioRad Protein Assay. Protein carbonyls were then derivatized with 2,4-dinitrophenylhydrazine (DNPH) to form dinitrophenol (DNP). DNP-derivatized protein samples were analyzed using dot blots and antibodies against DNP.

Immunoblotting

Western blots were performed as previously described (Caudle et al., 2006). Briefly, bilateral striata were homogenized in 1 ml buffer containing 300 mM sucrose, 10 mM HEPES and 1 μg/ml of leupeptin, aprotinin, and pepstatin. Additionally, 1% BME was added to prevent further oxidation for later use in OxyBlot studies. The addition of BME had no affect on western blot studies (unpublished observations). Samples (15 μg) were subjected to polyacrylamide gel electrophoresis on 10% precast NuPage gels (InVitrogen, Carlsbad, CA) and electrophoretically transferred to a polyvinylidene difluoride membrane. Nonspecific sites
were blocked in 7.5% nonfat dry milk in TBS. Membranes were then incubated in primary antibody overnight at 4°C, rinsed, and incubated in appropriate secondary antibody for one hour at RT and signal was detected by enhanced chemiluminescence. The chemiluminescent signal was captured on an Alpha Innotech Fluorchem 8800 (San Leandro, CA) imaging system and stored as a digital image. Densitometric analysis was performed and calibrated to co-blotted dilutional standards of striata from all control samples. Membranes were then stripped for 15 min at 25°C with Pierce Stripping Buffer (Rockford, IL) and reprobed for subsequent proteins.

**Synaptosomal 3H-Dopamine Uptake and 3H-WIN 35,428 Binding**

Dopamine uptake studies were performed as described previously (Caudle et al., 2006; Elwan et al., 2005). Briefly, crude synaptosomes were prepared from fresh striatal tissue and incubated in assay buffer (4 mM Tris, 6.25 HEPES, 120 mM NaCl, 5 mM KCl, 1.2 mM CaCl₂, 1.2 mM MgSO₄, 0.6 mM ascorbic acid, 5.5 mM glucose, 10 μM pargyline; pH 7.4) containing a saturating concentration of dopamine (1 μM final concentration) and a tracer amount of 3H-dopamine (20 nM). A single saturating concentration of dopamine was chosen to assess effects of dieldrin on the Vₘₐₓ of DAT, since we have previously shown no significant effect on Kₘ (unpublished observations). Uptake was allowed to proceed for 3 min at 37°C, and then terminated by the addition of ice-cold buffer and rapid vacuum filtration over GF/B filter paper using a Brandel harvester (Gaithersburg, MD). Filters were washed twice more with buffer, allowed to air dry, and placed in scintillation vials containing 10 ml of Econoscent (Fisher Scientific, Pittsburgh, PA) for scintillation counting (Beckman LS-6500, Fullerton, CA). Uptake rates were calculated as specific uptake (total uptake minus non-specific uptake), with non-specific uptake defined by the inclusion of 10 μM nomifensine. Following determination of synaptosomal protein concentration (Bradford, 1976), uptake rates were calculated as pmol/mg/min protein and expressed as percentage of control values.

Determination of 3H-WIN 35,428 binding to DAT was performed as previously described (Elwan et al., 2006). Binding studies were conducted with crude synaptosomes and a single concentration (10nM) of 3H-WIN 35,428 in 25 mM sodium phosphate buffer (125 mM NaCl, 5 mM KCl; pH 7.4) for one hour at 4°C in 96-well plates. Incubations were terminated by rapid vacuum filtration onto GF/B filter plates and radioactivity was determined by liquid scintillation counting. Non-specific binding was determined by the inclusion of 10 μM nomifensine and specific binding was calculated as the total binding (incubated without 10 μM nomifensine) minus non-specific binding (incubated with nomifensine). Data were calculated as fmol/mg protein and expressed as percentage of control values.

**Immunohistochemistry and Stereology**

Immunohistochemistry and stereology were performed as previously described (McCormack et al., 2002). Briefly, after removal of the mouse brains, midbrain blocks were immersion-fixed in 4% paraformaldehyde and cryoprotected in sucrose. Serial coronal sections (40 μm thick) were cut on a cryostat, collected in cryopreservative, and stored. For stereological cell counting, midbrain sections were immunostained with an antibody against TH (1:800; Pel Freez Biologicals, Rogers, AR) and counterstained with 0.5% Cresyl violet. After delineation at low magnification, every sixth section throughout the substantia nigra pars compacta was sampled at high magnification (Stereo Investigator, MicroBrightfield, Williston, VT). Neurons were counted using the optical fractionator method, an unbiased quantitative technique that is independent of neuronal size and shape and any conformational changes in the tissue (McCormack et al., 2002; West et al., 1991).

For tyrosine hydroxylase (TH) immunoreactivity in organotypic cultures, slices were fixed on the membrane in cold 4% paraformaldehyde and then rinsed in phosphate-buffered saline (PBS). Slices were then rinsed with Tris-buffered saline (TBS) followed by treatment with 3%
H₂O₂ in TBS for 10 min and further rinsing with TBS. Slices were incubated in 10% normal goat serum (NGS) in TBS with 0.5% Triton-X (TBS-tx) for 30 min, followed by overnight incubation at 4°C in the same TBS-tx solution with the mouse monoclonal antibody to TH (1:2000, Chemicon, Temecula, CA). The following day, slices were rinsed with TBS and incubated in Cy3-conjugated goat anti-mouse secondary antibody (1:200, Jackson ImmunoResearch, West Grove, PA) in 10% NGS/TBS-tx for one hour. Slices were again washed in TBS followed by a 30 min incubation in 10 mM cupric sulfate to reduce autofluorescence. Slices were rinsed once more in TBS and then mounted and coverslipped on slides using Aquamount aqueous mountant (Lerner Laboratories, Pittsburgh, PA). Sections were imaged using fluorescence microscopy with Axiovision software (Zeiss, Oberkochen, Germany).

Statistical analysis

Results were expressed as the mean ± SEM. Raw data were analyzed using one-way analysis of variance (ANOVA) followed by post-hoc analysis with Student Newman-Keuls test (SNK). Statistical significance is reported at the p < 0.05 level.

RESULTS

Previous studies have shown that dieldrin induces generation of reactive oxygen species (ROS) in a dopaminergic cell model (Kitazawa et al., 2001). Thus, we sought to examine the effects of direct application of dieldrin in a physiologically-relevant organotypic slice culture model of the rat midbrain to determine if the parent compound had an effect. Midbrain organotypic slices are advantageous because they maintain the cytoarchitecture of the region including glia and other supporting cells and allow examination of direct effects of dieldrin on the brain. Following a one week exposure to either 1 or 10 μM dieldrin, carbonyl formation was increased by 37% and 50% respectively (p<0.05; Supplemental Fig. 4). No overt toxicity was observed by LDH cell death assay (data not shown).

Based upon data shown in Supplemental Figure 4, we assessed whether dieldrin also affected the intact dopaminergic system in vivo. Eight-week old C57BL/6J male mice were intraperitoneally dosed with 0, 0.3, 1 or 3 mg/kg dieldrin every three days for 30 days. The 0.3 mg/kg dose did not show any significant changes on any of the analyses performed (data not shown) and thus was not included in subsequent analyses. No overt signs of toxicity, defined as tremor, seizures, stereotyped movements, excessive lethargy, gait abnormalities or twitching were observed following administration of dieldrin. There were also no significant weight changes in any of the animals (data not shown).

To verify the environmental relevance of dosing levels, we performed lipid extraction and GC analysis of mouse brain tissue to determine the levels of dieldrin that accumulated in the brain at the end of the 30-day exposure. Analysis revealed a dose-dependent increase in the amount of dieldrin in the tissue. There was no dieldrin detectable in the control animals whereas 0.375 ± 0.041 and 0.722 ± 0.094 ng/mg tissue were detected in the 1 and 3 mg/kg dosage groups, respectively (p < 0.01 and p < 0.001, respectively; overall p=0.002; n=4).

To determine the effect of dieldrin exposure on levels of dopamine and its metabolites, DOPAC and HVA, in the striatum, HPLC analysis was performed (Table 1). DOPAC and HVA levels were both significantly decreased as compared to control at the highest dose (31.7% and 29.2% decreases respectively, p<0.05), and DOPAC levels were significantly decreased from control at 1 mg/kg (24.9% decrease, p<0.01). Dose-dependent decreases in dopamine were not statistically significant, however the overall treatment effect was significant (p=0.048). Additionally, the DOPAC/DA ratio was significantly decreased by 32.5% from control with
1 mg/kg exposure (p<0.01) and by 23.5% with 3 mg/kg exposure (p<0.05). The HVA/DA ratio was decreased by 16.6% and 18.3% following 1 and 3 mg/kg exposures, respectively.

Based on previous work that reports increased formation of cysteinyl-catechols following disruption of dopamine-handling (Berman et al., 1996), we examined the levels of cys-DA and cys-DOPAC in the striatum by HPLC. Levels of cys-DA were undetectable in the control and 1 mg/kg groups but found at levels well above our lower detection limit following 3 mg/kg exposure. Cys-DOPAC levels were significantly increased by 85% and 241% following 1 mg/kg (p<0.05) and 3 mg/kg (p<0.001) dieldrin, respectively (Fig. 1a).

Because cysteinyl-adducts can result from the oxidation of dopamine to form ROS and quinones, we hypothesized that there would also be increased markers of oxidative stress in the striatum. Indeed, striatal (STR) carbonyl formation increased by 57% and 90% following 1 and 3 mg/kg exposures, respectively (p<0.05), whereas no increases in carbonyl formation were observed in the cortex (CTX), hippocampus (HIPPO), midbrain (MB), hypothalamus (HYPO), or cerebellum (CBL; Fig. 1b). Furthermore, we demonstrated that the redox potential (E0) of antioxidant, glutathione, was elevated from −238.2 ± 1.5 mV in the control group to −226.3 ± 2.1 mV at the highest dose (p<0.01, Fig. 1c) and the total levels glutathione were decreased by 53% in the striatum of mice dosed with 3 mg/kg dieldrin (p<0.01).

Based on previous work showing increased α-synuclein expression in response to oxidative stress (Hashimoto et al., 2002) and another study that reported induction of α-synuclein fibrillization following dieldrin exposure (Uversky et al., 2002), we examined levels of α-synuclein expression in the striatum. Immunochemical analysis revealed that α-synuclein protein expression was increased by 29.8% and 24.7% following 1 and 3 mg/kg doses, respectively (p<0.001, Fig 2). α-Synuclein expression was not altered in the CTX, HIPPO, MB, HYPO, or CBL (Supplemental Fig. 5).

The dopamine transporter (DAT) is a highly sensitive indicator of early dopaminergic dysfunction in PD (Innis et al., 1993; Miller et al., 1997; Seibyl et al., 1995). Thus, DAT-mediated dopamine uptake and the number of DAT binding sites in the striatum were determined. DAT-mediated dopamine uptake in striatal synaptosomes was decreased by 9% and 21% following the 1 and 3 mg/kg exposures, respectively (p<0.05 at highest dose, Fig 3a). There was also a significant decrease in the number of DAT binding sites following 1 and 3 mg/kg exposures (12% and 15%, p<0.01, Fig 3b), and a significant decrease in DAT protein expression as measured by immunoblotting (26.8% at highest dose, Fig 3c). To determine whether the decreases in DAT function and levels were due to decreased transcription, real time-polymerase chain reaction (rt-PCR) was performed. Analysis revealed no changes in DAT mRNA (data not shown). Additionally, the observed decreases in DAT levels were not due to cell loss. Stereologic analysis of TH-immunoreactive and total number of cell bodies in the SNc and immunohistochemical analysis of TH protein levels revealed no significant differences (Fig 4). There was also no significant change in TH activity observed (data not shown). The amount of dieldrin present in the brain was positively correlated to carbonyl levels (r²=0.913) and inversely correlated to dopamine-uptake and DAT-binding (r²=0.921 and 0.878, respectively). In addition, carbonyl formation was also inversely correlated to dopamine-uptake and DAT-binding (r²=0.964 and 0.978, respectively) supporting the relationship between dieldrin exposure and oxidative stress within the dopaminergic system.

To assess the specificity of dopaminergic dysfunction in the striatum, we measured levels of the GABA-transporter (GAT-1), a marker of GABAergic striatal neurons (Borden, 1996). Western immunoblotting showed no significant difference in protein expression of GAT-1 (Supplemental Fig 6A). To further address specificity of these effects for both the striatum and the dopamine system, we examined DAT in other brain dopaminergic regions and the glutamate
transporter (GLT) in glutamatergic regions including the striatum. No changes in protein expression were observed in the CTX, HIPPO, MB, HYPO, CBL or STR (Supplemental Fig 6B).

Alterations in locomotor gait are a key feature of PD. Previous work from our lab has shown that changes in gait, as measured by stride length, are significantly correlated to dopamine loss (Tillerson et al., 2002). Analysis of mice from all treatments revealed no significant changes in stride length (data not shown).

**DISCUSSION**

Exposure to pesticides has been consistently shown to be associated with increased risk of Parkinson’s disease (Ascherio et al., 2006; Frigerio et al., 2006), but the specific compound(s) responsible for the association has not been determined. Previous studies have shown elevated levels of dieldrin in post-mortem brains of PD patients (Corrigan et al., 2000; Fleming et al., 1994) and we have recently generated data showing a similar association in a U.S. population (Hatcher and Miller, unpublished observations). Dieldrin usage in the U.S. peaked in 1966 and its production was banned in 1974 (ATSDR, 2002). Although the levels of dieldrin that were detected in the mice in the present study were higher than those found in human patients, these measurements were taken directly after exposure as opposed to decades after peak exposure in the PD population. With an estimated dieldrin half-life of approximately one year in the human body (Hunter et al., 1969), the dieldrin levels observed in post-mortem human brain samples were, at early stages of disease, likely much closer to the levels detected in the treated mice

The half-life of dieldrin in mice and rats has been estimated to be between 1–10 days in previous studies (WHO-IPCS, 1989) and approximately three days in this study (Supp. Fig 1). The half-life of dieldrin in sheep is 96–116 days (Paton & Petterson, 1997), in cows it is 52–231 days (Casteel et al., 1993), and in humans it is 141–592 days (average 369 days; Hunter et al., 1969). Additionally, there is evidence that induction of the cytochrome P450 CYP1A1 is higher in rat liver cells than in human liver cells following exposure to organochlorine pesticide-containing mixtures (Vamvakas et al., 1996). In animals orally dosed with dieldrin, Müller and colleagues report that the total amount of dieldrin that was excreted in chimpanzees after 10 days was only 16.2% of the amount excreted by mice further supporting the idea of differential metabolism (Muller et al., 1979; WHO-IPCS, 1989). The species differences in the half-life of dieldrin are thus likely due to a combination of slower rates of metabolism and higher body fat percentages in larger mammals.

In this study, we report that, following a 30-day exposure to dieldrin, there were detectable cysteinyl-dopamine levels in the mice receiving the highest dose of dieldrin but not in the control or lower dose group. Levels below the limit of detection in non-pooled control animals are consistent with reports from other labs (Fornstedt-Wallin & Bergh, 1995; Hastings et al., 1996; LaVoie & Hastings, 1999). Levels of cys-DOPAC were significantly elevated in both exposure groups as well. These findings suggest that dieldrin is causing disruption of dopamine compartmentalization leading to increased cytosolic dopamine, and increased oxidative stress. Interestingly, dopamine depletion in PC-12 cells has been shown to attenuate dieldrin-induced ROS production (Kitazawa et al., 2001). Additionally, the antioxidants SOD and MnTBAP, a SOD mimetic, have been shown to attenuate dieldrin-induced oxidative stress lending further support to the idea of oxidative stress contributing to the neurotoxicity of dieldrin (Kitazawa et al., 2001, 2003). We also report that dieldrin exposure results in a significant increase in carbonyl levels and a decrease in the level of total glutathione in the striatum which may, in part, explain the increased oxidative damage seen following exposure. The increased redox potential of glutathione also reveals a more oxidative environment following dieldrin exposure.
We showed that direct application of dieldrin to midbrain slice cultures increases oxidative damage suggesting that dieldrin is the active neurotoxicant. Despite the widespread distribution of dieldrin throughout the fatty tissue of the brain, it appears that the nigrostriatal system is particularly susceptible to the effects of dieldrin exposure. Previous studies have shown the ability of dieldrin to preferentially cause dopamine release from syntaptosomes that is independent of its GABAergic inhibition (Kirby et al., 2002). In addition, in vitro studies from our lab show that physiologically-relevant concentrations of dieldrin do not directly inhibit DAT or the vesicular monoamine transporter 2 (VMAT2) – two key regulators of dopamine homeostasis (data not shown). The nigrostriatal system is particularly susceptible to damage following dopamine mishandling due to its low antioxidant capacity and precarious oxidative state. In addition to the normal generation of ROS during synthesis and metabolism of dopamine, which result in the formation of hydrogen peroxide, auto-oxidation and ROS- or metal-catalyzed oxidation of cytosolic dopamine to form dopamine-quinones can cause further oxidative damage by reacting with sulphydryl groups to form cysteinyld- adducts. Because cysteinyd residues are often present in the functional regions of many proteins, formation of dopamine-cysteinyld adducts at these sites can lead to serious alterations in protein function (Fornstedt & Carlsson, 1989; Hastings et al., 1996).

In this study, the increased oxidative stress following in vivo exposure to dieldrin was also highly correlated to decreased DAT levels as measured by [3H]-WIN 35,428 binding and immunoblotting and decreased DAT-mediated [3H]-dopamine uptake. It is unlikely that this alteration is benign given the other evidence for oxidative damage and dopamine alterations in the striatum. One potential mechanism for these decreases in DAT levels and activity is the direct oxidative modification of the transporter itself. Several studies have reported the ability of ROS to decrease the levels and function of DAT (Huang et al., 2003; Page et al., 1998; Park et al., 2002) and that Cys342-residue is essential for the proper functioning of DAT (Park et al., 2002). Thus, cysteinyld-adduct formation at this site could be a potential reason for reduced levels and function of DAT. Specifically, dopamine-quinones have been shown to react with this residue and alter DAT binding and function (Whitehead et al., 2001). Additionally, Daniels and Amara demonstrated the rapid internalization of DAT following PKC activation (1999) leading to decreased DAT on the plasma membrane. PKC-mediated internalization also appears to reduce recycling of DAT pools and increase degradation via the lysosomal pathway (Daniels & Amara, 1999; Loder & Melikian, 2003), thereby decreasing the total DAT levels in the cell. Interestingly, dieldrin exposure in cells can lead to the activation of a specific PKC isoform (Kanthasamy et al., 2003; Lederac et al., 2005) which could explain the decreased DAT expression seen in the striatum.

DAT is one of the fundamental regulators of dopamine storage and homeostasis. Perturbation of dopamine homeostasis can predispose neurons to damage (Miller et al., 1999b), potentially by altering dopamine handling thereby increasing the susceptibility of these cells to oxidative damage and toxic insult. Additionally, reductions in DAT levels in mice have been correlated to early damage to the dopamine nerve terminal (Miller et al., 1999a; Uhl, 1999) and decreases in radiolabeled tags of DAT, as measured by single photon emission computerized tomography (SPECT), have been correlated to the progression of dopamine nerve terminal loss in PD, even before clinical symptoms appear (Chou et al., 2004; Frost et al., 1993; Prunier et al., 2003). We have also demonstrated that DAT reduction appears to be a precursor to toxicity in mice treated with polychlorinated biphenyls (PCBs) that are known to damage the dopamine system (Caudle et al., 2006). Dopaminergic neurons in the nigrostriatal system appear to undergo a ‘dying-back’ process in PD such that the nerve terminals are damaged and die before death of the cell body itself (Lach et al., 1992). In our exposure model, we report increased oxidative damage and dopaminergic and DAT dysfunction in the terminals without significant changes or cell death in the midbrain – a pattern consistent with this dying-back process of dopaminergic cell death seen in PD. Because symptoms of PD do not appear until 70–80% of
striatal dopamine nerve terminals are lost (Dauer & Przedborski, 2003), there is a substantial presymptomatic interval during which these terminals are degenerating. Any event or exposure that threatens to accelerate the loss of dopaminergic neurons has the potential to increase the chance of a person developing PD within their lifetime, perhaps before they would die of another cause.

Loss of dopamine is the key pathophysiologic feature of PD (Olanow & Tatton, 1999). While the decreases in dopamine seen in this study did not reach statistical significance for the doses used, there was a statistically significant overall treatment effect. Additionally, longer exposure paradigms and higher dosages can ultimately cause decreased dopamine levels and dopaminergic cell death in the SNc (Heinz et al., 1980; Sharma et al., 1976; Wagner & Greene, 1978). However, it was our intent to evaluate subtle and early effects of this pesticide. Neither decreased synthesis nor increased degradation can explain the reduction in dopamine levels in the striatum as TH activity was not affected and levels of DOPAC and HVA are significantly decreased as well. Most likely, the incorporation of dopamine into cysteinyl-adducts reduces the dopamine signal detected by HPLC and decreased the formation of its metabolites, DOPAC and HVA, as well. Previous work in our lab has shown that decreased stride length of MPTP-treated mice is strongly correlated to decreases in DAT levels as well as a greater than 50% loss of dopamine (Tillerson et al., 2002). Alterations in gait, such as decreased stride length, are another hallmark feature of PD (Blin et al., 1990). Since there was only a 14% reduction in dopamine at the highest dose of dieldrin, we did not anticipate a significant change in stride length.

Collectively, the increase in cysteinyl-catechols and decrease in DAT demonstrate the preferential neurotoxic action of dieldrin on the dopamine nerve terminals. However, the decrease in total glutathione and increase in markers of oxidative stress may suggest that the dysfunction is not confined to the dopamine terminals in the striatum. It is possible that the oxidative alterations seen are dramatic in the dopamine terminals, a relatively small proportion of the striatum. Several studies have shown that damage specific to the dopamine terminals following insults such as methamphetamine or MPTP can significantly increase protein carbonyl levels in the striatum (Gluck et al., 2001; Munoz et al., 2006). Additionally, it is possible that the specific dysfunction in the dopamine neurons sets off a cascade of oxidative stress or inflammatory modulators that involve other striatal components. Other studies have reported post-synaptic changes in the striatum following damage specific for the dopamine neurons (Gnanalingham et al., 1995; Hallett et al., 2005; Todd et al., 1996).

In addition to loss of dopamine, the presence of α-synuclein in Lewy bodies is another pathologic feature of PD (Spillantini et al., 1997). Here, we show that dieldrin significantly increases α-synuclein expression in the striatum. Increased α-synuclein expression is thought to be a response to oxidative stress and transgenic mice over-expressing α-synuclein exhibit loss of dopaminergic terminals in the striatum and motor impairments (Masliah et al., 2000). Triplication of the non-mutated α-synuclein gene in humans has been shown to be sufficient to cause a familial form of PD in humans (Singleton et al., 2003). Additionally, dieldrin has been shown to cause fibrillization of α-synuclein in a cell-free system (Uversky et al., 2002) and increased cytosolic dopamine has been postulated to promote the accumulation of toxic α-synuclein protofibrils (Rochet et al., 2004). Sun and colleagues also report of dieldrin exposure leading to the abnormal aggregation of α-synuclein in a mesencephalic dopaminergic cell line over-expressing the human α-synuclein gene (2005). Thus, the ability of dieldrin to increase α-synuclein could provide an additional connection between pesticide exposure and PD.

In summary, we demonstrate that dieldrin exposure in mice results in altered dopamine metabolism, including an increase in the oxidative byproducts of dopamine, cysteinyl-
catechols. Together with the reduced levels and increased redox potential of glutathione, these reactive intermediates lead to oxidative damage and a reduction of dopamine terminal markers in the striatum. These data demonstrate the ability of environmentally-relevant concentrations of dieldrin to cause oxidative damage in the nigrostriatal dopamine system. Collectively, these data suggest that pesticides, such as dieldrin, do not need to overtly kill nigral neurons but can exert their deleterious effects by promoting pathogenesis in an already susceptible population of neurons.

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

**Acknowledgements**

This work was supported by the Emory Collaborative Center for Parkinson’s Disease Environmental Research (CCPDER) U54ES012068 (GWM), Woodruff Health Sciences Center Fund (GWM) Georgia Tech Foundation (KDP) and other NIH grants F32ES013457 (JRR), F30ES014141 (JMH), R21ES013828 (JRR), ES10806 (DAD), ES12077 (DAD) and ES09047 (DPJ). We would like to thank the NIMH Chemical Repository for their generous donation of the cysteinyl-catechol standards.

**References**


Fig 1. Dieldrin exposure for 30 days increased oxidative stress in the striatum

(A) Cysteinyl-dopamine levels were below the detection limit (BDL) in control and 1 mg/kg animals but present in highest dose group. Cysteinyl-DOPAC levels were increased by 85% and 241% in the striatum of mice dosed with 1 mg/kg and 3 mg/kg dieldrin, respectively (*p<0.05, ***p<0.001; overall p<0.0001). Mean values ± SEM for cys-DA levels was 0.165 ± 0.061 pmol/mg tissue for the 3mg/kg group. Mean values ± SEM for cys-DOPAC levels were 0.100 ± 0.014, 0.185 ± 0.025, and 0.341 ± 0.020 pmol/mg tissue for control, 1, and 3 mg/kg, respectively (n=4 mice per treatment). Limit of detection for cys-DA and cys-DOPAC were 0.0292 pmol and 0.0234 pmol, respectively, and are lower than what is reported in the literature.

(B) Carbonyl formation was increased 57% and 90% in the striatum (STR) of mice dosed
intraperitoneally with 1 or 3 mg/kg dieldrin, respectively. Data are presented as percentage of control values and represent mean + SEM (n=4 per treatment). (*p<0.05; overall p=0.0074). Cortex (CTX), Hippocampus (HIPPO), Midbrain (MB), Hypothalamus (HYPO), Cerebellum (CBL). (C) The redox potential ($E_h$) for glutathione was increased in the striatum of mice dosed with 3 mg/kg dieldrin (**p<0.01; overall p=0.0041). Mean values ± SEM for redox potentials were $-238.2 \pm 1.5$, $-233.4 \pm 1.9$, and $-226.3 \pm 2.1$ mV for control, 1, and 3 mg/kg, respectively (n=4 mice per treatment).
Fig 2. Dieldrin exposure for 30 days increased α-synuclein expression in the striatum

(A) Striatal α-synuclein expression increased by 29.8 and 24.7% following 1 or 3 mg/kg exposure, respectively, as measured by western immunoblotting. (***p<0.001; overall p < 0.0001). Data are presented as percentage of control values and represent mean ± SEM (n=3–4 per treatment). Representative western blots of α-synuclein (B; 19 kDa) and α-tubulin (C; 55 kDa) to ensure equal protein loading.
Fig 3. Dieldrin exposure for 30 days decreased striatal dopamine transporter levels and function

(A) 1 and 3 mg/kg exposures resulted in a 12.4% and 14.7% decrease in $^{3}$H-WIN35,428 binding to DAT, indicating a decrease in DAT levels. Reductions in DAT levels and increased carbonyl formation are highly correlated ($r^2=0.978$). Overall $p=0.0017$. Mean values ± SEM for binding were 841.0 ± 22.0, 737.0 ± 21.4, and 718.0 ± 17.3 fmol/mg protein for control, 1, and 3 mg/kg, respectively. (B) There was a 9.1% and 21.2% decrease in DAT-mediated $^{3}$H-dopamine uptake following 1 and 3 mg/kg exposure, indicating decreased DAT function. There is a strong correlation between reductions in dopamine uptake and increase carbonyl formation ($r^2=0.964$) Overall $p=0.0296$. Mean values ± SEM for uptake were 251.2 ± 8.0, 228.5 ± 1.4, and 198.0 ± 8.1 pmol/mg/min for control, 1, and 3 mg/kg, respectively. (C) Dose-dependent decrease in
striatal DAT protein expression as measured by western immunoblotting (16.4%, and 26.9% for 1 and 3 mg/kg, respectively; overall p=0.0389) Data are presented as percentage of control values and represent mean ± SEM (n=3–4 per treatment). (For all graphs, *p<0.05, **p<0.01). Representative western blots of DAT (D; 75 kDa) and a-tubulin (E; 55 kDa) to ensure equal protein loading.
Fig 4. Dieldrin exposure for 30 days did not cause changes in TH expression or the number of total or TH-positive neurons in the SNc.
(A) There is no significant neuronal loss in the SNc. Data expressed as mean ± SEM (n=4).
(B) There is no significant change in TH levels as measured by western immunoblotting. Data are presented as percentage of control values and represent mean ± SEM (n=3–4 per treatment). Representative western blots of TH (C; 62 kDa) and a-tubulin (D; 55 kDa) to ensure equal protein loading.
Table 1

Striatal dopamine and metabolite levels in control and dieldrin exposed mice.

<table>
<thead>
<tr>
<th></th>
<th>DA</th>
<th>DOPAC</th>
<th>HVA</th>
<th>DOPAC/DA</th>
<th>HVA/DA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>25.04 ± 0.56</td>
<td>1.106 ± 0.10</td>
<td>3.046 ± 0.45</td>
<td>0.0436 ± 0.002</td>
<td>0.1162 ± 0.010</td>
</tr>
<tr>
<td>1 mg/kg</td>
<td>24.46 ± 0.31</td>
<td>0.720 ± 0.25**</td>
<td>2.370 ± 0.13</td>
<td>0.0294 ± 0.001**</td>
<td>0.0969 ± 0.005</td>
</tr>
<tr>
<td>3 mg/kg</td>
<td>22.75 ± 0.78</td>
<td>0.755 ± 0.05**</td>
<td>2.159 ± 0.10*</td>
<td>0.0333 ± 0.003**</td>
<td>0.0950 ± 0.004</td>
</tr>
</tbody>
</table>

Striatal dopamine and metabolite levels are decreased in mice following 30 day dieldrin exposure. Levels were measured using an HPLC equipped with μECD. There are significant dose-dependent decreases in DOPAC and HVA. Monoamine levels expressed as ng/mg tissue. Data expressed as mean ± SEM (n = 3–4 per treatment). DOPAC/DA and HVA/DA are presented as ratios of raw data.

* p<0.05,

** p<0.01 versus control; one-way ANOVA followed by SNK. Overall p-values: DA, 0.0477; DOPAC, 0.0035; HVA, 0.0433, DOPAC/DA, 0.0024, HVA/DA, 0.0933.