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Disruption of dopamine transport by DDT and its metabolites

Jaime M. Hatcher^{a,b}, Kristin C. Delea^{a,b}, Jason R. Richardson^c, Kurt D. Pennell^{a,d,e}, and Gary W. Miller^{a,b,*}

Jaime M. Hatcher: jaime.hatcher@gmail.com; Gary W. Miller: gary.miller@emory.edu

^aCenter for Neurodegenerative Disease, Emory University School of Medicine, Atlanta, GA, United States

^bDepartment of Environmental and Occupational Health, Rollins School of Public Health, Emory University, Atlanta, GA, United States

^cEnvironmental and Occupational Health Sciences Institute and Department of Environmental and Occupational Medicine, University of Medicine and Dentistry-New Jersey/Robert Wood Johnson Medical School, Piscataway, NJ, United States

^dSchool of Civil and Environmental Engineering, Georgia Institute of Technology, Atlanta, GA, United States

^eDepartment of Neurology, Emory University School of Medicine, Atlanta, GA, United States

Abstract

Epidemiological studies suggest a link between pesticide exposure and an increased risk of developing Parkinson's disease (PD). Although studies have been unable to clearly identify specific pesticides that contribute to PD, a few human studies have reported higher levels of the organochlorine pesticides dieldrin and DDE (a metabolite of DDT) in post-mortem PD brains. Previously, we found that exposure of mice to dieldrin caused perturbations in the nigrostriatal dopamine system consistent with those seen in PD. Given the concern over the environmental persistence and reintroduction of DDT for the control of malaria-carrying mosquitoes and other pests, we sought to determine whether DDT and its two major metabolites, DDD and DDE, could damage the dopamine system. *In vitro* analyses in mouse synaptosomes and vesicles demonstrated that DDT and its metabolites inhibit the plasma membrane dopamine transporter (DAT) and the vesicular monoamine transporter (VMAT2). However, exposure of mice to either DDT or DDE failed to show evidence of nigrostriatal damage or behavioral abnormalities in any of the measures examined. Thus, we report that *in vitro* effects of DDT and its metabolites on components of the dopamine system do not translate into neurotoxicological outcomes in orally exposed mice and DDT appears to have less dopamine toxicity when compared to dieldrin. These data suggest elevated DDE levels in PD patients may represent a measure of general pesticide exposure and that other pesticides may be responsible for the association between pesticide exposure and PD.

*Corresponding author at: Emory University, Center for Neurodegenerative Disease, Whitehead Biomedical Research Building 505K, 615 Michael Street, Atlanta, GA 30322, United States. Tel.: +1 404 712 8582; fax: +1 404 727 3728.

Keywords

Parkinson's disease; Pesticides; Organochlorines; DDE; Dichlorodiphenylethane

1. Introduction

Epidemiological studies suggest a link between pesticide exposure and an increased risk of developing Parkinson's disease (PD; Ascherio et al., 2006; Frigerio et al., 2006; Gorell et al., 1998; Le Couteur et al., 1999; Priyadarshi et al., 2001, 2000; Semchuk et al., 1991, 1992). These findings are supported by numerous animal studies that have identified potential mechanistic links between pesticides and PD pathogenesis (Betarbet et al., 2000; Bloomquist et al., 2002; Caudle et al., 2005; Hatcher et al., 2007; Kitazawa et al., 2001, 2003; McCormack et al., 2005; Miller et al., 1999; Purkerson-Parker et al., 2001; Richardson et al., 2006; Thiruchelvam et al., 2003). Additionally, higher levels of pesticides have been identified in post-mortem PD brains versus age-matched controls (Corrigan et al., 1998, 2000; Fleming et al., 1994; Pennell et al., 2006).

DDT and DDE are dichlorodiphenylethanes, one of the three classes of organochlorine pesticides (Kamrin, 1997). It was first used to control vectors, such as mosquitoes, of malaria and typhus in military areas (Rogan and Chen, 2005). Like other organochlorines, DDT and its metabolites such as DDD and DDE, are highly lipophilic ($\log K_{ow} = 6.91, 6.02,$ and 6.51 , respectively) and resistant to degradation leading to their bioaccumulation and biomagnification in the environment (ATSDR, 2002). While there are numerous studies on DDT's health effects in animals, the limited exposure information in humans is primarily from occupational exposures of DDT-spray applicators or those who work in DDT manufacturing facilities (ATSDR, 2002). DDT and its metabolites have been reported to have a wide range of effects including endocrine disruption, carcinogenesis, neurologic sequelae, and immunologic dysfunction (ATSDR, 2002; Rogan and Chen, 2005; Turusov et al., 2002). However, it was ecologic concern over environmental persistence, bioaccumulation, and interference with reproduction in birds that largely led to the banning of DDT in Sweden, the United States, the United Kingdom, and other developed countries in the early 1970s (ATSDR, 2002; Rogan and Chen, 2005; Turusov et al., 2002). However, into the 1980s, DDT continued to be exported from the U.S. for use in developing countries where insect control remained a cogent concern (IARC, 1991; Turusov et al., 2002). Since the banning of DDT in several countries in the 1970s, the levels of DDT and its metabolites in the environment and population of developing countries have declined. However, due to the persistence of these compounds in the environment and food chain, exposure continues to occur on a daily basis.

Based on the analysis of pesticides levels in human tissue samples, we first examined dieldrin with exposure studies in mice and reported several changes in the nigrostriatal dopamine system that are consistent with changes seen in PD, including increased oxidative damage, decreased dopamine transporter (DAT) expression and function, and increased alpha-synuclein expression in the striatum (Hatcher et al., 2007). In light of these findings and the persistence of DDT and its metabolites, we sought to perform similar studies looking to assess effects of DDT compounds on the nigrostriatal dopamine system and dopamine

homeostasis. The reintroduction of DDT for use in the control of malaria and the World Health Organization's reversal of its 30-year ban on DDT use (Anon., 2006; Gunasekaran et al., 2005; WHO, 2006) has reignited human health concerns associated with DDT and its daughter products. In an effort to address the potential neurologic risk of elevated DDE levels in brain tissue from PD brains, we examined the effects of DDT, and its two major metabolites, DDD and DDE, on various measures of dopamine transport including cellular, vesicular, synaptosomal, and *in vivo* models.

2. Methods

2.1. Materials

Analytical grade p,p'-DDD, p,p'-DDE, and p,p'-DDT were obtained from ChemService Inc. (West Chester, PA). ³H-dopamine (58 Ci/mmol) was purchased from Perkin-Elmer Life Sciences (Boston, MA). The sources of immunochemical reagents are as follows: rat monoclonal antibody to DAT (cat #MAB369), rabbit polyclonal antibody to TH (cat #AB152), rabbit polyclonal antibody to GAT-1 (cat #AB1570W), 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) secondary antibody, 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–Aldrich (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA).

2.2. 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 orally gavaged with vehicle (methoxytriglycol; $n = 12$) and treated mice were orally gavaged with p,p'-DDT (1 or 3 mg/kg in methoxytriglycol) or p,p'-DDE (1, 3, or 6 mg/kg in methoxytriglycol) every 3 days for 30 days. These doses were chosen based on previous experiments examining exposure to the organochlorine dieldrin (Hatcher et al., 2007) in order to achieve similar mouse brain pesticide concentrations. Three days following the last treatment, mice were sacrificed by live decapitation and brains removed for further analysis.

2.3. Cell culture

All media and supplements were purchased from CellGro/Mediatech (Verndon, VA) unless otherwise noted. SK-N-MC cells (ATCC; Manassas, VA) stably expressing DAT (SK-DAT;

Ramachandiran et al., 2007; Stephans et al., 2002) were cultured at 37 °C and 5% CO₂ in minimum essential media supplemented with 10% fetal bovine serum (Atlanta Biologicals; Atlanta, GA), 50 U/ml penicillin, 50 µg/ml streptomycin, 2 mM L-glutamine, 1 mM sodium pyruvate, and 1% nonessential amino acids. Dopaminergic SN4741 cells were generously provided by Dr. J.H. Son (Son et al., 1999). Cells were grown in Dulbecco's minimal essential media with high glucose supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 0.5% glucose, 25 U/ml penicillin, and 25 µg/ml streptomycin. Cells were grown to 60–70% confluence at 33 °C and 5% CO₂ to minimize differentiation.

2.4. DCF assay

The presence of intracellular reactive oxygen species (ROS) production was measured by the oxidation of 6-carboxy-2,7-dichlorodihydrofluorescein diacetate (carboxy-H₂DCFDA; Molecular Probes; Eugene, OR) to its fluorescent products in SN4741 cells. The assay was performed according to manufacturer's protocol. Briefly, cells were grown overnight in 96-well, black-wall plates. Cells were rinsed once with warm PBS and then incubated in 10 µM DCF for 15 min at 37 °C in the dark. Cells were then washed in PBS again before the addition of 10 or 100 µM final concentrations of H₂O₂, dieldrin, DDD, DDE, or DDT in the media described above without phenol red. All organochlorines were dissolved in DMSO with a final DMSO concentration in treatment media of less than 1%. DCF fluorescence was measured at an excitation/emission of 485/535 nm using a Wallac Victor-3 MultiLabel Plate Reader (Perkin-Elmer; Waltham, MA).

2.5. Pesticide extraction/analytical methods

The tissue extraction procedure was based on methods of Corrigan et al. (2000) and Grandjean et al. (2001) and performed as previously described (Hatcher et al., 2007). Frozen frontal cortex mouse tissue (~150 mg) was added to 5 ml of a 1:1 mixture of hexane and acetone in an amber vial. Pesticide concentrations from this brain region are considered representative because organochlorines are evenly distributed throughout the brain (Hatcher et al., 2007). The tissue was homogenized Tissue Tearor™ (Biospec Products, Bartlesville, OK) and the vials were placed in a sonication bath for 15 min, vortexed for an additional 2 min and then allowed to incubate at room temperature overnight. The supernatant was separated by centrifugation for 10 min at 1250 × 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 45 °C in an exhaust fume hood with an analytical nitrogen evaporator (Ogranomation; Berlin, MA). The dry residue was weighed and dissolved in 2 ml of 1:1 hexane:acetone. The 2 ml volume, plus two 1 ml rinses, were transferred to a 25 ml solid-phase extraction column (Alltech Assoc., Model no. 227950, Deerfield, IL), 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 at 45 °C with nitrogen gas as described above. The residue was 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 PTFE-lined screw caps and stored at 4 °C in the dark prior to analysis.

The sample extracts were analyzed using a Model 6890N gas chromatograph (GC; Agilent Technologies, Palo Alto, CA) equipped with micro electron capture detector (μ ECD). Separation was achieved using a 30 m $\times$ 0.25 mm DB-5 column (J&W Scientific, Folsom, CA) with an internal film thickness of 0.25 μ m. The injector was operated in splitless mode at 200 °C, with helium as the carrier gas, and an injection volume of 1 μ l. At least five calibration standards were prepared in hexane over the relevant concentration range and analyzed at the beginning and end of each run. Chromatograph peaks were identified by comparison of retention times with independent standards and confirmed by analysis of selected samples using an Agilent 6890N GC equipped with an Agilent 5975 inert mass selective detector (MSD) (Hatcher et al., 2007). The minimum detection limit for DDD, DDE, and DDT was 0.3 ng/g and 0.1 ng/ml in tissue and extract, respectively. Recovery efficiencies ranged from 94 to 100% based on the extraction of nontreated tissue samples spiked with either DDD, DDE, or DDT at concentrations of approximately 10 μ g/l.

2.6. DAT-mediated ^3H -dopamine uptake

DAT-mediated dopamine uptake was measured essentially as described by Richardson et al. (2005). Briefly, SK-DAT cells were plated at 1×10^5 cells per well in 24-well trays and treated with various of organochlorines for 30 min. Cells were washed with 1 ml uptake buffer (4 mM Tris, 6.25 mM HEPES, 120 mM NaCl, 5 mM KCl, 1.2 mM CaCl_2 , 1.2 mM MgSO_4 , 5.6 mM D-glucose, and 0.5 mM ascorbic acid, pH 7.2) and incubated in 0.5 ml uptake buffer containing 0.15 μ M of ^3H -dopamine for 10 min at 37 °C. Nonspecific uptake was determined in the presence of 10 μ M nomifensine. Uptake was terminated by aspirating the uptake buffer and washing each well twice with 1 ml ice-cold uptake buffer. Cells were lysed by 1% sodium hydroxide and transferred into vials containing 10 ml of scintillation fluid and counted the following day using a Beckman LS6500 scintillation counter (Beckman Instruments; Fullerton, CA).

2.7. Synaptosomal ^3H -dopamine release

Synaptosomal dopamine release assays were performed as described by Kirby et al. (2002). Briefly, synaptosomes were prepared as described above and dopamine uptake with 100 nM ^3H -dopamine was allowed to proceed for five minutes at 37 °C. Synaptosomes were then centrifuged at $10,000 \times g$ for 15 min to remove excess dopamine and pellet was resuspended in assay buffer. Synaptosomes were then incubated in control or experimental compounds for 30 min. Following incubation, dopamine release was 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 Econoscint (Fisher Scientific, Pittsburgh, PA) for scintillation counting (Beckman LS-6500, Fullerton, CA). Percentage of dopamine release was calculated as a comparison to both ^3H -DA-loaded synaptosomes that were immediately measured or synaptosomes with similar incubation times to examine basal dopamine release.

2.8. VMAT2-mediated ^3H -DA uptake

Vesicular dopamine uptake was performed as previously described (Caudle et al., 2007). Whole brains were homogenized in buffer (4 mM HEPES, 0.32 M sucrose, pH 7.4). Homogenates were centrifuged at $1000 \times g$ for 10 min, and the resulting supernatant was centrifuged at $20,000 \times g$ for 20 min. The resulting pellet was resuspended in 1.6 ml of 0.32 M sucrose before being transferred to a glass/Teflon homogenizer containing 6.4 ml of water and subjected to 10 up-and-down strokes by hand. All contents of the homogenizer were then poured into a tube containing 1 ml each of 250 mM HEPES and 1 M potassium tartrate and inverted to mix. The mixture was then centrifuged at $20,000 \times g$ for 20 min, and the resulting supernatant was placed in an ultracentrifuge tube and centrifuged at $120,000 \times g$ for 2 h. Vesicles were resuspended in 1.8 ml of buffer (100 mM potassium tartrate, 25 mM HEPES, 0.1 mM EDTA, 0.05 mM EGTA, 1.7 mM ascorbate, pH 7.4). Uptake assays used 300 μl of vesicle solution for each treatment, 100 nM cold dopamine with 2% ^3H -dopamine as a tracer, 2 mM ATP, 10 μM tetrabenazine (Sigma, St. Louis, MO) to define specific uptake, and the appropriate treatment concentration. Samples were incubated for 10 min at 30 °C followed by the addition of [^3H] dopamine and further incubation for 5 min at 30 °C. The assay was terminated by addition of 5 ml of ice-cold assay buffer before filtration through 0.5% polyethylenamine-soaked Whatman GF/F filters (Brandel, Gaithersburg, MD). Filters were then placed in scintillation fluid and counted using a Beckman LS6500 (Beckman Instruments, Fullerton, CA). Dopamine uptake velocity was expressed as pmol dopamine/mg of protein/min, and the IC_{50} was calculated by nonlinear regression using GraphPad Prism 4.0 software (San Diego, CA).

2.9. Vesicular ^3H -dopamine release

Vesicular dopamine release assays were performed as described by Partilla et al. (2006). Briefly, vesicles were prepared as described above and uptake with 100 nM ^3H -dopamine was allowed to proceed for five minutes at 37 °C. Unlabeled dopamine was used to calculate background signal and ATP was omitted as a negative control to reduce the amount of ^3H -dopamine that was loaded for subsequent release. Vesicles were then incubated in control or experimental compounds for 30 min. Following incubation, dopamine release was terminated by the addition of ice-cold buffer and rapid vacuum filtration over GF/F 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). A small aliquot of vesicles was directly injected into scintillation vials immediately after loading to determine total loaded dopamine. Release was calculated as a comparison to ^3H -DA-loaded vesicles with similar incubation times to examine basal dopamine release.

2.10. Behavioral assays

To analyze locomotor activity, mice were placed in polycarbonate locomotor boxes (25.4 cm $\times$ 50.8 cm $\times$ 25.4 cm) and horizontal distance quantified over time using Noldus Ethovision 3.0 (Wageningen, Netherlands). The Noldus system uses video input from a spatially defined arena and records the coordinates of six animals simultaneously at a rate of six Hertz. Locomotor activity was quantified in five minute blocks for a period of 2 h. Stride

length was determined as previously described (Guillot et al., in press). Briefly, animals were first trained to walk across a clean sheet of paper into their home cage without stopping. Following training, the forepaws of each mouse were placed in black ink and length of forepaw steps during normal walking (in a straight line only) was measured. Animals were returned to their home cage upon completion of the task. Stride length was determined by averaging the distance between the middle toe of the first step to the heel of the second step on the same side of the body, for at least four clear steps.

2.11. High performance liquid chromatography (HPLC)

Monoamine levels were determined in the striatum as previously described (Hatcher et al., 2007). Briefly, dissected left striata were sonicated in 0.1 M perchloric acid containing 347 μ M sodium bisulfite and 134 μ M EDTA. Homogenates were centrifuged at $15,000 \times g$ for 20 min at 4 °C, the supernatant removed, and filtered through a 0.22 μ m filter by centrifugation at $15,000 \times g$ for 20 min. The supernatants were then analyzed for levels of dopamine, DOPAC, and HVA using a Waters Alliance HPLC (Milford, MA) with an eight-channel coulometric electrode array and expressed as μ g/mg tissue. Quantification was made by reference to calibration curves prepared with individual monoamine standards over relevant concentrations.

2.12. Immunoblotting

Western blots were performed as previously described (Caudle et al., 2006; Hatcher et al., 2007). Briefly, bilateral striata were homogenized in 1 ml buffer containing 300 mM sucrose, 10 mM HEPES and 1 μ g/ml of leupeptin, aprotinin, and pepstatin. Samples (15 μ g) were subjected to polyacrylamide gel electrophoresis on 10% precast NuPage gels (Invitrogen, Carlsbad, CA) and electrophoretically transferred to a polyvinylidene difluoride (PVDF) 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 1 h at RT and signal was detected by enhanced chemiluminescence using SuperSignal West Dura Extended Duration Substrate (Pierce; Rockford, IL). 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.

2.13. Carbonyl detection

Protein carbonyls were measured in tissue using the Oxyblot™ Protein Oxidation Detection Kit (Chemicon, Temecula, CA) according to manufacturer's protocol and as previously described (Caudle et al., 2007; Hatcher et al., 2007). Briefly, tissue was collected as described for immunoblotting and 1% β -mercaptoethanol (BME) was added to prevent further oxidation. 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 on PVDF membranes and kit antibodies against DNP and imaged as described above.

2.14. Statistical analysis

Results were expressed as the mean $\pm$ S.E.M. 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. All statistical analyses were performed using GraphPad Prism 4.0 software (San Diego, CA).

3. Results

To examine the effects of DDT and its metabolites on VMAT2-mediated dopamine uptake, mouse striatal vesicular preparations were treated with 0.1–100 μ M DDD, DDE, DDT, or dieldrin. Vesicular uptake studies revealed that DDD, DDE, and DDT inhibited VMAT2-mediated 3 H-dopamine (Fig. 1). The IC_{50} for each compound ranged from 8.9 to 14.4 μ M (Table 1). Dieldrin did not show any inhibition of VMAT2 at the concentrations tested (up to 100 μ M).

Next, we wanted to determine if the DDT compounds affected human DAT (hDAT) by assessing the response of SK-N-MC cells that had been stably-transfected with DAT (SK-DAT; Ramachandiran et al., 2007). This model have been used extensively to study DAT function (Elwan et al., 2006; Jones et al., 2004; Pifl et al., 2001; Ramachandiran et al., 2007; Stephans et al., 2002). Treatment of cells with 1, 10, or 100 μ M DDD for 30 min caused 27.3, 65.9, and 89.7% reductions in dopamine uptake, respectively ($p < 0.001$) (Fig. 2). Treatment with 10 or 100 μ M DDT caused 20.4 and 67.0% decreases in dopamine uptake, respectively ($p < 0.001$), while 10 and 100 μ M DDE caused 33.7% ($p < 0.01$) and 69.5% ($p < 0.001$) decreases, respectively.

Based on our previous studies showing that exposure of mice to the organochlorine dieldrin can disrupt dopamine homeostasis, leading to increased cysteinyl-catechol levels in the striatum due to oxidation of cytosolic dopamine (Hatcher et al., 2007), we measured the ability of DDD, DDE, and DDT to cause release of dopamine from isolated vesicles using a protocol based on that of Partilla et al. (2006). Isolated vesicles allow for the direct analysis of vesicular and VMAT2 function. Treatment of vesicles preloaded with dopamine with 100 μ M DDD, DDE, or DDT resulted in a 20.3, 14.0, 18.8% release of dopamine, respectively ($p < 0.05$; Fig. 3). Exposure of vesicles to 1 or 10 μ M DDD, DDE, or DDT did not result in dopamine release (data not shown). We also measured dopamine release in synaptosomes using a method published by Kirby et al. (2002). Synaptosomes provide a relatively intact presynaptic architecture that allows for analysis of nerve terminal function. Exposure of synaptosomes to 10 or 100 μ M DDD for 30 min in resulted in release of 17.6 and 51.6% dopamine, respectively (Fig. 4). Exposure to either 100 μ M DDE or DDT caused a 31.4 or 32.4% release of dopamine, respectively. In comparison, exposure to 1, 10, or 100 μ M amphetamine resulted in a 38.8, 63.9, or 75.0% release of dopamine. GBR-12935 (100 μ M), an inhibitor of DAT, caused a 90% dopamine release and incubation of synaptosomes with a combination of 100 μ M GBR-12,935 and 100 μ M of either DDD, DDE, or DDT did not cause further dopamine release (data not shown).

Based on many studies that have shown that disruption of dopamine storage can lead to oxidative stress (Caudle et al., 2007; Hatcher et al., 2007; Rabinovic et al., 2000; Spencer et

al., 2002), we measured intracellular formation of oxidative species following exposure to the various DDT compounds. Because of the potential contribution of cytoplasmic dopamine to oxidative stress, we chose a cell line (SN4741) that had a more dopaminergic phenotype (Son et al., 1999). Exposure of SN4741 cells to 100 μ M DDD, DDE, or DDT for up to 24 h did not result in significantly increased intracellular oxidative stress as measured by dichlorofluorescein (DCF) fluorescence (Fig. 5). Exposure to 10 or 100 μ M dieldrin for 24 h resulted in 23.0 and 50.5% increases in DCF fluorescence, respectively. By comparison, 10 or 100 μ M H₂O₂ resulted in a 14.6 and 361.1% increase in DCF fluorescence, respectively. There were no differences in cytotoxicity between equal concentrations of compounds as determined by LDH assay (data not shown).

The increased levels of DDE in post-mortem brain tissue and our *in vitro* studies warranted further study in an *in vivo* mouse model. Eight-week old C57BL/6J male mice were orally dosed with 0, 1 or 3 mg/kg DDE or DDT daily for 30 days. No overt signs of toxicity, defined as tremor, seizures, stereotyped movements, excessive lethargy, gait abnormalities or twitching were observed following administration of DDT or DDE. There were also no significant weight changes in any of the animals (data not shown). Table 2 shows compound concentrations in the brain tissue of mice treated with either 1, 3, or 6 mg/kg DDE or 1 or 3 mg/kg DDT for 30 days. The 6 mg/kg dose was added to the DDE regimen in order to reach brain levels that were similar to or in excess of what we observed in previous studies with dieldrin (up to 0.722 ± 0.094 ng/mg tissue), which has been shown to cause nigrostriatal damage in line with that seen in PD (Hatcher et al., 2007). There were no detectable changes in the locomotor tests, open field activity and stride length (Fig. 6). HPLC-analysis of striatal dopamine and metabolites did not reveal any differences in dopamine, DOPAC, or HVA levels in 3 mg/kg DDT or 6 mg/kg DDE mice versus controls (Table 3). Western immunoblotting of several dopaminergic markers in the striatum including tyrosine hydroxylase (TH), DAT, and VMAT2 revealed no detectable differences in striatal expression between control mice and mice treated with DDE or DDT, regardless of dose (Fig. 7). We also did not detect any changes in expression of alpha-synuclein (Fig. 8) nor did we detect increases in markers of oxidative stress, including carbonyl-formation and 3-nitrotyrosine, in the striatum of mice treated with DDT or DDE (Fig. 9).

4. Discussion

Based on epidemiologic studies reporting an association of pesticide exposure with PD, the recent reintroduction of DDT for the control of malaria-carrying mosquitoes (WHO (World Health Organization), 2006), and previous studies reporting increased levels of the DDT metabolite, DDE, in post-mortem PD brains, we sought to examine the ability of DDT and its metabolites to induce damage in the nigrostriatal dopamine system. There is significant epidemiological evidence supporting an association between pesticide exposure and an increased risk of PD (Ascherio et al., 2006; Kanthasamy et al., 2005; Le Couteur et al., 1999; Priyadarshi et al., 2000; Semchuk et al., 1991, 1992). However, the specific compounds that contribute to this risk have yet to be identified.

DDT usage peaked in 1962 and was banned from use in the United States in 1972 (ATSDR, 2002). Even with greater than three decades since the use of DDT and related compounds,

there are reports of elevations of DDE in post-mortem PD brains in U.S. and U.K. population (Corrigan et al., 2000; Pennell et al., 2006). The current population of PD patients (>50 years old) would have been exposed to these compounds during their peak usage (1960s–1970s). Estimates suggest that while DDT levels might be completely excreted from the body 10–20 years after complete cessation of exposure, DDE levels are likely to persist indefinitely (Smith, 2001; Turusov et al., 2002). However, exposure is ongoing and the population is continually exposed to DDT and its metabolites with the FDA total diet study estimating a mean daily intake of 9–44.8 ng of DDT and related compounds per kilogram of body weight in the U.S. (ATSDR, 2002; Gunderson, 1995a,b). Furthermore, since DDT is metabolized to DDE, it is possible that DDT is not being completely excreted from the body but is instead dechlorinated to form DDE. In addition, these compounds are, by design, neurotoxic with four proposed mechanisms of action including inhibition of potassium transport and closure of sodium channels, inhibition of both Na^+/K^+ and $\text{Ca}^{2+}/\text{Mg}^{2+}$ ATPases, and inhibition of calcium calmodulin binding with neurotransmitter release (Klaassen, 2001) which all serve to make neurons hyperexcitable (Klaassen, 2001; Matsumura, 1985). With the combination of these factors and the reintroduction of DDT usage, the potential for human exposure and bioaccumulation of these compounds remains highly relevant.

In the current study, we sought to examine the effects of DDD, DDE, or DDT on components of the nigrostriatal dopamine system that is affected in PD. We selected a range of concentrations that were relevant to, or slightly higher than, the levels to which humans may have been exposed (Chu et al., 2003; Corrigan et al., 1998, 2000; Fleming et al., 1994). This same approach, utilized in our dieldrin exposure studies, resulted in several pathological changes that were comparable to those seen in PD (Hatcher et al., 2007). While we increased the brain levels in mice to levels that are higher than those seen in previous post-mortem PD brain studies, the measurements in human tissue are typically obtained decades after peak exposure. Given the four decades since peak exposure, it is likely that, at that time, the brain levels of DDE in these PD patients were much closer to the levels detected in the exposed mice.

In vitro, we examined the effects of DDD, DDE, and DDT on both DAT and VMAT2 handling of dopamine. DDT and related compounds appeared to cause dopamine release from preloaded synaptosomes. However, in this assay, there is a normal release and reuptake of preloaded dopamine at baseline (data not shown). The pattern of release caused by DDT and its metabolites was similar to the decreased reuptake seen with GBR-12935, a DAT inhibitor. This finding, in addition to the lack of additive effect of GBR-12935 plus one of the DDT compounds, suggested that DDT and its metabolites actually reduced reuptake of basal dopamine release from the cell by inhibiting DAT, as opposed to directly causing dopamine release (Fig. 4). This is supported by the decreased DAT-mediated ^3H -dopamine uptake in SK-N-MC cells stably transfected with hDAT (Fig. 2).

Additionally, all three compounds were capable of inhibiting vesicular VMAT2 function with IC_{50} in the low micromolar range of 8.9–14.4 μM (Table 1). Inhibition of VMAT2 could lead to increased cytoplasmic levels of dopamine. Disruption of dopamine handling and increased cytosolic dopamine has consistently been associated with adverse effects in

cells and animals, including oxidative stress and the formation of catechol cysteinyl-adducts (Berman et al., 1996; Carlsson and Fornstedt, 1991; Caudle et al., 2007; Fornstedt et al., 1989; Fornstedt and Carlsson, 1989; Hatcher et al., 2007; LaVoie and Hastings, 1999; Montine et al., 1997; Smythies and Galzigna, 1998; Spencer et al., 2002) and the oxidative nature of dopamine has been identified as a susceptibility factor in the death of dopaminergic neurons (Halliwell, 2006; Halliwell and Gutteridge, 1999; Jenner, 1998, 2003; Olanow and Tatton, 1999). Interestingly, in cells treated with DDD, DDE, or DDT, there was no evidence of intracellular oxidative stress as measured by DCF fluorescence, but increased intracellular ROS were seen in the dieldrin treated cells (Fig. 5). This is supported by Chun and colleagues who report the same finding with SN4741 cells exposed to dieldrin for 15 h (Chun et al., 2001).

Based on our *in vitro* data demonstrating inhibition of two key regulators of dopamine homeostasis, we thought it necessary to examine the nigrostriatal effects of administration of DDT or its metabolite, DDE, in mice. However, when mice were orally-exposed to either DDE or DDT for 30 days, there was no evidence of neurochemical changes in the nigrostriatal system that are seen with other toxicants associated with PD such as decreases in striatal dopamine levels, decreases in the dopaminergic cell markers DAT, VMAT2, or TH, or increases in alpha-synuclein expression. There was also no evidence of increased oxidative stress as measured by markers of general oxidation (carbonyls and 3-NT). Furthermore, there were no alterations in stride length or open field activity, two behavioral measures that are commonly used for detection of nigrostriatal dysfunction. The *in vivo* measures examined were all negative despite brain concentrations of the pesticides reaching higher levels than those previously seen in our dieldrin studies (Hatcher et al., 2007). However, it is possible that, with higher doses, longer exposures, older animals, or more vulnerable strains, neurotoxicity could occur. It is also possible that while the DDT compounds are reaching the brain, they are either inactive or at ineffective concentrations when they reach DAT or VMAT2, or, that they are sequestered into other systems with higher affinities for the compounds. Alternatively, it is possible that the concentrations achieved in the mouse brain (~65–2910 ppb for DDE) were not high enough to elicit the same response seen at the estimated concentrations utilized *in vitro* (~320–32,000 ppb for DDE), although the lowest and middle doses *in vitro* (~320 and 3200 ppb) were well within range of the levels seen in the mouse brain tissue. Furthermore, we observed significant alterations in vesicular handling of dopamine at concentrations as low as 1 μ M DDD, suggesting that we should have seen some effect *in vivo* if the effective concentration reached this concentration at of the transporter. Metabolism of the compounds is unlikely to contribute to the lack of observable effect in mice as significant levels of the parent compounds were detected in the brains of the mice as well as in the post-mortem brain tissue of humans.

The environmental persistence and bioaccumulation of DDT have long raised both health and environmental concerns, which have been reignited with the reintroduction of DDT into usage. While DDT and its metabolites alter the function of dopamine transporters *in vitro*, unlike dieldrin, they do not appear to evoke neurotoxicological manifestations in treated mice at brain concentrations higher than would be expected to be seen in human brain.

Therefore, we conclude that DDE and DDT are less likely than dieldrin to be associated with Parkinson's disease-related pathology in exposed populations.

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Fig. 1. Effects DDT, DDE, or DDD on vesicular VMAT2-mediated ^3H -dopamine uptake. DDT, DDE, and DDD exposure for 5 min inhibited vesicular dopamine uptake *in vitro*. The IC_{50} values were 8.9, 11.7, and 14.4 μM for DDT, DDE, and DDD, respectively. Dieldrin did not inhibit VMAT2 (100 μM).

Fig. 2.

DAT-mediated ^3H -dopamine uptake in SK-DAT cells. All three DDT compounds inhibited DAT-mediated ^3H -dopamine uptake at the 100 μM level ($p < 0.001$) after 30 min while DDT inhibited uptake at 10 μM ($p < 0.001$) and DDD significantly inhibited uptake at 10 μM ($p < 0.001$) and as low as 1 μM ($p < 0.05$). Data are presented as percentage of control values and represent mean $\pm$ S.E.M. Control values were 823.5 ± 20.3 fmol dopamine/100,000 cells ($n = 3$ runs with triplicates in each run). Significance reported at $p < 0.05$. (*) Significantly different than control, (**) significantly different than 1 μM concentration of same compound, (***) significantly different than 10 μM concentration of same compound.

Fig. 3.

³H-Dopamine release from isolated vesicles. Treatment with 100 µM DDD caused significant release of preloaded ³H-dopamine from vesicles ($p < 0.05$). This release was comparable to that caused by 100 µM reserpine. None of the organochlorines were significantly different from each other. Data are presented as percentage of control values and represent mean $\pm$ S.E.M. Control values were 144.9 ± 6.6 fmol dopamine per mg vesicular protein ($n = 3$ runs with triplicates in each run). AMPH, Amphetamine; TBZ, tetrabenazine. * $p < 0.05$ versus control.

Fig. 4. ³H-Dopamine release from pre-loaded synaptosomes. 100 µM concentrations of any of the three DDT compounds or dieldrin caused significant release of preloaded ³H-dopamine ($p < 0.001$) while DDD caused release at 10 µM ($p < 0.001$). This was comparable to that caused by 1 µM amphetamine. Combination of 100 µM GBR with 100 µM of DDD, DDE, or DDT did not cause greater dopamine release than GBR-12,935 alone (data now shown). Data are presented as percentage of control values and represent mean $\pm$ S.E.M. Control values were 2.1 ± 0.03 pmol dopamine per mg synaptosomal protein ($n = 3$ runs with triplicates in each run). Significance reported at $p < 0.05$. (*) Significantly different than control, (**) significantly different than 1 µM concentration of same compound, (***) significantly different than 10 µM concentration of same compound.

Fig. 5.

Intracellular oxidative stress as measured by DCF fluorescence in SN4741 following exposure to DDD, DDT, or DDE over 24 h. Exposure of SN4741 cells to 10 or 100 μM dieldrin significantly increased intracellular oxidative stress. DDT and related compounds did not cause a significant increase in intracellular DCF fluorescence. 10 and 100 μM H₂O₂ were used as positive controls. (Data are representative of three independent experiments.) * $p < 0.05$ versus control.

Fig. 6. Locomotor analyses in mice treated with DDE or DDT three days after the last exposure. There were no changes in (A) open field locomotor activity over 2 h or (B) stride length in mice treated with any dose of DDE or DDT.

Fig. 7.

Striatal expression of dopaminergic markers three days after the last exposure. (A) Exposure of mice to DDT or DDE for 30 days did not alter expression of dopaminergic markers, DAT, VMAT2, or TH, in the striatum. Data are presented as percentage of control values and represent mean $\pm$ S.E.M. ($n = 4$ per treatment). (B) Representative Western blots of TH (62 kDa), VMAT2 (75, 55, 45 kDa), DAT (75 kDa), and α -tubulin (55 kDa) to ensure equal protein loading.

Fig. 8.

Striatal expression of α -synuclein three days after the last exposure. (A) DDT or DDE exposure for 30 days did not alter α -synuclein expression in the striatum. Data are presented as percentage of control values and represent mean $\pm$ S.E.M. ($n = 4$ per treatment). (B) Representative Western blots of α -synuclein (19 kDa) and α -tubulin (55 kDa) to ensure equal protein loading.

Fig. 9. Striatal levels of oxidative stress markers three days after the last exposure. (A) DDT or DDE exposure for 30 days did not increase carbonyl or 3-nitrotyrosine (3-NT) formation in the striatum. Data are presented as percentage of control values and represent mean $\pm$ S.E.M. ($n = 4$ per treatment). Representative dot blots of (B) Carbonyls, 3-NT, and α -tubulin to ensure equal protein loading.

Table 1

VMAT2-mediated ^3H -dopamine uptake IC_{50} values for DDD, DDE, and DDT

Compound	IC_{50} (μM)
p,p'-DDD	8.9 ± 1.2
p,p'-DDT	11.7 ± 1.3
p,p'-DDE	14.4 ± 1.3
Dieldrin	>100

VMAT2-mediated ^3H -dopamine uptake was measured in vesicles prepared from whole mouse brain. (Each IC_{50} was determined from three independent runs.)

Table 2

Levels of DDD, DDE, and DDT in mouse brain following dosing with either 1 or 3 mg/kg DDT or 1, 3, or 6 mg/kg DDE

Treatment	Metabolites detected (ppb)		
	DDD	DDE	DDT
Control	–	–	–
DDT 1.0	–	–	316.2 ± 24.9
DDT 3.0	176.7 ± 21.5	–	733.6 ± 90.5
DDE 1.0	–	64.9 ± 39.4	–
DDE 3.0	–	159.0 ± 17.7	–
DDE 6.0	–	2910.8 ± 541.5	–

Pesticide levels were measured in cortical tissue via GC. Levels expressed as ng/mg (ppb) tissue. (–) Non-detectable. Data expressed as mean ± S.E.M. ($n = 4$).

Table 3

Striatal dopamine and metabolite levels in control and exposed mice

	DA	DOPAC	HVA
Control	19.680 ± 0.204	1.512 ± 0.027	2.251 ± 0.041
DDT 3.0	19.230 ± 0.664	1.556 ± 0.084	2.318 ± 0.053
DDE 6.0	19.868 ± 0.379	1.423 ± 0.024	2.345 ± 0.087

Striatal dopamine and metabolite levels are unchanged in mice following 30-day exposure to the highest doses of DDT and DDE (3 and 6 mg/kg, respectively). Levels were measured using an HPLC equipped with electrochemical detection. Monoamine levels expressed as ng/mg tissue. Data expressed as mean ± S.E.M. ($n = 3-4$ per treatment).