Published in final edited form as:

Neurotoxicology. 2014 September ; 0: 279–287. doi:10.1016/j.neuro.2014.07.008.

Developmental exposure to the organochlorine insecticide endosulfan damages the nigrostriatal dopamine system in male offspring

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Abstract

The contribution of environmental toxicants to the etiology and risk of Parkinson's disease (PD) has been clearly established, with organochlorine insecticides routinely shown to damage the nigrostriatal dopamine pathway. Although PD is generally considered an adult onset disease, it has been postulated that exposure to environmental contaminants or other factors early in life during critical periods of neurodevelopment could alter the dopaminergic circuit and predispose individuals to developing PD. Recent epidemiological evidence has found exposure to the organochlorine insecticide endosulfan to be a risk factor for PD. However, the specific dopaminergic targets or vulnerable developmental time points related to endosulfan exposure have not been investigated. Thus, we sought to investigate dopaminergic neurotoxicity following developmental exposure to endosulfan as well as following an additional challenge with MPTP. Our *in vitro* findings demonstrate a reduction in SK-N-SH cells and ventral mesencephalic primary cultures after endosulfan treatment. Using an *in vivo* developmental model, exposure to endosulfan during gestation and lactation caused a reduction in DAT and TH in the striatum of male offspring. These alterations were exacerbated following subsequent treatment with MPTP. In contrast, exposure of adult mice to endosulfan did not elicit dopaminergic damage and did not appear to increase the vulnerability of the dopamine neurons to MPTP. These findings suggest that development during gestation and lactation represents a critical window of susceptibility to endosulfan exposure and development of the nigrostriatal dopamine system. Furthermore, these exposures appear to sensitize the dopamine neurons to additional insults that may occur later in life.

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Keywords

Dopamine; Endosulfan; GABA; Neurodevelopment; Nigrostriatal; Parkinson's disease

Introduction

Parkinson's disease (PD) is a progressive neurodegenerative disorder characterized by loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc) of the midbrain, resulting in subsequent loss of dopaminergic projections and dopamine in the striatum. These reductions manifest in the clinical symptoms comprised of bradykinesia, resting tremor, cogwheel rigidity and a postural instability (Fahn, 2003). Although mutations in specific genes have been shown to participate in the etiology of PD, the genetics accounts for only 5–10% of all PD cases, suggesting an additional role for exogenous or environmental factors in the etiopathogenesis of the disease (Tanner *et al.*, 1999). Research over the last several decades has provided a wealth of support for exposure to environmental chemicals, including organochlorine insecticides as a risk factor for PD (Hatcher *et al.*, 2008). Indeed, levels of organochlorine compounds, such as dieldrin and β -HCH have been found to be elevated in the brain tissue and serum, respectively, of PD patients and have been shown in laboratory studies to damage the nigrostriatal dopamine system (Corrigan *et al.*, 1996; Corrigan *et al.*, 2000; Kitazawa *et al.*, 2001; Kitazawa *et al.*, 2004; Hatcher *et al.*, 2007; Richardson *et al.*, 2009; Richardson *et al.*, 2011). In addition to these compounds, a recent epidemiological study also identified exposure to the organochlorine insecticide, endosulfan, as a risk factor for PD (Rhodes *et al.*, 2013). Like other organochlorines, endosulfan is extremely resistant to degradation and breakdown, thus allowing for repeated or continual exposure to the human population. As a result, endosulfan has been demonstrated to accumulate in significant levels in human tissue, including fat, liver, kidney, and brain. In addition, high levels of endosulfan have been recorded in the cord blood and breast milk of pregnant women (Moreno Frias *et al.*, 2004; Jimenez Torres *et al.*, 2006). This raises particular concern for the exposure of the developing fetus to endosulfan, both during gestation as well as postnatally, and the effect that this exposure may have on development of the nervous system.

Although PD is considered an age-related neurodegenerative disease, with the major clinical manifestations occurring in the 6th decade of life, alteration to the biological processes that contribute to these deficits may begin much earlier in life, suggesting a possible role for an early life exposure that leaves the nigrostriatal dopamine system vulnerable to future insults (Martyn and Osmond, 1995; Cory-Slechta *et al.*, 2005a; Cory-Slechta *et al.*, 2005b). Rats exposed to a single dose of the inflammatory bacteriotoxin lipopolysaccharide during gestation elicited damage to the dopamine system in offspring that was potentiated following subsequent adult exposure to 6-OHDA or rotenone (Ling *et al.*, 2004a; Ling *et al.*, 2004b). Similarly, prenatal exposure to the fungicide, maneb, increased the susceptibility of dopamine neurons to damage following exposure to the herbicide paraquat in adulthood (Barlow *et al.*, 2007). These findings suggest that exposure to environmental compounds during critical periods of neurodevelopment may explicitly damage dopamine neurons or increase the vulnerability of these neurons to future exposures to neurotoxic compounds.

Previous work has shown exposure to the organochlorine insecticides heptachlor and dieldrin cause significant alterations to the nigrostriatal dopamine system of offspring exposed throughout gestation and lactation (Caudle *et al.*, 2005; Richardson *et al.*, 2006; Richardson *et al.*, 2008). This damage is further exacerbated following a subsequent exposure to the dopaminergic neurotoxin, MPTP. Given these findings and the contribution of endosulfan exposure to PD risk, we hypothesized that perinatal exposure to endosulfan would damage the nigrostriatal dopamine system in offspring and make these neurons more vulnerable to future exposure to MPTP.

Materials and Methods

Chemicals and Reagents

α -Endosulfan was purchased from Accustandard (New Haven, CT). Hibernate A and Hibernate A- Calcium were purchased from BrainBits (Springfield, IL). B27, DNase1, and Neurobasal A were purchased from Life Technologies (Carlsbad, CA). Papain was obtained from Sigma (St. Louis, MO). Dispase II was purchased from Roche (Nutley, NJ). The BCA protein assay kit was obtained from Pierce (Rockford, IL). Aphidicolin was purchased from A.G. Scientific (San Diego, CA). Monoclonal anti-rat dopamine transporter and polyclonal anti-rabbit tyrosine hydroxylase were purchased from EMD Millipore (Billerica, MA). Monoclonal mouse-anti tubulin antibody was purchased from Sigma (St. Louis, MO). Mouse anti-GABA_A 2 α receptor subunit was purchased from Synaptic Systems (Germany) and mouse anti-MAP2 antibody was purchased from Abcam (San Francisco, CA). Secondary antibodies conjugated to fluorescent tags were obtained from Life Technologies (Grand Island, NY). SuperSignal West Dura Extended duration substrate and stripping buffer were obtained from Pierce.

Culturing and Treatment of SK-N-SH Cells

Cells were cultured in DMEM F12 media supplemented with 100 units/mL penicillin, 100 units/mL streptomycin and 10% fetal bovine serum. Cells were cultured at 37°C in a humidified atmosphere with 5% CO₂ and propagated according to the protocol provided by the supplier. When cells were confluent they were passaged to working concentrations in the appropriate culture plate for treatment with endosulfan. Cell death was assessed using the WST-1 Cell Proliferation assay. Following treatment for 72h with 100, 200, 300, 400 μ M of endosulfan dissolved in DMSO, 10 μ L/well of Cell Proliferation Reagent WST-1 was added to cells and incubated for 3h at 37°C and 5% CO₂. Cytotoxicity was then measured by enzymatic cleavage of the tetrazolium salt WST-1 to a water-soluble formazan dye detected by spectral absorbance. Viable cells form more formazan than less viable cells. Spectral absorbance was measured at 450 nm on an Epoch BioTek microplate spectrophotometer and analyzed using Gen5 software (2.0) and GraphPad software.

Primary Culture of Mesencephalic Neurons

Ventral mesencephalic neuron cultures were generated as previously described (Bradner *et al.*, 2013). Briefly, ventral mesencephalic neuron cultures were prepared from postnatal mice (postnatal day 1–3). Mouse brains were dissected in ice cold Hibernate A supplemented with B27. Following isolation of the relevant region and the removal of

meninges, tissue pieces were chemically treated with a dissociation solution containing Papain (1 mg/ml), Dispase II (1.2 units/ml), and DNase 1 (1 µl/ml) dissolved in Hibernate A- Calcium for 20 min at 37°C and gently agitated every 5 min. Tissue was then rinsed in plating media containing Neurobasal-A, 10% heat inactivated fetal bovine serum, pen-strep, and mechanically dissociated using gentle trituration. Cells were plated on poly-d-lysine pre-coated 96 well plates at 40,000 cells per well. Plating media was removed and immediately switched to Neurobasal-A based culture media containing B27, 1% L-glutamine and 1% penicillin-streptomycin after 2h, *in vitro*. The following day, culture media containing aphidicolin (1 µg/ml) was added to reduce the proliferation of glial cells in culture. Approximately one half of the culture media from each well was replaced every 4 days. Primary cultures were treated on day 8 *in vitro* with five concentrations of endosulfan (0, 15, 20, 25, 30 µM) dissolved in DMSO and then diluted to working concentrations in cell culture media. For all control and endosulfan treatment experiments the final concentration of DMSO was <0.01% and no toxicity was observed at this percentage. After 24h, cells were fixed in 4% PFA for 20 min and incubated overnight in rabbit anti-TH and mouse anti-MAP2 at 4°C. The following day, cultures were incubated with fluorescent secondary antibodies, goat antirabbit 488 and goat anti-mouse 572 for 1h at room temperature. After staining with DAPI, cells were rinsed and stored in PBS. Images of treated cultures were obtained using an Array Scan VTI HCS (Cellomics; Pittsburgh, PA). Forty-nine contiguous fields were taken per well and TH+ neurons were identified and measured using the neuronal profiling bioapplication from Thermo Scientific. Statistical significance between the control and treatment groups for neuron count was determined using GraphPad analysis software.

Animals and Treatment

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

Female mice were randomly assigned to treatment groups and were orally dosed with 0 or 1 mg/kg endosulfan dissolved in corn oil vehicle and mixed with peanut butter every 2 days for 2 weeks prior to introducing male mice for breeding. Control mice received an equivalent amount of corn oil vehicle in peanut butter. Mice were monitored to ensure total consumption of the treatment dose, which generally occurred within 10 min. Oral exposure was chosen since the most likely route of exposure to endosulfan in the current human population is through ingestion of contaminated food. The chosen dosage is 7.36-fold less than the acute oral LD₅₀ in mice NOAEL (Smith, 1991). Peanut butter was chosen as the method of exposure to reduce stress to the dam during gestation. Stress from repeated injections via oral gavage during gestation has been shown to alter GABA_A subunit development (Liu *et al.*, 1997). Dosing continued on the same schedule throughout gestation and lactation and dams were allowed to give birth and litters were culled to 6–8 pups/litter on PND 1, to ensure standardized nutritional availability. On PND 21 pups were separated

by litter and by sex and received standard care, including maintenance on a 12:12 light/dark cycle with food and water available *ad libitum* until approximately 12 weeks of age male mice were given two subcutaneous injections of saline or 10 mg/kg of MPTP 12h apart. Male mice were chosen as the focus of these experiments as PD appears to be more prevalent in men than women. Animals were sacrificed by live decapitation one week following the second MPTP injection and tissue from the striatum was collected for analysis. Each litter was considered as an individual unit of analysis (N=6–8).

To assess the effect of adult exposure to endosulfan, twenty, eight-week-old male C57BL/6J mice were purchased from Charles River Laboratories (Wilmington, MA). Mice were housed in a 12:12 light/dark colony with food and water available *ad libitum*. Mice were orally gavaged with either 0 (control) or 1 mg/kg of endosulfan dissolved in corn oil vehicle daily for 30 days. This concentration did not elicit any overt health effects including weight loss, convulsions or death. One day following the 30-day dosing regimen, mice were given two subcutaneous injections of saline or 10 mg/kg of MPTP 12h apart. Animals were sacrificed one week following the second MPTP injection and tissue from the striatum was collected for analysis.

Immunohistochemistry in Mouse Midbrain

To demonstrate the colocalization of the GABA_A 2 α receptor subunit with dopaminergic neurons located in the substantia nigra pars compacta of the midbrain, untreated male mice were processed as described previously (Caudle *et al.*, 2007). Briefly, whole brains were immersion fixed in 4% paraformaldehyde and serially sectioned at 40 μ m. Sections were incubated with mouse anti-GABA_A 2 α receptor subunit, and rabbit anti-TH overnight and then incubated in fluorescently conjugated secondary antibodies against mouse and rabbit IgG, raised in goats for 1h at room temperature. Images were captured at 20 $\times$ magnification using a Zeiss Axio Imager M2 microscope with fluorescent capabilities and merged using Image J software available from NIH.

Western Blot Analysis

Western blots were used to quantify the amount of dopamine transporter (DAT), tyrosine hydroxylase (TH), GABA transporter 1 (GAT1), GABA decarboxylase 67 (GAD67), vesicular glutamate transporter (vGlut), and α -tubulin present in samples of striatal tissue from treated and control mice. Analysis was performed as previously described (Caudle *et al.*, 2006; Caudle *et al.*, 2007). Briefly, striata samples were homogenized and samples subjected to polyacrylamide gel electrophoresis and electrophoretically transferred to polyvinylidene difluoride membranes. Nonspecific sites were blocked in 7.5% nonfat dry milk in Tris-buffered saline and then membranes incubated overnight in a monoclonal antibody to the N-terminus of DAT at 4°C. DAT antibody binding was detected using a goat anti-rat horseradish peroxidase secondary antibody (1:10,000) and enhanced chemiluminescence. The luminescence signal was captured on an Alpha Innotech Fluorochem imaging system and stored as a digital image. Densitometric analysis was performed and calibrated to coblotting dilutional standards of pooled striata from all control samples. Membranes were stripped for 15 min at room temperature with Pierce Stripping

Buffer and sequentially reprobed with α - tubulin (1:1,000) and TH (1:1,000) antibodies. α -Tubulin blots were used to ensure equal protein loading across samples.

Statistical Analysis

All analysis was performed on raw data for each treatment group by one-way or twoway ANOVA. Post hoc analysis was performed using Tukey's post hoc test. Significance is reported at the $p < 0.05$ level.

Results

Effects of endosulfan on a dopaminergic neuroblastoma cell line, SK-N-SH

Our initial investigation into the neurotoxic effects of endosulfan utilized the SK-N-SH neuroblastoma cell line, which has been shown to exhibit dopaminergic properties, which made it a useful model to further evaluate the general neurotoxic effects of endosulfan on dopamine neurons (Richards and Sadee, 1986; Kidd and Schneider, 2010). Cells were exposed to increasing concentrations of endosulfan for 72 hrs, which elicited a dose-dependent reduction in cell viability ranging from a 45% loss with 100 μ M to an 80% loss following treatment with 400 μ M of endosulfan (Figure 1). These data demonstrate the neurotoxic effects of endosulfan on a dopaminergic cell line and helped to further inform our additional evaluation of endosulfan in a more complex *in vitro* model of dopaminergic neurons.

Neurotoxicity of endosulfan on primary cultured dopamine neurons

In order to gain a better understanding of the effects endosulfan has on dopaminergic neurons we isolated neurons from the ventral mesencephalon, a region enriched in dopamine neurons shown to degenerate in PD (Fahn, 2003). Using tyrosine hydroxylase (TH), a key enzyme involved in the synthesis of dopamine, as our marker of dopamine neurons we found that an initial treatment with 15 μ M endosulfan did not cause any change in the viability of dopaminergic neurons in our cultures. However, exposure to increasing concentrations of endosulfan resulted in significant reductions in the number of TH+ neurons in our model (Figure 2). Not surprisingly, when compared, dopaminergic neurons isolated from mouse brain were significantly more sensitive to the neurotoxic effects of endosulfan than SK-N-SH cells, supporting the utility of a more elaborate *in vitro* approach to assess the impact of endosulfan on dopaminergic neurons.

Effects of perinatal endosulfan exposure on dopaminergic neurons in male offspring

As the impact of *in utero* exposure to neurotoxic compounds has been shown to damage dopamine neurons, we generated an *in vivo* developmental exposure model in order to evaluate the effects of endosulfan on the developing nigrostriatal dopamine system. To achieve this model, pregnant mice were treated with endosulfan throughout gestation and lactation and male offspring were evaluated at 3 months of age for alterations to specific proteins involved in normal functioning of the dopamine system. Dams treated with endosulfan or control did not display any overt signs of toxicity and showed no adverse effects on pup growth rate or other general health endpoints (data not shown). Additionally, treatment with endosulfan did not influence sex ratios of the litters or numbers of pups per

litter (data not shown). Previous data has shown that endosulfan elicits its neurotoxicity through potent binding to and blockade of GABA_A receptors (Casida, 1993). As we were interested in the effects of endosulfan on dopamine neurons in the nigrostriatal dopamine system, we first confirmed that dopaminergic neurons expressed relevant levels of these receptors. Coupling immunofluorescent dual-labeling of TH⁺ neurons that represent dopaminergic neurons in the SNpc with the GABA_A 2 α receptor subunit showed an extensive colocalization of both proteins within the SNpc (Figure 3). Specificity of our labeling can be further seen by the defined edge that delineates the border of the ventral SNpc from the substantia nigra pars reticulata, which is predominated by GABAergic neurons and devoid of TH⁺ neurons.

Further assessment of the nigrostriatal dopamine circuit focused on the striatum and evaluation of specific dopaminergic proteins known to be altered in PD. Developmental exposure to 1 mg/kg/day of endosulfan elicited a 38% reduction in the expression of the DAT in the striatum, compared with control offspring. A similar 25% decrease was also seen in striatal TH expression compared with control treated offspring (Figure 4). In a separate cohort of mice we next investigated whether exposure to endosulfan would increase the vulnerability of dopamine neurons to additional chemical insults. To test this hypothesis we treated control and endosulfan exposed animals with the dopaminergic neurotoxin, MPTP, and assessed alterations to the expression of DAT and TH in the striatum. Interestingly, exposure to a mild intoxication with MPTP elicited an exacerbation of reductions to each of these proteins. As seen in Figure 5, treatment with MPTP caused a well-established 60% reduction of DAT in control mice that showed an even greater decrease when endosulfan treated mice received MPTP. A similar 47% decrease in TH was seen in control mice treated with MPTP. This reduction was elaborated in endosulfan treated mice, which showed a 60% reduction of striatal TH following exposure to MPTP. Interestingly, these reductions in DAT and TH were not accompanied by loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc) as determined by unbiased stereological cell counts (data not shown). Furthermore, these alterations appeared to be selective for dopamine neurons in the striatum as no changes in expression of specific GABAergic or glutamatergic proteins was observed (Figure 6). These results demonstrate the effects of developmental exposure to endosulfan to sensitize the nigrostriatal dopamine neurons to future insults that impact the viability of these neurons.

Effects of endosulfan exposure on dopaminergic neurons in adult mice

Finally, we were interested if the alterations to the nigrostriatal dopamine system were effects of developmental exposure and possible disruption of the dopamine neurons during critical periods of development. Thus, to investigate this hypothesis we exposed endosulfan-naïve adult male mice to 1 mg/kg/day of endosulfan for 30 days and evaluated alterations to the nigrostriatal dopamine system in a similar manner to the male offspring who were exposed to endosulfan during development. In contrast to the developmentally exposed mice, adult mice treated with endosulfan did not demonstrate any alterations to the expression of DAT and TH. Although we did not observe any alterations to these proteins, we hypothesized that these neurons may still harbor alterations, however undetectable with our methods that could render them vulnerable to an additional insult. Using the same

exposure paradigm as in the developmentally exposed mice, we treated control and endosulfan exposed adult mice with MPTP and performed an additional assessment of DAT and TH in the striatum. As expected, MPTP elicited a well-defined reduction in both DAT and TH in the control treated mice. However, treatment of the endosulfan-exposed mice with MPTP did not cause an exacerbated reduction in DAT or TH, as previously seen in the developmentally exposed mice (Figure 7). Similar to the developmentally exposed mice, no changes in GABAergic or glutamatergic proteins in the striatum were observed (Figure 8). These findings suggest that exposure to endosulfan during critical periods of development of the nigrostriatal dopamine system can have significant repercussions for the general development of key dopaminergic proteins in this circuit as well as leaving them vulnerable to additional damage when challenged.

Discussion

Exposure to pesticides has repeatedly been identified as a major contributor to the risk of PD development (Hatcher *et al.*, 2008). Increasingly, the impact of exposure to pesticides during critical periods of neurodevelopment could be an integral factor in damage to the nigrostriatal dopamine system that increases the vulnerability of these neurons to additional chemical or traumatic insults over time (Cory-Slechta *et al.*, 2005a; Cory-Slechta *et al.*, 2005b). Our study focused on addressing these specific issues by evaluating the neurotoxic effects of the organochlorine insecticide, endosulfan on dopamine neurons using *in vitro* approaches in addition to exposure during perinatal and adult time periods. Our findings demonstrate that endosulfan is selectively neurotoxic to dopamine neurons in the striatum, especially following exposure during development, when compared with a similar exposure during adulthood. These findings suggest that dopamine neurons are uniquely susceptible to damage by endosulfan during key periods of neurodevelopment and this exposure could explicitly contribute to PD pathogenesis as well as facilitate additional dopaminergic pathology by future insults.

As this investigation was focused on evaluating the neurotoxic effects of endosulfan on the nigrostriatal dopamine system our initial assessment found exposure of the dopaminergic neuroblastoma cell line, SK-N-SH to be vulnerable to endosulfan. Our findings are in agreement with a previous study that utilized the SH-SY5Y neuroblastoma cell line and exposed these cells to endosulfan for 16 hours (Jia and Misra, 2007). Our findings were then elaborated to a primary culture model of the nigrostriatal dopamine system in mice, which demonstrated a significant loss of dopamine neurons in the SNpc following treatment with endosulfan. Although primary cultured neurons have been used previously to evaluate the GABAergic effects of endosulfan (Pomes *et al.*, 1994; Huang and Casida, 1996; Rosa *et al.*, 1996; Vale *et al.*, 2003), these are the first data to utilize this experimental platform to assess the vulnerability of specific neuronal populations to endosulfan.

The most critical findings from our investigation demonstrated gestational and lactational exposure to endosulfan elicited significant reductions in dopaminergic proteins previously shown to be damaged in PD. In our study, developmental exposure to endosulfan caused substantial losses in expression of the striatal DAT and TH in male offspring. These findings are extremely interesting considering the pivotal contribution GABA and the GABA_A

receptors provide in mediating various aspects of neurogenesis, neuronal migration and synaptic formation, especially development of dopamine neurons and dopaminergic circuitry (Lauder *et al.*, 1998; Okada *et al.*, 2004; Represa and Ben-Ari, 2005). The importance of GABAergic signaling in the monoamine neurotransmitter system has previously been demonstrated as antagonism of the GABA_A receptors disrupts monoaminergic neuron formation in the brainstem (Liu *et al.*, 1997).

The alterations to the nigrostriatal dopamine system seen in our study coupled with the known trophic effects of GABAergic signaling in the developing nervous system is especially interesting when compared with previous work that demonstrated an increase in DAT, TH, and VMAT2 expression in the striatum following perinatal exposure to other organochlorine insecticides, dieldrin and heptachlor (Caudle *et al.*, 2005; Richardson *et al.*, 2006; Richardson *et al.*, 2008). The difference in response of the developing nigrostriatal dopamine system among these compounds is not clear, as they are all members of the cyclodiene insecticide family and exert their neurotoxicity by targeting the central nervous system and inhibiting the GABA_A receptor-mediated chloride flux (Casida, 1993). However, one possibility for the differential effect on the development of the dopamine system following exposure to different organochlorines could lie in the way in which each compound specifically interacts with the GABA_A receptor. While endosulfan has been shown to interact specifically with the $\beta 3$ subunit of the GABA_A receptor, dieldrin preferentially exerts its effects through interaction with the $\gamma 2$ receptor subunit (Ratra and Casida, 2001; Ratra *et al.*, 2001). Although both are extensively expressed on dopamine neurons in the SNpc (Okada *et al.*, 2004), their binding and relative potencies at the GABA_A receptor could have differential effects on GABAergic signaling in the dopamine circuit.

Developmental exposure to endosulfan also appeared to increase the vulnerability of the nigrostriatal dopamine neurons to additional toxic insults, such as MPTP. As seen in our study, exposure to MPTP resulted in significantly greater reductions in DAT and TH in the endosulfan treated offspring. While the precise mechanisms that underlie this increased vulnerability remain to be clarified, it is possible that the alterations in DAT and TH in the endosulfan-treated animals significantly compromised the integrity of the dopaminergic neurons and enhances their susceptibility to further insults that specifically target the dopamine neuron. Similar increases in toxicity following a subsequent challenge have been previously observed using other animal models of developmental exposure to PD-relevant toxicants. Indeed, exposure to the fungicide, maneb, during critical periods of neurodevelopment followed by exposure to the herbicide, paraquat in adulthood elicited a significantly exaggerated alteration in the nigrostriatal dopamine system (Barlow *et al.*, 2007).

Finally, these findings motivated our research to tease out the importance of developmental exposure to endosulfan by investigating the potential for endosulfan to elicit similar disruption to the nigrostriatal dopamine system in adult male mice that had not been exposed during development. In contrast to our developmentally exposed animals, these mice did not demonstrate an overt disruption to the nigrostriatal dopamine system. Furthermore, these neurons did not appear to be more susceptible to treatment with MPTP. These data demonstrate that the time frame of exposure to endosulfan plays a critical role in the

ultimate damage and vulnerability of the nigrostriatal dopamine system. Again, this susceptibility could be directly attributed to alterations during critical periods of developmental of the dopamine circuit as a result of disruption to the normal GABAergic signaling by endosulfan. Indeed, as this neurodevelopmental process has been extensively curtailed in the adult mouse brain, the issue of altering the expression and function of key dopaminergic proteins in the striatum through attenuated GABA signaling may not significantly contribute to dopaminergic damage.

In sum, our findings provide data that demonstrates the neurotoxic potential of endosulfan exposure on the nigrostriatal dopamine system. These data suggest that exposure to endosulfan during defined periods of neurodevelopment cause substantial alterations to dopamine neurons that sensitizes these cells to further insults. Moreover, this work further supports the contribution of developmental exposure to organochlorine insecticides in PD, and emphasizes the importance of developmental neurotoxicity testing in evaluating the role of developmental exposure to neurotoxic compounds.

Acknowledgments

This work was supported by National Institutes of Health grants [R00ES017477 and P01ES016731 to W.M.C.]

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Highlights

- Pesticide exposure during neurodevelopment contributes to Parkinson's disease.
- Developmental exposure to endosulfan disrupts the nigrostriatal dopamine system.
- Endosulfan causes vulnerability of dopamine neurons to further insults.
- Adult exposure does not affect the nigrostriatal dopamine system.
- Developmental exposure to toxicants may underlie future neurological deficits.

Figure 1.

Treatment with endosulfan reduces cell viability in SK-N-SH dopaminergic neuroblastoma cells. SK-N-SH cells were grown to confluency and treated with increasing concentrations of endosulfan for 72 hrs. Alterations in cell viability were then evaluated using the WST-1 cell proliferation assay. A significant reduction in cell viability was observed at each concentration of endosulfan. Columns represent percent change from DMSO control. Data represent the mean $\pm$ SEM of 12 experimental replicates per treatment group performed over 3 separate experiments. ***Values significantly different from control ($p < 0.001$).

Figure 2.

Exposure of ventral mesencephalic dopamine neurons to endosulfan caused a reduction in the number of TH+ neurons. Treatment of mesencephalic cultures with endosulfan caused significant reduction in TH+ neurons beginning with 20 μ M. The number of TH+ neurons was further reduced with treatment of 25 and 30 μ M of endosulfan. Images below represent TH+ neurons following exposure to DMSO or 30 μ M endosulfan for 24hrs. Green = TH+ neurons. Blue = DAPI nuclear stain. Columns represent the percent change from DMSO. Data represent the mean $\pm$ SEM of 4 experimental replicates per treatment group performed

across 3 separate experiments. ***Values significantly different from DMSO control ($p < 0.001$).

Figure 3.

GABA_A receptors colocalize with dopamine neurons in the substantia nigra pars compacta. The expression of GABA_A receptors on dopaminergic neurons in the SNpc was evaluated using immunofluorescence. Neurons from control animals were stained for the GABA_A 2α receptor subunit as well as TH, showing a clear colocalization of these proteins in the SNpc. A lack of overlap between these two proteins can be seen in the substantia nigra pars reticulata, which is enriched in GABAergic neurons but not dopaminergic neurons. Green = TH. Red = GABA_A 2α receptor subunit.

Figure 4.

Developmental endosulfan exposure caused reductions in the DAT and TH in the striatum of male offspring. Female mice were administered 0 (control) or 1 mg/kg endosulfan throughout gestation and lactation and striatal DAT (A and C) and TH (B and C) protein expression was evaluated by immunoblot in male offspring at 16 weeks of age. Data represent mean $\pm$ SEM (6–8 animals each from a different litter per treatment group). *Values for animals that are significantly different from controls ($p < 0.05$). **Values for animals that are significantly different from controls ($p < 0.01$).

A.

B.

Figure 5.

Developmental exposure to endosulfan increases the vulnerability of dopamine neurons to MPTP. Female mice were administered 0 (control) or 1 mg/kg endosulfan throughout gestation and lactation. At 16 weeks of age, male mice were administered 2×10 mg/kg MPTP and evaluated 7 days later for alterations to the nigrostriatal dopamine system. (A) Treatment with MPTP caused a significant reduction in striatal DAT expression that was further exacerbated in the endosulfan exposure group. (B) A similar reduction of striatal TH was also seen. Data represent mean $\pm$ SEM (6–8 animals each from a different litter per treatment group). ***Values significantly different from control or endosulfan only group ($p < 0.001$). \$Values significantly different from endosulfan group ($p < 0.001$). #Values significantly different from control + MPTP group ($p < 0.05$).

Figure 6.

Developmental exposure to endosulfan and challenge with MPTP does not affect the expression of GABAergic or glutamatergic markers in the striatum. Female mice were administered 0 (control) or 1 mg/kg endosulfan throughout gestation and lactation. At 16 weeks of age, male mice were administered 2×10 mg/kg MPTP and evaluated 7 days later for alterations to the striatum. (A) Exposure to endosulfan and MPTP did not cause alterations in the expression of striatal GAT1. (B) Exposure to endosulfan and MPTP did not cause alterations in the expression of striatal GAD67. (C) Exposure to endosulfan and MPTP did not cause alterations in the expression of striatal vGlut. Data represent mean $\pm$ SEM of 6 animals.

A.

B.

Figure 7.

Adult exposure to endosulfan does not alter expression of DAT and TH in the striatum. Two month old male mice were exposed to 0 (control) or 1 mg/kg/day of endosulfan for 30 days. A subset of mice were administered 2×10 mg/kg MPTP and evaluated 7 days later for alterations to the nigrostriatal dopamine system. (A) Exposure to endosulfan did not cause reduction in striatal DAT. Treatment with MPTP significantly reduced expression of DAT in the striatum that were not dependent upon exposure to endosulfan. (B) Exposure to endosulfan did not cause reduction in striatal DAT. Treatment with MPTP significantly reduced expression of TH in the striatum that were not dependent upon exposure to endosulfan. Data represent mean $\pm$ SEM of 6 animals. **Values for animals that are significantly different from respective control or endosulfan only group ($p < 0.01$). ***Values significantly different from respective control or endosulfan only group ($p < 0.001$).

Figure 8.

Adult exposure to endosulfan does not alter expression of GABAergic or glutamatergic proteins in the striatum. Two month old male mice were exposed to 0 (control) or 1 mg/kg/day of endosulfan for 30 days. A subset of mice were administered 2×10 mg/kg MPTP and evaluated 7 days later for alterations to the striatum. (A) Exposure to endosulfan and MPTP did not cause alterations in the expression of striatal GAT1. (B) Exposure to endosulfan and MPTP did not cause alterations in the expression of striatal GAD67. (C) Exposure to endosulfan and MPTP did not cause alterations in the expression of striatal vGlut. Data represent mean $\pm$ SEM of 6 animals.