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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.

1. 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 nigtrostriatal dopamine system (Corrigan et al., 1996, 2000; Kitazawa et al., 2001, 2004; Hatcher et al., 2007; Richardson et al., 2009, 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

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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, 2005b). Rats exposed to a single dose of the inflammatory bacteriotoxin lipopolysaccaride 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, 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, 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.

2. Materials and methods

2.1. 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-$GABA_A$ 2α 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.

2.2. 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% CO_2 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 72 h with 100, 200, 300, 400 μM of endosulfan dissolved in DMSO, 10 μl/well of Cell Proliferation Reagent WST-1 was added to cells and incubated for 3 h at 37 °C and 5% CO_2. 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.

2.3. 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 days 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 2 h, *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 24 h, 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 anti-rabbit 488 and goat anti-mouse 572 for 1 h 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.

2.4. 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