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William Wyatt Wilson Date

Pesticides and Parkinson's disease: Attributable Risk of Occupational Exposure and Neurochemical Analysis of Sub-Chronic Environmental Exposure

By

William Wyatt Wilson

Master of Science in Public Health

Environmental Health and Epidemiology

_____

W. Michael Caudle, PhD
Committee Chair

_____

Kyle Steenland, PhD
Committee Member

_____

Paige Tolbert, PhD
Committee Member

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William Wyatt Wilson

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Thesis Committee Chair: W. Michael Caudle, PhD

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Abstract

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By William Wyatt Wilson

Background: The causes of Parkinson's disease (PD) are not well understood. In the past 30 years, epidemiological studies have emerged associating occupational and environmental pesticide exposure with PD. However, exposure misclassification and a lack of understanding the underlying biological mechanisms in pesticide exposure, particularly lipophilic pesticides that are potential toxins to the developing nervous system, obscure a reliable association with PD.

Objective: This study is designed to assess the epidemiologic factors in determining the attributable risk percent of PD from occupational exposure to pesticides as well as to determine the neurochemical mechanism underlying developmental exposure to the recently banned insecticide, endosulfan.

Methods: Using US Census data from 1990 and 2011 as well as a Job Exposure Matrix, occupational exposure to pesticides was generated. Risk-ratios from two meta-analyses were then used to calculate a range of attributable risk percentages (AR) for PD. Next, endosulfan toxicity was assessed in the SK-N-SH dopaminergic cell line and in primary culture neurons from the ventral mesencephalon. Finally, the impact of endosulfan was evaluated using mice developmentally exposed to endosulfan during gestation and lactation. Animals were challenged with MPTP to evaluate toxicity on the dopaminergic system in the striatum. Immunoblotting was performed to determine the effect of endosulfan on various neuronal proteins.

Results: Using never vs. ever occupationally exposed to pesticides, we found that the AF ranged from 6.3 – 13.41% depending on the mRR and Census data used. High vs. low occupational exposure yielded an AF of 3%. Endosulfan was toxic to SK-N-SH cells and primary cultured neurons elicited markers of oxidative stress. Developmental exposure to endosulfan did not cause significant modulation of dopaminergic proteins in the striatum. However, several cortical proteins were significantly altered following endosulfan exposure.

Discussion: While the range of the attributable risk fraction for PD varies, it underscores the uncertainty in assessing occupational exposure to pesticides. Furthermore, assessment of developmental exposure to endosulfan indicates that environmental exposure disrupts processes integral to the neural transmission in the cortex. Future studies should continue to research the effects induced by endosulfan on the brain and their association with neurological diseases such as schizophrenia.

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INTRODUCTION

Parkinson's disease: background, pathology and general risk factors

Parkinson's disease (PD) is a progressive and chronic neurodegenerative disorder that affects the central nervous system. PD primarily belongs to a group of diseases called motor system disorders, which are the result of dopaminergic cell loss in the brain (NINDS, 2013). Patients with PD also exhibit several non-motor symptoms including sensory deficits and cognitive difficulties (Barnett-Cowan *et al.*, 2010). The disease was first clinically characterized in 1817 by Dr. James Parkinson in *Essay on the Shaking Palsy* where he described patients with abnormal posture, gait, resting tremor and diminished muscle strength. Since then, the clinical presentation of PD has evolved to comprise four characteristics: tremor (involuntary movement), rigidity (velocity-independent muscle stiffness), bradykinesia (slowed movement) and impaired imbalance (Dauer and Przedborski, 2003). These motor defects are collectively referred to as Parkinsonism.

Currently, there are no blood or laboratory tests available to diagnose sporadic development of PD (NINDS, 2013). Pathologically, however, PD is characterized by the loss of dopamine-containing neurons in the substantia nigra *pars compacta* (SNpc), concomitant loss of dopamine input in the striatum, and depigmentation of the locus ceruleus and autonomic dysfunction (Hatcher *et al.*, 2008). Diagnosis can occur post mortem by the presence of Lewy bodies – dense inclusions of the protein α-synuclein bound to ubiquitin – inside of dopaminergic neurons of the patient (Baba *et al.*, 1998; Davie, 2008). It has become recently accepted that PD pathology is staged by the distribution Lewy bodies and the deposition of α-synuclein, beginning in the olfactory

bulb and lower brain stem and eventually spreading to cortical regions (Dexter and Jenner, 2013). Despite this expansive process, the underlying mechanism causing propagation is not known despite some hypothesizing that adjacent unaffected neurons could act as a "seed" in a prion-like perpetuation of α-synuclein misfolding (Dunning *et al.*, 2012).

Additional processes, particularly related to the loss of striatal dopamine, have also been researched. Deregulation of dopamine handling has been investigated through altered concentrations of dopamine transporter (DAT) and vesicular monoamine transporter 2 (VMAT2) since such protein irregularities are characteristic of brain regions in patients with PD (Miller *et al.*, 1999a; Richardson *et al.*, 2006). Oxidative stress on the dopaminergic system following autoxidation of dopamine has also provided a mechanistic understanding of PD development. The occurrence of oxidative stress is supported by both postmortem studies as well as studies that demonstrate the capacity of oxidative stress and oxidizing toxins to induce nigral cell degeneration (Jenner, 2003). While increased lipid/protein oxidation and enhanced cellular concentrations of the glutathione redox antioxidant system (GSH/GSSG) in the SNc have indicated that oxidative stress is a contributing factor to PD development, the exact origin and degree of etiologic contribution to PD remains a contested subject (Ambani *et al.*, 1975; Dexter and Jenner, 2013; Go *et al.*, 2007).

Globally, PD is the most common neurodegenerative movement disorder, affecting greater than 1% of individuals over 60 years of age (de Lau and Breteler, 2006). In the United States alone, the mean prevalence of PD in the population over the age of 65 is 1.6%, but is expected to increase as populations age (Wright Willis *et al.*, 2010).

Reported global standardized incidence rates of PD are between 8-18 per 100,000 person-years (de Lau and Breteler, 2006).

PD is epidemiologically linked with several risk factors. As with most neurodegenerative disorders, age is one of the clear characteristics that increases risk of PD (Kieburtz and Wunderle, 2013). While Parkinson's disease affects approximately 1% of individuals above the age of 60, it affects nearly 4% of individuals above the age of 80 (de Lau and Breteler, 2006). The mean prevalence of PD in 2005 for age groups 65-69, 70-74, 75-79 and 80-84 were 553.52, 1,093.74, 1,881.39, and 2,757.02 per 100,000 Medicare beneficiaries (Wright Willis, Evanoff, Lian, Criswell and Racette, 2010). A 2003 study of the Kaiser Permanente Medical Care Program of Northern California estimated the incidence of newly diagnosed Parkinson's disease by age. They reported a clear age dependency with rising incidence rates over the age of 60 years, with only 4% of cases being under the age of 50 years. Maximal incidence rates for men and women combined were highest in the age group of 80-89 years (IRR=119.0 per 100,000) compared to other decades (Van Den Eeden *et al.*, 2003).

Sex is also a strong risk factor with men having higher incidence rates of PD than women. A meta-analysis performed by Wooten et al. reported an average male to female incidence rate ratio of 1.49 (Wooten *et al.*, 2004). While there have been discrepancies targeting the heterogeneity of this ratio (Taylor *et al.*, 2007), the most rational interpretations focus on the potential protective characteristics of estrogen (Fleming *et al.*, 1994) as well as lifestyle differences between men and women that would preferentially predispose males to other putative risk factors for PD (Baldereschi *et al.*, 2003). Socioeconomic status (SES) as a risk factor for PD has also been investigated on

the basis that groups with lower socioeconomic status often have higher rates of non-communicable, chronic conditions. Area-level census data on average household income and residential postal codes from hospital abstracts in the Manitoba Centre for Health Policy and Research Data Repository were collected in a population-based case-control study. Physician-diagnosed PD cases were tied through their electronic records. In urban regions, the lowest income quintile (370.2 PD patients per 1000,000 individuals) had a significantly greater prevalence when compared to the highest quintile (251.7 PD patients per 100,000) (Lix *et al.*, 2010).

Consumer behaviors have also been documented with differential risk for PD. Multiple studies have reported that exposure to tobacco reduces the risk of PD. A meta-analysis of 44 case-control studies and 4 cohort studies reported a pooled risk-ratio of 0.59 (95% CI: 0.54-0.63) for dichotomous exposure of ever smokers to never smokers. This finding maintained its preventative association when never smokers were compared to past smokers (RR=.80; 95% CI: 0.69-0.93) and current smokers (RR=0.39; 95% CI: 0.32-0.47) (Hernan *et al.*, 2002). To bolster their findings, most of the studies reported a dosage effect through an assessment of pack-years that showed persons with higher pack-years conferred less risk than persons with lower pack-years. A more recent case-control study found that patients who smoked were more likely to not have Parkinson's disease than those who did not smoke (OR=0.6; 95% CI: 0.4-0.9) after matching cases by age, sex and region of residency (Galanaud *et al.*, 2005). The biological basis for tobacco's protective effect may be related the dopaminergic pathway as nicotine may stimulate dopamine release, act as an antioxidant or alter the activity of monoamine oxidase B (de Lau and Breteler, 2006). Nevertheless, a causal mechanism has yet to be determined.

Caffeine consumption also appears to reduce the risk of PD. The meta-analysis performed by Hernan et al. also reported a pooled risk ratio (RR) from 8 case-control studies and 5 cohort studies of 0.69 (95% CI: 0.59-0.80) for coffee drinkers compared to non-coffee drinkers (Hernan, Takkouche, Caamano-Isorna and Gestal-Otero, 2002). Moreover, a dose-response relationship similar to tobacco exposure was established showing that a greater number of coffees consumed led to a reduction in the risk of PD. Ascherio et al. (2012) reported that caffeine consumption offered a protective effect against PD incidence after adjusting for age, smoking and alcohol intake (RR = 0.43; 95% CI: 0.26-0.71) (Palacios *et al.*, 2012). While smoking and coffee consumption are considered highly correlated behaviors, both of the implicated active compounds, nicotine and caffeine, also act on the same dopaminergic reward system.

While Parkinson's disease appears to have a genetic cause, current research suggests that only 5%-10% of classical PD can be etiologically attributed to monogenic mutations (Dauer and Przedborski, 2003). Several families have been identified with distinct Mendelian inheritance of monogenetic mutations including α synuclein, Parkin, DJ-1, PINK1 and LRRK2 – many of which may make up a specific category of parkinsonian syndromes that are often atypical for PD (de Lau and Breteler, 2006; Hardy *et al.*, 2003). Despite an extensive review of these genes, a significant gap in etiological explanation has led researchers to focus on exogenous exposures. This argument was aided after a study reported that, of 71 monozygotic and 90 dizygotic twins, PD incidence rates for pairs after 50 years of age were not significantly different (RR=1.39; 95% CI: 0.63 – 3.1) (Tanner *et al.*, 1999).

Parkinson's disease and pesticides: epidemiology

It was not until 1983 when Langston and Ballard discovered that drug users who had intravenously injected a synthetic analog of Demerol contaminated with 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine (MPTP) sporadically developed parkinsonian traits, which were levodopa responsive, did researchers begin to hypothesize that environmental exposures may increase PD risk (Langston, 2002). Subsequent studies have demonstrated that MPTP selectively damages dopaminergic cells in the substantia nigra by entering astrocytes and converting to its active metabolite, MPP^+ (1-methyl-4-phenylpyrdinium), to enter the dopaminergic neuron and exert its toxicity (Hatcher, Pennell and Miller, 2008; Langston, 2002). Moreover, MPP^+ bears uncanny structural similarity to paraquat, a popular herbicide sprayed to kill green plant tissue upon contact, implicating environmental toxins may contribute to PD risk. In fact, MPP^+ is a marketed herbicide under the trade name cyperquat.

Since the discovery, researchers have investigated pesticide exposure as a potential risk factor for Parkinson's disease. A recent study reported on 46 studies (39 case-control, 4 cohort and 3 cross-sectional) investigating pesticide exposure and PD (van der Mark *et al.*, 2012). Using random effects meta-analysis, they found an overall meta-risk ratio (mRR) of 1.62 (95% CI: 1.40-1.88) for PD in persons ever exposed to pesticides compared to persons never exposed to pesticides. However, due to differences in exposure definitions (occupational versus non-occupational use, ever/never versus high/low exposure) between individual studies, van der Mark's meta-analysis reported a large degree of heterogeneity (I^2=63.7%). Results were subsequently stratified by exposure to specific pesticides in an attempt to attenuate meta-analysis heterogeneity. Non-occupational and occupational exposure to herbicides (mRR=1.40; 95% CI: 1.08 –

1.81) and insecticides (mRR=1.50; 95% CI: 1.07 – 2.11) yielded significantly increased risk for PD. Stratification by fungicide exposure did not yield a significant meta-estimate (mRR=0.99; 95% CI: 0.71 – 1.40) (van der Mark, Brouwer, Kromhout, Nijssen, Huss and Vermeulen, 2012). Alternatively, studies were stratified by occupational and/or non-occupational exposure (mRR=1.69; 95% CI: 1.38 – 2.06), and studies of occupational exposure only (mRR=1.52; 95% CI: 1.23 – 1.89). Only 3 studies estimated risk of non-occupational exposure only with an mRR of 1.18 (95% CI: 0.86 – 1.63).

Ultimately, these stratifications did not illuminate the cause of heterogeneity in van der Mark's analysis. Including occupational exposure of pesticides with non-occupational exposure resulted in little variation of the overall mRR point estimate. The most suggestive indication of study heterogeneity came after stratification by exposure method of self-reported exposure (n=36) and job-title (n=3), although resulting point estimates were not significantly different (van der Mark, Brouwer, Kromhout, Nijssen, Huss and Vermeulen, 2012). The small set of studies using reported job-titles for exposure assessment ultimately reported a higher mRR compared to self-reported pesticide exposure. While recall bias - differential over-reporting of exposure by cases leading to more false-positives and a greater risk estimate than the true estimate – supports the opposite of the observed trend, the difference is possibly explained by occupational exposures being often more acute and frequent compared to environmental exposures (van der Mark, Brouwer, Kromhout, Nijssen, Huss and Vermeulen, 2012). An equally likely explanation is that all subjects are not able to reliably report pesticide exposure (Daniels *et al.*, 2001) or, in another scenario, patients with PD are co-maligned with dementia, which would lead to under-reporting of previous pesticide exposure.

Lastly, it is worth noting that stratification by pesticide type is an important methodology in analysis (and even better in study design) since pesticide exposure often comprises a mixture of pesticide types, all of which have varied mechanisms of action that exert possible toxic effects (Hatcher, Pennell and Miller, 2008).

Of the 46 case-control studies reviewed, van der Mark's analysis included several notable studies, some yielding conflicting associations between pesticide exposure and PD. In 2009, Tanner et al. performed a multi-center matched case-control study with 519 incident cases comparing lifelong occupational and job task histories with Parkinsonism ($2 \geq$ cardinal signs). Prior exposure to pesticides was defined categorically and inferred from a detailed history of job duties including, but not limited to, pesticide use, painting, machining, cleaning and degreasing (Tanner *et al.*, 2009). Unconditional logistic regression yielded an OR=1.90 (95% CI: 1.12 – 3.21) for occupational pesticide use. A year later, however, Firestone et al. (2010) completed another case-control study with 404 cases comparing self-reported work histories with Parkinsonism ($2 \geq$ cardinal signs) and found contrary results. Incident PD cases were asked about occupations held longer than 6 months as well as their workplace exposure to various industrial toxicants. Their unconditional logistic regression found no association of prior job-related pesticide exposures or farming work with PD (OR=1.0; 95% CI: 0.72 – 1.49) (Firestone *et al.*, 2010).

The discrepancy between the two point estimates is likely attributable to a variety of factors, all of which underscore the delicacy of an accurate classification of pesticide exposure and PD. Controls from Tanner et al. (2009) were non-primary relatives of cases who were identified from referral centers. While this method improved clinical

diagnosis, their results may not apply to a more heterogeneous population, which was the sampling reservoir from which Firestone et al. (2010) identified controls. Furthermore, despite the argument that the inclusion of atypical parkinsonism potentially captured more cases etiologically attributable to toxicant exposure, parkinsonism diagnosis relies on clinical criteria who specificity only approaches 80% (Hughes *et al.*, 1992). Overall, these features coupled with the possibility that categorization of job-related exposure may have differed substantially helps explain how susceptible these studies are to bias.

Given that the majority of case-control studies are potentially subject to large degrees of methodological bias (selection bias (control selection), recall bias, misclassification of retrospective exposure and/or clinical misclassification of PD), cohort studies may offer a more accurate assessment of pesticide exposure and risk of PD. A 2012 meta-analysis of cohort studies (n=12) investigated the association between pesticide exposure and PD using random-effects modeling and found significantly increased risk for PD (RR=1.28; 95% CI: 1.03 – 1.59) that did not vary substantially when omitting studies with extreme weights (Van Maele-Fabry *et al.*, 2012). Similar to van der Mark's meta-analysis, a high degree of heterogeneity was reported (I^2 = 74%) suggesting that the overall estimate should be considered with caution. Stratification of studies by exposure assessment that relied on job categories as surrogates for pesticide exposure slightly reduced heterogeneity (I^2=61; n=8) but yielded non-significant decreased risk of PD (mRR=1.23; 95% CI: 0.99 – 1.53). Despite the observed decrease in risk estimate which contrasts from the increased risk of occupational exposure seen in van der Mark's analysis, Li et al. (2009) comprised the majority of weight (over 77%) generating Van Maele-Fabry's risk estimate. Stratification by pesticide type could only

be performed for herbicides and fungicides but resulted in non-significant risk, which greatly impacted heterogeneity. These stratified results were too scarce to permit stable conclusions.

Nonetheless, stratification by pesticide type has recently been encouraged in the scientific community. Research has suggested that classes of pesticides differentially affect the processes that are putatively responsible for PD – oxidative stress, interference with dopamine transporters, mitochondrial dysfunction, promotion of α-synuclein fibrillation, and inflammation (Brown *et al.*, 2006). Hatcher et al. (2008) completed a thorough biological and scientific analysis of specific pesticides that are putatively implicated in increasing risk for PD development. To summarize briefly, fungicides, herbicides, and a sub-class of insecticides called organochlorines all warrant further investigation as potential contributors to PD development. Research has demonstrated that mice exposed to organochlorines, dieldrin and heptachlor, cause alteration of dopaminergic processes (Richardson, Caudle, Wang, Dean, Pennell and Miller, 2006; Richardson *et al.*, 2008) while *in vivo* exposure to the herbicide paraquat has shown to cause 20%-30% selective dopamine neuronal loss in the SNpc (Manning-Bog *et al.*, 2003). Furthermore, these pesticides wide commercial use in occupational settings, long half-life in the environment and strong bioavailability strengthen the epidemiologic plausibility of contributing to PD pathogenesis (Hatcher, Pennell and Miller, 2008).

Of the 5.2 billion pounds of pesticides used in 2007, 2,018 million pounds were herbicides (39%), 955 million pounds were insecticides (18%) and 519 million pounds were fungicides (10%) (Grube, 2011). Generally, these figures are increased for prior decades. Insecticides were used before the turn of the 20th century and their use has

remained relatively constant from 1955-1970 (Hayes, 1975). From 1965 to 1975 metric tons of insecticides stayed about the same. Their use then dropped to about 60% of the 1965-75 levels and stayed about the same until 1985 (Zilberman *et al.*, 1991). Since 1976, insecticide use has become a smaller proportion of the total amount of pesticide used dramatically declined due to introduction of pyrethroids and integrated pest management, until in the 1990s insecticides are only about 10% of all pesticides. Herbicides were introduced in 1947. In contrast to insecticides, herbicide use doubled from 1953 to 1964, and from 1964 to 1982, herbicide use increased eight times. Fungicides were introduced in 1943. Their use remained stable during 1970s and 80s (Lin, 1995).

With the understanding that these classes of pesticides affect PD pathogenesis through different mechanisms, an increasing number of studies have begun to investigate specific pesticides and their role in PD development. Using the California Department of Pesticide Regulation system, Wang et al. (2011) conducted a case-control study of Parkinson's disease and environmental exposure to maneb, ziram and paraquat using geographic information system modeling (GIS) of residencies matched for age, age at diagnosis, sex, ethnicity, education and smoking status. Following unconditional logistic regression, residential exposure to maneb yielded an odds ratio of 1.71 (95% CI: 1.06 – 2.77), an odds ratio of 0.77 (95% CI: 0.50 – 1.17) when residentially exposed to paraquat and an odds ratio of 1.13 (95% CI: 0.70 – 1.82) when residentially exposed to ziram (Wang *et al.*, 2011).

Pesticides and PD: organochlorine toxicology

Organochlorine pesticides offer the most threatening case for a class of pesticide contributory to PD development. They were heavily used during the 1950s – 1970s and, despite specific organochlorines being passed out by the EPA, their low volatility, chemical stability and high lipophilicity allow them to remain and bioaccumulate in the environment (Hatcher, Pennell and Miller, 2008). Thus, public health concern has grown as individuals outside of occupations using pesticides had not been previously considered exposed. Weisskopf et al. (2010) investigated the presence of persistent organochlorines in human and risk of PD in a nested case-control study within the Finnish Mobile Clinic Health Examination Survey. Increasing concentrations of dieldrin were associated with increased odds of PD (OR per IQR 1.95; 95% CI 1.26 – 3.02; p = 0.003) but none of the other organochlorines studied were associated with PD (Weisskopf *et al.*, 2010).

The residual environmental presence of pesticides has pushed researchers to examine the possible effects of exposure further *in utero*, driven from findings that concentrations of organochlorines have been documented in infant serum of exposed mothers (Schaalan *et al.*, 2012). Low-level perinatal exposure of the organochlorine, dieldrin, in mice during gestation and lactation disrupts dopaminergic neurochemistry in mouse offspring and renders them more vulnerable to MPTP toxicity (Richardson, Caudle, Wang, Dean, Pennell and Miller, 2006). Compared to control mice, exposed mice were found to have significantly reduced concentrations of striatal dopamine. Additionally, offspring exposed to dieldrin had higher concentrations of MPP+, the metabolite of the environmental toxin responsible for sporadic PD development, MPTP. These mice were found to have long-term enhancement of dopaminergic proteins,

dopamine transporter (DAT) and vesicular monoamine transporter 2 (VMAT2), in the striatum when exposed to dieldrin during gestation and lactation.

This same developmental study design was repeated in an investigation of the effects of the organochlorine heptachlor on dopaminergic proteins in the brain. Low concentrations of heptachlor were administered to female mice 2 weeks before, during and 3 weeks after pregnancy. Compared with control offspring, exposed mice had similarly enhanced concentrations of DAT and VMAT2. Accordingly, exposed offspring who were administered MPTP experienced greater neurotoxicity as evidenced by a greater loss of striatal dopamine and increased levels of alpha-synuclein (Richardson, Caudle, Wang, Dean, Pennell and Miller, 2008). These alterations in the dopamine system are important insofar as they provide insight into a possible biological pathway that environmental toxins take to affect PD development. They emphasized the mismanagement of dopaminergic proteins and the inability to properly store dopamine – a cornerstone to the pathophysiology of PD. It was also shown that changes in the DAT:VMAT2 ratio can greatly affect the vulnerability to the Parkinsonism-inducing toxin, MPTP (Richardson, Caudle, Wang, Dean, Pennell and Miller, 2006; Richardson, Caudle, Wang, Dean, Pennell and Miller, 2008). As organochlorines continue to persist in the environment, it is critical to investigate whether their residual exposure contributes to the sporadic development of idiopathic PD.

Endosulfan and PD

Beginning in July 2010, the EPA began phasing-out an acutely toxic organochlorine insecticide called endosulfan. It was first registered in 1956 to be used on a variety of vegetables and fruits, on cotton, and on ornamental plants (EPA, 2010).

Crops with the highest use between 2006 and 2008 included apple, cotton, cucurbit and tomato and its application is usually to target a variety of pests including leafhoppers, Colorado potato beetles, and cabbage worms (Galatone, 2009). As an insecticide, endosulfan is acutely poisonous. The EPA classifies endosulfan as a Category I: "Highly Acutely Toxic" chemical based on reported oral LD_{50} values ranging from 18 – 160 mg/kg in rats and 7.36 mg/kg in mice. Dermal exposures have also demonstrated significant toxicity with reported LD_{50} values in rats ranging from 78 to 359 mg/kg (EPA, 2012). Mechanistically, endosulfan primarily affects the nervous system by blocking γ-amino-butyric acid ($GABA_A$) gated chloride channels. Because $GABA_A$ receptors are the principal inhibitory neuroreceptors in the mammalian brain, antagonism of GABAergic neurons causes generalized stimulation in the central nervous system (CNS) (Silva and Gammon, 2009). Endosulfan poisoning thus increases parasympathetic activity of the CNS causing tremors, hyperactivity, vomiting and convulsions. Despite an understanding of the underlying mechanism for acute toxicity, little is understood about the effects of sub-chronic exposure of endosulfan.

Chemically, technical grade endosulfan is a yellow-brown color with a molecular weight of 406.96 g/mol. Commercially available endosulfan is a mixture of two isomers, α-endosulfan (64-67%) and β-endosulfan (29-32%) (Silva and Gammon, 2009). Its relatively high vapor pressure makes it highly volatile and, thus, very mobile in the environment - significant amounts evaporate from soil and leaf surfaces soon after application (Galatone, 2009). As a result, endosulfan is one of the most abundant organochlorine pesticides found in the Arctic region and has also been detected in the Great Lakes, various mountainous regions as well as other areas thousands of miles from

use areas (EPA, 2010). Accordingly, endosulfan's lipophilic structure allows it to readily bioaccumulate in the fatty tissue of terrestrial and aquatic animals. Morris et al. (2008) found concentrations of α- and β-endosulfan in ice-algae, phytoplankton, zooplankton, marine fish and ringed seals at concentrations ranging from 0.1-2.5 ng/g lipid (Morris, 2008). Additional studies have shown that endosulfan mixtures (both isomers) have the high potential for bioaccumulation in biota as indicated by an average log *K*ow value of 4.74 for α-isomer and 4.79 for the β-isomer, respectively (Morris, 2008).

Given the burden of evidence suggesting the biopersistence of endosulfan, prolonged, chronic exposures is also a major concern. Concentrations as low as 10mg/kg/day have been shown to cause death in mice after 15 days (Smith, 1991). Doses of 5 mg/kg/day in female mice caused liver enlargement and, at a lower but more sustained dose of 0.1mg/kg/day for 78 weeks, researchers found damage to dam reproductive organs (Institute, 1978). More significantly, endosulfan bears a nearly identical structure to both heptachlor and dieldrin, suggesting that it would generate similar developmental and degenerative effects to dopaminergic cells *in utero*. Despite the beginnings of a lengthy phase-out process, understanding developmental effects of residual endosulfan exposure has the potential to elucidate mechanisms contributing to neurodegenerative diseases such as Parkinson's disease.

Hypothesis for current research

Hypothesis 1: Using a job exposure matrix, the attributable risk fraction of PD from adult occupational pesticide exposure has decreased from 1990 to 2011.

Null Hypothesis 1: The attributable risk to occupational exposure to pesticides will stay the same.

Hypothesis 2: The short-term effects of exposure to endosulfan and its metabolites, endosulfan alcohol and endosulfan sulfate, on both primary and immortalized neuronal cell lines will results in significant cell death, altered expression of dopaminergic proteins and increased oxidative stress through a decrease in cellular GSH:GSSG concentration.

Null Hypothesis 2: *In vitro* endosulfan exposure will not change levels of dopaminergic proteins nor alter cellular oxidation concentrations.

Hypothesis 3: Following a developmental dosing paradigm for mice, there will be a significant alternation of specific dopaminergic proteins in the striatum as well as greater susceptibility to the neurotoxin MPTP.

Null Hypothesis 3: There will be no change in dopaminergic proteins following the developmental dosing scheme.

METHODS

Occupational survey data

The proportion of individuals occupationally exposed to pesticides presently as well as 20 years prior was determined using the 1990 US Census Survey and the 2011 American Community Survey (ACS). The US Census Survey provides information on social, economic and geographic data and is administered over a ten year period to non-institutionalized civilians. The survey is administered using a probability selected sample of approximately 60,000 occupied houses over a semi-continuous period of 16 months after which they are dropped from the sample permanently. Response is mandated by law and participation rates are 100%. Please refer to (Bureau, 2011a) for an in-depth description of the sampling methodology. In consideration of PD development, 1990

Census data was chosen following research that has shown the average development of PD takes 20-30 years (Poewe, 2006).

The ACS is an ongoing statistical survey that randomly samples approximately 3.3 million households per year comprising a representative sample of the United States (Commerce, 2011). Surveys are administered continuously throughout the year and all surveys conducted in that year are combined to make the ACS for that year. There appears to be no statistical bias in seasonal estimates. Response to the American Community Survey is also required by law ensuring its 97.6% response rate. In this regard, the ACS is an appropriate substitute to the 2010 Census Survey as data from the latter has yet to be publicly released. Refer to (Bureau, 2011b) for an in-depth description of sampling procedures used.

Both Census and ACS data files were downloaded from IPUMS-USA, University of Minnesota, online (Ruggles, 2010). Given the larger volume of the 1990 US Census Survey, we chose a 5% sample of 1990 US Census survey. Data from the 5% sample was a 1-in-20 national random and weighted sample of the complete 1990 Census survey population. Refer to Ruggles et al. (2010) for an in-depth description of the IPUMS-USA sampling procedures used.

Files were extracted using WinGZip v1.0.0 (build 21) and analyzed using Microsoft Excel and SAS 9.3 (Cary, NC).

Data sets, job exposure matrix and job codes

Datasets from the ACS and the US Census surveys contained information on an individual's occupation title, duration of employment, hours worked at occupation and household weight based on sample size. While both data sets included employment

status information on unemployed and new workers, these observations were excluded from our analysis. Both surveys were considered to be self-reported and neither survey contained questions about pesticide use, application or exposure at any point.

To quantify the proportion of individuals exposed to pesticides occupationally, a Job Exposure Matrix that had previously been used in a 2001 study looking at occupational pesticide exposure and pancreatic cancer was obtained from Professor Trish Stewart of the NIH/NCI (Ji *et al.*, 2001). Since the 2001 publication, the matrix has been augmented to quantify exposure probability, frequency and intensity using an ordinal number system based on quartile categories of exposure (Appendix A, personal communication Patricia Stewart (NCI/NIH)). Ordinal values were assigned for 3 pesticide types: Insecticides, Herbicides and Fungicides. These numerical assignments fluctuated with calendar decade as new pesticide regulations came into effect.

1990 Census occupations are coded with the 3-digit Census Code system. 2011 ACS codes contain a more detailed set of codes from the 2010 Census that are 4 digits. Thus, while the 2011 ACS codes are more detailed than the 1990 Census codes, particularly within the information technology, healthcare, printing, and human resources occupation categories, they were ultimately accounted for during the coding upgrade in 2010 (Commerce, 2011). 1990 and 2011 survey occupations related to the general occupation title in the JEM were grouped under the JEM occupation title. All survey occupations under the same JEM title received the same exposure score. Furthermore, frequency counts of occupation were weighted using sample weight for each person in the survey, which was the inverse probability of that person being in the sample surveyed. Use of these weights results in estimates of the number of adults in a given

occupation in the entire adult population (18+ years). These weighted frequencies for survey occupations were then summed with other occupations, which were grouped together in the same JEM occupation title. This procedure gave the overall frequencies of persons in the US population in JEM specific occupations. All analyses excluded missing values and unemployed individuals.

Proportion ever exposed to pesticides

For the current proportion of workers ever exposed to pesticides, survey occupations in the 2011 ACS receiving a JEM probability of exposure greater than zero were qualified as "ever" exposed while survey occupations receiving no probability of exposure (i.e., occupations not grouped under a JEM occupation title) were qualified as "never" exposed. Individual frequency counts of occupations for "ever" exposed were then summed over the total number of observations in each data set. 1990 Census data was analyzed using the same method as the 2011 ACS data.

Proportion of "high" exposure compared to "low" exposure occupations

To determine the proportion of occupations exposed to a "High" proportion of pesticides, the ordinal values delegated to herbicide, insecticide, and fungicide probability were averaged to obtain an overall pesticide exposure probability score for each survey occupation. Herbicide, insecticide and fungicide ordinal values for intensity and frequency of exposure were also averaged to obtain an overall score for exposure intensity and frequency. Average scores for probability, frequency and intensity were then summed to a maximum overall score of 12. Any occupation receiving a score above 6 was designated as "High" exposed; occupations receiving a score of 6 or below were designated as "Low" exposed.

Meta-analysis estimate and attributable risk fraction

To determine the risk factor attributable to PD by "ever" occupational pesticide exposure in the 1990 Census survey, two meta-risk ratios (mRR) were used after a review of two recent meta-analysis by van der Mark et al. (2011) and van Maele-Fabry et al. (2012). The former considered 39 case-control, 4 cohort and 3 cross-sectional studies while the latter was restricted to 12 cohort studies, which are possibly subject to fewer biases than case-control studies. Both overall summary risk-ratios from van der Mark et al. (2011) (mRR= 1.62; 95% CI: 1.40-1.88) and van Maele-Fabry et al. (2012) (mRR = 1.28; 95% CI: 1.03-1.59) were considered in the analysis.

Percentages of persons ever occupationally exposed to pesticides were then used to calculate the risk attributable to pesticide exposure through equation 1:

$$AF_{pop} = p_p(RR - 1)/(p_p(RR - 1) + 1) \quad (1)$$

where p_p is the percentage of the *total population* exposed to pesticides (Steenland and Armstrong, 2006). Accordingly, this procedure was repeated for the 2011 ACS.

In addition, those ever exposed were divided into two groups for a separate attributable risk calculation incorporating different levels of exposure. Occupations receiving a summed score of average pesticide frequency, intensity and probability that was greater than 6 were considered highly exposed to pesticides. Occupations receiving a summed score equal to or less than 6 were considered low exposed. This attributable risk may be more accurate than using ever versus never exposure because it takes into account the different exposure levels within the "ever exposed" category. Percentages of persons highly exposed to pesticides and persons with low occupational pesticide exposure were included in an analysis using equation 2, which calculates the risk

percentage of Parkinson's disease attributable to occupational pesticide exposure across different levels:

$$AF_{pop} = \sum p_{p,i}(RR_i - 1)/[\sum p_{p,i}(RR_i - 1) + 1] \quad (2)$$

where $p_{p,i}$ is the proportion of individuals exposed by level *i* and RR_i indicates the risk-ratio specific to level *i* (Steenland and Armstrong, 2006). The summary risk-ratios for high level and low level of exposure, respectively, were taken from the sensitivity analysis in van Maele-Fabry et al. (2012), in which they re-calculated their mRR by excluding the influence of individual studies one by one with replacement (Van Maele-Fabry, Hoet, Vilain and Lison, 2012). We used the upper and lower limit of this sensitivity range for the mRR (1.20 and 1.36 respectively) as our RRs for low and high pesticide exposure.

Chemicals and reagents for neurochemical assessment of endosulfan

Endosulfan was purchased from AccuStandard (New Haven, CT). Endosulfan diol was purchased from Santa Cruz Biotechnologies, Inc. (Santa Cruz, CA). Endosulfan sulfate was purchased from Sigma Aldrich (St. Louis, MO). SK-N-SH cells were purchased from American Type Culture Collection (ATCC, Manassas, VA). Hibernate A and Hibernate A- Calcium were purchased from BrainBits (Springfield, IL). B27, DNase1, and Neurobasal A were purchased from Life Technologies (Life Technologies). Papain was obtained from Sigma (St. Louis, MO). Dispase II was purchased from Roche (Nutley, NJ). Aphidicolin was purchased from A.G. scientific (San Diego, CA). Whatman GF/F filter papers were obtained from Brandel, Inc. (Plantation, FL). DMEM/F12 media was purchased from Lonza (Walkersville, MD). The BCA protein assay kit was obtained from Pierce (Rockford, IL). Monoclonal anti-rat

dopamine transporter and polyclonal anti-rabbit tyrosine hydroxylase antibodies were purchased from EMD Milipore (Billerica, MA) or Pel Freez Biologicals (Rogers, AR). Polyclonal anti-rabbit VMAT2 antibodies were generated by Covance to the C-terminal sequence in mouse (CTQNNVQPYVGDDEESESD). Monoclonal anti-mouse α-tubulin antibodies were purchased from Sigma Aldrich (St. Louis, MO). Mouse anti-MAP2 antibodies were purchased from Abcam (San Francisco, CA). Rabbit anti-GABA transporter 1 (GAT1) and vesicular GABA transporter (vGAT) were purchased from Synaptic Systems (Germany). Rabbit-NMDA, Mouse-GABA(A) receptor, Mouse anti-Glial Fibrillary Acid Protein (GFAP) was purchased from Sigma-Aldrich (St. Louis, MO). Secondary antibodies conjugated to horseradish peroxidase were obtained from Jackson Immunoresearch Laboratories (West Grove, PA). Secondaries conjugated to fluorescent tags were obtained from Life Technologies. SuperSignal West Dura Extended duration substrate and stripping buffer were obtained from Pierce.

In vitro analysis of endosulfan and metabolites on SK-N-SH neuronal cell lines

Cells were cultured in DMEM F12 media supplemented with 100 units/ml pencillin and 100 units/ml streptomycin and 100mmol/ml of L-glutamine and 10% fetal bovine serum. Cells were cultured at 37^0C in a humidified atmosphere with 5% CO_2 and propagated according to the protocol provided by ATCC. When cells were confluent, they were passaged to 40,000 cells per well in 96-well plates at 100 µl for treatment with endosulfan, endosulfan sulfate and endosulfan diol. Cell death was assessed using the WST-1 Cell Proliferation assay. Following treatment for 24 hours with 100, 200, 300, 400, 500 and 600 µM of endosulfan, endosulfan sulfate and endosulfan diol, 10 µl/well of Cell Proliferation Reagent WST-1 was added to cells and incubated for 3 hours at

37^0C 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 nanometers on an Epoch BioTek microplate spectrophotometer and analyzed using Gen5 software (2.0) and GraphPad software. WST-1 assay was also repeated with the above treatments for 72 hours.

In vitro analysis of endosulfan as an oxidative stressor

SK-N-SH cells cultured in complete media (10% FBS in DMEM) were grown to 80% confluence. Cells were then washed in HBSS twice, followed by incubation with 10% FBS in DMEM or 10% FBS. Cells were then plated on 96-well plates with increasing concentrations of endosulfan (31.25, 62.5, 125, 250, 500µM respectively) for 1 hour with DMSO control. GSH and GSSG were quantified by HPLC with fluorescence detection and used to calculate the steady-state redox potential values using the Nernst equation, as described previously (Jones, 2002). For reactive oxygen species detection by dichlorofluroescin (DCF) oxidation, endosulfan treated SK-N-SH cells were washed with KRH buffer, incubated with DCF-DA at 50 µmol/L for 4 hours (37C, 5% CO_2) and washed. DCF fluorescence from each well was measured with a plate reader (Go and Jones, 2005).

In vitro analysis of endosulfan and metabolites on ventral mesencephalon cells

The protocol used has been modified from Bradner et al. (2013). Briefly, ventral mesencephalic neuron cultures were prepared from postnatal mice (postnatal day 1-3). 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 dissociation solution, containing Papain (1mg/ml), Dipase II (1.2U/ml), and DNase 1 (1ul/ml) dissolved in Hibernate A- Calcium for 20 mins at 37^0C and gently agitated every 5 minutes. Tissue was then rinsed in plating media, containing Neurobasal-A and 10% heat inactivated fetal bovine serum, 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 hours, *in vitro*. The following day, culture media containing aphidicolin (1ug/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 various concentrations of endosulfan I dissolved in DMSO. After 24 hours, cells were fixed in 4% PFA for minutes and incubated overnight in rabbit anti-TH, chicken anti-MAP2, and mouse anti-NeuN at 4^0C. The following day, cultures were incubated with fluorescent secondary antibodies, goat anti-rabbit 488, anti-chicken 647, and anti-mouse 594 for 1 hour at room temperature. Cells were rinsed and stored in PBS. Images of treated cultures were taken using an Array Scan VTI HCS (Cellomics; Pittsburgh, PA). Forty-nine contiguous fields were taken per well and TH+ neurons were counted and analyzed using GraphPad analysis software.

In vivo analysis of developmental exposure to endosulfan

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 administered 1mg/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 with 10 minutes. 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 LD_{50} in mice (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 ended upon weaning of the pups on postnatal day (PND) 21. Mice were then separated by litter and by sex into separate cages until 12 weeks of age.

MPTP administration

At 12 weeks of age, male and female offspring of control and endosulfan treated animals were administered 2 injections of saline or 10mg/kg MPTP subcutaneously (s.c.) 10 hours apart (total dosage being 20 mg/kg) and sacrificed 7 days after the second injection (Tillerson *et al.*, 2002). MPTP is a known dopaminergic neurotoxicant that reliably causes loss of dopaminergic markers. For this study, MPTP was used in order to

challenge the dopamine system in order to unmask potential underlying damage to the nigrostriatal dopamine system.

Neurochemical analysis of developmental exposure to endosulfan

Western blots were used to quantify the amount of dopaminergic proteins, DAT, TH, and VMAT2, as well as GABAergic proteins, GAT1, vGAT, GABA(A) receptor, NMDA receptor and GFAP, present in samples of cortical and striatal tissue from treated and control mice. Samples were homogenized and subjected to polyacrylamide gel electrophoresis and electrophoretically transferred to polyvinylidene difluoride membranes. Non-specific binding sites were blocked in 7.5% nonfact dry milk in Tris-buffered saline and then membranes incubated overnight in a monoclonal antibody to the N-terminus of DAT. DAT antibody binding (1:5,000) 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 coblotted dilutional standards. Membranes were stripped for 15 minutes at room temperature with Pierce Stripping Buffer and sequentially reprobed with α-tubulin (1:5,000), GAT1 (1:5,000), vGAT (1:5,000), GFAP (1:5,000), GABA(A) receptor, NMDA receptor, TH (1:1,000) and VMAT2 (1:10,000) antibodies. α-Tubulin blots were used to ensure equal protein loading across samples.

Wet-lab statistical analysis

Litter was considered the smallest unit of analysis, with each litter representing an independent replication (n=4-6 litters per treatment). Neurochemical data were analyzed using two-tailed Student's t-test or 1-way ANOVA. When a significant *F* was

determined, post hoc comparisons were performed using the Student-Newman Keuls (SNK) test. Statistical significance is reported at the $P \leq 0.05$ level unless otherwise noted.

RESULTS

Risk attributable to pesticide exposure in ever exposed vs. never exposed

The attributable risk of a 5% randomized sample of the 1990 Census and the 2011 American Community Survey was analyzed using a Job Exposure Matrix (JEM) developed by Trisha Stewart (NCI/NIH) (Table 1). The percentage of adult persons "ever" exposed occupationally to pesticides, including herbicides, fungicides and insecticides, was determined to be 23.55% in 1990 and 24.98% in 2011, virtually unchanged. Here, we will use and common percentage of 24% for both periods, and present attributable risks which are common to each period. Using an analysis with equation 1, Table 2 shows the risk of Parkinson's disease that is attributable to any occupational pesticide exposure in 1990 as well as in 2011 using two different meta-risk ratios from van Maele-Fabry et al. (2012) and van der Mark et al. (2011). Using a common percentage (24%) for "ever" occupationally exposed in both 1990 and 2011 and the overall mRR (1.28) from van Maele-Fabry et al. (2012), we found an AF_p of 6.30% and 6.54%. Accordingly, using the overall mRR (1.62) from van der Mark et al. (2011) we found an AF_p of 12.74% in 1990 and 13.41% in 2011.

Risk attributable to pesticide exposure using levels of exposure (high and low)

In the 1990 Census Survey, 9.73% of persons occupationally exposed to pesticides were considered highly exposed and 13.82% were considered low exposed (Table 3). These percentages yielded an AF_{pop} of 3.04% using the risk-ratios from van

Maele-Fabry's sensitivity analysis (Table 4). Likewise, in the 2011 ACS dataset, 10.99% were considered high exposed and 15.99% were considered low exposed after JEM analysis. These percentages generated an AF_{pop} of 3.27%, which is slightly increased compared to the calculated percentage for in the 1990 Census Survey (Table 4). Ultimately, the attributable risk fraction fluctuates within the range of 3% - 13% given different epidemiologic parameters and classification schemes.

Neurochemical results of endosulfan exposure

Since we were interested in further evaluating the specific neurological effects of the recently banned organochlorine, endosulfan, and its metabolites on the dopamine system, we first assessed the neurotoxic properties of these compounds using the SK-N-SH neuroblastoma cell line, which is known to exhibit dopaminergic properties (Richards and Sadee, 1986). These characteristics make it an ideal cell line resembling those cells affected in patients with Parkinson's disease.

In vitro 24 hr endosulfan exposure is cytotoxic to SK-N-SH cells

Cells were treated with increasing concentrations of endosulfan, endosulfan diol and endosulfan sulfate dissolved in cell culture media in 0.1% DMSO for 24 hours. Exposure to the parent-compound endosulfan resulted in a statistically significant decrease in formazan formation from DMSO control at concentrations of 200uM and greater ($F=27.45$; $p<0.05$), indicating dose-dependent cytotoxicity (Figure 1). SK-N-SH exposure to both endosulfan diol and endosulfan sulfate did not result in any statistically significant increase in cytotoxicity at an alpha level of 0.05. These data demonstrate the ability of the parent-compound endosulfan, but not its metabolites, to be an efficient neurotoxicant in a dopaminergic cell line. These results were subsequently used to

further guide our dosing paradigm in additional *in vitro* models using 72hr exposure to endosulfan and its metabolites as well as exposure to primary cells from the ventral mesencephalon.

In vitro 72hr endosulfan and metabolite exposure is cytotoxic to SK-N-SH cells

To monitor chronic exposure of endosulfan and its metabolites on immortalized dopaminergic cells, cytotoxicity of toxicant exposure for 72 hours on SK-N-SH cells was analyzed. Again, cells were treated with increasing concentrations of endosulfan, endosulfan diol and endosulfan sulfate in cell media (0.1% DMSO) for 72 hours. WST-1 assay was used to quantify spectral absorbance from formazan formation. Exposure to the parent-compound endosulfan resulted in a significant decrease in formazan formation from DMSO control at concentrations of 100uM and greater ($F=71.86$; $p<05$) (Figure 2). Additionally, 72 hour exposure to endosulfan trends toward a dose-dependent relationship with cytotoxicity, particularly at lower concentrations of 100uM, 150uM and 200uM. SK-N-SH exposure to both endosulfan diol and endosulfan resulted in similar significant reductions in cell viability, but lacked a trend toward dose-dependency.

In vitro endosulfan exposure modulates GSH and GSSG levels in SK-N-SH cells

To examine the effects of endosulfan exposure on the redox state of cellular GSH/GSSG, SK-N-SH cells were exposed to increasing concentrations of endosulfan for 4 hours. HPLC fluorescent detection saw significant decreases in GSH levels at all applied endosulfan concentrations when compared to DMSO control ($F=18,824$; $p=0.0001$) (Figure 3A). Accordingly, HPLC analysis demonstrated a significant increase in GSSG, the oxidized state of GSH, in SK-N-SH at all concentrations of endosulfan compared to DMSO control ($F=11,776$; $p=0.0001$) (Figure 3B). The reduction potential

(E_h) for GSH/GSSG in SK-N-SH cells was calculated using the Nernst equation at all concentrations of *in vitro* endosulfan application. Figure 3C shows that there was a significant increase of cellular reduction potential (E_h) of GSH/GSSG by endosulfan exposure of 31.25μM or greater for 4 hours (31.25μM Depletion, -240 ± 9.2 mV; F=3.739; p=0.0001), indicating cells exposed to endosulfan maintained a greater oxidation state than DMSO control. Lastly, the DCF-DA assay showed that SK-N-SH cells exposed to endosulfan for 1 hour had significantly increased concentrations of ROS species at concentrations of 125 μM and greater of endosulfan (Figure 3D).

In vitro 24hr endosulfan exposure is cytotoxic to ventral mesencephalon neurons

We then sought to elaborate the dopaminergic effects of endosulfan by assessing its neurotoxicity on primary cultured neurons isolated from the dopamine-rich mesencephalic region of neonatal WT C57BL/6J mice. A dose-dependent reduction in TH + neurons (stained green) was observed in cultures, with 20uM and higher concentrations demonstrating a significant loss in TH+ neurons in the cultures (Figure 4). WT cultures exposed to endosulfan also demonstrated increased neurite length up to 17.5 uM after which point neurite length decreased significantly (Figure 4). Interestingly, significant increases in neurite length at concentrations of 15 uM and 17.5 uM were occurring in the absence of any significant TH neuron loss. At 20uM, significant TH neuron cytotoxicity occurred concomitantly with a reduction in neurite growth. Additionally, endosulfan concentrations of 30uM demonstrated significant reduction in TH+ neurons.

In vivo endosulfan exposure reduces striatal DAT and TH levels in mothers

We next assessed the effects of residual environmental exposure of endosulfan on the nigrostriatal dopamine system through a developmental paradigm. Specifically, we analyzed the effect of endosulfan ingestion on cortical and striatal proteins involved in dopaminergic homeostasis in both mothers and their pups. Schaalan et al. (2012) demonstrated that organochlorine concentrations are detectable in serum of infants exposed *in utero* and, furthermore, that maternal serum concentrations of organochlorines are correlated with neonatal concentrations.

Before assessing alterations to the dopaminergic system in offspring, we analyzed endosulfan insult in mothers of offspring (n=14). As seen in Figure 5, ingestion of sub-chronic concentrations of endosulfan (1 mg/kg) every other day beginning 2 weeks prior until birth significantly reduces striatal levels of TH ($T=2.557$; $p=0.0251$) and also marks a similar trend in reducing striatal levels of DAT although not significantly ($T=1.739$; $p=0.1076$). These results suggest that sub-chronic exposure to endosulfan through ingestion alters the homeostasis of dopamine handling in the striatum.

In vivo developmental endosulfan exposure modulates striatal dopaminergic proteins in offspring

Next, we assessed alterations in striatal dopaminergic proteins in offspring exposed to endosulfan *in utero* and during lactation. Developmental endosulfan exposure resulted in little fluctuations in relative striatal concentrations of DAT, TH and VMAT2 (Figure 6A, C, and E). These alternations became more pronounced, however, after stratifying the analysis by sex (Figure 6B, D, and F). Males exhibited significantly increased striatal TH levels compared to female controls despite having no difference between treatments within sex ($F=7.260$; $p=0.0424$). Male offspring also had increased levels striatal DAT despite being non-significant ($F=0.8611$; $p=0.2816$). Additionally,

levels of β-Actin were only significantly different after stratifying by sex (Figure 6F). These results suggest that differences in sex contribute to the damage insult allowed by endosulfan exposure on developing neurons.

In vivo developmental endosulfan exposure does not significantly exacerbate toxicity to MPTP in striatum

We next assessed if developmental endosulfan exposure facilitated greater alterations in striatal dopaminergic proteins after a moderate dose (2x10mg/kg) of MPTP was administered. While offspring injected with MPTP experienced significant ~50% reductions in DAT ($F=12.29$, $p<0.0001$) and TH (compared to those who received saline injections ($F=3.220$, $p<0.0424$), there was no significant difference between levels of dopaminergic proteins in control MPTP mice versus endosulfan exposed MPTP mice (Figure 7).

In vivo developmental endosulfan exposure significantly reduces cortical levels of DAT and TH in offspring

An assessment of male cortical tissue samples from developmentally endosulfan exposed offspring was conducted after striatal tissue analysis failed to offer significant findings. While reductions in dopaminergic proteins in the cortex is not pathologic in patients experiencing PD, alterations to the cortical dopaminergic system has been studied in other chronic developmental disorders such as schizophrenia and autism (Lewis and Sweet, 2009; Nakamura *et al.*, 2010). Figure 7 shows that developmental endosulfan exposure in male offspring revealed significant reductions in relative cortical DAT levels ($t=4.158$; $p=0.002$) as well as relative cortical TH levels ($t=2.893$; $p=0.0160$).

In vivo developmental endosulfan exposure significantly modulates cortical concentrations of GABAergic proteins in male mice

Western blotting was also performed to assess GABAergic proteins in the cortex of male offspring with the rationale that mechanisms of acute exposure to chlorinated hydrocarbon act predominately at the GABA(A) receptor. While it is known that acute concentrations of endosulfan block the Cl^- channel linked to the GABA(A)-receptor (Silva and Gammon, 2009), chronic developmental exposure has yet to be assessed. Accordingly, male offspring exposed to endosulfan through our developmental dosing paradigm had significant reductions in cortical concentrations of vGAT ($t=2.59$, $p=0.238$) and GAT1 ($t=2.41$, $p=0.0329$), suggesting that chronic endosulfan exposure affects the homeostatic management of the neurotransmitter, GABA, in the cortex (Figure 9A and B). Mice developmentally exposed to endosulfan had significant increases in GFAP levels in the cortex ($T=2.991$, $p=0.0202$), indicating increased astrogliosis in the cortex – a marker of neurological inflammation (Figure 9C). Lastly, these male offspring also had significantly increased levels of GABA(A) post-synaptic receptor ($T=3.414$, $p=0.0066$) (Figure 9D).

In vivo developmental endosulfan exposure significantly modulates cortical concentrations of glutamatergic proteins in male mice

Cortical concentrations of glutaminergic proteins were also quantified using Western immunoblot with the rationale that pyramidal neurons, comprising75% cortical neurons, utilize the excitatory neurotransmitter, glutamate (Lewis and Sweet, 2009). Here, we report that male offspring exposed to endosulfan through our developmental dosing paradigm had significantly greater concentrations of vesicular glutamate transporter, vGlut, when compared to controls ($T=7.68$; $p=0.0001$) (Figure 10A). vGlut transports glutamate neurotransmitters into synaptic vesicles to be re-released into the synapse. Developmentally exposed mice also had significantly reduced concentrations of

the post-synaptic NMDA receptor (T=4.15, p=0.0013), which mediates the excitatory input to the dorsal lateral prefrontal cortex (DLPFC) deep layer 3 pyramidal neurons (Figure 10B).

DISCUSSION

To reiterate, the current investigation considers the epidemiologic features of exposure assessment and meta-analysis in establishing a valid attributable fraction estimate of PD from occupational exposure to pesticides. Additionally, we demonstrate the toxicological effects that the environmentally persistent organochlorine insecticide, endosulfan, exerts on the developing neurological system using an *in vivo* mouse model. Developmental exposure to endosulfan mimics the route experienced by humans who are exposed to environmentally exposed to sub-chronic concentrations of organochlorines. While our initial hypothesis focused on Parkinson's disease, we report that developmental exposure to endosulfan significantly alters dopaminergic, GABAergic and glutaminergic processes in the cortex, suggesting that prolonged exposure to endosulfan *in utero* may contribute to pathogenesis of schizophrenia, or other neurological disorders mediated by alteration to the normal functioning of the frontal cortex.

Epidemiological assessment of attributable risk calculations

Langston and Ballard's 1983 discovery that injection of MPTP caused sporadic development of PD led researchers to investigate the association between PD and pesticide exposure, bearing its structural similarity with a commercial pesticide, paraquat. Unfortunately, an accurate assessment of overall pesticide exposure is a complex issue. Persons can be exposed to residual concentrations of certain types of pesticides that linger in the environment due to their lipophilicity and chemical stability (Morris, 2008).

These exposures may also occur in locations distant from the initial source of pesticide application as many pesticides have high vapor pressures and are easily transported. Additionally, persons can be exposed to pesticides occupationally if used on the job, which may vary by frequency, duration and intensity. Lastly, the variety of pesticide types may obscure a clear mechanistic relationship between exposure and disease, particularly when considering that many pesticides greatly differ in their mode of action, uptake in the body, metabolism and elimination from the body (Freire and Koifman, 2012; Hatcher, Pennell and Miller, 2008).

The shortcomings of accurately quantifying pesticide exposure are further compounded by imperfect study designs. The majority of epidemiologic evidence on pesticides and PD is frequently based on case-control studies, which have notorious weaknesses for the investigation of exposure that occurred decades before PD onset (Freire and Koifman, 2012). In particular, case-control studies that rely on self-reported exposure are susceptible to recall bias, which can be differential with respective to both disease and exposure. Individuals with disease who are aware of the disease epidemiology are likely to over-report their exposure profiles, leading to differential bias away from the null. It has also been suggested that patients with PD are paradoxically either more aware of the potential risk factors for disease when compared to non-diseased or conversely less likely to remember their exposure status since many PD patients can be maligned with comorbidities affecting the memory (Hancock *et al.*, 2008).

While some biases are difficult to avoid in a certain study type, others can be ameliorated. Our study aims to emulate the scheme proposed by Ji et al. (2001) by investigating occupational exposure to pesticides through implementation of a Job

Exposure Matrix. Although occupational matrices risk over-reporting exposure in the workplace, in that not everyone in a job is likely to be exposed (MacFarlane *et al.*, 2009), misclassification is nondifferential and would, at most, lead to a conservative underestimation of the association between exposure and disease (Costello *et al.*, 2009). Here, we show that the change in the attributable risk fraction (AF) of PD from pesticide exposure fluctuates largely with the use of point estimates generated in 2 different meta-analyses as well as with the method of estimating the proportion of the population exposed.

In individuals ever exposed to pesticides versus individuals never exposed to pesticides, estimated to be 24% in the 1990 and 2011 surveys, the use of van der Mark et al.'s meta-risk ratio (mRR=1.62; 95% CI:1.40 – 1.88) yielded the highest AR_p of 12.74% in 1990 and 13.41% in 2011, respectively. Inspection of van der Mark's meta-analysis showed that most studies (36 out of 39) were based on self-reported exposure to pesticides, defined as ever versus never use, or as regular versus non-regular use (van der Mark, Brouwer, Kromhout, Nijssen, Huss and Vermeulen, 2012). Since recall bias likely biases results in an over-reporting of exposure by PD cases, one should carefully consider the validity of the associations in the individual studies, especially those that had risk ratios greater than 6 (Peterson et al., 2008; Herishanu et al., 2011; Golbe et al., 1990) and that were accompanied by large confidence intervals. This concept is underscored in the large degree of heterogeneity (I^2=64%) in the overall mRR.

Accordingly, the mRR from van Maele-Fabry's analysis yielded an AF_p of 6.18% and 6.54% for the 1990 Census Survey and the 2011 American Community Survey, respectively. The attributable risk for PD by occupational exposure to pesticides was

virtually unchanged between data sampling. Like van der Mark et al., there is a large degree of heterogeneity in van Maele-Fabry's mRR (I^2=74%). The reduction in AR_p from van der Mark's estimate is due in part to the incorporation of a retrospective cohort study based on job title performed by Li et al. (2009), which found a standardized incidence ratio of 1.06 (95% CI: 0.99 – 1.13). The size of the study, 2,143 exposed cases and 12,594 total participants, accounted for nearly 77.29% of the weight used to determine the mRR in van Maele-Fabry's analysis (Van Maele-Fabry, Hoet, Vilain and Lison, 2012). Thus, while van Maele-Fabry's overall risk-ratio estimate appears to be more conservative quantitatively than van der Mark, it risks the possibility of being overly influenced by one study.

Exposure assessment is a critical issue in epidemiologic research and a significant portion of heterogeneity among studies may most likely be the result of exposure misclassification. Again, MacFarlane et al. (2009) has demonstrated that considering all agricultural jobs as exposed versus unexposed creates a strong potential for non-differential misclassification (MacFarlane, Glass and Fritschi, 2009). Extending and echoing MacFarlane's argument to classify jobs with more than one level of exposure, occupations that were categorized as ever exposed in the first analysis were next stratified into groups of high exposure and low exposure. To do this, we used the upper (RR=1.36) and lower bounds (RR=1.20) of the sensitivity analysis for the cohort studies comprising van Maele-Fabry et al. (2012).

The attributable risk percentage for high vs. low pesticide exposure in both years was ultimately smaller (3%) than all of the attributable risk fractions calculated using ever vs. never quantification. This finding was the result of having slightly less than half

of the population ever exposed classified as low exposed (41.32% in 1990 and 44.0% in 2011, respectively) and, thus, subjected to the effects of a low mRR of 1.20. This evaluation, however, makes perhaps the best attempt at proper control of misclassification bias and unreliability of vastly heterogeneous mRRs. In individuals ever exposed to pesticides versus individuals never exposed to pesticides, one should also note that jobs such as fire-fighters, railroads workers, cashiers, and animal maintenance jobs including veterinarians were also considered "ever" exposed regardless of how small the level of exposure was. The potential for misclassification with this approach is high and considering many agricultural related jobs as pesticide exposed is likely to result an overestimation of exposure (MacFarlane, Glass and Fritschi, 2009).

There were several limitations of the assessment of attributable risk fraction of PD from pesticide exposure. Using meta-risk ratio estimates developed from van der Mark and van Maele-Fabry required an assumption of portability between the source population (the study generating the statistic) and the target population (the US Census data) (Steenland and Armstrong, 2006). Portability hinges on a strict definition of no confounding or interaction between source and target population, but one should not exclude this possibility given our use of meta-analyses. However, the homogenous assessment of exposure between both the source population (occupational exposure via JEM) and target population (the JEM in our analysis) improves the validity of exchangeability, particularly when considering that many prospective studies in van Maele-Fabry et al. (2012) used occupational matrices to model exposure.

Toxicological investigation of developmental exposure to endosulfan

While epidemiological studies have repeatedly indicated that pesticide exposure is a significant risk for PD, a mechanistic link between increased risk and pesticide exposure has yet be established. The immense heterogeneity of chemical properties within types of pesticides adds to the complexity of determining a standardized risk estimate and, moreover, an overarching neurochemical mechanism responsible for increasing PD risk (Tanner *et al.,* 2011; Wang *et al.*, 2011). Here, we report the neurochemical effects of a specific class of pesticides through a laboratory analysis of the recently EPA-banned organochlorine pesticide, endosulfan.

Our rationale for investigating the toxicological effects of *in vivo* developmental endosulfan exposure was predicated on the findings that *in vitro* endosulfan exposure to the immortalized SK-N-SH cell line causes cytotoxicity. Previous research has shown that *in vitro* exposure to endosulfan causes significant cell death in the SH-SY5Y dopaminergic cell line at concentrations of 100μM and greater compared to DMSO control (Jia and Misra, 2007a). Using an alternative cell line with similar requisite dopaminergic properties (synthesis of dopamine, neuromelanin formation and expression of DAT and VMAT2 (Segura-Aguilar, 2011)), we were able to reproduce the findings from Jia and Misra (2007) following 24-hour exposure to endosulfan. While SK-N-SH cell death reached significance at 200uM (n=6), the relative difference in cytotoxic concentrations may be attributed to a greater statistical precision (n=8), a different assay or a different cell line in the study by Jia and Misra (2007).

To our knowledge, *in vitro* exposure to the metabolites of endosulfan, endosulfan sulfate and endosulfan diol, has not been studied using a dopaminergic cell line. Given that endosulfan is metabolized into more hydro-soluble endosulfan diol and sulfate,

which have been found in adipose tissue, placenta and human milk of fertile women (Cerrillo *et al.*, 2005), it is worthwhile to investigate the potential cytotoxic properties compared to the parent compound. 24-hour exposure to metabolites endosulfan diol and endosulfan sulfate did not result in any significant cytotoxicity at concentrations ranging up to 600µM. However, the bio persistent properties of endosulfan and its metabolites warranted an investigation of its cytotoxic effects under more chronic conditions. 72-hour exposure to the parent compound, endosulfan, resulted in significant decreases in cell viability at the same concentrations of the 24-hour assay. In contrast, however, 72-hour SK-N-SH exposure to endosulfan sulfate resulted in significant cell death at all concentrations of 100µM or greater. These results suggest that prolonged exposure to the endosulfan metabolite, endosulfan sulfate, induces cytotoxicity similar to the initial insult by the parent compound endosulfan.

In an effort to explore the possibility that oxidative stress mediates endosulfan exposed SK-N-SH cytotoxicity, cellular glutathione (GSH), oxidized glutathione (GSSG) and reactive oxygen species (ROS) were quantified. The autoxidation of dopamine has provided rationale for oxidative stress as a potential mechanism underlying dopaminergic cell death. Indeed, enhanced cellular concentrations of the glutathione redox antioxidant system are present in the tissue of postmortem PD patients as well as of toxicant exposed *in vivo* nigral cells (Ambani, Van Woert and Murphy, 1975; Dexter and Jenner, 2013; Jenner, 2003). Furthermore, recent research has demonstrated that 1hr exposure to endosulfan concentrations as low as 100µM results in significant intracellular production of reactive oxygen species (ROS) in SH-SY5Y cells quantified by DCF-DA assay (Jia and Misra, 2007b). Additionally, Jia and Misra found that 100µM exposure to

endosulfan results in significantly greater intracellular superoxide anion production in SH-SY5Y cells, which is a marker of increased oxidized glutathione (GSSG) levels (Jia and Misra, 2007b).

Following *in vitro* SK-N-SH exposure to endosulfan, our results report significant reductions in GSH levels, significant increases in GSSG levels and subsequent increases in cellular reduction potentials (E_h) at all concentrations (31.25µM - 500µM) when compared to controls. A trend in increasing reactive oxygen species was observed in our DCF-DA assay. Our findings align with previous research performed by Jia and Mirsa (2007) and, suggest that oxidative stress may be a contributory mechanism through which endosulfan exerts its cytotoxicity.

Previous research has investigated the toxicological effects of endosulfan on primary neuronal cell cultures. Endosulfan exposure to primary neuronal cultures from cerebral cortices (CN) results in significant modulation of the endocrine response system through modulation of Estrogen Receptor-alpha (ERα) and induction cell proliferation of MCF-7 human breast cancer-derived cells (Briz *et al.*, 2011). Rosa et al. (1996) reported that 2-hour endosulfan exposure to primary cerebellar granule cells produced significant cytotoxic results. Quantified using two assays, iodide staining and LDH leakage testing, they found that 200µM of endosulfan resulted in significant cell death (Rosa *et al.*, 1996). Similarly, we used a primary neuronal cell line derived from the ventral mesencephalon (VMES) of WT mice and found a significant dose-dependent reduction in cell viability following endosulfan exposure. Significant cell death was witnessed at concentrations of 20µM and greater. Accordingly, primary cultures were analyzed for developmentally abnormalities as a result of endosulfan exposure. Figure 4 shows that neurite length

increased significantly from control at concentrations of 15µM and 17.5µM. In concentrations of 20µM and greater of endosulfan, the same concentration at which cellular cytotoxicity reached significance, neurite length decreased significantly from previous concentrations. This observation reveals a potential mechanistic relationship between neurite length and cell death: endosulfan exposure may precipitate the arborization observed in exposed cells, which, at larger concentrations, may not be supported by cellular infrastructure and result in increased cytotoxicity.

Following the results from *in vitro* exposure to endosulfan, we demonstrate that *in vivo* developmental exposure to sub-chronic concentrations of endosulfan modulates the dopaminergic, GABAergic and glutaminergic systems. Alterations to catecholamine systems, including dopamine, serotonin, GABA, and norepinephrine, are pathologically representative of a multitude of neurological disorders such as Parkinson's disease (Liu, Morrow, Devaud, Grayson and Lauder, 1997; Miller, Erickson, Perez, Penland, Mash, Rye and Levey, 1999a; Richardson, Caudle, Wang, Dean, Pennell and Miller, 2006), but also other disorders including schizophrenia (Lewis and Sweet, 2009; Mittleman *et al.*, 2008). Previous studies that have investigated the putative toxic effects of persistent organochlorines on the developing nervous system have found significant increases in striatal concentrations of dopamine transporter (DAT) and tyrosine hydroxylase (TH) (Richardson, Caudle, Wang, Dean, Pennell and Miller, 2006; Richardson, Caudle, Wang, Dean, Pennell and Miller, 2008). These two proteins, among others, are critical to dopamine homeostasis; their pathological modulation in postmortem PD patients implicates pesticides as a contributory factor in the neurodegenerative process. With regards to endosulfan specifically, a 2007 study found that developmental exposure to

endosulfan injected intraperitioneally resulted in significantly decreased levels of striatal dopamine, but only after these pups were re-exposed to endosulfan in adulthood. This same group (twice-exposed mice) was found to have significantly reduced acetylcholinesterase activity in the cortex following developmental and, then later, adult exposure (Jia and Misra, 2007c). The results of this study suggest that developmental exposure to pesticides, and endosulfan specifically plays a role in alteration of dopaminergic proteins and exacerbates the neurodegenerative effects of environmental pesticide exposure to pesticides later in life.

In our present study, we assessed the neurodegenerative effects of developmental exposure to endosulfan following a modified scheme outlined in Richardson et al. (2008). Departing from intraperitioneal injection, our developmental dosing paradigm follows an oral ingestion of endosulfan dissolved in corn oil to mimic the route of human exposure in the environment. We observed that offspring of endosulfan mothers exposed to subchronic concentrations of endosulfan showed minimal differences in striatal DAT and TH levels compared to control offspring. Any trend towards a significant difference in striatal dopaminergic protein levels in the striatal tissue of offspring appeared entirely related to sex after stratification. Endosulfan exposed females exhibited only minimal change in striatal DAT or TH levels when compared to female controls. Males, accordingly, exhibited a more pronounced change by treatment type though still not significant. Since estrogen has been demonstrated to be neuroprotective against many dopaminergic neurotoxicants (Miller *et al.*, 1998), our results suggest that the absence of estrogen in males contributes to this trend, however slight. This observation is consistent with epidemiologic literature - gender differences have consistently observed in PD in the

human population, with males having a higher incidence (de Lau and Breteler, 2006; Wright Willis, Evanoff, Lian, Criswell and Racette, 2010).

Since the first observations that heptachlor increased DAT levels in mice (Miller *et al.*, 1999b), there has been speculation that the alteration of dopaminergic proteins following organochlorine exposure would result in increased susceptibility of dopamine neurons to endogenous neurotoxic dopamine metabolites or exogenous neurotoxicants that use DAT to facilitate a biochemical gateway into the neuron (Richardson, Caudle, Wang, Dean, Pennell and Miller, 2006). Mice with a genetic deletion for DAT have been shown to be resistant to the exogenous toxicant, MPTP, (Gainetdinov *et al.*, 1997) while over expression of DAT leads to enhanced neurotoxicity (Donovan *et al.*, 1999). To test if developmental endosulfan exposure resulted in increased susceptibility of the dopamine system, MPTP was administered (2 x 10 mg/kg, s.c.) to male and female offspring who were developmentally exposed to endosulfan. Here, we found that MPTP injection did not produce significant differences in striatal concentrations of DAT or TH in endosulfan exposed mice when compared to control mice that were also administered MPTP. These results suggest that endosulfan exposure does not increase susceptibility to PD development from exogenous neurotoxicants.

While these results suggest that subchronic endosulfan exposure *in utero* does not alter dopamine handling or exacerbate susceptibility to PD development in the striatum, we did not rule out the possibility of exposure effects in the cortex. Indeed, the pathological hallmark of Parkinson's disease, the loss of dopamine neurons in substantia nigra *pars compacta*, is mainly mediated through the striatum, but abnormalities in the cortex of PD patients have also been found. These characteristics include the

accumulation of Lewy bodies and cortical basal impairment that affect both cognitive and executive functioning, characteristic of fully developed PD (Davie, 2008). Additionally, disruption of catecholamine management in the cortex is pathologically characteristic of other developmental neurological disorders including schizophrenia. While multifaceted and complex, schizophrenia diagnosis typically comprises a core set of cognitive deficits, such as impaired memory, that are associated with alterations in dorsal prefrontal cortex (DLPFC) (Lewis and Sweet, 2009). Furthermore, given that a clear mode of transfer has yet to be established for the 80% of genetically inherited cases, researchers have implicated gene-environment interactions and even environmental agents capable of altering cortical circuitry alone as contributory to schizophrenia development (Lewis and Sweet, 2009; Shelton *et al.*, 2012). Thus, a subsequent aim of investigating catecholaminergic modulation in the cortex following developmental exposure to endosulfan was to assess the markers representative of schizophrenia pathology.

Our subsequent and final analysis shows that developmental endosulfan exposure produces significant modulation of dopaminergic, GABAergic and glutaminergic proteins in the cerebral cortex of male mice (Figures 8 – 10). Dopaminegic proteins, DAT and TH, were significantly reduced following Western immunoblot (Figure 8). As these proteins are integral in the management of dopamine transmission, their deficit could contribute to working memory impairments in individuals with schizophrenia because memory depends on activation of DA D1 receptors in the DLPFC (Lewis and Sweet, 2009). Furthermore, a combination of decreased DA innervation of the DLPFC and DA hypoactivity has been implicated to lead to reduced extracellular DA levels and decreased DA D1 receptor stimulation (Abi-Dargham *et al.*, 2002).

Accordingly, we also reported that the glutamatergic protein, vGlut, was significantly increased and that the post-synaptic NMDA receptor was significantly reduced following developmental exposure to endosulfan (Figure 10). Research has suggested that NMDA receptors located on the dendritic spines of pyramidal neurons in the cortex of schizophrenic patients are reduced, which is supported by the findings that NMDA antagonists replicate clinical aspects of schizophrenia in humans (Lewis and Sweet, 2009). Whether or not the increase in cortical levels of vGlut is consistent with this theory remains to be understood. Quite possibly, increased levels of vGlut would sequester glutamate preferentially in the presynaptic neuron, thereby attenuating a normal rate of neurotransmitter release and subsequent NMDA binding.

Our findings in the excitatory glutaminergic system are followed by subsequent modulations of GABAergic proteins in the cortex of developmentally exposed mice (Figure 9). Here, we report that vGAT and GAT1 were significantly reduced while the post-synaptic GABA(A) receptor was significantly increased. These GABAergic alterations also align with putative schizophrenia circuitry. Lewis and Sweet (2009) argue that deficient excitatory output from DLPFC pyramidal neurons (consequence of reduced glutamatergic activity reported above) might reduce GABA neurotransmission. Levels of mRNA encoding GAT1, a GABA transporter responsible for reuptake of released GABA into nerve terminals, are also decreased in the DLPFC in schizophrenia (Hashimoto *et al.*, 2008). Finally, increased expressions of GABA(A) receptor is another pathological marker of individuals with schizophrenia and is theorized to arise from a compensatory response to deficient GABA release (Gonzalez-Burgos *et al.*, 2009). Ultimately, the catecholaminergic alterations in the cortex following developmental

exposure to endosulfan strongly suggest that subchronic exposure to endosulfan is contributory to the neurodegenerative markers found in patients with schizophrenia

CONCLUSION

We find that the population attributable risk for occupational pesticide exposure and PD is likely to be small, on the order of 3% - 13%. This relatively low percentage stems primarly from the fact that occupational pesticide exposure is relatively rare in the United States. Nevertheless, attributable risks of this size carry an important health burden.

The analysis here demonstrates that the attributable risk percents can vary substantially with small adjustments in risk-ratio estimates and with the method of calculating the percentage exposed (taking into account or not different levels of exposure). This feature underscores the importance of accurately quantifying exposure of environmental and occupational exposures such as pesticides.

While previous work has analyzed changes in the catecholinergic system following developmental endosulfan exposure via intravenous or intraperitioneal injection (Jia and Misra, 2007c; Scremin *et al.*, 2011), our findings provide information on the neurochemical effects of endosulfan following ingestion - a route of exposure similar to the environmental experience of the human population. These results suggest that endosulfan exposure *in utero* preferentially alters cortical dopaminergic, GABAergic and glutaminergic biochemistry in a manner is contributory to the neurodegenerative process underlying schizophrenia.Given that neurodegenerative diseases often inflict a large economic and increasing morbidity in the United States, this information should

support the further vigilance and reduction of harmful toxicants that are able to persist in the environment after their application.

REFERENCES

NINDS (2013). *Parkinson's disease Information Page: National Institute of Neurological Disorders and Stroke (NINDS).* Accessed January 4, 2013 2013.

Barnett-Cowan, M., Dyde, R. T., Fox, S. H., Moro, E., Hutchison, W. D., and Harris, L. R. (2010). Multisensory determinants of orientation perception in Parkinson's disease. *Neuroscience* **167**(4), 1138-50, 10.1016/j.neuroscience.2010.02.065.

Dauer, W., and Przedborski, S. (2003). Parkinson's disease: mechanisms and models. *Neuron* **39**(6), 889-909.

Hatcher, J. M., Pennell, K. D., and Miller, G. W. (2008). Parkinson's disease and pesticides: a toxicological perspective. *Trends in pharmacological sciences* **29**(6), 322-9, 10.1016/j.tips.2008.03.007.

Baba, M., Nakajo, S., Tu, P. H., Tomita, T., Nakaya, K., Lee, V. M., Trojanowski, J. Q., and Iwatsubo, T. (1998). Aggregation of alpha-synuclein in Lewy bodies of sporadic Parkinson's disease and dementia with Lewy bodies. *The American journal of pathology* **152**(4), 879-84.

Davie, C. A. (2008). A review of Parkinson's disease. *British medical bulletin* **86**, 109-27, 10.1093/bmb/ldn013.

Dexter, D. T., and Jenner, P. (2013). Parkinson disease: from pathology to molecular disease mechanisms. *Free radical biology & medicine*, 10.1016/j.freeradbiomed.2013.01.018.

Dunning, C. J., Reyes, J. F., Steiner, J. A., and Brundin, P. (2012). Can Parkinson's disease pathology be propagated from one neuron to another? *Progress in neurobiology* **97**(2), 205-19, 10.1016/j.pneurobio.2011.11.003.

Miller, G. W., Erickson, J. D., Perez, J. T., Penland, S. N., Mash, D. C., Rye, D. B., and Levey, A. I. (1999a). Immunochemical analysis of vesicular monoamine transporter (VMAT2) protein in Parkinson's disease. *Experimental neurology* **156**(1), 138-48, 10.1006/exnr.1998.7008.

Richardson, J. R., Caudle, W. M., Wang, M., Dean, E. D., Pennell, K. D., and Miller, G. W. (2006). Developmental exposure to the pesticide dieldrin alters the dopamine system and increases neurotoxicity in an animal model of Parkinson's disease. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* **20**(10), 1695-7, 10.1096/fj.06-5864fje.

Jenner, P. (2003). Oxidative stress in Parkinson's disease. *Annals of neurology* **53 Suppl 3**, S26-36; discussion S36-8, 10.1002/ana.10483.

Ambani, L. M., Van Woert, M. H., and Murphy, S. (1975). Brain peroxidase and catalase in Parkinson disease. *Archives of neurology* **32**(2), 114-8.

Go, Y. M., Ziegler, T. R., Johnson, J. M., Gu, L., Hansen, J. M., and Jones, D. P. (2007). Selective protection of nuclear thioredoxin-1 and glutathione redox systems against oxidation during glucose and glutamine deficiency in human colonic epithelial cells. *Free radical biology & medicine* **42**(3), 363-70, 10.1016/j.freeradbiomed.2006.11.005.

de Lau, L. M., and Breteler, M. M. (2006). Epidemiology of Parkinson's disease. *Lancet neurology* **5**(6), 525-35, 10.1016/S1474-4422(06)70471-9.

Wright Willis, A., Evanoff, B. A., Lian, M., Criswell, S. R., and Racette, B. A. (2010). Geographic and ethnic variation in Parkinson disease: a population-based study of

US Medicare beneficiaries. *Neuroepidemiology* **34**(3), 143-51, 10.1159/000275491.

Kieburtz, K., and Wunderle, K. B. (2013). Parkinson's disease: evidence for environmental risk factors. *Movement disorders : official journal of the Movement Disorder Society* **28**(1), 8-13, 10.1002/mds.25150.

Van Den Eeden, S. K., Tanner, C. M., Bernstein, A. L., Fross, R. D., Leimpeter, A., Bloch, D. A., and Nelson, L. M. (2003). Incidence of Parkinson's disease: variation by age, gender, and race/ethnicity. *American journal of epidemiology* **157**(11), 1015-22.

Wooten, G. F., Currie, L. J., Bovbjerg, V. E., Lee, J. K., and Patrie, J. (2004). Are men at greater risk for Parkinson's disease than women? *Journal of neurology, neurosurgery, and psychiatry* **75**(4), 637-9.

Taylor, K. S., Cook, J. A., and Counsell, C. E. (2007). Heterogeneity in male to female risk for Parkinson's disease. *Journal of neurology, neurosurgery, and psychiatry* **78**(8), 905-6, 10.1136/jnnp.2006.104695.

Fleming, L., Mann, J. B., Bean, J., Briggle, T., and Sanchez-Ramos, J. R. (1994). Parkinson's disease and brain levels of organochlorine pesticides. *Annals of neurology* **36**(1), 100-3, 10.1002/ana.410360119.

Baldereschi, M., Di Carlo, A., Vanni, P., Ghetti, A., Carbonin, P., Amaducci, L., Inzitari, D., and Italian Longitudinal Study on Aging Working, G. (2003). Lifestyle-related risk factors for Parkinson's disease: a population-based study. *Acta neurologica Scandinavica* **108**(4), 239-44.

Lix, L. M., Hobson, D. E., Azimaee, M., Leslie, W. D., Burchill, C., and Hobson, S. (2010). Socioeconomic variations in the prevalence and incidence of Parkinson's disease: a population-based analysis. *Journal of epidemiology and community health* **64**(4), 335-40, 10.1136/jech.2008.084954.

Hernan, M. A., Takkouche, B., Caamano-Isorna, F., and Gestal-Otero, J. J. (2002). A meta-analysis of coffee drinking, cigarette smoking, and the risk of Parkinson's disease. *Annals of neurology* **52**(3), 276-84, 10.1002/ana.10277.

Galanaud, J. P., Elbaz, A., Clavel, J., Vidal, J. S., Correze, J. R., Alperovitch, A., and Tzourio, C. (2005). Cigarette smoking and Parkinson's disease: a case-control study in a population characterized by a high prevalence of pesticide exposure. *Movement disorders : official journal of the Movement Disorder Society* **20**(2), 181-9, 10.1002/mds.20307.

Palacios, N., Gao, X., McCullough, M. L., Schwarzschild, M. A., Shah, R., Gapstur, S., and Ascherio, A. (2012). Caffeine and risk of Parkinson's disease in a large cohort of men and women. *Movement disorders : official journal of the Movement Disorder Society* **27**(10), 1276-82, 10.1002/mds.25076.

Hardy, J., Cookson, M. R., and Singleton, A. (2003). Genes and parkinsonism. *Lancet neurology* **2**(4), 221-8.

Tanner, C. M., Ottman, R., Goldman, S. M., Ellenberg, J., Chan, P., Mayeux, R., and Langston, J. W. (1999). Parkinson disease in twins: an etiologic study. *JAMA : the journal of the American Medical Association* **281**(4), 341-6.

Langston, J. W. (2002). The Impact of MPTP on Parkinson's disease Research: Past, Present and Future. In *Parkinson's Disease: Diagnosis and Clinical Management* (S. Factor, Weiner, W., Ed.) Eds.). Demos Medical Publishing.

van der Mark, M., Brouwer, M., Kromhout, H., Nijssen, P., Huss, A., and Vermeulen, R. (2012). Is pesticide use related to Parkinson disease? Some clues to heterogeneity in study results. *Environmental health perspectives* **120**(3), 340-7, 10.1289/ehp.1103881.

Daniels, J. L., Olshan, A. F., Teschke, K., Hertz-Picciotto, I., Savitz, D. A., and Blatt, J. (2001). Comparison of assessment methods for pesticide exposure in a case-control interview study. *American journal of epidemiology* **153**(12), 1227-32.

Tanner, C. M., Ross, G. W., Jewell, S. A., Hauser, R. A., Jankovic, J., Factor, S. A., Bressman, S., Deligtisch, A., Marras, C., Lyons, K. E., Bhudhikanok, G. S., Roucoux, D. F., Meng, C., Abbott, R. D., and Langston, J. W. (2009). Occupation and risk of parkinsonism: a multicenter case-control study. *Archives of neurology* **66**(9), 1106-13, 10.1001/archneurol.2009.195.

Firestone, J. A., Lundin, J. I., Powers, K. M., Smith-Weller, T., Franklin, G. M., Swanson, P. D., Longstreth, W. T., Jr., and Checkoway, H. (2010). Occupational factors and risk of Parkinson's disease: A population-based case-control study. *American journal of industrial medicine* **53**(3), 217-23, 10.1002/ajim.20788.

Hughes, A. J., Daniel, S. E., Kilford, L., and Lees, A. J. (1992). Accuracy of clinical diagnosis of idiopathic Parkinson's disease: a clinico-pathological study of 100 cases. *Journal of neurology, neurosurgery, and psychiatry* **55**(3), 181-4.

Van Maele-Fabry, G., Hoet, P., Vilain, F., and Lison, D. (2012). Occupational exposure to pesticides and Parkinson's disease: a systematic review and meta-analysis of cohort studies. *Environment international* **46**, 30-43, 10.1016/j.envint.2012.05.004.

Brown, T. P., Rumsby, P. C., Capleton, A. C., Rushton, L., and Levy, L. S. (2006). Pesticides and Parkinson's disease--is there a link? *Environmental health perspectives* **114**(2), 156-64.

Richardson, J. R., Caudle, W. M., Wang, M. Z., Dean, E. D., Pennell, K. D., and Miller, G. W. (2008). Developmental heptachlor exposure increases susceptibility of dopamine neurons to N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)in a gender-specific manner. *Neurotoxicology* **29**(5), 855-63, 10.1016/j.neuro.2008.05.007.

Manning-Bog, A. B., McCormack, A. L., Purisai, M. G., Bolin, L. M., and Di Monte, D. A. (2003). Alpha-synuclein overexpression protects against paraquat-induced neurodegeneration. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **23**(8), 3095-9.

Grube, A., Donaldson, D., Kiely, T., Wu, L. (2011). Pesticide Industry Sales and Usage. In (B. a. E. A. Division, Ed.) Eds.), pp. 41. U.S. Environmental Protection Agency, Washington, DC.

Hayes, W. J. (1975). *Toxicology of Pesticides*. Lippincott, Williams and Wilkins, New York, NY.

Zilberman, D., Schmitz, A., Casterline, G., Lichtenberg, E., and Siebert, J. B. (1991). The economics of pesticide use and regulation. *Science* **253**(5019), 518-22, 10.1126/science.253.5019.518.

Lin, B. (1995). *Pesticide and Fertilizer Use and Trends in US Agriculture*. Diane Publishing Company, Darby, PA.

Wang, A., Costello, S., Cockburn, M., Zhang, X., Bronstein, J., and Ritz, B. (2011). Parkinson's disease risk from ambient exposure to pesticides. *European journal of epidemiology* **26**(7), 547-55, 10.1007/s10654-011-9574-5.

Weisskopf, M. G., Knekt, P., O'Reilly, E. J., Lyytinen, J., Reunanen, A., Laden, F., Altshul, L., and Ascherio, A. (2010). Persistent organochlorine pesticides in serum and risk of Parkinson disease. *Neurology* **74**(13), 1055-61, 10.1212/WNL.0b013e3181d76a93.

Schaalan, M. F., Abdelraouf, S. M., Mohamed, W. A., and Hassanein, F. S. (2012). Correlation between maternal milk and infant serum levels of chlorinated pesticides (CP) and the impact of elevated CP on bleeding tendency and immune status in some infants in Egypt. *Journal of immunotoxicology* **9**(1), 15-24, 10.3109/1547691X.2011.606432.

EPA (2010). *Endosulfan Phase-Out.* Available at: http://www.epa.gov/oppsrrd1/reregistration/endosulfan/endosulfan-agreement.html2013.

Galatone, V. (2009). Endosulfan: Canada's submission of information specified in Annex E of the Stockholm Convention pursuant to Article 8 of the Convention. In (C. M. Division, Ed.) Eds.), pp. 20.

EPA, U. (2012). *Endosulfan (CASRN 115-29-7).* Available at: http://www.epa.gov/iris/subst/0235.htm.

Silva, M. H., and Gammon, D. (2009). An assessment of the developmental, reproductive, and neurotoxicity of endosulfan. *Birth defects research. Part B, Developmental and reproductive toxicology* **86**(1), 1-28, 10.1002/bdrb.20183.

Morris, A. D., Muir, D.C.G., Solomon, K.R., Teixeira, C., Epp, J., Fisk, A.T., Letcher, R., Wang, X. (2008). Current-use pesticide bioaccumulation in Canadian Artic ringed seal. *Artic Change 2008.*

Smith, A. G. (1991). Chlorinated Hydrocarbon Insecticides. In *Pesticide Toxicology* (W. J. Hayes, Laws, E. R., Ed.) Eds.), Vol. 6. Academic Press Inc., New York.

Institute, N. C. (1978). Bioassay of Endosulfan for Possible Carcinogenicity. *Technical Report Series No. 62.* 53.

Bureau, U. S. C. (2011a). *Current Population Survey (CPS): Methodology.* Available at: http://www.census.gov/cps/methodology/2013.

Poewe, W. (2006). The natural history of Parkinson's disease. *Journal of neurology* **253 Suppl 7**, VII2-6, 10.1007/s00415-006-7002-7.

Commerce, D. o. (2011). *American Community Survey*2013.

Bureau, U. S. C. (2011b). *American Community Survey: Survey Methodology Main.* Available at: http://www.census.gov/acs/www/methodology/methodology_main/2013.

Ruggles, S., Alexander, J. T., Genadek, K., Goeken, R., Schroeder, M., Sobek, M., (2010). Integrated Public Use Microdata Series: Version 5.0 [Machine-readable database]. In (U. o. Minnesota, Ed.) Eds.), Minneapolis.

Ji, B. T., Silverman, D. T., Stewart, P. A., Blair, A., Swanson, G. M., Baris, D., Greenberg, R. S., Hayes, R. B., Brown, L. M., Lillemoe, K. D., Schoenberg, J. B., Pottern, L. M., Schwartz, A. G., and Hoover, R. N. (2001). Occupational exposure to pesticides and pancreatic cancer. *American journal of industrial medicine* **39**(1), 92-9.

Steenland, K., and Armstrong, B. (2006). An overview of methods for calculating the burden of disease due to specific risk factors. *Epidemiology* **17**(5), 512-9, 10.1097/01.ede.0000229155.05644.43.

Jones, D. P. (2002). Redox potential of GSH/GSSG couple: assay and biological significance. *Methods in enzymology* **348**, 93-112.

Go, Y. M., and Jones, D. P. (2005). Intracellular proatherogenic events and cell adhesion modulated by extracellular thiol/disulfide redox state. *Circulation* **111**(22), 2973-80, 10.1161/CIRCULATIONAHA.104.515155.

Liu, J., Morrow, A. L., Devaud, L., Grayson, D. R., and Lauder, J. M. (1997). GABAA receptors mediate trophic effects of GABA on embryonic brainstem monoamine neurons in vitro. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **17**(7), 2420-8.

Tillerson, J. L., Caudle, W. M., Reveron, M. E., and Miller, G. W. (2002). Detection of behavioral impairments correlated to neurochemical deficits in mice treated with moderate doses of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine. *Experimental neurology* **178**(1), 80-90.

Richards, M. L., and Sadee, W. (1986). Human neuroblastoma cell lines as models of catechol uptake. *Brain research* **384**(1), 132-7.

Lewis, D. A., and Sweet, R. A. (2009). Schizophrenia from a neural circuitry perspective: advancing toward rational pharmacological therapies. *The Journal of clinical investigation* **119**(4), 706-16, 10.1172/JCI37335.

Nakamura, K., Sekine, Y., Ouchi, Y., Tsujii, M., Yoshikawa, E., Futatsubashi, M., Tsuchiya, K. J., Sugihara, G., Iwata, Y., Suzuki, K., Matsuzaki, H., Suda, S., Sugiyama, T., Takei, N., and Mori, N. (2010). Brain serotonin and dopamine transporter bindings in adults with high-functioning autism. *Archives of general psychiatry* **67**(1), 59-68, 10.1001/archgenpsychiatry.2009.137.

Freire, C., and Koifman, S. (2012). Pesticide exposure and Parkinson's disease: epidemiological evidence of association. *Neurotoxicology* **33**(5), 947-71, 10.1016/j.neuro.2012.05.011.

Hancock, D. B., Martin, E. R., Mayhew, G. M., Stajich, J. M., Jewett, R., Stacy, M. A., Scott, B. L., Vance, J. M., and Scott, W. K. (2008). Pesticide exposure and risk of Parkinson's disease: a family-based case-control study. *BMC neurology* **8**, 6, 10.1186/1471-2377-8-6.

MacFarlane, E., Glass, D., and Fritschi, L. (2009). Is farm-related job title an adequate surrogate for pesticide exposure in occupational cancer epidemiology? *Occupational and environmental medicine* **66**(8), 497-501, 10.1136/oem.2008.041566.

Costello, S., Cockburn, M., Bronstein, J., Zhang, X., and Ritz, B. (2009). Parkinson's disease and residential exposure to maneb and paraquat from agricultural applications in the central valley of California. *American journal of epidemiology* **169**(8), 919-26, 10.1093/aje/kwp006.

Jia, Z., and Misra, H. P. (2007a). Exposure to mixtures of endosulfan and zineb induces apoptotic and necrotic cell death in SH-SY5Y neuroblastoma cells, in vitro. *Journal of applied toxicology : JAT* **27**(5), 434-46, 10.1002/jat.1218.

Segura-Aguilar, J. (2011). Catecholaminergic Cell Lines for the Study of Dopamine. In *Cell Culture Techniques* (M. Aschner, Ed.) Eds.), Vol. 56, pp. 383-401. Springer Science and Business Media.

Cerrillo, I., Granada, A., Lopez-Espinosa, M. J., Olmos, B., Jimenez, M., Cano, A., Olea, N., and Fatima Olea-Serrano, M. (2005). Endosulfan and its metabolites in fertile women, placenta, cord blood, and human milk. *Environmental research* **98**(2), 233-9, 10.1016/j.envres.2004.08.008.

Jia, Z., and Misra, H. P. (2007b). Reactive oxygen species in in vitro pesticide-induced neuronal cell (SH-SY5Y) cytotoxicity: role of NFkappaB and caspase-3. *Free radical biology & medicine* **42**(2), 288-98, 10.1016/j.freeradbiomed.2006.10.047.

Briz, V., Molina-Molina, J. M., Sanchez-Redondo, S., Fernandez, M. F., Grimalt, J. O., Olea, N., Rodriguez-Farre, E., and Sunol, C. (2011). Differential estrogenic effects of the persistent organochlorine pesticides dieldrin, endosulfan, and lindane in primary neuronal cultures. *Toxicological sciences : an official journal of the Society of Toxicology* **120**(2), 413-27, 10.1093/toxsci/kfr019.

Rosa, R., Rodriguez-Farre, E., and Sanfeliu, C. (1996). Cytotoxicity of hexachlorocyclohexane isomers and cyclodienes in primary cultures of cerebellar granule cells. *The Journal of pharmacology and experimental therapeutics* **278**(1), 163-9.

Mittleman, G., Goldowitz, D., Heck, D. H., and Blaha, C. D. (2008). Cerebellar modulation of frontal cortex dopamine efflux in mice: relevance to autism and schizophrenia. *Synapse* **62**(7), 544-50, 10.1002/syn.20525.

Jia, Z., and Misra, H. P. (2007c). Developmental exposure to pesticides zineb and/or endosulfan renders the nigrostriatal dopamine system more susceptible to these environmental chemicals later in life. *Neurotoxicology* **28**(4), 727-35, 10.1016/j.neuro.2007.04.003.

Miller, D. B., Ali, S. F., O'Callaghan, J. P., and Laws, S. C. (1998). The impact of gender and estrogen on striatal dopaminergic neurotoxicity. *Annals of the New York Academy of Sciences* **844**, 153-65.

Miller, G. W., Kirby, M. L., Levey, A. I., and Bloomquist, J. R. (1999b). Heptachlor alters expression and function of dopamine transporters. *Neurotoxicology* **20**(4), 631-7.

Gainetdinov, R. R., Fumagalli, F., Jones, S. R., and Caron, M. G. (1997). Dopamine transporter is required for in vivo MPTP neurotoxicity: evidence from mice lacking the transporter. *Journal of neurochemistry* **69**(3), 1322-5.

Donovan, D. M., Miner, L. L., Perry, M. P., Revay, R. S., Sharpe, L. G., Przedborski, S., Kostic, V., Philpot, R. M., Kirstein, C. L., Rothman, R. B., Schindler, C. W., and Uhl, G. R. (1999). Cocaine reward and MPTP toxicity: alteration by regional variant dopamine transporter overexpression. *Brain research. Molecular brain research* **73**(1-2), 37-49.

Shelton, J. F., Hertz-Picciotto, I., and Pessah, I. N. (2012). Tipping the balance of autism risk: potential mechanisms linking pesticides and autism. *Environmental health perspectives* **120**(7), 944-51, 10.1289/ehp.1104553.

Abi-Dargham, A., Mawlawi, O., Lombardo, I., Gil, R., Martinez, D., Huang, Y., Hwang, D. R., Keilp, J., Kochan, L., Van Heertum, R., Gorman, J. M., and Laruelle, M. (2002). Prefrontal dopamine D1 receptors and working memory in schizophrenia. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **22**(9), 3708-19, 20026302.

Hashimoto, T., Bazmi, H. H., Mirnics, K., Wu, Q., Sampson, A. R., and Lewis, D. A. (2008). Conserved regional patterns of GABA-related transcript expression in the neocortex of subjects with schizophrenia. *The American journal of psychiatry* **165**(4), 479-89, 10.1176/appi.ajp.2007.07081223.

Gonzalez-Burgos, G., Rotaru, D. C., Zaitsev, A. V., Povysheva, N. V., and Lewis, D. A. (2009). GABA transporter GAT1 prevents spillover at proximal and distal GABA synapses onto primate prefrontal cortex neurons. *Journal of neurophysiology* **101**(2), 533-47, 10.1152/jn.91161.2008.

Scremin, O. U., Chialvo, D. R., Lavarello, S., Berra, H. H., and Lucero, M. A. (2011). The environmental pollutant endosulfan disrupts cerebral cortical function at low doses. *Neurotoxicology* **32**(1), 31-7, 10.1016/j.neuro.2010.12.001.

TABLES

Table 1. Job Exposure Matrix of Occupational Exposure to Pesticides

Occupation	Avg. Pesticide Probability	Avg. Pesticide Intensity	Avg. Pesticide Frequency	Summed Avg. Parameters	Ever=1; Never=0	High=1; Low=0
Farmers	3	1	4	8	1	1
Gardeners/ Landscapers	2.7	4.0	1.7	8.3	1	1
Janitor (all industries)	0.7	4.0	0.7	5.3	1	0
Food Handlers, Washers, Pickers	2.0	1.0	3.0	6.0	1	0
Waiters	3.0	1.0	3.3	7.3	1	1
Dishwashers	3.0	1.0	3.3	7.3	1	1
Maintenance, laborers and material handlers in the food industry	2.0	1.0	3.0	6.0	1	0
Butchers	3.0	1.0	3.0	7.0	1	1
Cashiers	3.0	1.0	3.0	7.0	1	1
Hotel Workers	1.7	3.0	1.0	5.7	1	0
Property Managers	1.7	3.0	1.0	5.7	1	0
Chemical Manufacturing	1.0	3.7	4.0	8.7	1	1
Teachers, Janitors	2.5	1.0	2.5	6.0	1	0
Carpet	2.7	1.0	3.0	6.7	1	1
Drug Store	3.0	1.0	1.0	5.0	1	0
Hardware Store	3.0	1.0	2.0	6.0	1	0
Carpenter	3.0	1.0	2.0	6.0	1	0
Lumber	3.0	1.0	2.0	6.0	1	0
Paper industry	1.0	1.0	1.0	3.0	1	0
Recreation workers / Golf Maintainence	3.0	2.0	3.0	8.0	1	1
Firefighters	2.0	1.0	3.0	6.0	1	0
Railroads workers	2.7	1.0	2.0	5.7	1	0
Animal maintainence	2.0	1.5	3.5	7.0	1	1

Table 2. Attributable Risk of Pesticide Exposure in Ever vs. Never Exposure

Survey	Study Source	mRR	CI	$P_{p,\ target}$	AR_p (%)
1990 Census	Van Maele-Fabry (2012)	1.28	1.03 - 1.59	0.240	6.30
	van Der Mark (2011)	1.62	1.40 - 1.88	0.240	12.74
2011 ACS	Van Maele-Fabry (2012)	1.28	1.03 - 1.59	0.240	6.54
	van Der Mark (2011)	1.62	1.40 - 1.88	0.240	13.41

Table 3. Percentage of Exposure by Level

Survey	Exposure Level	Frequency	% of Total Observations	% of Total Exposed
1990 Census	High	13,847,987	9.73	41.32
	Low	19,668,125	13.82	58.68
	All Exposed	33,516,112	23.55	100.00
2011 ACS	High	19,933,702	10.99	44.00
	Low	25,365,944	13.99	56.00
	All Exposed	45,299,646	24.98	100.00

Table 4. Total AF_p of High/Low Pesticide Exposure

Survey	Exposure Level	*i*	mRR	P_p (%)	AR_p (%)
1990 Census	High	2	1.36	9.73	3.04
	Low	1	1.20	13.82	
2011 ACS	High	2	1.36	10.99	3.27
	Low	1	1.20	13.99	

FIGURES

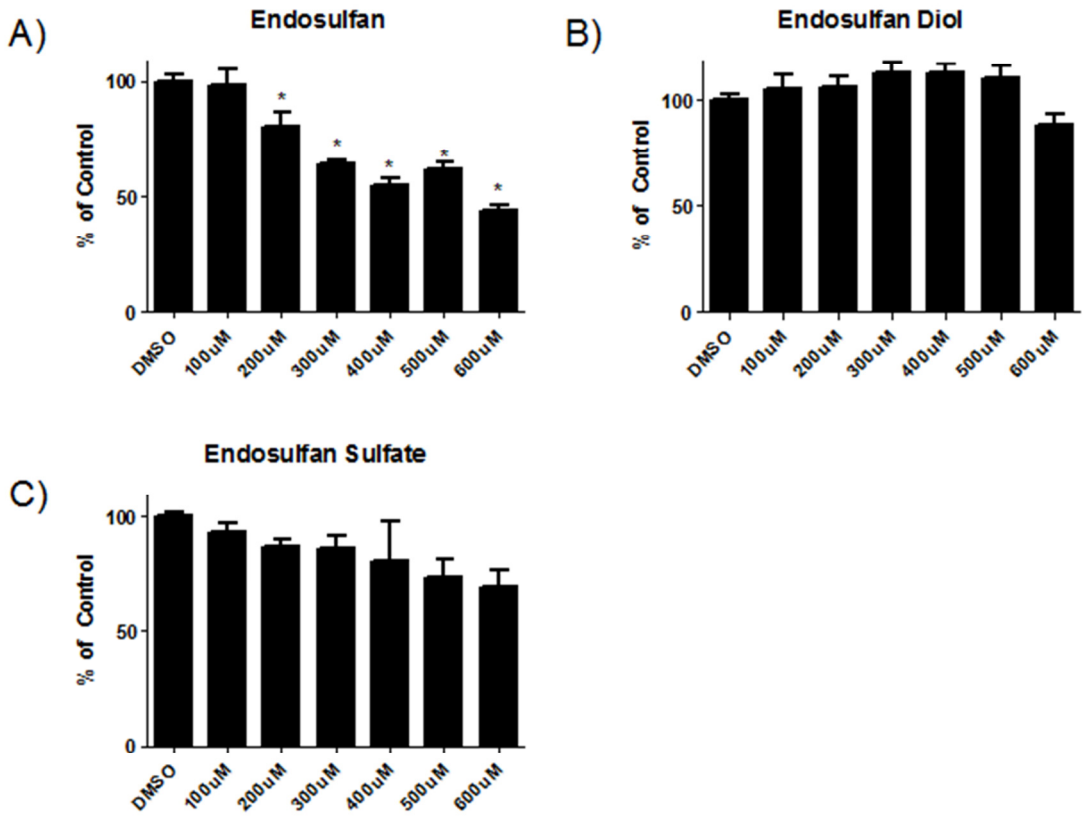

Fig. 1. 24 hour exposure to A) endosulfan significantly decreases cell viability of the immortalized, dopaminergic SK-N-SH cell line at concentrations of 200µM and greater. 24 hour exposure to B) endosulfan diol did not significantly decrease cell viability of SK-N-SH cells at concentrations up to 500µM while 24 hour exposure to C) endosulfan sulfate resulted in non-significant decrease in SK-N-SH viability at concentrations of 100µM and greater. *Indicates groups are significantly different from DMSO control by one-way ANOVA after post-hoc test at α=0.05 (n=6).

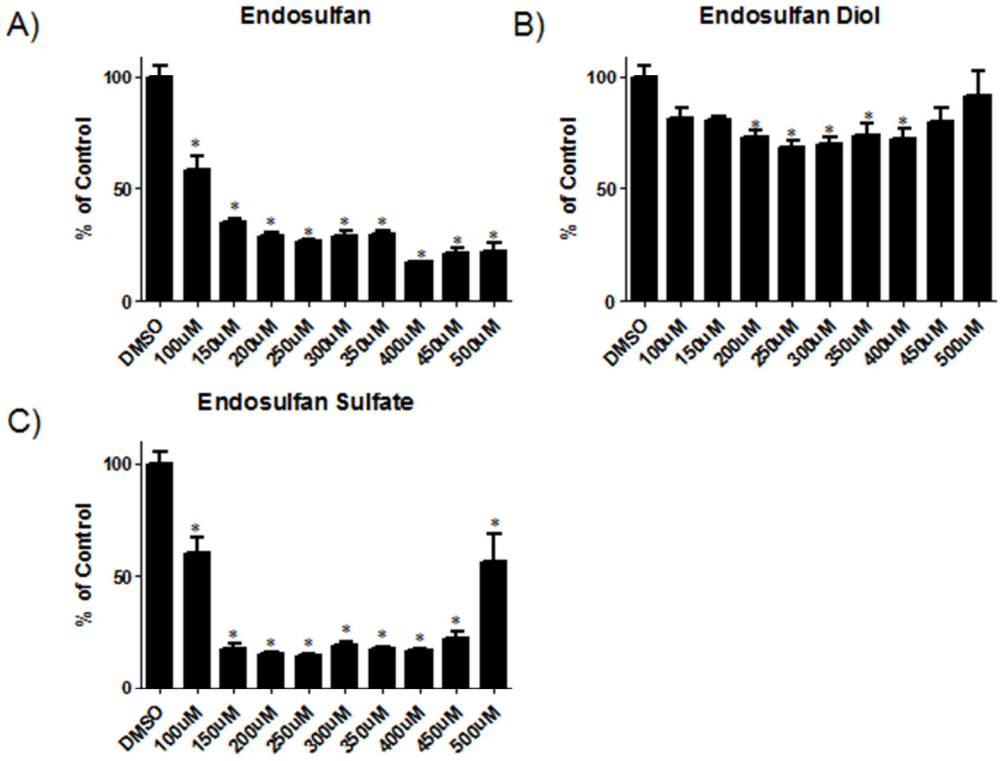

Fig 2. 72 hour exposure to A) endosulfan significantly decreases cell viability of the immortalized, dopaminergic SK-N-SH cell line at concentrations of 100µM and greater. 72 hour exposure to B) endosulfan diol significantly decreased cell viability of SK-N-SH cells only at concentrations of 200µM to 400µM. Greater concentrations of endosulfan diol did not produce significant cell death. 72 hour exposure to C) endosulfan sulfate resulted in significant decrease in SK-N-SH viability at concentrations of 100µM and greater, but witnessed a non-linear dose-response relationship as indicated by the less significant cell loss at 500µM. *Indicates groups are significantly different from DMSO control by one-way ANOVA after post-hoc test at α=0.05 (n=6).

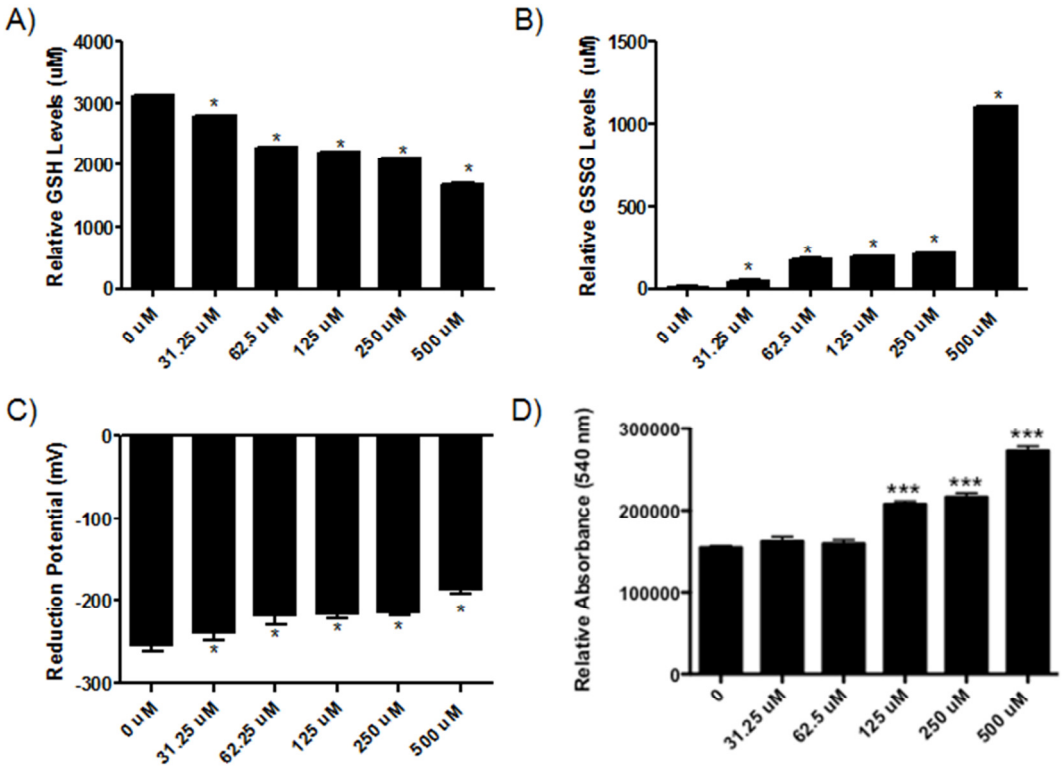

Fig 3. Effect of endosulfan exposure on SK-N-SH cellular GSH, GSSG, GSH/GSSG reduction potential (E_h) and relative ROS levels. 4 hour *in vitro* SK-N-SH exposure to 31.25, 62.25, 125, 250, and 500µM of endosulfan, respectively, results in A) significantly decreased levels of cellular GSH and B) significantly increased levels of cellular GSSG. C) Cells experienced an increased (more positive) reduction potential (E_h) for GSH/GSSG at all concentrations of endosulfan ($F=39.34$, $p=0.0001$). D) 1 hour exposure SK-N-SH exposure to 31.25, 62.25, 125, 250, and 500µM of endosulfan resulted in a significant increase in relative absorbance of ROS via DCF-DA fluorescence. *Indicates groups are significantly different from DMSO control by one-way ANOVA after post-hoc test at $\alpha=0.05$.

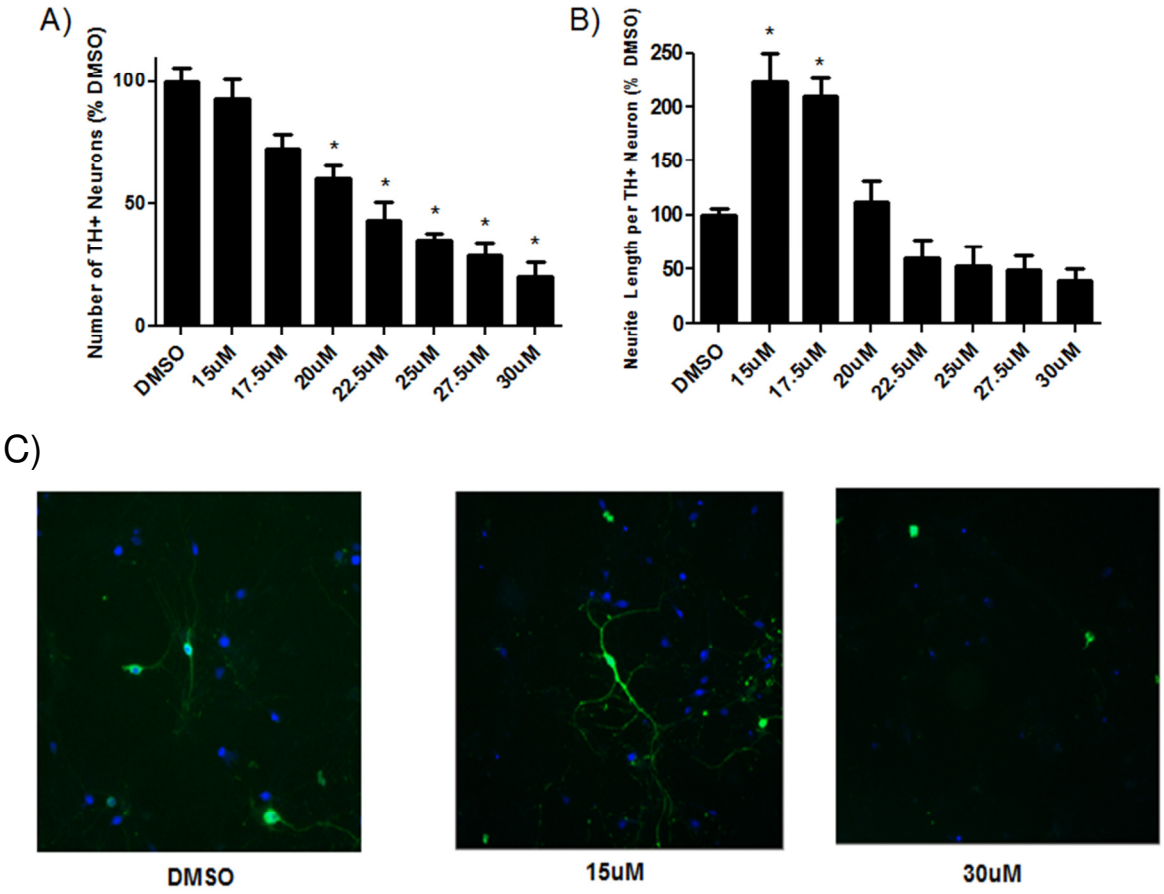

Fig 4. 24 hour exposure of ventral mesencephalic primary cultures from WT C57BL/6J mice to endosulfan shows a reduction in the number of TH + neurons. A) Treatment of ventral mesencephalic cultures from WT animals caused a significant reduction in the number of TH+ neurons at 20µM and greater when treated with endosulfan for 24 hours. Columns represent the percent change from DMSO control. Data represent the mean ± SEM of 6 replicates per treatment group. B) Treatment of ventral mesencephalic cultures from WT animals caused a significant increase in neurite length at 15µM and 17.5µM endosulfan only. *Indicates values for treatments that are significantly different from DMSO control after post-hoc test at α=0.05. C) Representative ventral mesencephalic cultures stained for TH+ from WT mice and treated with DMSO, 15µM and 30µM endosulfan.

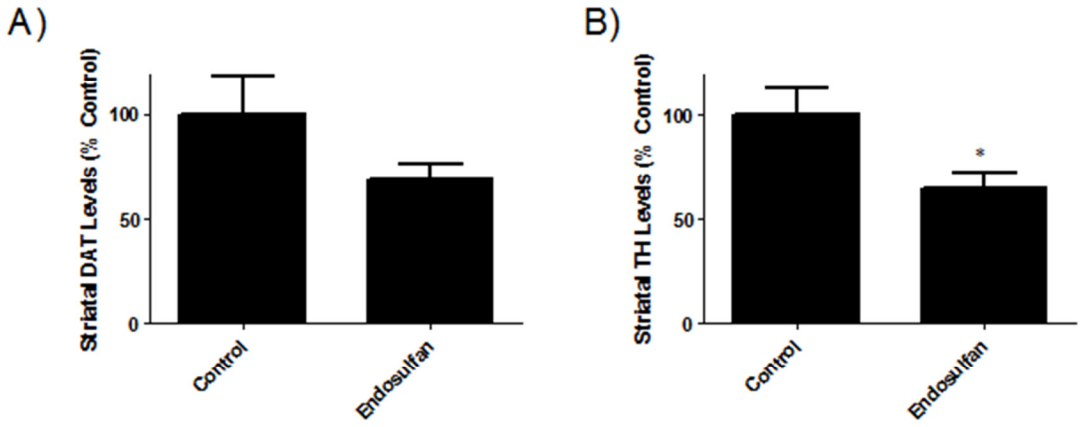

Fig 5. Striatal DAT and TH levels in WT mothers following sub-chronic exposure to 1 mg/kg endosulfan. A) Exposure of WT mice moms to endosulfan caused a non-significant reduction in striatal dopamine transporter protein concentration. B) Exposure of WT mice moms to endosulfan caused a significant reduction of striatal tyrosine hydroxylase protein concentration. Columns represent relative values (% of Control) ± SEM (6-8 animals per group). *Indicates values for treatments that are significantly different from control after one-way ANOVA analysis followed by post-hoc test at α=0.05.

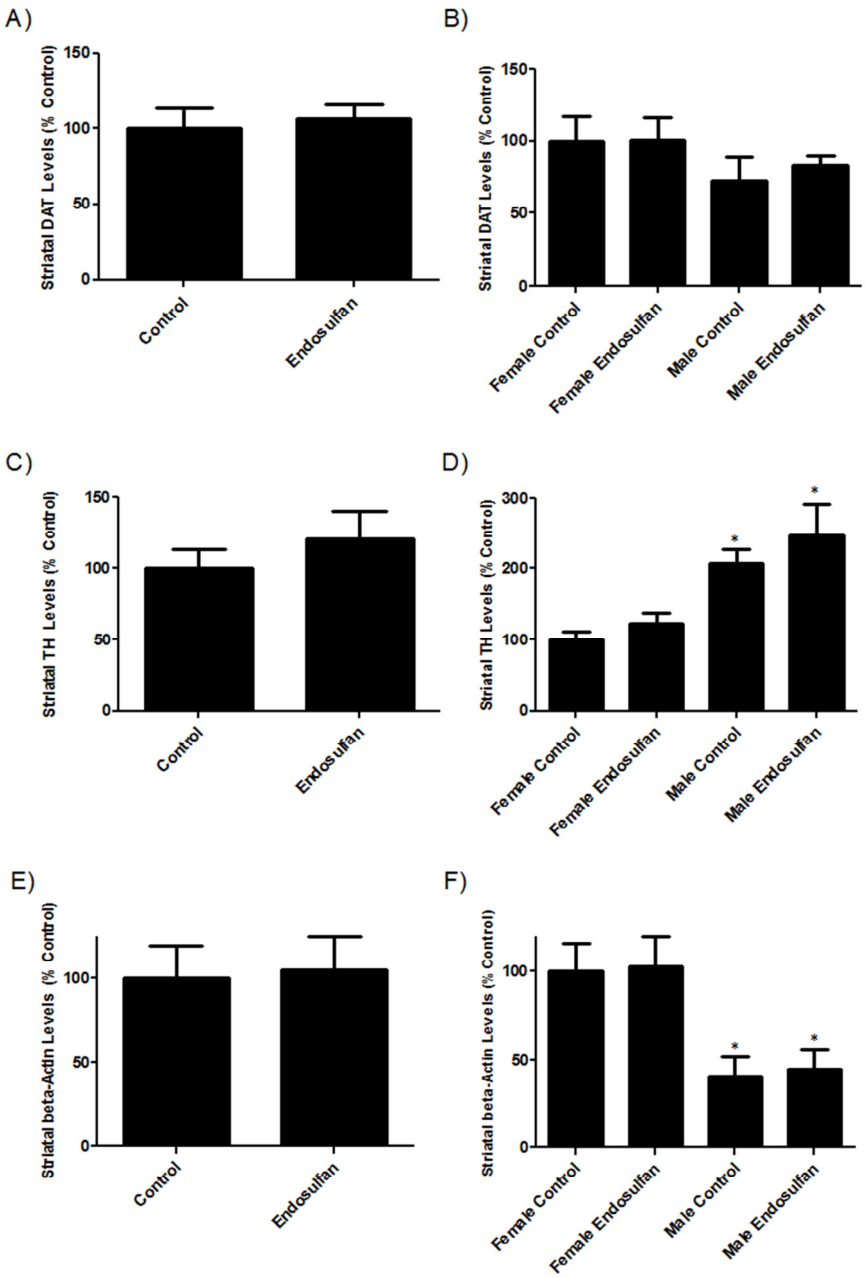

Fig 6. Developmental endosulfan exposure results in non-significant modulation of striatal dopaminergic proteins DAT and TH. Female mice were administered 1 mg/kg endosulfan throughout gestation and lactation. Levels of dopaminergic proteins were determined in striatal tissue samples in male and female offspring. A) and E) Developmental exposure resulted in non-significant modulation of DAT and β-Actin while B) stratification of treatment by sex revealed a non-significant reduction in relative DAT levels and F) a significant reduction in β-Actin in male mice compared to female mice. C) Developmental exposure resulted in a non-significant increase in striatal TH levels while D) stratification of treatment by sex revealed a significant increase in relative TH concentration compared to female control offspring but not significant difference between treatments within sex. *Indicates values for treatments that are significantly different from female control after one-way ANOVA analysis followed by post-hoc test at α=0.05

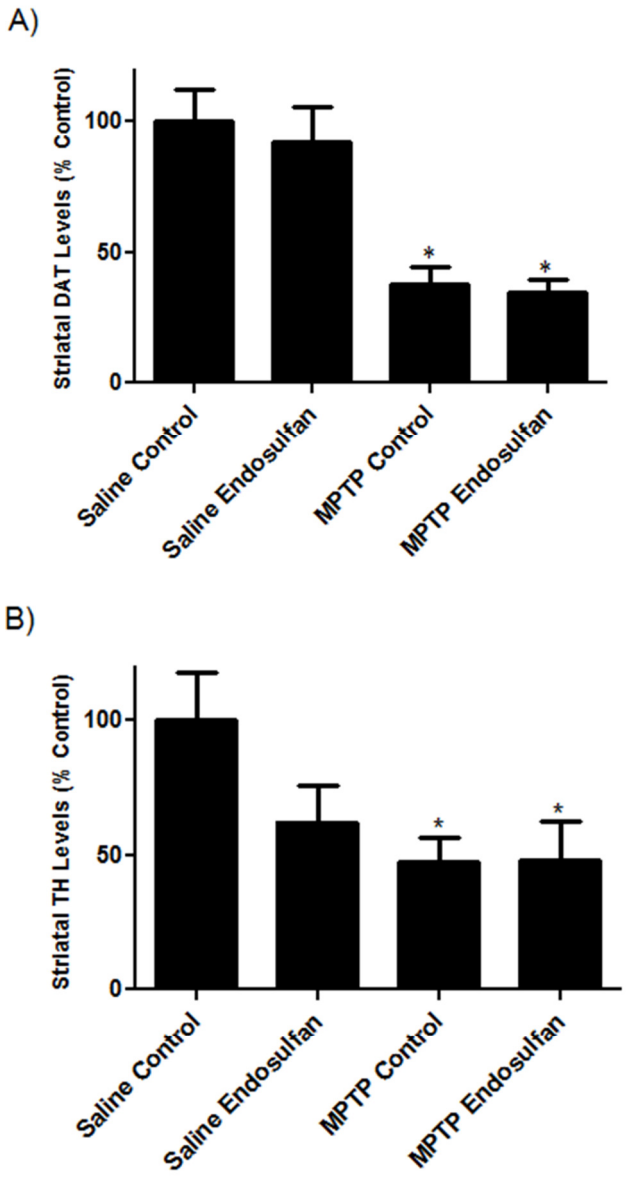

Fig 7. Developmental exposure to endosulfan does not significantly alter MPTP toxicity in the striatum compared to controls. Female mice were administered 0 (control) or 1 mg/kg endosulfan throughout gestation and lactation and striatal levels of DAT and TH protein were determined by Western immunoblot. Male and female offspring were then administered 2x10 mg/kg of MPTP s.c. and striatal levels of A) DAT and B) TH were measured. Developmental endosulfan exposure did not result in any increased reduction in striatal proteins after MPTP administration compared to unexposed offspring given MPTP. *Indicates values for treatments that are significantly different from saline-injected controls after one-way ANOVA analysis followed by post-hoc test at α=0.05

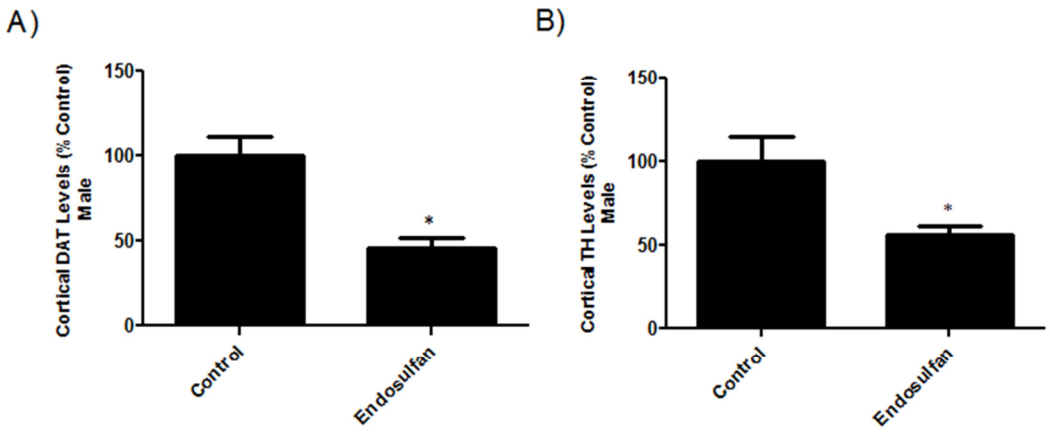

Fig 8. Developmental exposure to endosulfan significantly reduces DAT and TH in cortex of male offspring. Female mice were administered 0 (control) or 1 mg/kg endosulfan throughout gestation and lactation and cortical levels of DAT and TH protein in male offspring were determined by Western immunoblot. Developmental exposure significantly A) reduces relative DAT levels in male cortical tissue and B) reduces relative TH levels in male cortical tissue. *Indicates values for treatments that are significantly different from controls after Student's T-Test analysis followed by post-hoc test at α=0.05.

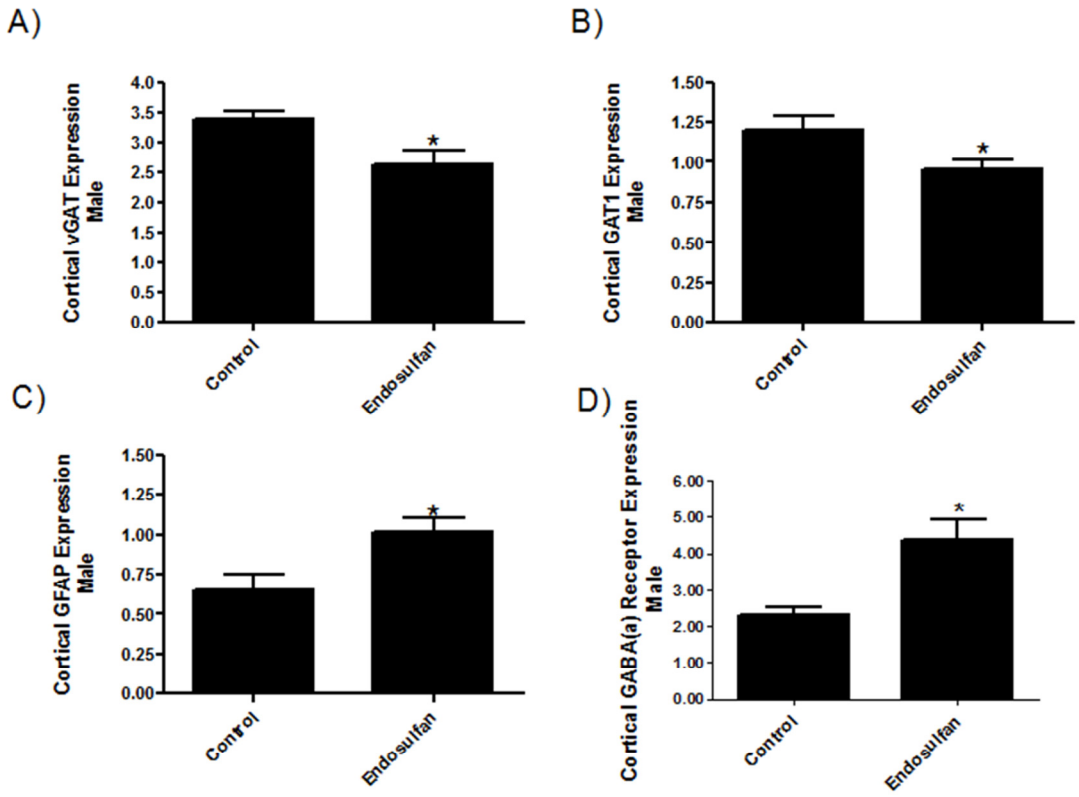

Fig 9. Developmental exposure to endosulfan significantly reduces vGAT, GAT1 expression and increases GFAP and GABA(a) receptor expression in the cortex. Female mice were administered 0 (control) or 1 mg/kg endosulfan throughout gestation and lactation. Levels of cortical vGAT, GAT1 and GFAP of offspring were determined by Western immunoblot. Developmental endosulfan exposure yielded significant decreases in cortical concentrations of A) vGAT and B) GAT1 in male offspring. Developmental endosulfan exposure also yielded a significant increase in cortical C) GFAP and D) GABA(a) receptor concentrations in male offspring. *Indicates values for treatments that are significantly different from controls after Student's T-Test analysis followed by post-hoc test at α=0.05

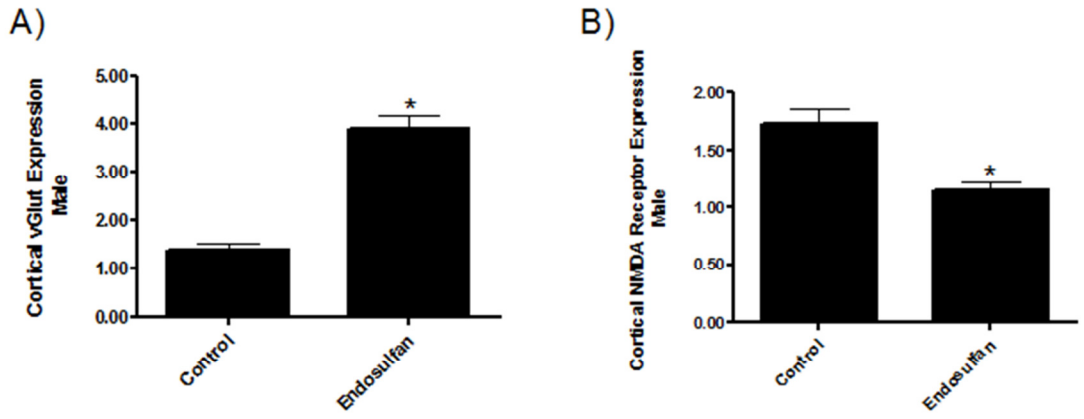

Fig 10. Developmental exposure to endosulfan significantly reduces NMDA receptor expression but increases vGlut expression in the cortex of male offspring. Female mice were administered 0 (control) or 1 mg/kg endosulfan throughout gestation and lactation and cortical levels of vGlut and NMDA receptors in offspring were determined by Western immunoblot. A) Developmental endosulfan exposure yielded significant increases in cortical vGlut. B) Developmental endosulfan exposure yielded a significant decrease in cortical NMDA receptor concentrations in male offspring. *Indicates values for treatments that are significantly different from controls after Student's T-Test analysis followed by post-hoc test at α=0.05