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Disruption of neuronal circuitry following exposure to chlorinated organophosphate flame retardants: Implications for neurological disease

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Abstract

Disruption of neuronal circuitry following exposure to chlorinated organophosphate flame retardants: Implications for neurological disease
By Rebecca M. Coleman

Introduction: Many environmental contaminants are proven or suspected to cause neurotoxicity resulting in brain damage, neurobehavioral issues, memory and motor dysfunction, decreased reaction times, and other neurological symptoms. Chemicals such as PCBs and PBDEs have been banned or phased out from use as they are suspected as neurotoxicants. TDCPP was introduced to replace these flame-retardants, and it is detected in dust as it leaches from products and accumulates in the environment. The neurotoxic potential of TDCPP is unknown.

Objective: This study is designed to determine whether chlorinated organophosphate flame retardant exposure disrupts neuronal circuitry and contributes to the onset of neurological disease.

Methods: I exposed three chlorinated organophosphate flame retardant compounds to SK-N-SH cells to assess cytotoxicity, and TDCPP was selected for further study. I exposed primary culture neurons from the ventral mesencephalon and frontal cortex to TDCPP, followed by a 30-day animal study using 30mg/kg/day TDCPP by oral gavage to adult male mice. Animals were challenged with MPTP to evaluate TDCPP's impact to the dopaminergic neurons of the striatum.

Results: Exposure of SK-N-SH cells to TDCPP resulted in a dose-dependent cytotoxicity, with significant reduction in cell viability. Primary neuron cultures from the ventral mesencephalon were more susceptible to damage by TDCPP and also showed a dose-dependent toxicity. Primary neurons from the frontal cortex were also susceptible and showed significant reduction in neurite length, whereas VMES neurite length was not affected. The animal study showed that while MPTP alone elicited a significant reduction in DAT expression in the striatum, MPTP challenge following TDCPP exposure resulted in even greater reduction in DAT, suggesting that TDCPP exposure compromises the neurons.

Discussion: This study demonstrates that exposure of *in vitro* models to TDCPP causes a dose-dependent cytotoxic effect as seen in reduction of viable SK-N-SH cells and in reduction of TH+ and MAP+ neurons in primary culture. MPTP exposure *in vivo* also results in reductions in TH and DAT, which are exacerbated by treatment with TDCPP. These results suggest that TDCPP may be a neurotoxicant and warrants further investigation to better understand pathways of exposure and neuronal damage.

Disruption of neuronal circuitry following exposure to chlorinated organophosphate flame retardants: Implications for neurological disease

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TABLE OF CONTENTS

Figures:

Introduction

Anatomy

Neurological diseases and disorders can occur when the brain is injured by chemicals that occur in the environment. These chemicals may be ingested, absorbed, or inhaled, and cause damage to the delicate systems and pathways of the brain. The dopamine system is one such susceptible system that can be affected by exposure to hazardous chemicals. Dopamine is a neurotransmitter produced in the striatum of the brain that influences mood, cognition, and motor function. Parkinson's disease and mood disorders like schizophrenia have been associated with disruption or alteration of the dopamine system as well as with exposure to industrial toxicants such as polybrominated diphenyl ethers (PBDEs) (Bradner et al., 2012; Feldman R, 1997). The exact mechanisms through which the dopamine system is damaged and these neurological symptoms occur are not entirely understood.

Dopamine is a monoamine neurotransmitter present in several brain regions, with the largest concentrations in the corpus striatum. This area of the brain receives input from the substantia nigra pars compacta and plays a critical role in the function of body movements. When this area of the brain is damaged and these dopaminergic neurons degenerate, mental illness (schizophrenia), locomotor dysfunction (Parkinson's disease), and other neurological disorders develop. Dopamine is also involved in motivation, reward, and reinforcement of behavior; many drugs of abuse act on dopaminergic synapses in the central nervous system (*Neuroscience*, 2008).

Dopamine synthesis begins with the amino acid tyrosine, which is converted by tyrosine hydroxylase (TH) into dihydroxyphenylalanine (DOPA) by addition of a hydroxyl group. A carboxyl group is then cleaved from DOPA to make dopamine. In

dopaminergic neurons, the synthesis ends here, but in other areas of the brain dopamine is converted into norepinephrine by the addition of a hydroxyl group, which then may be further converted into epinephrine by addition of a methyl group. Dopamine is synthesized in the cytoplasm of the presynaptic terminal, loaded into synaptic vesicles via the vesicular monoamine transporter 2 (VMAT2), and released into the synapse. Dopamine is then taken up into nerve terminals by sodium-dependent dopamine transporters (DAT). Substances such as cocaine produce psychotropic effects by binding to DAT and inhibiting its action, increasing the amount of dopamine in the synapse, resulting in overstimulation of the nerves (*Neuroscience*, 2008).

The various pathways through which the dopaminergic system may be damaged are not well understood, but one hypothesis is that injury involves a mechanism of oxidative stress. Electron transfer reactions can convert molecular oxygen to hydrogen peroxide and other free radicals, which are extremely reactive with other compounds. These reactions can result in extensive cell injury and death, as is seen in many neurodegenerative conditions (Feldman R, 1997). Loss of glutathione (GSH) and oxidative damage are thought to be an early signal to events leading to apoptotic cell death. Depletion of mitochondrial GSH has also been shown to increase generation of reactive oxygen species (ROS), disruption in mitochondrial membrane potential, and subsequent loss of mitochondrial function, also leading to cell death. There is also evidence that a reduction in GSH is a normal part of aging, which leads us to believe that a more raid depletion of GSH could lead to the onset of neurodegenerative diseases. Previous work has also shown evidence that dopamine depletion resulting from damage to dopamine neurons causes an increase in dopamine production from the remaining

neurons, which results in increased demand and use of GSH, leading to increased formation of glutathione disulfide (GSSH) and ROS, potentially causing further neurodegeneration (Schulz, Lindenau, Seyfried, & Dichgans, 2000). Glucose and glutamine are also major metabolic fuels in cultured cells. Removal of intracellular glucose results in depletion of cellular ATP, which results in a chain of events leading to apoptosis. Glutamine is also important for the metabolism of rapidly dividing cells and GSH synthesis. Reduced glutamine results in energy depletion, which leads to lower GSH concentration and increases incidence of apoptosis (Go et al., 2007).

Models of neurodegenerative diseases have been developed to better understand how they occur and develop. 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) was "accidentally" discovered as one of these useful models when a group of heroin addicts injected themselves with a meperidine analog mistakenly contaminated with MPTP during synthesis. Dozens of these addicts developed, within hours to days, irreversible symptoms of Parkinson's disease, eventually becoming completely rigid and immobile. Autopsy later revealed the substantial degradation of dopaminergic neurons of the substantia nigra pars compacta; these neurons were also seen to continue degrading years after exposure. While the mechanisms of action are not identical, MPTP is a widely used inducer of Parkinson's symptoms in animals and is used to investigate Parkinson's pathogenic mechanism, as well as the potential for other environmental and occupational exposures to cause similar damage (*Casarett and Doull's Toxicology: The Basic Science of Poisons*, 2008).

Environmental Exposures

Many environmental contaminants have been shown or are suspected to cause neurotoxicity. Neurotoxicity can result in brain damage, neurobehavioral issues, memory and motor dysfunction, decreased reaction times, and many other neurological symptoms (London et al., 2012). Many halogenated organophosphate compounds, such as pesticides and flame retardants, are found to cause these problems in children and adults (London et al., 2012). Flame retardant chemical additives are used to reduce flammability of resins and polymers found in furniture, mattresses, various electronics, car seats, strollers, and some baby clothing. Because these are additive chemicals and are not integrated into the products, the flame retardant compounds are able to leach out of the products and into the environment in detectable amounts. Exposure to flame retardants occurs frequently through direct contact, as well as leaching of the compounds from protected materials into the environment (Alaee, Arias, Sjodin, & Bergman, 2003; Stapleton et al., 2009). Toxicological information on many of these compounds is unfortunately limited. Thousands of chemicals are continually and repeatedly introduced into the environment through their production, distribution, use, breakdown, disposal, and recycling, many of which have unknown fates and effects on the environment, including plants, animals, and humans. Some of these chemicals have recently been recognized for their potential harm and persistence, and are thus becoming the focus of toxicological studies.

Various industrial toxicants have been identified as potentially neurotoxic and were subsequently banned from use. PCBs were commonly used as flame retardants, and in oils and paints, until it was banned in 1977 for carcinogenicity and other reproductive, immune, nervous, and endocrine system effects (Environmental Protection

Agency, 2013b). In the 1990s studies showed that PCB exposure for twenty weeks significantly reduced basal ganglia dopamine concentrations in nonhuman primates. Experiments using similar concentrations of PCBs and then removing the animals from the exposure for almost a year showed that the reduced dopamine concentrations did not improve, even though the serum PCB levels were dramatically lower, suggesting long-term, potentially permanent changes in dopamine function (Seegal, 2003). Another study in rodents found that exposure to the PCB congener Aroclor induced a marked disruption of dopamine function in the striatum, as well as a dose-dependent reduction in DAT and VMAT2 levels (Caudle et al., 2006). The congener profile and concentrations in this study were similar to those seen in postmortem human brain samples (Hatcher-Martin et al., 2012).

When PCBs were banned from manufacture in the late 1970s, PBDEs began to replace PCBs as flame retardant additives in manufacturing of electronic equipment, carpeting, furniture, and home insulation. As before, little to no information existed about PBDE toxicity. PBDEs were found to leach into the environment, bioaccumulating and causing adverse effects in animals. They were subsequently phased out using both mandatory and voluntary bans, but PBDE congeners are still commonly detected in various products, environments, and humans (Environmental Protection Agency, 2013a). Previous work has associated detection of PBDEs in breast milk to incidence of cryptorchidism and low birth weight (Chao, Wang, Lee, Wang, & Papke, 2007). Another found an association between PBDEs in household dust and hormonal abnormalities in men (Meeker, Johnson, Camann, & Hauser, 2009). A more recent study addressed the effects of a specific congener of PBDE, called DE-71, on neuronal cells *in*

vitro and *in vivo*. DE-71 exposure was found to elicit significant reductions in DAT and VMAT2 expression in the striatum, as well as decreases in striatal dopamine and in locomotor activity. Animals that were deficient in VMAT2 (a key transporter of dopamine) were also used and were found to have a greater decrease in striatal dopamine and locomotor activity than those with normal VMAT2. These results provide further evidence that this neurotoxicity is a result of damage to the striatal dopamine system by the PBDE congener DE-71 (Bradner et al., 2012).

Chlorinated organophosphate compounds (Cl-OPs) were introduced to replace some uses of PBDEs. However, the biological effects of Cl-OP exposure have not been extensively studied. These compounds are of extreme interest as they display similar physiochemical properties as PBDEs which are known to disrupt the nervous system (Dishaw et al., 2011; Marklund, Andersson, & Haglund, 2005). Children are of great concern for exposure because the compounds tend to accumulate in dust, and children spend more time on or near the floor, put items in their mouths, and ingest the compounds. Since the phase-out of PBDEs, replacement flame retardant additives have been widely implemented in a wide range of products, with little to no research done to determine toxicity or safety of such compounds.

Several studies have assessed the occurrence of Cl-OP flame retardants in furniture and its subsequent detection in household dust. In one such study, 26 samples of foam from household sofas, chairs, mattress pads, pillows, and sound proofing equipment were tested for flame retardant compounds using gas chromatography coupled with mass spectrometry. Of these, 24 contained tris(1,3-dichloro-2-propyl) phosphate (TDCPP), and four contained tris(2-chloropropyl) phosphate (TCPP) in

concentrations similar to those previously reported for PBDEs in polyurethane foam, indicating that these two compounds are indeed common replacements for PBDE flame retardants. TDCPP is structurally similar to tris(2,3-dimbromopropyl) phosphate (Tris-BP), which was banned from use in children's clothing in 1977 when it was suspected as a mutagenic, carcinogenic, and absorbed by the children wearing the clothing (Stapleton et al., 2009).

Other common environments have also been considered as potential routes for concentrated flame retardant exposure. One study evaluated indoor environmental flame retardant and plasticizer concentrations. Acknowledging the bulk application of organophosphate flame retardants in various resins, textile finishes, polyesters, and fluids, samples were taken from inside homes, day care centers, office buildings, prisons, hospitals, stores, and other public buildings. OP flame retardants and plasticizers were detected in various amounts in all samples. Chlorinated OPs were the most abundant and were also found in air samples. TDCPP was found in the highest levels in the hospitals, presumably because of its use in mattress materials (Marklund et al., 2005). Allen et al. more recently tested 19 commercial airplanes (manufactured from 1986-2008, from five different manufacturers) for flame retardant chemicals. In 100% of the dust samples collected from carpets and air vents on the planes, flame retardant chemicals including PBDEs and TDCPP were detected in significant amounts (Allen et al., 2013). All of the findings are concerning and support the need for research to determine whether these OP flame retardant compounds are neurotoxic.

Few studies have investigated the toxic effects of TDCPP. One used PC12 cells, which are often used to examine *in vitro* neurotoxicity by reproducing key mechanisms

and features of *in vivo* developmental toxicity of OP pesticides, and found a significant dose-dependent reduction in DNA synthesis, indicating a decrease in cell viability. This study also compared TDCPP's effects to those of chlorpyrifos (CPF), a known neurotoxicant, and concluded that similar *in vitro* exposures would likely result in similar cognitive and behavioral deficits as seen with CPF. (Dishaw et al., 2011) Another study examined exposure of primary avian neuronal cells to TDCPP and TCPP, as both compounds have been detected in numerous free-living avian species. TDCPP was found to decrease the viability of cells at concentrations greater than 10uM (Crump, Chiu, & Kennedy, 2012).

The Environmental Protection Agency has an extensive, yet still incomplete, report of the hazards of TDCPP. It has shown evidence of acute oral, and dermal toxicity, as well as inhalational, dermal, and eye irritation upon acute exposures in various animals. It is characterized by existing data to be a reproductive toxicant, to interfere with prenatal development, to be neurotoxic, to have chronic toxicity over prolonged exposure, and to cause gene mutations and DNA damage *in vivo*. These hazards are of great concern, given the ample evidence presented in the exposure of humans, and particularly children, to this compound and others like it. This thesis project will provide further support for the continued thorough research of polychlorinated flame retardant chemicals and will support the need for the development and use of safer products (Environmental Protection Agency, 2005).

This project has provided information on the neurotoxicity of compounds presently used in flame retardants whose toxicity to humans is unknown. It has also given

me the opportunity to learn and improve upon a wide array of important laboratory skills that will be essential to my future career as a public health researcher.

Methods

Selection of TDCPP for treatment. Polychlorinated organophosphate compounds are suspected neurotoxins; previous work has shown that many of these compounds are consistently found in dust samples in significant amounts (Stapleton et al., 2009). We chose three similar and often detected compounds, tris(1-chloropropyl)phosphate (TCPP), tris(2-chloroethyl)phosphate (TCEP), and tris(1,3-dichloro-2-propyl)phosphate (TDCPP) (Figure 1), for treatment with culture and primary cells to assess cytotoxicity.

Cytotoxicity assay. Human-derived neuroblastoma SK-N-SH cells were cultured in DMEM/F-12 media (1:1 mixture) with 10% fetal bovine serum (FBS) and 1% Penicillin streptomycin on culture dishes. Cells were incubated at 37°C with 95% oxygen and 5% carbon dioxide. Media was changed every 3-4 days and cells were split when confluence exceeded 90%. Cells were seeded at a density of 40,000 cells per well in a 96-well plate, incubated for 24-hours, then treated with varying concentrations of TDCPP prepared in drug culture media, as well as DMSO as a control, for 24 hours. A WST-1 colorimetric assay was used to determine cytotoxicity; cells were treated with cell proliferation reagent WST-1 for three hours, and absorbances were measured on a Biotek Epoch plate reader at the one and three hour marks.

Primary culture. Primary cells were collected as described by (Bradner et al., 2012). Ventral mesencephalic and cortical neuron cultures were prepared from postnatal mice (postnatal day 1-3). Mouse brains were dissected in ice cold Hibernate A

supplemented with B27. Relevant regions were isolated, meninges removed, and tissue pieces were chemically treated with a dissociation solution containing Papain (1mg/ml), Dispase II (1.2U/ml), and DNase 1 (1ul/ml) dissolved in Hibernate A- Calcium for 20 min at 37°C and gently agitated every 5 min. Tissue was rinsed in plating media containing Neurobasal-A, 10% heat inactivated fetal bovine serum, pen-strep, and mechanically dissociated using gentle trituration. Cells were plated on poly-d-lysine pre-coated 96-well plated at 40,000 cells per well. Plating media was removed and immediately switched to Neurobasal-A *in vitro*. Culture media containing aphidicolin (1ug/ml) was added the following day to reduce proliferation of glial cells in culture. Approximately one-half of the culture media from each well was replaced every 4 days. Cultures were treated on day 8 with varying concentrations of TDCPP dissolved in cell culture media, incubated for 1, 4, or 6 days, and then fixed in 4% paraformaldehyde (PFA).

Immunocytochemistry. Primary cell cultures were fixed in preparation for staining with 2% paraformaldehyde (PFA) solution and then in 4% PFA. Tissue was then blocked in a 1% NGS, 0.1% Triton X-100, 1% BSA, 1X TBS solution, rinsed with TTBS (1X TBS + 0.1% Triton), and incubated with primary antibodies (anti-mouse TH, anti-rabbit synapsin, and anti-chicken MAP2) at 4°C overnight. Tissue was then rinsed in TTBS and incubated in fluorescent secondary antibodies in a 1% NGS, 1% BSA, 0.1% Triton X-100 TBS solution at a dilution of 1:400. Cultures were then analyzed using an Array Scanner.

Detection of Reactive Oxygen Species. Procedure performed as previously described (Go et al., 2007). SK-N-SH cells were treated with TDCPP and examined by

HPLC with fluorescence detection for GSH and GSSG quantification. For reactive oxygen species (ROS) detection by DCF oxidation, cells were cultured in 96-well plates with or without glucose- and glutamine-free media for 1 hour and DCF fluorescence was measured.

Animal study. Procedure performed as previously described by (Bradner et al., 2012). Twenty male C57BL/6J mice were acquired and split randomly into two groups of ten for control and of ten for treatment (purchased from Charles River). Mice were housed in a 12:12 light-dark colony with food and water 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 approved by the Institutional Animal Care and Use Committee at Emory University. Control mice received oral gavage of 25uL corn oil for 30 days; treatment mice received oral gavage of 30 mg/kg TDCPP dissolved in corn oil vehicle for 30 days. Oral exposure was chosen because humans are commonly exposed to TDCPP through the incidental ingestion of contaminated dust (Stapleton et al., 2009). Mice were sacrificed one day after the last dose and brain tissue was collected and immediately frozen in liquid nitrogen, and stored at -80°C until analysis.

1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). MPTP injections were administered to five control and five treatment mice immediately following the 30-day TDCPP dosing regimen. Mice received subcutaneous injections of saline or 10mg/kg of MPTP 12 hours apart. Animals were sacrificed one week after the second MPTP injection and tissue from the striatum and cortex was collected for analysis.

Western Blot Analysis. Procedure was performed as previously described by (Bradner et al., 2012). Tissue from striatum was briefly homogenized to prepare for Western immunoblotting. Samples were run on a polyacrylamide gel electrophoresis on a NuPAGE 10% Bis-Tris gel (Invitrogen, Carlsbad, CA). Samples were electrophoretically transferred to a polyvinylidene difluoride (PVDF) membrane. Non-specific sites were blocked with 7.5% nonfat dry milk in Tris-buffered saline solution. Antibodies for DAT, TH, and tubulin were used to determine the expression of each protein in tissue. Membranes were incubated overnight in monoclonal primary antibodies for each of the proteins of interest. Primaries were detected using anti-rat, -mouse, and –rabbit horseradish peroxidase secondary antibodies and enhanced chemiluminescence. Luminescence signal was captured on a Biorad Chemidoc.

Immunohistochemistry. Tissue staining and cell counts were performed as described previously (Caudle, et al., 2007). Briefly, striatal sections from TDCPP-treated mice were immersion fixed in 4% paraformaldehyde and serially sectioned at 40 um. Sections were incubated with a monoclonal anti-DAT overnight and then incubated in a biotinylated goat anti-rabbit secondary antibody for 1 hour at room temperature. Visualization was performed using DAB for 1 min 45 sec at room temperature. After DAB sections were dehydrated and coverslipped. Relative intensity of regions of interest was measured.

RESULTS

The purpose of this study is to assess the potential neurotoxicity of the chlorinated organophosphate flame retardant additives. The three most prominent Cl-OP compounds

as determined by previous studies, including TCPP, TCEP, and TDCPP, were exposed individually to a neuroblastoma cell line. These findings were elaborated to investigate TDCPP exposure to primary cultured neurons from the ventral mesencephalon and frontal cortex of postnatal pups. Finally, the dopaminergic effects of TDCPP were assessed in mice exposed to 30mg/kg TDCPP for 30 days, followed by a challenge with the dopaminergic neurotoxin MPTP.

The three most commonly detected Cl-OP compounds are TCPP, TCEP, and TDCPP. I initially sought to investigate the comparative neurotoxicity of these three compounds to SK-N-SH neuroblastoma cells, which retain many characteristics of dopamine neurons. Cytotoxicity was assessed by the WST-1 cell viability assay. Cells treated with increasing concentrations of TDCPP showed a dose-dependent significant increase in toxicity compared to DMSO control treatment. Figure 1A and B demonstrates that with TCPP and TCEP treatments, no significant reduction in cell viability occurs. In Figure 1C a 20% reduction in the number of viable cells is seen at 125uM TDCPP; at 175uM TDCPP, a 67% reduction; and at 225uM TDCPP, a 90% reduction in viable cells is seen. Cells were also treated for 24 hours and 72 hours, but no apparent difference in toxicity was observed (data not shown). These results implicate TDCPP as a neurotoxicant that warrants further investigation.

Potential mechanisms involved in TDCPP-mediated cytotoxicity using several oxidative stress endpoints in SK-N-SH cells were examined next. First, a general measure of oxidative stress was conducted using HPLC to quantify GSH and GSSG in the striatum. GSH is oxidized to GSSH by hydrogen peroxide (H_2O_2), a reactive oxygen species (Feldman R, 1997). In Figure 2A a significant reduction ($p=0.32$) is seen in the

oxidation of GSH to GSSG, as compared to DMSO control. A significant decrease ($p=0.0006$) in striatal GSH is seen in Figure 2B, which may indicate an early step in the process leading to apoptosis. DCF fluorescence is an assay that uses DCFH oxidation to DCF and fluorescence to measure the presence of ROS generation; Figure 2C shows a significant increase of almost 50% ($p<0.0001$) in DCF fluorescence as compared to DMSO control, indicating an increase in reactive oxygen species detected. These results taken together point to an increase in presence of reactive oxygen species in the cells, which is thought to lead to antioxidant defense and eventual cell damage and/or death.

In order to determine if TDCPP was selectively toxic to the dopamine system, cells cultured from the dopamine-rich ventral mesencephalon were treated for 24 hours with varying concentrations of TDCPP. Stereological software was used to count ventral mesencephalic (VMES) neurons stained for TH and MAP2. A significant decrease in cell viability is seen by significant decrease in the number of VMES neurons expressing TH beginning at 20uM TDCPP and MAP2 beginning at 40uM TDCPP compared to DMSO control (Figure 4A and B). No significant effect is seen on the length of TH+ or MAP2+ neurite length, suggesting that TDCPP has a cytotoxic effect overall, but does not initially impact the length of the neurites. These primary treatments were also at much lower concentrations than the neuroblastoma cell treatments (significant reduction seen at 20uM and 40uM in primaries, as opposed to 125uM in SK-N-SH cells), indicating that primary cells are much more vulnerable to harm by TDCPP.

The effects of TDCPP on the frontal cortex, a non-dopaminergic brain region, were investigated. Significant reduction in cortical neurons expressing MAP2 was seen in neurons exposed to 40uM TDCPP and greater (compared to DMSO control).

Interestingly, significant reductions in neurite length were observed at lower concentrations (20um), suggesting an increase vulnerability of the neuronal processes relative to the neuronal cell body. Experiments using longer exposure times (48 and 72 hours) were also conducted with no apparent change in toxicity observed (data not shown). These observations in VMES and cortical neurons suggest that dopaminergic and non-dopaminergic neurons are susceptible to damage by TDCPP exposure. They also suggest that the mechanism of action of TDCPP varies between the brain regions, as reduction in neurite length was seen in cortical neurons but not in VMES neurons.

Given the evidence supporting selective toxicity to the dopamine system provided by primary culture results, the impact of TDCPP on the dopaminergic system was analyzed using Western Blot of tissue from animals treated with TDCPP for 30 consecutive days. The effects were obtained using various antibodies for DAT (70 kDA) and TH (60 kDA). Tubulin (50 kDA) was included to ensure even loading and to normalize during quantification (data not shown). Western blot analysis determined that, compared to vehicle control (corn oil) treatment, TDCPP treatment alone did not elicit a significant change in expression of striatal DAT or TH. Following the 30-day TDCPP experiment, an MPTP challenge was also conducted on half of the animals to further investigate the damage elicited by TDCPP. Western blot analysis showed that control treatment followed by MPTP treatment resulted in a 12.9% reduction in TH expression and a 24% reduction in DAT expression. MPTP following TDCPP treatment resulted in 64% reduction in DAT expression, as well as a 44% reduction in TH expression (Figure 6A and B).

To further verify the results from the western blotting, immunohistochemistry was performed on striatal brain slices from the animals to determine the impact of TDCPP exposure on the dopaminergic system. Slices were stained for DAT (Figure 7) and relative intensity of the regions of interest was performed. The graph showing the relative intensities provides further validation for the reduction in DAT expression we can visually see in the striatal slices. These results are consistent with a decrease in striatal dopaminergic neuron expression following exposure to MPTP, and this reduction appears to be exacerbated by prior exposure to TDCPP. In the striatum there is a significant decrease in expression of DAT after treatment with TDCPP/MPTP (65% reduction, $p<0.05$) compared to both saline control treatment and TDCPP control, indicating an effect of MPTP on striatal DAT that is exacerbated by TDCPP.

DISCUSSION

This study was conducted to determine the neurotoxicity of polychlorinated organophosphate flame retardants, which have been introduced to replace PBDEs in many industrial and commercial uses. This is the first time these effects have been investigated using SK-N-SH cells and primary mouse neurons, as well as an animal model. Using the available literature, three such compounds were found to be present in most environmental assessments and in significant concentrations, and were thus chosen for preliminary comparison. This study demonstrates that exposure of *in vitro* models to TDCPP causes a dose-dependent cytotoxic effect as seen in reduction of viable SK-N-SH cells and in reduction of TH+ and MAP2+ neurons in primary culture. MPTP exposure *in*

vivo is also seen to result in reductions in TH and DAT, which are exacerbated by treatment with TDCPP.

Initially seeking to evaluate the effects of TDCPP on dopaminergic neurons, exposure of SK-N-SH cells was examined first. Similar to previous studies using PC12 cells and TDCPP, as well as a study investigating TDCPP exposure to avian neuronal cells, a dose-dependent reduction in cell viability was observed. While acknowledging that the cells used in this study are different from PC12 and avian cells, I was interested to find that they elicited similar responses at similar exposure levels. In my study, however, a series of experiments using SK-N-SH cells and various concentrations, as well as various lengths of time (24, 48, and 72 hours), did not elicit a significant toxic effect from TCEP or TCPP. TDCPP elicited a significant reduction in cell viability at 125uM and higher, and was chosen for further study.

Oxidative stress is suspected to play an important role in neuronal damage. Three measures of oxidative stress were examined here, including glutathione, glutathione disulfide, and the DCF fluorescence assay. Cellular GSH is reduced by the reactive oxygen species hydrogen peroxide to GSSH, and reduced GSH is thought to be an essential step in a cascade leading to apoptosis. GSH depletion is also seen in Parkinson's disease, although the specific mechanism through which these changes occur is not well understood (Schulz et al., 2000). The GSH redox potential was found to decrease, showing a decrease in GSH and increase in GSSH, thus indicating the presence of reactive oxygen species. Striatal GSH levels decreased by 23% after TDCPP exposure as compared to DMSO control. The DCF assay showed an almost 50% increase in ROS after TDCPP treatment as compared to DMSO control. All of these measurements

suggest that significant changes in reactive oxygen species production are occurring following TDCPP exposures that are involved in dopaminergic cell damage.

Having established an effective dosing paradigm, investigation continued with cultured primary ventral mesencephalic neurons by measuring TH+ and MAP2+ neurons after exposure. TDCPP elicited a significant reduction in expression of both TH+ neurons at a lower concentration than was seen in SK-N-SH cells. TH is the enzyme that catalyzes the first step of dopamine and is used to identify all dopamine neurons, in culture. MAP2 is a microtubule-associated protein found in dendrites and cell bodies, and detection of MAP2 is used to identify all neuronal populations. TDCPP exposure to VMES neurons resulted in significant reduction of MAP2+ neurons at 40uM. However, no reduction was seen in the length of TH+ or MAP2+ neurites in the VMES, suggesting that a mechanism of action involving a location other than the synapse of the neurons is involved. These results also indicate that the primary VMES neurons are much more sensitive to such exposure, compared to the SK-N-SH cell model. Primary culture neurons are fully functioning and thus present more opportunities and mechanisms for toxicants to disrupt. In contrast, SK-N-SH cells are much simpler and are thus not as susceptible to damage by TDCPP.

Exposure to cortical neurons, which are from a non-dopaminergic area of the brain, resulted in a significant reduction in MAP2+ neurons exposed to 40uM TDCPP, which was similar to the reduction of MAP2 expression in the VMES. In the cortex, however, there was also a significant reduction in neurite length at 20uM TDCPP, whereas the VMES neurite length was not significantly affected at any concentration measured. These results could point to a vulnerability of the neuronal processes of the

cortex compared to the VMES. The differences between the neurite length effects may also suggest a mechanism of action somewhere on the cell body in the VMES rather than in the synapse, which is perhaps different from action in the cortex. This is the first experiment to report these findings, so further study is needed to verify these results. Neurotoxicity of TDCPP should be explored further using other methods of cell damage detection and quantification.

In the present study, a 30-day *in vivo* study demonstrated that oral exposure to TDPP did not elicit a significant reduction in striatal expression of DAT or TH compared to control as measured by Western blotting (Figure 6). TDCPP exposure followed by an MPTP challenge resulted in a significant reduction in DAT expression as compared to control. Striatal damage was further analyzed using immunohistochemistry using staining for DAT and quantitative comparison of relative intensity of the regions of interest (ROI) (Figure 7). Treatment with saline followed by MPTP challenge elicited a significant reduction of about 80% compared to control, as was expected. MPTP treatment following TDCPP exposure elicited an even greater reduction in DAT expression. These results indicate that TDCPP is somehow compromising the dopamine neurons and resulting in greater harm from MPTP exposure. As stated before the mechanism for this action remains unknown. The striatum is a crucial dopamine-rich area that is often associated with neurological diseases such as Parkinson's disease and addiction. The results from this study implicate TDCPP as a possible dopaminergic neurotoxicant. A previous study has suggested that TCEP acts as a GABA antagonist and increases ambulatory activity in mice (Umezu, Yonemoto, Soma, & Suzuki, 1998), and another has shown that TCEP resulted in neurotoxicity in the hippocampal region in rats (Tilson, Veronesi, McLamb, &

Matthews, 1990). This study, however, is the first to assess dopaminergic neurotoxicity of TDCPP in mice.

This study provides novel information that supports the need for future studies to further characterize the specificity of neuronal damage and the mechanisms through which this damage occurs. TDCPP is one of many environmental contaminants present that are persistent and have unknown toxicological effects. Further study of TDCPP and similar chemicals will provide essential insight into environmental exposures and resulting neurotoxic damage, as well as ways to prevent such contamination and exposure in the future. Finally, although this study was conducted in adult animals, it appears that human children are the most vulnerable population to Cl-OP exposure, usually through ingestion of house dust or possibly prenatally. It has been well established that exposure to neurotoxic compounds during critical periods of neurodevelopment can have deleterious effects on multiple neurobehavioral endpoints, including cognition and risk for neurological disease. Thus, future studies should evaluate the developmental effects of TDCPP exposure and elaborated their focus to consider other brain regions and neuronal proteins that could be altered following exposure.

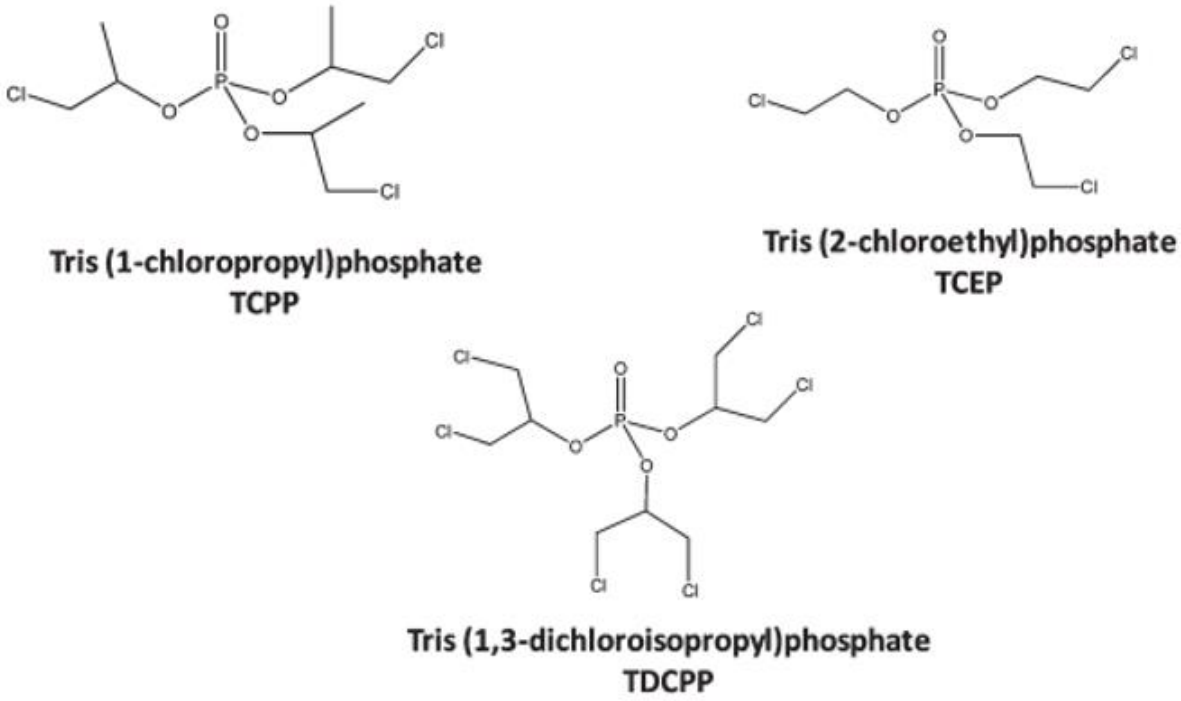

Figure 1: Chemical structures of commonly detected chlorinated organophosphate flame retardant compounds (Stapleton et al., 2009)

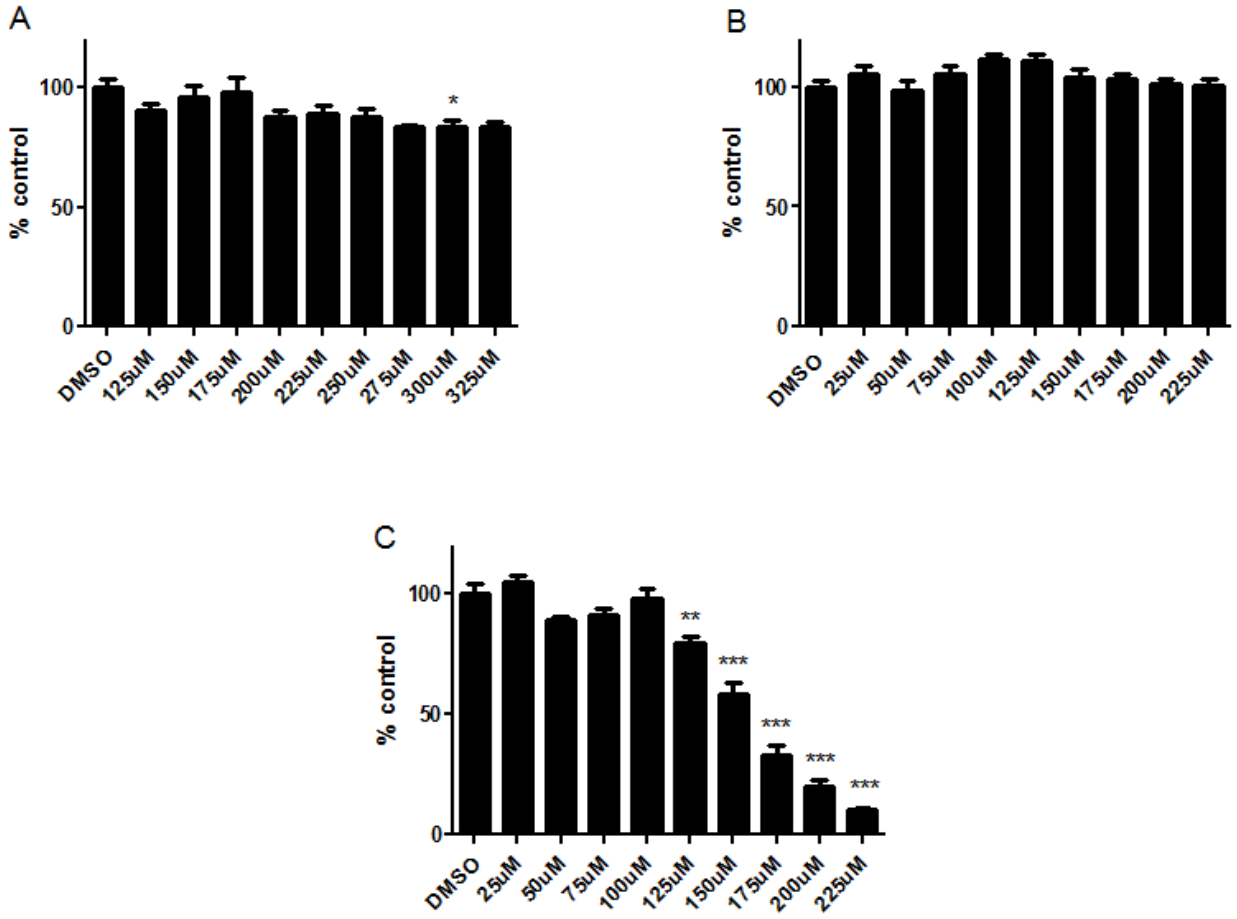

Figure 2: Comparison of TCPP, TCEP, and TDCPP 48-hour SK-N-SH treatment: A) TCPP and B) TCEP had no significant toxicity, compared to C) TDCPP, which elicited significant toxic effect. A one-way ANOVA and Tukey's Multiple Comparison test were used for statistical analyses.

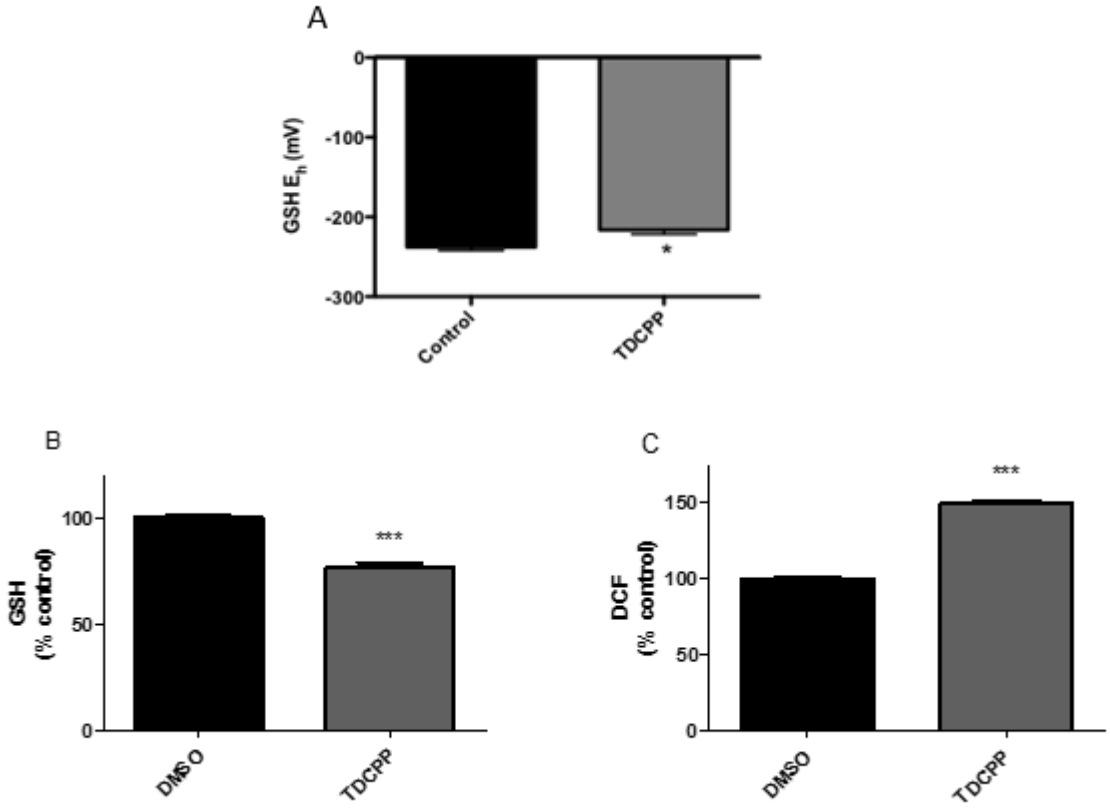

Figure 3: Detection of Oxidative Stress generated by TDCPP treatment: A) GSH:GSSG redox potential showing increased oxidative stress in treatment compared with DMSO control ($p=0.0315$); B) GSH produced by cells treated compared with DMSO control ($p=0.0006$); C) DCF assay indicating production of reactive oxygen species ($p<0.0001$). Unpaired t-test was used for statistical analyses.

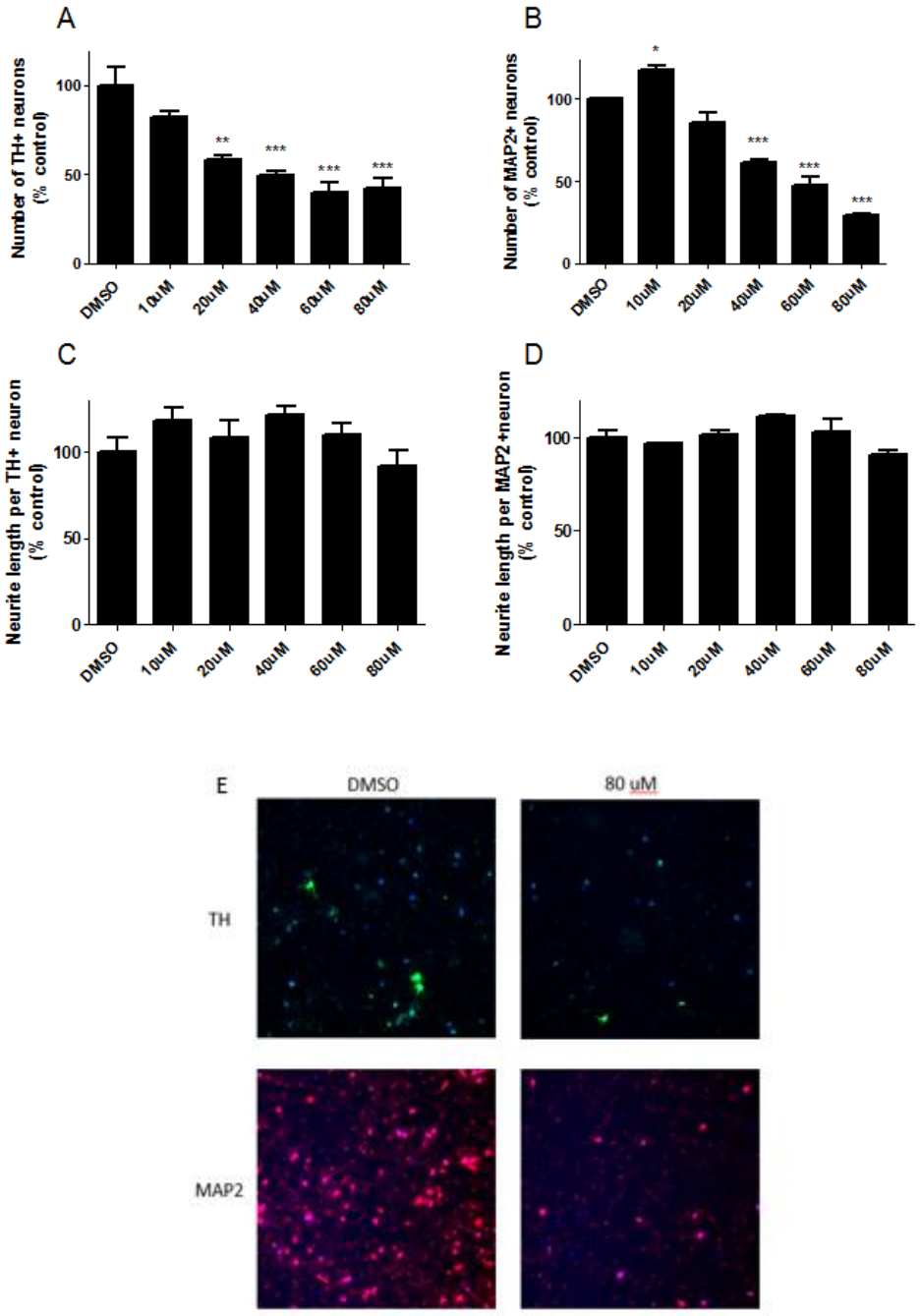

Figure 4: TDCPP treatment in ventral mesencephalic neurons. A) Number of TH+ neurons as percent of DMSO control following exposure to TDCPP ($p<0.0001$); B) Number of MAP2+ neurons as percent of DMSO control following exposure to TDCPP ($p<0.0001$); C) Neurite length of TH+ neurons as percent of DMSO control following exposure to TDCPP; D) Neurite length of MAP22+ neurons as percent DMSO control following exposure to TDCPP. One-way ANOVA and Tukey's Multiple Comparison test used for statistical analysis; E) Fluorescent staining of TH and MAP of VMES neurons treated with DMSO or 80uM TDCPP.

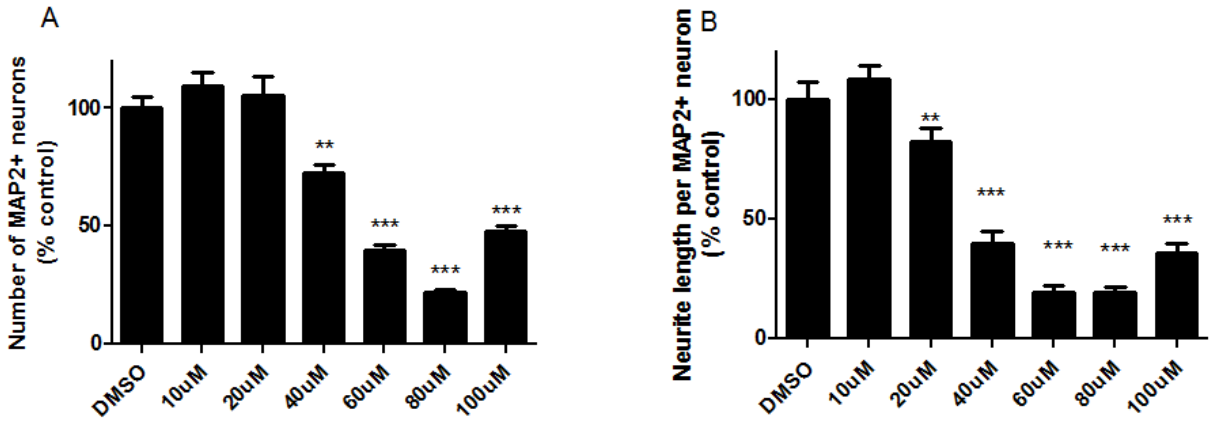

Fig. 5: TDCPP treatment in cortical neurons. A) Neuron count after exposure to TDCPP as percent of DMSO control ($p<0.0001$). B) Neurite length per neuron after exposure to TDCPP as percent of DMSO control ($p<0.0001$). One-way ANOVA and Tukey's Multiple Comparison test used for statistical analysis.

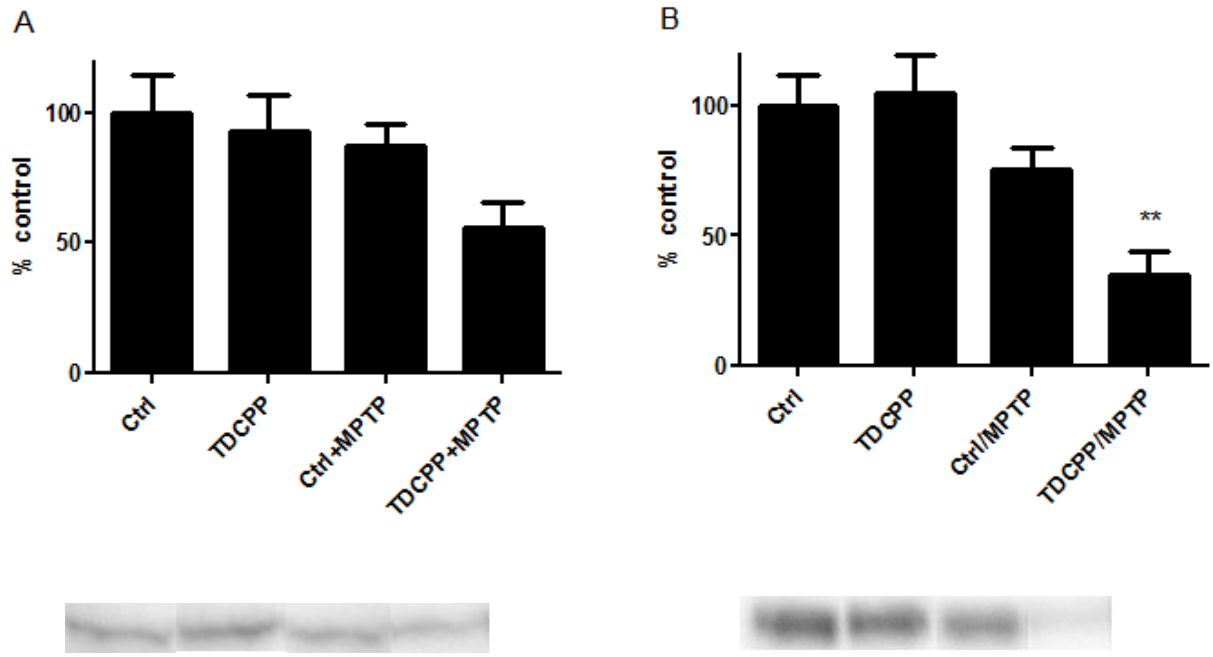

Figure 6: Western blot analysis of TDCPP treatment *in vivo*. A) TDCPP, Control+MPTP, and TDCPP+MPTP treatments result in decrease in percentage of TH expression as compared to DMSO control; a representative Western blot image is shown. B) TDCPP, Control+MPTP, and TDCPP+MPTP treatments result in statistically significant decrease in percentage of DAT expression as compared to DMSO control ($p<0.05$); a representative Western blot image is shown.

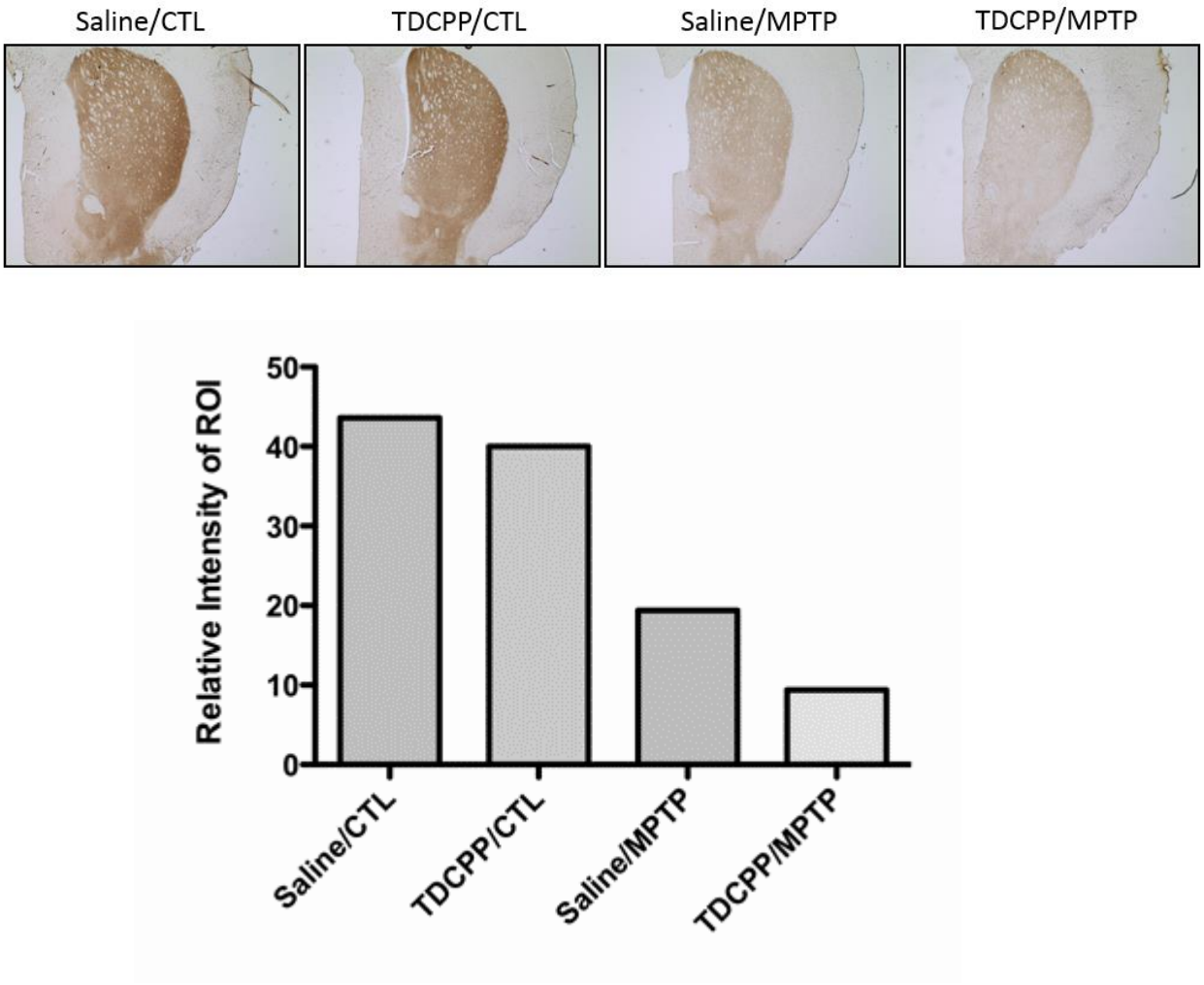

Figure 7: Immunohistochemistry of striatal dopaminergic neurons. A) 40 micron slices of striatal tissue from TDCPP-treated mice, stained for DAT; B) Relative intensity of regions of interest of tissue slices in (A)

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