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Permethrin pesticide-induced alterations in the dopaminergic system across time points and genders

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B.S. Chemistry
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Abstract

Permethrin pesticide-induced alterations in the dopaminergic system across time points and genders

By Jennifer Michelle Leveille

Dopamine (DA) is one of many neurotransmitters in the central nervous system. The dopaminergic system and dopamine are responsible for such bodily functions as motivational and emotional behavior, attention, executive function, reward, control of involuntary and rapid motor function, and neurosecretion associated with the rhythm of light, biological clock, and reproduction. Major regulators of the dopaminergic system are tyrosine hydroxylase (TH), dopamine transporter (DAT), vesicular monoamine transporter 2 (VMAT2), and D1-D5 receptors. Great interest in the dopaminergic system in humans results from the fact that dysfunctions of the system can result in serious neurological disorders, such as Parkinson's disease (PD), attention deficit hyperactivity disorder (ADHD), addiction, and schizophrenia. It has long been known that exposure to pesticides and insecticides can induce such neurobehavioral effects in many species, including humans. Pyrethroid pesticides, including permethrin, are now the most commonly used pesticides for residential pest control and public health purposes. Previous studies have shown disruption to the dopamine system by permethrin and other pyrethroids, yet little is known about the selectivity and duration of exposure of these pesticides. Using both male and female mice, four exposure groups at day 1, day 8, and day 15 were used vs controls, where mice were sacrificed 24-hours after exposure. The protein levels of DAT, TH, and VMAT2 were then analyzed by Western blot. Analysis found a nearly 70% increase of DAT expression levels at day 15 in male mice ($P=0.006$) and a statistically significant difference in male day 15 mice vs female day 15 mice ($P <0.0001$), but no statistically significant increase in female mice. TH levels did not reach significance in either gender of mice, however, an initial decrease was followed by an increasing trend across exposure days. VMAT2 levels were found to be statistically significant in male day 15 vs female day 15 mice ($P < 0.03$), but no significance was found across exposure days. This study found a significant gender difference to permethrin exposure, and significant levels of DAT increase in male mice, helping elucidate further toxicity of permethrin pesticides, despite the belief that they are innocuous, warranting further research.

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Introduction

Dopamine (DA) is one of many neurotransmitters in the central nervous system. The dopaminergic (DArgic) system and the neurotransmitter dopamine are responsible for many basic bodily functions, such as motivational and emotional behavior of humans and animals, attention, executive function, reward, control of involuntary as well as rapid motor function, and neurosecretion associated with the rhythm of light, biological clock, and reproduction (Jones et al. 1998; Greengard 2001; Torres 2006; Money et al. 2013). The bulk of DA-releasing projections to the forebrain, including major inputs targeted to the nucleus accumbens (NAcc), prefrontal cortex, and striatum, arise from midbrain neurons located in the ventral tegmental area (VTA) and the substantia nigra (SN) (Lindvall et al. 1978). The nigrostriatal projection contains about 80% of the brain's dopamine, and projects from cell bodies in the pars compacta of the SN (midbrain) to terminals that innervate the dorsal striatum (containing the caudate and putamen). Nigrostriatal projections play an important role in movement control (Korchounov et al. 2010), learning, and memory. The mesocorticolimbic projection projects from the ventral tegmental area (VTA/midbrain) to the ventral striatum (containing the nucleus accumbens). The mesocorticolimbic dopamine system is associated primarily with its role in reward processing and motivated behavior (Roseberry et al. 2015).

Dopamine neurons are involved in many important brain functions, in which DA is synthesized, stored, and released into the synapse. The major regulators of the dopaminergic system are tyrosine hydroxylase (TH), the dopamine transporter (DAT), vesicular monoamine transporter 2 (VMAT2), and D1-D5 receptors. Tyrosine hydroxylase hydroxylates tyrosine to L-DOPA, where DOPA is then converted to

dopamine by the aromatic amino acid decarboxylase (Daubner et al. 2011). Once inside the presynaptic nerve terminal, dopamine is transported into small synaptic vesicles by the vesicular monoamine transporter (VMAT2) (Liu et al. 1992), where synthesis continues. Influx of calcium causes the emptying of the vesicles into the synaptic cleft, and the nervous signal is passed on (Daubner et al. 2011) to the postsynaptic neuron. In the synapse, dopamine exerts its effects through activation of either postsynaptic or presynaptic dopamine receptors (D1–D5) (Sibley 1999). Clearance of DA from the synapse by DAT is the primary mechanism, besides diffusion, for terminating DA signaling (Giros and Caron 1993). As an integral presynaptic neuron membrane protein, DAT is widely expressed in the dopamine neurons, and plays a key role in determining the duration of action of dopamine by rapidly taking up extracellular dopamine into presynaptic terminals after release and putting it back into the cytosol, thereby terminating dopamine signaling. Dopamine is then packaged back into vesicles by VMAT2, where more can be synthesized for the next transmission.

Great interest in the dopaminergic system in humans primarily results from the fact that dysfunctions of the system, i.e., hypo- or hyperfunction, result in serious neurological disorders (Krzymowski and Stefanczyk-Krzymowska 2015), such as Parkinson's disease (PD), attention deficit hyperactivity disorder (ADHD), addiction, and schizophrenia. Because dopamine is an important regulator in many physiological functions including locomotion, cognition, affect, hormone secretion, motivating behavior, memory, and reward, dysfunction has been linked extensively to development of these neurologic diseases. Loss of dopamine in the striatum is a cause of the loss of motor control seen in Parkinson's patients (Lotharius and Brundin 2002). Neuroimaging

studies have found increased DAT levels in ADHD patients using a variety of imaging modalities (Krause 2008). The etiology of ADHD is still unclear but is mainly associated with disturbances in dopamine rigid activity (Takeda et al. 2014). From a mechanistic standpoint, these behavioral alterations appear to be driven by disruption of the dopamine system, including elevated DAT levels, lower synaptic dopamine, and increased D1 dopamine receptor levels (Richardson et al. 2015). Reduced dopamine in the pre-frontal cortex and disinhibited striatal dopamine release is seen in schizophrenic patients (Kienast and Heinz 2006).

It has long been known that exposure to pesticides and insecticides can induce such neurobehavioral effects in rodents, as well as in others species, including humans (Eriksson and Fredricksson 1991; Ray and Cremer 1979; Shettgen et al. 2002). Although experimental studies have demonstrated that exposure to pyrethroid pesticides may disrupt the integrity of brain dopaminergic system (Karen et al. 2001; Nasuti et al. 2007; Kou and Bloomquist 2007), and several comprehensive reviews of pyrethroid toxicity, metabolism, and actions are available (Shafer et al. 2005; Kolaczinski and Curtis 2004; Fry and Ray 2006), data regarding the potential developmental neurotoxicity of these compounds is limited.

Pyrethroid pesticides, including permethrin, deltamethrin, and cypermethrin among others, are synthetic analogues of pyrethrins, which are natural chemicals found in chrysanthemum flowers. The basic pyrethroid structure consists of an acid and an alcohol moiety, with an ester bond, where changes have been progressively introduced to increase their insecticidal potency and decrease their sensitivity to air and light (Saillenfait et al. 2015). They are now the most commonly used pesticides for residential

pest control and public health purposes (including control of vector-borne diseases) where they are increasingly being used in agriculture, with biomonitoring studies confirming widespread exposure to one or more (Perez et al. 2010). Introduced into widespread use more than three decades ago, they have grown to represent 18% of the dollar value of the world insecticide market (Pickett 2004). As a class, pyrethroids exert their toxicity primarily through binding to sodium channels and prolonging the opening of the channel, resulting in neuronal hyperexcitability (Soderlund et al. 2002). Although pyrethroid pesticides are often considered a "safer" choice because they are generally not as acutely toxic as organophosphates (Casida and Durkin 2013), animal studies indicate that exposure to pyrethroids may not be benign (Wagner-Schuman et al. 2015).

The nomenclature of Type I and Type II was proposed for subgroups of pyrethroids based on their syndromes of intoxication (Lawrence and Casida 1982) and their chemical structures, signs of poisoning in insects, and actions on insect nerve preparations (Gammon et al. 1981). Permethrin, a Type II pyrethroid, lacks the alpha-cyano group present in the Type I pyrethroids deltamethrin and cypermethrin, and is called a non-cyano pyrethroid. Pyrethroids induce the sodium channels to close slower than normal, resulting in a gradual decaying inward sodium current after termination of membrane depolarization, known as sodium tail current (Flannigan et al.1985). The alpha-cyano pyrethroids induce short trains of nerve impulses and repetitive firing in the peripheral nerves. The non-cyano pyrethroids induce long trains of nerve impulses without repetitive firing, but causing quickly reversible suppression of the nerve action potential that results in a long lasting depolarizing after potential. While there seem to be notable differences in the two groups, various studies have documented similar modes of action

and potency between the two. Symington's group was able to show that five of the six Type II compounds (including deltamethrin) and permethrin, a Type I compound, were potent enhancers of both calcium uptake and neurotransmitter release (Soderlund et al. 2013), and with similar metabolism pathways. Although experimental studies have demonstrated that exposure to pyrethroid pesticides may disrupt the integrity of brain dopaminergic system (Karen et al. 2001; Nasuti et al. 2007; Kou and Bloomquist 2007), and several comprehensive reviews of pyrethroid toxicity, metabolism, and actions are available (Shafer 2005; Kolaczinski and Curtis 2004; Fry and Ray 2006), data regarding the potential neurotoxicity of these compounds is limited.

There has been limited research assessing the toxicity of pyrethroid pesticides despite their widespread use. Pesticides accumulated through the food chain and environmental exposures are identified as one of the main risk factors leading to psychiatric disorders and neurodegenerative diseases (Parron et al. 2011; Zhang et al. 2006). And while pyrethroid insecticides have not been extensively evaluated for long-term neurotoxic effects after low-level exposure in humans, experimental data has raised concerns about the safety of prenatal and early childhood exposures (Shafer et al. 2005). Exposure during development can pose greater risks, where a large dependence to the acute toxicity of pyrethroids are at least an order of magnitude more sensitive than adults to pyrethroids (Shafer et al. 2005).

Decrease in the binding of dopamine receptors was observed in the corpus striatum of developing rats prenatally exposed to fenvalerate, a Type II synthetic pyrethroid (Malaviya et al. 1993). Mice exposed to the Type I pyrethroid pesticide deltamethrin during development exhibit several features reminiscent of ADHD, including elevated

dopamine transporter (DAT) levels, hyperactivity, working memory and attention deficits, and impulsive-like behavior (Richardson et al. 2015). A biomonitoring study by Shelton et al. showed that children of mothers residing near pyrethroid applications just to conception or during the third trimester were at greater risk of developmental delay and autism spectrum disorder. These results suggest disturbances in dopaminergic pathways which are more pronounced during the "growth spurt" period may lead to a functional delay in brain maturation (Malaviya et al. 1993). This is exceptionally alarming considering the increasing concentration of pyrethroid metabolites found in the urine of U.S. children, according to the NHANES.

Similar results, but with varying consequences, have been shown in studies on adult mice exposed to pyrethroid pesticides. Parkinson's disease (PD) is a disabling neurodegenerative disorder characterized by the loss of nigrostriatal dopamine neurons and the formation of intraneuronal inclusions, termed Lewy bodies (Olanow and Tatton 1999). Studies have demonstrated that repeated exposure of mice to the pyrethroid pesticides, deltamethrin and permethrin, results in increased synaptosomal dopamine uptake (Karen et al. 2001; Gillette and Bloomquist 2003), seemingly due to functional up-regulation of dopamine uptake by increased levels of DAT protein (Elwan et al. 2006). It has been reported that repeated exposure (3 injections over 2 weeks) of mice to two commonly used pyrethroid pesticides, deltamethrin (3 mg/kg) and permethrin (0.8 mg/kg), increases DAT-mediated dopamine uptake by 31 and 28%, respectively (Elwan et al. 2006). Data has also demonstrated that alterations of DAT expression could greatly affect the vulnerability of the dopamine neuron to neurotoxins such as MPTP or methamphetamine (Gainetdinov et al. 1997; Donovan et al. 1999). In addition, the

brain regions most vulnerable to parkinsonism-inducing toxin MPTP and those most affected by PD display the highest levels of DAT expression (Miller et al. 1999; Uhl 1998).

Repeated exposure to deltamethrin has also been shown to decrease tyrosine hydroxylase mRNA and protein expression, as well as hydroxylase activity in adult male rats (Liu et al. 2006). Tyrosine hydroxylase (TH) is the rate-limiting enzyme responsible for converting tyrosine to L-DOPA in the dopamine synthesis pathway. The pathophysiology of Parkinson's disease (PD) is largely due to the nigrostriatal dopaminergic system, with a decrease in TH activity, TH synthesis and TH mRNA in the striatum of PD and animal experimental models (Feve 2012). VMAT 2, shown to decrease in pyrethroid exposure studies, transports cytoplasmic dopamine into vesicles for storage and release and protects it from oxidation (Fon et al. 1997; Sora et al. 1998). VMAT2 dysfunction, or abnormal expressions, leads to dopamine oxidation, thereby free radical generation, one of the major causes of the nigrostriatal neurodegeneration (Xu et al. 2005).

While these data provide critical support for the sensitivity of the dopamine system to pyrethroid exposure, important questions regarding the selectivity of these effects for gender remain to be determined. Additionally, understanding the duration of exposure will further highlight the neurotoxicity of pyrethroid insecticides on the dopamine circuit, where data on long-term exposures is lacking.

Materials and Methods

Analytical grade (purity ≥ 98%) permethrin was obtained from ChemService Inc. (West Chester, PA). The rat monoclonal antibody to DAT was purchased from Chemicon

(Temecula, CA) and the monoclonal anti-mouse α-tubulin was purchased from Sigma (St. Louis, MO). The goat anti-rat secondary antibody was purchased from ICN (Costa Mesa, CA) and the goat anti-mouse secondary antibody was from Bio-Rad (Hercules, CA). Super Signal West substrate and stripping buffer were obtained from Pierce (Rockford, IL). All other reagents were obtained from Sigma or Fisher Scientific (Pittsburgh, PA).

Eight-week-old female and male C57BL/6J mice purchased from Jackson Laboratory (Bar Harbor, ME, USA) were used. Mice were maintained on a 12:12 light-dark cycle with food (Purina Rodent Chow #5001; Research Diets, New Brunswick, NJ, USA) and water available ad libitum. All procedures were conducted in accordance with the U.S. National Institutes of Health (NIH) Guide for Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee at Emory University and Rutgers–Robert Wood Johnson Medical School.

Study Design

Animal Treatment

A total of 40 mice (20 male and 20 female) were used for these experiments. Control male and female mice were injected intraperitoneally with vehicle (corn oil; n = 10) and treated mice were injected with permethrin (0.8 mg/ kg; n = 6) three times over a 2-week period (Days 1, 8, and 15) as described previously (Kirby et al. 1999; Miller et al. 1999b; Gillette and Bloomquist 2003). One day following the last treatment (Day 2, 9, and 16), a subset of mice (5 male and 5 female) were sacrificed and striatal tissue was dissected out and prepared for assay as described below.

Immunoblotting

Western blots were performed as previously described (Richardson and Miller 2004). Briefly, samples (48 μg) were subjected to SDS PAGE on 10% precast NuPage gels (InVitrogen, Carlsbad, CA). Samples were electrophoretically transferred to a polyvinylidene difluoride membrane, and non-specific sites were blocked in 7.5% nonfat dry milk in Tris-buffered saline (135 mM NaCl, 2.5 mM KCl, 50 mM Tris, and 0.1% Tween 20, pH 7.4). Membranes were then incubated in a monoclonal antibody (Chemicon, Temecula, CA) to the N-terminus of DAT (Miller 1997). Antibody binding was detected using a goat anti-rat horse-radish peroxidase secondary antibody (ICN, Costa Mesa, CA) and enhanced chemiluminescence. The chemiluminescent signal was captured on an Alpha Innotech Fluorchem 8800 (San Leandro, CA) imaging system and stored as a digital image. Membranes were then stripped for 15 min at 25°C with Pierce Stripping Buffer and reprobed with TH and then again for VMAT2 binding and imaging. Densitometric analysis was performed and calibrated to co-blotted dilutional standards of pooled cells from all control samples. Membranes were then stripped again for 15 min at 25°C with Pierce Stripping Buffer and reprobed with a monoclonal α-tubulin antibody to ensure equal protein loading across samples.

Statistical Analysis

Results were expressed as the mean ± SEM. In instances where data were presented as percentage of control, all statistical procedures were performed on the raw numbers. Data were analyzed by one- or two-way analysis of variance (ANOVA). If a significant F was determined by ANOVA, post hoc analysis was performed with Dunnett's test. Statistical significance is reported at the $P \leq 0.05$ level.

Consistent with previous studies, we will attempt to assess the role of permethrin exposure in the up-regulation of DAT and of alterations in dopaminergic synaptic proteins, including TH and VMAT2, over several time points and across genders. Previous work has shown gender differences to pyrethroid exposure, however, the timing of dosage is experimental. The goal of this study is to help elucidate the specificity of exposure to further highlight the neurotoxicity of pyrethroid insecticide permethrin on the dopamine system.

Results

Consistent with previous studies, we attempted to assess the role of permethrin exposure in the up-regulation of DAT and of alterations in the dopaminergic synaptic proteins, including TH and VMAT2, over several time points and across genders. Previous work has shown gender differences to pyrethroid exposure, however, the timing of dosage is experimental. The goal of this study is to help elucidate the specificity of exposure to further highlight the neurotoxicity of pyrethroid insecticide permethrin on the dopamine system.

Permethrin exposure increases dopamine transporter levels

Several previous studies have demonstrated increased dopamine transporter expression levels to pyrethroids; therefore, we injected mice with permethrin at 0.8 mg/kg over three time periods, where we expected to see an increase in expression of DAT protein in the striatum of exposed mice. This, however, is the first study assessing the effect of gender to permethrin exposure over several time points. Studies from the Miller laboratory and others have demonstrated that certain pesticides increase levels of the dopamine transporter (DAT), an integral component of dopaminergic neurotransmission and a

gateway for dopaminergic neurotoxins (Elwan et al. 2005), but only in male mice. Due to the lack of data on duration of exposure to permethrin across genders and over time points, we injected both male and female mice across three time points and proteins were analyzed for each in hopes of determining the effects of multiple exposures on the dopamine system. At eight weeks of age, striatal DAT protein levels were significantly increased by permethrin in a dose-related manner, where D15 male mice increased by nearly 70% over controls. Figure 1 shows striatal DAT expression across the three dosage groups of days 1, 8, and 15 (D1, D8, D15) for both male and female mice. This graph shows an overall increase in DAT expression levels across the male dosing groups, with an interesting, though nominal, decrease for male D1 mice from controls (saline). As expected, female mice showed no significant increase in any dosing group from controls, however, a significant increase was seen between day 1 and day 8. At dosage day 15, there was a statistically significant increase of DAT expression levels, at roughly 67%, in male mice from

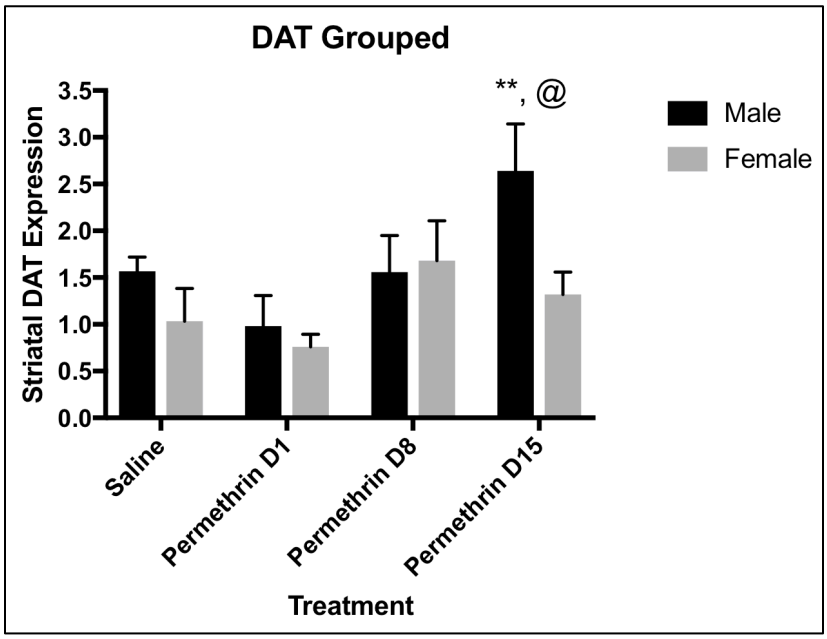

Figure 1
Pesticide exposure increases in DAT expression levels for male and female mice, grouped. Exposure to permethrin at 0.8 mg/kg increased levels in the striatum of male mice greater than in female mice as determined by Western blot.
**** = statistically significant difference of male mice D15 from male saline mice (P=0.006)**
@ = statistically significant difference between male D15 and female D15 mice (P < 0.0001)

controls (P=0.006). There was also a statistically significant permethrin x sex interaction (P <0.0001), with male increases almost three times that of the female. Figure 2 further illustrates the results seen for DAT protein expression levels in both male and female mice after exposure to permethrin, where significance can be seen between saline and D15 male mice, and D1 and D8 female mice. Again, there is shown an increasing trend of DAT expression from D1-D15 male mice. As expected, there also appears to be a gender effect in the change of DAT expression levels between male and female mice, which is consistent with previous studies.

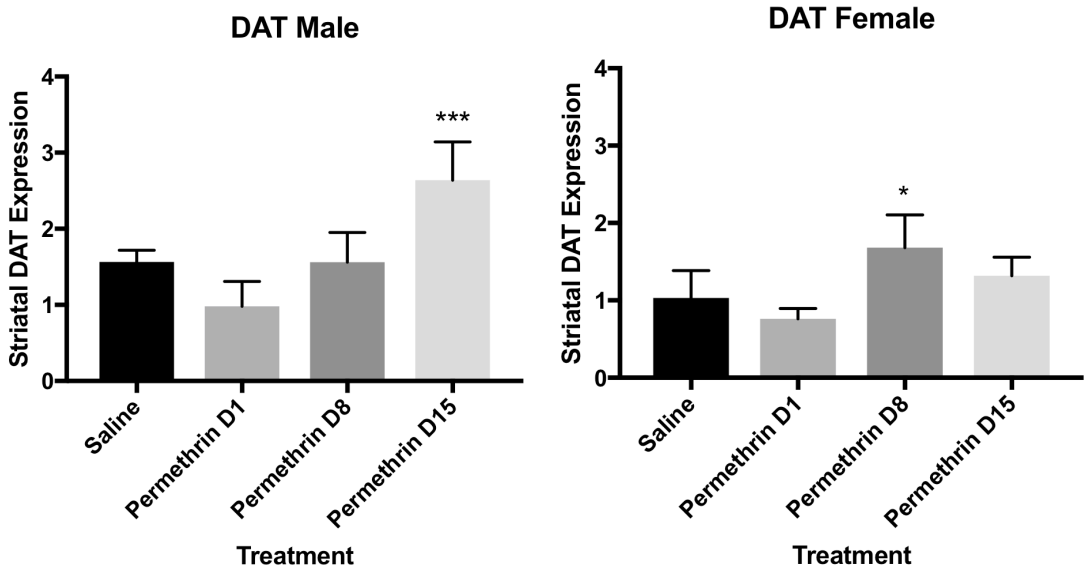

Figure 2

Pesticide exposure changes in striatal DAT expression levels for male and female mice, not grouped.
***** = statistically significant difference in male mice D15 from male saline (P=0.009)**
*** = statistically significant difference in female mice D8 from female mice D1 (P=0.05).**

Effect of permethrin exposure on TH levels

Data analyzing the effect of permethrin on Tyrosine Hydroxylase (TH) levels is extremely limited, and this study provides the first of its kind across time points and genders. In studies by Liu et al, TH was shown to decrease in the striatum of mice

exposed to the pyrethroid deltamethrin, highlighting its toxicity beyond DAT. Both Figure 3, grouped by gender, and Figure 4, not grouped by gender, show no statistically significant difference in TH expression levels across the three dosing groups for both male and female mice in the current study. It is important to note, however, that while large variability is seen in the data as depicted by the wide error bars, there appears to a slight decrease in TH overall from the saline control group as seen in previous studies. The large variability seen in the tyrosine hydroxylase results could possibly be due to the small sample size or the antibody used for the procedure.

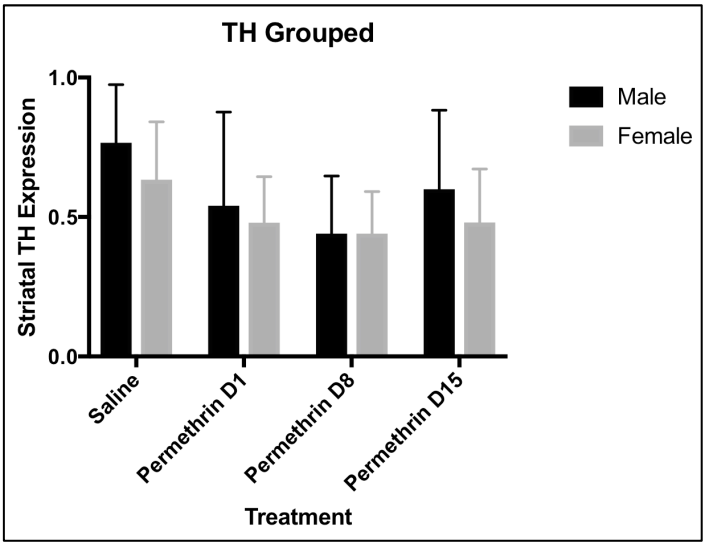

Figure 3

Pesticide exposure changes in striatal TH expression levels for male and female mice, grouped. Trend indicates overall decrease in TH levels across D1, D8, D15 male and female mice, however, no significance was reached. Error bars are much wider than the DAT/VMAT2 results.

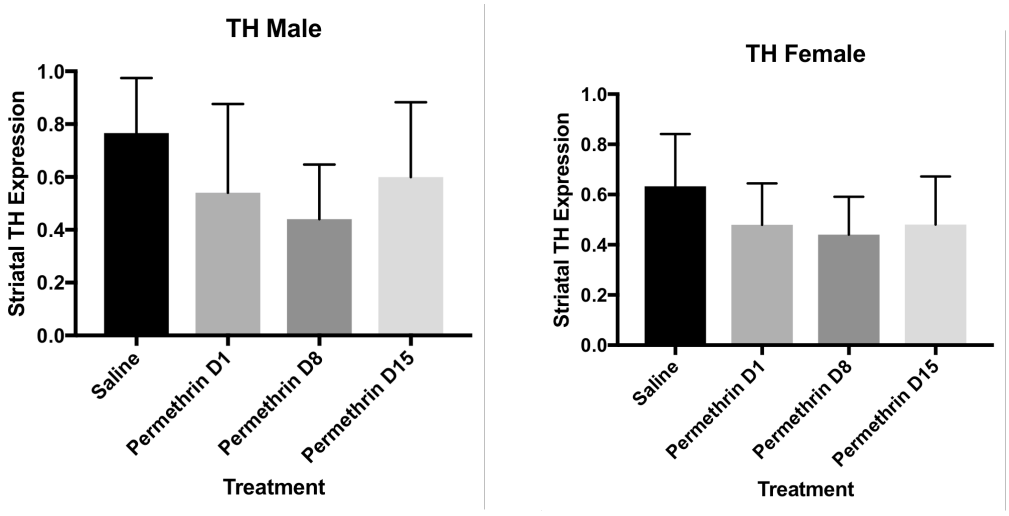

Figure 4

TH expression level changes for male and female mice, not grouped.

Effect of permethrin exposure on VMAT2 levels

There are also limited studies available assessing the effect of pyrethroid, particularly permethrin, exposure on VMAT2 levels in mice, and none across genders. Studies that have assessed the effect of pyrethroid exposure on the change in VMAT2 levels have shown a decrease in the protein. Again, to assess the change in levels of VMAT2 protein in the brain of mice across genders, mice were injected with 0.8 mg/kg of permethrin over three time periods and analyzed by Western blot. Figure 5 shows striatal VMAT2 expression across the three dosage groups of days 1, 8, and 15 (D1, D8, D15) for both male and female mice. There was a statistically significant difference between male and female D15 mice, showing permethrin x sex interaction ($P < 0.03$). There is no statistically significant increase or decrease of D1, D8, or D15 male or female mice from saline controls. Interesting to note is the decrease seen in male mice from controls to D1, and the trend of increasing VMAT2 expression levels in male mice from D1 through D15. Figure 6 is included to show that no significance was reached by each gender group alone, only indicating a permethrin x gender difference in exposure between male and female mice.

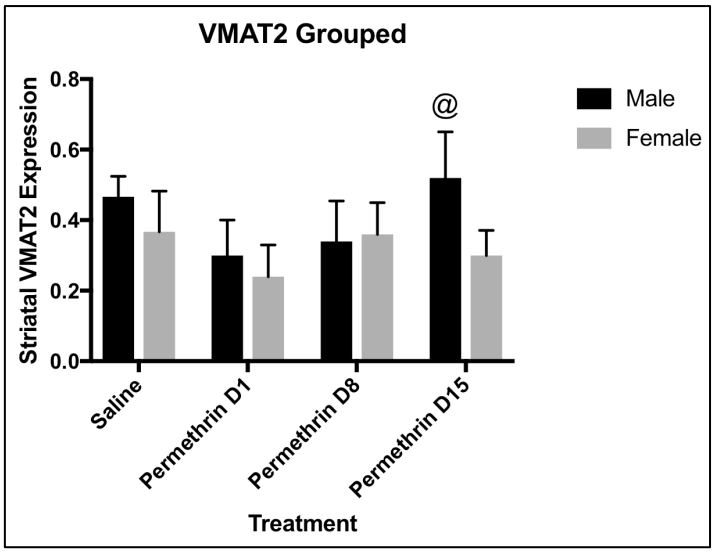

Figure 5
Pesticide exposure changes in striatal VMAT2 expression levels for male and female mice, grouped. There is a gender interaction seen for D15 mice (P <0.03).

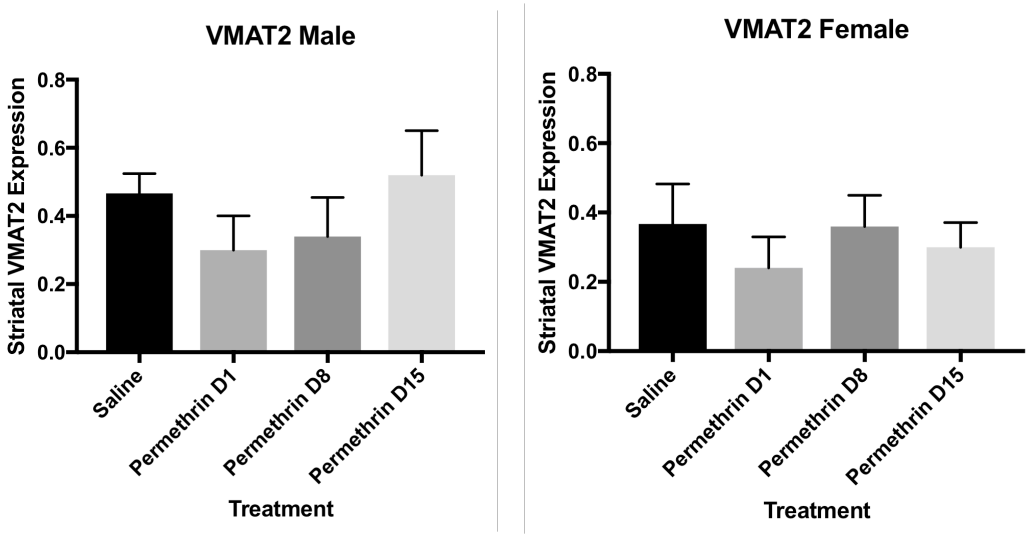

Figure 6

Pesticide exposure changes in VMAT2 expression levels for male and female mice, not grouped. Neither gender had a statistically significant change in levels, however, an overall decreasing trend is seen until male D15 mice.

Discussion

Pyrethroid pesticides have been used for more than 40 years and account for 25% of the worldwide insecticide market (Shafer et al. 2005). Thought to be environmentally labile, pyrethroids are highly lipophilic and by easily crossing the blood–brain-barrier they can reach the central nervous system at concentrations that can be potentially neurotoxic (Nasuti et al 2013). The main metabolites of pyrethroids have frequently been detected in urine samples from the general population, confirming widespread exposure of children and adults to one or more pyrethroids (Saillenfait et al. 2015), where some studies have shown the presence of permethrin metabolite 3-PBA in upwards of 98% of individuals.

Concerning neurodevelopment, data from 1999-2002 from the NHANES has shown that children between 6 and 11 years of age had higher urinary metabolites of pyrethroids than adults, which has shown to be increasing since the phase-out of other types of pesticides over the last decade. Exposure to pyrethroids during development has been

shown to target both the pre- and post-neuronal synapse, which is a vulnerable target for disruption and can contribute to neurodevelopmental disorders. A number of epidemiological studies have also found that there is a close association between pyrethroid exposure and neurodegenerative diseases such as Parkinson's, that developmental exposure to pyrethroids is associated with behavioral difficulties such as ADHD, and evidence is beginning to shown an association of pesticide exposure and addiction.

While no specific mechanism has been identified for the increase of DAT protein by these compounds, multiple studies have indicated that they potentially do not act directly on DAT. Chemicals known to cause dopamine release, like amantidine and the organo-chlorine pesticide heptachlor, can increase DAT expression (Gordon et al. 1996; Miller et al. 1999; Page et al. 2000; Kirby et al. 2002). If this were to be sustained over time, one would expect that the elevated extracellular dopamine would increase the expression of the dopamine transporter in an attempt to clear and recycle dopamine. Indeed, deltamethrin has been demonstrated to cause dopamine release from pre-loaded synaptosomes (Kirby et al. 1999; Bloomquist et al. 2002). Also, in mice showing an elevation in DAT expression following a developmental exposure to the pyrethroid pesticide, deltamethrin, a concomitant increase in the striatal D1 (30%) and D2 receptor (60%) is also observed. This suggests that the D1 receptor changes found in deltamethrin exposed mice may be a result of up-regulation in response to increased DAT numbers and subsequently decreased extracellular DA levels (Richardson et al. 2015).

Another possible mechanism of up-regulation of DAT, and changes in other dopaminergic proteins, lies in transcription of genes associated with dopaminergic

proteins. This could occur through histone modification and/or methylation of other genes involved in dopamine production, such as Nurr1 (Fedeli et al. 2017). Nurr1, a transcription factor belonging to the orphan nuclear receptor family, regulates genes involved in dopamine neurotransmission such as TH and vesicular monoamine transporter 2 (VMAT2), where reduction in Nurr1 gene expression has been reported in PD, schizophrenia, and manic depression (Bensinger and Tontonoz 2009). Since Nurr1 transcription can be enhanced by membrane depolarization, the release of dopamine or the blockage of sodium channels as seen to occur in pyrethroid exposure, up-regulation of Nurr1 could lead to increased expression of DAT (Elwan et al. 2005). A study by Fedeli et al. which assessed the role of genetic modification of Nurr1 in permethrin-exposed rats found that, although no difference in Nurr1 promoter methylation was measured in control and treated groups at different ages, Nurr1-body methylation cannot be excluded as well as the involvement of other epigenetic mechanisms like histone modifications at H3 and H4 as previously reported for dieldrin (Feng et al. 2015). Studies aimed to clarify these aspects are in progress.

In the present study, DAT expression levels increased significantly in the striatum of day 15 male mice injected with permethrin, at a concentration known to be comparable to low-level chronic exposures seen in humans. Dysfunction of dopaminergic proteins in the brain is highly linked to neurodegenerative diseases and is known to produce neurobehavioral deficits in humans. Alterations to DAT expression levels in the brains of developing mice, corroborated with epidemiological studies of mothers and children exposed to pyrethroids, have been linked to neurodevelopmental diseases and behavioral disorders such as ADHD. In this study, significance was reached at exposure day 15,

where it could be hypothesized that this trend would continue if exposure were to continue, leading to an even greater dysfunction and overall decrease of dopamine in the brain. This could help in answering the questions regarding neurodegenerative diseases and neurobehavioral deficits seen in epidemiological studies of pyrethroid pesticides, where studies such as this show their effect on the dopamine system.

We also saw statistically significantly difference in male and female mice at day 15, indicating an effect of gender. In some, though very limited, studies on alternative types of pesticides and other pyrethroids, male but not female mice were also significantly changed from controls. Increased expression of DAT protein in the brain of males, though not fully understood, has been highly correlated with development of neurological disorders such as Parkinson's disease, addiction, and schizophrenia. These changes in the brain of male, but not female, mice would indicate that males are more susceptible to pesticide insult than are females. One hypothesis for this difference is the role of sex hormones in drug metabolism, where females potentially metabolize pesticides at a quicker rate. Male gender has also been demonstrated to be a risk factor for PD (Van den Eeden et al. 2003) and pesticide exposure has been found to be associated with increased risk of PD in men but not women (Baldereschi et al. 2003), although this is not the case in all studies (Ascherio et al. 2006). The role of gender preference of dopaminergic effects observed here and other studies suggests that further research is warranted in the role of gender and dopaminergic function.

The role of TH in the synthesis of dopamine in the brain, along with permethrin's affinity for the dopamine system, makes this an important protein to analyze as well. Various studies have shown some decrease in TH levels in the brain, others have shown

an increase in TH expression levels in the brain over time, while still some have shown little to no change. These disparities could be due to sample size differences, difference in study design, and potentially inadequate statistical analysis. In this study, no statistically significant changes were seen in the levels of TH. However, looking at the trend, there appears to be an initial decrease in TH levels, followed by an increase at D15, in both the male and female mice. The study by Kou and Bloomquist, assessing long-term exposure of permethrin on the dopaminergic system, found an up-regulation of TH. It was hypothesized that enhanced requirement for dopamine synthesis occurred due to increased dopamine release via nerve firing through modification of sodium channel function (Soderlund et al. 2002). This could indicate that TH is more sensitive to dysfunction of dopaminergic neurons (Kou and Bloomquist 2007). Should permethrin exposure cause TH expression levels to significantly decrease, it could be hypothesized that an overall decrease of dopamine synthesis would occur in the striatum of exposed individuals. Whether permethrin acts directly on TH or some other indirect mechanism is involved, decreased TH activity in the dopaminergic system in the striatum has been highly linked to Parkinson's disease, due to its role in the rate of dopamine synthesis. An overall decrease of dopamine in the system could mean that pyrethroids can be linked to the neurodegenerative diseases neurobehavioral deficits seen in epidemiological studies.

Studies that have assessed VMAT2 changes in the dopaminergic system of exposed mice have generally reported decreased expression levels of the protein in the brain. DAT and VMAT2 are known to be main regulators of the cytosolic DA concentration, where DAT takes up extracellular dopamine into the cytosol, and VMAT2 sequesters cytosolic dopamine into intracellular vesicles (Xiong et al. 2017). Lower levels of VMAT2 causes

increased levels of the neurotransmitter dopamine in the cytosol of the neuron, where it can be come toxic and cause degeneration of the neuron. Dopamine is a known endogenous cytotoxin to dopamine neurons if not properly sequestered. VMAT2 is also known to protect dopamine neurons through vesicular sequestration of toxic metabolites so that reduced VMAT2 expression could induce dopamine neurons' loss in Parkinson's disease (Caudle et al. 2007). Though there appears to be a trend of increasing expression levels of VMAT2 in both the male and female mice exposed to permethrin, the findings in the current study found no statistically significant change in VMAT2 expression levels across the dosage days for either male or female mice. There was a permethrin x sex interaction, where D15 male and female mice where found to be significantly different ($P < 0.03$). This could indicate the same protective effect for females seen in the expression levels of DAT, and should warrant further research.

In summary, this is the first report that a permethrin x sex interaction exists in exposed mice. While this study only analyzed the striatum of the mice exposed, it would be beneficial to analyze alternative brain regions, specifically the frontal cortex, which involves a separate dopaminergic pathway. This dopaminergic system is associated with reward and motivating behavior, and studies assessing permethrin's role in its function could help elucidate those effects. Additional proteins of interest should also be evaluated for changes in response to permethrin exposure, especially the post-synaptic D1 and D2 receptors that are involved in signal transduction of dopamine and in regulation of motor output and locomotor activity. These receptors have also been shown to be sensitive to pesticide exposure and have had a gender preference to exposure. In response to exposure to deltamethrin, D1 and D2 expression levels have increased, causing behavioral

alterations in male mice only. Importantly, studies have shown that D1 receptors have remained higher in the NAc of males throughout adolescence and into adulthood (Anderson and Teicher 2000). While our sample size was small, further research should be conducted with larger sample sizes, in search of additional effects of the specificity of the pesticide permethrin on the dopaminergic system. This is especially imperative considering the prevalence of exposure to pyrethroid pesticides, the attitudes regarding its toxicity, and the danger of its effects on the brain.

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