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Shreya Bandlamudi April 7, 2021

LRRK2 and Environmental Effects on Dopamine Systems within the Gut-Brain Axis: A Parkinson's Disease Pathology Focus

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Abstract

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Parkinson's disease (PD) is progressive, neurodegenerative disorder characterized by dopaminergic neuron degeneration in the susbtantia nigra pars compacta (SNpc) region of the midbrain, gut dysregulatory symptoms, and dopamine pathway dysregulation. Although the causes of PD are unknown, about ~5% of causes are attributable to genetic mutations, the most common being the G2019S mutation of the LRRK2 (Leucine Rich Repeat Kinase 2) gene, which is known to contribute to the development of inflammatory phenotypes. An overwhelming majority of PD diagnoses are due to a combination of genetic and environmental risk factors. Among these, a known environmental risk factor for PD includes pyrethroid pesticide exposure which is known to have negative neuroregulatory effects. This project seeks to explore the effects of environmental exposure to pesticides combined with genetic predisposition to PD in the LRRK2 genotypic background on dopamine systems within the brain and gut. We looked at experimental groups of mice treated with pesticide exposure or vehicle controls who either overexpressed the mutant form of LRRK2 (G2019S), overexpressed the wildtype form of LRRK2 (WTOE), or expressed wildtype LRRK2 (B6). In these mice, we analyzed dopaminergic neuron degeneration in the SNpc using stereology. We additionally looked in wildtype mice who were exposed to either pyrethroid pesticide or vehicle control for mRNA expression of dopamine-related genes, the LRRK2 gene, and cholinergic neurons (CHAT) as a marker of enteric function via qPCR in parts of the gut as well as in the SNpc and striatum. There were no significant changes observed in numbers of dopaminergic neurons in the SNpc across treatment groups or genotypes, however this part of the

study was significantly limited by its low power. We found increased expression of VMAT2 in the striatum of pyrethroid treated animals and decreased expression of CHAT and LRRK2 in the proximal large intestine of pyrethroid treated animals. Overall, our results need more powered analyses and further data to conclude that there are changes in dopamine systems due to LRRK2 mutation and environmental insult. However, the preliminary results provide groundwork into future analyses on the gut-brain connection of dopamine systems in eliciting PD pathology.

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Table of Contents

I. Introduction and Background……1-10

A. Parkinson's Disease: An Overview……1-3

B. Dopamine Systems…….3-4

C. The Gut-Brain Axis……4-5

D. The Microbiome Connection……..6-7

E. LRRK2……7-9

F. Pyrethroid Pesticides…….9-10

II. Objective and Project Questions……..11

III. Specific Aims and Methods……12-16

IV. Figures and Results……..17-24

V. Discussion and Future Directions……25-30

VI. List of Figures……..31-32

VII. References……33-35

Introduction

Parkinson's Disease: An Overview

Parkinson's disease (PD) is one of the most common chronic diseases in the world and the second-most common neurodegenerative disease in the world[1]. It is estimated that between 5 and 35 individuals per 100,000 individuals worldwide are diagnosed with Parkinson's per year[1]. Within North America prevalence increases to about 572 individuals per 100,000[2]. Diagnosis of this disease before age 50 is rare and accounts for less than 5% of cases, however, upwards of 60% of cases can be accounted for in individuals aged 65-79[3]. This marked increase in incidence with age can be observed in global populations as Parkinson's disease affects about 5% of the population aged 85 or older[4]. In addition to the increased incidence of Parkinson's with age, there is also evidence of sex-based differences[1]. In many populations, there is a greater incidence of PD in men over women and this difference generally increases with age as well[1, 3]. This difference has been observed to be about a two-fold increase in incidence in men vs women and about a 60% increased occurrence of PD-related pathologies in men over women[1, 5]. There are also race and ethnicity-based variances in incidence of PD with evidence of higher rates in Hispanic populations and Non-Hispanic White populations within the United States, Inuit and Native American populations, and Ashkenazi Jewish populations within and outside of Israel[1, 3]. Contrastingly, Asian populations both within and outside the United States have been found to have lower rates of incidence[2].

Despite these variable and widespread incidences of Parkinson's disease, there are several, consistent markers at a pathophysiological level. PD is classified as a neurodegenerative disease and as such its most prominent characterizations is degeneration of dopaminergic neurons in the substantia nigra pars compacta region (SNpc) of the midbrain as well as aggregation of misfolded

α-synuclein protein—Lewy bodies— within neurons[1, 6]. The loss of dopaminergic neurons within the substantia nigra is a visible phenomenon. When comparing diseased brain cross sections to healthy ones, the characteristically darkened SNpc region is more faded in appearance in PD patients[1]. The midbrain is the primary region of the brain containing dopamine-producing neurons, so this loss of neurons leads to many of the characteristic movement-related motor deficits observed in PD patients[1].

Clinically, PD is characterized by both motor and nonmotor symptoms. Motor symptoms include tremors, bradykinesia (slowed movement), akinesia (impaired voluntary movement), rigidity, slowed speech, and balance issues among other deficits[7]. Motor deficits are the characteristic symptoms that come to mind with Parkinson's disease, however non-motor symptoms often manifest far before motor symptoms and official diagnosis[8, 9]. These include insomnia, olfactory problems, depression, gut dysregulation, and constipation among others and appear first in the pre-motor or prodromal stage of Parkinson's[8, 10]. Constipation is one of the most common symptoms of PD patients and about 80% of individuals report longer than average gastrointestinal transit time[8]. These non-motor symptoms, especially constipation and gastrointestinal dysfunction, can be reported years before official PD diagnosis making them possible early indicators of neurodegenerative disease[9].

Although there are many symptoms, like the aforementioned, that can be early indicators of Parkinson's Disease, the etiopathology is yet to be understood. We know that aging is the most prominent risk factor for PD as individual's risk for the disease increases exponentially after age 60[11]. Additionally, 5-10% of PD cases can be accounted for by genetic factors including mutations that predispose individuals to disease pathology[12]. These mutations target a variety of systems, including dopamine pathways, to induce PD-pathology but exact mechanisms are yet to be

understood[12]. Given the relatively small percentage of genetically attributable PD cases, we look to other factors to account for more than 90% of cases. Relatively recent studies into environmental factors like pollution and pesticides have generated some excitement about neurotoxicity and systemic toxicity contributing to PD pathology[13]. Perhaps a more common or applicable entryway to looking at PD pathology is via some combination of gene-environment interactions compounding effects we see from genetic mutation and environmental exposure. To gain further understanding into idiopathic PD, it is important to consider and explore these factors.

Dopamine Systems:

Dopamine (DA) is a neurotransmitter found and synthesized primarily in the brain but also has functions in the periphery[14]. It is a chemical messenger used by neurons to communicate to other neurons, and functions in reward pathways and motor pathways[14]. Dopamine synthesis takes place in neuron cell bodies and starts as the amino acid tyrosine which is then modified by the rate-limiting enzyme tyrosine hydroxylase (TH) to form the L-DOPA precursor[14]. L-DOPA is then modified by dopa decarboxylase (DDC) to form cytosolic dopamine within the neuron's cytosol[14]. This dopamine is then packaged into vesicles via a transporter protein known as vesicular monoamine transporter 2 (VMAT2) for delivery to the neuron terminal where exocytosis releases the dopamine into the synaptic cleft between neurons[14]. Once released, DA molecules traverse the synapse to bind to dopamine receptors on the other neuron triggering the signal propagation down the pathway[14]. The leftover DA is then taken back up into the releasing neuron via the dopamine transporter (DAT) protein[14]. Specifically, the substantia nigra pars compacta (SNpc) and ventral tegmental area (VTA) are the primary regions of the brain that contain neuron cell bodies capable of synthesizing DA[15]. It's from these regions that most of the main dopaminergic pathways project

to other parts of the brain and exert motor control and reward functions. Loss of about 50-70% of dopaminergic neurons in the SNpc results in motor dysfunction and deficits characteristic of Parkinson's Disease; this indicates why dopamine as a neurotransmitter is of particular interest in discerning PD pathologies[12].

There are four main dopamine pathways of the central nervous system: the mesolimbic, mesocortical, nigrostriatal, and tuberohypophyseal[16]. Each of these pathways projects from the midbrain into different parts of the brain. The midbrain is the primary location of dopaminergic neurons and includes the substantia nigra pars compacta (SNpc) and ventral tegmental area (VTA) [15]. The mesolimbic pathway transmits dopamine from the VTA to the striatum including the nucleus accumbens or "reward center" of the brain[16]. The mesocortical pathway transmits dopamine from the VTA to the prefrontal cortex[16]. The nigrostriatal pathway transmits dopamine from the SNpc to the striatum including the caudate nucleus—which is known to regulate motor function and is implicated in Parkinson's Disease[15]. The main pathway implicated in Parkinson's Disease is the nigrostriatal[15]. Loss of dopaminergic neurons in this region—a part of a group of nuclei most affected in PD known as the basal ganglia— can lead to the motor deficits observed in PD pathology[15].

The Gut-Brain Axis:

Although the main dopamine systems primarily function in the central nervous system, there is also innervation throughout the gut[17]. In addition to the central and peripheral nervous systems, there exists the enteric nervous system (ENS) innervating the gut, which is sometimes referred to as the "second brain" [17]. There is a bidirectional communication network between the central nervous system and the enteric nervous system which includes the autonomic nervous

system and hypothalamic-pituitary-adrenal axis (HPA)[17]. This communication network is colloquially known as the gut-brain axis[17]. This cross talk between the CNS and ENS is mediated by the vagus nerve and sympathetic nerves of the spinal cord[18]. The vagus nerve belongs to the parasympathetic division of the autonomic nervous system and innervates the gut extrinsically[18]. Within the gut ENS, there are two main layers: the submucosal plexus and myenteric plexus[19]. The submucosal plexus is further divided into the inner, intermediate, and outer layers which control secretion and blood flow[19]. The ENS self-modulates intrinsically, but it also receives extrinsic modulation via the vagus nerve which synapses within the gut onto muscle and ENS neurons in the myenteric plexus[18].

There is evidence that the enteric nervous system of the upper gastrointestinal (GI) tract is made up of about 20% dopaminergic neurons and about 6% within the lower GI tract[18]. This is important in the context of PD because this indicates that there are dopamine pathways involved in the gut and potentially in the bidirectional communication between the gut and brain. Additionally, there is evidence of almost every neurotransmitter found in the central nervous system, also being found in the gut enteric system; this includes acetylcholine, nitric oxide, and other catecholamines[19]. These neurotransmitters are present in the tissues of the gut as part of the enteric nervous system, but there is also evidence of production of neurotransmitters—including dopamine—by gut bacteria within the lumen of the gut[20]. The microbiome connection to the gut-brain axis is an essential one and helps us understand how this crosstalk takes place.

The Microbiome Connection:

The gut microbiome refers to the population of microbes and their genes residing within our GI tract[22]. This microbiome has been shown to be vital for gastrointestinal function, and there is more research investigating its role in neurodevelopment and gut-brain axis crosstalk[21, 22]. Specifically, the enteroendocrine cells lining the lumen of the gut regulate and receive signals from the microbiota within the gut lumen to relay via the gut-brain axis and vagus nerve[23]. This communication is still under investigation especially as there is literature suggesting the production of neurotransmitters, including dopamine and other catecholamine, by the microbiome which contributes to the microbiome-nervous system connection[21]. The microbiome can produce local neurotransmitters like serotonin, GABA, norepinephrine, and dopamine that interact with the enteric nervous system and have effects on the central nervous system[17, 20].

The microbiome in particular seems to be an important regulator of central and enteric nervous system signaling through its local neurotransmitters[18]. For example, research utilizing germ-free (GF) and specific-pathogen free (SPF) mice saw an increase in luminal levels of dopamine in SPF mice and a decrease in GF mice indicating the importance of the gut microbiome in production and maintenance of these neural signaling molecules[20]. The microbiome has clear functionality in the gut, but it exerts important effects in the brain as well. Specifically, in the brain striatum, there is evidence of increased dopamine and noradrenaline turnover among other markers of serotonergic pathways in GF mice[24]. Studies indicate that changes in dopamine markers and other neural markers, specifically in the striatum, as a consequence of microbial colonization implicates the gut microbiome as a regulator of neural pathways and motor circuits[24]. However, the way in which this mechanistically happens and factors that may affect the microbiome extrinsically and intrinsically is still an area of study.

In context of Parkinson's Disease, there have been studies and meta-analyses into the gut microbiome of PD-patients and healthy controls to elucidate a difference in the colonies of bacteria present. Throughout several studies, the most consistent difference between PD-patient gut flora and that of healthy controls was an increase in the genera *Lactobacillus*, *Akkermansia*, and *Bifidobacterium*, and a decrease in the *Lachnospiraceae* family and *Faecalibacterium* genus[25]. These changes in gut microbiota reveal interesting implications for neural pathways and the gut-brain axis and could also lead to pro-inflammatory pathologies within the gut contributing to this dysbiosis. Specifically, because inflammatory diseases like Crohn's Disease (CD) and constipation often precede PD diagnosis, these microbiome differences can provide insight into the development of non-motor symptoms of PD[9].

LRRK2:

One of the most commonly discussed genes when considering PD-risk is the LRRK2 gene. The LRRK2 (Leucine Rich Repeat Kinase 2) gene is expressed in innate and adaptive immune cells within the periphery, including peripheral macrophages, and in the brain—like microglia[26]. Its kinase activity functions in a variety of cellular processes and signal transduction pathways including, but not limited to, endocytosis, vesicle-trafficking and growth, endolysosomal pathways, neurite outgrowth, and trans-golgi network related processes[27-29]. Only about 5-10% of Parkinson's Disease cases can be accounted for solely by genetic factors, however within this, the most common genetic mutation is the G2019S mutation of LRRK2[28]. This mutation is a glycine to serine point mutation in the kinase domain causing a gain of kinase activity[28]. This mutation makes up about 4% of familial and 1% of sporadic PD cases, and there is increased penetrance with age increasing from 50% at age 50 to over 70% by age 79[28]. Epidemiologically, the highest

incidence of the G2019S mutation amongst PD patients manifests in Ashkenazi Jewish (10-30%) and North African Berber (35-40%) populations as carriers of this mutation[28].

At a cellular level, LRRK2 has kinase activity so the G2019S mutation exacerbates this activity and can cause pro-inflammatory pathologies both in the brain and periphery[30]. Studies show that reducing, or removing, expression of LRRK2 in microglial cells—cells making up the brain's immune system—decreases pro-inflammatory cytokine levels[30]. In this way, there seems to be neuroinflammatory consequences of LRRK2 mutation and this can also be seen in the periphery—specifically the gut.

There have been several studies exploring the link between PD and Inflammatory Bowel Disease (IBD) including Crohn's Disease (CD) and Ulcerative Colitis (UC). Meta-analyses found that IBD, CD, and UC patients had a 46%, 28%, and 30%, respectively, increase in risk for PD[31]. Additionally, the same studies found that when individuals with these inflammatory bowel conditions are treated with anti-inflammatory treatment, there is a decreased risk for PD[32]. This has interesting implications for PD under the context that the most common non-motor symptoms of PD are bowel inflammatory ones can that manifest decades before PD diagnosis[8]. Interestingly, more recent literature examines the role of LRRK2 as a pro-inflammatory mediator in linking PD and IBD[32]. For example, there has been an increase in LRRK2 mRNA levels found in the intestinal inflamed tissues of CD patients as well as an increase in LRRK2 expression in immune cells (B and T cells and monocytes) in PD patients compared to healthy controls[32]. Contrastingly, there is recent literature suggesting that LRRK2 kinase activity confers a potentially protective effect against gut infection by pathogenic bacteria like Salmonella *typhimurium* and *Listeria monocytogenes*, suggesting an interesting link between LRRK2 and the gut microbiome as well[33]. However, more studies are needed to interpret what this means in the context of inflammation and

PD. Given the ongoing link between inflammatory intestinal diseases like IBD, CD, and UC and PD, LRRK2 seems to be emerging as an important and interesting factor potentially mediating inflammatory phenotypes in PD pathologies.

Pyrethroid Pesticides:

We know that a very small percentage of idiopathic PD diagnoses can be accounted for solely by genetic factors, so looking at synergistic effects of the environment and genes is important in this context. Pesticides and related toxins in particular are of interest in associating with PD diagnosis incidence. Among other toxins like poly-chlorinated biphenyls (PCBs), MPTP (1-methyl 4-phenyl 1,2,3,6-tetrahydropyridine), and dieldrin (insecticide), there have been meta-analyses indicating that pesticide exposure in general can increase risk of PD by over 50%[34-37]. In this way, pesticide exposure emerges as a predictor of PD diagnosis and consistent risk factor.

Specifically, pyrethroids are a class of synthetic insecticides derived from chrysanthemums and are the most commonly used pesticide across the United States[38]. There have been reports and evidence of these pyrethroids being present in urine after consuming foods like wheat, semolina, and fruits from areas treated with it, as well as having a half-life in the body's adipose tissues of up to 2.5 days [38]. Individuals are most commonly exposed to pyrethroids through ingestion or skin contact[38]. There are two classes of pyrethroids: Type I and Type II[39]. Both Type I and Type II disrupt the voltage-gated sodium (Na+) channels required for proper neuron functionality and action potential propagations, while type II pyrethroids simultaneously inhibit the GABA-A receptor to further disrupt and prolong action potentials[39]. Additionally, pyrethroid exposure within the gut seems to change microbiome composition relative to controls with reductions in *Bacteroides, Prevotella,* and *Porphyromonas* species and increases in *Enterobacteriaceae* and

Lactobacillus[40]. What this means in context of PD-related bowel symptoms is yet to be explored but indicates that pyrethroids have systemic effects.

In context of PD, pyrethroid and other environmental toxicant exposure paradigms seem to indicate dysregulation within dopamine systems following exposure. For example, there is evidence of dopamine system disruptions and nervous system toxicity after exposure to toxins like PCBs, MPTP, and dieldrin[34-37]. After developmental exposure to the insecticide dieldrin, there is evidence of dopamine disruptions in the form of dose-dependent increases in DAT and vesicular monoamine transporter 2 (VMAT2) mRNA and protein levels[37]. This disruption extends to the enteric nervous system with evidence of dopaminergic neuron loss in the gut after exposure to MPTP (1-methyl 4-phenyl 1,2,3,6-tetrahydropyridine), a hallmark parkinsonian selective neurotoxin found in synthetic drugs[36]. Additionally, we see a reduction in DAT and VMAT2 levels in the brain striatum after exposure to PCBs[35]. Additionally, pyrethroid exposure in specific show similar effects. For example, repeated exposure to deltamethrin (3mg/kg) increased dopamine uptake mediated by dopamine transporter protein (DAT) by 31%, and permethrin (0.8mg/kg) exposure does the same at a 28% increase[41]. Additionally, studies of oral pyrethroid exposure at high doses indicate changes to the brain dopamine systems in the form of increased dopamine turnover in the brain striatum[42]. In this way, environmental toxicants have systemic effects on dopamine systems, however the way in which these disruptions can lead to PD pathology remains unknown. Understanding these additional environmental exposures helps us understand the extent to which these toxicant exposures are ingrained in everyday life, and how they can have chronic, toxic consequences in the nervous system throughout the brain and gut and lead to PD pathology.

Objective and Project Questions

The overarching objective of this project is to identify the effects of pyrethroid exposure coupled with genetic predisposition to Parkinson's disease pathology on dopaminergic pathways in the gut and in the brain as it relates to Parkinson's disease etiology. We did this by measuring gene expression levels of dopamine-related genes and Parkinson's disease-related genes both in the brain and in the gut. We additionally quantified levels of dopaminergic neurons within the midbrain—specifically, the substantia nigra pars compacta (SNpc) region. By measuring gene expression of dopamine markers like the dopamine transporter protein (DAT), tyrosine hydroxylase (TH), and vesicular monoamine transporter 2 (VMAT2) we assessed endogenous changes associated with both intraperitoneal and oral pyrethroid pesticide exposure. While still preliminary, this assessment provided valuable preliminary insight into the gut-brain axis interactions and how pyrethroid exposures affect connected dopamine systems. This objective gives way to the primary project questions of:

- How do genetic and environmental risk factors for Parkinson's disease compound to contribute to Parkinson's disease-pathology in the gut and brain?
- How do indicators of dopamine systems (DAT, TH, VMAT2) change within the gut and brain followed by oral exposure to pyrethroid pesticides?
- Does the LRRK2 gene expression change in the gut?
- What is the role of the gut-brain axis in mediating pyrethroid-induced toxicity?

We approached answering these questions via specific aims accompanied by robust experimental design.

Specific Aims and Methods

Aim 1: Determine the interaction of chronic pyrethroid exposure with LRRK2 gene products in G2019S (mutant overexpressing) vs WTOE (wildtype overexpressing) vs B6 (wildtype) mice as it pertains to dopaminergic neurodegeneration in the substantia nigra of the brain.

Experimental Design Overview:

Three different genotypes of transgenic mice were divided into two experimental groups with cypermethrin delivery or vehicle (control) delivery. The three genotypes were B6, WTOE, and G2019S mice. The B6 littermates express only endogenous levels of LRRK2 levels and served as controls. The WTOE, or wild-type overexpression, mice overexpress the non-mutant, or wild-type, LRRK2 form. The G2019S mice overexpress the mutant form of LRRK2. These three cohorts underwent 16 weeks of exposure to 15mg/kg cypermethrin or a control vehicle of corn oil through intraperitoneal injection twice a week. After this period of chronic pesticide exposure, the mice were humanely sacrificed. One hemisphere of the brain was then collected for immunohistochemical staining and stereology.

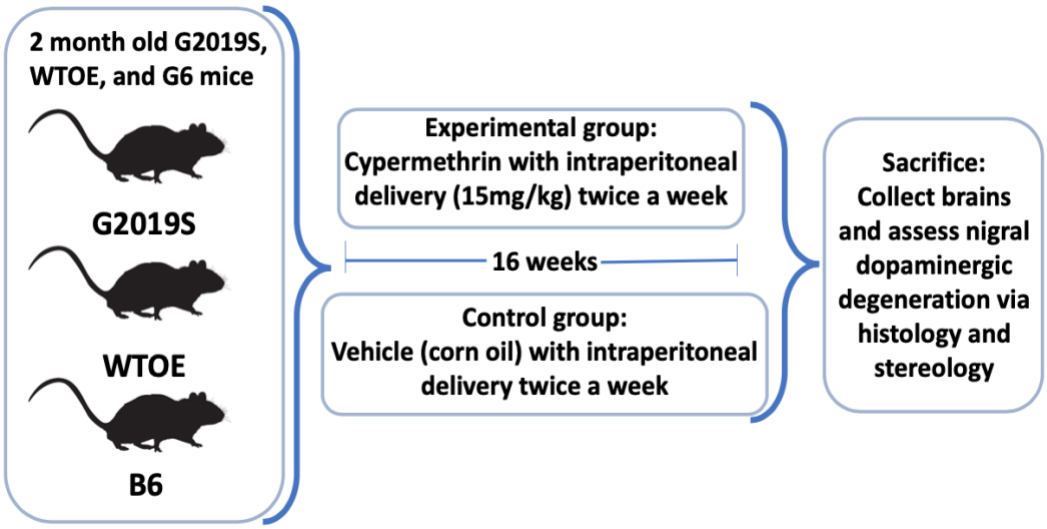


Figure 1. Aim 1 Experimental Design Timeline – Chronic, High-Dose Cypermethrin Intraperitoneal Injection Paradigm

Animals:

Bacterial artificial chromosome transgenic mouse lines were used. These mouse lines either overexpressed the G2019S mutant form of the LRRK2 gene (G2019S) or overexpressed the wildtype LRRK2 gene (WTOE). Littermate controls were used as wildtype controls expressing only endogenous levels of LRRK2 (B6). Two-month old male and female mice were used for this experiment.

Histology:

After humane sacrifice and perfusion (in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals approved by Institutional Animal Care and Use Committee at Emory University), the mouse brains were collected and preserved. The brain blocks were fixed in 4% paraformaldehyde for 24 hours and equilibrated in 30% sucrose in phosphate-buffered saline (PBS)—all at 4°C. The brain sections were then sliced using a Leica cryostat into 40µm coronal sections. Single hemisphere sections containing the substantia nigra pars compacta (SNpc) were then incubated with a polyclonal anti-tyrosine hydroxylase (TH) antibody overnight followed by TH signal amplification with ABC (Avidin-Biotin Complex) Elite kit. Sections were then incubated in secondary antibody for 1 hour at 4°C to mark dopaminergic neurons. 3,3-diaminobenzedene (DAB) was used for development. Sections then underwent cresyl violet counterstaining using a 0.1% aqueous cresyl violet solution to stain Nissl to visualize neuron nuclei and distinguish cell loss followed by destain and xylene and ethanol dehydration[43].

Stereology Quantification

After staining the sections were mounted onto slides and coverslipped after drying. Out of the sliced and stained sections, every third section was counted. Each sliced section was visually assessed and assigned a bregma level. The bregma point is the intersection of the coronal and

sagittal skull sutures, and the bregma level is a numerical measure of brain depth from rostral to caudal. A mouse brain atlas was used to assigned bregma levels. There were six sections per animal with consistent and chronological bregma levels across animals to allow for inter-animal comparison. The bregma levels were ordered from more rostral to more caudal in the SNpc. The stereology method of quantification involved hand-drawing clear boundaries encompassing the SNpc and counting cells using the optical fractionator method in StereoInvestigator analysis software for stereological estimations and parameters set to minimize coefficient of error. After assigning bregma and delineating the SNpc region as per mouse atlas guidelines at low magnification, counting took place at 40x magnification using immersion oil. The quantification is carried out by persons blinded to treatment groups in order to maintain objectivity.

Statistics:

Graph Pad Prism was used to make comparisons across treatment groups. A one-way analysis of variance (ANOVA) was used to compare genotypic groups and an unpaired t-test to compare treatment groups. A p-value of less than 0.05 was considered as significant.

Aim 2: Identify the effects of low-dose, sub-chronic oral pyrethroid exposure on dopaminergic systems in the gastrointestinal tract and brains of wildtype mice

Experimental Design Overview:

Wildtype mice were divided into two experimental groups: one with an oral gavage of 3 mg/kg deltamethrin and another with an oral gavage of vehicle (corn oil) every week for 3 weeks. After the dosing period, the mice were then humanely sacrificed, and GI tract and brain tissue and is collected for analysis. The duodenum, ileum, cecum, proximal large intestine, and distal large intestine were collected, and the midbrain and striatum were dissected out. These tissues samples

were analyzed for changes in markers of dopamine-related pathways relevant for PD-pathology such as DAT, TH, VMAT2, and LRRK2 genes.

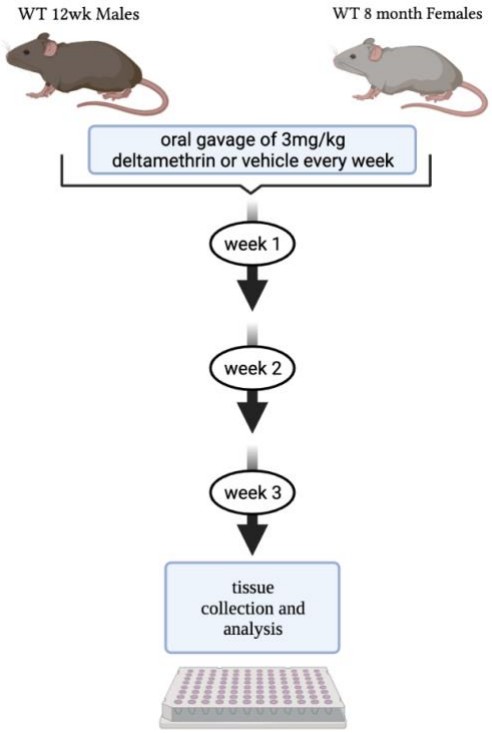


Figure 2. Aim 2 Experimental Design Timeline – Sub chronic, Low Dose Deltamethrin Oral Gavage Paradigm

Animals:

The wildtype mouse line was used with a B6 background. Mouse brain and gut tissue was then collected after humane sacrifice (in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals approved by Institutional Animal Care and Use Committee at Emory University).

RNA Extraction:

Gut tissue, midbrain tissue, and striatal tissue was homogenized in cold trizol (TriReagent) using a sonicator. The homogenous mixture was then mixed with chloroform followed by ethanol and centrifuged to allow separation into aqueous and organic layers. The RNA was then extracted

using Zymo Miniprep Kit spin columns and reagents (Zymo Research). Then, the TURBO DNA-free Kit (Thermofisher) was used for Routine DNase Treatment with related reagents. The RNA was then quantified and assessed for purity using a Nanodrop 2000 spectrophotometer.

cDNA Synthesis:

Following the manufacturer's protocol, RNA was reverse transcribed using iScript cDNA Synthesis Kit (Bio-Rad) with cDNA synthesis 5x reaction buffer, reverse transcriptase, and PCR-grade water.

Quantitative real-time PCR (qPCR):

qPCR was performed using an ABI Prism 7900 HT Fast Real-time PCR system in a 384-well plate. Samples were run in triplicates with a per-well reaction volume of 12 µL made up of SYBR Green, oligonucleotide forward and reverse primers, and cDNA. Primers used were previously validated. Threshold of Cycles (Ct) values were normalized to Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH)—housekeeping gene—values.

Statistics:

Graph Pad Prism was used to make comparisons across treatment groups. An unpaired t-test was used to make comparisons between treatment groups with a p-value of less than 0.05 being considered as significant.

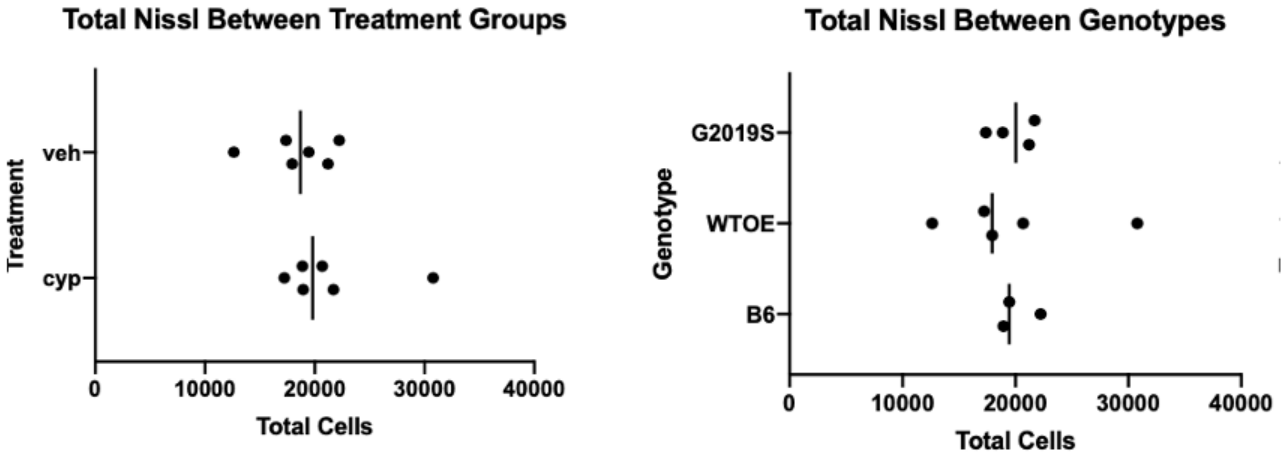
Figures and Results

Overall, through our experimental analysis we looked for changes in dopaminergic neuron levels using stereology after treatment with cypermethrin and coupled with the PD genetic risk gene—LRRK2. We additionally looked in wildtype animals for changes in gene expression of dopamine-related (VMAT2, DAT, TH) markers, cholinergic (CHAT) markers, and LRRK2 in gut and brain regions following oral deltamethrin exposure. We found no significant differences in dopaminergic neuron levels across treatment groups or genotypes through our stereological paradigm. However, after oral exposure to deltamethrin in the sub chronic exposure paradigm using wildtype mice, we found a significant increase in VMAT2 expression in the striatum, and a significant decrease in CHAT and LRRK2 expression in the proximal large intestine of deltamethrin treated mice.

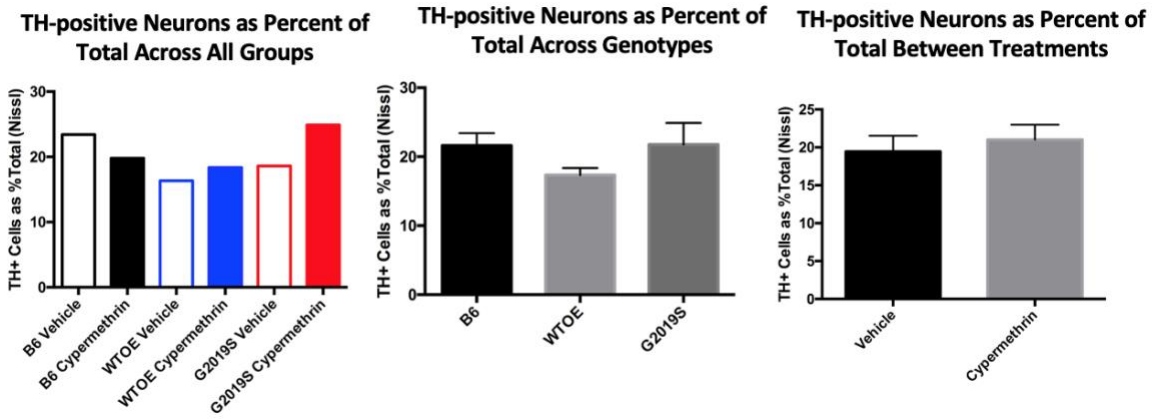
Aim 1: Assessment of total and TH+ neurons across treatment groups and genotypes

Using stereology methods, we counted total neurons and TH+ (tyrosine hydroxylase positive) neurons within the SNpc to quantify dopaminergic neurons relative to total neurons to test PD-applicable neurogenerative effects following chronic cypermethrin exposure in 2-month-old mice with different genotypic backgrounds predisposing them to PD-like pathology. B6 wildtype background and vehicle delivery were used as controls. As shown in **Figures 3-4**, when comparing total neuron counts in the SNpc across treatment groups (cypermethrin (cyp) vs vehicle (veh) delivery) and across genotypes (G2019S vs WTOE vs B6), a one-way ANOVA revealed no significant baseline differences in cell counts across genotypes ($p= 0.7871$) and an unpaired t-test indicated the same between treatments ($p=0.0928$). As shown in Figure 5-7, A comparison of TH-positive neurons as a percent of total neurons (Nissl) across all treatment and genotypic groups; a one-way ANOVA indicated no significant differences ($p=0.4705$) Middle: a comparison of TH-

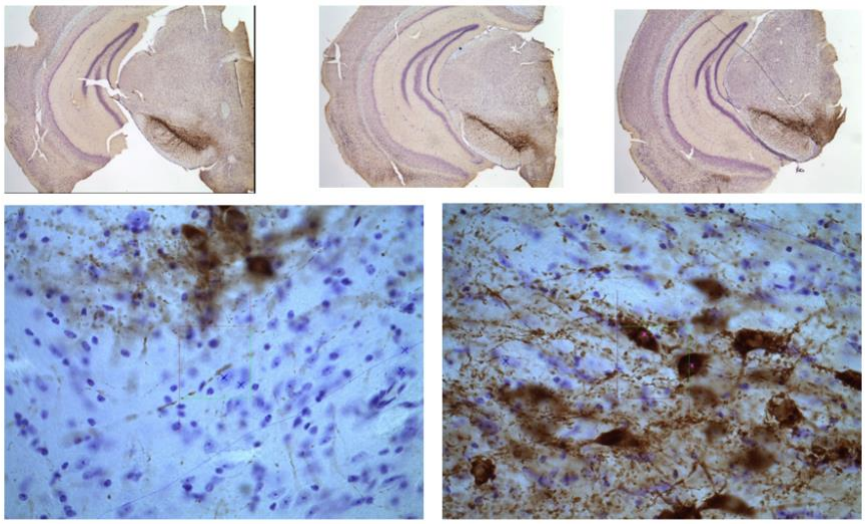
positive neurons as a percent of total neurons (Nissl) across genotypes; one-way ANOVA indicated no significance (p=0.3853). Right: A comparison of TH-positive neurons as a percent of total neurons (Nissl) across treatment groups; unpaired t-test indicates no significance (p=0.6172).



Figures 3 & 4. Total number of Nissl-stained neurons in the SNpc across treatment groups (cypermethrin (cyp) vs vehicle (veh) delivery) and across genotypes (G2019S vs WTOE vs B6). A one-way ANOVA was performed and revealed no significant baseline differences in cell counts across genotypes (p= 0.7871) and an unpaired t-test indicated no significant differences between treatments (p=0.0928).



Figures 5-7. Left: TH-positive neurons as a percent of total neurons (Nissl) in the SNpc across all treatment and genotypic groups. A one-way ANOVA indicated no significant differences (p=0.4705) Middle: TH-positive neurons as a percent of total neurons (Nissl) in the SNpc across genotypes. A one-way ANOVA indicated no significance (p=0.3853). Right: TH-positive neurons as a percent of total neurons (Nissl) in the SNpc across treatment groups. An unpaired t-test indicates no significance (p=0.6172)

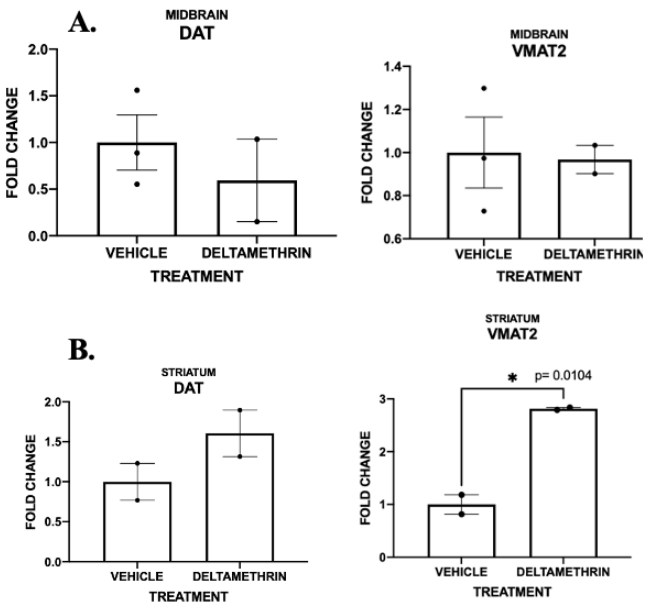


Images 1-3 and 4-5. **Representative images of stereology**. **Images 1-3 (top row):** 2x magnified left brain hemispheres containing the substantia nigra (SNpc) of B6, G2019S, and WTOE mice (from left to right) all treated with cypermethrin injection. Bregma levels around 3.18 to 3.28. **Images 4-5 (bottom row):** 40x magnified substantia nigra (SNpc) region of left brain hemispheres with counting frame (red and green square) and markers over dopaminergic neurons (right image) and nondopaminergic neurons (left image).

Stereology counting within the substantia nigra of either cypermethrin treated or vehicle treated animals within each genotypic group indicated no significant differences. This protocol was completed in parallel with the aim 2 methods. Data collected under aim 2 was gene expression data relative to GAPDH (housekeeping gene) via qPCR methods. We looked in the ileum (small intestine), proximal large intestine, and distal large intestine within the gut, and the midbrain and striatum regions of the brain for TH, DAT, VMAT2, CHAT, and LRRK2 targets.

Aim 2: Assessment of gene expression of VMAT2, DAT, TH, LRRK2, and CHAT in the gut and brain following oral deltamethrin exposure in wildtype mice

In our comparison of vehicle-treated vs deltamethrin treated animals we looked at markers of dopamine function in the midbrain and striatum and found a significant increase in VMAT2 expression in the striatum of deltamethrin treated mice as shown in **Figure 8** (p= 0.0104). However, all other brain region comparisons were not significant for dopamine markers.

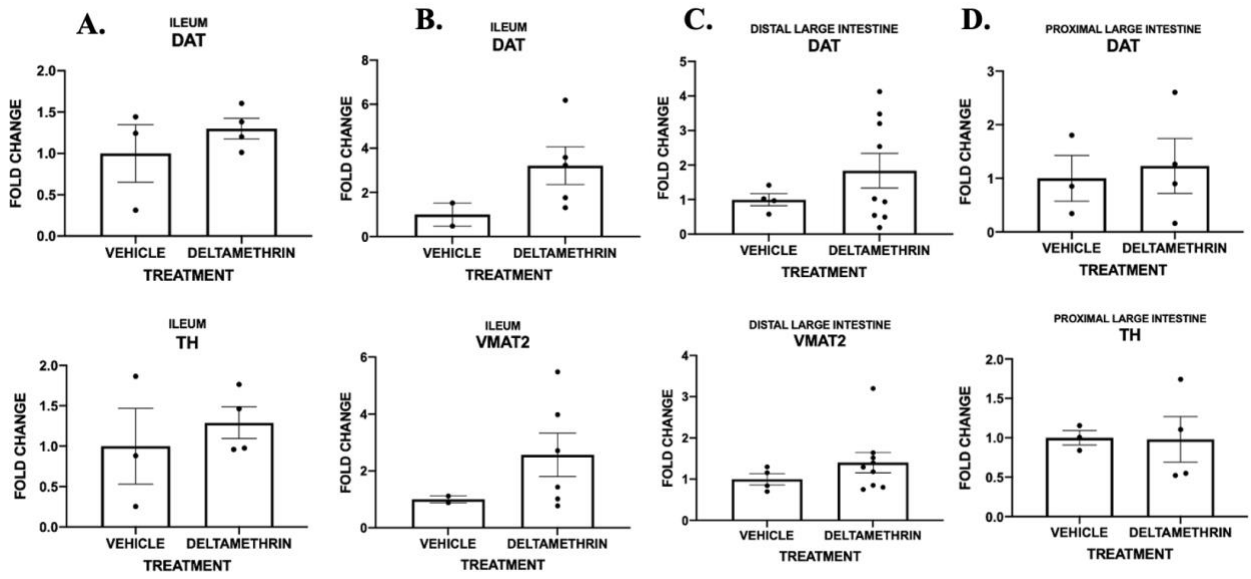


<u>Figure 8.</u> mRNA Expression of Dopamine-Related Markers (DAT and VMAT2) Relative to GAPDH Expression in the Midbrain (row A) and Striatum(row B). Unpaired t-tests for each comparison indicate no significance in all cohorts except when comparing VMAT2 in the striatum with p=0.0104. qPCR samples from WT 12-week-old male mice. P-values in clockwise direction are as follows: p=0.4779, p=0.8916, p=0.0104, p=0.2436.

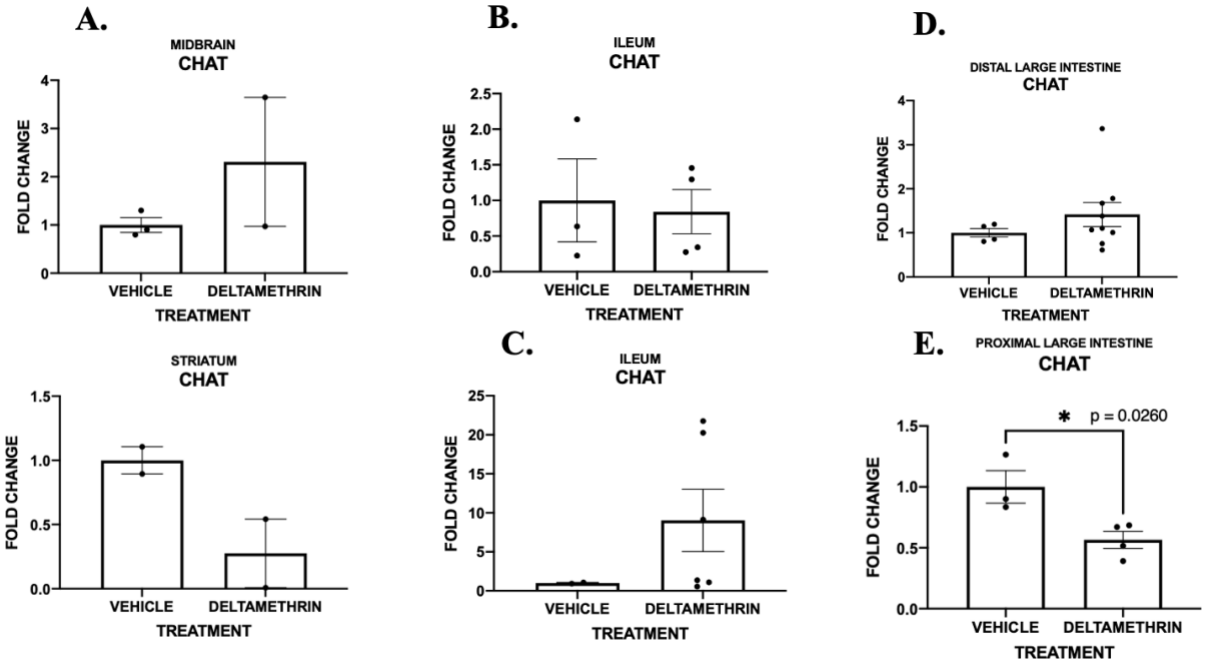
We additionally looked in the small intestine (ileum) and large intestine (proximal and distal) for markers of dopamine function. Although there were some increased in deltamethrin treated groups of DAT and VMAT2 expression, unpaired t-tests indicated these were not significant as shown in **Figure 9**. There were not significant differences between deltamethrin treated and vehicle treated animals in expression of DAT, TH, or VMAT2 for the analyzed tissues within the small intestine and large intestine of the gut as shown in **Figure 9**. Both 8-month-old female mice and 12-week-old male mice were used for these analyses.

We looked at CHAT expression as an indicator of gut function and motility and brain cholinergic pathways. Specifically, CHAT is involved in movement of gut contents and embryonic depletion of CHAT has been shown to cause reduced colon transit, intestinal dysbiosis, and death in mice[44]. There were no significant differences in deltamethrin versus vehicle treated animals in the brain or small intestine, but there was a significant decrease in CHAT expression ($p=0.0260$) in the proximal large intestine of the deltamethrin-treated WT 12-week-old male mice as per unpaired t-tests as shown in **Figure 10**.

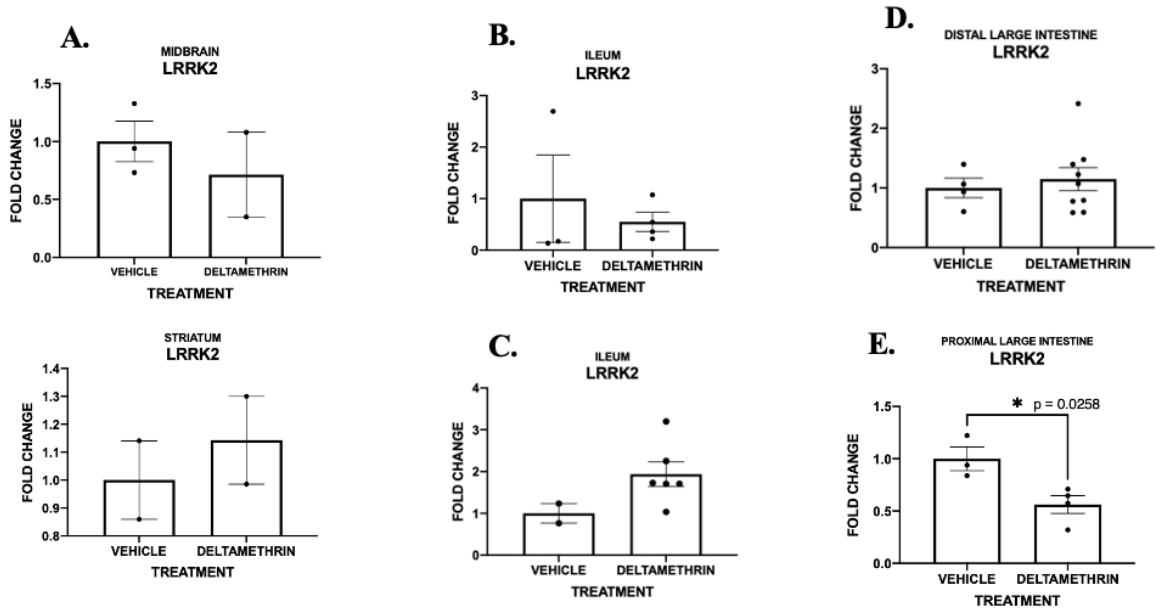
We also looked at LRRK2 expression and although we found no significant changes in brain regions, we saw a significant decrease ($p=0.0258$) in expression in the proximal large intestine of deltamethrin-treated 12-week old male, wildtype mice as shown in **Figure 11**.



<u>Figure 9</u>. mRNA Expression of Dopamine-related Genes (DAT, TH, VMAT2) in the Small and Large Intestines Relative to GAPDH. Column A: A comparison of DAT and TH expression levels in the ileum of WT 12-week-old male mice treated with vehicle or deltamethrin. Not significant differences in DAT or TH, respective p-values are p=0.4009 and p= 0.5518. **Column B:** A comparison of DAT and VMAT2 expression levels in the ileum of WT 8-month-old female mice treated with vehicle or deltamethrin. Not significant differences in DAT or VMAT2, respective p-values are p= 0.1894and p= 0.3038. **Column C:** A comparison of DAT and VMAT2 expression levels in the distal large intestine of WT 18-month-old female mice treated with vehicle or deltamethrin. Not significant differences in DAT or VMAT2, respective p-values are p=0.3015 and p= 0.3236. **Column D**: A comparison of DAT and TH expression levels in the proximal large intestine of WT 12-week-old male mice treated with vehicle or deltamethrin. Not significant differences in DAT or TH, respective p-values are p=0.7549 and p=0.9559 .



<u>Figure 10.</u> mRNA Expression of CHAT Relative to GAPDH in the Brain and Gut. **Column A:** mRNA expression of CHAT in the midbrain and striatum of WT 12-week old male mice indicate no significant differences between treatment groups with respective p-values of p=0.2877 and p=0.1275 . **B:** mRNA expression of CHAT in the ileum of WT 12-week-old male mice indicate no significant differences between treatment groups with a p-value of p=0.8058. **C:** mRNA expression of CHAT in the ileum of WT 8-month-old female mice indicate no significant differences between treatment groups with a p-value of p= 0.3146 . **D:** mRNA expression of CHAT in the distal large intestine of WT 8-month-old female mice indicate no significant differences between treatment groups with a p-value of p= 0.3514 . **E:** mRNA expression of CHAT in the proximal large intestine of WT 12-week-old male mice indicates a significant decrease in deltamethrin treated animals compared to vehicle controls with a p-value of p= 0.0260 .



<u>Figure 11.</u> mRNA Expression of LRRK2 Relative to GAPDH in the Brain and Gut. Column A: mRNA expression of LRRK2 in the midbrain and striatum of WT 12-week-old male mice indicate no significant differences between treatment groups with respective p-values of p=0.4782 and p=0.5689 . **B:** mRNA expression of LRRK2 in the ileum of WT 12-week-old male mice indicate no significant differences between treatment groups with a p-value of p= 0.5697 . **C:** mRNA expression of LRRK2 in the ileum of WT 8-month-old female mice indicate no significant differences between treatment groups with a p-value of p= 0.1414 . **D:** mRNA expression of LRRK2 in the distal large intestine of WT 8-month-old female mice indicate no significant differences between treatment groups with a p-value of p= 0.6468 . **E:** mRNA expression of LRRK2 in the proximal large intestine of WT 12-week-old male mice indicates a significant decrease in deltamethrin treated animals compared to vehicle controls with a p-value of p=0.0258 .

Discussion and Future Directions

In the current study, we investigated the effects of genetic and environmental risk factors for PD on dopamine systems within the gut and brain through the experimental paradigms of peripheral (Aim 1) and oral (Aim 2) exposure to pyrethroid pesticides and genetic predisposition to PD. Much of our data lacks the statistical significance and power to draw concrete conclusions, however it is important to examine these preliminary analyses to elucidate what it might means for PD-pathology.

A major limitation of our stereological analyses used in Aim 1 was its low power. Sufficiently powering this experimental design would likely provide insight into PD-pathology in the brains of mice genetically predisposed to PD. Specifically, we would be better able to quantify dopamine degeneration in the substantia nigra pars compacta (SNpc) region of the midbrain to look for hallmark PD-related degeneration across different treatment groups and different genotypes. Current data does not indicate a significant difference in TH-positive cells relative to total neurons across the mutant LRRK2 (G2019S), wildtype LRRK2 overexpressing (WTOE), or wildtype genotypes (B6), as well as between cypermethrin-treated and vehicle-treated groups. However, given that LRRK2 overactivity is known to have inflammatory functions in the periphery, this has interesting implications for the gut-brain axis communication. Within the stereology paradigm, we found comparable baseline levels of total neurons indicating no observable difference in the number of neurons in the SNpc across genotypes and between treatment groups. However, this relationship cannot be stated with certainty due to the low power of the study.

In aim 2, we orally gavaged both male and female wildtype mice at 12-weeks and 8-months old, respectively, to investigate the effects of sub-chronic oral pyrethroid delivery to determine

pyrethroid ability to disrupt the gut microbiome, dopamine pathways in the enteric and central nervous systems, and potential inflammatory pathways[38, 40, 45]. In this paradigm, we examined mRNA expression levels of dopamine-marker genes like DAT, TH, and VMAT2 relative to GAPDH. We also looked at expression of CHAT as a marker of intestinal function and motility and cholinergic neurons within the brain. Finally, we looked at LRRK2 expression to elucidate endogenous changes in LRRK2 expression that may accompany pyrethroid exposure.

When examining dopamine-related markers like DAT, TH, and VMAT2, we found no significant difference in expression between treatments throughout the gut tissues. We found that within the midbrain and striatum, there were no significant differences in DAT expression between treatment groups, however there was a significant increase in VMAT2 expression in the striatum of deltamethrin treated animals. VMAT2 functions in dopamine synthesis and release to uptake cytosolic dopamine into vesicles for release into the synapse[14]. However, it also is expressed in all monoamine-releasing neurons and functions in the sequestering and release of other monoamines in addition to dopamine[46]. In this way, increased expression of this gene could mean increased transporters present within neurons, but this could also be acting on serotonin, noradrenaline, adrenaline, and histamine pathways as well[46]. Because we are looking specifically in the brain striatum, this includes dopaminergic and serotonergic neurons so the increased VMAT2 expression we see may not be specific to dopaminergic pathways and additional protein analyses and coupling with dopamine-specific markers is needed[46]. However, in dopaminergic neurons, should increased VMAT2 gene expression reflect increased VMAT2 protein production, this would increase the rate at which dopamine is taken into vesicles and could potentially lead to an excessive release of dopamine into the synapse. Because dopamine is prone to reactive oxygen species, this could have neurotoxic effects and be detrimental to healthy individuals[47].

Contrastingly, increased VMAT2 could also have the potential to increase efficiency of dopamine movement which could help counteract dopaminergic neuron loss in PD-pathology. There is evidence of this in the literature as well, with a mouse model expressing a 200% increase in VMAT2 results in increased dopamine vesicular capacity by 56% and increased dopamine release by 84% within the striatum[48]. This form of modulation seems to be an exciting prospect for PD-therapy. Although there is evidence in the literature of VMAT2 downregulation in the striatum decreasing dopamine levels and dysregulating dopamine systems, this deltamethrin-mediated increase in VMAT2 could have neurotoxic effects leading to systemic dopamine dysregulation as well in healthy individual, or a potential rescue function in PD-patients[49]. In order to confirm increased translation of the gene and protein presence, Western blot analyses would be necessary.

The choline acetyltransferase (CHAT) gene was analyzed as a marker of gut function and motility as cholinergic pathways are the primary excitatory pathways innervating the enteric nervous system[50]. Additionally, the striatum in the brain contains the highest levels of cholinergic neurons and evidence indicates that changes in striatal cholinergic cell populations affects motor control[51]. These cholinergic pathways in the brain are additionally involved in fine tuning processes of brain function[51]. We found a general increase in the midbrain and a general decrease of CHAT levels in the striatum of deltamethrin treated animals. Coupled with the significant increase in VMAT2 levels in the striatum which could potentially increase dopamine release and evidence that dopamine inhibits acetylcholine release in the striatum, reduced CHAT levels we see in the striatum might be a consequence of the inhibitory effects of increased dopamine from increased VMAT2[52]. However, these trends cannot be validated due to a lack of statistical significance and more data is needed to validate this. In the gut, there was no statistically significant difference in CHAT levels between treatments within ileum or distal large intestine

tissues. However, there was a significant decrease in CHAT expression within the proximal large intestine of deltamethrin treated animals. Literature indicates that reduced CHAT levels in the gut has negative consequences including decreased motility of stools through the intestine and microbiome dysbiosis[44]. Consequently, this decrease in CHAT within the gut could be indicative of gut dysregulation as a consequence of pyrethroid exposure. However, additional research is needed to determine whether there is decreased protein as well, if it is actually pyrethroid-mediated CHAT-reduction, and how this reduction potentially impacts the enteric nervous system. However, this preliminary insight is interesting especially in context of many of the non-motor symptoms of PD being gut dysregulatory (i.e. constipation and Crohn's Disease) ones[8].

The LRRK2 gene is a known risk gene for PD. In the peripheral pyrethroid injection model in Aim 1, we looked at genetic predisposition to PD in the form of G2019S genotypic models. In the oral exposure model used in Aim 2, we take a more gut-mediated perspective. We looked in the midbrain and striatum for changes in LRRK2 expression, but there were no statistically significant differences. This was also true within the ileum and distal large intestine. In the proximal large intestine, there was a significant decrease in LRRK2 expression relative to GAPDH in the deltamethrin treated animals. LRRK2 is known to be expressed throughout the body, however there is evidence of increased expression on peripheral monocytes as well as B and T cells in PD phenotypes[53]. Additionally, its mutant form (G2019S) is also implicated in inflammatory bowel disease like Crohn's Disease[28, 32]. Additionally, LRRK2 is involved in a variety of cellular processes like autophagy and vesicle trafficking[28]. Given this information, a decrease in LRRK2 expression in the gut of pyrethroid-treated animals seems to suggest potential pyrethroid-mediated toxic effects and disruption in the normal function of gut cells. However, in context of PD, if an individual with the G2019S mutation had decreased levels of the LRRK2 gene,

they may not be subject to the full extent of the inflammatory and kinase activity of the mutant gene. This could potentially confer a protective effect against PD-pathology. However, additional research including Western blots are needed to test for translated protein levels to explore the exact interaction that takes place between these pyrethroid pesticides and LRRK2 within cells.

Overall, there seem to be some deltamethrin-mediated changes to the dopamine systems of the brain striatum, effects on cholinergic systems within the gut, and potential changes to the PD-risk gene, LRRK2, also within the gut. All of these mRNA expression levels need to be coupled with protein data from Western blot analyses to further solidify our understanding of the observed changes. Furthermore, performing stereological analyses at a higher power would allow for more statistically sound conclusions. For future directions of this project, conducting oral pyrethroid exposure assessments using genetically predisposed mice with the G2019S mutation and WTOE genetic background might help better elucidate the role of the gut-brain axis and how dopamine systems throughout the central and enteric nervous systems can interact to give rise to PD pathology. Additionally, administering low doses of deltamethrin oral gavage over a more chronic time period would better replicate real-world environmental toxicant—particularly pyrethroid—exposures. To bolster the genetic-environmental aspect of this project, introducing another genetic background predisposed to PD could be a long-term goal. For example, the SNCA gene is another PD-risk gene giving risk to alpha-synuclein aggregations which then form Lewy bodies which are hallmark indicators of Parkinson's Disease[54].

Overall, the results of this study serve as important preliminary insights into Parkinson's Disease pathogenesis as it relates to genetic and environmental risk factors in context of the gut-brain axis. It has real-world significance as pesticides and other toxicants are ingrained into daily life, and it is important to examine the long-term effects these exposures can have on individuals.

Although our data is preliminary, the significant differences in VMAT2 in the brain and changes in cholinergic neuron and LRRK2 expression within the gut provide insight into the potentially pyrethroid-mediated effects on the gut-brain axis as it pertains to PD-pathology.

List of Figures and Images

Figure 1. Aim 1 Experimental Design Timeline – Chronic, High-Dose Cypermethrin Intraperitoneal Injection Paradigm.……..…….12

Figure 2. Aim 2 Experimental Design Timeline – Sub chronic, Low Dose Deltamethrin Oral Gavage Paradigm.…….…….15

Figure 3. A comparison of total neuron counts across treatment groups (cypermethrin(cyp) vs vehicle (veh) delivery) ……..……..….17

Figure 4. A comparison of total neuron counts across genotypes (G2019S vs WTOE vs B6)….17

Figure 5. A comparison of TH-positive neurons as a percent of total neurons (Nissl) across all treatment and genotypic groups.……...…….17

Figure 6. A comparison of TH-positive neurons as a percent of total neurons (Nissl) across genotypes.……..……...….17

Figure 7. A comparison of TH-positive neurons as a percent of total neurons (Nissl) across treatment groups.……...….17

Image 1. Representative images of stereology……..……18

Image 2. Representative images of stereology……..……18

Image 3. Representative images of stereology……..……18

Image 4. Representative images of stereology……..……18

Image 5. Representative images of stereology……..……18

Figure 8. mRNA Expression of Dopamine-Related Markers (DAT and VMAT2) Relative to GAPDH Expression in the Midbrain and Striatum.……..…….19

Figure 9. mRNA Expression of Dopamine-related Genes (DAT, TH, VMAT2) in the Small and Large Intestines Relative to GAPDH.……..……...…20

Figure 10. mRNA Expression of CHAT Relative to GAPDH in the Brain and Gut……..…..21

Figure 11. mRNA Expression of LRRK2 Relative to GAPDH in the Brain and Gut……..…22

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