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Examining gene-environment interactions and synaptic effects of Parkinson's disease-linked pesticides using a Drosophila model

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Examining gene-environment interactions and synaptic effects of Parkinson's disease-linked pesticides using a *Drosophila* model

A dissertation submitted in partial satisfaction of the

requirements for the degree Doctor of Philosophy

in Molecular Toxicology

by

Ciara Ann-Marie Martin

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ABSTRACT OF THE DISSERTATION

Examining gene-environment interactions and synaptic effects of Parkinson's disease-linked pesticides using a *Drosophila* model

by

Ciara Anne-Marie Martin

Doctor of Philosophy in Molecular Toxicology

University of California, Los Angeles, 2014

Professor David E. Krantz, Chair

Parkinson's disease (PD) is a neurodegenerative disorder that affects millions of people worldwide and is characterized by a loss of dopaminergic neurons along with other deficits. While rare inheritable forms of PD exist, the vast majority of cases are sporadic and their etiology remains poorly understood. Epidemiological studies have increasingly linked exposures to environmental toxins, including a variety of pesticides, to an increased risk of contracting PD. However, it remains unclear as to the mechanism by which these pesticides cause neurotoxicity and the extent to which individual genetic variability plays a role. Alterations in the vesicular monoamine transporter (VMAT) gene, which packages dopamine and other amines into synaptic vesicles, have been shown to affect PD susceptibility in animal models and epidemiological studies alike. Using live-imaging techniques at the *Drosophila melanogaster* neuromuscular junction, I investigated the functional consequences of altered VMAT trafficking at an intact synapse. I show that mutations in putative VMAT trafficking domains result in abnormal protein localization during synaptic vesicle exo- and endocytosis, in a manner that is dependent on nerve

terminal identity. Using the same approach, I show that the PD-linked pesticide ziram differentially disrupts neuronal activity and synaptic vesicle cycling at functionally distinct nerve terminals. In particular, I demonstrate that endocytosis at aminergic nerve terminals is particularly sensitive to both disruption of VMAT trafficking motifs and exposure to ziram, and that ziram appears to selectivity induce the spontaneous depolarization of these terminals. Additionally, using traditional toxicological approaches, I developed a novel model of paraquat and maneb induced PD in *Drosophila* and demonstrate the general utility of this model for interrogating specific mechanisms implicated in PD etiology. This dissertation discusses my research using *Drosophila* to address basic biological questions fundamental to understanding gene-environment interactions implicated in PD.

The dissertation of Ciara Ann-Marie Martin is approved by

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2014

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Chapter 1 is a version of a paper in press with the *Journal of Neuroscience* and is titled "The redistribution of *Drosophila* vesicular monoamine transporter mutants from synaptic vesicles to large dense core vesicles impairs amine-dependent behaviors." Co-authors include Anna Grygoruk, Audrey Chen, Hakeem Lawal, Hao Fei, Gabriel Gutierrez, Traci Biederman, Rod Najibi, Richard Hadi, Amit Chouhan, Niall Murphy, Felix Schweizer, Greg Macleod, Nigel T. Maidment, and David Krantz. Chapter 2 is a version of a paper currently in preparation for submission titled, "The Parkinson's disease linked pesticide, ziram, causes synaptic dysfunction *in vivo* and has differential effects at functionally distinct terminals." Co-authors include Audrey Chen, Nathan Martin, Felix Schweizer, and David Krantz. Chapter 3 is a version of a paper currently in review with the journal *Neurotoxicology*, titled "Synergistic effects of paraquat and maneb on dopamine cell death in a *Drosophila* model of chronic toxin exposure."

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CA Martin, DE Krantz. The Study of Neurotransmitter Transporters Using the Model Organism *Drosophila Melanogaster*. *Neurochemistry International*.

A Grygoruk, A Chen, HO Lawal, **CA Martin**, G Gutierrez, H Fei, T Biederman, R Najibi, R Hadi, A Chouhan, N Murphy, F Schweizer, G Macleod, N Maidment, DE Krantz. The redistribution of *Drosophil*a vesicular monoamine transporter mutants from synaptic vesicles to large dense core vesicles impairs amine-dependent behaviors. *The Journal of Neuroscience*.

In press

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In preparation

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2011, 2013 Cold Spring Harbor Neurobiology of Drosophila Meeting

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2012 Gordon Research Conference on Synaptic Transmission

2011, 2014 Drosophila Research Conference

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Introduction:

To pesticide-linked Parkinson's disease and to the synaptic neurobiology of the *Drosophila* neuromuscular junction

Pesticide exposures and Parkinson's disease

Parkinson's disease (PD) is characterized by the irreversible loss of dopamine (DA) neurons in the substantia nigra. PD manifests clinically as the progressive deregulation of essential functions such as movement, sleep, mood, smell, and digestion that are controlled by DA and other amines. In sporadic PD, which accounts for the vast majority of cases, various genetic and/or toxic insults are thought to accumulate over the years causing deregulation of DA neurons that eventually pushes them beyond their threshold for survival (Shlomo, 1996). In the late 80's and early 90's epidemiological studies began linking pesticide exposures to age-related neurological diseases, such as PD (Barbeau, 1987; Koller, 1990). Global pesticide use is now in the range of billions of pounds per year, with annual use of active pesticide ingredients in the U.S. at 857 million pounds in 2007 alone (Grube, 2011). The combination of these widespread pesticide exposures with the aging population, which is more susceptible to neurodegenerative disease, poses a significant public health risk.

The idea that there may be a link between environmental toxins and PD gained headway in the early 1980's when several young adults in Santa Clara, California contracted rapid-onset, irreversible PD; the cause of PD in these individuals was eventually traced back to their use of an incorrectly synthesized recreational drug with the toxic byproduct 1-methyl-4-phenyl-1,2,3,6-tertrahydropyridine (MPTP) (Weingarten, 1988). Both MPTP exposure and sporadic PD lead to the irreversible loss of DA neurons, however, sporadic PD progresses over decades while MPTP elicits its effects immediately. The toxic metabolite of MPTP, MPP^+, has a similar chemical structure to the widely used agriculture pesticide paraquat and these similarities lead researchers to explore the connection between pesticide exposures and PD incidence (Weingarten, 1988). In

the late 80's and early 90's epidemiological studies began reporting a correlative increase in PD rates in rural farming areas with heavy pesticide use (Barbeau, 1987; Koller, 1990). These early studies, however, relied heavily on patient recall and did not have enough data to determine if it was pesticide exposure in general or a particular pesticide that was responsible for the association with PD.

The Parkinson's Environment and Gene study (PEG) at the University of California, Los Angeles (UCLA) has recently developed a geographical information system (GIS) based approach to estimate human pesticide exposure (Costello et al., 2009). This approach utilizes a combination of California Pesticide Use Reporting records, land-use maps and geo-coded residential historical locations to generate a database of unbiased exposure information. This powerful tool has allowed researchers to single out individual pesticides that increase PD risk. Individual pesticides found to significantly increase PD risk include paraquat, maneb, and benomyl (Costello et al., 2009; Fitzmaurice et al., 2013; Wang et al., 2011). PEG also demonstrated that combined exposure to multiple pesticides could further increase PD risk. For instance, while exposure to paraquat + maneb or paraquat + ziram substantially increases PD risk (increases of 40% and 80%, respectively), combined exposure to paraquat + maneb + ziram results in a ~200% increase in risk (Costello et al., 2009).

Several animal models of PD have been developed that substantiate the PEG epidemiological findings linking pesticide exposures to PD. Exposure to benomyl kills DA neurons in primary culture and in a zebrafish model (Fitzmaurice et al., 2013). Paraquat's DA neurotoxicity has been validated in invertebrate and rodent models alike (Chaudhuri et al., 2007; Liou et al., 1996); and in rodents, paraquat's toxicity is clearly potentiated by maneb exposure (Cicchetti et al., 2005; Thiruchelvam, 2000). To our knowledge, no studies had investigated the

potential synergism of paraquat and maneb in *Drosophila* prior to my work discussed in Chapter 1. I developed a fly model of paraquat and maneb induced DA cell loss that provided a platform for the investigation of multiple gene-environment interactions implicated in PD.

PEG was the first study to identify ziram as a factor in PD etiology and subsequent rodent studies have demonstrated ziram's ability to selectively kill DA neurons *in vitro* and to lead to motor deficits *in vivo* (Chou et al., 2008; Wang et al., 2011). In Chapter 3, I discuss how I used live imaging at the *Drosophila* neuromuscular junction (NMJ) to investigate the effects of ziram at an intact synapse.

Not all persons exposed to pesticides develop PD, suggesting genetic background plays a significant role in the disease progression. Several genetic variations have been shown to either increase risk of PD or be protective against PD including alterations in the genes encoding α-synuclein, aldehyde dehydrogenase, the vesicular monoamine transporter (VMAT), and the dopamine transporter (DAT), and proteins involved in iron regulation (Fitzmaurice et al., 2014; Gatto, 2010; Glatt et al., 2006; Rhodes and Ritz, 2008; Ritz, 2009). I investigated these potential gene environment interactions using my paraquat and maneb model, discussed in Chapter 1.

The neurotoxic mechanisms of action linking pesticides to PD are unclear

The relationship between paraquat and PD has been very well studied due to paraquat's structural similarity to MPP^+. Ironically, MPP^+ appears to elicit its toxicity in a different manner than paraquat; the toxicity of MPP^+ is attributed to its inhibition of mitochondrial complex I while paraquat's toxicity is attributed to its robust induction of oxidative stress (Miller, 2007). Initial studies of paraquat in the rodent failed to reproducibly replicate DA neurotoxicity and lead researchers to look at the effects of multiple environmental risk factors in combination (Liou et

al., 1996; Minnema et al., 2014; Naylor et al., 1995). Maneb was an obvious candidate due to its widespread use and overlap in spray locations with paraquat, and maneb exposure was indeed found to potentiate paraquat's DA neurotoxicity in rodent models (Thiruchelvam, 2000). The paraquat and maneb model of PD is now a well-established model of PD in rodents and has proven to be reproducible in multiple laboratories (Cicchetti et al., 2005; Li et al., 2005; Saint-Pierre et al., 2006; Srivastava et al., 2012; Thiruchelvam, 2000).

Questions still linger though regarding paraquat's mechanism of action. The effects of paraquat exposure appear to be hormetic, as paraquat exposure has also been shown to extend lifespan of *C. elegans* on a calorie-restricted diet and in *Drosophila* lacking the inheritable PD gene *PARKIN* (Bonilla et al., 2002; Schulz et al., 2007). Thus, low doses of paraquat may be beneficial (likely through the activation of antioxidant gene transcription pathways) while high doses appear to pose insurmountable toxicity. It has been proposed that the oxidative potential of DA is what makes DA neurons more susceptibility to paraquat toxicity (Sutachan et al., 2012), however, the toxicity caused by elevated DA levels is reduced in flies exposed to paraquat, suggesting other mechanisms of action are at play (Chaudhuri et al., 2007).

Both the PD linked pesticides maneb and ziram are dithiocarbamates, which contain sulfur groups that can react readily with biologic macromolecules (Rath, 2011). Maneb is an ethylene-bis-dithiocarbamate (EDBC) fungicide containing manganese. In humans, exposure to excessive manganese results in 'manganism' a disease reminiscent of, and often misdiagnosed as, PD (Martinez-Finley et al., 2013). Maneb has also been shown to elicit mitochondrial inhibition and disruption of dopamine homeostasis (Fitzmaurice et al., 2014; Leiphon and Picklo Sr, 2007; Zhang et al., 2003), however, what mechanisms exactly are responsible for its association with PD are unclear. It is clear however, that maneb has the ability to damage the DA

system as exposure to maneb alone in both rodents and *C.elegans* has been shown to cause DA cell loss (Negga et al., 2012; Zhang et al., 2003).

In addition to paraquat and maneb, ziram has also been linked to PD but less is known regarding its potential neurotoxic mechanisms of action (Wang et al., 2011). Reports thus far have shown ziram to disrupt immune function, cause genotoxicity and have toxic reproductive effects (Cilievici, 1983; Franekić J, 1994; Giavini, 1983; Hemavathi, 1993; Li et al.; Tripathy, 1989). Ziram contains zinc and can be metabolized into carbon disulfide, both of which have been linked to neurodegenerative diseases (Huang, 2004; Jomova et al., 2010). Recent experiments demonstrate the ability of ziram to selectively decrease survival of primary DA neurons, and cause motor dysfunction *in vivo*; however, the DA neuron loss stereotypical of PD was not observed (Chou et al., 2008). Additionally, ziram's ability to inhibit aldehyde dehydrogenase (ALDH) and components of the ubiquitin proteasome system (UPS) have been proposed as potential neurotoxic mechanisms of action (discussed in further detail below) (Chou et al., 2008; Fitzmaurice et al., 2014).

ALDH converts the toxic dopamine metabolite, DOPAL, to the less toxic metabolite, DOPAC, and therefore plays a role in dopamine homeostasis (Florang, 2006). The dithiocarbamate moiety found in ziram and maneb has been shown to inhibit ALDH, in addition several other non-dithiocarbamate pesticides have also been shown to inhibit ALDH (Fitzmaurice et al., 2013; Leiphon and Picklo Sr, 2007; Shen, 2000, 2001) Recently, variants in the ALDH gene have been linked to an increased risk of PD in humans (Fitzmaurice et al., 2014), further suggesting that ALDH plays a role in PD pathogenesis.

Interestingly many pesticides, including ziram, maneb and benomyl, have been shown to inhibit proteasome activity *in vitro* (Wang et al., 2006). Recent *in vitro* experiments have established that ziram does not inhibit the proteasome directly but instead interferes with the targeting of proteins to the proteasome via inhibition of the E1 ligase (Chou et al., 2008; Rinetti and Schweizer, 2010). E1 ligase is responsible for activating ubiquitin, which is then attached to a protein substrate. The location and number of ubiquitin molecules attached act as a tag, labeling a protein for cellular signaling events or for degradation by the proteasome. Altering these processes can result in accumulation and aggregation of proteins and/or the deregulation of cellular processes such as synaptic plasticity and learning and memory (Fioravante and Byrne, 2011). The ubiquitin proteasome system is beginning to emerge as one of the key pathways involved in PD etiology, however, the extent and significance of its involvement remains to be determined (Ebrahimi-Fakhari et al., 2012).

The majority of PD studies focus on DA cell loss, however, it is unclear how neuronal function is disrupted prior to this cell loss. The ability of a neuron to fire is a unique, defining and essential property, and thus a logical place to look for neurotoxic properties unique to neurons. Exposure to paraquat and maneb, or manganese alone, has been shown to disrupt neuronal firing patterns in primary neurons and in rodent *in vivo* models, however, how these phenotypes contribute to PD onset is unclear (Chen et al., 2011; Horvath et al., 2012; Martella et al., 2011). With regards to ziram, exposure in primary neuron cultures disrupts normal synaptic function in a manner similar to chemical proteasomal inhibition (Rinetti and Schweizer, 2010). More specifically, treatment of hippocampal neurons with ziram or MG132 (a reversible proteasome inhibitor) caused an increase in the frequency but not amplitude of spontaneous release events, suggesting a presynaptic mechanism of action (Rinetti and Schweizer, 2010). The

extent to which ziram exposure may disrupt synaptic activity and neuronal integrity *in vivo,* however, is not clear. Using genetic reagents expressed presynaptically, I demonstrate for the first time the effects of ziram on synaptic vesicle cycling and calcium dynamics at an intact synapse (Chapter 2).

Dopamine homeostasis, Parkinson's disease and the vesicular monoamine transporter (VMAT)

DA metabolites are highly reactive and the deregulation of DA sequestration and release has been implicated in PD (Bozzi and Borrelli, 2006; Choi et al., 2005). Free cytosolic DA is believed to raise the baseline level of oxidative stress in DA neurons thereby increasing their sensitivity to additional oxidative stressors. Multiple proteins are involved in regulating DA homeostasis, including VMAT. VMAT loads aminergic neurotransmitters, including DA and serotonin, into secretory vesicles prior to their extracellular release. Over-expression of VMAT has been demonstrated to protect against the selective DA neurotoxins rotenone and MPTP (Guillot and Miller, 2009; Lawal et al., 2010; Liu et al., 1992a; Liu et al., 1992b). Likewise, mice with lowered VMAT expression exhibit enhanced DA cell loss upon exposure to MPTP, implicating a possible role for VMAT in pesticide-linked PD (Takahashi et al., 1997; Taylor et al., 2011). A gain-of-function VMAT haplotype has also been identified that has been shown to be protective for PD in epidemiological studies (Glatt et al., 2006).

The number of neurotransmitter transporters present on a synaptic vesicle (SV) determines the quantity of neurotransmitter loaded into the SV (Edwards, 2007). Thus, altering VMAT localization or protein levels could potentially affect amine release. Our lab has previously identified several VMAT trafficking motifs that regulates its localization *in vitro*

(Grygoruk et al., 2010a). In Chapter 1, I address the implications of altering VMAT trafficking motifs *in vivo*, with a particular focus on the process of synaptic vesicle cycling.

In addition to its role packaging DA, VMAT is also required for the packaging of serotonin and noradrenalin. While the hallmark of PD is the loss of DA neurons in the substantia nigra, serotinergic and noradrenergic neurons are also affected in the disease progression and non-motor symptoms of PD are accredited to their deregulation (Politis et al., 2012; Szot, 2012; Taylor et al., 2009). The involvement of serotonin, noradrenalin and dopamine in PD pathology highlights the importance of proper VMAT functioning in aminergic neurons, which will be addressed in Chapters 2 & 3.

The study of neurobiology using Drosophila

The low cost, short lifespan and genetic accessibility of the fruit fly, *Drosophila melanogaster*, make it an excellent model system. The GAL4-UAS genetic system is particularly useful, as it allows cell-type specific over expression of any transgene of interest, which can also be temporally controlled. Briefly, the GAL4 transcription factor is placed under the control of an endogenous promoter, or driver gene, such that GAL4 is only expressed in a subset of cells. The UAS (upstream activating sequence) is fused upstream of the gene for which expression is desired, and transcription will only occur in the cells for which the GAL4 is expressed and able to bind with the UAS. Thousands of GAL4 'drivers' exist, each with a unique expression pattern; for the purposes of this paper I will focus on the neuronal drivers, such as elav-GAL4 which is a pan-neuronal driver, Ddc-GAL4 which drives expression in dopaminergic and

serotinergic cells, and Tdc2-GAL4 which drives octopaminergic and tyraminergic cells (Cole et al., 2005a; Li et al., 2000; Robinow and White, 1991).

A large degree of homology exists between the *Drosophila* and mammalian genomes and the fundamental molecular components regulating basic neuronal functions, such as synaptic transmission, are largely conserved. Neurotransmitter transporters, such as VMAT and the dopamine transporter (DAT), are present in the fly and exhibit similar sensitivity as their mammalian counterparts to chemical inhibition by reserpine and amphetamine respectively (Greer et al., 2005a; Pizzo et al., 2013). It is important to note, however, is that amine synthesis in the fly differs slightly from mammals. The mammalian synthesis pathway is linear: tyrosine is hydroxylated to become DOPA which is decarboxylated to give DA. Furthermore, DA is hydroxylated to norepinephrine which is methylated to produce epinephrine (Medina et al., 2003). The fly synthesis pathway differs in that it is two pronged: tyrosine can be either decarboxylated or hydroxylated. The hydroxylation pathway is similar to mammals, however, decarboxylation of tyrosine is used in flies to produce tyramine which is converted by tyramine beta-hydroxylase into octopamine (closely related to norepinephrine) (Budnik and White, 1987). In the fly, DA is essential for cuticle synthesis, thus the expression of a transgene in dopamine positive cells may not just affect the DA neurons relevant to PD, but could potentially have lethal effects due to DA's role in cuticle development.

The fly DA system has proven to be sensitive to many of the same environmental toxins and genetic alterations that result in PD in rodent models and in humans (Lawal et al., 2010; Ming, 2010). One advantage of using the fly to study neurodegenerative disease is that the end of life, when degeneration occurs, can be observed within weeks to months, not months to years as with mammalian models. Additionally, the vast array of genetic tools and the ease of drug

administration in the fly make the study of multiple genetic variations and/or multiple environmental exposures feasible.

The *Drosophila* neuromuscular junction (NMJ) has proven particularly useful for electrophysiological, genetic and chemical interrogations of an intact synapse. The musculature and synaptic characteristics of the NMJ are understood in depth. Synaptic transmission and synaptic plasticity were first genetically examined at the fly NMJ (Ganetzky and Wu, 1986; Jan and Jan, 1976), and this preparation has been used extensively to understand essential and key components of neurophysiology. For example, experiments in the fruit fly were the first to demonstrate *in vivo* that synaptotagmin acts as the calcium sensor during synaptic transmission (Yoshihara and Littleton, 2002). Live-imaging approaches, such as calcium or pH sensitive forms of GFP, now allow for high resolution and real time monitoring of neuronal processes such as synaptic vesicle cycling. In Chapters 2 & 3 I utilize these powerful tools at the NMJ to determine the synaptic effects of both altering *Drosophila* VMAT trafficking domains and of exposure to the pesticide ziram.

Chapter 1:

Synergistic effects on dopamine cell death in a *Drosophila* model of chronic toxin exposure

Abstract

The neurodegenerative effects of Parkinson's disease (PD) are marked by a selective loss of dopaminergic (DA) neurons. Epidemiological studies suggest that chronic exposure to the pesticide paraquat may increase the risk for PD and DA cell loss. However, combined exposure with additional fungicide(s) including maneb and/or ziram may be required for pathogenesis. To explore potential pathogenic mechanisms, I have developed a *Drosophila* model of chronic paraquat exposure. I find that while chronic paraquat exposure alone decreased organismal survival and motor function, combined chronic exposure to both paraquat and maneb was required for DA cell death in the fly. To initiate mechanistic studies of this interaction, I used additional genetic reagents to target proposed pesticide targets, including reagents affecting the ubiquitin proteasome system, genes involved in the regulation of dopamine homeostasis, and other PD-linked genes. Overexpression of mutant human alpha-synuclein, which causes a form of inheritable PD in humans, increased susceptibility to paraquat and maneb induced mortality and DA cell loss in our fly model. Additionally, genetic inhibition of E1 ubiquitin ligase, but not the proteasome itself, increased DA cell death in combination with maneb but not paraquat. These studies establish a model for long-term exposure to multiple pesticides, and suggest that some pesticide interactions relevant to PD may involve inhibition of ubiquitin-dependent processes independent of the proteasome.

1.0 Introduction

The hallmark of Parkinson's disease (PD) is the progressive loss of dopaminergic (DA) neurons in the substantia nigra. Rare, familial forms of PD indicate a pathogenic role for

disruption of the ubiquitin proteasome system (UPS) and mitochondrion (Lee et al., 2010; Olanow and McNaught, 2006). Epidemiological studies demonstrate an increased risk for PD with chronic exposure to specific pesticides, and suggest additive and potentially synergistic effects between the herbicide paraquat and the fungicides maneb and ziram (Costello et al., 2009; Wang et al., 2011). Similarly, rodent models demonstrate that the combined effects of paraquat and maneb exceed that of each toxin alone (Srivastava et al., 2012; Thiruchelvam, 2000). We are using the model organism *Drosophila melanogaster* to investigate the mechanisms underlying these and other genetic interactions potentially relevant to sporadic PD (Lawal et al., 2010; Lawal et al., 2014).

Current *Drosophila* models of combined pesticide exposure are limited and the combined effects of paraquat plus maneb in the fly are not known. Furthermore, existing models of paraquat administration in *Drosophila* are acute in nature and cause DA cell death within hours to days (Bonilla et al., 2002; Chaudhuri et al., 2007; Hosamani and Muralidhara, 2013). Rodent models of paraquat exposure are also relatively acute, and generally conducted over the course of several weeks, a relatively small fraction of the rodent's lifetime (Liou et al., 1996; McCormack et al., 2002). Thus, neither current fly nor rodent models replicate the chronic time course of exposure seen in many patient populations (Costello et al., 2009). Moreover, most if not all *Drosophila* acute paraquat studies have utilized sucrose as a vehicle (Chaudhuri et al., 2007; Humphreys et al., 1993; Legan et al., 2008), which may increase both oxidative stress and mortality in the fly (Rzezniczak et al., 2011).

One potential target of pesticides associated with increased risk for PD is the proteasome and the UPS (Ebrahimi-Fakhari et al., 2012; Wang et al., 2006). A hallmark of PD pathology is protein aggregation and several inheritable forms of PD involve UPS genes ((Ebrahimi-Fakhari

et al., 2012), suggesting that proteasome dysfunction plays a role in the etiology. Alpha-synuclein, a gene linked to both sporadic and heritable forms of PD, is a key component of the protein inclusions characteristic of PD and epidemiological studies show individuals with variants in alpha-synuclein to be at increased risk of pesticide-linked PD (Gatto, 2010). The effects of proteasome inhibition on DA cell death, however, remain poorly understood (Kordower et al., 2006; Matsui et al., 2010; McNaught et al., 2004; Shin et al., 2011). Furthermore, ubiquitin is involved in the regulation of many cellular processes other than proteasomal degradation, including endocytosis, axon guidance, and synaptic strength (Hamilton and Zito, 2013; Schmidt and Finley, 2014).

In addition to UPS disruption, disruption of DA homeostasis is also implicated in pesticide-linked PD. DA is highly reactive and increased levels of cytosolic DA are thought to be involved in the etiology of PD (Bisaglia et al., 2013). Several pesticides, including ziram, have been shown to inhibit aldehyde dehydrogenase (ALDH), a mitochondrial enzyme that degrades oxidative metabolites of DA and other toxic species (Doorn et al., 2014; Wey et al., 2012), and individuals with decreased ALDH function appear to be more susceptible to pesticide-linked PD (Fitzmaurice et al., 2014). Similarly, the DA transporter (DAT) gene is responsible for reuptake of DA from the synaptic cleft and appears to also regulate individual susceptibility to pesticide-linked PD (Ritz, 2009). The vesicular monoamine transporter (VMAT) removes DA from the cytosol by packaging it into secretory vesicles and epidemiological studies show that variants in VMAT may be neuroprotective against pesticide-linked PD (Glatt et al., 2006; Guillot and Miller, 2009; Lawal et al., 2010).

It is not clear which of these mechanisms may contribute to the toxic effects of specific pesticides in DA neurons and how they may interact (Thrash et al., 2007; Wang et al., 2011).

One potential strategy to address these questions is to genetically "mimic" proposed mechanisms of action and determine if this activity is toxic to DA cells. The genetic tools available in *Drosophila* are particularly useful for this approach. I show here that chronic exposure to maneb plus paraquat, but neither toxin alone, cause DA cell death in the fly and that these effects are only observed in extensively aged flies. I find that that over-expression of mutant human alpha-synuclein increases mortality and induces early DA cell loss in paraquat and maneb exposed flies. Additionally, using genetic mimics of ziram's proposed activity I demonstrate that some synergistic effects may reflect inhibition of ubiquitination pathways independent of the proteasome.

2.0 Materials and Methods

2.1 Drosophila strains and maintenance

Drosophila strains were maintained on standard molasses agar media at 25°C with a 12-hour light/dark cycle. Male flies, 2-4 days post-eclosion were used for all experiments. Transgenic UAS lines expressing the "degron" *CL1* (Pandey et al., 2007) and the dominant negative proteasomal subunits *Pros26* and *Prosβ2* (Belote and Fortier, 2002) were gifts of Paul Taylor (U. Penn) and John Belote (Syracuse U.) respectively. F_1 progeny derived from genetically crossed *Ddc-GAL4* and *UAS-Pros26; UAS-Prosβ2* homozygous lines were used for proteasome inhibition experiments. Flies containing a null mutation in the *Drosophila Aldh* gene generated by imprecise P element excision (*$Aldh^{24}$,* (Fry and Saweikis, 2006)) were a gift from James Fry (U. Rochester). To ensure that previously reported effects of *$Aldh^{24}$* were not due to spurious mutations, *$Aldh^{24}$* was outcrossed for ten generations into a Canton S. wild type genetic background (Simon, 2003). To follow the recessive null allele, PCR was used to amplify a region

of the *Aldh* gene as defined by the primers *aldh-a* (also used in (Fry and Saweikis, 2006)) gttcttctga cagcacttgt and *aldh-31b* tcaattaaat cgaacgaacg c. The outcrossing and PCR of the $Aldh^{24}$ construct was conducted by George Lawless, a technician our laboratory.

The dvmat1 loss of function P-insertion allele lacking expression of *Drosophila* vesicular monoamine transporter (DVMAT), $dVMAT^{l}$ and also known as *l(2)SH0459/ Cyo*, has been previously described (Simon et al., 2009). F_1 progeny derived from genetically crossed $Aldh^{24}$ and $dVMAT^{l}$ flies were used to assess gene-environment interactions in trans-heterozygous flies. UAS-human-alpha-synuclein-A30P lines (Feany, 2000) were used to develop the homozygous Ddc-gal4; UAS-human alpha-synuclein-A30P stock which was used for all alpha-synuclein experiments. The dopamine transporter (DAT) mutant, *fumin*, was a generous gift from Rob Jackson (Tufts University).

To generate transgenes expressing RNAi directed against E1 ligase (uba1), a 725 bp fragment was excised from a *uba1* cDNA (gh24511, *Drosophila* Genomics Resource Center, Indiana) via digestion with Sal I, and inserted into the intermediate vector pGEM-11 (Promega, Madison WI). This construct was digested with EcoRI – NotI and the fragment representing *uba1* inserted into the symmetrically transcribed RNAi vector sympUAST (Giordano et al Genetics 2002) for injection into *Drosophila* embryos (Bestgene Chino Hills, CA). The E1 RNAi constructs were also developed by George Lawless.

2.2 In vivo assay of proteasome inhibition

Wandering 3rd instar larva of the genotype *Ddc-GAL4/UAS-GFP-CL1* and *Ddc-GAL4/UAS-GFP-CL1;UAS-E1-RNAi/+* were dissected in PBS and fixed for 40 minutes in a 4% paraformaldehyde solution. The supraesophageal ganglion (the brain) and ventral nerve cord

were then mounted on glass slides using Aquamount (PolySciences, Warrington, PA) and visualized using a Zeiss Pascal LSM 5 confocal microscope with a 40x /0.75 EC Plan-Neofluar objective. Z-stack projections of the entire brain and ventral nerve cord were collected and used to count GFP-positive cells. All imaging conducted by Dr. Hakeem Lawal.

2.3 Toxin administration

Stock solutions of paraquat (400 mM) (Sigma Aldrich, St. Louis, MO) were made in distilled water. Maneb (100mM) and ziram (200mM) (Chem Services, West Chester, PA) were solubilized in DMSO; all stocks were aliquoted, stored at -20ºC and discarded after one freeze-thaw cycle. Stock solutions of toxins were mixed into molten molasses-agar media under a fume hood at the indicated final concentrations, and used within one week. DMSO was added to food containing paraquat so that all treatment groups were exposed to 1% DMSO in the media.

2.4 Survival

Flies were cultured in standard food containing the indicated concentration of toxin(s) and passed onto a fresh food + toxin mixture every 2-3 days for 60 days. Experiments were initiated with 40 flies/vial (n=6 vials/ condition) and deaths were recorded at each passage.

2.5 Negative geotaxis

Drosophila motor function was assessed using a climbing (negative geotaxis) assay (Benzer, 1967). *Drosophila* were passed onto fresh media (with toxin(s)) at 24 hours and 2 hours prior to the assay and climbing was assessed in the dark to eliminate phototactic effects. Six vials with 30-40 flies each were assessed per treatment group. For each assay, flies were loaded into 0.7 x 10 cm vials affixed to a standard countercurrent apparatus (Benzer, 1967). After one-

minute acclimation to the dark, the apparatus was tapped on the benchtop and the flies allowed 15 sec to climb from the bottom vial to the top vial. The performance index (PI) for climbing was calculated as the percentage of flies that climbed to the top vial within 15 seconds. Climbing ability was quantified and graphed as a performance index (PI), where PI represents [(Flies at the top vial after 15 sec./total number of flies in bottom+top vials)*100].

2.6 Immunolabeling and DA cell counts

Adult *Drosophila* brains were fixed in 4% paraformaldehyde then probed with mouse anti-tyrosine hydroxylase primary antibody (Immunostar, Hudson, WI) and a secondary goat anti-mouse Alexa 555 antibody (Molecular Probes, Grand Island, NY) as described previously (Lawal et al., 2010). DA neurons of the PPL1 cluster (Coulom and Birman, 2004; Lawal et al., 2010) were visualized and on an AxioSkop Zeiss upright microscope with a 40x /0.75 EC Plan-Neofluar objective by manually adjusting the plain of focus through entire brain. For all experiments, DA cells were counted by an observer blind to the genotype of the samples. Images for Figures 2a-c and 3a-b were collected on a Zeiss Pascal LSM 5 confocal microscope with a 40x /0.75 EC Plan-Neofluar objective.

3.0 Results

3.1 Paraquat and paraquat plus maneb exposures result in impaired survival and motor deficits

To assess possible dosage regimens for the chronic application of paraquat, we quantified survival of adult male *Drosophila* exposed to 0, 4, 6, or 8 mM paraquat in standard molasses cornmeal agar media for four weeks (Figure 1a; conducted by Dr. Hakeem Lawal). Survival was significantly lower in all paraquat treated groups (log rank test, Bonferroni correction for

multiple comparisons; $p < 0.01$), and after four weeks of exposure was 80%, 36%, 0% and 0% at each dose, respectively. Since 4 mM represented the highest tested dose of paraquat that allowed for survival of extensively aged flies, we used this dose for subsequent experiments. The maximum lifespan of a fly under standard culture conditions is 50-80 days with progressive defects in behavioral performance beginning at 10-14 days (Grotewiel et al., 2005; Simon et al., 2006). We estimate that chronic exposure over 4 weeks in the fly is comparable to exposure of rodents for 12 to 18 months or human patients for several decades, through the end of middle age. "Old age" in flies aged may be considered $\geq$ 5-6 weeks (Grotewiel et al., 2005).

To initially assess the potential effects of combined chronic exposure to both paraquat and maneb exposure, I first tested survival. Over the course of 8 weeks (60 days), survival of adult male flies was reduced by paraquat or paraquat + maneb compared to maneb alone or vehicle (DMSO) (log-rank test, Bonferroni correction for multiple comparisons; $p < 0.01$, Figure 1b). Maneb exposure alone showed a modest increase in lifespan compared to vehicle alone (log-rank test, Bonferroni correction for multiple comparisons; $p < 0.01$).

To test motor function in flies exposed to paraquat and/or maneb I used a well- described negative geotaxis (climbing) assay (Benzer, 1967). No effects on motor function were observed after either 24 or 96 hours of pesticide exposure (data not shown). However, after one-week, both the paraquat and paraquat + maneb treated groups exhibited significantly impaired climbing ability (Figure 1c, one-way ANOVA post-hoc Bonferroni; $p < 0.05$).

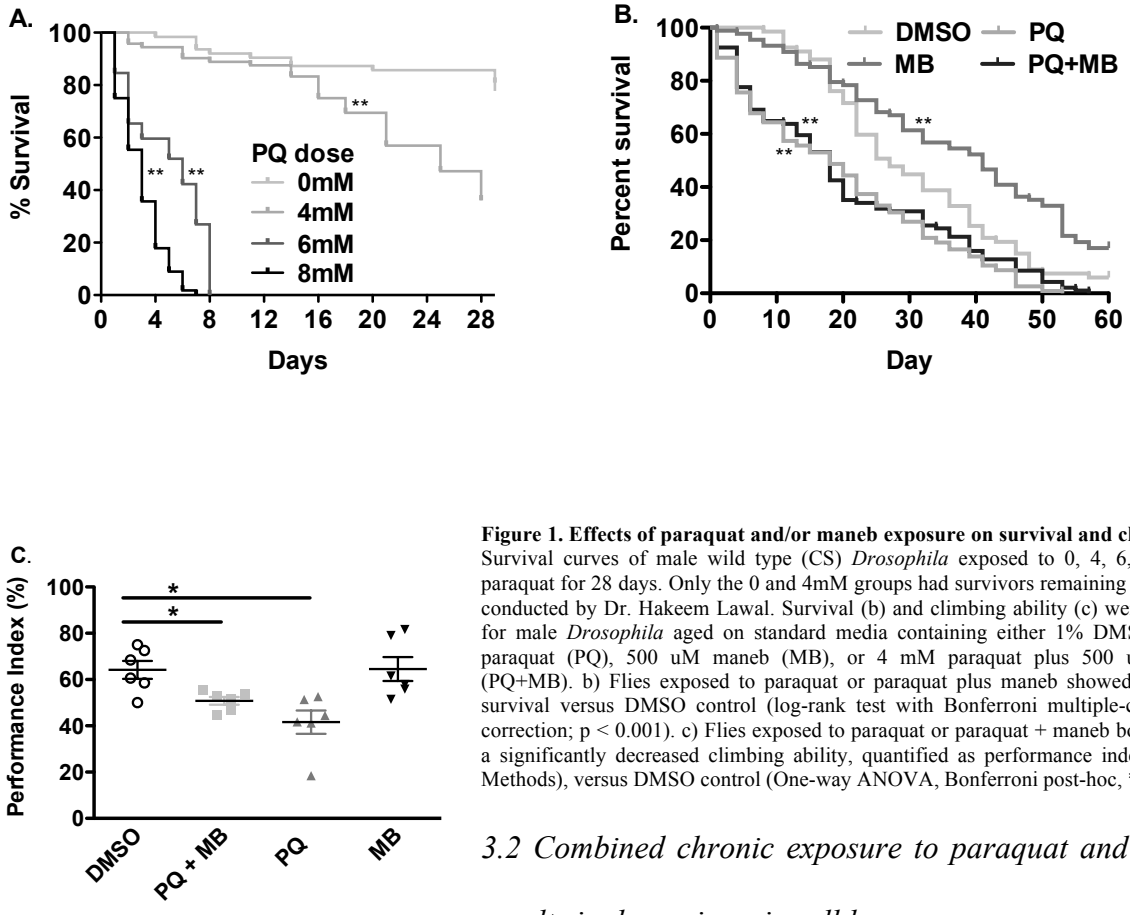


Figure 1. Effects of paraquat and/or maneb exposure on survival and climbing. a) Survival curves of male wild type (CS) *Drosophila* exposed to 0, 4, 6, or 8 mM paraquat for 28 days. Only the 0 and 4mM groups had survivors remaining at 28 days, conducted by Dr. Hakeem Lawal. Survival (b) and climbing ability (c) were assessed for male *Drosophila* aged on standard media containing either 1% DMSO, 4 mM paraquat (PQ), 500 uM maneb (MB), or 4 mM paraquat plus 500 uM maneb (PQ+MB). b) Flies exposed to paraquat or paraquat plus maneb showed decreased survival versus DMSO control (log-rank test with Bonferroni multiple-comparison correction; $p < 0.001$). c) Flies exposed to paraquat or paraquat + maneb both showed a significantly decreased climbing ability, quantified as performance index (PI, see Methods), versus DMSO control (One-way ANOVA, Bonferroni post-hoc, $*p < 0.05$).

3.2 Combined chronic exposure to paraquat and maneb results in dopaminergic cell loss

I assessed DA cell loss in *Drosophila* exposed to pesticides by counting DA neurons in the PPL1 cluster as described (Coulom and Birman, 2004; Lawal et al., 2010) (Figure 2a-f). DA cell loss was not detected after four weeks exposure to paraquat, maneb, or both, compared to vehicle alone (Figure 2c). By contrast, I observed significant loss of DA neurons at six weeks in flies treated with paraquat + maneb, but neither paraquat nor maneb individually (Figure 2d, one-way ANOVA, post-hoc Bonferroni, $p < 0.05$). We have previously tested the effects of ziram in *Drosophila* and failed to detect any significant decrease in survival in flies with chronic exposure (Lawal et al., 2010). Similarly, I report here that ziram did not potentiate the loss of DA cells

caused by exposure to paraquat or maneb (Figure 2e).

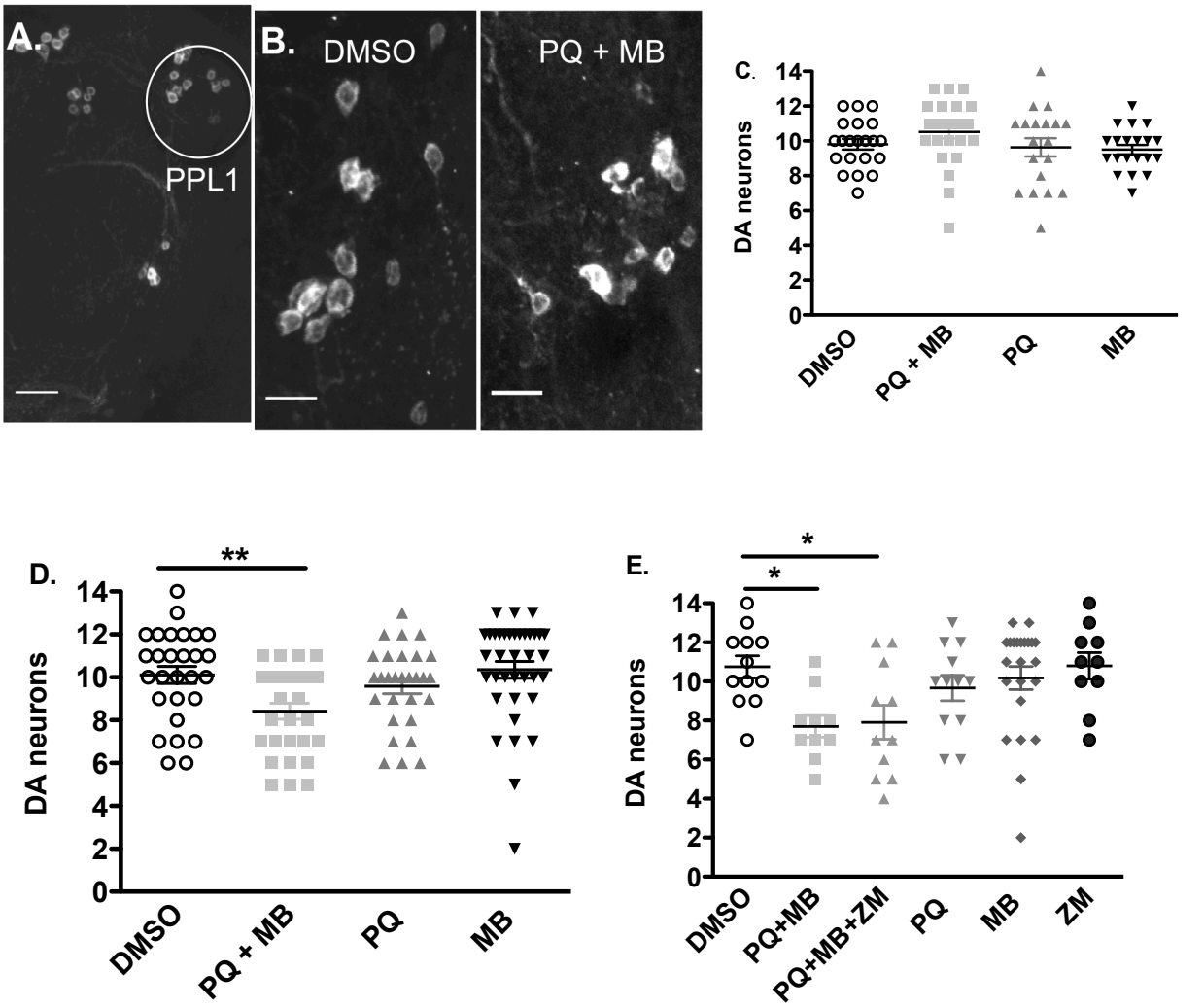


Figure 2. Chronic paraquat and maneb exposure results in dopaminergic neuron loss. Male flies were incubated on standard media containing DMSO 1%, paraquat 4 mM, maneb 500 uM, ziram 1mM, paraquat 4 mM plus maneb 500 uM, or paraquat 4mM plus maneb 500 uM plus ziram 1mM for four © or six weeks (a-b, d, e). a) One half of a bilaterally symmetric wild type, adult fly brain is shown labeled with anti-tyrosine hydroxylase antibody. The PPL1 cluster is highlighted. Dopaminergic neurons of the PPL1 cluster after six weeks of exposure to either DMSO control (b) or 4mM paraquat (PQ) plus 500 uM maneb (MB) (these representative images show 12 and 8 neurons, respectively). Scale bars: a, 20 microns; b, c, 10 microns. c) No significant change in the number of dopaminergic neurons in PPL1 were observed with any pesticide treatment after four weeks of exposure (One-way ANOVA, Bonferroni post-hoc, $p > 0.05$). d) Flies treated with both paraquat and maneb for six weeks show a significant loss of dopaminergic neurons (One-way ANOVA, Bonferroni; post-hoc $p < 0.05$). fe As in b, exposure to paraquat + maneb resulted in a significant loss of DA cells at 6 weeks. The addition of ziram (ZM) to paraquat + maneb treatment did not result in a further increase in cell loss. Ziram treatment alone did not differ from vehicle controls (DMSO, One-way ANOVA, Bonferroni post-hoc, $p < 0.05$).

3.3 Inhibition of E1 ligase alters maneb's toxicity but not that of paraquat

Inhibition of ubiquitin E1 ligase by the pesticide ziram has been proposed to kill DA cells *in vitro* (Chou et al., 2008). We speculated that a genetic approach might be used to directly test the potentially neurotoxic effects of ziram's proposed mechanism of action, the inhibition of ubiquitin E1 ligase. We therefore developed an RNAi construct to knockdown E1 ligase expression. To validate the *in vivo* biological effects of the E1 RNAi expression, I used a previously characterized transgene expressing a GFP labeled proteasome substrate or "degron" (*UAS-CL1*) (Pandey et al., 2007). The E1 RNAi and degron transgenes were co-expressed in DA and serotinergic cells using the *Ddc-GAL4* driver (Li et al., 2000). The use of this driver allowed visualization of a discrete number of stereotypically positioned cells (Figure 3a-d). The number of labeled cells in larva co-expressing E1 RNAi was increased two-fold compared to those expressing the degron alone (Figure 3e). These data support the idea that E1 RNAi construct reduced ubiquitination and the subsequent degradation of the GFP marker via proteasomal degradation.

Flies expressing *UAS-E1 RNAi* using either *Ddc-GAL4* or *TH-GAL4* die as pupae (not shown). Therefore, to investigate the potential neurotoxic effects of E1 knock down in adults, I tested additional drivers including *elav-GAL4* (Robinow and White, 1991). Pan neuronal expression of E1 RNAi using *elav-GAL4* allowed survival through adulthood but showed a decreased life span with minimal survival after four weeks (not shown).

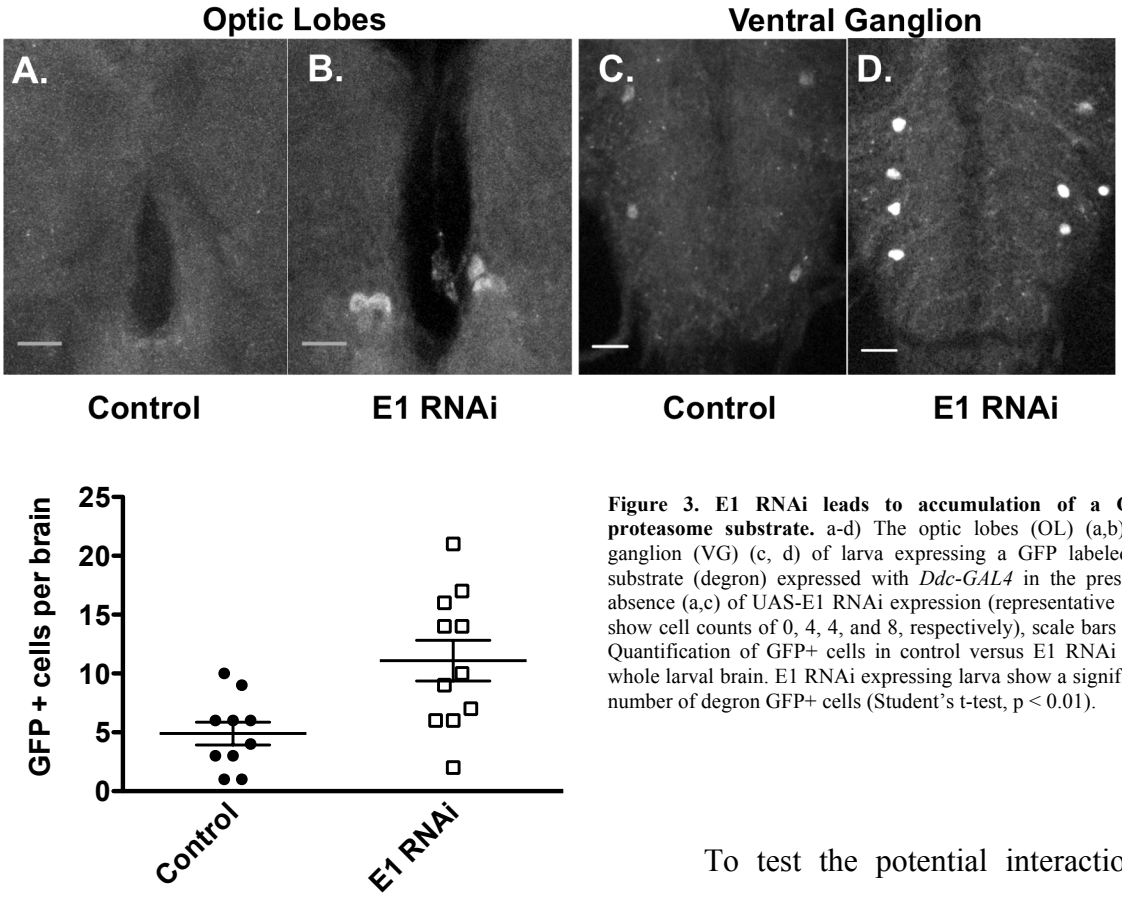
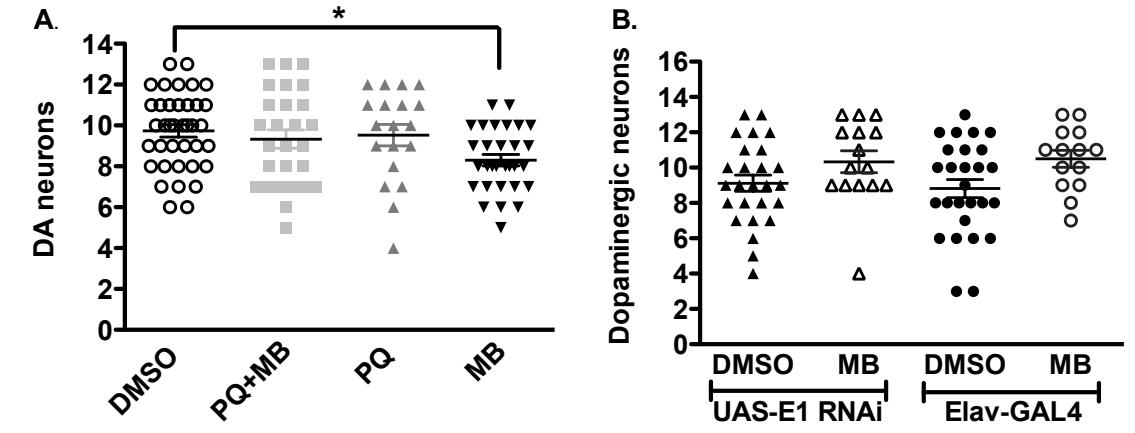


Figure 3. E1 RNAi leads to accumulation of a GFP labeled proteasome substrate. a-d) The optic lobes (OL) (a,b) and ventral ganglion (VG) (c, d) of larva expressing a GFP labeled proteasome substrate (degron) expressed with *Ddc-GAL4* in the presence (b,d) or absence (a,c) of UAS-E1 RNAi expression (representative images in a-d show cell counts of 0, 4, 4, and 8, respectively), scale bars 20 micron. c) Quantification of GFP+ cells in control versus E1 RNAi conditions in whole larval brain. E1 RNAi expressing larva show a significantly higher number of degron GFP+ cells (Student's t-test, $p < 0.01$).

To test the potential interaction of E1 knockdown with additional pesticides, I exposed flies expressing *UAS-E1 RNAi* with *elav-Gal4* to paraquat, maneb or paraquat + maneb for four weeks (Figure 4a). I was unable to perform cell counts at six weeks as in Figure 2d due to the decrease in lifespan. Neither paraquat alone nor paraquat + maneb combined significantly reduced DA cells numbers relative to controls exposed to vehicle alone. By contrast, I observed significant loss of DA neurons in flies expressing E1 RNAi and exposed to maneb for four weeks (Figure 4a, One-way ANOVA, post-hoc Bonferroni; p-value < 0.05). The magnitude of the loss was similar to that observed in wild type flies exposed to paraquat + maneb for six weeks (see Figure 2e). To control for non-specific insertional effects of the transgenes, I similarly tested maneb exposure in flies containing either *UAS-E1-RNAi* alone or *elav-Gal4* alone. I did not detect a loss of DA neurons under these conditions, consistent with the idea that the expression

of E1 RNAi was specifically responsible for the cell loss (Figure 4b).



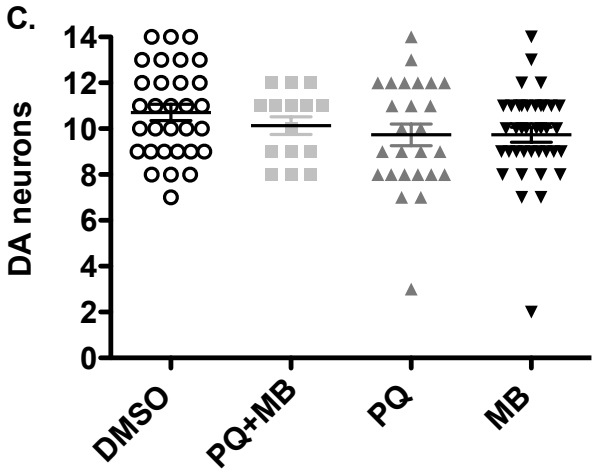


Figure 4. Interactions of paraquat and/or maneb with genetic alterations of the ubiquitin proteasome system a) Four week pesticide exposure in E1 RNAi expressing flies results in significantly lower dopaminergic neuron counts for maneb treated groups versus DMSO control (One-way ANOVA, Bonferroni post-hoc; $p < 0.05$). b) Genetic controls for a) exposed to either DMSO (filled symbols) control or maneb (open symbols) (One-way ANOVA, Bonferroni post-hoc $p > 0.05$) c) Genetic inhibition of the Pros26 and Pros β2 proteasomal subunits in dopaminergic cells did not show an interaction with any of the indicated pesticide treatments.

3.4 Inhibition of the proteasome does not synergize with maneb

Conjugation to ubiquitin can have multiple effects including targeting of proteins to the proteasome or autophagosome, regulation of endocytosis, maintenance of synaptic function and alterations in mitochondrial dynamics (Chin et al., 2010; DiAntonio and Hicke, 2004; Schapira, 2011; Schmidt and Finley, 2014). To test whether proteasome inhibition would mimic the neurotoxic effects of E1 ligase inhibition, I used two previously characterized dominant-negative proteasome subunits, Pros26 and Prosβ2 (Belote and Fortier, 2002). Importantly, both constructs are temperature sensitive and show inhibitory effects at the non-

permissive temperature (30 C) but not at lower temperatures (e.g. 18 C) (Belote and Fortier, 2002). Flies constitutively expressing one copy of *Ddc-Gal4* and each of the *Pros* subunits consistently died as 3rd instar larva (not shown). To circumvent the lethal effects of proteasome inhibition, cultures were maintained at 18 C throughout development and until 2-4 days post eclosion. The adult flies were then incubated at the non-permissive temperature (30 C) for four weeks in media containing DMSO control, paraquat, maneb, or paraquat + maneb (Figure 4c). In contrast to the results obtained with E1 knockdown, I did not detect a loss of DA cells with proteasomal inhibition in combination with exposure to paraquat, maneb or paraquat + maneb. These data suggest that the DA cell loss seen in the presence of maneb plus E1 ligase inhibition may be due to effects of ubiquitination other than decreased protein degradation by the proteasome.

3.5 Gene-environment interactions in an aldehyde dehydrogenase null

In addition to its effects on the E1 ligase, ziram (along with several other pesticides) has been shown to inhibit aldehyde dehydrogenase (ALDH), which is responsible for the degradation of reactive dopamine species (Fitzmaurice et al., 2013; Wey et al., 2012). I therefore tested the potential neurotoxic effects of a previously identified mutation in the *Drosophila* ortholog of mammalian ALDH2 (Fry and Saweikis, 2006). *Drosophila Aldh* null mutants show a reduced lifespan and increased sensitivity to ethanol (Fry and Saweikis, 2006). However, I did not detect a DA cell loss at four weeks of age in the absence of additional toxins or in *Aldh* null flies exposed to paraquat and/or maneb (Figure 5a).

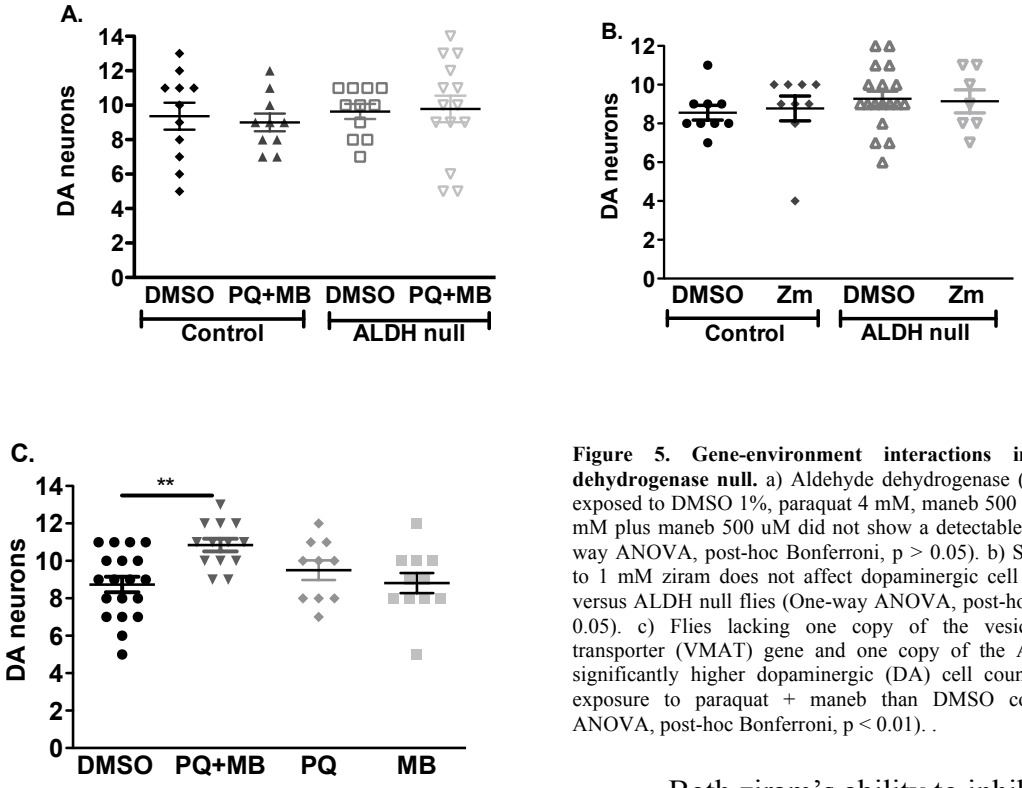


Figure 5. Gene-environment interactions in an aldehyde dehydrogenase null. a) Aldehyde dehydrogenase (ALDH) null flies exposed to DMSO 1%, paraquat 4 mM, maneb 500 uM, or paraquat 4 mM plus maneb 500 uM did not show a detectable interaction (One-way ANOVA, post-hoc Bonferroni, $p > 0.05$). b) Six week exposure to 1 mM ziram does not affect dopaminergic cell counts in control versus ALDH null flies (One-way ANOVA, post-hoc Bonferroni, $p > 0.05$). c) Flies lacking one copy of the vesicular monoamine transporter (VMAT) gene and one copy of the ALDH gene have significantly higher dopaminergic (DA) cell counts after 3 weeks exposure to paraquat + maneb than DMSO controls (One-way ANOVA, post-hoc Bonferroni, $p < 0.01$). .

Both ziram's ability to inhibit E1 ligase and its ability to inhibit ALDH have been implicated in its link to PD (Chou et al., 2008; Fitzmaurice et al., 2014); additionally, other possible mechanisms of action may exist. I looked to see if exposure to ziram would synergize with loss of ALDH. However, I do not report any DA cell loss at after six weeks of 1 mM ziram treatment versus control (Figure 5b).

Like ALDH, the vesicular monoamine transporter (VMAT) also regulates dopamine homeostasis; VMAT packages dopamine into synaptic vesicles and increased levels of VMAT have been shown to be neuroprotective against pesticide induced DA cell loss in invertebrates and mammals alike (Glatt et al., 2006; Guillot and Miller, 2009; Lawal et al., 2010). I asked if alterations in both VMAT and ALDH combined could induce DA degeneration. It was technically infeasible to assess pesticide effects in homozygous null flies for both ALDH and VMAT, due to the short lifespan of VMAT null flies and to genetic constraints. Thus, I used the

F_1 cross of VMAT and ALDH null flies to determine if transheterozygous progeny, each lacking one copy of VMAT and one copy of ALDH, were more susceptible to paraquat and/or maneb exposure. Surprisingly, I found that exposure to paraquat and maneb combined, but neither pesticide alone, significantly increased DA cell counts versus control (Figure 5c; One-way ANOVA, post-hoc Bonferroni; $p < 0.01$).

3.6 Overexpression o f mutant human alpha-synuclein enhances paraquat and maneb toxicity

Alpha-synuclein has been linked to both sporadic and inheritable forms of PD and constitutes one of the main components of Lewy bodies, the protein inclusions typical in the brains of PD patients (Corti et al., 2011; Gatto, 2010). While *Drosophila* do not have an alpha-synuclein gene, expression of wild-type and mutant human alpha-synuclein have both been shown to result in PD-like phenotypes (Feany, 2000). Epidemiological studies have shown that individuals with variants in the alpha-synuclein gene are at an increased risk of contracting PD when exposed to pesticides (Gatto, 2010). I asked if overexpression of mutant human alpha-synuclein would synergize with my paraquat and maneb model.

Using heterozygous F_1 progeny from a Ddc-GAL4 and UAS-human-alpha-synuclein-A30P cross, I found no significant baseline DA cell loss at six weeks of age, nor did I see any synergy in flies exposed to paraquat, maneb or paraquat+maneb (data not shown). To achieve higher gene dosage, I developed a homozygous fly stock (+;Ddc-GAL4;UAS-human alpha-synuclein A30P) and conducted survival and DA cell count experiments. I found no baseline decrease in survival in homozygous alpha-synuclein overexpressing flies versus control with UAS alone; I did find, however, that flies over-expressing alpha-synuclein had significantly lower survival versus UAS control when exposed to paraquat+maneb (Figure 6a; One-way

ANOVA, Bonferroni correction for multiple comparisons; $p < 0.01$). I next conducted DA neuron counts in UAS controls and alpha-synuclein overexpressing flies after two or four weeks exposure to DMSO control or paraquat+maneb. At two weeks of exposure, control flies had significantly lower DA neuron counts when treated with DMSO than when treated with paraquat + maneb, however, alpha-synuclein flies had significantly lower cell counts when exposed to paraquat + maneb versus DMSO (One-way ANOVA, post-hoc Bonferroni $p < 0.05$). After four weeks of exposure, we found again only alpha-synuclein overexpressing flies to have significant loss of DA neurons when exposed to paraquat + maneb versus DMSO (Figure 6b; One-way ANOVA, post-hoc Bonferroni; $p < 0.05$).

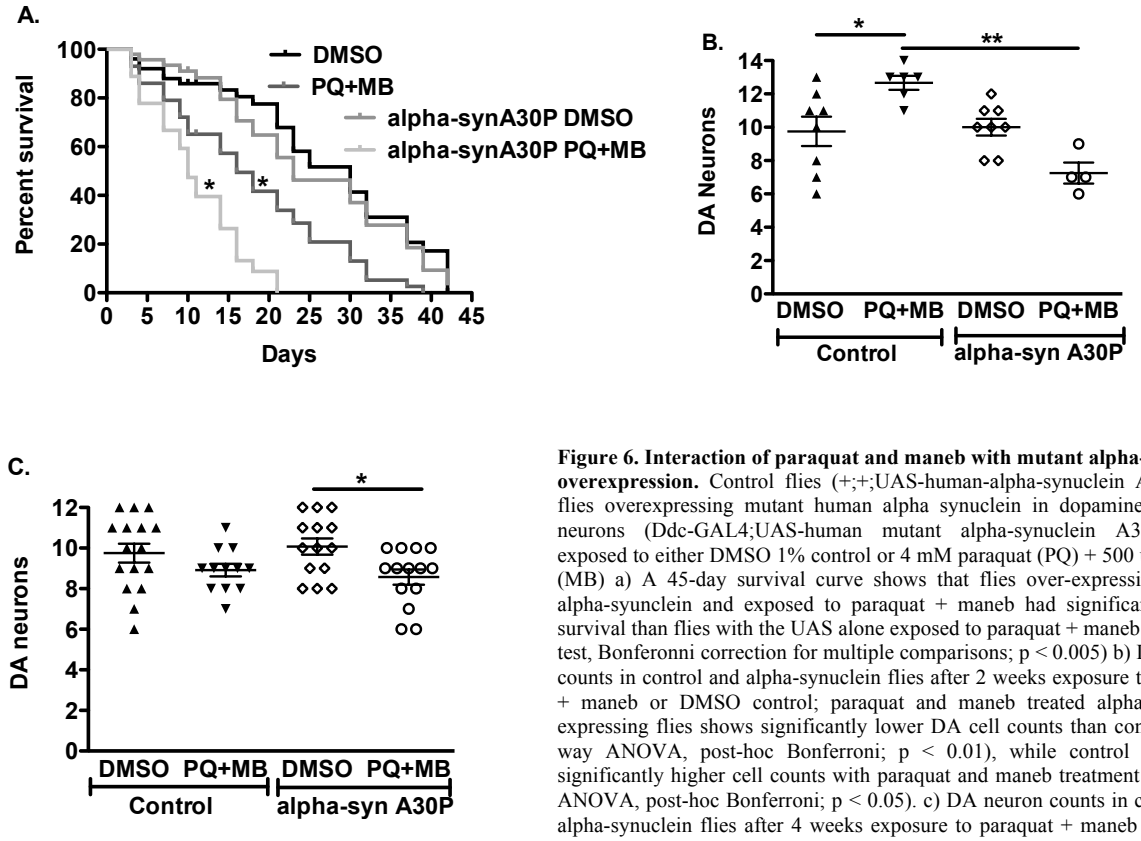


Figure 6. Interaction of paraquat and maneb with mutant alpha-synuclein overexpression. Control flies (+;+;UAS-human-alpha-synuclein A30P) and flies overexpressing mutant human alpha synuclein in dopaminergic (DA) neurons (Ddc-GAL4;UAS-human mutant alpha-synuclein A30P) were exposed to either DMSO 1% control or 4 mM paraquat (PQ) + 500 uM maneb (MB) a) A 45-day survival curve shows that flies over-expressing mutant alpha-syunclein and exposed to paraquat + maneb had significantly lower survival than flies with the UAS alone exposed to paraquat + maneb (Log-rank test, Bonferonni correction for multiple comparisons; $p < 0.005$) b) DA neuron counts in control and alpha-synuclein flies after 2 weeks exposure to paraquat + maneb or DMSO control; paraquat and maneb treated alpha-synuclein expressing flies shows significantly lower DA cell counts than control (One-way ANOVA, post-hoc Bonferroni; $p < 0.01$), while control flies have significantly higher cell counts with paraquat and maneb treatment (One-way ANOVA, post-hoc Bonferroni; $p < 0.05$). c) DA neuron counts in control and alpha-synuclein flies after 4 weeks exposure to paraquat + maneb or DMSO control; paraquat and maneb treated alpha-synuclein expressing flies shows significantly lower DA cell counts than DMSO treated flies, while no effect of paraquat and maneb treatment is observed in control flies (One-way ANOVA, post-hoc Bonferroni; $p < 0.05$).

3.7 The dopamine transporter mutant, fumin, does not exhibit increased paraquat sensitivity

The DA transporter (DAT) is responsible for reuptake of DA from the synaptic cleft into the cytosol and variants in the human DAT gene have been shown to increase PD risk in individuals exposed to pesticides up to three-fold (Ritz, 2009). Thus, I hypothesized that disruption of DAT function would induce enhanced DA cell loss in pesticide-exposed flies. *Fumin*, a *Drosophila* DAT (*dDAT*) mutant, has been previously characterized and lacks *dDAT* activity (Kume et al., 2005). For my DAT gene-environment interactions studies, I focused on paraquat exposure alone as paraquat is a proposed substrate of DAT and, in rodents with decreased DAT function the susceptibility to paraquat toxicity is reduced (Rappold et al., 2011). I reduced the dose of paraquat 10-fold from our standard dosing, to 400 uM, in order to achieve sustained survival as 2 mM and 4 mM doses killed *fumin* flies rapidly. At this dose, wt and *fumin* flies exhibited no difference in survival, whether aged on control food or food containing 400 uM paraquat (Figure 7a). After seven weeks of exposure I conducted DA cell counts and found no significant cell loss in between wt or *dDAT* groups, regardless of exposure type (Figure 7b, One-way ANOVA; $p > 0.05$).

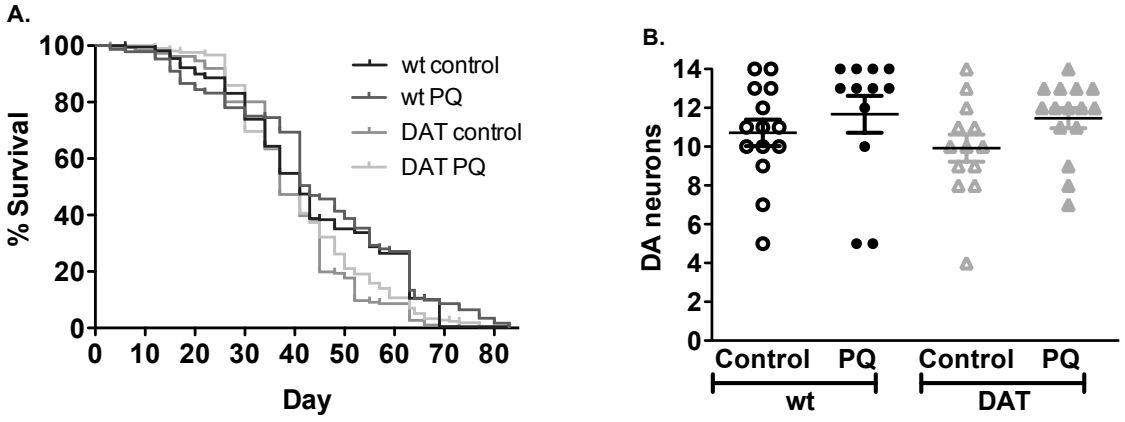


Figure 7. Interaction of paraquat with a dopamine transporter mutant. Both wild type flies and flies lacking expression of the dopamine transporter (DAT) gene, *fumin*, were exposed to vehicle alone or paraquat 400 uM A) No difference in survival was observed between untreated wt versus *dDAT* mutant flies; additionally, paraquat had no effect on survival of either wt or *dDAT* flies (Log-rank test, Bonferroni correction for multiple comparisons; $p > 0.0125$) b) Dopaminergic cell counts for wt and *dDAT* flies exposed to vehicle or 400uM paraquat for 7 weeks demonstrate no significant neuron loss between wt and *dDAT* flies, treated or untreated (One-way ANOVA, post-hoc Bonferroni; $p > 0.05$).

4.0 Discussion

Idiopathic Parkinson's disease (PD) may require 'multiple-hits' and disruption of more than one molecular pathway, by either environmental exposure or genetic variation (Sulzer, 2007). For example, in rodent models, the toxicity of paraquat to dopamine (DA) neurons is dramatically potentiated by maneb (McCormack et al., 2002; Miller, 2007; Minnema et al., 2014; Naylor et al., 1995; Thrash et al., 2007). However, the mechanisms by which these and other agents interact remain obscure (Cicchetti et al., 2005; Li et al., 2005; Thiruchelvam, 2000). To address this question I have developed a chronic exposure model for paraquat and maneb neurotoxicity in the fly. The ease of genetic manipulation in the fly and their short lifespan makes the fly a useful model organism for the study of chronic neurodegenerative processes. Importantly, flies demonstrate the DA cell loss characteristic of PD both by transgenic expression of human PD genes or in the presence of environmental toxins (Lawal et al., 2010; Ming, 2010).

I report that exposure to either paraquat or paraquat and maneb combined results in significantly impaired climbing ability after only one week of exposure and an overall decline in survivorship. Importantly, I also demonstrate that six-week exposure to the pesticides paraquat and maneb combined, but neither toxin alone, is associated with DA cell death in the fly. Although a precise comparison to human aging is impossible, the functional decline and proportion of average life span suggests that a 6 week old fly may be comparable to 65-70 year old human (Grotewiel et al., 2005). Interestingly, I did not detect a difference in DA cell death at 4 weeks, which we estimate to be similar to late middle age in humans (Grotewiel et al., 2005; Simon et al., 2006). Prior studies of paraquat exposure in flies show DA cell loss with relatively high doses of paraquat (10-20 mM) administered in sucrose (Chaudhuri et al., 2007; Lawal et al.,

2010); however, in this paradigm flies die within four days of exposure and sucrose itself may be toxic under these conditions (Rzezniczak et al., 2011). Importantly, pathological changes in PD patients usually manifest decades after exposure to high dose pesticide (Goldman, 2014). We suggest that our model of chronic exposure may be useful to test the long-term effects of other pesticides, and the mechanisms by which paraquat, maneb and other pesticides and genetic risk alleles may interact.

Here I have also investigated the neurotoxic mechanisms proposed for ziram, a dithiocarbamate extensively used in the central valley of CA and with annual national use approaching 2 million pounds (Shackleford, 2003). In human populations, ziram exposure alone increases PD risk 37%; concomitant exposure to paraquat increases risk further to 82%, and exposure to paraquat, maneb and ziram combined increases PD risk 209% (Wang et al., 2011). As shown here and in previous studies (Lawal et al., 2010), ziram itself does not affect the survival, behavior or DA cell counts in the fly. We therefore used a genetic approach to target pathways inhibited by ziram (Chou et al., 2008; Fitzmaurice, 2012).

Ziram has been shown to inhibit aldehyde dehydrogenase (ALDH) *in vitro* and ALDH can detoxify DOPAL, a reactive byproduct of dopamine metabolism (Fitzmaurice et al., 2013). We hypothesized that knockout of ALDH might be toxic to DA neurons and potentiate the affects of paraquat and/or maneb exposure. However, we did not detect any loss of DA neurons in flies lacking ALDH alone, nor do we observe any increase in cell loss in ALDH null flies exposed to paraquat and/or maneb for four weeks. Both ALDH and VMAT regulate dopamine homeostasis, and lower activity of either is thought to increase PD risk (Fitzmaurice et al., 2014; Glatt et al., 2006; Taylor et al., 2011). Thus, I hypothesized that transheterozygous flies, lacking one copy of VMAT and one copy of ALDH, may exhibit enhanced sensitivity to paraquat and

maneb. Surprisingly, transheterozygous flies showed an increase in the number of DA cells after three weeks of exposure to paraquat and maneb (*For discussion see below).*

Multiple isoforms of ALDH are present in both flies and humans, and their role in DA metabolism is poorly understood (Chakraborty and Fry, 2011; Marchitti et al., 2007). The mitochondrial ALDH2 and cytosolic ALDH1a1 are thought to be the primary isoforms responsible for DA breakdown in human cells, however, involvement of other ALDH isoforms has not been ruled out (Marchitti et al., 2007). Similarly, the specific roles for particular isoforms of ALDH in the fly have not been fully elucidated (Chakraborty and Fry, 2011). Therefore, our data must be interpreted with caution and further experiments will be needed to rule out a role for ALDH in neurotoxic processes in *Drosophila*.

Deregulation of the Ubiquitin Proteasome System (UPS) has been proposed as a possible pathogenic mechanism in both sporadic and genetic forms of PD (Olanow and McNaught, 2006), and multiple pesticides, including ziram, are known to inhibit components of the UPS (Wang et al., 2006). Ziram specifically inhibits the E1 ligase (Chou et al., 2008), the first enzyme of the biochemical cascade required for protein conjugation to ubiquitination (Kleiger and Mayor, 2014). To knockdown E1 expression, I expressed an RNAi transgene directed against the *Drosophila* E1 ligase. I then exposed flies with pan-neuronal expression of E1 RNAi to either paraquat, maneb or both pesticides combined and assessed DA cell loss. In conjunction with E1 knockdown, I observed significant DA cell loss at 4 weeks in flies exposed to maneb but not in other treatment groups. Decreased survival of flies expressing E1 RNAi prevented similar cell counts at the later six week time point used in my experiments with wild type flies. It is interesting to note that I could detect DA cell loss by 4 weeks, approximately late middle age, in

flies expressing a genetically susceptibility but not the wild type strain. These observations may be relevant to gene-environment interactions in human genetic populations at risk for PD.

One downstream effect of E1 inhibition would be decreased targeting of proteins for proteasomal degradation. I therefore postulated that the interaction between E1 inhibition and maneb could result from a decrease in protein degradation via the proteasome. To test this hypothesis, I used temperature sensitive proteasomal subunits to directly inhibit the proteasome and exposed flies to maneb and/or paraquat. In contrast to E1 knockdown, I did not observe an interaction between direct proteasome inhibition and either paraquat or maneb. It is possible that more dramatic inhibition of proteasome dysfunction would have a detectable effect. However, previous studies demonstrate the ability of these constructs have a wide range of robust effects, including alterations in organ cell development and fate, regulation of synaptic activity, and neuroblast mitosis (Neuburger et al., 2006; Schweisguth, 1999; Speese et al., 2003) Therefore, I postulate that the neurotoxic effects I observe with E1 inhibition plus maneb may result from disrupting ubiquitin-dependent processes other than the UPS.

Ubiquitin functions other than proteasomal degradation that have been implicated in PD pathogenesis including modifications of the aggresome-autophagy pathway, mitochondria fission and mitophagy, and alpha-synuclein aggregation (Beyer and Ariza, 2013; Chin et al., 2010; Schapira, 2011). We do not yet know whether any of these processes are disrupted by E1 RNAi + maneb. However, comparison of my results using paraquat + maneb and E1 + maneb suggests a possible relationship between ubiquitination pathways and paraquat. Since either paraquat exposure or E1 inhibition suffice to confer neurotoxic effects in combination with maneb, it is possible that paraquat and E1 inhibition can disrupt the same biochemical pathway(s).

Alpha-synuclein is linked to both sporadic and genetic forms of PD and epidemiological studies show that variants of alpha-synuclein increase PD risk in pesticide exposed individuals (Corti et al., 2011; Gatto, 2010). I overexpressed human alpha-synuclein, with the A30P mutation linked to hereditary PD (Corti et al., 2011), in the DA neurons of flies that I exposed to either DMSO control or paraquat and maneb. I found no difference in survival or DA cell counts in wt versus mutant flies on control food, however, overexpression of mutant human alpha-synuclein resulted in significantly decreased survival and decreased DA cell counts in paraquat and maneb treated flies. These data taken together with the epidemiological findings (Gatto, 2010) support the hypothesis that pesticide exposure may interact with alpha-synuclein in some manner that results in increased PD risk.

Unlike our findings with alpha-synuclein, several of the gene-environment interactions I investigated did not induce any DA cell loss. DAT, the dopamine transporter, is responsible for the reuptake of dopamine from the synaptic cleft. Certain variants in the DAT gene increase PD risk in pesticide-exposed individuals (Ritz, 2009) and it is proposed that paraquat is a substrate of DAT, allowing for its accumulation in the cytosol of DA neurons (Rappold et al., 2011). In line with this hypothesis, rodents with decreased DAT function are less sensitive to paraquat toxicity (Rappold et al., 2011). I exposed DAT mutant flies that lack DAT function (Kume et al., 2005) to chronic low dose paraquat (400 uM), as higher doses caused high mortality rates. At this dose, we did not observe a decrease in survival or DA cell counts after seven weeks of exposure in either control or paraquat treated groups.

It remains unclear why transheterozygous flies lacking one copy of VMAT and one copy of ALDH exhibit neuroprotection against age-related decline in DA cell counts. Similarly, in my alpha-synuclein experiments I saw increased DA counts in the genetic control group treated with

paraquat + maneb versus DMSO. It is also unclear why treatment with chronic exposure to maneb alone increased lifespan and why I observed loss of DA neurons with E1 inhibition + maneb but not E1 inhibition + maneb and paraquat. It is possible that I simply failed to detect some toxic effects. However, in some cases, limited exposure to toxins can be protective against other insults (Calabrese, 2008). Exposure to paraquat paradoxically increased the life span in *C. elegans* on a calorie restrictive diet (Schulz et al., 2007) and in flies expressing decreased levels of the PD-associated protein parkin (Bonilla-Ramirez et al., 2013). A similar phenomenon may have occurred under some of the conditions used in our studies.

4.0 Conclusions

In the 'multiple-hit' model of sporadic PD, inhibition of at two least pathways are hypothesized to be necessary for pathogenesis and the apparent lack of individual risk factors to cause disease (Sulzer, 2007). However, multiple genetic and environmental risk factors have been identified and it remains unclear which combinations may be most relevant. I have shown that under the chronic exposure conditions used here, paraquat and maneb combined, but neither paraquat nor maneb alone, cause DA cell loss at six weeks. I have further exploited this chronic exposure paradigm to show synergistic effects of maneb and a genetic mimic of ziram, E1 inhibition. Additionally, mirroring the epidemiological data, I show the ability of alpha-synuclein and paraquat + maneb exposure to synergize, enhancing DA cell loss. The interactions we observe may be relevant to human populations at risk for PD.

Chapter 2:
Characterization of vesicular monoamine transporter trafficking mutants at the *Drosophila* neuromuscular junction

Abstract

The vesicular monoamine transporter (VMAT) is responsible for packaging monoamine neurotransmitters, such as dopamine and serotonin, into synaptic vesicles (SVs) and large dense core vesicles (LDCVs) prior to exocytosis. SVs are present at the active zone and are responsible for the release of amines into the synapse, while LDCVs are responsible for the extrasynaptic release of amines from both the nerve terminal and somatodendritic sites. Some of the signals and pathways responsible for sorting VMAT to SVs and LDCVs have been examined *in vitro* and in cell culture models, however, the regulation of VMAT localization *in vivo* remains unclear. The *Drosophila* neuromuscular junction (NMJ) preparation provides a useful platform for the study of VMAT trafficking at an intact synapse. We developed transgenic *Drosophila* VMAT (DVMAT) lines expressing either wild type (wt) *Drosophila* VMAT (DVMAT), or one of two trafficking mutations, a point mutation in a tyrosine based trafficking motif (Y600A), or a C-terminus truncation containing multiple putative trafficking domains (delta 3). Wt, Y600A and delta 3 DVMAT all co-label extensively with SV and LDCV markers, suggesting that in the absence of putative trafficking motifs DVMAT can still localize to secretory vesicles. Next, we generated wt, Y600A and delta 3 DVMAT pHluorin constructs tagged with a pH-sensitive form of GFP or 'pHluorin' allowing for the high-resolution visualization of DVMAT exo- and endocytosis. By taking advantage of the NMJ preparation, which contains both aminergic and glutamatergic synapses within the same muscle segment, we could directly compare the effects of altered VMAT trafficking at different nerve terminals. We report that alterations in DVMAT trafficking domains led to dramatic alterations in VMAT exo- and endocytosis in a manner that was dependent on nerve terminal identity, suggesting potential differences in trafficking machinery between functionally distinct nerve terminals.

1.0 Introduction

Vesicular neurotransmitter transporters, such as the vesicular monoamine transporter (VMAT), are responsible for packaging neurotransmitters into secretory vesicles prior to their release into the synapse (Blakely and Edwards, 2012; Lawal and Krantz, 2013). VMAT is a highly conserved protein and its substrates include monoamines such a dopamine (DA) and serotonin, which regulate a wide range of processes, from mood to motor function (Lawal and Krantz, 2013). Deregulation of aminergic circuits has been implicated in a variety of disease states such as depression, epilepsy and Parkinson's disease (PD) (Kurian et al., 2011). Loss of VMAT is lethal and decreased amounts of VMAT protein results in a variety of behavioral and pathological phenotypes including loss of DA neurons, decreased motor function, and increased susceptibility to DA neurotoxins in the mouse (Takahashi et al., 1997; Taylor et al., 2011). Likewise, increased expression of VMAT has been shown to be neuroprotective against DA cell loss in pesticide-induced animal models of PD (Guillot and Miller, 2009; Lawal et al., 2010) and, in the human population, to decrease PD risk in pesticide-exposed women (Glatt et al., 2006).

VMAT's important role requires the tight cellular regulation of both VMAT quantity and localization. We have previously described two mutants in the carboxy-terminal trafficking domain of *Drosophila* VMAT, the first containing a point mutation (Y600A), and the second containing a more severe deletion of the terminal 23 amino acids (delta 3) (Grygoruk et al., 2010a). The Y600A mutant contains a alanine substitution of a tyrosine based sorting motif ($Y_{600}XXY_{603}$) (Grygoruk et al., 2010a), while the delta 3 mutant lacks the $Y_{600}XXY_{603}$ motif completely, in addition to other known and predicted endocytosis signals thought to be involved in de novo trafficking to SVs and recycling to SVs following endocytosis at the synapse (Fei et al., 2008; Greer et al., 2005a; Grygoruk et al., 2010a). Both the Y600a and delta 3 mutants

exhibit endocytosis defects in non-neuronal S2 cells and in the neuronal cell line DmBG2C6 (Grygoruk et al., 2010a). In addition, both the Y600A and delta 3 mutants show decreased sorting to SVs *in vivo* (Grygoruk et al., 2010a). However, the manner in which mutations in DVMAT trafficking domains may alter DVMAT localization to the nerve terminal and how they may affect DVMAT exo- and endocytosis at the synapse *in vivo* remains unclear.

We sought to determine the effects of alterations in DVMAT trafficking domains *in vivo* using the *Drosophila* neuromuscular junction (NMJ) as a model synapse. The ease of genetic manipulation, short life span and low cost of *Drosophila* makes it an appealing organism for addressing these types of questions. Traditional studies at the fly NMJ have focused on the excitatory and glutamatergic Type Ib terminals, which are large, easy to visualize, and illicit post synaptic responses that can be monitored electrophysiologically (Budnik, 2006; Frank et al., 2006). The NMJ also contains the modulatory and octopaminergic Type II terminals, which endogenously express DVMAT and cannot be monitored electrophysiologically as their postsynaptic receptors are not ionotropic (Budnik, 2006; Monastirioti et al., 1995). Additionally, the vesicle composition between the two terminal types differs substantially; Type II terminals contain roughly equal numbers of synaptic vesicles (SVs) and large dense core vesicles (LDCVs), while Type I terminals primarily utilize SVs (Atwood et al., 1993; Jia et al., 1993). Here, using live-imaging techniques, we characterized the effects of DVMAT trafficking mutations at the canonical Type Ib terminals and the aminergic Type II terminals.

We developed transgenic fly lines with a pH sensitive form of GFP, or 'pHluorin' tag, on a lumenal loop of wild type, Y600A or delta 3 DVMAT and expressed them pan-neuronally at the NMJ. The pHluorin construct allows for the high-resolution visualization of DVMAT exo- and endocytosis, due to the pH gradient between the synaptic vesicle lumen and the synaptic

cleft. We quantified exo- and endocytosis in response to a mechanical stimulus at both Type Ib and Type II terminals expressing the wt or mutant DVMAT pHluorin constructs. We report that DVMAT exo- and endocytosis is significantly and differentially disrupted by trafficking mutations at functionally distinct nerve terminals. Our data demonstrate for the first time *in vivo* that disruptions in DVMAT trafficking motifs significantly alter VMAT exocytic and endocytic cycling during neuronal firing, and that these phenotypes are dependent on nerve terminal identity.

2.0 Materials and Methods

2.1 Generation and use of Drosophila transgenes and mutants

For insertion of the superecliptic Green Fluorescent Protein (GFP) pHluorin moiety into the lumenal loop of DVMAT, an XmaI(c|ccggg) site was first introduced into the cDNA construct pMT-DVMAT-A-HA sequence (Greer et al., 2005b) using the Quikchange Site-Directed Mutagenesis Kit (Strategene). cDNA representing the pHluorin moiety was inserted into the XmaI site to replace the HA tag and the resultant fragment ligated to sequence encoding: 1) the wt DVMAT C-terminal region; 2) the Y600A mutation or 3) the Delta 3 mutation. Each pHluorin tagged DVMAT variant was subcloned into the pExp-UAS expression vector (Exelixis), followed by injection into the *white*^{1118} strain (BestGene *Drosophila* Embryo Injection Services, Chino Hills, CA or Rainbow Transgenic Flies, Newbury Park, CA). The pHluorin transgene generation was conducted by Dr. Audrey Chen, a former graduate student in the laboratory.

Lines expressing both wild type and mutant forms of HA-tagged *UAS-DVMAT* transgenic fly lines on chromosomes 2 and 3 have been described previously (Chang et al., 2006; Grygoruk

et al., 2010b). Note that all *UAS-DVMAT* transgenes used here encode the neuronal isoform of DVMAT (DVMAT-A); a distinct RNA splice variant, DVMAT-B, is expressed in a small subset of histamine-containing glia in the adult visual system (Romero-Calderón et al., 2008) and is not pertinent to the studies described here. The drivers *elav-Gal4* (Robinow and White, 1988) and *Tdc2-Gal4* (Cole et al., 2005b) were used to express wild type and trafficking mutant DVMAT constructs as indicated in the text. Additional UAS lines used as markers included GFP-tagged versions ANF and syt1 proteins (Rao et al., 2001; Wang et al., 2007).

2.2 Immunofluorescent labeling

Larval body wall fillets (used to visualize the neuromuscular junction (NMJ)) were prepared in phosphate buffered Saline (PBS) from wandering third instar larvae. The fillet (including the larval brain+nerve cord) or brain+nerve cord alone were fixed in 4% paraformaldehyde (40 minutes, ambient temperature), washed in PBS (x3) and PBS-T (PBS with 0.1% Trition X detergent), incubated for one hour at room temperature in PBS-T plus 5% normal goat serum (NGS) ("blocking solution") and overnight in PBS-T 5% NGS with mouse anti-HA (1:500, Covance) and rabbit anti-GFP primary antibodies (1:500, Invitrogen). After washing, preparations were incubated in the secondary antibodies goat anti-mouse Alexa 555 (1:1000, (Jackson Immunoresearch Labs) and donkey anti-rabbit Alexa 488 (1:1000) (Jackson Immunoresearch Labs) in PBS-T 5% NGS. Washed brains were mounted onto slides using Aquamount solution (Thermo Scientific). Images were acquired using a Zeiss Plan-Apochromat 63x oil immersion objective (NA 1.4) and Zeiss 710 confocal scanning microscope. Ms. Ciara Martin conducted staining and Dr. Hakeem Lawal conducted the confocal imaging.

2.3 pHluorin imaging

For imaging studies, third instar larvae were dissected in chilled Ca^{2+}-free HL3.1 saline (Feng et al., 2004) adjusted to pH 7.32. Measurements of exo- and endocytosis were performed in "recording solution": HL3.1 solution (pH 7.32) supplemented with 2.0 mM calcium (to allow normal rates of exo-endocytosis) and 0.7mM L-glutamic acid (to block muscle contraction for imaging). Fresh stock solutions of L-glutamic acid (LGA) (Sigma, St. Louis, MO) were made weekly. LGA and calcium were added to HL3.1 on the day of experiment. All images were acquired on a Zeiss Axio Examiner Z1 microscope using a cooled back-illuminated electron-multiplying CCD camera (Andor iXon3 897, South Windsor, CT) and a DG4 light source (Sutter, Novato, CA) with a GFP Brightline® Filter Set (Semrock, Rochester, NY). A Zeiss Achroplan water-immersion objective (100x, 1.0 N.A.) was used to image the NMJ. Data acquisition for Type IB terminals was 20 images/sec. For the longer time course of Type II terminals data was acquired at 10 images/sec. For quantification of exo- and endocytosis, individual Type Ib or or Type II boutons from (abdominal segment A4, muscle 13) were tracked with the MeasureStack plugin in NIH ImageJ. Boutons that moved in or out of focus during image acquisition were discarded prior to analysis. To correct for photobleaching, prestimulus F values were used to fit single exponential and the resulting curve subtracted from the entire time series. DF/F was calculated as $(F_{peak} - F_{baseline}) / F_{baseline}$, where F_{peak} is the average of the 10 frames during the 0.5 s after cessation of the 40 Hz stimulus, and $F_{baseline}$ is the average of the 10 frames (0.5s duration) prior to stimulus. To quantify endocytosis rates in Type Ib terminals, DF/F values over the first 30 seconds following the peak value at stimulus offset were fit to a single exponential using the equation $Y= (Y_0 - Y_p)^{-kx}$ where Y_0 represents the peak after the stimulus and Y_p represents the asymptotic, plateau value of Y at $t=\infty$ (GraphPad Prism) and k is the rate constant of decay. Endocytosis rates are represented as the time constant t (1/k) . For

Type II terminals, DF/F was calculated as above for Type Ib. For some traces from Type II boutons expressing the DVMAT-Y600A mutant (see text), we observed minimal decay during the recording period, thus prohibiting calculation of a time constant. Therefore, to compare endocytosis across genotypes we calculated the decrease in relative fluorescence $(DF_{decrease}/F)_t$ at increasing times following the stimulus using the equation Decay $DF_t=(F_{peak} - F_t) / F_{baseline}$, with F_{peak} as above and F_t the value at 10seconds following the peak, or 12 sec after initial stimulus at time 0 indicated in Fig 3. pHluorin recordings at the Type Ib terminals (Figure 3 a-d) were conducted by Dr. Audrey Chen and Type II terminals (Figure 3e-h) were conducted by Ms. Ciara Martin.

3.0 Results

3.1 DVMAT trafficking mutants correctly localize to secretory vesicles

We first determined if *Drosophila* VMAT (DVMAT) trafficking domain mutations disrupt the localization of DVMAT to nerve terminals of the fly neuromuscular junction (NMJ). We tested two DVMAT mutants, Y600A and delta 3, that contain known and proposed trafficking domains; the Y600A mutant has a tyrosine to alanine mutation in a known tyrosine based sorting motif ($Y_{600}XXY_{603}$), and the delta 3 mutant has a more severe 23 amino acid C-terminal truncation that includes three putative trafficking domains (Grygoruk et al., 2010a) (Figure 1; work conducted by Dr. Anna Grygoruk).

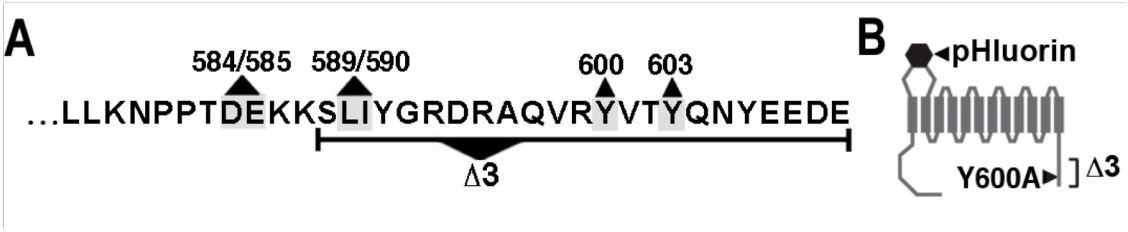


Figure 1. DVMAT pHluorin trafficking mutants (A) The DVMAT C-terminus. The final 34 amino acids of the 39 amino acid, cytosolic C-terminal domain of DVMAT are shown. The extent of the Delta 3 deletion and the sites of the predicted dileucine motif (residues 589/590 in the DVMAT sequence) upstream acidic residues (584-585) and the tyrosine-based motif (Y600 and Y603) are indicated (B) The cartoon represents the DVMAT protein and shows the locations of the lumenal pHluorin insert, and both the Delta 3 deletion and the Y600A mutation within the cytosolic C-terminus of DVMAT. These mutant fly stocks were made by a former postdoc in the laboratory, Dr. Anna Grygoruk, as was the figure design.

We conducted immunofluorescent labeling to determine the baseline, subcellular localization of wt and mutant DVMAT. The Tdc-Gal4 driver was used to achieve expression in octopaminergic and tyraminergic cells. We focused on the somata of the midline octopaminergic neurons in the larval ventral nerve cord, as these are easy to visualize and their processes form the Type II terminals at the neuromuscular junction (NMJ) (Monastirioti et al., 1995). A GFP tagged Syt1 (Syt1-GFP) construct and antibody to GFP was used to mark synaptic vesicles (SVs) and other secretory vesicles (Zhang et al., 2002). We co-expressed either wild type (wt), Y600A, or delta 3 HA-tagged DVMAT with Syt1-GFP and assessed co-localization (all crosses and immunolabeling conducted by Ms. Ciara Martin). Confocal imaging of the preparations (conducted by Dr. Hakeem Lawal) did not suggest any gross deficits in DVMAT localization to Type II nerve terminals of the fly NMJ (Figure 2b, e, h). Partial co-localization of both wt and mutant DVMAT with Syt1 was observed, suggesting all three can target to secretory vesicles to some degree (Figure 2a-i).

Using this same approach, we expressed the UAS-ANF-GFP transgene, which encodes a chimera of Atrial Naturetic Factor, to mark large dense core vesicles (LDCVs) (Rao et al., 2001; Wong et al., 2012) and assessed co-localization with both wt and mutant DVMAT constructs.

Staining was detected both in the nerve terminal (not shown) and somata, where the individual puncta could be detected more clearly (Figure 2J-R). Both wt and mutant DVMAT co-localize extensively, suggesting that both the Y600A and delta 3 mutants are capable of sorting to LDCVs despite the absence of known trafficking motifs.

3.2 DVMAT trafficking mutant pHluorin recordings at glutamatergic terminals

We chose to first examine the synaptic effects of our DVMAT-pHluorin trafficking mutants at conventional Type Ib terminals (at muscle 13, segment A4). Type Ib terminals are glutamatergic, excitatory in nature, and elicit a postsynaptic response that can be measured electrophysiologically (Budnik, 2006; Frank et al., 2006). The electrophysiological accessibility of Type Ib terminals, coupled with their large size (which allows for easy live imaging), has resulted in their almost exclusive use for neurobiological studies.

Using a pH sensitive form of GFP, or 'pHluorin', we generated UAS constructs with a pHluorin tag placed on a lumenal loop of wt, Y600A or delta 3 DVMAT (construct generation was conducted by Dr. Audrey Chen). The pH gradient between the synaptic vesicle lumen and the synaptic cleft results in pHluorin fluorescence during exocytosis and subsequent quenching during endocytosis that can be quantified using live imaging techniques.

Using the Elav-GAL4 driver (Robinow and White, 1991) we pan neuronally expressed our DVMAT pHluorin constructs (wt, Y600A, Delta 3) at the *Drosophila* NMJ. We then mechanically stimulated the presynaptic nerve to fire 80 action potentials, at a frequency of 40hz, during which we conducted live imaging at Type Ib terminals (Figure 3a). Photobleaching and background were accounted for (see Methods). Exocytosis was quantified as delta F/F [(Fpeak-

Fbaseline)/Fbaseline)] and endocytosis rate was quantified as the tau value for a non-linear single exponential fit of the 30 seconds following endocytosis.

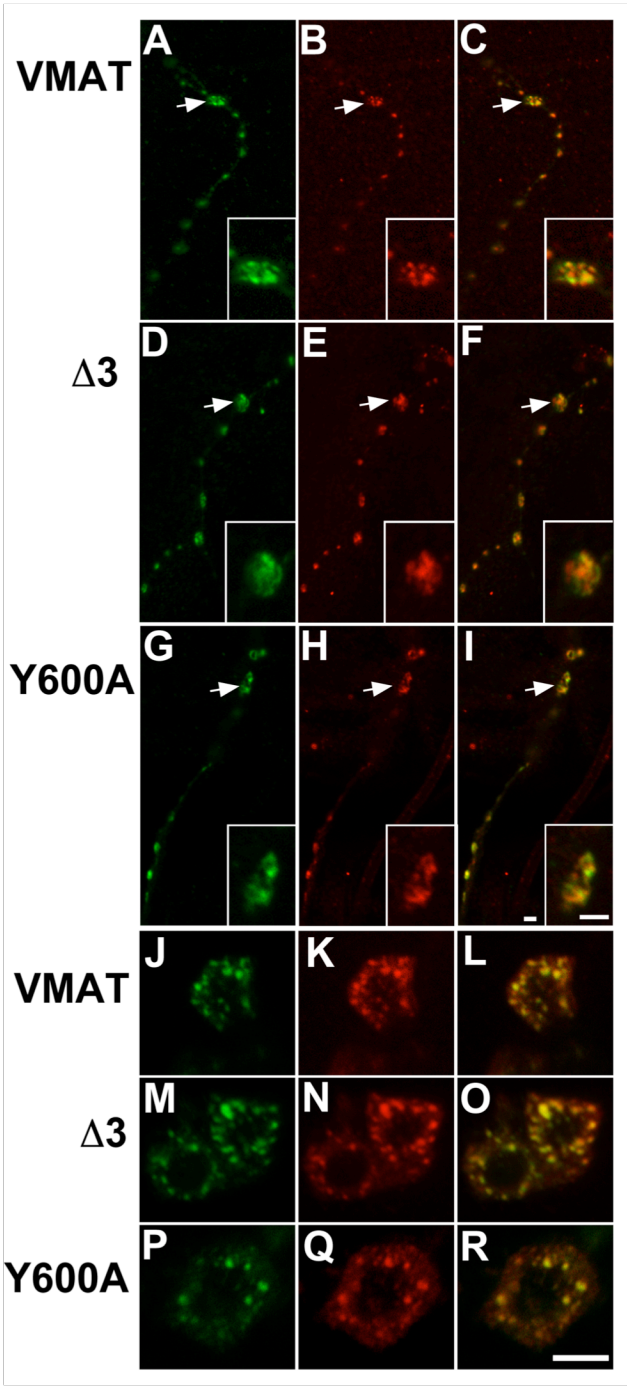


Fig. 2. Immunolabeling of wt and mutant DVMAT. The *Tdc2-Gal4* driver was used to express the HA-tagged, *UAS-DVMAT* transgenes encoding DVMAT-wt (A-C, J-L), DVMAT-Delta 3 (D-F, M-O) and DVMAT-Y600A (G-I, P-R) with either *UAS-syt1-GFP* (A-I) or *UAS-ANF-GFP* (J-R). Body wall fillets containing Type II NMJ terminals expressing the transgenes (A-J) or larval nerve cords (J-R) were co-labeled with a monoclonal antibody to the HA tag in DVMAT and an antiserum to GFP to detect either syt1-GFP or ANF-GFP, followed by the appropriate secondary antibodies to label DVMAT red and the markers green. Processes on either muscle 7 or 13 were used to visualize expression in Type II nerve terminals in A-I. Scale bars A-I (shown in I): 2 microns; J-R (shown in R): 5 microns. Preparations and staining conducted by Ciara Martin; imaging conducted by Dr. Hakeem Lawal.

We report that the Y600A trafficking mutant does not exhibit any exocytic or endocytic defect relative to wt (Figure 3B-D) at Type Ib terminals. The more severe delta 3 mutation, however, significantly disrupts both exocytosis and endocytosis at these terminals. With regards to exocytosis, delta 3 mutants exhibit approximately half that of wt, with ~10% increase in fluorescence vs. ~22% in wt (one-way ANOVA, post-hoc Bonferroni; $p < 0.01$). In addition to a decrease in exocytosis, we report that endocytosis occurs at a much slower

rate in the delta 3 mutants, taking about twice as long as wt (tau of ~8s for delta 3 and ~4s for wt; One-way ANOVA, post-hoc Bonferroni; $p < 0.01$). These data suggest that motifs contained within the delta 3 deletion region, other than the $Y_{600}XXY_{603}$ motif, are necessary for efficient exo- and endocytosis at Type Ib terminals. All Type Ib recordings were conducted by Dr. Audrey Chen.

3.3 DVMAT trafficking mutant pHluorin recordings at octopaminergic terminals

The NMJ preparation contains Type Ib ('big' and glutamatergic), Type Is ('small' and glutamatergic), Type II (octopaminergic) and Type III (peptidergic) terminals (Jia et al., 1993), however, to our knowledge no studies have assessed the behavior of the Type II terminals where VMAT is endogenously expressed. I investigated whether expression of DVMAT-pHluorin trafficking mutants at their endogenous Type II terminals would result in similar patterns of exocytic and endocytic disruption as at Type Ib terminals. Using the same techniques and constructs as in Type Ib terminals (Figure 3A-D), I quantified exocytosis and endocytosis at Type II terminals for the wt, Y600A, and delta 3 DVMAT-pHluorin constructs (Figure 4E-H). Interestingly, I report that exocytosis and endocytosis of DVMAT-pHluorin trafficking mutants is dramatically different at functionally distinct nerve terminals. Delta 3, but not Y600A, exhibited decreased exocytosis at Type Ib terminals (Figure 3C), yet neither mutant had a delta F/F value significantly different from wt at Type II terminals (Figure 3G, One-way ANOVA; $p > 0.05$). Additionally, both the Y600A and the delta 3 mutants showed severe reduction in endocytosis at Type II terminals (Figure 3F, H; One-way ANOVA; $p < 0.05$). I also report that endocytosis of wt DVMAT at is generally slower at Type II terminals than Type Ib, similar to studies conducted in mammalian cultures of aminergic versus non-aminergic neurons in culture

(Onoa et al., 2010).

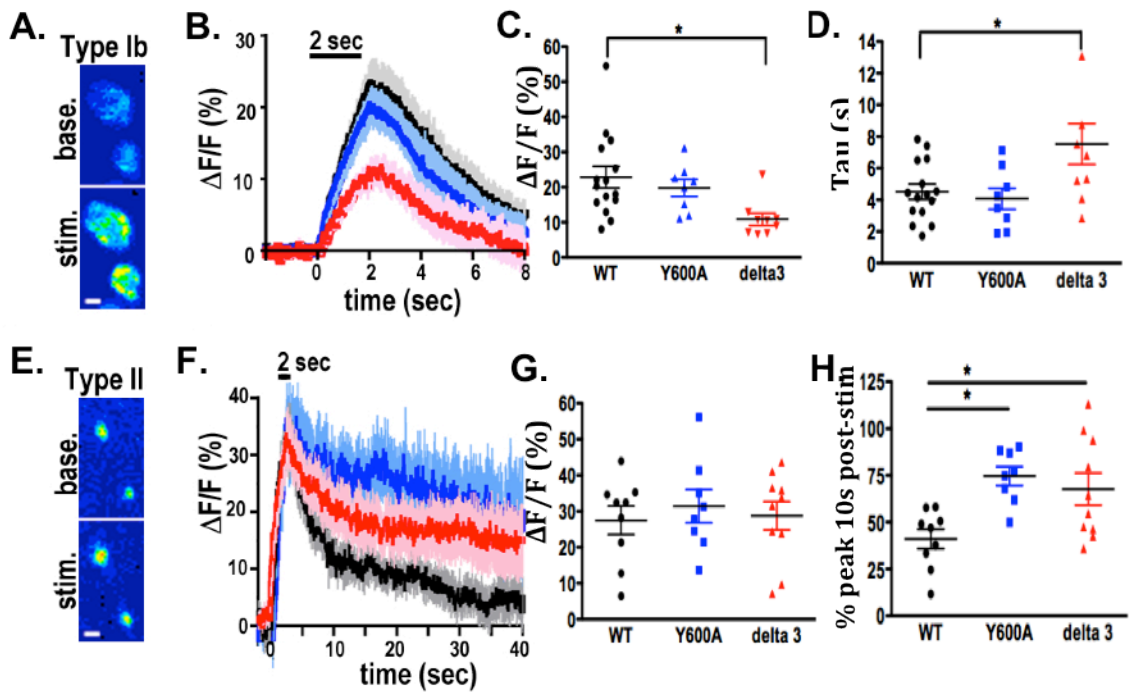


Figure 3. Delta 3 DVMAT displays slowed endocytosis at the larval NMJ (A) False color images of Type 1b terminals at baseline and following stimulation. Scale bar: 1 micron. (B) Summed traces of DVMAT-wt (black, n=15), delta 3 (red, n=10), and -Y600A (blue, n=8) in Type Ib terminals. Bar indicates 2 sec stimulus (10 mV, 40 Hz). (C) Peak levels of exocytosis (DF/F) were reduced in DVMAT-delta 3 relative to DVMAT-wt ("VMAT"); DVMAT-Y600A showed an intermediate level of DF/F. For all panels, *p<0.05, **p<0.01 compared to DVMAT-wt, 1-way ANOVA, Bonferroni post-test. (D) The time constant for endocytosis (t, see methods) was elevated in DVMAT-Delta 3 but not DVMAT-Y600A (n=8) compared to DVMAT-wt (E) False color images of Type II terminals at baseline and following stimulation. Scale bar 1 micron. (F) Summed traces of DVMAT-wt (black, n=10), Delta 3 (n=9), and -Y600A (blue, n=8) in Type II terminals. Bar indicates 2 sec stimulus (10 mV, 40 Hz) as in panel D. (Note difference in x axes in panel H versus D). (G) Peak levels of exocytosis (DF/F) for DVMAT-wt, -Delta 3 and -600A did not detectably differ (H) Relative rates of endocytosis were calculated as the decay from peak DF/F, measured at 12 sec followong the initial stimulus (10 sec following the peak DF/F). (see Methods). Both DVMAT-Delta 3 and -Y600A differ from DVMAT-wt. (Data from panels A-D was collected by Dr. Audrey Chen and panels E-H by Ms. Ciara Martin).

Discussion

Vesicular neurotransmitter transporters, such as the vesicular monoamine transporter (VMAT), are responsible for the packaging of neurotransmitter into secretory vesicles prior to vesicle fusion with the plasma membrane. Regulation of both the quantity and localization of VMAT is essential for maintaining healthy neuronal function, and disruption of VMAT function

has been linked to a variety of diseases including Parkinson's disease, schizophrenia and depression (Kurian et al., 2011; Wimalasena, 2011). Alterations in putative VMAT trafficking domains has been shown to cause delayed endocytosis *in vitro* and also to disrupt sorting to synaptic vesicles *in vivo (Grygoruk et al., 2010a)*. We sought to investigate the functional consequences of disrupting VMAT trafficking *in vivo* using the model synapse of the *Drosophila* neuromuscular junction (NMJ).

We investigated the effects of altering VMAT trafficking using a point mutant of a tyrosine based sorting motif (Y600A) and a C-terminus truncation mutant that lacks multiple sorting domains (delta 3) in *Drosophila* VMAT (DVMAT) (Grygoruk et al., 2010a). First we verified localization of the trafficking mutants using immunolabeling techniques and report no gross deficits in localization to either glutamatergic Type Ib or octopaminergic Type II terminals of the fly NMJ. Both wt and mutant VMAT co-localized with markers for secretory vesicles and large dense core vesicles (LDCVs), suggesting that in the absence of the affected sorting motifs DVMAT can at least partially localize to synaptic vesicles (SVs) and LDCVs.

Using a pH-sensitive form of GFP, or 'pHluorin', we developed wt, Y600A and delta 3 DVMAT-pHluorin constructs that allow for live imaging of DVMAT exocytosis and endocytosis at the fly NMJ. We monitored fluorescence in wt, Y600A and delta 3 DVMAT-pHluorin constructs after mechanical stimulation of the presynaptic nerve in the standard fly NMJ preparation. The NMJ prep is unique in that it allows for the side-by-side visualization of excitatory, glutamatergic Type Ib nerve terminals and modulatory, octopaminergic Type II terminals. Despite this, no studies to our knowledge have previously compared the synaptic activity of these two synapses using live-imaging techniques.

We first interrogated the effects of DVMAT mistrafficking at the canonical Type Ib boutons. The Y600A point mutant exhibited no phenotype at Type Ib terminals, however, the more dramatic delta 3 mutant had significantly decreased exocytosis, in addition to a delayed rate of endocytosis. These data suggest that trafficking motifs, other than $Y_{600}XXY_{603}$ motif, that are absent in the delta 3 mutant play an important role in normal DVMAT cycling at Type Ib terminals.

Using the exact same approach taken at Type Ib terminals, we examined the synaptic effects of altering DVMAT trafficking domains at Type II terminals. Type II terminals utilize octopamine as a neurotransmitter, the invertebrate homolog of noradrenalin, instead of glutamate as at Type Ib and Is terminals (Monastirioti et al., 1995). Type Ib terminals modulate fast synaptic transmission, contain mostly SVs, and possess iontropic postsynaptic receptors, while Type II terminals are more modulatory in function, have roughly equal number SVs and LDCVs and possess G-protein coupled receptors that cannot be monitored electrophysiologically (Budnik, 2006; Jia et al., 1993). We hypothesized that these functional distinctions between Type Ib and Type II terminals may result in the differential trafficking of DVMAT. Using the same approach as we took at Type Ib terminals, we assessed both wt and DVMAT trafficking mutants at Type II terminals. Contrary to our findings at Type Ib terminals where we found delta 3 to exhibit an exocytic defect, we report that that neither the Y600A nor the delta 3 mutants exhibited any disruption in exocytosis at Type II terminals. While at Type Ib terminals only delta 3 had a defect in endocytosis, at Type II terminals the rate of endocytosis is dramatically decreased in both the Y600A and the delta 3 mutants. Thus, the signals required for the initial sorting of DVMAT to secretory vesicles and for sorting after an exocytic event appear to differ between terminal types. Our data suggest that the Y600A domain has an important role for

DVMAT sorting at Type II terminals but is not required at Type Ib terminals. In conclusion, the Y600A and delta 3 DVMAT mutations result in distinct exo- and endocytic phenotypes that are specific to nerve terminal type, suggesting that different molecular machinery is involved in these processes at each terminal.

Chapter 3

Ziram differentially affects synaptic function at distinct nerve terminals of the *Drosophila* neuromuscular junction

Abstract

Multiple populations of aminergic neurons are affected in Parkinson's disease (PD), with serotonergic and noradrenergic loci responsible for many non-motor symptoms. Environmental toxins, such as the dithiocarbamate fungicide ziram, significantly increase the risk of developing PD and the attendant spectrum of both motor and non-motor symptoms. The mechanisms by which ziram and other environmental toxins increase the risk of PD, and their potential effects on aminergic neurons remain unclear. To investigate the effects of ziram on synaptic function in aminergic neurons, we used *Drosophila melanogaster* and live-imaging at the larval neuromuscular junction (NMJ). In contrast to nearly all other studies of this model synapse, we imaged presynaptic function at both glutamatergic Type Ib and aminergic Type II boutons, the latter responsible for storage and release of octopamine, the invertebrate equivalent of noradrenalin. To quantify the kinetics of exo- and endo- cytosis we employed an acid-sensitive form of GFP fused to the *Drosophila* vesicular monoamine transporter (DVMAT-pHluorin). An additional genetic probe (GCaMP) was used to visualize intracellular calcium flux. We find that at glutamatergic Type Ib terminals, exposure to ziram increases exocytosis but has a less pronounced effect on endocytosis. By contrast, at octopaminergic Type II terminals, ziram has no detectable effect on exocytosis but dramatically inhibits endocytosis of DVMAT-pHluorin. In addition, ziram causes bursts of calcium influx at aminergic nerve terminals. These events are sensitive to tetrodotoxin and thus appear to represent spontaneous depolarizations not seen in glutamatergic terminals. We speculate that the differential effects of ziram may be relevant to the dysfunction of aminergic neurons in PD.

1.0 Introduction

Parkinson's disease (PD) is a prevalent neurodegenerative disease best known for movement deficits and dopaminergic (DA) neuron loss in the substantia nigra (Corti et al., 2011). However, other populations of aminergic neurons are also affected in PD and are responsible for many non-motor symptoms of PD such as depression, insomnia and gastrointestinal dysfunction (Kuhn et al., 2011; Politis et al., 2012; Taylor et al., 2009). It remains unclear why aminergic neurons are particularly susceptible to the pathogenic mechanisms of PD. Moreover, few disease models have directly compared the function of aminergic versus non-aminergic neurons.

While several heritable forms of PD have been identified, the vast majority of cases are sporadic, suggesting the possibility that environmental exposures play a role in disease etiology. Recent epidemiological data demonstrate that exposure to the fungicide ziram increases the risk of PD ~40%; risk is further increased to three-fold in individuals exposed to ziram in addition to the herbicide paraquat and the fungicide maneb (Wang et al., 2011). Previous studies have demonstrated that ziram, maneb and paraquat can selectively kill aminergic neurons *in vitro* and *in vivo* (Chou et al., 2008; Cicchetti et al., 2005; McCormack et al., 2002; Meco, 1994). However, it remains unclear why these environmental toxins show relatively selective neurotoxic effects for aminergic cells or the mechanisms by which they increase the risk for PD.

Several direct protein targets for ziram binding have been proposed based on *in vitro* biochemical studies. These include the enzyme aldehyde dehydrogenase (ALDH) (Fitzmaurice, 2012), and E1 ligase (Chou et al., 2008), the first enzyme in the biochemical cascade responsible for protein ubiquitination (Kleiger and Mayor, 2014). Ziram directly inhibits both of these

enzymes, and the downstream effects of inhibition are postulated to either directly compromise neuronal viability or interact with other cellular factors to cause neurodegeneration. The link(s) between the direct effects of ziram binding to specific protein targets and downstream neurodegenerative processes are not known. It is possible that inhibition of ubiquitous enzymes such as E1 and ALDH2 cause equally broad effects on the function of a variety of cell types. However, this would not explain their toxicity to neurons or their relative specificity for aminergic neurons (Chou et al., 2008; Wang et al., 2011). We therefore hypothesized that different types of neurons might respond differently to ziram exposure.

Synaptic transmission is a unique and defining feature of most neurons and therefore a logical site to search for neurotoxic processes specific for neurons. Many sub-populations of neurons have distinctive pre- and/or postsynaptic specializations that clearly affect their function (Yu and Goodrich, 2014). Thus, changes in synaptic function represent a potential link between the biochemical effects of pesticides or other pathogenic factors relevant to PD and their apparent ability to target specific subsets of neurons (Chen et al., 2011; Martella et al., 2011; Xu et al., 2011). However, the effects of environmental toxins on synaptic function are poorly understood, and it is not known whether different types of synapses might be differentially affected by pesticides or other environmental toxins.

Previous studies in primary mammalian cultures have demonstrated that ziram increases the frequency of spontaneous depolarization events (minis) recorded in postsynaptic hippocampal neurons (Rinetti and Schweizer, 2010). Although this effect is most likely due to a presynaptic increase in synaptic vesicle fusion with the plasma membrane, all studies to date have employed post-synaptic electrophysiological assays to quantify these events, and the affect of ziram on pre-synaptic terminals has not been directly tested. In addition to increasing mini

frequency, ziram has been shown to increase intracellular calcium *in vitro* (Sook Han et al., 2003). The mechanism(s) by which ziram increases mini frequency or calcium levels are not clear. It is possible that these effects are caused by changes in ubiquitination, e.g. of presynaptic proteins responsible for exo- and endocytosis, but ALDH or additional targets may also play a role (Chou et al., 2008; Fitzmaurice et al., 2014).

The *Drosophila* larval neuromuscular junction (NMJ) represents a potentially useful model synapse to explore the effects of pesticides on synaptic function. Unlike mammals, flies release glutamate rather than acetylcholine as a fast-acting acting excitatory neurotransmitter to some synapses at the NMJ. Glutamatergic synapses at the fly NMJ include Type Ib (big) and Is (small) (Atwood et al., 1993; Jan and Jan, 1976; Jia et al., 1993). Importantly, the larval NMJ contains several other types of synapses that are not glutamatergic. These include peptidergic Type III, and Type II terminals that store and release the aminergic neurotransmitter octopamine. Octopamine is both structurally and functionally similar to mammalian norepinephrine (Atwood et al., 1993; Jia et al., 1993; Monastirioti et al., 1995). Relatively few studies of the fly NMJ have focused on Type II terminals in part because octopamine, like most mammalian amines, does not activate ionotropic receptors and cannot be followed via postsynaptic electrophysiological recordings. Other methods to quantify presynaptic function such as FM dyes or genetic probes might be used to study aminergic Type II terminals, but surprisingly, to our knowledge this has not been reported.

Here I have used genetic probes for vesicle recycling and calcium flux to compare the effects of ziram on aminergic Type II versus glutamatergic Type Ib nerve terminals. I report unexpected differences in their respective responses to toxin exposure. These differences may be

relevant to the neurotoxic affects of ziram on mammalian aminergic neurons and the observed increased risk for PD.

2.0 Materials and Methods

2.1 Fly husbandry

Flies were reared on standard molasses yeast agar at room temperature. For pHluorin imaging, male larva of genotype elav-GAL4 (X);+;UAS-DVMAT-pHluorin (III) were used for all experiments, except for the E1 RNAi experiments. To generate transgenes expressing RNAi directed against E1 ligase (uba1), a 725 bp fragment was excised from a *uba1* cDNA (gh24511, *Drosophila* Genomics Resource Center, Indiana) via digestion with Sal I, and inserted into the intermediate vector pGEM-11 (Promega, Madison WI). This construct was digested with EcoRI – NotI and the fragment representing *uba1* inserted into the symmetrically transcribed RNAi vector sympUAST (Giordano et al Genetics 2002) for injection into *Drosophila* embryos (Bestgene Chino Hills, CA). E1 RNAi constructs were generated by George Lawless. For E1 RNAi pHluorin experiments, F_1 progeny were used from crosses of female virgin elav-GAL4;+;UAS-DVMAT-pHluorin flies to male +;+;UAS-E1-RNAi flies, and as genetic controls, F_1 progeny were used from female virgin elav-GAL4;+;UAS-DVMAT-pHluorin flies crossed to male Canton S. (wild type) flies.

For calcium imaging experiments, male larva of genotype elav-GAL4/+;UAS-GCAMP6m/+;+ were used for all experiments (Chen et al., 2013).

2.2 pHluorin imaging

The construction of the DVMAT-pHluorin transgene is discussed in detail in the methods of Chapter 2. For all recordings, segment A4 muscle 13 was selected for visualization and analysis. Third instar larvae neuromuscular junction (NMJ) fillets were dissected in chilled HL3.1 Ca^{2+}-free media (final mM concentration: NaCl 70, KCl 5, $MgCl_2$ 4, $NaHCO_3$ 10, trehalose 5mM, sucrose 115, HEPES 5) adjusted to pH 7.32. Stock solutions of 10 mM ziram were made in DMSO, aliquoted, stored at -20ºC and discarded after one freeze-thaw cycle; stocks of 10 mM lactacystin (Sigma, St. Louis, MO) were made in the same manner but solubilized in water. Fresh stocks of L-glutamic acid (LGA) (Sigma, St. Louis, MO) were made weekly, and fresh stocks of HL3.1 were made monthly. LGA, $CaCl_2$, ziram, lactacystin and DMSO were added to HL3.1 on the day of experiment. The fillets were incubated for 45 minutes at 18ºC in 'recording solution' (HL3.1 supplemented with 2 mM calcium and 700 uM L-glutamic acid) and containing either 20 uM ziram (Chem Services, West Chester, PA) or DMSO (Sigma, St. Louis, MO) as a control (final DMSO concentration 0.2%) or for the lactacystin experiments, 100 uM lactacystin or water vehicle as control. After incubation, the preps were washed 3x with chilled recording solution and imaged.

Nerve terminals were imaged using a Zeiss Axio Examiner Z1 microscope and Zeiss Achroplan water-immersion objective (100x, 1.0 N.A.) fitted with a cooled back-illuminated electron-multiplying CCD camera (Andor iXon 897, South Windsor, CT) and a capture rate of 20 frames/second.. A DG4 light source (Sutter, Novato, CA) with a GFP Brightline® Filter Set (Semrock, Rochester, NY) was used for illumination. To stimulate exocytosis in selected boutons, a suction electrode was used to stimulate a single nerve root (10 V, 40 hz for 2 seconds). Exocytosis was quantified as $\Delta F/F$ $[(F_{peak} - F_{baseline}/(F_{baseline})]*100$, where F_{peak} is the average fluorescence for the ten frames (0.5s) after the end of the 2 sec stimulus period and

$F_{baseline}$ is the average of the ten frames immediately preceding stimulus. Endocytosis rate was calculated at Type Ib terminals as the tau value for a non-linear single exponential fit to the 30 seconds following stimulus. We observed minimal decay in many of the endocytic curves for Type II terminals, prohibiting the calculation of tau by fitting a non-linear exponential curve. We therefore quantified the decrease in fluorescence due to endocytosis as % of F_{peak} at t = 10sec post stimulus (negative % values were dropped).

2.3 Calcium imaging

To monitor intracellular calcium concentrations, a calcium sensitive form of GFP, UAS-GCAMP6 (Chen et al., 2013) was expressed pan-neuronally using the *elav-GAL4* driver on the x chromosome (Robinow and White, 1991). For baseline recordings, larvae were dissected as for pHluorin experiments, and either 1) preincubated in ziram for 45 minutes (18 C) followed by washing in recording solution or 2) imaged during continuous incubation in recording solution containing either 20 uM ziram (0.2 % DMSO) or DMSO alone at ambient temperature (see Results). Nerve roots were stimulated (10 V, 40 Hz) and imaging performed as for pHluorins. Due to differences in the kinetics of the pH and calcium sensitive GFP reagents, ΔF/F was calculated for GCAMP as as $[(F_{peak} - F_{baseline}/(F_{baseline})]*100$, with F_{peak} representing the average of four frames prior to and after stimulus (0.4 sec total) (Macleod, 2012) and $F_{baseline}$ representing the average of the ten frames prior to stimulus. A custom script written in the R programming language (Gnu Operating System) was used to quantify peak amplitude, frequency, and the rise time and decay of spontaneous calcium events. Amplitude was normalized to baseline and quantification was conducted over a 30-60 sec time range. For experiments involving tetrodotoxin (TTX), larval fillets were imaged in recording solution plus 0.2% DMSO +/- 20 uM ziram without TTX, then washed 3x with recording solution containing either 20 uM ziram or 20

uM ziram plus 1 uM TTX. After incubation in this solution for an additional 5 minutes, the preparations were imaged for $\geq$ 1.2 minutes.

3.0 Results

3.1 Ziram exposure at glutamatergic terminals results in aberrant exo- and endocytosis

To determine how ziram exposure affects the function of presynaptic nerve terminals I used fillet preparations of *Drosophila* 3rd instar larvae (Jan and Jan, 1976). The exposed abdominal musculature and neuromuscular junctions (NMJs) were pre-incubated in 20 μM ziram for 45 minutes at 18°C then washed and imaged in 'recording solution' (HL3.1 supplemented with 2mM calcium and 7mM L-Glutamic acid). The nerve root innervating a selected segment was stimulated to induce exocytosis, and the preparation was imaged before, during and after the stimulus. I first recorded DVMAT-pHluorin exo- and endocytosis at the canonical Type Ib excitatory nerve terminals (Figure 1a,b). Ziram exposure at Type Ib terminals caused a significant increase in exocytosis relative to preparations incubated in vehicle alone (Figure 1c. n= 23-25 per treatment; Student's t-test, $p < 0.05$). I also observed a modest decrease in the rate of endocytosis, quantified as an increase in the tau value of a non-linear single exponential curve fit (see Methods) from 4.85 seconds to 6.12 seconds (Figure 1d; n = 23-24 per treatment; Student's t-test, $p < 0.01$). Thus, at glutamatergic, Type 1b terminals ziram significantly increases the exocytotic processes, but modestly retards endocytosis.

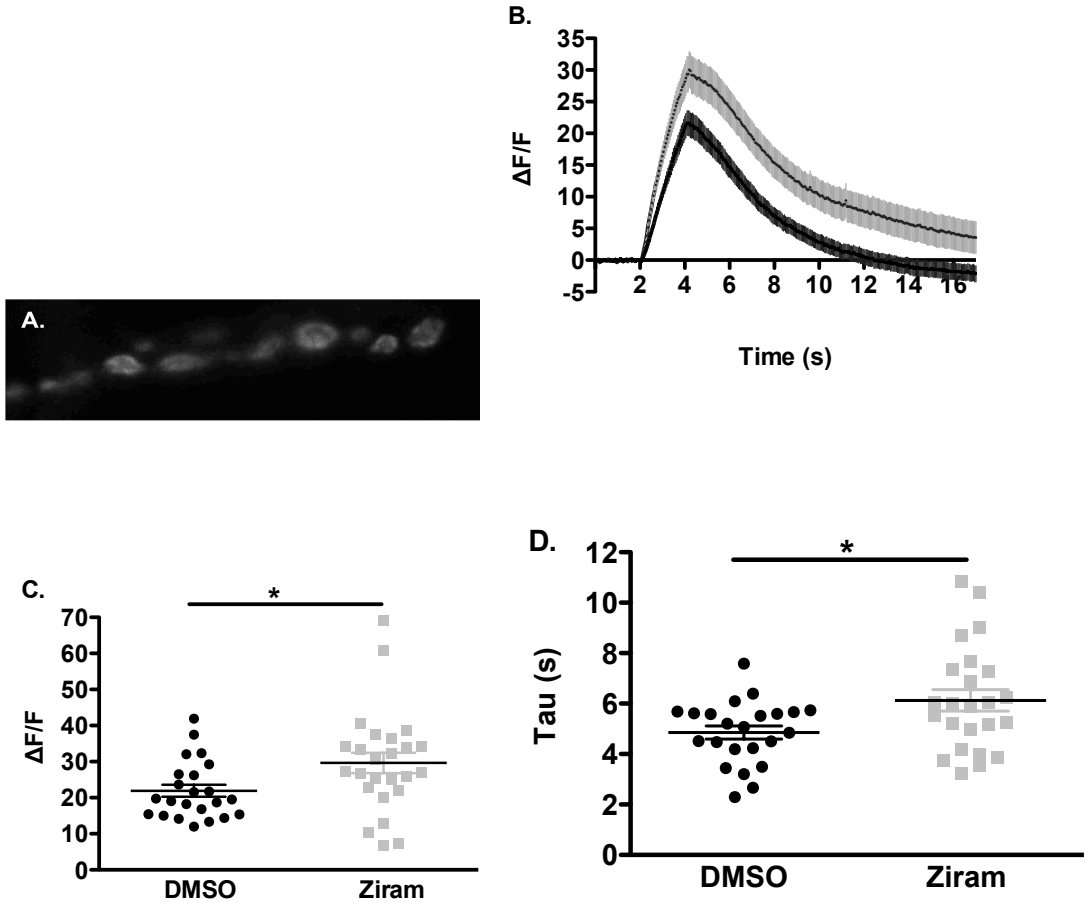


Figure 1. Exposure to ziram at excitatory glutamatergic terminals results in aberrant exo- and endo-cytosis. The standard fly 3rd instar NMJ prep was exposed for 45min to 20 uM ziram followed by wash and imaging at Type Ib boutons. pHluorin fluorescence was recorded in response to a 40 hz 2 second stimulus a) Representative image of baseline pHluorin fluorescence at Type Ib boutons b) Averaged trace of pHluorin recordings at treated (light grey trace) and control boutons (dark grey trace) (n=23-25 per treatment) c) ΔF/F at control and treated boutons (Student's t-test; n = 23-25; $p < 0.05$) d) Tau of a single non-linear exponential fit to the 30 sec trace following end of stimulus for both treated and control groups (Student's t-test; n = 23-25; $p < 0.01$).

3.2 Ziram exposure at aminergic terminals disrupts endocytosis but not exocytosis

To examine how ziram exposure affects synaptic function at Type II terminals, I again exposed larval fillets to ziram, followed by stimulation of the nerve root in the absence of ziram and imaging exactly as described for Type Ib terminals (Figure 2b). In contrast to Type Ib terminals, exocytosis at Type II terminals exposed to ziram does not detectably differ from DMSO controls (Figure 2c; n = 18-20 per treatment; Student's t-test, $p > 0.05$). Endocytosis at Type II terminals was generally slower than Type Ib in control conditions, with the average

return to baseline occurring at 18.6s versus 12.6s (Figures 1d, 2d). The endocytic curves at Type II terminals were variable, sometimes exhibiting almost no decay at all, thus preventing an accurate fit for a non-linear exponential curve as for Type Ib. Thus, unlike Type Ib, I present my data only as the % of the F_{peak} value at t = 10 seconds. I report that endocytosis is significantly slowed after exposure to ziram, with ziram treated controls showing a decay at t = 10 seconds post-stimulus to 47.75% of peak versus 28.83% in controls (Figure 2c,d; n = 14-17 per treatment; Students t-test, $p < 0.05$). In sum, my data demonstrates that after exposure to ziram Type II terminals exhibit a pronounced delay in endocytosis without a detectable increase in exocytosis.

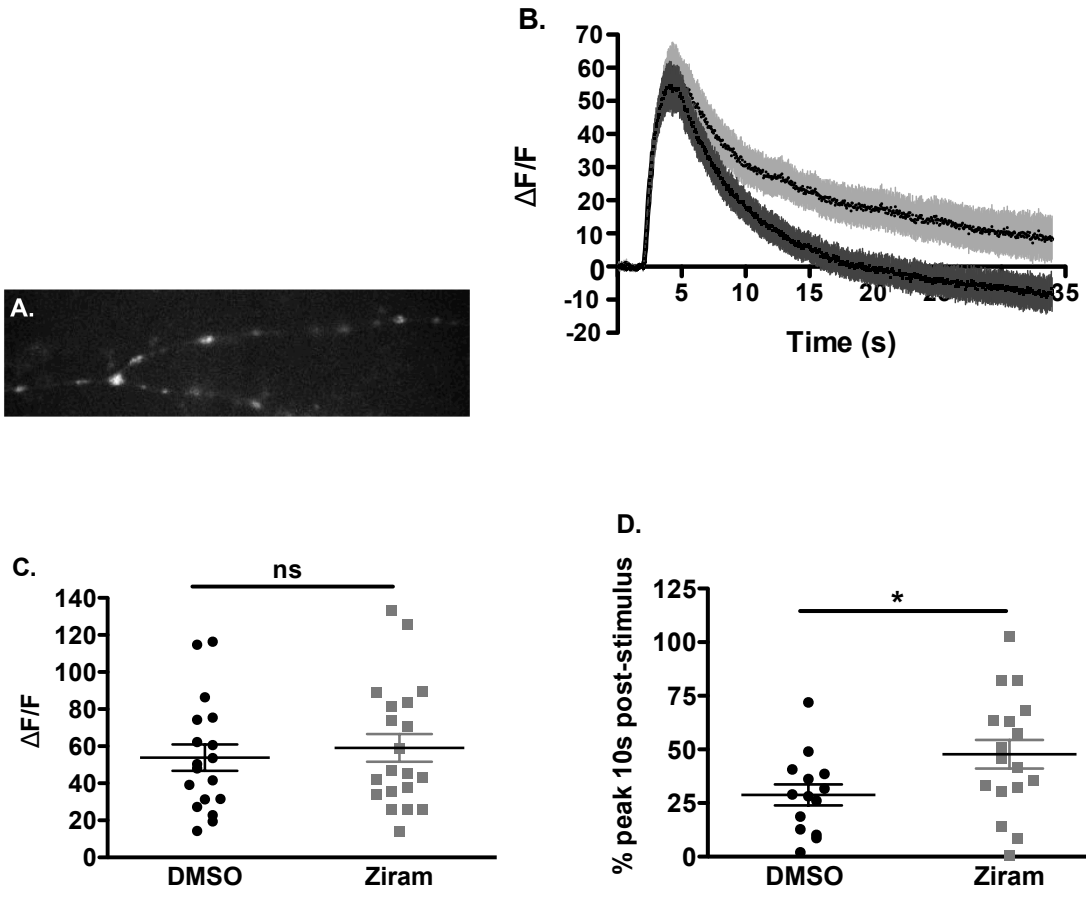


Figure 2. Exposure to ziram at modulatory, aminergic terminals results in aberrant endocytosis. The standard fly 3rd instar NMJ prep was exposed for 45min to 20 uM ziram followed by wash and imaging at Type II boutons. pHluorin fluorescence was recorded in response to a 40 hz 2 second stimulus a) Representative image of baseline pHluorin fluorescence at Type II boutons b) Averaged trace of pHluorin recordings at treated (light grey) and control (dark grey) boutons (n=18-20 per treatment) c) delta F/F at control and treated boutons (Student's t-test; n=18-20 per treatment; $p > 0.05$) d) Value shown is the % of peak fluorescence at t = 10 seconds post stimulus (n = 14-17; Student's t-test; $p < 0.05$).

3.3 Inhibition of components of the Ubiquitin Proteasome System does not mimic ziram's effect at Type Ib terminals

Ziram is known to inhibit the ubiquitin activating E1 ligase, leading to downstream inhibition of the proteasome (Chou et al., 2008). Similar to the effect I see for ziram exposure on exocytosis at Type Ib boutons, exposure to proteasome inhibitors (under the same exposure conditions, 45 min at 18 C followed by wash) has been shown to increase evoked response at the fly NMJ using electrophysiological approaches (Speese et al., 2003). Thus, we hypothesized that the increase in exocytosis, and perhaps the decrease in endocytosis, we observed at Type Ib terminals may be due to ziram's downstream effects on the proteasome. However, using a similar protocol as Speese et al. I did not observe any change in exocytosis after 100 uM lactacystin treatment (n = 18-19 per treatment; Student's t-test, $p > 0.05$). While our protocols both involve 45 minute incubation at 18 C followed by wash, my recordings were conducted at room temperature in 2 mM calcium in HL3.1 (final mM concentration: NaCl 70, KCl 5, $MgCl_2$ 4, $NaHCO_3$ 10, trehalose 5mM, sucrose 115, HEPES 5) while their recordings were conducted at 18C in 0.4 mM calcium and in a saline solution with a different composition (final mM concentration: 128 NaCl, 4 MgCl2, 2 KCl, 70 sucrose, 5 HEPES) (Speese et al., 2003); these differences may account for the different effects we observe on synaptic vesicle release upon lactacystin exposure. We found ziram exposure to decrease the rate of endocytosis at Type Ib terminals (Figure 1D), however, under the same conditions lactacystin exposure actually increased the rate of endocytosis (Figure 3C; Student's t-test, $p < 0.05$). Taken together, these

data suggest that ziram's downstream affect on the proteasome is not responsible for the increase in exocytosis we observe in ziram-treated Type Ib terminals.

We next hypothesized that ziram's affect on E1 ligase, not its downstream affect on the proteasome, may be responsible for the increase in exocytosis we observe at Type Ib terminals. To address this I expressed an E1 RNAi construct (see Methods; construct developed in our lab by George Lawless) at the NMJ and conducted pHluorin recordings (Figure 3D). F_1 progeny of elav-gal4;+; UAS-VMAT-pHluorin female virgins crossed to +;+; UAS-E1-RNAi males were used to achieve progeny with E1 knockdown at Type Ib terminals and F_1 progeny of elav-gal4;+; UAS-VMAT-pHluorin female virgins crossed to Canton S. wild type males were used as genetic controls. We found no significant difference in exocytosis in flies expressing E1 RNAi versus control (Figure 3E, n = 9-12; Student's t-test, $p > 0.05$); if anything, there is a trend towards decreased exocytosis, the opposite of what we observe at ziram exposed Type Ib terminals. Additionally, we did not find any significant difference in the % peak values at 10 second post-stimulus of Type Ib terminals with E1 knockdown versus control (Figure 3F, n = 9-12; Student's t-test, $p > 0.05$) (tau values were not used as several curves could not be accurately fit by a single non-linear exponential curve). These data suggest that ziram's ability to inhibit E1 ligase may not be responsible for the effects of ziram we observe at Type Ib terminals. However, it must be kept in mind that the knockdown of E1 RNAi is constitutive, not acute like the ziram exposure, and compensatory mechanisms may exist.

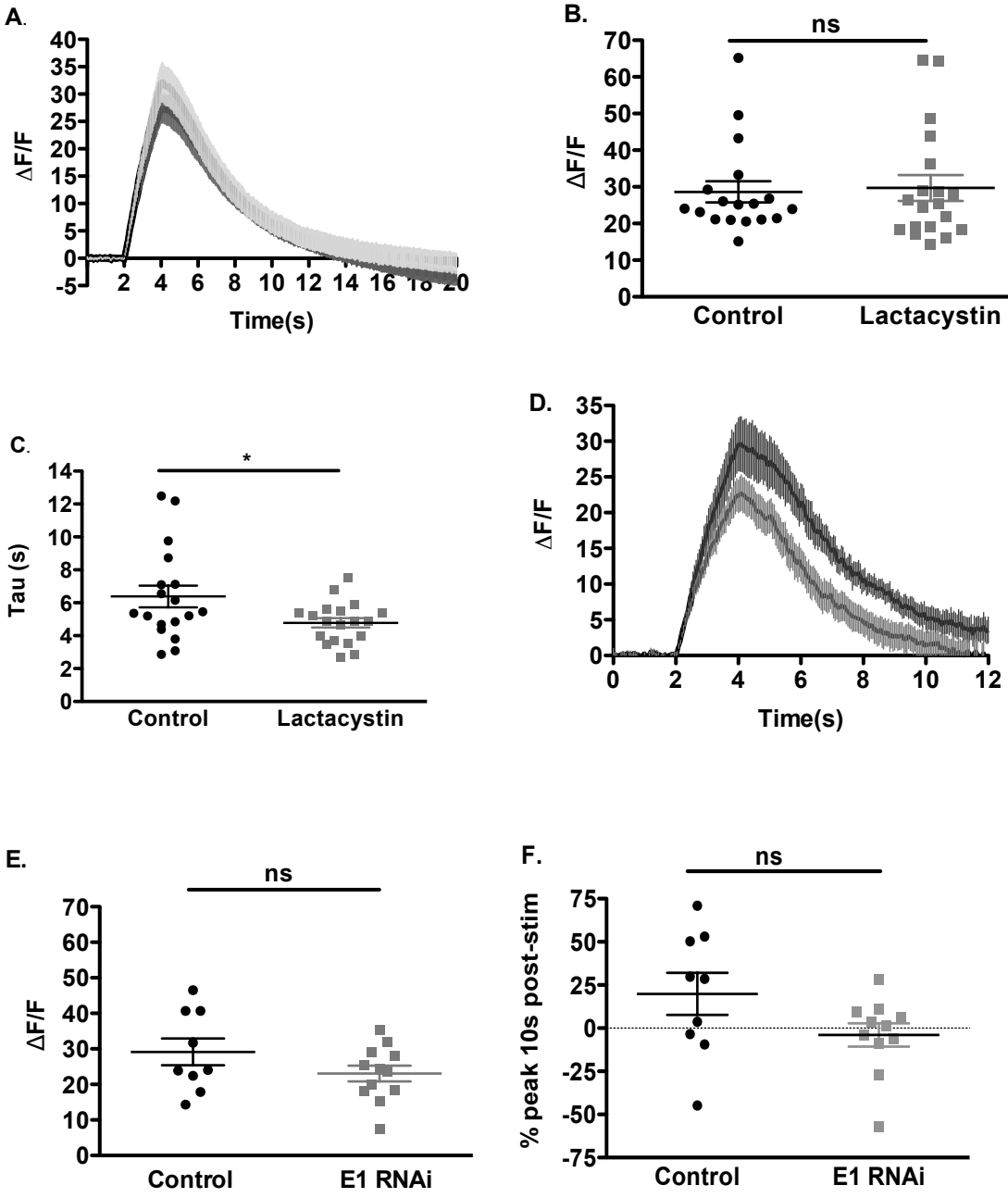
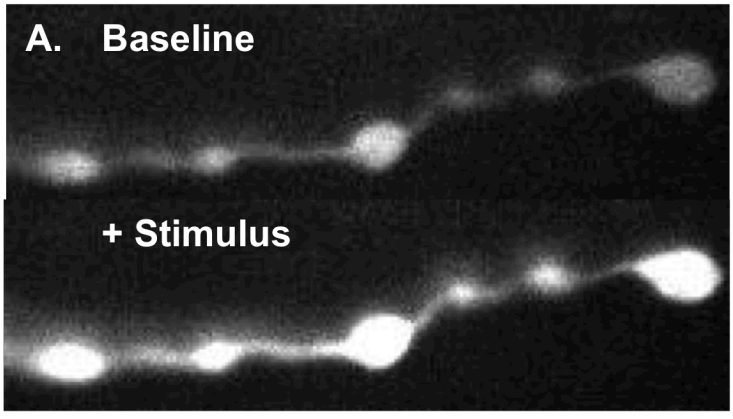


Figure 3. Proteasome inhibition does not mimic ziram's action at Type Ib terminals. The standard fly 3rd instar NMJ prep was exposed for 45min to 100 uM lactacystin followed by wash and imaging at Type Ib boutons. pHluorin fluorescence was quantified in response to a 40 hz stimulus for 2 seconds a) Averaged Type Ib terminal traces of treated and control conditions (n=18-19 per treatment) b) delta F/F at control (dark grey) and treated (light grey) boutons (n=18-19 per treatment; Student's t-test, $p > 0.05$) c) Tau of a single non-linear exponential fit to the 30 sec trace following end of stimulus for both treated and control groups (Student's t-test; n = 18-19; $p < 0.05$).d) Averaged Type Ib terminal traces of treated and control conditions (n=9-12 per treatment) e) delta F/F at control and E1 RNAi expressing boutons (n=9-12 per treatment; Student's t-test, $p > 0.05$) f) Decay for both control and E1 RNAi expressing groups (Student's t-test; n = 9-12 per treatment; $p > 0.05$), shown as the % of peak fluorescence at t = 10 sec post-stimulus.

3.4 Ziram exposure does not alter calcium influx during exocytosis at Type Ib terminals

Since ziram exposure has been shown to increase levels of intracellular calcium in PC12 cells (Sook Han et al., 2003) and calcium is tightly coupled to the release of secretory vesicles, I hypothesized that the increase in exocytosis I observed in Type Ib terminals exposed to ziram may be due to higher baseline calcium concentrations or an increase in calcium influx during stimulation. To address this, I expressed a calcium sensitive form of GFP (GCAMP6) at the larval NMJ and conducted live-imaging after exposure to 20 uM ziram for 45 minutes followed by recording in the absence of ziram (Figure 4a), as described for pHluorin recordings. Exposure to ziram did not result in a detectable increase in intracellular calcium during evoked exocytosis at Type Ib boutons (Figure 4c, n=11 per treatment; Student's t test, $p > 0.05$), nor did it alter baseline calcium levels (Figure 4d, n = 11 per treatment; Student's t test, $p > 0.05$). These data suggest that increases in calcium influx during exocytosis or baseline calcium levels are not responsible for the increase in exocytosis we observe at ziram-treated Type Ib terminals.



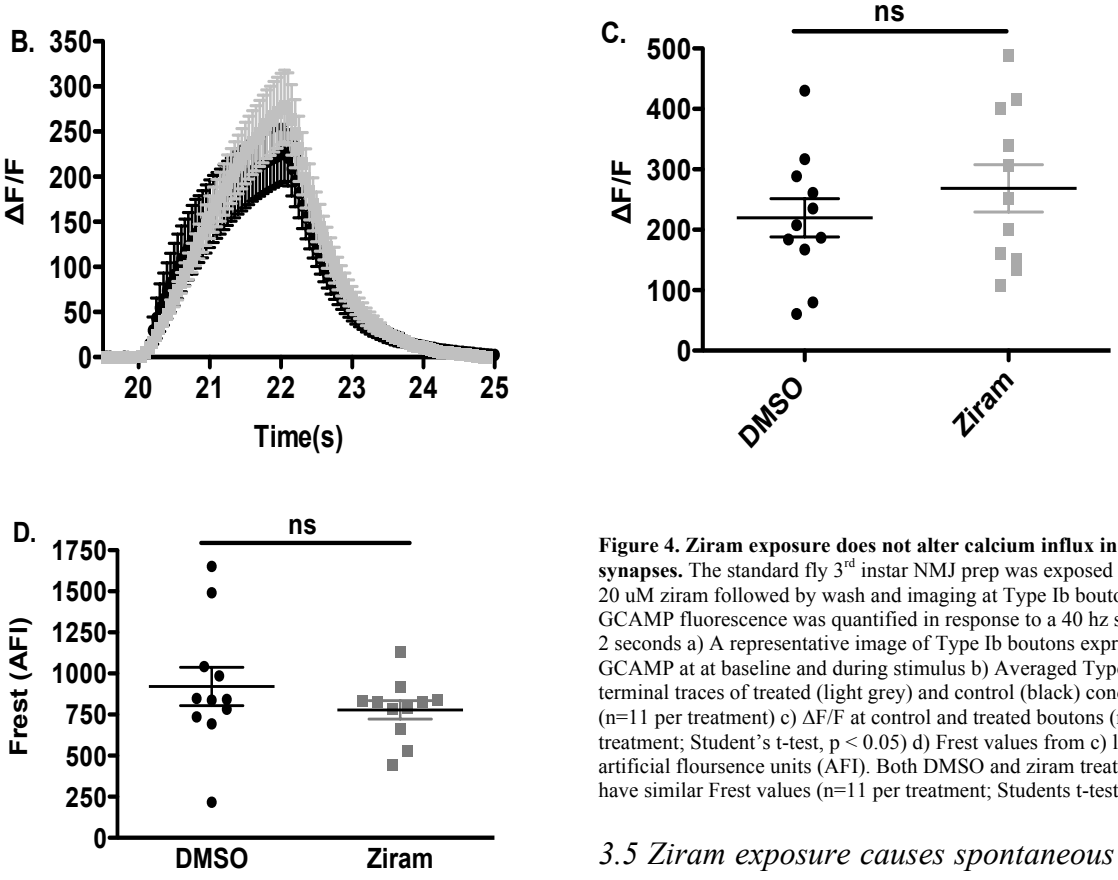


Figure 4. Ziram exposure does not alter calcium influx in excitatory synapses. The standard fly 3rd instar NMJ prep was exposed for 45min to 20 uM ziram followed by wash and imaging at Type Ib boutons. GCAMP fluorescence was quantified in response to a 40 hz stimulus for 2 seconds a) A representative image of Type Ib boutons expressing GCAMP at at baseline and during stimulus b) Averaged Type Ib terminal traces of treated (light grey) and control (black) conditions (n=11 per treatment) c) ΔF/F at control and treated boutons (n=11 per treatment; Student's t-test, $p < 0.05$) d) Frest values from c) listed in artificial flourscence units (AFI). Both DMSO and ziram treated preps have similar Frest values (n=11 per treatment; Students t-test, $p > 0.05$).

3.5 Ziram exposure causes spontaneous calcium events in aminergic, but not glutamatergic, terminals

While conducting baseline and evoked calcium recordings following pre-incubation in ziram at 18C, I observed occasional spontaneous and synchronized 'flickering' of GCAMP6 fluorescence in aminergic Type II terminals but never in Type Ib terminals (not shown). To further explore this phenomenon, I performed additional recordings during continuous ziram exposure at ambient temperature. Fillets expressing GCAMP6 were continuously incubated in 20 uM ziram while simultaneously imaging the nerve terminals. Spontaneous calcium events were consistently observed in Type II terminals within 5-10 minutes of ziram exposure in all experiments. Remarkably, spontaneous calcium events were never observed in Type Ib terminals in any recording. Type Is terminals are also glutamatergic but slightly smaller than 1b and I did

not observe any spontaneous events at Type 1s either. Quantification of spontaneous events in Type II terminals after 10 minutes of incubation in ziram showed a frequency of 8.01 ± 1.03 events/minute, with an amplitude of 47.63 ± 5.10 in AFI (artificial fluorescence intensity) and rising and falling slopes of 156.66 ± 22.29 and -40.44 ± 5.65 AFI/sec, respectively (all data shown as mean ± SEM), (n = 5). Calcium peaks were not induced by vehicle exposure alone (Figure 5c; n = 8).

I hypothesized that ziram exposure may increase baseline calcium levels in Type II boutons, thus lowering their firing threshold and potentially leading to the spontaneous calcium events I observed. I conducted recordings of baseline calcium fluorescence at the NMJ both prior to and following 15 minute exposure to either DMSO control or 20 uM ziram exposure. I report that neither DMSO control nor ziram treated preps exhibit a change in baseline calcium during continuous exposure to ziram (Figure 5e, One-way ANOVA, post-hoc Bonferroni; $p > 0.05$). Next, I stimulated Type II terminals before and after 15 min of DMSO control or ziram treatment to determine if their were any changes in calcium levels during evoked response, however, I did not find in significant difference between the delta F/F values of DMSO or ziram treated preps before or after 15 minutes of treatment (Figure 5f, One-way ANOVA, post-hoc Bonferroni; $p > 0.05$). These data suggest that changes in calcium levels are not responsible for the spontaneous events we observe at Type II terminals upon ziram exposure.

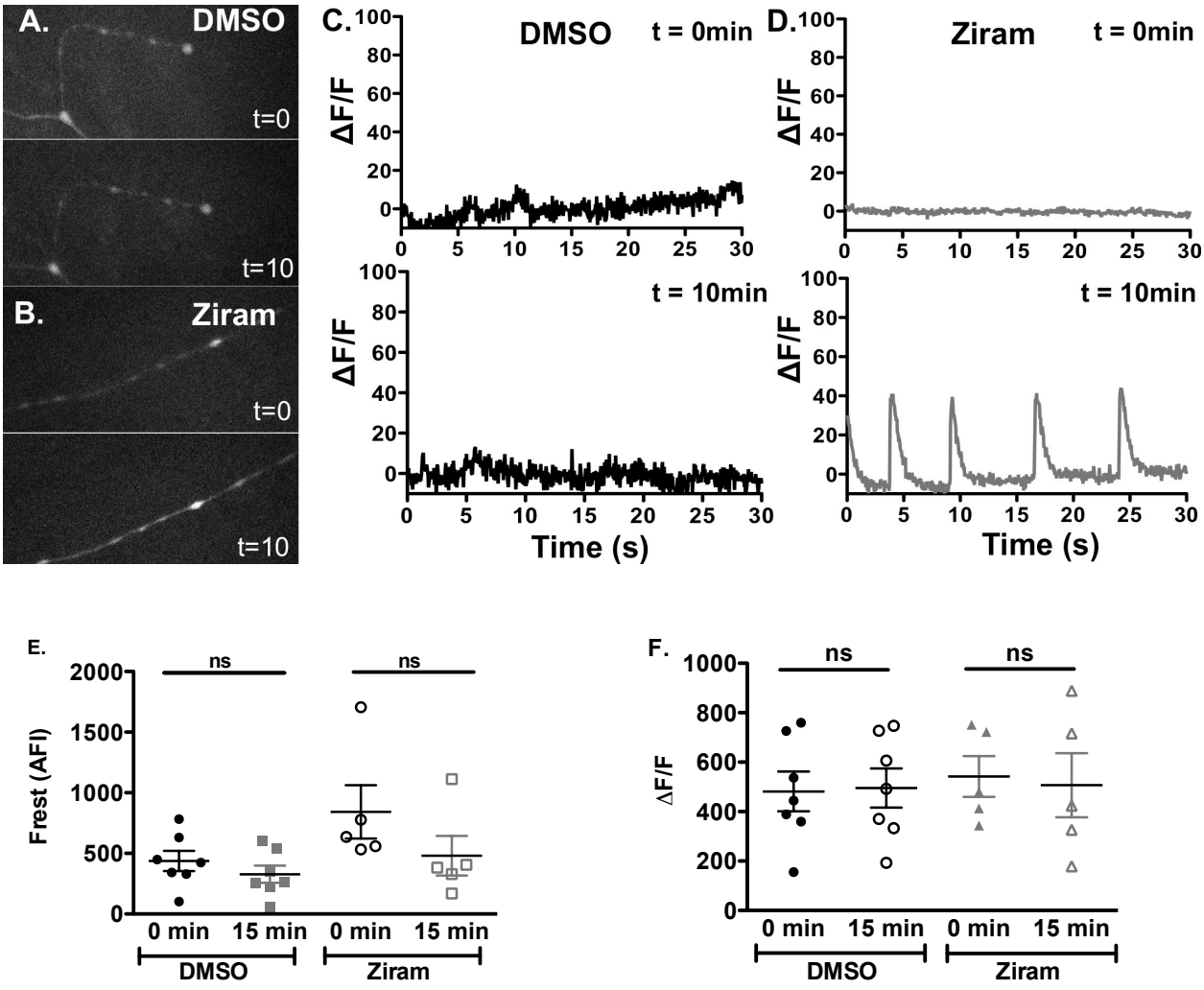


Figure 5. Ziram exposure causes spontaneous calcium events in aminergic terminals. GCAMP fluorescence was quantified in a standard fly 3rd instar NMJ prep continuously exposed to 20 uM ziram. Representative image of baseline GCAMP fluorescence of a) control and b) treated Type II terminals at t=0 and t=10min. For b) boutons are shown during the peak of a spontaneous event. c) Representative trace of DMSO and d) ziram treated preps at t=0 and t=10min at Type II terminals e) Frest values for preps in the continuous presence of DMSO or ziram at t= 0 and t= 15min f) delta F/F values from a 40hz, 2 sec stimulus after 0 or 15 min treatment with continuous presence of DMSO 0.2% or ziram 20uM

Further characterization of the calcium transients revealed that they occurred in all of the observed boutons within a single process (Figure 4b). Since I observed a signal throughout the process, I hypothesized that this might be caused by activation of cationic channels and the depolarization of the entire process. To test this hypothesis, I performed additional experiments using tetrodotoxin (TTX), which blocks Na channels. Larval fillets were incubated in ziram 20 uM for 10 minutes and imaged to confirm the presence of spontaneous synchronized calcium evens; I then replaced the solution with ziram + tetrodotoxin (TTX) and incubated for 5 minutes.

We observe that TTX blocks the spontaneous calcium events observed at aminergic terminals exposed to ziram (Figure 6b; n =3). In summary, we find that exposure to ziram at the NMJ causes spontaneous depolarization of aminergic, but not glutamatergic, nerve terminals.

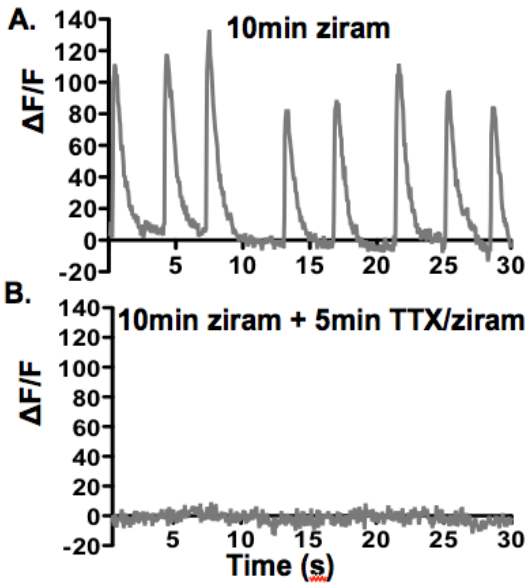


Figure 6. Tetrodotoxin blocks ziram-induced spontaneous calcium events at aminergic terminals. Representative trace of prep treated with a) 10 min of 20 uM ziram followed by b) ziram 20 uM in combination with tetrodotoxin 1uM for 5 min.

Discussion

Epidemiological studies have demonstrated an increase in Parkinson's disease (PD) in human populations exposed to the pesticide ziram, but the mechanism remains unclear. To investigate the mechanisms by which ziram may disrupt neuronal function, I have used the *Drosophila* neuromuscular junction (NMJ) as a model synapse. To our knowledge, the fly NMJ has not been used previously to examine the effects of putative neurotoxins. Importantly, our studies also differ from nearly all previous experiments using the NMJ because we have directly compared the synaptic physiology of two distinct types of nerve terminals: aminergic Type II terminals and glutamatergic Type Ib. This comparison is relevant to the pathophysiology of PD because several subtypes of aminergic neurons appear to be sensitive to the neurodegenerative processes that characterize this disease, including dopaminergic neurons of the substantia nigra, noradrenergic neurons of the locus coeruleus and serotonergic neurons in the raphe nuclei (Kuhn et al., 2011; Politis and Loane, 2011; Politis et al., 2012; Szot, 2012; Taylor et al., 2009). I observe striking differences in the affect of ziram on

Type Ib versus Type II and speculate that these differences may be relevant to neurotoxic effects of ziram in mammals and perhaps PD patients.

Using a DVMAT-pHluorin transgene and live-imaging techniques, I report that ziram treatment delays endocytosis in both Type Ib and Type II terminals but has much more dramatic effects in Type II terminals. In addition, I observe an increase in exocytosis in Type Ib but no detectable change in exocytosis in Type II upon ziram exposure. Using another transgene to image calcium dynamics, I did not observe an increase in baseline or evoked calcium levels suggesting that the increase in exocytosis I observe at Type Ib was not due to increased calcium influx. However, I observed a striking increase in spontaneous calcium events in Type II but not Type 1B. Since these events are blocked by tetrodotoxin they are likely to represent spontaneous depolarization events in processes containing Type II boutons. Importantly, these events cannot be detected electrophysiologically via post-synaptic recording at Type II synapse due to the metabotropic nature of aminergic receptors. More generally a comparison between Type II and Type Ib terminals cannot be conducted electrophysiologically, and the function of Type II boutons have therefore received relatively little attention. We anticipate that further use of both pHluorins and Ca imaging at Type II may be used to address a variety of questions.

The differences I observed between aminergic and non-aminergic terminals could conceivably be specific for the larval NMJ and idiosyncratic to differences between Type Ib and Type II terminals. For example, Type Ib terminals are deep and enveloped by membrane invaginations known as the by subsynaptic reticulum (SSR), whereas Type II terminals are closer to the surface with little surrounding SSR (Atwood et al., 1993). However, many other differences between Type Ib and II terminals appear to extend to other systems. For example, similar to the fly NMJ, the vast majority of aminergic synapses perform neuromodulatory roles

and the release of neurotransmitter activates GPCRs, whereas most glutamatergic synapses mediate fast synaptic transmission via ionotropic receptors.

It remains unclear how the proposed molecular targets of ziram could account for the differences I observe. Ziram has been shown to inhibit E1 ligase, the enzyme responsible for ubiquitin activation prior to protein conjugation (Chou et al., 2008). Ubiquitin conjugation to proteins can result in a myriad of downstream effects, including proteasomal degradation and regulation of protein function. Ubiquitination has been shown to play an important role in regulating the function of some synaptic proteins (DiAntonio and Hicke, 2004).

We speculate that the differences we observe between Type Ib and Type II boutons may represent general models for the behavior of aminergic versus non-aminergic cells. If so, it is possible that the differential effects of ziram on Type Ib vs Type II are relevant to the sensitivity of aminergic pathways to the pathophysiologic processes that cause PD. We suggest that both mammalian cells and the fly NMJ will be useful models to investigate the underlying mechanisms.

Conclusions

The main aim of my dissertation work was to examine gene-environment interactions implicated in Parkinson's disease (PD) using *Drosophila melanogaster,* commonly known as the fruit fly*,* as a model organism. Idiopathic PD is a complex disease of which the etiology is still poorly understood. Current models suggest that a variety of life-long environmental exposures, to potentially toxic agents such as pesticides, combine with an individual's genetic background to increase disease risk (Shlomo, 1996). Recent epidemiological studies have been able to identify particular pesticides and genetic variants that independently, or sometimes in combination, increase PD risk (Fitzmaurice et al., 2014; Gatto, 2010; Ritz, 2009).

Due to its short life span, the fly is a particularly attractive model for studying neurodegenerative diseases such as PD. The genetic tractability and low cost of the fly model allows for the study of gene-environment interactions or combinatorial toxicity in a manner that is not feasible in mammalian models. In addition, the model synapse of the fly neuromuscular junction (NMJ) allows for the study of the synaptic effects of genetic or chemical disruption at an intact synapse. Recent advances in live-imaging techniques allow for the real-time visual monitoring of neuronal activity and synaptic vesicle cycling at the fly NMJ. Through live-imaging and traditional toxicological approaches, my work has demonstrated the utility of the fruit fly for assessing the complex multi-variable gene-environment interactions implicated in PD.

The mechanisms behind the selective loss of dopamine (DA) neurons in PD are still not understood. The reactive potential of DA itself has received a great deal of focus, with the thought that increased levels of cytosolic DA can lead to cell death (Bisaglia et al., 2013). The vesicular monoamine transporter (VMAT) is responsible for packaging dopamine into synaptic

vesicles prior to release, and upregulation of VMAT has been shown to protect against toxin-induced DA cell loss in invertebrates and mammals alike (Glatt et al., 2006; Guillot and Miller, 2009; Lawal et al., 2010). VMAT regulation may also play a significant role in other diseases linked to disruption of aminergic circuits, such as depression, addiction, and schizophrenia (Wimalasena, 2011).

Our lab has developed *Drosophila* VMAT (DVMAT) mutants with alterations in putative trafficking domains in order to understand how DVMAT trafficking and localization is regulated *in vivo;* in particular, we have focused on a C-terminal deletion mutant (delta 3) that lacks multiple sorting motifs and a point mutant in a known tyrosine based trafficking motif (Y600A) (Grygoruk et al., 2010a). We developed a DVMAT construct tagged with a pH-sensitive form of GFP, or 'pHluorin' in both mutant and wild type DVMAT. The pH gradient between the acidic vesicle lumen and relatively basic synaptic cleft allows for visualization of the pHluorin construct during vesicle fusion and reuptake, or exo- and endocytosis, respectively.

While prior work in our lab focused on identifying the motifs responsible for DVMAT sorting and on characterizing the downstream behavioral consequences of mislocalization, my work focused on how alterations in trafficking motifs could alter DVMAT cycling at the synapse during vesicle exo- and endocytosis. We show here that the delta 3 mutant, but not the Y600A mutant, exhibits exocytic and endocytic defects at the canonical Type Ib glutamatergic synapse of the fly NMJ (work conducted by Dr. Audrey Chen). These data suggest that the Y600A motif is not needed for correct DVMAT exo- and endocytosis at Type Ib terminals, but that other motifs absent in the delta 3 mutant are. Little is known about the Type II octopaminergic nerve terminals where DVMAT is endogenously expressed. Type II terminals are morphologically and functionally distinct from Type Ib terminals, as Type II terminals contain more large dense core

vesicles (LDCVs) (which are involved in the extrasynaptic release of both neuropeptides and neuorotransmitters), while Type Ib terminals contain more synaptic vesicles (SVs) (which are located at the active zone and are thus responsible for the release of neurotransmitter into the synaptic cleft) (Jia et al., 1993).

We hypothesized that mutations in DVMAT trafficking domains may have different phenotypes at functionally distinct nerve terminals. Thus, I developed flies expressing the DVMAT pHluorin constructs at Type II octopaminergic terminals and assessed exo- and endocytosis in both wt, Y600A and delta 3 DVMAT. Interestingly, disruption of the DVMAT trafficking motifs in both the Y600A and delta 3 mutants at aminergic terminals does not result in an exocytic defect, contrary to what I observed with delta 3 at Type Ib. Additionally, both the Y600A and delta 3 mutants exhibit profound disruption of endocytosis at Type II terminals. We note also that the kinetics of wt VMAT endocytosis at Type Ib and Type II terminals is distinctly different, with endocytosis occurring slower at Type II terminals. Together these data suggest that the mechanisms regulating the synaptic cycling of VMAT at Type Ib and Type II terminals are distinct. Future work in the lab will focus on identifying differences in the molecular machinery regulating VMAT exo- and endocytosis at terminals with distinct vesicle populations.

Synaptic transmission is one of the defining feature of neurons, however, little data exists on how environmental toxins, such as pesticides, may affect this most basic and essential function. I took advantage of our VMAT-pHluorin construct to determine whether pesticides linked to PD could have selective toxicity at functionally distinct nerve terminals. I exposed the fly NMJ prep to ziram, a pesticide only recently linked to PD whose neurotoxic mechanism(s) of action are poorly understood, and examined its effect on exo- and endocytosis. Similar to our findings with VMAT trafficking mutants, I found different sensitivity of Type Ib and Type II

terminals to ziram exposure. Type Ib terminals had an increase exocytosis and a subtle slowing of endocytosis after ziram expsure, while Type II terminals exhibited no exocytic phenotype but a robust slowing of endocytosis.

I then used the genetically encoded calcium indicator, GCAMP, to determine if ziram's effects on exocytosis at Type Ib terminals were due to increased calcium influx. I did not observe an increase in calcium influx during exocytosis in exposed preps, nor did I find an increase in baseline calcium levels, suggesting that changes in calcium dynamics are not responsible for the observed increase in exocytosis. However, while conducting these recordings I made the serendipitous discovery that ziram exposure induced spontaneous calcium events *only* at Type II terminals. These events appeared to be action potentials, as they were coordinated across all boutons and sensitive to TTX. Ongoing investigations in the lab are examining the molecular mechanisms behind this phenomenon. Using genetic and chemical reagents we are taking a multi-prong approach, examining whether other dithiocarbamates, ubiquitin proteasome inhibitors, or various ion channel inhibitors can elicit or block these coordinated calcium events at Type II terminals.

In addition to these live-imaging approaches, I used traditional toxicological techniques to examine gene-environment interactions implicated in PD. I developed a paraquat and maneb induced model of PD in the fly. While paraquat and maneb models of PD exist in the rodent, I demonstrate the utility of the fly model for examining multiple gene-environment interactions in tandem and for building an understanding of the underlying mechanisms of action responsible for pesticide-linked PD. In particular, I show that paraquat and maneb exposure combined, but not individually, lead to DA cell loss that is only observable at the end of life, after long term exposures.

Epidemiological studies demonstrate the ability of ziram exposure to synergize with exposure to paraquat and maneb, leading to a further increase in PD risk (Wang et al., 2011). However, upon chronic exposure to ziram alone or in tandem with paraquat and maneb, I did not observe any increased DA cell loss in the fly at the doses and time points tested. This may be due to differences in the toxicodynamics properties, such as absorption or distribution, of ziram in the fly. Ziram has been shown to inhibit E1 ligase, a component of the ubiquitin proteasome system (UPS), and it has been suggested that it is this mechanism of action that is responsible for ziram's link to PD (Chou et al., 2008). I genetically mimicked ziram's inhibition of E1 using an RNAi developed in our lab and found that E1 knockdown synergized with maneb, but not paraquat, to cause significant DA cell loss. One downstream effect of E1 inhibition is inhibition of proteasome function. I genetically knocked down proteasome function but did not find any synergy with paraquat and/or maneb exposure on DA toxicity. These data suggest that other effects of E1 inhibition, besides proteasome inhibition, may be contributing factors to ziram's neurotoxicity. Ongoing research by other lab members has demonstrated the robustness of my paraquat and maneb model in the fly and suggests that this model can be used also to screen potential neuroprotective agents.

In conclusion, I show that the fly is a model organism that can be used to investigate a wide range of disease states, from the effects on synaptic function to whole organism effects on survival, motor function and cell loss.

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