A GENETIC AND NEUROCHEMICAL ANALYSIS OF DOPAMINE REGULATION IN
DROSOPHILA MELANOGASTER OF HUMAN NEURODEGENERATIVE DISORDERS
AND NEUROINFLAMMATION

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ABSTRACT

Dopamine, a critical neurotransmitter, regulates dozens of vital biological functions within the human brain and throughout the body, and therefore disruptions in dopamine signaling result in a wide range of behavioral, psychological and movement-related disorders. In this report, I evaluate the role of dopamine production and trafficking in two of the most common neurological movement disorders, Parkinson’s disease and Torsion Dystonia. As we age, a naturally occurring process known as neurodegeneration results in the gradual loss of neuron structure and function over time. Due to this compromised cellular integrity, neurons become considerably more susceptible to oxidative stress, mitochondrial damage, and protein misfolding and aggregation. In Parkinson’s disease and many other neurodegenerative disorders, aging remains the greatest factor contributing to disease onset. In both neurodegenerative disorders, such as Parkinson’s disease, and neurodevelopmental conditions, such as Dystonia, dopamine regulation is significantly altered, causing severe motor and mobility defects. Here, I use the model system Drosophila melanogaster to investigate genetic, environmental and dietary factors contributing to the disruption of dopamine signaling and transmission. I also present a novel method for the quantification of cellular signals related to neuroinflammation, a sophisticated immune cascade activated in the central nervous system in response to neuron stress or damage. Our work has not only proved robust evidence for the validation of our previously published results, but has also introduced novel genetic interactions and molecular pathway that strongly influence the development and progression of Parkinson’s disease and Dystonia pathology. More
broadly, these findings contribute new insight into the mechanistic networks contributing to the general understanding of dopamine regulation and disorders.
LIST OF ABBREVIATIONS AND SYMBOLS

BH4  Tetrahydrobiopterin or 6(R)L-erythro-5,6,7,8-tetrahydrobiopterin
Catsup  Catecholamines up
°C  Celsius
DA  Dopamine
DAT  Dopamine transporter
dDAT  Drosophila dopamine transporter
Dcp-1  Death caspase 1
DDC  DOPA decarboxylase
DOPAC  3,4-Dihydroxy-phenylacetic acid
dTH  Drosophila tyrosine hydroxylase
GFP  Green fluorescent protein
GTP  Guanosine triphosphate
GTPCH  GTP cyclohydrolase
HPLC  High pressure liquid chromatography
iNOS  Inducible nitric oxide synthase
IL  Interleukin
JNK  c-Jun NH2-terminal kinases
kDa  kilo Dalton
L-DOPA  3, 4-dihydroxy-L-phenylalanine
L-NAME  N-nitro-L-arginine methyl ester
MAO  Monoamine oxidase
MAPKs  Mitogen activated protein kinases
mg  Milligram
ml  Milliliter
mM  Millimolar
MPP+  1-methyl-4-phenylpyridinium
MPTP  1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
NADP  Nicotinamide Adenine Dinucleotide Phosphate
NF-κβ  Nuclear factor kappa beta
NH₂PPP  Dihydroneoperin triphosphate
nm  Nanometer
NO  Nitric oxide
NOS  Nitric oxide synthase
O₂⁻  Superoxide radicals
PBS  Phosphate buffered saline
PBT  Phosphate buffered saline with Tween 20
PCD  Programmed cell death
PD  Parkinson’s disease
PI3K  Phosphoinositide 3-kinase
PKB  Protein kinase B
PKC  Protein kinase C
ple  Pale
PQ  N',N'-dimethyl-4,4'-bipyridinium dichloride or Paraquat
Pu  Punch
repo  Reverse polarity
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<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
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<tr>
<td>SN</td>
<td>Substantia nigra</td>
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<tr>
<td>SNpc</td>
<td>Substantia nigra pars compacta</td>
</tr>
<tr>
<td>TDC</td>
<td>Tyrosine decarboxylase</td>
</tr>
<tr>
<td>TH</td>
<td>Tyrosine hydroxylase</td>
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<tr>
<td>VMAT</td>
<td>Vesicular monoamine transporter</td>
</tr>
<tr>
<td>VTA</td>
<td>Ventral tegmental area</td>
</tr>
<tr>
<td>mg</td>
<td>Microgram</td>
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<td>µl</td>
<td>Microliter</td>
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First and foremost, I’d like to thank my parents, Rifat and Sana Ajjuri. I owe them an immeasurable debt of gratitude for all of the years of selfless support and sacrifice that they have provided to be sure that I have all I need to be able to find my own path. Also, a special thanks to my siblings, Dana, Sammy and Hana, without whom none of this would have been possible. The unconditional and unwavering love and support from my family have made me who I am today and I could not be luckier than to have them on my side.

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In recognition of my committee—you have all shaped not only the development of my research but my personal development as well, as a scientist and as a young adult.

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“Lovers don’t finally meet somewhere; they are inside each other all along.” – Rumi.

I cannot imagine a path that did not lead me to her.

A huge thank you to Emily’s parents and family, to my friends, my loved ones and to all those who’ve shared in a moment along the way.
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CHAPTER ONE
AN INTRODUCTION INTO DOPAMINE REGULATION AND RELATED DISORDERS
IN HUMANS AND THEIR *DROSOPHILA* MODELS

DISSERTATION BACKGROUND AND OVERVIEW

*The broad impact of dopamine in the human body*

The regulation of the neurotransmitter dopamine (DA) is essential for a multitude of vital functions within the human brain and throughout the body. Commonly known for its role in pleasure and reward-seeking behaviors, DA production and regulation are widely studied in relation to understanding addictive behaviors. However, many other disorders have been linked to dysfunctions in DA homeostasis. These disorders include multiple psychological conditions, such as schizophrenia, depression, bipolar disorder, and attention deficit hyperactivity (ADHD), as well as movement disorders, most notably torsion dystonia and Parkinson’s disease (PD).

Dopaminergic neurons account for a relatively small portion of total neurons in the human brain-(around 400,000 (Schultz, 2007) out of 100 billion (Lange, 1975)), and are concentrated in the ventral tegmental area (VTA), nucleus accumbens, frontal cortex, substantia nigra, striatum and the posterior hypothalamus (Fig. 1.1). Within these regions, however, there are two largely distinct dopamine response pathways- one regulating reward sensations and another controlling body movement. In the first, DA production occurs in the VTA and upon stimulation is released into the prefrontal cortex and nucleus accumbens, giving a sensation of pleasure (Matsumoto and
Hikosaka, 2009). Disruptions in this pathway are associated with mood regulatory disorders, schizophrenia, and drug-seeking behaviors in addiction. In the second pathway, responsible for movement, dopamine is produced in the substantia nigra and then transmitted to the striatum. Defects in this network result in movement disorders, most notably the tremors and mobility deficiencies found in PD pathology (Christine and Aminoff, 2004). The dopaminergic neurons found in the hypothalamus releases DA as a neuroendocrine signaling molecule into the pituitary gland, and also playing a role in pubescent reproductive development in both males and females (Ben-Jonathan and Hnasko, 2001).

Figure 1.1. Regions of the human brain containing dopamine neurons. (figure credit: Okinawa Institute of Science and Technology Graduate University. “Unless otherwise noted, images are free for anyone to re-use, and should be credited to OIST.”)
Dopamine structure, biosynthesis and metabolism

**Figure 1.2. The structure of dopamine.** Dopamine, or IUPAC 4-(2-aminoethyl)benzene-1, 2-diol, is comprised of a catechol group, and an amine side chain. The molecular formula of DA is C₈H₁₁NO₂ and its molecular weight is 153.17844 g/mol.

In the catecholamine family, which also includes epinephrine (adrenaline) and norepinephrine, DA is a monoamine molecule consisting of a benzene ring containing two hydroxyl side groups (-OH) and an amine side chain (1-2). In humans, DA makes up around 50% of the total catecholamines in the body and acts as the precursor to both norepinephrine and epinephrine, in addition to having neuromodulating functions of its own (Benes, 2001). DA biosynthesis occurs when phenylalanine hydroxylase (PAH) with cofactor tetrahydrobiopterin (THB) converts L-Phenylalanine into L-Tyrosine. Following which, L-Tyrosine is converted into L-3,4-dihydroxyphenylalanine (L-DOPA) by Tyrosine Hydroxylase (TH) and THB. Lastly, L-DOPA is converted into DA by DOPA Decarboxylase (DDC), also called aromatic L-amino acid decarboxylase (AADC) (Fig. 1.3). Important to note here is that, due to its polar nature, DA is not lipid soluble and therefore unable to cross the Blood-Brain Barrier (BBB). However, L-
DOPA, the immediate precursor to DA, is able to pass through the BBB via its L-amino acid transporter (LAT)-mediated binding and transport across the membrane.

Figure 1.3. Summary of dopamine biosynthesis in *Drosophila melanogaster*. *Punch*, encoding for GTP cyclohydrolase, is required for the synthesis of tetrahydrobiopterin (BH₄) by converting guanosine triphosphate (GTP) to dihydronopterin triphosphate (H₂NPPP). BH₄ then acts as a necessary cofactor with tyrosine hydroxylase (TH) in the synthesis of the dopamine (DA) precursor, L-3,4-dihydroxyphenylalanine (L-DOPA). In the final step of DA synthesis, L-DOPA is converted into DA by the enzyme DOPA decarboxylase (DDC).

**Dopamine synaptic transmission and reuptake**

Due to the highly-reactive nature of DA (Graham et al., 1978), the tightly-regulated control of DA homeostasis is critical for healthy dopaminergic neuron health and function. Catsup, or Catecholamines-up, modulates the biosynthesis of DA through the negative regulation of two rate-limiting steps. First, Catsup inhibits GTP Cyclohydrolase, preventing the production of tetrahydrobiopterin (BH₄), which acts as a necessary cofactor for TH. Second, Catsup inhibits
TH activity directly, reducing the production of DA precursor L-DOPA (Fig. 1.3). When L-DOPA is converted to DA by DDC, DA is packaged in synaptic vesicles via Vesicular

![Figure 1.4. Cartoon of a presynaptic dopaminergic neuron.](image)

Once dopamine is produced, cytosolic dopamine is packaged into cellular vesicles via vesicular monoamine oxidase (VMAT), and these vesicles then fuse to the nuclear membrane and release DA into the synapse. The presence of unpackaged dopamine in the cytosol results in the metabolism of DA molecules by monoamine oxidase (MAO) into non-toxic metabolites such as DOPAC and toxic byproducts, such as hydrogen peroxide ($H_2O_2$), oxygen free radical ($O_2^-$) and DA-quinone. The production of these toxic metabolites results in cellular oxidative stress and are thought to contribute to neurodegeneration of DA neurons in PD pathology.

Monoamine Transporter (VMAT). Under normal conditions, a threshold dependent increase in cellular $[Ca^{2+}]$ triggers exocytosis of DA into the synaptic cleft (Mundorf et al., 1999; 2000),
where DA either binds to receptors on the post-synaptic neuron, reenters the pre-synaptic neuron via DAT, or undergoes degradation by synaptic enzymes or CNS immune cells known as glia.

However, when VMAT function is reduced or when dopamine is produced in excess, free cytosolic DA is oxidized by Monoamine Oxidase (MAO) into the stable metabolite 3,4-Dihydroxyphenylacetic acid (DOPAC), and the toxic by-products hydrogen peroxide (H$_2$O$_2$), oxygen free radicals and reactive DA-quinone (Fig. 1.4) (Kuhn et al., 2006; Miller et al., 1999; Pedrosa and Soares-da-Silva, 2002; Stokes et al., 2000). The accumulation of these toxic molecules result in cellular oxidative stress, disrupted DA regulation and, potentially, neuron death (Hastings et al., 1996; Spencer et al., 1998). This cascade of dopamine toxicity is thought to play a major role in the pathology of Parkinson’s disease, in which dopaminergic neurons are preferentially targeted and degraded. The exact mechanisms leading to the dysfunction of DA regulation or packaging remains largely unknown.

Neurodegeneration, Parkinson’s disease and neuroinflammation

Neurodegeneration is a naturally occurring process characterized by the gradual loss of neuron integrity and function over time. As we age, neurons become considerably more susceptible to oxidative stress, mitochondrial damage, and protein misfolding and aggregation. Disruptions in pathways related to aging, however, result in neurodegenerative conditions such as Alzheimer’s, Huntington’s and Parkinson’s Disease (PD). Although many varying genetic and environmental factors have been shown to contribute to the onset of these disorders, aging remains the greatest factor affecting the development and progression of neurodegenerative disorders. In PD, about 5-15% of cases are considered to be hereditary, while the causes of over 85% of PD cases remain
unknown. Furthermore, of the familial conditions, only around 30% can be confidently contributed to one or more of the six monogenic factors that have been discovered (Klein and Westenberger, 2012). While several environmental toxins, such as soil bacteria, or toxicants, such as the herbicide paraquat, have been identified to potentially contribute to or exacerbate PD pathology, the remaining ~95% of PD cases are thought to result from a combination of both unidentified genetic factors and/or exogenous triggers.

As dopaminergic neurons begin to degenerate in PD pathology, they release cellular signals that initiate a complex innate immune response known as neuroinflammation. Under basal conditions, this network of inducers, detectors and effectors maintain tissue health by rapidly responding to infection, insult or injury. When this response is triggered in mammalian brain due to a minor insult, such as cellular oxidative stress associated with normal aging, or major damage, such as neuron damage occurring from acute exposure to a chemical toxin, phagocytic surveillance cells known as microglia are activated and migrate to the site of injury. These scavenger-like immune cells, which are similar to macrophage in the periphery, then begin a sophisticated signaling cascade between themselves and the damaged neuron, as well as with astrocytes, another glial cell that acts as support for the typically robust microglial response. Once activated, the microglia begin a process of inflammation to stimulate the immune system and promote tissue repair [Fig. 1.5] (Glass et al., 2010).

However, under conditions of chronic insult as in neurodegenerative diseases like Parkinson’s disease (PD), Alzheimer’s disease and amyotrophic lateral sclerosis, inflammation becomes
chronic and can cause significant damage to healthy tissue as well. Recent studies have shown that this persistent, aggressive response by the immune system may in fact be leading to an over-

(Figure adapted from Glass, C. et al., (2010) and Julie Lotharius & Patrik Brundin (2002))

**Figure 1.5. The mammalian neuroinflammatory response network as understood in Parkinson’s disease.** As dopaminergic neurons are damaged, whether due to aging, introduction of toxic chemicals or protein aggregation (eg. alpha-synuclein misfolding), the neuron typically begins to secrete an apoptotic or necrotic signal which is then received by microglia, phagocytic immune cells that act as a surveillance system in the mammalian brain. After microglial activation, a signaling cascade occurs between the microglia and both the damaged neuron as well as support immune cells known as astrocytes. Together, these immune cells stimulate the apoptosis of the damaged neuron and then phagocytose the dying cell. (Glass et al., 2010; Lotharius and Brundin, 2002)
excitatory reaction that exacerbates the disease state rather than ameliorating the condition (Auvin et al., 2007; Cunningham et al., 2005; Iravani et al., 2002; Yong, 2010).

In Drosophila melanogaster, or fruit flies, this innate immune response is genetically well-conserved, but is not without some critical differences. First, although some researchers may disagree, flies are not credited with having an adaptive immune system, but maintain a sophisticated innate immune system. Secondly, without an organized blood vessel system, the immune response is mediated through the hemolymph, a circulatory network that acts as analogous to the blood and intestinal fluid systems found in mammals and other vertebrates. Thirdly, the Drosophila immune system does not have microglia, but does contain a class of phagocytes known as hemocytes.

Hemocytes have been previously identified as macrophage-like cells that migrate to the site of injury (Babcock et al., 2008; Wood et al., 2006), and undergo phagocytosis during septic shock (Vlisidou et al., 2009), introduction of a parasite (Foley, 2003) or viral infection (Costa et al., 2009). Like macrophage, phagocytic hemocytes express nitric oxide synthase (NOS) and produce NO, a critical signaling component of mammalian innate immunity (Carton et al., 2009; Foley et al., 2003). All of these studies, however, have only been able to demonstrate hemocyte responses during embryonic development and in larva.

That said, our lab has observed robust, hemocyte-mediated neuroinflammation in Drosophila adult brains (Daigle & Hall, et al., manuscript in preparation), a previously unknown phenomenon. Therefore, although the mechanisms and genetic factors associated this
neuroinflammatory response have not yet been clearly defined in *Drosophila*, with the use of the complex genetic tools available and the conservation of cellular and mechanistic properties of this immune network, this model has the capacity to serve as a promising utility for investigating the dynamic inflammation network triggered by neurological disease. The following chapters describe studies of the interactions of multiple genetic and environmental factors with the molecular pathways that modify dopamine biosynthesis and regulation.

(Figure adapted from Illingworth, www.mbm.leeds.ac.uk, 2007)

**Figure 1.6. Mechanism of nitric oxide production, a cell-signalling component of immune response.** L-Arginine serves as the immediate precursor in the nitric oxide synthase (NOS)-mediated production of NO. In this reaction, NADPH and 2 molecules of oxygen are required to produce one molecule of NO, with by-products NADP⁺, L-citrulline and two molecules of water. Tetrahydrobiopterin (BH₄) is acts as a necessary cofactor for NOS activity.

In Chapter Two, we explore the relationship between insulin signaling on Parkinson’s disease. The IGF-1/insulin-like signaling (IIS) pathway has been shown to dramatically affect lifespan
and longevity in multiple animal models and therefore may provide key insight into the pathology of age-dependent neurodegenerative diseases. In this study, we investigate two components of the IIS pathway in relation to a familial, genetic form of PD and assess the impact each has on dopamine production and homeostasis.

Next, in Chapter Three, we describe the development and validation of a novel assay to quantify the neuroinflammatory response triggered in *Drosophila* as a result of genetically or chemically-induced neuron stress. Neuroinflammation is widely studied in respect to neurodegeneration and oxidative damage, but the triggers, magnitude, duration and consequences of the inflammatory response warrant further and more thorough investigation. This assay allows for *Drosophila* researchers to implement a highly sensitive, reproducible and customizable approach to quantify the inflammatory response and elucidate specific inducers or inhibitors of that network in order to better understand its components and their effect on disease models.

In Chapter Four, we investigate the relationship between Catecholamines-up, a critical regulator of dopamine biosynthesis and synaptic transport, and alpha-synuclein (α-syn) A30P, a genetic mutation identified in PD familial studies. We have previously reported that knocking down Catsup expression is neuroprotective in *Drosophila* against the neurotoxic herbicide paraquat. In this study, we assess the consequence of knocking down Catsup protein levels on dopamine production, packaging and trafficking in flies expressing WT and mutant α-syn, and explore the directly and indirect effects of mutations in Catsup on VMAT function and α-syn- induced dopaminergic neurotoxicity.
Lastly, in Chapter Six, we describe the relationship between of GTP cyclohydrolase, a protein responsible for a rate-limiting step in the synthesis of dopamine, and TOR1A (DYT1), which causes the most common early-onset dystonia, a debilitating neuromuscular disorder. We model dystonia in *Drosophila*, which contain a torsin-related gene, *dtorsin*, to demonstrate the dominant-negative effects of human *torsinA ΔE* on locomotion, dopamine production and expression of GTPCH.

Individually, each chapter demonstrates novel understanding of dopamine regulatory components in the onset of or response to neurological diseases and disorders using *Drosophila melanogaster* as a model system. Together, they provide an in depth and multidimensional view of the intricate networks of genes responding to and modifying processes related to human disease in an intact nervous system.

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CHAPTER TWO

GLUCOSE METABOLISM AND INSULIN SIGNALING MODULATE α-SYNUCLEIN-
INDUCED DOPAMINERGIC NEUROTOXICITY IN DROSOPHILA MELANOGASTER

The following project was initiated as a collaboration between Adam Knight in the Caldwell Lab and Gavin Daigle in the O’Donnell lab during their master’s research. Following preliminary experiments in Drosophila done by Gavin Daigle concluding that mutations in both chico and pgi resulted in aberrant dopamine pools, the remainder of all Drosophila experiments were completed by Rami Ajjuri. This work includes: Fig. 2.1A-H, Fig. 2.5F, Fig 2.6A-H, and Suppl. Movies 1-3. All of the proceeding figures were prepared by Rami Ajjuri and Kim Caldwell. All methods, results and data analyses related to Drosophila experiments were written and edited by Rami Ajjuri and Janis O’Donnell.

ABSTRACT

Neurodegenerative diseases represent an increasing burden in our aging society, yet the underlying metabolic factors influencing onset and progression remain poorly defined. The relationship between impaired IGF-1/insulin-like signaling (IIS) and life-span extension represents an opportunity to investigate the interface of metabolism with age-associated neurodegeneration. Using data sets of established DAF-2/IIS-signaling components in Caenorhabditis elegans, we conducted systematic RNAi screens in worms to select for daf-2-associated genetic modifiers of α-synuclein misfolding and dopaminergic neurodegeneration, two clinical hallmarks of Parkinson’s disease. An outcome of this strategy was the identification of GPI-1/GPI, an enzyme in glucose metabolism, as a daf-2-regulated modifier that acts independent of the downstream cytoprotective transcription factor DAF-16/FOXO to modulate neuroprotection. Subsequent mechanistic analyses using Drosophila and mouse primary neuron cultures further validated the conserved nature of GPI neuroprotection from α-synuclein proteotoxicity. Collectively, these results support glucose metabolism as a conserved functional node at the intersection of proteostasis and neurodegeneration.

INTRODUCTION

Parkinson’s Disease (PD) is an age-dependent neurodegenerative disease characterized by the accumulation of α-synuclein (α-syn) within Lewy bodies and the selective loss of dopamine (DA) neurons in the substantia nigra. While human genetic and genomic studies have illuminated various contributors to disease pathology, aging remains the single most definitive risk factor for the development of PD (Amaducci and Tesco, 1994). Therefore, an unresolved issue is the underlying molecular relationship between genetic factors influencing aging-associated
metabolic changes and the loss of DA neurons associated with PD.

The IGF-1/insulin-like signaling (IIS) pathway has been implicated in human aging (Suh et al., 2008) and neurodegeneration (Cohen and Dillin, 2008). This pathway has been studied extensively in the nematode *Caenorhabditis elegans* (*C. elegans*), where mutation of DAF-2, the sole worm insulin/IGF-1 receptor, doubles lifespan and protects against a wide variety of cellular stressors. This effect is mediated through activation of DAF-16, the only FOXO/forkhead transcription factor (FOXO) in worms, which regulates the expression of numerous cytoprotective genes. In flies, reduced IIS via mutations in *chico*/insulin receptor substrate (IRS) extend lifespan and provide stress resistance (Clancy et al., 2001); phenotypes also are mediated by FOXO/ FKHR (Yamamoto and Tatar, 2011). Similarly, mutations in *Drosophila* insulin-like receptor, the homolog of DAF-2, also extend lifespan in adult flies (Tatar et al., 2001). While the IIS pathway is comparably simplified in worms and flies, it is important to note that this signaling cascade is nearly identical to that in mammals (Taguchi and White, 2008). This provides the exciting opportunity to utilize age-modified animals to enrich for gene candidates involved in neurodegenerative disease processes.

Among proteotoxicity models in worms, reduced IIS has demonstrated protection against amyloid-β (Aβ), polyglutamine-repeat (polyQ) proteins, and SOD-1 (Morley et al., 2002; Cohen et al., 2006; Boccitto et al., 2012). Similarly, expression of insulin-degrading enzyme in *Drosophila* suppresses Aβ neurotoxicity (Tsuda et al., 2010). We previously established worm models in which two pathological hallmarks of PD, α-syn- induced DA neurodegeneration (Cao et al., 2005) and toxicity (Tsuda et al., 2010). We previous can be visualized and assayed. Similarly, we have established a *Drosophila* model for DA neurodegeneration, which takes
advantage of its greater neuronal complexity to develop neurochemical and behavioral markers of PD-related degeneration (Chaudhuri et al., 2007). Here, we combine these established assay systems with the wealth of existing data on aging in these distinct models as a synergistic platform to uncover a relationship between metabolic change and neurodegeneration.

In this study, we report that reduced IIS suppresses α-syn toxicity and DA neurodegeneration in Drosophila and C. elegans. We performed a large-scale RNAi screen in C. elegans to reveal 60 distinct genetic modifiers associated with this protection. Subsequent bioinformatic and functional analyses of these candidates identified GPI-1/GPI, a key enzyme in glucose metabolism, as a potent modifier of α-syn misfolding and DA neurodegeneration in C. elegans. We also found that mutation of Pgi/GPI in Drosophila elevated a neuroinflammatory signal, disrupted DA homeostasis, and induced DA neurodegeneration. Lastly, this effect was translated to mammals, as Gpi1/GPI knockdown resulted in α-syn accumulation and neurotoxicity in mouse primary cortical neurons. These data from worms, flies, and mice collectively advance our understanding of proteotoxicity in the context of neurodegeneration, with glucose metabolism as a conserved unifying feature.

RESULTS

Reduced IIS Suppresses α-Syn-Induced DA Neurodegeneration in Drosophila

Although the IIS pathway is highly conserved in worms, flies, and mammals, a direct link between the IIS pathway and proteotoxicity or neurodegeneration has not previously been demonstrated in Drosophila. Chico/IRS mutations extend Drosophila lifespan and provide stress resistance (Clancy et al., 2001). To determine whether reduced IIS modulates α-syn toxicity in flies, we evaluated DA neurons in the CNS of α-syn- expressing adults heterozygous for a
mutant *chico* allele. Quantification of these neuron clusters at day 1 (24–48 hr post-eclosion) revealed no significant variations in the number of DA neurons for any genotype (Figure S1A available online). At day 20, *chico* mutants maintain wild-type (WT) levels of DA neurons in all clusters (Figures 2.1A–2.1C). In contrast, flies expressing human α-syn showed significant declines in neuron numbers in all DA neuron clusters. However, the presence of the *chico* mutation suppressed α-syn- induced DA neuron loss (Figures 2.1A–2.1E).

**IIS Mediates DA Homeostasis and Inflammatory Signaling in Drosophila**

To investigate the effect of *chico* on DA homeostasis, we quantified cellular DA levels and turnover. At day 1, α-syn flies showed no significant change in DA pools relative to the WT control (Figure S1B). Interestingly, *chico* mutant flies and *chico* mutant- α-syn flies exhibited elevated levels of DA when compared to either WT or α-syn flies alone (Figure S1B). By day 20, all genotypes except α-syn have experienced a slight, age-dependent reduction in DA (compare Figures 2.1F and S1B), while the slight, age-dependent reduction in DA (compare Figures (Figure S) *chico* mutation rescued the effect of α-syn on DA (Figure 2.1F).

We then quantified the ratio of DOPAC:DA, which we have shown to be elevated in *Drosophila* DA neurons prior to degeneration (Chaudhuri et al., 2007). Analysis of 1-day-old flies revealed no significant change in DA turnover in *chico* or *chico*; α-syn versus the control, but elevated DA turnover was evident in α -syn flies (Figure S1C). At 20 days post-eclosion, control flies had a slight increase in DA turnover associated with normal aging, while *chico* mutants remained virtually unchanged (Figure 2.1G). Thus, flies expressing α-syn exhibit elevated DA turnover, which is partially rescued in the *chico*; α-syn strain (Figure 2.1G).
We tested whether there was an accompanying inflammatory response associated with these phenotypes. Using a modified Griess reagent assay (Ajjuri and O’Donnell, 2013), we determined the levels of secreted nitrites, a stable metabolite of NO, as a measure of relative inflammatory response. At day 1, α-syn flies displayed an increase in NO levels, while chico and chico; α-syn showed little variation from controls (Figure S1D). A strong increase in NO levels was observed in α-syn flies after 20 days of aging, which was significantly reduced in chico; α-syn transgenic flies (Figure 2.1H). Taken together, these findings suggest the Drosophila IIS pathway can influence α-syn -induced DA neurotoxicity.

**IIS Pathway Modulates α-Syn-Induced DA Neurodegeneration in C. elegans**

Next, we investigated whether the link between the IIS pathway and α-syn toxicity represents an evolutionarily conserved mechanism that may be applicable toward understanding PD. We performed genetic crosses to generate daf-2 mutant worms that overexpress human α-syn and GFP in DA neurons. At day 7, we found that only 15% of α-syn worms displayed the WT complement of six anterior DA neurons (Figures 2.2A and 2.2D) compared to worms expressing GFP alone, which have 100% WT DA neurons (Figure 2.2C) (Hamamichi et al., 2008). Strikingly, 40% of daf-2; α-syn worms exhibited complete retention of DA neurons (Figures 2.2A and 2.2E). A well-characterized downstream component of the IIS pathway is DAF-16/FOXO, which functions as a key regulator of numerous cytoprotective genes (Murphy et al., 2003). We examined daf-16 mutants that overexpress α-syn + GFP in DA neurons. As predicted, daf-16 mutation enhanced neurodegeneration (Figure 2.2A). Surprisingly, we observed an intermediate level of neuroprotection in daf-2; daf-16 double mutants overexpressing α-syn + GFP in DA neurons (Figure 2.2A). We verified that the genetic markers (unc-75 and dpy-1) used
in these mutants did not affect the DA neurodegeneration phenotype by examining the balancers alone (Figure 2.2A) and by crossing α-syn into the same daf-2 and daf-16 alleles balanced with different genetic markers (Figure S2A). Our findings that daf-2 mutation dramatically suppressed
Figure 2.1. *chico* Mutation Protects against Age-Dependent \( \alpha \)-syn Toxicity in DA Neurons

DA neurons in the brains of 20-day-old adult males were visualized by expressing UAS-GFP tyrosine hydroxylase (TH)-Gal4.

(A) DA neurons were counted in each of the following DA subtype regions: anterior PAL (protocerebral anterolateral); posterior protocerebral posterior medial 1 (PPM1), PPM2, and PPM3; and posterior PPL1 and PPL2 (protocerebral posterolateral). Values are averages of 15 brains per genotype.

(B–E) Representative images of brains used for neuron counts with insets of the PPL1 region.

(F and G) DA and DOPAC pools in 20-day-old male heads. Values are averages of assays from three independent head extractions and three technical replicas for each extract, per genotype. Pools are quantified as ng per fly head.

(H) Nitric oxide synthase activity in brains of 20- day-old males was measured using a modified Griess Reagent assay. Results are displayed as mM concentration of nitrites in incubation medium. Values are averages of three replications of 20 fly brains per replication and three technical replicas per genotype. Genotypes tested were as follows: TH > GFP (UAS-GFP/+; TH-GAL4/+), chico (UAS-GFP/chico; TH-GAL4/+), \( \alpha \)-syn (UAS-GFP/+; TH- GAL4/UAS-\( \alpha \)-syn), and chico; \( \alpha \)-syn (UAS-GFP/ chico; TH-GAL4/UAS- \( \alpha \)-syn). All values compared to TH > GFP controls unless otherwise indicated. \( *p < 0.05; **p < 0.01; ***p < 0.001; \) one-way ANOVA with a Dunnett’s post hoc test. Error bars indicate ±SEM.

neurodegeneration, *daf-16* mutants enhanced neurodegeneration, and *daf-2; daf-16* double mutants suppressed neurodegeneration (although not to the same extent as *daf-2* mutants) indicate that DAF-16 is not the only genetic component responsible for DA neuroprotection. Additional pathways downstream of DAF-2 must contribute to neuroprotection.

**DA Neurodegeneration at Biological versus Chronological Aging in Worms**

Associated with *daf-2* mutant lifespan extension is a delayed rate of aging. We questioned whether *daf-2*-mediated neuroprotection at day 7 (chronological aging) might result from slower development than WT (N2) control animals. We performed lifespan assays to determine biological aging (mean lifespan) and found that it was 20 days for WT; \( \alpha \)-syn worms while it
was 40 days for *daf*-2; α-syn worms (data not shown). Interestingly, we found that *daf*-2 was no longer neuroprotective at biological aging (Figures 2.2B and S2B). These findings, together with our results demonstrating IIS-mediated neuroprotection at chronological aging in worms (Figures 2.2A and S2A) and flies (Figure 2.1), suggest that the metabolic changes associated with reduced IIS are responsible for protection against α-syn proteotoxicity and DA neurodegeneration.

**RNAi Screen for IIS-Mediated Protection against α-syn Misfolding**

We next sought to identify the genetic factors responsible for this IIS pathway-mediated protection against α-syn by generating a *daf*-2 strain that overexpresses α-syn::GFP in muscle cells. Misfolded α-syn::GFP fusion protein accumulated in the cytoplasm of N2 worms (Figure 2.2F), while in *daf*-2 mutants the fusion protein was almost undetectable (Figure 2.2G). We reasoned that *daf*-2 mutation likely promoted degradation of the fusion protein rather than affecting the α-syn::GFP expression level (Figures 2.2J and 2.2K). We then knocked down *daf*-16 and *hsp*-16.11 (positive controls) via RNAi in *daf*-2; α-syn::GFP worms and discovered that GFP inclusions reproducibly returned in these animals (Figures 2.2H and 2.2I).

To conduct the RNAi screen, we compiled a list of candidate genes and/or proteins with clear human orthologs that are upregulated in the *daf*-2 background (Murphy et al., 2003; McElwee et al., 2004; Halaschek-Wiener et al., 2005; Dong et al., 2007) and genes that mediate *daf*-2 lifespan extension (Samuelson et al., 2007). We included genes upregulated upon pan-neuronal overexpression of α-syn (Vartiainen et al., 2006) and previously identified genetic modifiers of α-syn misfolding and toxicity in worms (Hamamichi et al., 2008; Kuwahara et al., 2008; van Ham et al., 2008). In total, we assayed 625 targets in triplicate and identified 60 genes that, when knocked down by RNAi, reproducibly enhanced α-syn::GFP accumulation in the *daf*-2 mutant
Figure 2.2. IIS Pathway Modulates α-Syn Aggregation and α-Syn -Induced DA Neurodegeneration in C. elegans

(A–E) IIS pathway modulates α-syn-induced DA neurodegeneration.

(A) IIS pathway modulates α-syn-induced DA neurodegeneration at chronological aging (day 7) in worms. daf-2 mutation dramatically suppressed α-syn -induced DA neurodegeneration while the daf-16 mutation enhanced α-syn- induced DA neurodegeneration. Notably, the daf-2; daf-16 double mutation moderately suppressed α-syn-induced DA neurodegeneration at chronological aging. White bars indicate controls of the genetics background of the normal (N2), daf-2, and daf-16 mutations.

(B) The daf-2 mutation did not suppress α-syn- induced DA neurodegeneration at biological aging (mean lifespan: day 20 for WT and day 40 for daf-2 mutants). Three independent trials were performed (n = 90 total), and positives were considered significant if p < 0.05 via Student’s t test.

(C–E) Representative images of DA neurons in Pdat-1::GFP (C), Pdat-1::GFP + Pdat-1::tFP + (D), and Pdat-1::GFP + Pdat-1::tFP + PPdaf-2; dpy-1 mutant (E). Arrowheads denote normal neurons; arrows denote degenerated neurons.

(F–L) IIS pathway modulates α-syn aggregation. (F) In the WT background, α-syn::GFP accumulates in the cytoplasm of muscle cells. (G) In the daf-2 mutant background, the α-syn::GFP fusion protein is completely degraded. This daf-2 mutant + α-syn::GFP strain was used in an RNAi screen. (H and I) RNAi knockdown of daf-16 and hsp-16.11 resulted in a return of α-syn::GFP aggregates in the daf-2 background. (J) Semi-quantitative RT-PCR demonstrates that daf-2 mutation and RNAi do not affect mRNA level of α-syn::GFP. (K) Western blot confirms the degradation of the fusion protein in the daf-2 mutant background. (L) Summary of the 60 positive candidates that, when knocked down by RNAi in a daf-2 mutant + α-syn::GFP strain, reproducibly enhanced α-syn::GFP accumulation. Positive candidates were categorized using KOG and/or GO annotations. Error bars indicate ±SD.

Examination of IIS-Mediated α-Syn Modifiers in Additional Proteostasis Models

We next determined whether the 60 candidates were specific to α-syn misfolding or are more general effectors of proteostasis. A previous study identified genetic modifiers of polyQ background (Tables S1 and S2; Figure 2.3A). These positive hits were categorized into functional groups using KOG and GO annotations (Figure 2.2L; Table S2).
aggregation (Nollen et al., 2004), but none of those reported were identified in the daf-2 background. We generated daf-2 worms that overexpress Q82::GFP in muscle cells and, consistent with a previous report (Morley et al., 2002), found that reduced IIS suppressed polyQ aggregation (Figures 2.3B and 2.3C). We then performed RNAi of the 60 candidates to identify genes that, when knocked down in the daf-2 + Q82::GFP background, enhanced polyQ aggregation (Figures 2.3A–2.3E). We discovered that 11/60 modifiers of α-syn misfolding also affected polyQ aggregation (Figure 2.3F; Table S2).

We further investigated whether any of the 60 candidates modified amyloid-beta (Aβ) peptide toxicity. To evaluate these targets, an established worm model that utilizes temperature-sensitive induction of Aβ-induced paralysis (Dostal and Link, 2010) was employed. We found that 8/60 targets enhanced Aβ-induced paralysis (Figures S3A and S3B; Table S2).

In total, we found that 18/60 α-syn effectors also modified polyQ or Aβ misfolding; one gene (Y45F10B.9, an uncharacterized zinc-finger protein) impacted misfolding in all three models. Thus, many of these genes appear to be general age-associated modifiers of proteostasis that may affect susceptibility to a variety of protein misfolding diseases.

**The Majority of α-Syn Modifiers Act Independent of DAF-16/FOXO**

Since DAF-16 is a key regulator of numerous cytoprotective genes and was found to mediate α-syn misfolding (Figure 2.2H) and DA neurodegeneration (Figure 2.2A), we investigated whether the 60 identified effectors were DAF-16 dependent. We performed genetic crosses to generate daf-2; DAF-16::GFP worms to visualize the distribution of DAF-16 within cells. Consistent with previous reports (Lin et al., 2001), we observed predominantly nuclear localization of DAF-16 in
untreated and empty vector (EV) control RNAi-fed animals and increased cytoplasmic
distribution of DAF-16 in daf-18 RNAi-fed animals, in accordance with daf-18 negative
regulation of DAF-16 activity (Figure 2.3G–2.3I). We performed RNAi against each of the 60
targets and examined the knockdown effect on DAF-16 localization. Surprisingly, we found that
only 11/60 candidates altered the distribution of DAF-16 within worms, including 3/17
metabolism-categorized candidates (Figures 2.3A and 3J; Table S2). For example, a metabolic
candidate, gpi-1, when knocked down, did not have an overt effect on DAF-16::GFP localization
in the daf-2 background (Figure 2.3I; Table S2). Thus, while some candidates may function as
DAF-16-dependent effectors of α-syn misfolding, the majority of these modifiers appear to
modulate proteotoxicity through distinct mechanisms.

In order to further define mechanistic pathways that may be enriched in our screens, we analyzed
our 60 – modifiers using IPA, which relies on the Ingenuity Pathway Knowledge Base. Table S3
shows the five most significant functional networks of α-syn modifiers identified by IPA
analysis. The leading network included functional categories of energy production and nucleic
acid metabolism. We further investigated the most significant functional network by visualizing
its interaction with daf-2/IGFR and daf-16/FOXO (Figure S4). We found that 21 proteins from
our initial 60 α-syn modifiers interact within this network, revealing an important role for
metabolism in age-associated α-syn toxicity. We hypothesize this subset of functional modifiers
mediate metabolic changes connected to PD.

**Examination of IIS-Mediated α-syn Modifiers in C. elegans DA Neurons**

Next we assessed whether our 60 candidate genes modify α-syn- induced DA neurodegeneration
independent of the daf-2 mutation. Our reasoning was 2-fold: (1) most candidates were not daf-
dependent, and (2) as described in Figure 2.2A, \( daf-2; daf-16 \) double mutants only decrease \( \alpha\)-syn- induced DA neurodegeneration by 13%, compared to a 30% decrease in the WT background, suggesting there are additional pathways impacting neuroprotection in parallel to DAF-16. We utilized a worm strain that enables DA neuron-selective RNAi with transgenic animals expressing \( \alpha\)-syn (and GFP) in DA neurons to knock down the 60 IIS-related candidates (Harrington et al., 2012). We found that 30/60 candidates upon RNAi treatment significantly enhanced DA neurodegeneration (Figure 2.4A–2.4E; Table S2). Interestingly, 10/30 positives were involved in the energy production and metabolism IPA network (Figure S3), including glucose-6-phosphate isomerase (\( gpi-1/GPI \)), a key enzyme in glycolysis. Interestingly, when GPI-1 is secreted by cancer cells, it can also serve as a cytokine to activate autocrine motility factor (AMF) signaling (Haga et al., 2000). It was therefore significant that the receptor for AMF (\( hrdl-1/AMFR \)) was also identified in this screen. Accordingly, both \( gpi-1 \) and \( hrdl-1 \) RNAi enhanced \( \alpha\)-syn- induced DA neurotoxicity (Figures 2.2L and 2.4A–2.4D; Table S2). Conversely, overexpression of \( gpi-1 \) and \( hrdl-1 \) in DA neurons resulted in significant protection from \( \alpha\)-syn- induced neurotoxicity (Figures 2.4F–2.4I).

**GPI-1 Protects DA Neurons in Parallel to DAF-16**

To investigate the mechanism for GPI-1-mediated neuroprotection, we performed combinatorial RNAi against \( daf-2 + gpi-1 \) and \( daf-2 + hrdl-1 \) to determine whether \( daf-2 \)-mediated neuroprotection is dependent on these gene products. Combinatorial RNAi eliminated the neuroprotection phenotype conferred by \( daf-2 \) RNAi (Figure 2.5A). Notably, neuroprotection decreased 79% in \( daf-2; gpi-1 \) double RNAi versus 30% in \( daf-2; daf-16 \) double mutants (Figures 2.5A versus 2.2A), suggesting the cellular events involving \( gpi-1 \) provide greater protection than the DAF-16 pathway. We also found that combinatorial RNAi against \( daf-16 + gpi-1 \) enhances DA
neurodegeneration compared to gpi-1 RNAi alone (Figure 2.5B), providing further evidence that gpi-1 is functioning independently of daf-16. We validated this result with quantitative RT-PCR and found that gpi-1 RNAi did not significantly impact the expression level of two known endogenous DAF-16 transcriptional targets, sod-3 and mtl-1 (Robida-Stubbs et al., 2012). Similar results were observed in the WT genetic background (Figure 2.5C).

**GPI-1 Influences Protein Homeostasis through Glycolysis**

We tested whether GPI-1 could regulate the folding of endogenous metastable proteins by using worm strains carrying temperature-sensitive (ts) mutations unc-15(e1402) and unc-52(e669su250), encoding paramyosin and perlecan, respectively. At permissive temperature, these ts metastable proteins fold and function properly, while at elevated temperature the proteins misfold and induce motility defects (Gidalevitz et al., 2006). Figure 2.5D shows that gpi-1 RNAi at permissive temperature significantly (p < 0.01) reduced the motility of young adult unc-15 and unc-52 worms by 46% and 37%, respectively, suggesting a role for GPI-1 in maintaining global proteostasis.

To study the underlying mechanism impacting proteostasis, we inquired whether GPI-1 neuroprotection involved glycolysis, since this enzyme is involved in its initial steps. Treatment with 2-deoxyglucose (DOG), a glucose analog that blocks glycolysis, enhanced α-syn -induced DA neurodegeneration in worms (Figure 2.5E) and α-syn- induced mobility defects in flies (Figure 2.5F). Importantly, combinatorial treatment of worms with DOG + gpi-1 RNAi did not enhance α-syn- induced DA neurodegeneration compared to gpi-1 RNAi alone (Figure 2.5E). In addition, excess glucose suppressed α-syn -induced mobility defects in Drosophila (Figure 2.5F; Movies S1, S2, and S3). Lastly, we quantified ATP levels in worms treated with gpi-1 RNAi and found these were unaffected (Figure S5), implying certain glycolytic metabolites may be
Figure 2.3. Secondary Screening of Positive Candidates

(A) Summary of secondary RNAi screens.

(B) In the WT background, Q82::GFP accumulates in the cytoplasm of muscle cells.

(C) Q82::GFP aggregation is suppressed in daf-2 mutants, with approximately 9 ± 2 aggregates per worm.

(D and E) RNAi knockdown of daf-16 or hsf-1 in daf-2 mutant background caused a return of the formation of Q82::GFP aggregates, with averages of 18 ± 1 and 23 ± 6 aggregates per worm, on average. We used the daf-2 + Q82::GFP worms (C) to screen for modifiers of polyglutamine aggregation.

(F) Summary of the candidates that, when knocked down in daf-2 mutant background, significantly enhanced polyQ aggregation. Two independent trials (n = 40 total) were performed, and positives were determined as significant if *p < 0.05; one-way ANOVA with Dunnett post hoc test.

(G) In daf-2 mutants treated with EV control RNAi, DAF-16::GFP is localized to the nucleus (arrow- heads).

(H) In daf-2 mutants treated with daf-18 RNAi. DAF-16::GFP is distributed evenly throughout the cytoplasm.

(I) Graph showing quantification of DAF-16::GFP localization in worms. Localization was scored as “nuclear” (light gray), “cytoplasmic” (black), or “both” nuclear and cytoplasmic (dark gray). In daf-2 mutants fed EV RNAi, DAF-16::GFP is predominantly localized to the nucleus. In daf-2 mutants exposed to the RNAi clone targeting daf-18 there is increased cytoplasmic distribution of DAF-16::GFP. We performed RNAi against all 60 positive candidates in daf-2; DAF-16::GFP worms and examined the effect of candidate knockdown on DAF-16 localization versus the DAF-16::GFP pattern obtained with mock, or EV, RNAi knockdown. One candidate from our screen, gpi-1 (RNAi), is displayed in this graph.

(J) Summary of candidates that, when knocked down, altered DAF-16::GFP localization. Two in- dependent trials (n = 40 total) were performed, and positives were determined as significant if p < 0.05 via chi-square test.

responsible neuroprotection. Along with prior studies (Schulz et al., 2007; Tauffenberger et al., 2012), these data provide strong evidence that GPI-1 mediates proteotoxicity via glycolysis.
Figure 2.4. IIS-Associated Modifiers of α-Syn Misfolding Also Affect α-syn -Induced DA Neurodegeneration

(A) Graph showing percentage of 6-day-old worms with normal DA neurons for two RNAi targeting controls (EV and *arg-7*) and two positive candidates from RNAi screen (*gpi-1* and *hrdl-1*). RNAi targeting each of the 60 candidates was performed in *sid-1* mutant worms overexpressing *Punc-119::sid-1 + Pdat-1::GFP + Pdat-1::α-syn*.

(B–D) Representative images of *Punc-119::SID-1 + Pdat-1::GFP + Pdat-1::α-syn* worms treated with EV RNAi (B) *hrdl-1* RNAi (C), and *gpi-1* RNAi (D). Arrowheads show intact DA neuron cell bodies. Arrows indicate areas where DA neurons have degenerated.

(E) Summary of candidates that, when knocked down, enhanced DA neurodegeneration in transgenic worms expressing *Punc-119::sid-1 + Pdat-1::GFP + Pdat-1::α-syn*.

(F) Graph showing the percentage of 7-day-old worms with normal DA neurons for *Pdat-1::GFP + Pdat-1::α-syn* worms overexpressing either *hrdl-1* or *gpi-1*.

(G–I) Representative images of *Pdat-1::GFP + Pdat-1::α-syn* worms (G), *Pdat-1::GFP + Pdat-1::α-syn + Pdat-1::hrdl-1* worms (H), and *Pdat-1::GFP + Pdat-1::α-syn + Pdat-1::gpi-1* (I).*p < 0.05, one-way ANOVA with Dunnett post hoc test. Error bars indicate ±SD.
Figure 2.5. GPI-1 Mechanistic Analyses

(A and B) Graphs depicting percentage of 6-day-old worms with normal neurons following combinatorial RNAi of daf-2 + EV control, gpi-1, or hrdl-1 (A) and combinatorial RNAi of daf-16 + EV control or gpi-1 (B) in Punc-119::sid-1 + Pdat-1::GFP + Pdat-1::a-syn C. elegans. *p < 0.05; **p < 0.01, one-way ANOVA with Dunnett post hoc test. (C) No induction of endogenous DAF-16 target genes in response to gpi-1 RNAi. Quantitative RT-PCR was performed with young adult daf-2 mutant or N2 WT worms expressing Punc-54::a-syn::GFP, treated with EV control RNAi, gpi-1 RNAi, or daf-16 RNAi (positive control). For each daf-16 target gene, its mRNA levels were normalized to the expression in worms treated with EV control RNAi. Values are means ±SD (n = 3 independent biological samples with 100 worms in each). P value was calculated by nonparametric one-way ANOVA. NS, not significant, compared with worms treated with EV control RNAi.

(D) gpi-1 RNAi at permissive temperature (16°C throughout the experiment) significantly reduced the motility of young adult unc-15(e1402) and unc-52(e669su250) worms. Animals were exposed to EV or gpi-1 RNAi from embryos. The movement of young adult worms were recorded and analyzed by WormLab3.0 with the crawling mode. Values are means ±SD (n = 3 independent experiments with 15–20 worms in each). **p < 0.01, unpaired Student’s two tailed t test with Welch correction.

(E) Graph showing percentage of 4-day-old worms with normal DA neurons. Punc-119::sid-1 + Pdat-1::GFP + Pdat-1::a-syn worms fed EV control RNAi or gpi-1 RNAi were treated with 0 mM, 5 mM, and 10 mM 2-DOG and then analyzed 24 hr after exposure. **p < 0.01, one-way ANOVA with Dunnett post hoc test. For (A)–(E), error bars indicate ±SD. (F) Mobility of a-syn expressing flies aged to 10 days and then fed 200 mM DOG or 1 M glucose for 5 days. *p < 0.05; **p < 0.01, one-way ANOVA with Bonferroni post hoc test. Error bars indicate ±SEM.
Pgi/GPI Mutation Induces DA Neurodegeneration in Drosophila

We sought to investigate whether a relationship between glucose metabolism, proteostasis, and neurodegeneration is conserved in other species. To this end, we first examined the link between IIS and the gpi-1 fly ortholog pgi. While heterozygous chico mutants had no deficiency in climbing behavior, heterozygous pgi mutants displayed a significant reduction in mobility relative to WT flies (Figure 2.6A). The presence of the mutant allele of chico in double heterozygotes was unable to rescue the climbing deficits of heterozygous pgi mutants. This result suggests that pgi acts downstream of chico or that the two genes function in genetically separate pathways.

Next we examined whether pgi modulates α-syn-induced DA neurodegeneration. A heterozygous pgi mutation was introduced into flies expressing α-syn and GFP in DA neurons, under the control of a TH-GAL4 driver. To investigate the effect of pgi on α-syn toxicity, we compared DA neuron numbers for the WT and heterozygous pgi mutant genotypes in GFP-expressing neurons, in the absence and presence of α-syn. No significant changes were observed DA neuron numbers for any genotype at day 1 post-eclosion (Figure S6A). However, we found a significant decline at day 20 in DA neuron numbers in pgi heterozygous mutant brains in the absence of α-syn (Figures 2.6B–2.6D). Similarly, α-syn, pgi\textsuperscript{WT} flies exhibited significant degeneration of DA neurons (Figure 2.6B). The combination of the pgi mutant allele with α-syn significantly reduced neuron numbers further in nearly all DA neuron clusters (Figures 2.6B–2.6D).
Figure 2.6. Pgi/GPI Mutation Enhances α-Syn-Induced DA Neuron Loss

(A) Mobility assays of heterozygous chico and pgi mutant alleles, individually and in combination, relative to WT flies. (B–D) DA neurons in the brains of 20-day-old adult males, visualized by TH-Gal4 driven GFP. (B) Neurons were counted in anterior and posterior DA neuron clusters. Values are averages of 15 brains per genotype. (C and D) Representative images of brains used for neuron counts with insets of the PPL1 region. (E and F) DA and DOPAC pools were measured in heads of 20-day-old male flies. Assay values were determined using three independent head extracts and three technical replicates for each genotype and calculated as ng per fly head. (G) Nitric oxide synthase activity in brains of 20-day-old males was measured using a modified Griess Reagent assay. Results are displayed as mM concentration of nitrates in incubation medium. Values are averages of three replications of 20 fly brains per replication and three technical replicates per genotype. Genotypes for strains tested are as follows: TH > GFP (UAS-GFP/+; TH-GAL4/+), Pgi (UAS-GFP/Pgi; TH-GAL4/+), α-syn (UAS-GFP/+; TH-GAL4/UAS-α-syn), and Pgi; α-syn (UAS-GFP/Pgi; TH-GAL4/UAS-α-syn). These assays were performed simultaneously with those in Figure 2.1 and employed the same TH>GFP controls. *p < 0.05; **p < 0.01; ***p < 0.001; one-way ANOVA followed by Dunnett’s post hoc test analysis. Error bars indicate ±SEM. (H) Western blot reveals that levels of the apoptosis factor death caspase-1 (DCP-1) in 20-day-old adult male heads was elevated 1.6-fold in the pgi mutant flies and reduced slightly in chico alone (0.83:1). Expression of chico and pgi together only slightly ameliorated DCP-1 protein expression relative to WT (1.23:1).
**PgI/GPI Mutation Exacerbates the Effects of α-syn on DA Metabolism and Nitric Oxide Production in Drosophila**

Since glucose restriction increases oxidative stress in worms (Schulz et al., 2007), we investigated whether the *pgi* mutation would result in cytosolic accumulation and oxidation of DA in fly brains. HPLC analysis of DA and DOPAC levels in fly heads revealed that even at day 1 post-eclosion *pgi* mutant flies possessed significantly lower DA levels (Figure S6B) and dramatically elevated DOPAC:DA ratios (Figure S6C). While α-syn alone did not result in significant diminution of DA pools or elevated DA turnover at day 1, it enhanced the effects of the *pgi* mutant allele on both DA loss and DOPAC production (Figures S6B and S6C). After aging to 20 days, DA turnover in *pgi* mutants increased nearly 2.5-fold (Figure 2.6F). At this age, α-syn flies displayed significantly reduced DA, further loss in combination with the *pgi* mutation, and an enhanced DOPAC:DA ratio (Figures 2.6E and 2.6F). These findings reveal a strong age-associated effect of *pgi* on DA regulation.

We assayed NO production in heterozygous *pgi* mutants alone and in the α-syn background. Both *pgi* mutants and α-syn-expressing flies individually displayed strong inflammatory responses as measured by NO production, and in combination, nearly a 4-fold increase in NO relative to control flies was observed at day 1 and day 20 post-eclosion (Figures S6D and 6G). Consistent with these data, levels of the apoptosis factor DCP-1, encoded by *death caspase-1*, were significantly elevated in *pgi* mutant flies (Figure 2.6H). While *chico* alone showed a minor decrease in DCP-1 levels, compared to WT flies, expressing both *chico* and *pgi* together only slightly ameliorated DCP-1 protein expression triggered by the mutant *pgi* allele alone. In summary, α-syn expression and *pgi* mutation individually cause deleterious effects on DA neurons, and the combination of these two modifications is synergistic in their damage to the
adult *Drosophila* brain.

**Knockdown of Gpi1/GPI in Mouse Primary Cortical Neurons**

To determine if the effect of *gpi-1/pgi* knockdown translated to a mammalian system, mouse neuronal cortical cultures were transduced with lentiviral constructs expressing a short hairpin RNA (shRNA) sequence targeted against mouse *Gpi1*. Infection of neurons at a multiplicity of infection (MOI) of 1 resulted in over 90% knockdown of GPI1 protein, compared to cells infected with a scrambled shRNA sequence (scr), when assessed at 7 days post-infection (dpi) (Figure 2.7A). Neurotoxicity was determined by immunofluorescence analysis of neurofilament protein, a sensitive assay that measures the degeneration of neurites. Infection with lenti-*Gpi1*-shRNA at MOI 0.5 resulted in significant neurotoxicity compared to both non-transduced (N.T.) and scrb-infected neurons, when assessed at both 7 and 9 dpi (Figure 2.7B). Increasing the viral titer to an MOI of 1 further enhanced the neurotoxic effect (Figure 2.7C).

We subsequently characterized the effect of *Gpi1* knockdown on α-syn solubility in neuronal cultures by sequential biochemical extraction. Neurons from *Gpi1*-shRNA-transduced neurons were sequentially extracted in 1% Triton X-100, 2% SDS, then 70% formic acid (FA), and compared to extracts from scrb-shRNA-infected neurons. Western blot analysis revealed that *Gpi1* knockdown significantly reduced the levels of Triton X-100-soluble monomeric α-syn migrating at 18kDa by 50%, while increasing the levels of soluble high molecular weight (HMW) oligomers by 3-fold (Figures 2.7D–2.7H). Additionally, *Gpi1* knockdown appeared to cause a dramatic accumulation of Triton X-100 insoluble α-syn (~5 fold increase) extracted in SDS and FA-counting fractions (Figures 2.7D–2.7F and 2.7I). The solubility changes of α-syn were confirmed with two antibodies that were generated against unmodified α-syn (syn 202 and
SNL-1), as well mAb syn 505, which was generated against oxidized/nitrated α-syn (Duda et al., 2002) and recognizes cross-linked species of the protein; thus, syn 505 preferentially detects misfolded forms of α-syn (Waxman et al., 2008). These results indicate that GPI1 depletion results in neurotoxicity and concomitantly causes the accumulation of soluble α-syn oligomers and insoluble species.

DISCUSSION

The wealth of information that has accumulated from decades of elegant research in model systems has led to lists of genetic factors influencing lifespan and healthspan (Kenyon, 2010; Gems and Partridge, 2013). Likewise, human genetic studies have also generated an expanding catalog of heritable and candidate susceptibility factors for PD. We set out to better define the molecular intersection of aging and PD in the context of established genetic modifiers by taking advantage of preexisting data sets and the respective strengths of select model systems. Without question, the IIS pathway is central to understanding the organismal control of metabolism. We show here that reduced IIS suppresses α-syn misfolding and neurotoxicity in flies and worms. Moreover, we identify a conserved DAF-16/FOXO-independent mechanism through which the IIS pathway integrates metabolic regulation with proteotoxicity and DA neurodegeneration.

GPI-1 is upregulated in daf-2 mutants (Dong et al., 2007) and functions independently of DAF-16/FOXO to modulate neurodegeneration (Figure 2.4). Interestingly, GPI-1 RNAi/mutation extends lifespan in C. elegans (Schulz et al., 2007) and influences lifespan in Drosophila (Lai et al., 2007). While this relationship is seemingly paradoxical, it highlights the distinction between chronological aging and neuronal dysfunction and health. An expression level change in GPI-1 in daf-2 mutant worms does not necessarily contribute to the further lifespan extension and may
reflect a compensatory mechanism activated by a reduction in DAF-2 signaling. Indeed, this was implied by our result that gpi-1 + daf-2 combinatorial RNAi abolished the protective effect of daf-2 RNAi (Figure 2.5A) and was echoed by the climbing assay in flies (Figure 2.6A). We also determined that GPI RNAi/mutation enhances DA metabolism (Figures 2.6F and S6C); induces an inflammatory response and apoptosis (Figure 2.6G, 2.6H, and S6D); and causes widespread protein aggregation and neurodegeneration in worms, flies, and mammalian primary neurons (Figures 2.4–2.7; Table S2), but it does not affect ATP production (Figure S5). Furthermore, we found that increased glucose metabolism via overexpression of GPI and glucose surplus suppressed α-syn-induced DA neurodegeneration in worms (Figure 2.4F) and flies (Figure 2.5F), respectively. Our data, together with Tauffenberger et al. (2012), support a role for GPI in mediating proteotoxicity and neurodegeneration via glucose metabolism.

In addition to its role as a cytoplasmic enzyme, GPI has an alternative role in cancer cells, where it acts as a ligand (GPI-1/AMF) in the AMF pathway. Its receptor, HRDL-1/AMFR, was also identified in our screen to modify α-syn misfolding and neurodegeneration (Figure 2.4). AMF/AMFR attenuate ER stress and apoptosis in cancer cells (Fu et al., 2011). However, HRDL-1 also functions as a homolog of HRD family of E3 ligases that regulate HMG-CoA reductase degradation. The identification of HRDL-1/AMRF activity in neuroprotection suggests a potential relationship between cholesterol biosynthesis and α-syn toxicity in PD. This is underscored by the results of Scherzer et al. (2003), in which changes in the expression of lipid metabolism genes represented a major response to α-syn expression in Drosophila. Albeit (presumably) mechanically distinct, these data are intriguing given the reported efficacy of stains in PD models (Bar-On et al., 2008). How an alternation of lipid metabolism contributes to α-syn misfolding and neurotoxicity remains to be determined. Nevertheless, glucose metabolism may
impact \( \alpha \)-syn toxicity through a mechanism involving the elevation of certain lipids that bind to \( \alpha \)-syn and facilitate the process of toxic oligomerization.

Importantly, three positive candidates from this study are associated with neurodegenerative disease in patients: C01A2.4/ CHMP2B (frontotemporal dementia and degeneration; Isaacs et al., 2011), \( gpd-2 \)/GAPDH (Parkinson’s, Alzheimer’s, and Huntington’s; Mazzola and Sirover, 2001), and C54D10.10/TFPI (Alzheimer’s; Piazza et al., 2012). Notably, we did not identify \( C.\) elegans orthologs of other known heritable PD genes among these modifiers. While this may seem surprising, in the context of reduced IIS, these findings were not completely unexpected.

Indeed, patients with monogenic forms of PD do not develop the disease until later in life, demonstrating a key role for the metabolic changes associated with aging in disease onset. Consistent with this point is the observation that energy-metabolism-associated genes are a key class of genes whose expression is modified as \( \alpha \)-syn-induced neurodegeneration progresses (Scherzer et al., 2003). Likewise, our screening paradigm involved analysis of different phenotypes (aggregation; degeneration) in distinct cell types (bodywall muscles; dopamine neurons). Thus, contextual differences in expression may account for an absence of select modifiers. These collective results highlight the utility of model systems in deciphering the integrated consequences of imbalances in protein management and metabolic networks on neuronal survival.

**EXPERIMENTAL PROCEDURES**

**Basic \( C.\) elegans Genetic and Biochemical Methods** Strain maintenance, genetic crosses, creation of transgenic nematodes, and supporting biochemical procedures were carried out using standard methods (see Supplemental Experimental Procedures for details).
Figure 2.7. Knockdown of Gpi1/GPI in Mouse Cortical Neurons Causes Neurotoxicity and α-syn Accumulation

(A) Neurons were transduced with lenti-Gpi1 shRNA or scrambled shRNA (scrb) at MOI 1 and harvested at 7 dpi. GPI1 protein levels were determined by western blot and densitometry. α-tubulin was used as a loading control (n = 4).

(B) Neurotoxicity was assessed by neurofilament measurement at dpi 7 and 9 in neurons infected at MOI 0.5.

(C) Neurotoxicity assessment at MOI 1 infection. (n = 4.)

(D–F) Sequential extraction analysis of dpi 7 neurons infected at MOI 0.5 using anti-α-syn antibodies syn 202, SNL-1, and syn 505. Reactivity with syn 505 reveals the presence of misfolded, oxidized/nitrated α-syn. Neural specific enolase (NSE) was used as a loading control.

(G–I) Densitometric quantification of various biochemical forms of α-syn (n = 2 for syn 202, n
= 2 for SNL-1, and n = 1 for syn 505). Values are the mean SEM; *p < 0.05.

**C. elegans RNAi Screening** Screening was performed using RNAi feeding clones (Geneservice, Cambridge). Twenty age-synchronized young adult F1 animals were analyzed per clone. The RNAi clones resulting in significant α-syn aggregation (80% of worms with increased quantity and size of aggregates) were scored as positive, and all positives were tested in triplicate. RNAi feeding procedures, assays, and worm strains are described in the Supplemental Experimental Procedures.

**C. elegans Secondary Screening of Candidate Genes from RNAi Screen** Nematode models of polyglutamine aggregation in bodywall muscle cells, Ab paralysis, DAF-16::GFP localization, and α-syn- induced DA neurodegeneration were examined as secondary screening assays. These models were interrogated using RNAi of the 60 gene candidates identified from the screen by exploring by the impact of gene knockdown on F1 offspring (versus EV RNAi). Polyglutamine aggregation and Ab paralysis worm models (daf-2; P_{unc-54}::Q82::GFP and P_{myo-3}::Ab1-42) were scored at the L3-stage for total number of aggregates or numbers of worms paralyzed, respectively. DA neurons were also examined for α-syn- induced neurodegeneration in 7-day-old animals (sid-1; P_{unc-119}::sid-1; P_{dat-1}:: α-syn; P_{dat-1}::GFP) that had been exposed to RNAi since embryonic stages. A worm was considered rescued when all six anterior DA neurons were intact had no visible signs of degeneration. Statistical analyses for RNAi experiments with Q82 aggregation, Ab paralysis, and DA neuron degeneration were performed using the one-way ANOVA with Dunnett’s post hoc test (p < 0.05) to compare control worms (fed RNAi bacteria not targeting any gene) with experimental worms (fed RNAi bacteria targeting candidate gene). **C. elegans** scored for DAF-16::GFP localization were L3 staged and had been exposed to RNAi since the embryonic stage (daf-2; P_{daf-16a/b}::GFP). For each worm, GFP localization was
scored as ‘‘completely nuclear,’’ ‘‘both nuclear and cytoplasmic,’’ or ‘‘completely cytoplasmic.’’ Two independent trials (n = 40 total) were performed, and positives were determined as significant if p < 0.05, via a chi-square test. Supplemental Experimental Procedures provide more details for all of these procedures.

**C. elegans ts Mutant Phenotype Assays** The nematode mutants, *unc-15* and *unc-52*, were treated with EV or *gpi-1* RNAi and maintained at permissive temperature. Behavioral analysis was recorded and analyzed using WormLab3.0 (MBF Bioscience) with Kalman smoothing. Three independent trials were conducted, each with 15–20 worms. An unpaired Student’s two-tailed t test with Welch correction was used to determine statistical significance (see Supplemental Experimental Procedures for details).

**DOG Analysis in C. elegans** DA neurodegeneration assays were performed on P*dat-1::α-syn*, P*dat-1::GFP* animals that were age-synchronized, exposed to 5 or 10 mM DOG for 24 hr, and analyzed at day 4. Three trials were performed (n = 90 animals/treatment); one-way ANOVA with Dunnett post hoc test.

**Ingenuity Pathway Analysis** The Ingenuity Pathway Analysis (IPA) software (Ingenuity! Systems; http://www.ingenuity.com) was used for distributing the 60 positive candidate genes from our primary screen into biological networks and for evaluation of functional significance.

**Basic Drosophila Genetic and Biochemical Methods** Strain maintenance, genetic crosses, and supporting biochemical procedures were carried out using standard methods (see Supplemental Experimental Procedures for details).

**Drosophila DA Neuron Quantification** DA neurons from adult males were dissected for
analysis either 1 or 20 days post-eclosion. These neurons were visualized by expression of GFP under the control of the DA neuron driver TH-Gal4, and samples were quantified following confocal microscopy imaging. Additional details are outlined in the Supplemental Experimental Procedures section.

*Drosophila DA Neuron HPLC and NO Synthase Analyses*  Monoamines from male heads were separated using HPLC as described in Chaudhuri et al. (2007). A modified Griess reagent assay described in Ajjuri and O’Donnell (2013) was used to quantify nitric oxide synthase activity. Male heads were also examined in this assay. For all experiments, genotypes were analyzed in triplicate and statistics were analyzed using one-way ANOVA followed by Dunnett post hoc test. See Supplemental Experimental Procedures for more details.

*Drosophila DOG and Glucose Feeding*  Male flies were aged to 10 days post-eclosion and then fed either standard corn syrup food or standard food supplemented with either 200 mM 2-DOG or glucose with a final concentration of 1 M continuously for 5 days. Statistics were analyzed using one-way ANOVA followed by Bonferroni post hoc test.

*Drosophila Climbing Assay*  Flies were anesthetized using cold coma, transferred in groups of ten to climbing vials, and allowed 45 min to recuperate. Vials were gently tapped before each trial, and climbing was scored by calculating the number of flies to climb 6 cm in 20 s. The results represent ten trials with three repetitions per trial. Statistical analyses were performed using one-way ANOVA with Bonferroni post hoc test.

*Mouse Cortical Cultures, Lentiviral Infection, and Neurotoxicity Analysis*  Murine neuronal cortical cells were obtained at embryonic day 17, as previously described (Tsika et al., 2010). Cells were seeded in 96-well plates at 50,000 cells/well and infected at 5 days in vitro.
(DIV) with lentiviral particles containing shRNA against *Gpi1* at either MOI 0.5 or 1. The cells were then fixed in 4% paraformaldehyde at the indicated time points. The staining and analysis procedures have been described in detail previously (Tsika et al., 2010). Neurotoxicity analysis was performed with two separate culture preparations, with four replicates for each preparation. One-way ANOVA with Tukey’s post hoc test was used to determine statistical significance, and p < 0.05 was considered significant. See Supplemental Experimental Procedures for details.

**Sequential Biochemical Extraction of Mouse Neuronal Cultures** 6,000,000 cells/condition were extracted for SDS-PAGE analyses using the methods described in the Supplemental Experimental Procedures section. The subsequent gels were transferred to polyvinylidene difluoride membranes and probed with anti-α-syn antibodies (syn 202, dilution 1:500, Covance; SNL-1, dilution 1:500, gift of Benoit I. Giasson, University of Pennsylvania; or syn 505, dilution 1:500, Invitrogen). Anti-Neural-specific enolase and anti-alpha-tubulin were used as loading controls. Primary antibodies were detected with anti-mouse or rabbit IgG conjugated to IRDye 680 or 800. For controls, blots were scanned after the blocking step to determine autofluorescent bands and also after the addition of secondary Ab alone. Any nonspecific bands detected were not included in densitometric analyses. Quantification of the Triton-soluble band migrating at 18 kDa was used for monomer measurements (syn 202 and SNL-1); quantification of aggregated forms of α-syn was done with syn 202, SNL-1, and syn 505 and repeated with separated culture preparations. A Student’s t test was used to determine statistical significance; p < 0.05 was considered significant.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes six figures, three tables, three movies, and Supplemental
Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.cmet.2014.04.017.

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REFERENCES


CHAPTER THREE
EXPLORING NITRIC OXIDE SYNTHASE-MEDIATED INFLAMMATION IN DOPAMINERGIC NEURODEGENERATION


ABSTRACT

Neuroinflammation is a complex innate immune response vital to the healthy function of the central nervous system (CNS). Under normal conditions, an intricate network of inducers, detectors, and activators rapidly responds to neuron damage, infection or other immune infractions. This inflammation of immune cells is intimately associated with the pathology of neurodegenerative disorders, such as Parkinson's disease (PD), Alzheimer's disease and ALS. Under compromised disease states, chronic inflammation, intended to minimize neuron damage, may lead to an over-excitation of the immune cells, ultimately resulting in the exacerbation of disease progression. For example, loss of dopaminergic neurons in the midbrain, a hallmark of PD, is accelerated by the excessive activation of the inflammatory response. Though the cause of PD is largely unknown, exposure to environmental toxins has been implicated in the onset of sporadic cases. The herbicide paraquat, for example, has been shown to induce Parkinsonian-like
pathology in several animal models, including *Drosophila melanogaster*. Here, we have used the conserved innate immune response in *Drosophila* to develop an assay capable of detecting varying levels of nitric oxide, a cell-signaling molecule critical to the activation of the inflammatory response cascade and targeted neuron death. Using paraquat-induced neuronal damage, we assess the impact of these immune insults on neuroinflammatory stimulation through the use of a novel, quantitative assay. Whole brains are fully extracted from flies either exposed to neurotoxins or of genotypes that elevate susceptibility to neurodegeneration then incubated in cell-culture media. Then, using the principles of the Griess reagent reaction, we are able to detect minor changes in the secretion of nitric oxide into cell-culture media, essentially creating a primary live-tissue model in a simple procedure. The utility of this model is amplified by the robust genetic and molecular complexity of *Drosophila melanogaster*, and this assay can be modified to be applicable to other *Drosophila* tissues or even other small, whole-organism inflammation models.

**INTRODUCTION**

Neuroinflammation is an intricate immune response that has been shown to be intimately associated with the pathology of a wide range of diseases, the majority of which are neurodegenerative disorders. Under basal conditions, this multifaceted network of peripatetic immune cells maintains tissue health through its rapid response to infection, plaques, or injury (Gehrmann et al., 1995; Dissing-Olesen et al., 2007). A neuroinflammatory response by the mammalian central nervous system can be triggered due to minor insults, such as cellular oxidative stress associated with normal aging, or major assaults, such as neuron damage occurring from acute exposure to a chemical toxin. When this induction occurs, phagocytic
surveillance cells known as microglia are activated and migrate to the site of neuronal cellular damage. These scavenger-like immune cells, which are similar to macrophage cells that exist in the periphery, then begin a sophisticated signaling cascade with the damaged neuron, as well as with astrocytes, supporting glial cells that act alongside microglia for the typically robust microglial response (Aloisi et al. 1997; Vincent et al., 1997; Rohrenbeck et al., 1999; Walsh et al., 2000; Aloisi et al., 2000). Once activated, the microglia begin a process of inflammation to stimulate the immune system and promote tissue repair (Gehrmann et al., 1995; Minghetti & Levi 1998).

Under conditions of sustained insult, as in neurodegenerative diseases such as Parkinson's disease (PD), Alzheimer's disease and amyotrophic lateral sclerosis, chronic inflammation causes significant damage to healthy tissue as well. Recent studies have shown that this persistent, aggressive response by the immune system may in fact be leading to an over-excitatory reaction that exacerbates the disease state rather than ameliorating the condition (Mrak & Griffin 2005; Zhang et al., 2005; Beers et al., 2011).

In *Drosophila melanogaster*, the innate immune response is genetically well-conserved. Although lacking a true adaptive immune network, *Drosophila* maintains a sophisticated innate immune system containing a class of phagocytes known as hemocytes. These immune cells have been previously identified as macrophage-like cells that migrate to the site of injury (Wood et al., 2006; Babcock et al., 2008), and undergo phagocytosis during septic shock (Vlisidou et al., 2009), introduction of a parasite (Foley & O’Farrell 2003), or viral infection (Costa et al., 2009).
Importantly, like mammalian macrophages, phagocytic hemocytes express nitric oxide synthase (NOS) and produce nitric oxide (NO), a critical signaling component of Drosophila immunity (Foley & O’Farrell 2003; Costa et al., 2009). All of these studies, however, have only been able to demonstrate hemocyte responses during embryonic development and in larva.

Our lab has observed a robust, hemocyte-mediated neuroinflammation in Drosophila adult brains, a previously unknown phenomenon (Daigle & Hall, et al., manuscript in preparation). This response was induced through exposure to the toxicant paraquat. Epidemiological studies by the National Institutes of Health have identified the widely-used herbicide as a potential risk factor in the onset of PD in humans (Inamdar et al., 2012). Research investigating paraquat treatment in mammalian models had already validated its neurotoxic properties and had shown the herbicide to result in Parkinsonian symptoms, such as motor abnormalities and selective neuron loss (Broderick et al., 2003; Desjardins et al., 2009, 2010). In Drosophila, treatment of paraquat leads to a wide range of Parkinsonian behavioral phenotypes as well as dopaminergic neuron death consistent with PD pathology (Ossowaska et al., 2006). Though the mechanisms and genetic factors associated with this neuroinflammatory response have not yet been fully elucidated in Drosophila, we have observed a conserved induction of NOS during neurodegeneration.

We have additionally detected a similar, albeit less dramatic, neuroinflammatory response using mutant genotypes with known neurotoxic sensitivity. Encoded by the gene Punch, GTP cyclohydrolase is the first and rate-limiting protein in the biosynthesis of BH4, which then acts as a necessary cofactor for NOS (McCormack et al., 2005; Chaudhuri et al., 2007). The loss-of-
function mutation $Pu^{223}$ causes a decline in the inflammatory response and heightened susceptibility to paraquat (Knight et al., 2014). With this protocol, we were able to quantify relatively subtle modifications of nitric oxide levels induced by this genetic variation.

To detect NO in our samples we use Griess reagent, a widely-accepted colorimetric method for measuring NO levels. This reagent indirectly measures relative NO abundance by detecting the presence of nitrites, one of two end products of nitric oxide production. The existing nitrite detection protocols using Griess reagent have been applied primarily for in vitro analyses or in cell culture, where NO diffuses into the culture medium. We initially developed a Griess reagent-based method for detecting NOS in crude Drosophila head homogenates (Knight et al., 2014). We have, however, found that method to be somewhat variable with altered conditions, presumably due to the instability, high reactivity and low relative concentration of NO in whole head tissues. Therefore, we sought to develop a method based on cell culture protocols that would allow for greater sensitivity and reliability in quantifying NOS activity. Here we describe a method conceptually similar to one previously employed to detect NOS in Drosophila larval Malpighian tubules (Iburg et al., 2013). In this procedure, we utilize whole brains that were dissected immediately following a toxin treatment to induce the inflammatory response. Then, we incubated these samples in insect culture medium to preserve the integrity of the tissue, and to enhance the sensitivity and reliability of a Griess reagent-based assay.

We anticipate that, with the use of the sophisticated genetic tools available, this model has the capacity to serve as a promising utility for investigating the dynamic inflammation/neurodegeneration network. With the ability to accurately quantify the activation and relative
intensity of the neuroinflammatory response, this novel assay creates a primary tissue culture capable of detecting secreted neuroinflammatory markers. This method offers small organism models of inflammation an inexpensive, highly sensitive technique for rapidly assaying whole-organism or live-tissue samples where previously lacking.

EXPERIMENTAL PROCEDURES

I. Paraquat Exposure

1. Maintain the cultures of the test flies on standard *Drosophila* culture medium at 25°C under equivalent culture densities consisting of approximately 35 females and 15 males.

2. To prepare feeding chambers, place a small amount of absorbent cotton at the bottom of an empty vial with filter paper directly above the cotton.

3. Anesthetize adult male flies 3-5 days post-eclosion using cold coma or CO₂. For the experiments described here, use males of genotype y* w¹¹¹⁸*.

   a. For cold coma separation, tap down feeding vials containing flies and then quickly remove stopper and transfer flies directly to a clean, empty vial, making sure to label properly.

   b. Once flies are successfully transferred to empty food vials, tap down vials then immediately place in ice bucket for 5 min or until flies are knocked out.

   **Note:** Do not completely submerge vial in ice. This can cause water or moisture to get into the vial and drown the flies. As another precaution, use fresh ice rather than melted ice in ice buckets.

   **Note:** Pay close attention to the number of flies needed for each assay and, when possible, add additional flies to each group as a precaution in the event of human
error, *e.g.* flies escaping during feeding or transferring. The nitric oxide detection assay, for example, requires 20 fly brains per sample set, so account for dissection skill-level and efficiency when feeding (*i.e.* novices should set up more than 20 flies in case of transferring or dissection errors).

4. Once flies are anesthetized, transfer each group into corresponding feeding vial as prepared above.

5. After the flies are fully awake in feeding vials, starve them for 1.5 hr and meanwhile, prepare solutions described below:

   a. Prepare a stock solution of 5% sucrose (control solution) by adding 5 g of sucrose into 100 ml of double-distilled water. Stir until completely dissolved and store at 4 °C.

      **Note:** Use 5% sucrose stock solution as the solvent for all subsequent feeding solutions.

   b. Prepare stock 10 mM paraquat stock solution using 5% sucrose as diluent and stored at 4 °C. Prepare all other concentrations using serial dilutions as needed.

      **CAUTION: PARAQUAT IS EXTREMELY TOXIC. REFER TO MSDS FOR SAFETY GUIDELINES AND PROPER HANDLING/DISPOSAL.**

   c. Prepare 10 mM stock solutions of *N*-Nitro-L-arginine methyl ester hydrochloride (L-NAME), *N*-nitro-D-arginine methyl ester hydrochloride (D-NAME) using 5% sucrose as diluent and store at 4° C. For co-incubations of L-NAME or D-NAME with paraquat administer each compound at a 1:1 ratio with paraquat concentration. Prepare all other concentrations using serial dilutions as needed.

   d. Note: Filter all solutions directly after preparation. Paraquat, D-NAME, and L-
NAME stocks should be used within one week of preparation to ensure experimental consistency.

e. Pipette 150 \( \mu l \) per day (or less depending on feeding duration) of the feeding solution onto the filter paper, being careful not to drown flies or let any escape.

II. Survival/Mortality Assay

1. Set up feeding chambers as previously described.
   Note: Use at least 3 layers of filter paper per vial to prevent desiccation.

2. Place 10 male flies 3-5 days post-eclosion in each vial and feed as directed above, being cautious to not let the filter paper dry out overnight.
   Note: Maintain vials at room temperature, away from direct sunlight to avoid accelerating evaporation of solutions.

3. Beginning the following day (designated "Day 1"), record number of flies dead per vial twice a day for 10 consecutive days.
   Note: Regulate both scoring and feeding to specific hours each day to decrease variation and provide higher consistency.

4. Calculate average survival using at least three independent replications of all groups.

III. Dissecting Adult Fly Brains and Incubation

1. Prior to dissections, pipette 50 \( \mu l \) of Grace’s Insect Medium into the appropriate number of microtiter plate and label suitably. Cover with plate lid until use.
   a. Note: Alternatively, sterile centrifuge tubes may also be used if preferred.

2. Anesthetize flies of one sample set using cold coma as described in steps 1.3.1-1.3.2.

3. Once flies are fully anesthetized, place several flies on a microscope slide
   a. Note: Begin with a small subset of flies if not experienced with dissections.
4. Decapitate the fly heads under a dissection microscope using forceps and a surgical blade or two forceps, discarding fly bodies when done.

5. Use a small amount of PBS as a dissection buffer; desiccation must be avoided.

6. Extract the full brain following the method of Williamson & Hiesinger (2010) carefully removing cuticle particulates and any non-brain tissue (e.g. eye pigment tissue) (See Figure 3.1).

7. Transfer the dissected brain into the appropriately-labeled well and repeat until there is a minimum of 20 brains per well.

8. Keep plates on ice during dissection period and do not exceed longer than 20-30 min total time between the start of dissecting and assay initiation.

   a. **Note:** More than 20 brains can be used so long as each group has the same number in each well. Minimum number of brains to achieve accurate reading may vary (refer to Discussion section for more detail).

9. Once the working set is complete, allow brains to incubate for 6 hr in 25 °C with light shaking.

10. Follow steps 1.2-1.6 for all remaining groups.

   a. **Note:** Record and adjust end time of incubation depending on differentials between when each sample set was completed.

**IV. Preparation During Incubation Period**

1. Prepare the Modified Griess reagent as directed by the manufacturer (see table) and keep in the dark until use. The amount of reagent needed is dependent on the amount of samples. For example, the Griess reagent will be added in a 1:1 ratio to the Grace's Medium (step 5.5). Therefore, 50 μl per sample set + 50 μl for the blank control should
be prepared.

2. Construct a nitrite standard curve using sodium nitrite in a serial dilution ranging from 0.78125-100 mM nitrite for a total of 8 concentrations.

   **Note:** This should be done for each experiment to ensure consistency and accuracy in sample concentration and Griess Reagent activity.

3. Additionally, prepare and label sterile 0.5 ml centrifuge tubes, two tubes for each treatment group and two for the control.

**V. Detection of Nitrite Levels**

1. Once the incubation period has ended, transfer the Grace's Medium (at least 45 µl) from the microtiter wells into the first set of centrifuge tubes corresponding to each group.

   **Note:** As previously noted, be sure to account for differences in incubation periods due to time taken between each set of dissections.

2. Optional Step: Add nitrate reductase and NADPH at this step to convert nitrates to nitrites. (This step was not carried out on the results shown)

   **Note:** A nitrate/nitrite assay may be conducted if desired with an aliquot of sample supernatant as described in assay kits.

3. Gently centrifuge (around 8,175 x g) for 2 min to pellet any brain or tissue particulates that may have been aspirated from the wells.

4. Pipette 30 µl of the supernatant from the first set of centrifuge tubes into the second set of corresponding tubes, making sure not to disrupt the pellet after spinning down the samples.

5. Add Griess reagent in a 1:1 ratio, incubate for 5-10 min and assay using NanoDrop spectrophotometer at 548 nm absorbance within 30 min.
**Note:** Samples can also be assayed using a microplate reader at an absorbance wavelength of 540 nm with 620 nm reference wavelength.

6. Repeat each reading a minimum of three times and average the readouts.

**Note:** Be sure to "blank" the machine between every 6-10 readings and before completion, run the blank control (Grace's Medium plus Griess reagent without brains) as a sample to use as a standard deviation.

7. Use the nitrite standard curve generated in step 4.2 to calculate relative nitrite levels of each sample.
   
   a. To calculate unknown concentrations, first plot the values of the known concentrations from the standard curve using absorbance (*i.e.* optical density) on the y axis and concentration on the x axis.
   
   b. Once all standard curve values are graphed, find the slope of the best fit line and solve the equation for x.

   **Note:** If serial dilutions are done properly, the best fit line should be linear or very close to it. If this is not the case, reconstruct standard curve. If problems persist, check all solutions, media and reagents for expiration and be sure equipment is working properly and within its range of detection.

   c. Next, simply substitute the absorbance values for y and solve for x, which represents concentration.

8. For additional information regarding the use of NanoDrop equipment, refer to Desjardins *et al.* (2009) and Desjardins & Conklin (2010).

**VI. Western Blot**

1. Feed 30 flies per condition as described above and decapitate heads, preferably using
liquid nitrogen and vortexing.

2. Homogenize whole heads on ice in 60 µl RIPA buffer with 2 mM dithiothreitol (DTT) and 1x protease inhibitor cocktail added just before use.

3. Spin down samples for 5 min at 8,175 x g in 4 °C.

4. Transfer supernatant to new centrifuge tubes.

5. Mix samples gently and pipette 17 µl of supernatant into centrifuge tubes containing 6.5 µl 4x LDS sample buffer and 2.5 µl 500 mM DTT.

   **Note:** Flash frozen remaining sample in liquid nitrogen and stored in -20 °C until use.

6. Mix samples well and heat at 70 °C for 10 min, mixing once mid-incubation.

7. While heating, assemble polyacrylamide gel electrophoresis (PAGE) running apparatus using precast 4-12% NuPage Bis-Tris minigel, MOPS SDS running buffer with 500 µl antioxidant in inner chamber.

8. Quick spin samples and allow to cool.

9. Load 25 µl into each lane.

10. Run the gel electrophoresis at 200 V on ice or in 4 °C for 45 min or until gel is complete.

11. Once electrophoresis is completed, transfer separated proteins from the gel to nitrocellulose membranes using iBlot Transfer Stacks and detect signal using Western Blot Detection Kit.

   **Note:** Antibodies used are as followed: primary antibodies mouse α-nNOS (1:250), mouse α-syntaxin (1:200); secondary antibodies (1:250) provided in Western Blot Detection Kit (mouse).

12. Detect chemiluminescent signal using western blot imaging system or X-ray film.
Note: Any standard immunoblot detection and quantification methods can be used for blot analysis.

RESULTS

Paraquat has been shown in numerous animal models, such as mice (Manning-Bog et al., 2002), rats (Ossowska et al., 2005) and fruit flies (McCormack et al., 2005), to induce neurological degeneration consistent with Parkinsonian-like pathology. We have previously reported that the anti-inflammatory antibiotic minocycline, when co-fed with paraquat, results in extended survival, reduced production of reactive oxygen species and rescue of dopaminergic neuron death (Inamdar et al., 2012). Therefore, as minocycline acts to suppress inflammation, we began investigating NOS, a key protein in the activation of the inflammatory response, and its role in modulating paraquat toxicity.

A paraquat toxicity curve was performed to establish an effective lethal dose and toxic treatment range, using paraquat concentrations between 1.25 and 10 mM (Figure 3.2A). We also assayed the effects of paraquat concentrations of 20 mM and 40 mM, but found that toxicity was too acute at these higher concentrations to accurately assess cellular responses to this oxidative stressor, and therefore, we have not included the results for these concentrations in this report.

Using L-NAME, a competitive NOS inhibitor, and D-NAME, the inactive isomer of NAME, we co-treated adult male flies with paraquat and one of the NAME isomers. Co-treatment of flies with paraquat and L-NAME resulted in a significant rescue of lifespan truncation caused by paraquat ingestion, while flies co-treated with paraquat and D-NAME showed no improvement in survival (Figure 3.2B), supporting the hypothesis that suppressing inflammation through the inhibition of NOS enhanced survival of paraquat-treated flies.
As further validation of a NOS-mediated paraquat response, we detected changes in NOS protein levels directly after paraquat exposure. NOS protein levels increased in a paraquat concentration-dependent manner (Figure 3.3A). When treating flies with 10 mM paraquat over exposure durations ranging from 6-30 hr, we observed an initial increase, then a decrease as exposure time increased (Figure 3.3B), consistent with the patterns of nitrite levels observed in our variant of the Griess assay (Figure 3.5B). These results are consistent with our reports that paraquat treatment causes induction of NOS and that inhibition of NOS or treatment with L-NAME or minocycline provides partial protection (Daigle & Hall et al., manuscript in preparation; Inamdar et al., 2012).

Figure 3.4A demonstrates a linear relationship between the increase in paraquat concentration and the magnitude of the inflammatory response as defined by the secretion and detection of NO. Under basal levels, heterozygous Punch mutants secreted slightly lower levels of NO, a variation too indiscernible to be determined using previous detection methods. When fed paraquat, a pronounced increase in NO levels were observed in the Punch mutant, however, significantly less than the wildtype treated flies (Figure 3.4B). The relative values of the wildtype and Punch flies can be accurately and reproducibly quantified, though the variation at untreated levels were extraordinarily subtle.

When working with toxins or chemicals, it is vital to establish both a time-of-exposure curve as well as a concentration-based toxicity curve, since these conditions can dramatically alter the sensitivity of the assay. For example, treatment with 5 mM paraquat results in maximum NO detection after 24 hr of exposure (Figure 3.5A), while exposure to 10 mM paraquat resulted in more rapid induction (maximum levels at 12 hr), but also more rapid decay of activity during extended exposures (Figure 3.5B). Although a higher concentration with quick induction might
seem preferable, a maximized response accelerates death of both neurons and possibly hemocytes to the point that reproducibility may become difficult.

NO molecules are unstable and highly reactive. A successful assay must therefore include incubation conditions that provide an optimal balance between continued induction of NOS and rapid turnover of NO. In order to define optimal incubation conditions, we assessed the effect of the time that dissected brains were incubated in the culture medium prior to adding the Griess reagent. We found that a 6 hr incubation at room temperature produced maximum nitrite levels in the subsequent Griess reaction (Figure 3.5C). We expect that variations in the incubation period may be needed to optimize detection for various models of inflammation. Careful establishment of all optimal parameters for the particular organism, genotype, and tissue being assayed, will be essential to achieve reliable and accurate results since the process is highly dynamic.

In particular, genetic variants and transgene expression models are expected to require substantial optimization, particularly with respect to developmental stage or age of adults.
**Figure 3.1. *Drosophila* adult brain.** Light microscopy of dissected adult male brain 2-3 days post-eclosion. MB = midbrain (central cortex), OL = optic lobe.
Figure 3.2. Paraquat results in a dose-dependent reduction in lifespan, mediated through nitric oxide synthase. Wild type male flies fed (A) 5% sucrose (control), and serial dilution of paraquat ranging from 1.25-10 mM concentration in 5% sucrose and (B) co-fed paraquat with L-NAME or D-NAME for 10 consecutive days. N = 3 groups of 10. Error bars = SEM.
Figure 3.3. Nitric oxide synthase protein levels increase with paraquat concentration and length of exposure. Immunoblot detection of nitric oxide synthase levels of flies treated with varying (A) paraquat concentrations (12 hr exposure) and (B) 10 mM paraquat with varying exposure durations. Protein levels were detected on nitrocellulose membranes using chemiluminescence and X-ray film exposure.
Figure 3.4. Nitric oxide detection levels after paraquat exposure. Male wild type (WT) flies 2-3 days post-eclosion were assayed following 12 hr feeding. (A) Concentration curve of paraquat concentrations. (B) Male Punch heterozygous mutants fed sucrose control and 10mM paraquat. N = 20 brains/group. Results represent 3 replications per group. Error bars = SEM. Statistical analyses were performed using one-way ANOVA with Dunnett's Multiple Comparison posttest (*, p < 0.05, **, p < 0.01, ***, p < 0.001).
Figure 3.5. Optimizing the detection of nitric oxide production by altering the length of paraquat exposure and time of tissue incubation. Relative nitric oxide levels on male brains 2-3 days post-eclosion following 12 hr treatment of (A) 5 mM paraquat and (B) 10 mM paraquat. (C) NO curve using varying tissue incubations periods following 12 hr, 10 mM paraquat feeding. Significance values were calculated relative to the following: (A, B) 5% sucrose controls for each exposure time (not shown in graph), (C) 5% sucrose controls for each incubation time (not shown in graph). N = 20 brains/group. Results represent 3 replications per group. Error bars = SEM. Statistical analyses were performed using one-way ANOVA with Dunnett's Multiple Comparison posttest (*, p<0.05, **, p<0.01, ***, p<0.001).
DISCUSSION

This method, though simple in approach, provides a cost-efficient, highly repeatable and exceedingly sensitive method for quantifying levels of NOS-mediated neuroinflammation. As shown in the Results section, there are many variables that can be adjusted to enhance or optimize the response in different induction models or organisms. Because this is a highly sensitive system, however, these variables may also result in highly variable outcomes if all aspects of the protocol are not handled carefully. When first testing using any model, we recommend setting up the parameters by testing variables, which include chemical concentration (Figure 3.4A), length of exposure (Figures 3.5A and 3.5B), and incubation duration (Figure 3.5C). Be mindful of other variables, such as the number of brains/tissue samples needed to achieve detection threshold and consistency, even when applying this method to similar Drosophila models. Additionally, in age-dependent genetic models of inflammation, extended time point optimization will be needed.

As mentioned above, due to the sensitive nature of the assay, inconsistencies in the protocol can yield unreliable results. With this in mind, all equipment and materials were sterilized prior to use. Due to the short incubation time, no antibiotics were added to the culture media in these experiments. However, if the protocol is adapted for longer incubation times or if bacterial growth is observed in cultures or media, typical culture antibiotics such as penicillin-streptomycin should be added and equipment should be re-sterilized. As in any chemical treatment experiments caution should be used to ensure that all groups are reared under well-controlled conditions, gender and age matched, and all groups receive exactly the same feeding conditions. Some example feeding conditions to consider include variations in concentrations,
availability and access to feeding source, duration of feeding and the time of the day that the feeding is administered. Detailed records for all experimental conditions and close attention to the natural feeding patterns of your model will help minimize variability. If mutant or transgenic strains that affect neural circuitry or behavioral patterns are employed an important control would include monitoring of feeding behavior.

Once again, while similar protocols are available in cell culture and lysates, one major advantage of this method is that the integrity of the tissue is maintained, therefore allowing virtually all cellular relationships within the brain to remain intact. This system represents a relatively natural, in vivo state of neuroinflammatory signaling and cellular secretion. Established in Drosophila, this technique can be easily tailored to other small model organisms, and is not limited to the central nervous system, as demonstrated by a similar application for NO signaling in a Malpighian tubule model for kidney function (Kean et al., 2002). The most significant advantage by far of this protocol is the ability to manipulate and adapt this assay to fit the needs and interests of the researcher or model system.

Through the use of a combined chemical-genetic approach, this assay has the potential to significantly enhance the ability of small model systems to investigate the mechanisms, genetic components and chemical modulators of inflammation.

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REFERENCES


CHAPTER FOUR

MODULATION OF α-SYNUCLEIN-INDUCED NEUROTOXICITY THROUGH CATSUP-DEPENDENT VMAT FUNCTION

The work in this chapter was initiated by Faiza Ferdousy and completed by Rami Ajjuri. Abstract, Introduction and Discussion sections were written by Rami Ajjuri and edited by Janis O’Donnell. Methods and Results were written by Rami Ajjuri, Faiza Ferdousy and edited by Janis O’Donnell. Figures 4.2C-D, 4.5A-B, 4.7B, 4.7D were done by Rami Ajjuri and Faiza Ferdousy; Figures 4.4A-F, 4.9A-B and 4.10A-D were completed by Rami Ajjuri. All remaining experiments were done by Faiza Ferdousy.

ABSTRACT

In the early stages of Parkinson’s disease (PD) pathology, dopaminergic neurons are primarily and preferentially targeted. When dopamine-producing neurons are lost, the dysregulation of their neurotransmitter, dopamine, results in severe motor and physiological defects. Consequently, a substantial focus in PD research is devoted to understanding dopamine homeostasis and the susceptibility of dopaminergic neurons. While several environmental and genetic factors of PD have been shown to alter dopamine homeostasis, there is much left unexplained regarding the exact mechanisms leading to dopaminergic neurotoxicity. We previously reported that Catsup, or Catecholamines-up, the *Drosophila* homolog of zinc transporter SLC39A7/KE4 plays a vital role in the negative regulation of dopamine production via the inhibition of GTP Cyclohydrolase and Tyrosine Hydroxylase, two rate-limiting enzymes
in the dopamine biosynthesis pathway. We have also shown that mutations in *Catsup* result in the partial rescue of paraquat-induced dopaminergic neurotoxicity and in the elevation of vesicular monoamine transporter (VMAT) activity, a critical regulator of neurotransmitter storage and release. Here, we evaluated whether knocking down *Catsup* could modify the dopaminergic neurotoxicity of α-synuclein and the α-synuclein A30P mutation, two commonly studied genotypes linked to familial PD. Our results strongly suggest that *Catsup*, through VMAT-regulated DA packaging, plays a central role in modulating the detrimental effects of α-synuclein expression in Parkinson’s disease pathology.

**INTRODUCTION**

Despite decades of research, the vast majority of Parkinson’s disease (PD) cases arise from unknown causes. In fact, only around 5-10% of PD cases are caused by familial inheritance, and over a dozen genes and agricultural chemicals have been linked to the onset of parkinsonian-like symptoms. One of the hallmark and earliest features of PD pathology is the loss of dopamine neurons. While many treatments focus on the restoration of the dopamine regulatory process or the reduction of neuron loss, before effective treatments can be implemented, one must consider that the underlining mechanisms for this early degeneration may vary from patient to patient depending on the causes of his/her onset and overall variation in genetic background.

The common factor in these cases, however, is that dopaminergic neurons are prejudicially affected long before symptoms arise. Therefore, a thorough understanding of the process by which dopamine is regulated may expose areas of potential mechanistic understanding and advanced treatment of PD cases with varying causes.
In previous publications (Chaudhuri et al., 2007; Wang et al., 2011), we have shown that \textit{Catecholamines-up (Catsup)}, the \textit{Drosophila} homolog of \textit{SLC39A7/KE4}, plays a critical role in dopamine production and cellular regulation and that mutations in \textit{Catsup} have the ability to modify neurotoxicity and motor defects following treatment of the neurotoxic herbicide paraquat. In this report, we have further characterized the impact of \textit{Catsup} on parkinsonian-like symptoms using genetic PD models, \textit{\alpha-synuclein} wildtype and A30P mutation.

Though its function remains unknown, \textit{\alpha-synuclein} (\textit{\alpha-syn} or \textit{SNCA}) is one of a few known monogenic causes of PD, which comprise approximately 30\% of hereditary cases (Klein and Westenberger, 2012) and the first of which to be discovered (Polymeropoulos et al., 1996; 1997). There are three known missense mutations of \textit{\alpha-syn}, all of which impair the amino-terminal domain of the natively-unfolded \textit{\alpha-syn} protein, consequently disrupting the association of \textit{\alpha-syn} with phospholipid membranes (Giasson et al., 2001). As these mutations modify the stability of \textit{\alpha-syn}, the conformation of membrane-bound \textit{\alpha-syn} is altered from \textit{\alpha}-helical to stable \textit{\beta} sheets, resulting in the production of toxic oligomers and fibrils (Bertoncini et al., 2005). One such mutation, A30P, not only disrupts the binding of \textit{\alpha-syn} to neuronal membranes but it also leads to a relocalization of \textit{\alpha-syn} away from the synapse in cultured neurons (Fortin, 2004). All of these mutant forms and excessive amounts of the wild type protein share the common outcome of increasing cytoplasmic oxidative load, with subsequent free radical damage to the cell.

Dopamine molecules are particularly prone to oxidation and therefore can accelerate the damage caused by mutant \textit{\alpha-syn} (Graham et al., 1978), if they accumulate in the cytoplasm where they
contribute to the formation of toxic radicals and reactive oxygen species (ROS) (Hastings et al., 1996; Spencer et al., 1998). A critical means of protecting the cell from this toxic process rests with regulation of the activity of vesicular monoamine transporter (VMAT2), which transports DA from the cytoplasm where it is susceptible to oxidation into synaptic vesicles. VMAT2 therefore is an important key to determining the resistance of dopaminergic neurons by reducing free-cytosolic DA before it is metabolized into toxic metabolites (Caudle et al., 2007; Mosharov et al., 2003; 2009).

Inhibition of VMAT2 has been shown to reduce animal mobility and survival of DA neurons (Satou et al., 2001) and induce neuronal oxidative stress in (Bilska et al., 2007; Fornstedt and Carlsson, 1989; Spina and Cohen, 1989). Recently, a study examining striatal tissue isolated from human PD patients found that defective VMAT2 function was the primary cause of disrupted DA transport into presynaptic vesicles, suggesting the dysfunction of VMAT2 as an initial and principal cause of DA neuron death in PD pathology (Pifl et al., 2014).

We demonstrate here that the expression of human wildtype and mutant \( \alpha \)-syn in \textit{Drosophila melanogaster} results in reduction of tyrosine hydroxylase activity and a subsequent decrease in dopamine neurotransmitter levels. We also observe an increase in the turnover of dopamine into the metabolite DOPAC, indicating a disruption in the vesicular packaging of cytosolic dopamine. However, when wildtype \( \alpha \)-syn or the A30P mutant form are expressed in a heterozygous \textit{Catsup} loss-of-function mutant background (\textit{Catsup}^{26}), these \( \alpha \)-syn- induced effects were rescued to near wildtype levels. Similarly, dopamine neurons in \textit{Catsup}^{26}/+ mutants expressing \( \alpha \)-syn wildtype or mutant A30P are significantly protected from neurodegeneration relative to
the \(\alpha\)-syn wildtype or A30P mutant genotypes. Furthermore, *Catsup* loss-of-function also significantly alleviates the sensitivity of \(\alpha\)-syn wildtype and A30P flies to reserpine, an inhibitor of VMAT. These findings support the conclusion that VMAT, activated by loss of Catsup, can sequester cytosolic DA at an accelerated rate, which then significantly reduces the toxic effects of synucleinopathies in DA neurons.

**EXPERIMENTAL PROCEDURES**

*Drosophila strains and culture maintenance:* All *Drosophila* stocks were maintained at 25°C on standard cornmeal-yeast-sugar media. Male flies of genotype +/UAS- GFP ; +/TH-Gal4 (indicated as UAS-GFP; TH-Gal4) were used as the control for all experiments since all other mutant genes and transgenes were moved into this background. The following strains were used in these experiments: *Catsup\(^{26}\)/UAS-GFP; +/TH-Gal4, +/UAS-GFP; UAS- \(\alpha\) synuclein wt/ TH-Gal4, +/UAS-GFP; UAS- \(\alpha\)-synuclein A30P/ TH-Gal4, *Catsup\(^{26}\)/UAS-GFP; UAS- \(\alpha\)-synuclein wt/ TH-Gal4, *Catsup\(^{26}\)/UAS- GFP; UAS- \(\alpha\)-synuclein A30P/ TH-Gal4.

**Reserpine exposure:** Adult flies were collected at 1-2 days post-eclosion and aged for 72 hr. Aged flies were then placed in feeding vials consisting of a base of filter-paper disc overlying absorbent cotton wool soaked with 15 mM of reserpine in a 5% sucrose solution. Feeding was continued for a period of 8-10 hr.

**Locomotion assay:** The mobility assay was conducted as described by Carbone et al. (Carbone et al., 2006) with a few modifications. For all genotypes, 20 males were collected 1-3 days after eclosion and aged for 48-72 hrs. The mobility of the fly was measured by ascertaining the
number of seconds the fly was in motion within that 45 sec period immediately following gentle, mechanical disturbance. Each of these flies was transferred to a vial containing 3-5 mL of standard media for 1 hr prior to the performing the mobility assay.

**Tyrosine hydroxylase assay:** TH activity of tissue extracts was determined as described in Vie et al., 1999 (Vié et al., 1999) with some modifications. Briefly, 50 fly heads were homogenized in 100 μl of cold homogenization buffer. Homogenates were centrifuged at 9,300 x g for 10 min. at 4°C; the protein concentration of the supernatant was determined using BioRad protein assay reagent (BioRad, Hercules, CA). Extracts were adjusted to equal protein concentration range (20-30 μg total protein) and 50 μl of the extract were mixed with 1 mM m-hydroxybenzylhydrazine dihydrochloride (NSD 1015; Sigma-Aldrich), 2 mM ferrous ammonium sulfate and 0.1% catalase. After incubation for 5 min. at 37°C, 200 μM L-tyrosine and 1 mM (6R)-5,6,7,8-tetrahydro-L-biopterin (6R-BH₄) were added to the mixture and incubated for another 20 min. at 37°C.

The reaction was stopped by adding 100 μl of stop solution, and the mixture was incubated on ice for 10 min. The assay mixture was centrifuged for 10 min. at 1,000 x g at 4°C, and the supernatant was filtered through 0.2 μm PVDF microspin filters (Analytical) and analyzed by HPLC.

**HPLC analysis:** DA, DOPAC and L-Dopa were separated by HPLC analysis using an ESA CoulArray Model 5600A high performance liquid chromatography system (Chelmsford, MA) and a Phenomenex Synergi 4 μm Hydro-RP column (4.6 X 150 mm) (Torrance, CA), according to the method of McClung and Hirsh (McClung and Hirsh, 1999). Heads from 75-200 adults
were extracted in 100-200 \( \mu l \) 0.1 M perchloric acid; extracts were filtered through 0.2 \( \mu m \) filters. Ten \( \mu l \) of each extract was injected for each sample. The mobile phase contained 75 mM sodium phosphate, pH 3.0, 1.4 mM octanesulfonic acid, 25 \( \mu M \) ethylene diamine tetraacetic acid, 100 \( \mu l/L \) triethylamine, and 7% acetonitrile. Separations were performed with isocratic flow at 1 ml/min. Amines were detected with ESA electrochemical analytical cell, Model 5011, (channel 1 at -50mV, channel 2 at 300 mV). Pool sizes were determined relative to freshly prepared standards (Sigma Chemicals, St. Louis, MO). Analysis was performed using ESA CoulArray software.

**Confocal Microscopy**: Adult flies of the aforementioned strains were collected at 4-5 days post-eclosion and aged for 20 days. Whole mount of dissected brains were observed for dopaminergic neuron number and morphology using a Leica TCS SP2 AOBS confocal microscope. Brain images were then coded and insets were selected based on anatomical regions of dopamine neuron subclasses. Images were then quantified using ImageJ Cell Counter plug-in using 15 replicates of each anatomical region per genotype. In brief, images were first converted into 16-bit images then the detection threshold was set to include visible neurons, regardless of pixel density. The results were graphed and analyzed using Graphpad Prism 5.0 (GraphPad Software, Dan Diego, CA USA) as described in respective figure legends.

**Catalase Assay**: Catalase enzyme activity was calculated using methods described in Chaudhuri *et al.* (Chaudhuri et al., 2007) with minor modifications. Briefly, 15 male flies of each genotype were aged 20 days and decapitated using liquid nitrogen. Fly heads were homogenized using 150 \( \mu l \) of 0.1 M sodium-potassium phosphate buffer, pH 7.0, containing 0.1 M Triton X-100 (Fisher Biotech, Hampton, NH). Samples were incubated at 4° C for 10 mins then centrifuged for 10
mins at 12,000 x g. A mixture of the reaction solution containing 48.65 mM hydrogen peroxide (H$_2$O$_2$) in 0.1 M sodium- potassium phosphate buffer (pH 6.8) and 20 μl of crude extract were combined to equal a 2.55 ml final volume. Catalase activity was measured by negative delta absorbance (230nm), using 62.4 as an H$_2$O$_2$ molar extinction coefficient (Nelson and Kiesow, 1972), where one unit of enzyme activity equals 1 mmol H$_2$O$_2$ decomposed/min.

**Nitric oxide synthase activity assay:** NOS activity is measured by the detection of nitrites using a modified Griess Reagent Assay described in Ajjuri & O’Donnell (Ajjuri and O'Donnell, 2013). Briefly, 20 brains of aged, male flies were incubated in 50 μl of Graces Insect Medium (Invitrogen) for 6 hrs. The medium was then aspirated, centrifuged and the supernatant was mixed in equal volume with Modified Griess reagent (Sigma-Aldrich). The mixture was stored for 10 mins at 25°C, then measured using a Biorad Nanodrop spectrophotometer at 548 nm. Absorbance values of each sample were then compared to a sodium nitrate standard curve, and then calculated as μM of nitrite/sample.

**Statistical analysis:** Data Analyses was done with GraphPad Prism (San Diego, CA), using one-way ANOVA (Bonferroni post-test) or two-tailed Student’s T-test, assuming equal variances. Details of analyses are presented in figure legends.
RESULTS

Heterozygous loss-of-function Catsup\textsuperscript{26} mutation and over-expression of \( \alpha \)-synuclein in dopaminergic neurons interact to modulate tyrosine hydroxylase activity

Normal \( \alpha \)-syn function has been shown to decrease TH activity \textit{in vitro} and reduce DA levels \textit{in vivo} (Kuwahara et al., 2006; Perez et al., 2002). To determine whether \( \alpha \)-syn had a similar effect on \textit{Drosophila} TH, we generated lines of flies expressing either \( \alpha \)-syn \textit{wt} or the A30P mutant form in dopaminergic neurons under the control TH-Gal4, with co-expression of GFP. All subsequent experiments were done using male flies. As early as 3-5 days post-eclosion, we observed significant reductions in the specific activity of TH (Fig. 4.1). Conversely, the loss of Catsup function, as first reported by Stathakis \textit{et al.} (Stathakis et al., 1999) and confirmed by Wang \textit{et al.} (Wang et al., 2011), dominantly leads to an elevation of specific TH enzyme activity as well as an increase of DA levels in adult \textit{Drosophila} CNS. This stimulation of DA synthesis also results in an increase of DA-related mobility, confirming that the excess DA was synaptically active (Chaudhuri et al., 2007). To determine whether these genes might interact functionally to modulate TH activity, we expressed human \( \alpha \)-syn \textit{wt} or human \( \alpha \)-syn A30P in dopaminergic neurons in a heterozygous Catsup\textsuperscript{26} mutant background and found that TH specific activity levels were elevated to normal levels in both \( \alpha \)-syn genotypes (Fig. 4.1). While neither loss of Catsup function or expression of \( \alpha \)-syn exhibited epistasis, it is clear that these genotypes can interact in a beneficial manner with respect to TH modulation.

Analysis of mobility, DA levels and DA turnover rates in \textit{Drosophila} expressing wild type \( \alpha \)-synuclein in the Catsup\textsuperscript{26} mutant background

Previously, we reported that DA pools at day 1 post-eclosion in \textit{Drosophila} expressing wild type \( \alpha \)-syn in dopaminergic neurons were indistinguishable from wild type controls, but by day 20
post-eclosion reduced DA pools were evident (Knight et al., 2014). As TH catalyzes the rate-limiting reaction in DA synthesis, we expected that the reduction of TH activity in the presence of α-synuclein at 3-5 days post-eclosion would lead to similarly diminished DA pools. However, we observed only a slight downward and insignificant trend in DA levels when α-syn

Figure 4.1. α-synuclein-dependent reduction in TH activity is rescued to normal levels by loss of Catsup function. Catsup^{26} mutants have higher TH activity levels than the UAS-GFP; TH-Gal4 flies, used as the control group. Expression of both α-syn wild type and α-syn A30P in the dopaminergic neurons reduced TH enzyme activity. Expression of both α-syn variants in the Catsup^{26} mutant background increases TH activity to levels insignificant from UAS-GFP; TH-Gal4. Enzyme activity assay were performed on flies aged for 3-5 days following eclosion (n=5). Statistical analysis was performed using one-way Anova. Error bars represent mean SEM. (* p<0.05, ** p<0.01, *** p<0.001).
was expressed in a wild type background. In contrast, DA pools are elevated in the heterozygous Catsup\textsuperscript{26} genotype. When α-syn is expressed in the Catsup\textsuperscript{26} background, a significant increase in DA (\sim 1.5 fold) relative to the DA pools of flies expressing α-syn\textit{wt} in a Catsup\textsuperscript{*} background was observed. This result suggests that reducing Catsup function can have a beneficial effect prior to the onset of clear neuronal dysfunction (Fig. 4.2A).

To assess whether the modulation of TH activity and DA pools culminated in parallel effects on movement, the longest recognized manifestation of PD, we assayed the mobility of these flies using a ‘time-in-motion’ method (Carbone et al., 2006). Previously, Kuwahara et al. (2006) reported that expression of the PD-associated mutant forms of α-syn, A30P and A53T, but not wild type α-syn, led to a reduction in DA-linked food seeking behavior in \textit{C. elegans} (Kuwahara et al., 2006). In \textit{Drosophila}, the expression of human α-syn leads to age-dependent deficits in DA-linked motor function beginning around 35 days post-eclosion in α-syn\textit{wt} flies, and 23 days in A30P mutant flies (Feany and Bender, 2000). Heterozygous Catsup\textsuperscript{26} mutant flies display increased mobility (Fig. 4.2C), confirming our previous results (Wang et al., 2011). While there is no significant loss of motor function in when α-syn\textit{wt} is expressed in 3-7 day old adult Catsup\textsuperscript{*} flies (Fig. 4.2C), α-syn\textit{wt} modulates the hyperactivity of Catsup\textsuperscript{26}/+ flies (Fig. 4.2C).

Interestingly, we observed that the expression of α-syn A30P negatively affects both DA levels (Fig. 4.2B) and mobility (Fig. 4.2D) in a Catsup\textsuperscript{*} background, in contrast to the effects of the wild type form of α-syn. While the heterozygous Catsup\textsuperscript{26} mutant background rescued DA levels in flies expressing α-syn A30P (Fig. 4.2B), the Catsup\textsuperscript{26} mutation was unable to rescue the deficits in mobility (Fig. 4.2D). We have previously observed a strong positive
Figure 4.2. Analysis of mobility and DA levels in *Drosophila* expressing α-synuclein in the *Catsup*\textsuperscript{26} mutant background. (A) Expression of α-syn \textit{wt} in the *Catsup*\textsuperscript{26} background modulates the effects of the *Catsup*\textsuperscript{26} mutation on DA levels. (B) Expression of α-syn A30P results in significantly reduced DA levels relative to those in the control, UAS-GFP; TH- Gal4. The *Catsup*\textsuperscript{26} mutation rescues DA levels when combined with α-syn A30P expression. Neurochemical assays were performed on samples of 100 heads (n=5) from flies aged between 3-5 days. (C) Mobility deficits were not observed when α-syn \textit{wt} was expressed in dopaminergic neurons, in comparison to UAS-GFP; TH- Gal4. However, when compared to α-syn \textit{wt} expression in the *Catsup*\textsuperscript{26} background, a significant decrease of mobility was observed. (D) Expression of α-syn A30P leads to a significant decrease of mobility, which is not elevated by expression in the *Catsup*\textsuperscript{26} background. The mobility assays were conducted with flies between the ages of 3-7 days old (n=20). Note that the results for the control and *Catsup*\textsuperscript{26} genotypes are identical in graphs A vs. B and C vs. D. The effects of expression of mutant and wildtype α-synuclein were assayed simultaneously, but separated graphically for clarity. Statistical analysis was performed using one-way ANOVA. Error bars represent mean SEM. (* p< 0.05, ** p< 0.01, *** p< 0.001).

correlation between TH activity, DA pools and mobility (Stathakis et al., 1999; Wang et al., 2011). Hyperactivity under conditions of elevated DA synthesis demonstrates that the excess DA is synaptically active, and we have reported previously that loss-of-*Catsup* function mutants have elevated VMAT activity (Wang et al., 2011), suggesting that the excess DA is packaged in
synaptic vesicles. We were surprised, therefore, to find that the rescue of TH activity and DA levels by the Catsup$^{26}$ mutation in $\alpha$-syn A30P flies was not accompanied by an improvement to mobility. As mobility is associated with the release of vesicular DA into the synapse, these results suggest that although reduction of Catsup expression increases overall dopamine levels, it is unable to restore wildtype regulation of DA synaptic transmission in the presence of the A30P mutant form of $\alpha$-syn.

Failure to rescue the mobility deficit could result either from inhibition of vesicular loading of DA by VMAT, or from interference with synaptic release. Due to the highly-reactive nature of cytosolic DA and the toxicity of its metabolites, an excess of DA in the presynaptic neuron resulting from defective VMAT-mediated DA transport into vesicles will lead to a significant increase in oxidative stress (Lawal et al., 2010; Wang et al., 2011). We first tested whether $\alpha$-syn A30P flies possessed elevated levels of cytosolic DA, we assessed the rate of DA turnover in the presence of mutant $\alpha$-syn. This was done by measuring the ratio of DA to DOPAC, a stable, non-toxic by-product of DA metabolism, which can then be used as an index of DA storage, reuptake and metabolism (Di Monte et al., 1996; Drolet et al., 2004). An elevated DA turnover rate (i.e. a greater DOPAC/DA ratio) indicates inefficient clearing of DA from the cytosol, where DA is degraded by monoamine oxidase (MAO) into DOPAC and ROS such as superoxide ($\text{O}_2^-$) and hydrogen peroxide ($\text{H}_2\text{O}_2$).
Figure 4.3. DA oxidation resulting from the expression of the wild type, but not the A30P mutant form of \(\alpha\)-syn can be fully rescued by the \textit{Catsup}^{26} mutation. Neither the \textit{Catsup}^{26} mutants nor the \textit{UAS-GFP; TH-Gal4} (wild type) flies showed appreciable levels of DOPAC, resulting in negligible ratios for DA turnover. Expression of both wt and A30P \(\alpha\)-syn leads to elevated DA turnover rates, which are a result of increased DOPAC levels. The \textit{Catsup}^{26} mutation completely rescues DA turnover rates when combined with \(\alpha\)-syn \textit{wt} and shows partial rescue with \(\alpha\)-syn A30P. Neurochemical assays were performed on samples of 100 heads (n=5) from flies aged between 3-5 days. Statistical analysis was performed using one-way Anova. Error bars represent mean SEM. (** p<0.001).
We hypothesized that if $Catsup^{26}/UAS$-GFP; UAS- $\alpha$-synuclein A30P/ $TH$- Gal4 flies were deficient in clearing and trafficking cytosolic DA, we should observe a significant increase in the DOPAC/DA ratio. In support of this hypothesis, we observed that expression of $\alpha$-syn mutant A30P resulted in significantly elevated DA turnover rates relative to control values ($UAS$-GFP; $TH$-Gal4) (Fig. 4.3). Even though DA pools were not yet significantly diminished in 3-7 day post-eclosion flies expressing the wild type form of $\alpha$-syn, we clearly detect early signs of impending neuron loss, here measured as elevated DA turnover. Flies expressing $\alpha$-syn wt in the $Catsup^{26}$ mutant background displayed a complete rescue of DA turnover rates (Fig. 4.3). This observation is consistent with our previous report (Wang et al., 2011) demonstrating that VMAT activity and therefore, vesicular transport of DA, is up-regulated with reduction of expression. In contrast, $Catsup^{26}$ mutant flies possessing $\alpha$-syn A30P showed only a partial amelioration in DA turnover rates (Fig. 4.3), since the reduction of TH activity, and in consequence, DA synthesis, by $\alpha$-syn A30P is fully rescued by reduction of $Catsup$ expression (Fig. 4.1). Since the DOPAC:DA ratio is only moderately suppressed by reduced expression of $Catsup$, we infer that the A30P mutant form of $\alpha$-syn is interfering with the transport function of VMAT wildtype or $Catsup^{26}$ mutant flies along with the elevation of DA turnover suggests that the A30P mutation disrupts the ability of VMAT to package cytosolic DA, consequently reducing the transmission of DA into the synapse.

**Loss of dopaminergic neurons induced by $\alpha$-synuclein expression is reduced in the $Catsup^{26}$ mutant background**

It has been previously reported that over-expression of $\alpha$-synuclein leads to loss of dopaminergic neurons in $Drosophila$, $C. elegans$, and mammalian cell lines (Auluck, 2001; Cao et al. 2005; Kuwahara et al., 2006; Xu et al., 2002). Previously, we have shown $Catsup^{26}$ mutants to be
neuroprotective for dopaminergic neurons in response to paraquat exposure, which, like α-

Figure 4.4. α-Synuclein wt or A30P expression has detrimental effects on neuronal health and survival that can be rescued by a loss-of-function Catsup26 mutation. The brains of male flies (aged to 25 days) were imaged using confocal analysis. (A) UAS-GFP/+; TH-Gal4/+ and (B) Catsup26/UAS-GFP; TH-Gal4/+ are used as controls. (C) UAS-GFP/+; UAS-α-synuclein wt/TH-Gal4 and (D) UAS-α-synuclein A30P/TH-Gal4, respectively show dopaminergic neuron degeneration indicated by decreased signal of GFP. (E) Catsup26/UAS-GFP; UAS-α-synuclein wt/TH-Gal4 and (F) Catsup26/UAS-GFP; UAS-α-synuclein A30P/TH-Gal4 exhibit an increase in neuron survival as well as morphology characteristic of typical neuron structure. Scale bar represents 50 μm.
Overall, the reduction of mobility observed when expressing $\alpha$-syn $A30P$ in either synuclein toxicity, causes an elevation in DA turnover (Chaudhuri et al., 2007). To determine whether these behavioral and neurochemical enhancements resulting from the knockdown of $Catsup$ are accompanied by improved neuronal health and survival of dopaminergic neurons, we used examined dopaminergic neurons expressing GFP driven by TH-Gal4 (Fig. 4.4). As synuclein-dependent loss of dopaminergic neurons is negligible prior to 20 days post eclosion despite clear evidence of neurochemical dysfunction, we aged male flies to 25 days post-eclosion prior to neuron imaging. We observed that, by day 25, expression of either the wild type or mutant forms of $\alpha$-synuclein in a $Catsup^+$ background (+/UAS-GFP; UAS- $\alpha$-synuclein wt/ TH-Gal4 and +/UAS-GFP; UAS- $\alpha$-synuclein A30P/ TH-Gal4, respectively) resulted in decreased GFP fluorescence (Fig. 4.4), as well as a significant loss of dopaminergic neurons across all the six neuron clusters examined (Fig. 4.5).

It is important to note that there was no significant difference in neuron loss between the $\alpha$-syn wt and $\alpha$-syn $A30P$ expressing flies, except in one cluster, PPL1, which also is the most paraquat- sensitive group of DA neurons (Fig. 4.5A-B). The differences in improvement of neuron survival in the $Catsup^{26}$ mutant background were insignificant between flies expressing wild type and mutant $\alpha$-synuclein, except in the PPM2 and PAL clusters, where the $Catsup^{26}$ mutation showed greater survival rates in response to $\alpha$-syn wt expression than $\alpha$-syn $A30P$ expression (Fig. 4.5A-B). Overall, the knockdown of $Catsup$ resulted in the significant rescue of dopaminergic neuron survival from $\alpha$-syn- induced toxicity.
Figure 4.5. *Catsup*^26^ mutation is neuroprotective for α-synuclein expression. Flies were aged to 25 days and six DA neuron clusters were counted individually. (A) When α-syn wt was expressed selectively in dopaminergic neurons, all DA neuron clusters showed a significant lose of neurons. The *Catsup*^26^ mutation rescues neuron survivorship of all six clusters. (B) α-syn A30P expression leads to a significant reduction in DA neuron numbers in all clusters except the PPM1 cluster. Expression in the *Catsup*^26^ mutant background rescues neuron survival in the PPM2, PPM3, PPL1 and PPL2 clusters but not in the PAL and PPM1 clusters. Statistical analysis was performed using one-way Anova. Error bars represent mean SEM. (* p < 0.05, ** p < 0.01, *** p < 0.001).
Figure 4.6. After reserpine treatment, DA levels are further reduced in flies expressing α-syn wt but not in flies expressing α-syn A30P. DA levels were determined following exposure to 15 mM reserpine for 8-10 hrs. For the UAS-GFP; TH-Gal4 flies, DA levels are decreased by approximately 1/3, but expression of α-syn wt led to a greater than 2 fold decrease of DA levels. No significant difference in DA levels was observed in observed +/UAS-GFP; UAS-α-synuclein A30P/TH-Gal4 flies after reserpine treatment. Neurochemical assays were performed on samples of 100 heads (n=5) from flies aged between 3-5 days. Statistical analysis was performed using one-way Anova. Error bars represent mean SEM. (** p< 0.01, *** p<0.001).
Catsup\textsuperscript{26} mutation partially alleviates \(\alpha\)-synuclein-dependent reserpine susceptibility

If our conclusion that differential rescue of \(\alpha\)-syn-induced DA turnover and related functions by the Catsup\textsuperscript{26} mutation is based upon differences in their effects on VMAT is correct, we predicted that treating flies with reserpine, an irreversible VMAT inhibitor, would result in increased sensitivity in \(\alpha\)-syn wt but little effect in A30P mutants. In agreement with this prediction, we found that \(\alpha\)-syn wt flies fed reserpine showed depressed DA levels (Fig. 4.6), while mutant A30P flies had no significant change. Two potential explanations for this result are that A) A30P mutant flies already suffer from such drastic DA deficiencies that inhibiting VMAT activity has little or no effect, or B) A30P protein is directly or indirectly affecting DA packaging or transmission in such a manner that further inhibition of VMAT is irrelevant in modifying DA levels.

As Catsup acts as a negative regulator of VMAT activity (Wang et al., 2011), reduced Catsup expression in the Catsup\textsuperscript{26} mutant flies should then result in increased VMAT activity. We found that both \(\alpha\)-syn wt and \(\alpha\)-syn A30P, when expressed in the Catsup\textsuperscript{26} mutant background, were less susceptible to the reserpine and displayed levels of DA and mobility near or above that of the wildtype controls (Fig. 4.7A-D). Additionally, the inhibition of VMAT via reserpine increased the rate of DA turnover for all genotypes tested relative to DOPAC/DA turnover in non-treated groups (Fig. 4.8 compared to Fig. 4.3, respectively). When expressed in the Catsup\textsuperscript{26} mutant background, turnover rates in both \(\alpha\)-syn wt and \(\alpha\)-syn A30P are drastically reduced (Fig. 4.8). However, expression of the A30P mutant causes significantly less rescue with Catsup knockdown than the expression of \(\alpha\)-syn wt in every reserpine-treatment assay, further evidence supporting the hypothesis that the A30P-induced defects are a result of interference with VMAT.
function.

Figure 4.7. Analysis of mobility, DA levels and DA turnover rates in *Drosophila* expressing α-synuclein in the *Catsup*26 mutant background after reserpine treatment. All flies were exposed to 15 mM reserpine for a period of 8-10 hrs prior to performing the assays. The *Catsup*26 mutation rescues DA levels (A) and increases mobility (B) from that observed with expression of α-syn wt alone. When α-synuclein A30P is expressed, both DA levels (C) and mobility (D) deficits are observed; these are subsequently rescued in the presence of the *Catsup*26 mutation. Neurochemical assays were performed on samples of 100 heads (n=5) from flies aged between 3-5 days. The mobility assays were conducted with flies between the ages of 3-7 days old (n=20). Statistical analysis was performed using one-way Anova. Error bars represent mean SEM. (* p < 0.05, ** p < 0.01, *** p < 0.001).
Figure 4.8. Analysis of mobility, DA levels and DA turnover rates in Drosophila expressing α-synuclein in the Catsup\textsuperscript{26} mutant background after reserpine treatment. Reserpine treatment in general increases DOPAC levels and, in turn, increase DA turnover rates. The Catsup\textsuperscript{26} mutants have reduced rates of DA catabolism, as indicated by the low DOPAC/DA ratios. Expression of both wt and A30P variants of α-syn leads to elevated DA turnover rates. When combined with the Catsup\textsuperscript{26}, α-syn wt expression DOPAC/DA ratios are significantly reduced from those observed with α-syn wt expression alone and UAS-GFP; TH-Gal4. Expression of α-syn A30P in the Catsup\textsuperscript{26} mutant background decreases DA turnover rates when compared to expression of α-syn A30P in the wildtype representative background, but is still significantly higher than UAS-GFP; TH-Gal4. Neurochemical assays were performed on
samples of 100 heads (n=5) from flies aged between 3-5 days. Statistical analysis was performed using one-way Anova. Error bars represent mean SEM. (* p< 0.05, *** p< 0.001).

DISCUSSION

Current PD literature indicates that α-syn toxicity is likely the result of elevated cellular ROS production. It was reported by Lee et al. (2001) that mutant forms of α-synuclein render cells more sensitive to oxidative stress and increases the level of protein and lipid oxidation. Xu et al. (2002) also observed that overexpression of α-syn in human fetal dopaminergic neurons lead to an accumulation of ROS and eventually apoptosis, whereas, expressing α-syn in non-dopaminergic neurons is neuroprotective (Xu et al., 2002). Additionally, the overexpression of the human Cu/Zn superoxide dismutase (SOD), which reduces the levels of cellular ROS, rescues the neurodegenerative and motor deficit phenotypes associated with expression of α-syn A30P (Botella et al., 2008). These studies provide a strong line of evidence for the crucial role played by oxidative stress in the neurodegenerative phenotypes observed in Parkinson’s disease.

As the highly-reactive nature of cytosolic free DA is able to generate a cascade of oxidative stress (Kuhn et al., 2006; Miller et al., 1999; Pedrosa and Soares-da-Silva, 2002; Stokes et al., 2000; Witt and Flower, 2006), we predict that the disruption of DA vesicular packaging mediated through VMAT is the source of accumulating cellular damage. Under both basal and disease conditions, VMAT offers neuroprotection by reducing free-cytosolic DA before it is metabolized into toxic metabolites (Caudle et al., 2007; Mosharov et al., 2003; 2009). Overexpression of VMAT has been shown to partially rescue the neurodegenerative phenotypes associated with parkin and α-syn- induced Parkinson’s disease in Drosophila (Sang et al., 2007;
Soon et al., 2007), as well as to enhance cytosolic DA sequestration when $\alpha$-syn $A30P$ is expressed (Park et al., 2007).

In this study, we report that a loss-of-function $Catsup^{26}$ mutant partially rescues the $\alpha$-syn-related behavioral and neurodegenerative phenotypes observed in $Drosophila$ by increasing DA biosynthesis and reducing the levels of cytosolic DA. Catsup, a transmembrane protein, has previously been shown to negatively modulate VMAT and TH activity (Stathakis et al., 1999; Wang et al., 2011). In such a capacity, Catsup is centrally positioned to regulate DA homeostasis $in\ vivo$. We found that although the mutation $Catsup^{26}$ was able to enhance TH activity (Fig. 4.1) and DA levels (Fig 4.2C) in $\alpha$-syn $A30P$ flies, it was unable to rescue mobility (Fig. 4.2D), indicating a disruption in the packaging and/or exocytosis of DA into the synapse. It is also important to note that, while both $\alpha$-syn $wt$ and $\alpha$-syn $A30P$ increase DA turnover rates (Fig. 4.3), $\alpha$-syn $A30P$ expression does not result in a dramatic reduction of TH activity, and that this reduction is fully rescued in the mutant $Catsup$ flies. From these findings, we can deduce that, while the production of DA is not severely altered, the transport and sequestering of DA in these $A30P$-expressing $Catsup^{26}$ mutant flies is impaired.
Figure 4.9. Following reserpine treatment, flies expressing heterozygous Catsup\textsuperscript{26} mutation are protected against dopaminergic neuron loss in \( \alpha \)-synuclein wildtype, but not the \( \alpha \)-synuclein A30P mutant, backgrounds. The brains of aged male flies exposed to reserpine (15mM for 8-10hrs) were imaged using confocal microscopy and quantified by individual DA neuron clusters. (A) Flies expressing \( \alpha \)-syn \textit{wt} in DA neurons are significantly more sensitive to VMAT inhibition in all DA neuron clusters compared to control flies. Flies expressing Catsup\textsuperscript{26}/+ mutation, however, show enhanced neuron survivorship of all six clusters. (B) \( \alpha \)-syn A30P expression results in a significant loss of DA neurons in all clusters and the expression of the Catsup\textsuperscript{26} mutation offers little to no protection against neurodegeneration. Statistical analysis was performed using one-way Anova. Error bars represent mean SEM. (* \( p < 0.05 \), ** \( p < 0.01 \), *** \( p < 0.001 \)).
Previously, we found that Catsup and VMAT co-localize in dopaminergic neurons, and that *Catsup*26 mutants are resistant to VMAT inhibitor reserpine and are able to reduce the DA turnover rates observed in VMAT mutants (Wang et al., 2011), indicating a functional interaction between Catsup and VMAT where Catsup is able to negatively modulate VMAT activity. To delineate the mechanism by which the *Catsup*26 mutation reduces DA turnover rates in α-syn- expressing flies, we treated all genotypes with reserpine. After reserpine exposure, levels of DA were reduced in the presence of α-syn wt, likely due to increased DA metabolism resulting from the reserpine-mediated inhibition of DA vesicular packaging. DA levels were not reduced in α-syn A30P flies, indicating that, even during conditions of reduced DA synthesis, α-syn A30P is still able to enhance DA metabolism.

One possible explanation for this differential effect is the ability of the A30P mutation to increase permeability of membranes *in vitro* (Volles et al., 2001, Rochet et al., 2004; Tsigelny et al., 2007). As previously stated, A30P is also able to disrupt the binding of α-syn to neuronal membranes and re-localize α-syn away from the synapse in cultured neurons (Fortin, 2004). Due to its location in the presynaptic neuron, its affinity for membranes and its ability to affect cellular DA levels, α-syn has been implicated in neurotransmitter release and regulation, and has been shown to promote DA release (Cabin et al., 2002; Murphy et al., 2000). This said, if α-syn is in fact promoting neurotransmitter function, and the A30P mutant is disrupting this function and relocating α-syn, the inhibition of VMAT activity via reserpine should have little effect on A30P mutants, which is consistent with our findings. In the Catsup26 mutant background, however, DA production is increased and Catsup inhibition of VMAT function is alleviated, allowing the partial restoration of DA vesicular packaging, as seen by reduced DA-to-DOPAC
turnover, even after reserpine exposure. Our findings strongly support the hypothesis that the unimpeded activity of VMAT resulting from loss-of-function Catsup expression is neuroprotective against α-synuclein–induced dopamine-mediated cytotoxicity.

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CHAPTER FIVE
MUTANT HUMAN TORSINA SUPPRESSES GTPCH EXPRESSION
IN A DROSOFLILA MODEL OF DYSTONIA


This is an Open Access article distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/3.0), which permits unrestricted use, distribution and reproduction in any medium provided that the original work is properly attributed. Author contributions are as follows: N.W.-I., R.R.A., B.W.H., O.M.D., J.M.O., and N.I. conceived and designed the experiments. N.W.-I., R.R.A., B.W.H., O.M.D., and N.I. performed the experiments. N.W.-I., R.R.A., B.W.H., O.M.D., J.M.O., and N.I. analyzed the data. N.W.-I., R.R.A., B.W.H., O.M.D., X.O.B., J.M.O., and N.I. wrote and edited the paper.

ABSTRACT

Dystonia represents the third most common movement disorder in humans with over 20 genetic loci identified. TOR1A (DYT1), the gene responsible for the most common primary hereditary dystonia, encodes torsinA, an AAA ATPase family protein. Most cases of DYT1 dystonia are caused by a 3 bp (DGAG) deletion that results in the loss of a glutamic acid residue (DE302/303)
in the carboxyl terminal region of torsinA. This torsinAΔE mutant protein has been speculated to act in a dominant-negative manner to decrease activity of wild type torsinA. *Drosophila melanogaster* has a single torsin-related gene, *dtorsin*. Null mutants of dtorsin exhibited locomotion defects in third instar larvae. Levels of dopamine and GTP cyclohydrolase (GTPCH) proteins were severely reduced in dtorsin-null brains. Further, the locomotion defect was rescued by the expression of human torsinA or feeding with dopamine.

Here, we demonstrate that human torsinAΔE dominantly inhibited locomotion in larvae and adults when expressed in neurons using a pan-neuronal promoter Elav. Dopamine and tetrahydrobiopterin (BH₄) levels were significantly reduced in larval brains and the expression level of GTPCH protein was severely impaired in adult and larval brains. When human torsinA and torsinAΔE were co-expressed in neurons in dtorsin-null larvae and adults, the locomotion rates and the expression levels of GTPCH protein were severely reduced. These results support the hypothesis that torsinAΔE inhibits wild type torsinA activity. Similarly, neuronal expression of a *Drosophila* DtorsinΔE equivalent mutation dominantly inhibited larval locomotion and GTPCH protein expression. These results indicate that both torsinAΔE and DtorsinΔE act in a dominant-negative manner. We also demonstrate that Dtorsin regulates GTPCH expression at the post-transcriptional level. This *Drosophila* model of DYT1 dystonia provides an important tool for studying the differences in the molecular function between the wild type and the mutant torsin proteins.
INTRODUCTION

Dystonia is the third most common movement disorder in humans, after essential tremor and Parkinson’s disease (Defazio, 2010). Dystonia comprises a group of movement disorders that are characterized by involuntary movements and abnormal postures. It is a complex disease involving at least 20 genetic loci in humans (Tarsy and Simon, 2006; Breakefield et al., 2008; Bruggemann and Klein, 2010).

One of the loci, TOR1A/DYT1, is responsible for most cases of early-onset dystonia and has been the most studied form of dystonia (Breakefield et al., 2001; Atai et al., 2012; Bragg et al., 2011). It is an autosomal dominant syndrome with onset between 5 to 28 years of age and low penetrance. The TOR1A gene encodes torsinA, a 332 amino acid protein from the AAA ATPase family. The torsinA protein is widely expressed in the body and is localized within the lumen of the endoplasmic reticulum and the nuclear envelope (Breakefield et al., 2008), but its function is still under study. A 3-bp (DGAG) deletion that removes one of a pair of glutamic acid residues (DE302/E303) in the carboxyl terminal region of torsinA causes the autosomal dominant dystonia phenotype (Breakefield et al., 2008; Bragg et al., 2011). TorsinA displays LAP1 and LULL1-dependent ATPase activity, while the torsinA ΔE protein is defective in this activation (Zhao et al., 2013). The torsinA ΔE (DE302/303) mutant protein has been speculated to act in a dominant-negative manner, so that the wild type function is reduced but not eliminated in the cells expressing both torsinA and torsinA ΔE, although this has never been clearly demonstrated (Breakefield et al., 2001; Breakefield et al., 2008).

Most AAA ATPase proteins form oligomeric complexes and use energy from ATP hydrolysis to regulate protein folding, membrane trafficking, and vesicle fusion (Neuwald et al., 1999; Vale,
Although torsinA is widely expressed in human tissue, it is considered to have a critical role in the central nervous system, where it is present in neurons at high levels during development and in adult life (Augood et al., 2003; Xiao et al., 2004; Vasudevan et al., 2006). In homozygous torsinA-knock-out mice, abnormal nuclear membrane morphology was observed in neurons, suggesting a functional role of torsinA in maintaining the normal structure of the nuclear envelope in the central nervous system (Goodchild et al., 2005). TorsinA has been shown to interact with nesprins, which are anchored in the outer nuclear envelope and form bridges to the cytoskeleton (Nery et al., 2008; Jungwirth et al., 2011; Atai et al., 2012), suggesting an important functional role of torsinA at the nuclear envelope, including nuclear polarization during cell migration (Nery et al., 2008). Recent studies also implicate torsinA in egress of Herpes simplex virus capsids (Maric et al., 2011) and large ribonucleoprotein particles (Jokhi et al., 2013) out from the nucleus into cytoplasm.

The fruit fly, Drosophila melanogaster, provides an excellent model system to study functions of human disease genes and has contributed to better understanding of many human diseases (Bellen et al., 2010). Drosophila has a single TOR1A-related gene, dtorsin (Torsin), at position 4C11 on the X chromosome (Ozelius et al., 1999; Breakefield et al., 2001; Wakabayashi-Ito et al., 2011). The dtorsin-encoded protein, Dtorsin, comprises 339 amino acids with 31.9% identity to human torsinA and also displays the characteristic features of the AAA ATPase gene family members (supplementary material Fig. S1) (Ozelius et al., 1999). We recently isolated dtorsin-null mutants and showed that hemizygous mutant third instar male larvae exhibited locomotion defects that were rescued by feeding dopamine (Wakabayashi-Ito et al., 2011). The dtorsin-null mutation was semi-lethal at the pupal stage with only less than 1% reaching adult stage. The dtorsin mutant exhibited a very strong genetic interaction with Pu (Punch: GTP cyclohydrolase:
GTPCH), the ortholog of the human gene underlying dopa-responsive DYT5a dystonia (GCH1) (Segawa, 2009). Moreover, biochemical analysis revealed a severe reduction of GTPCH protein and activity in dtorsin-null adults and larvae, as well as marked reduction in tetrahydrobiopterin (BH4), the terminal product of the GTPCH pathway. In contrast, levels of tyrosine hydroxylase (TH) protein, which catalyzes the rate-limiting step in dopamine production, were not affected, although dopamine pools were reduced (Wakabayashi-Ito et al., 2011). Since GTPCH is rate limiting for the synthesis of BH4, and BH4 is required by TH as a rate-limiting cofactor for dopamine synthesis in flies as in mammals (Krishnakumar et al., 2000), these data suggested that dtorsin plays a novel role in dopamine metabolism as a positive-regulator of GTPCH protein levels in Drosophila. Moreover, the wild type human torsinA cDNA expressed with the pan-neuronal promoter elavGAL4 rescued dtorsin-null male larval mobility with marked significance. These results demonstrated that the function of torsin in regulating larval locomotion is conserved between the fly and the human proteins (Wakabayashi-Ito et al., 2011). However, the fly dtorsin-null mutant is not an authentic DYT1 disease model system, since the dtorsin-null mutant line does not express any functional Dtorsin protein, while mutated torsinA protein is expressed together with normal torsinA in the DYT1 patients (Breakefield et al., 2001).

To investigate the molecular mechanism underlying the human disease caused by mutated torsinA protein using the fly system, we expressed human wild type torsinA and/or torsinAΔE cDNA using the pan-neuronal GAL4 driver, elavGAL4, in fly brains. We report here that expression of the human mutant form caused larval and adult locomotion defects, and severe reduction of GTPCH protein, dopamine, and BH4 levels in larval brains and adult heads. Moreover, co-expression of human torsinAΔE and the wild type human torsinA in dtorsin-null
males resulted in similar larval/adult locomotion and neurochemical defects, suggesting that the human torsinA ΔE exerts dominant-negative effects on human wild type torsinA protein in Drosophila neurons, as in human tissues. Furthermore, a comparable mutation in the Drosophila gene, dtorsinA ΔE also had a dominant-negative effect on larval locomotion and GTPCH protein level, as did human torsinA ΔE. Finally, we report that the relative amount of GTPCH RNA was similar in wild type and dtorsin-null adult male heads, suggesting that GTPCH protein levels depend on wild type dtorsin-activity at the post-transcriptional level. Our findings establish conclusively that torsinA ΔE dominantly inhibits the normal function of torsinA and Dtorsin including the regulation of GTPCH expression. These results demonstrate that Drosophila provides a powerful system for studying the molecular abnormalities caused by the torsinA ΔE mutation.

**EXPERIMENTAL PROCEDURES**

*Fly stocks* Flies were grown on standard medium containing cornmeal, yeast and agar at 25 °C in fly incubators with a constant humidity of 70% (Ashburner and Roote, 2007). ElavGAL4 transgenic strain was obtained from the Drosophila Stock Center (Bloomington, IN USA). The dtorsin- null lines, y w dtorsinKO13/FM7i, Act-GFP and w elavGAL4 dtorsinKO13/ FM7i, Act-GFP were described previously (Wakabayashi-Ito et al., 2011). The Punch-null line, PuZ22, was previously described (Mackay et al., 1985). The RNAi line for GTPCH (Pu) gene, v107296 (KK107763) (Dietzl et al., 2007), was obtained from Vienna Drosophila RNAi Stock Center (Vienna, Austria). This RNAi line has 514 nt hairpin sequences that target all three isoforms (Pu-RA, Pu-RB, and Pu-RC) of GTPCH (Pu) transcripts.
**UAS lines** dtorsinΔD and dtorsinΔD cDNA constructs were made from the wild type dtorsin cDNA using QuikChange II XL Site-Directed Mutagenesis kit (Agilent Technologies, Santa Clara, CA USA). Briefly, a 1.2 kb wild type dtorsin cDNA was cut from pUAST-dtorsin with EcoRI and NotI (Wakabayashi-Ito et al., 2011) and cloned between the EcoRI and NotI sites of pBluescript II KS (Agilent Technologies). Mutagenesis strand synthesis was done following the manufacturer’s protocol using two primers torp4aE3 (59-CTAATGGAGGAGTTTATTATGTCATGATT- TTTTGTTGTTTCGC-39) and torp4aE5 (59-GCGAACAACCAAAAAATCATTGACATA-ATAAACTCCTCCATTAG-39) to make dtorsin cDNA that lacks GAG (E306), and torp4aD3 (59-CTAATGGAGGAGTTTATTATCTCAATGATTTTTTGGTTG-TTCGC-39) and torp4aD5 (59-GCGAACAACCAAAAAATCATTGAGATAAATAACTCC-TCCATTAG-39) to make dtorsin cDNA that lacks GAC (D307), respectively. After confirming mutated sequences, the insert was again cut out with EcoRI and NotI and inserted between EcoRI and NotI sites of pUAST to produce pUAST-dtorsinΔD and pUAST-dtorsinΔD. The transgenic lines E12 (pUAST-dtorsinΔD transgene on the second chromosome), E21 (pUAST-dtorsinΔD on the third chromosome) and D19 (pUAST- dtorsinΔD on the third chromosome) were used for the experiments. A 1.0 kb human torsinA ΔE cDNA was amplified from pcDNA3- htorM (Hewett et al., 2000) by PCR using the following primers htor5 (59-GCGGGATCCATTCATGAAAGCTGGGCCGGGC-CGCTGCTGGGCCTGC-39) and htor3 (59-CTCGAGCAGGCGGCTCAATCATCGTAGTAATA-ATCTAACCGTGGT-39). The PCR product was digested with Acc65I and NotI and inserted between Acc65I and NotI sites of pUAST. Injections were performed by Genetic Services, Inc. (Cambridge, MA USA). The transgenic line #24 with UAS-
htorsinA ΔE transgene on the second chromosome was used for the experiments.

**Larval locomotion assay** The larval locomotion assay was done as described previously (Wakabayashi-Ito *et al.*, 2011). Briefly, a wandering third instar larva of a particular genotype was individually picked from the vial with a bamboo stick and placed at the center of a 100mm petri dish containing 0.7% agarose at room temperature placed on a light box. Larval locomotion was recorded for one minute using a Canon Powershot G7 digital camera attached to a stereoscopic microscope. Peristaltic frequency was counted manually using the Quicktime movie. The experiments were done in a double-blinded manner with only numbers assigned for each genotype. Peristaltic rates are usually highly reproducible with little variation for each genotype with relatively small SEM values. Since we were unable to get dtorsin-null homozygous females (Wakabayashi-Ito *et al.*, 2011), we used dtorsin-null males for locomotion assays. We did not observe any significant gender difference between males and females of wild type larvae in our locomotion assay.

**Adult locomotion assay** The adult locomotion assay was adapted from the method described previously (Carbone *et al.*, 2006). Flies were maintained at 25 °C and a 12 hour light–12 hour dark circadian cycle. At least 10 males of each genotype, aged 3–5 days post-eclosion, were assayed in a double-blind manner. Individual flies were placed in vials and allowed to acclimate to the vial for 1 hour prior to assay. The vials were subjected to a gentle mechanical disturbance, and then locomotion behavior was quantified as the number of seconds each fly spent in motion during a 45 second period. The experiments were done in a double-blinded manner with only numbers assigned for each genotype. Each assay was replicated five times per fly. All assays were completed at the same time of the day (12 pm–3 pm). The results are usually highly
reproducible with little variation with relatively small SEM values.

**HPLC analysis** Dopamine and BH4 were separated by HPLC using a CoulArray HPLC system (model 5600A; ESA, Chelmsford, MA USA) and a Synergi 4 mm Hydro-RP column (4.66150 mm; Phenomenex, Torrance, CA), as described (Chaudhuri et al., 2007). Brains of third instar larvae or heads of 48–72 hour post-eclosion adult flies were homogenized in 0.1 M perchloric acid. One hundred third instar larval brains or 75 to 200 adult heads were extracted in 100–200 ml of 0.1 M perchloric acid. Ten microliters of each extract were injected for each sample. Pool sizes were determined relative to freshly prepared standards (Sigma-Aldrich, St. Louis, MO USA). Analysis was performed using ESA CoulArray software.

**Western blot analysis** Detection of proteins in adult heads (Figs 5.2, 5.6; supplementary material Figs S3, S6, S7) was performed, as described (Wakabayashi-Ito et al., 2011). Briefly, fifty heads from adult males of each genotype were homogenized in 100 ml RIPA buffer [50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% NP40, 0.5% deoxycholate, 0.1% SDS (sodium dodecyl sulfate)] with Protein Inhibitor Cocktail (Roche Applied Science, Indianapolis, IN USA). The proteins (30 mg), which corresponded to approximately three adult heads, were separated by electrophoresis in 10% SDS-polyacrylamide gels and transferred to Protran BA85 (0.45 mm pore size) nitrocellulose membranes (Sigma-Aldrich). Membranes were blocked with 10% non-fat dry milk in TBST (20 mM Tris-HCl buffer, pH 7.6, 167 mM sodium chloride, 0.1% Tween 20) and incubated with antibodies in 5% non-fat dry milk in TBST. GTPCH protein was detected using affinity-purified polyclonal anti-GTPCH isoform A/C antibody (Chen et al., 1994) at 1:50,000 dilution. Human torsinA protein was detected using rabbit polyclonal anti-human torsinA TA-2 (Bragg et al., 2004) at 1:5000 dilution, Rabbit anti-actin antibody (Sigma-Aldrich) was used at
1:5000 dilution. The secondary antibody used was peroxidase-conjugated anti-rabbit IgG at 1:5000 dilution (Jackson ImmunoResearch, West Grove, PA USA). Signals were detected using Supersignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific, Waltham, MA USA).

For detection of proteins in larval brains (Fig. 5.3; supplementary material Figs S4, S5), fifteen whole male brains from each genotype were dissected from late third instar larvae in phosphate-buffered saline (PBS) and homogenized in 50 ml of RIPA lysis buffer (AMRESCO)/2 mM DTT/16 protease inhibitor cocktail (AMRESCO, Solon, OH USA) containing 2 mM EDTA added immediately before use. Forty ml of supernatant were mixed with 14.9 ml of NuPage LDS sample buffer (Life Technologies, Carlsbad, CA USA) and 5.7 ml of 500 mM DTT. Twenty mg of proteins per lane were separated by electrophoresis on 4–12% NuPage Bis-Tris mini gels (Life Technologies). Separated proteins were transferred into a nitrocellulose membrane, and blocked with 2.5% BSA in TBST. Rabbit anti-GTPCH isoform A/C was used at 1:8000, while mouse anti-syntaxin (8C3 supernatant, Developmental Studies Hybridoma Bank) (1:200) was used in 5% BSA in TBST. Horseradish peroxidase-conjugated anti-rabbit (1:20,000) and anti-mouse (1:20,000) IgG secondary antibodies (VWR International, Randor, CA USA) were prepared in 2.5% BSA in TBST.

After the reaction with the peroxidase substrate, the membranes were exposed with multiple exposure times and the optimal condition for densitometry measurement was determined. Relative densities of GTPCH bands (two major species, RA and RC, were combined) and human torsinA bands were quantified from scanned images of X-ray films by the NIH ImageJ 1.40 g software (“`gels`” function under “`analyze`” command; http://rsb.info.nih.gov/ij/docs/guide/146-30.html#sec: Analyze-Menu) using densities of actin bands or syntaxin bands as internal
standards.

**Isolation of RNA** Total RNA was extracted from thirty adult male fly heads suspended in 100 ml PBS/0.1% Triton X-100 using 800 ml TRI reagent (Molecular Research Center, Inc., Cincinnati, OH USA) and 80 ml of BCP (Molecular Research Center, Inc.) following the manufacturer’s protocol.

**qRT-PCR analysis** qRT-PCR was performed as described previously with minor modifications (Balaj et al., 2011). Total RNA (2 mg) was converted into cDNA with the Omniscript reverse transcription kit (Qiagen, Valencia, CA USA) using random primers, according to manufacturer’s recommendations, and a 1:10 fraction (corresponding to 2.5 ng reverse transcribed RNA) was used for qRT-PCR. All reactions were performed in a 20 ml reaction using Power SYBR Green PCR Master Mix (Life Technologies) and 320 nM of each primer. Amplification conditions consisted of: 1 cycle of 50 °C, 2 minutes; 1 cycle of 95 °C, 10 minutes; 40 cycles of 95 °C, 15 seconds; and 60 °C, 1 minute followed by a dissociation curve analysis of each amplicon on the 7000 ABI Prism PCR system (Life Technologies). Ct values were analyzed in auto mode. The Ct-values were normalized to the housekeeping gene Rpl32 (rp49) in each sample (Brown et al., 2009; Willis et al., 2010). The following primers were used for qRT-PCR: Rp49: F:59-CCCAAGGGTATCGAC AACAG-39; R:59-GTTCGATCCGTAACCGATGT-39; Pu: F:59-CGGA TAGTGATGGCCACGAG-39; R:59-AGTAGACGATACGAGCGTGC-39.

**Dopamine feeding assay** The dopamine-feeding assay was done with some modification, as described previously (Wakabayashi-Ito et al., 2011). Fifty females of w, elavGAL4, dtorsin KO/FM7i, Actin-GFP were mated with twenty-five males of w; UAS-dtorsin(A11)(II); UAS-dtorsin ΔD(12)(III) or w; UAS-htorsinA(#8), UAS-htorsinA ΔE(#24)(II).
Fifty Green Fluorescent Protein (GFP)-negative first instar larvae were transferred to 1.5 g Formula 4–24 Instant Drosophila Medium (Carolina Biological Supply Company, Burlington, SC USA) in 7 ml water or 7 ml of 20 mM dopamine hydrochloride (Sigma-Aldrich) solution. Experiments with other genotypes were performed similarly with the same number of larvae in each vial. Larval locomotion assays were performed as described above (Wakabayashi-Ito et al., 2011).

**Statistical analysis** Since we were comparing two groups with comparable genetic backgrounds with the only exception being that of the particular genotype on which we focused, an unpaired t test was used rather than ANOVA. Means of two groups were compared by an unpaired t test using the statistical software Graphpad Prism 5.0 (GraphPad Software, Dan Diego, CA USA).

**RESULTS**

**Human torsinA ΔE dominantly inhibits larval locomotion**

In the previous study, we analyzed the peristaltic frequency of third instar larvae to quantify the difference in locomotion between wild type and mutant. The wild type third instar larvae show approximately 55 muscle contraction cycles per minute when placed on 0.7% agarose plates at room temperature. These peristaltic rates are relatively easy to monitor and provide a sensitive and reliable way of quantifying larval locomotion (Song et al., 2007; Wakabayashi-Ito et al., 2011). Males of the null mutant, dtorsinKO13, exhibit approximately a ~50% decrease in peristaltic rates, 22.962.5 (n=28, p<0.0001) (Fig. 5.1A, column 5), compared to wild type (55.262.5, n=15) (Fig. 5.1A, column 1). As previously observed, the wild type human torsinA cDNA expressed with the pan-neuronal driver elavGAL4 rescued dtorsinKO13 male larval mobility to a very significant level (56.363.7, n=14, p<0.0001) (Fig. 5.1A, column 7) (Wakabayashi-Ito et al., 2011),
compared to dtorsin\textsuperscript{KO13} male larvae with the elavGAL4 transgene (27.261.1, n539) (Fig. 5.1A, column 6). By way of controls, the pan-neuronal expression of the wild type human torsinA cDNA in wild type flies had no effect on larval mobility (54.362.3, n515, p50.7) (Fig. 5.1A, column 3), compared to male larvae with elavGAL4 transgene alone (53.061.8, n59) (Fig. 5.1A, column 2). Similarly, the presence/absence of the elavGAL4 transgene had no effect on mobility in wild type (Fig. 5.1A, columns 1, 2) and dtorsin\textsuperscript{KO13} larvae (Fig. 5.1A, columns 5,6).

To examine the effect of mutated human torsinA\textsuperscript{ΔE} protein in flies, we expressed human torsinA\textsuperscript{ΔE} cDNA with the pan-neuronal elavGAL4 driver in wild type males (\textit{w dtorsin\textsuperscript{+}}). ElavGAL4/UAS-htorsinA\textsuperscript{ΔE} males exhibited a severe locomotion deficit, approaching that of the dtorsin-null mutant (26.763.4, n59, p<0.0001) (Fig. 5.1A, column 4, compared to column 2). This result demonstrates that pan-neuronal expression of human torsinA\textsuperscript{ΔE} protein has a negative effect on larval locomotion, similar to the dtorsin-null state in flies, and that it interferes with the function of endogenous Dtorsin.

While pan-neuronal expression of human wild type torsinA could rescue the locomotion deficit phenotype of dtorsin\textsuperscript{KO13} males (Fig. 5.1A, columns 6,7), human torsinA\textsuperscript{ΔE} was unable to do so (20.562.0, n521) (Fig. 5.1A, column 8). To determine whether the human torsinA\textsuperscript{ΔE} could inhibit wild type human torsinA, we co-expressed the human torsinA\textsuperscript{ΔE} cDNA with the wild type human torsinA cDNA in dtorsin\textsuperscript{KO13} male using the same elavGAL4 driver. Co-expression of human wild type torsinA and human torsinA\textsuperscript{ΔE} resulted in a significant inhibition of mobility (25.362.8, n514, p<0.0001) (Fig. 5.1A, column 9), compared to the rescue by human torsinA alone (Fig. 5.1A, column 7).
**Human torsinA ΔE dominantly inhibits adult locomotion**

We have also analyzed the locomotion activities in the adult stage to examine whether they were similarly affected by the neuronal expression of human torsinA ΔE. Adult flies, aged 3–5 days after eclosion, were placed in vials, subjected to a gentle mechanical disturbance, and then locomotion activities were quantified as the number of seconds each fly spent in motion during a 45 second period (Carbone et al., 2006). Adult wild type male flies (Canton S- B) spent approximately 21.860.9 seconds in motion (n547) (Fig. 5.1B, column 1). Adult male flies that were heterozygous for a lethal Punch (GTPCH gene) null mutation, Pu^22+ (Mackay et al., 1985), exhibit a significant reduction of locomotion activities with 16.760.8 seconds spent in motion (n544, p,0.0001) (Fig. 5.1B, column 2), compared to wild type (Fig. 5.1B, column 1). Similarly, adult males of the null mutant, dtorsin^KO13, exhibit a significant reduction of adult locomotion activities, (17.560.7 seconds, n564, p50.0002) (Fig. 5.1B, column 3), as observed in the third instar larvae. The wild type human torsinA cDNA expressed with the pan-neuronal driver elavGAL4 strongly rescued dtorsin^KO13 male adult locomotion activities (21.460.9 seconds, n577) (Fig. 5.1B, column 4), compared to dtorsin^KO13 adult males (Fig. 5.1B, column 3) (p50.01). The mutant form of torsinA ΔE was unable to rescue the adult locomotion defect (16.960.9 seconds, n569) (Fig. 5.1B, column 5) (p50.603, compared to column 3). Co-expression of the human torsinA ΔE with the wild type human torsinA cDNA in dtorsin^KO13 adult male resulted in a significant reduction of locomotion activities (14.660.9, n563) (Fig. 5.1B, column 6) compared to the rescue by human torsinA alone (Fig. 5.1B, column 4) (p,0.0001). These results demonstrate that adult locomotion activities in flies are dominantly inhibited by the neuronal expression of the mutant form of human torsinA.
Figure 5.1. Neuronal expression of human torsinA ΔE has a dominant-negative effect on larval and adult locomotion. (A) Peristaltic frequencies were counted for the wandering stage third instar larvae of the genotype: (1) y w/Y (wild type) male (n=515), (2) w elavGAL4/Y (wild type) male (n=59), (3) w elavGAL4/Y; UAS-htorsinA/+ male (n=515), (4) w elavGAL4/Y; UAS-htorsinA ΔE/+ male (n=59), (5) y w dtorsinKO13/Y (dtorsin-null) male (n=514), (6) w elavGAL4 dtorsinKO13/Y (dtorsin-null) male (n=539), (7) w elavGAL4 dtorsinKO13/Y; UAS-htorsinA/+ male (n=514), (8) w elavGAL4 dtorsinKO13/Y; UAS-htorsinA ΔE/+ male (n=521), (9) w elavGAL4 dtorsinKO13/Y; UAS-htorsinA ΔE/+ male (n=514). Results are expressed as the mean S.E.M. ***p<0.0001. (B) Adult locomotion activities were measured for the adults of the genotype: (1) Canton S-B (wild type) male (n=547), (2) PuZ22/+ (Pu null mutation) male (n=544), (3) w elavGAL4 dtorsinKO13/Y (dtorsin-null) male (n=564), (4) w elavGAL4 dtorsinKO13/Y; UAS-htorsinA/+ male (n=577), (5) w elavGAL4 dtorsinKO13/Y; UAS-htorsinA ΔE/+ male (n=569), (6) w elavGAL4 dtorsinKO13/Y; UAS-htorsinA ΔE/+ male (n=563). Results are expressed as the mean S.E.M. ***p<0.001, **p<0.05.

Human torsinA ΔE dominantly suppresses GTPCH expression

We have previously shown, and confirm here, that dtorsinKO13 males have a severe reduction of both the 45 kD (Pu-RA) and 43 kD (Pu-RC) isoforms of GTPCH protein in adult brains (Fig. 5.2A, lane 1 and 2; supplementary material, 2) (Wakabayashi-Ito et al., 2011). Males heterozygous for the embryo-lethal Pu null mutation, PuZ22/+ (Mackay et al., 1985), had a severe reduction of both Pu-RA and Pu-RC isoforms (supplementary material Fig. S2, lanes 1, 2), confirming that these two polypeptides are encoded by the GTPCH gene. To investigate whether human torsinAΔE has a similar effect on GTPCH, we prepared extracts from heads of dtorsinKO13 adult males expressing human torsinAΔE in neurons, dtorsinKO13 adult males expressing wild type torsinA in neurons, and dtorsinKO13 adult males expressing both human torsinA and human torsinAΔE in neurons, and compared GTPCH protein levels by western blot analysis (Fig. 5.2A, lanes 3–5; supplementary material Fig. S3A, columns 3–5). Pan-neuronal expression of human torsinAΔE in dtorsinKO13 adult males, confirmed by immunoblotting using an antibody specific to human torsinA (Bragg et al., 2004) (Fig. 5.2B, lane 3), revealed that the mutant human torsinA protein
Figure 5.2. Neuronal expression of human torsinA ΔE has a dominant-negative effect on GTPCH protein levels in adult brains. (A) Adult head extracts were analyzed by western blots. The membrane was probed with rabbit anti-GTPCH A/C (upper panel) and reprobed with rabbit anti-actin (lower panel). The genotypes are: (1) y w (wild type) males, (2) w elavGAL4 dtorsin\textsuperscript{K013/Y} (dtorsin-null) males, (3) w elavGAL4 dtorsin\textsuperscript{K013/Y}; UAS-htorsinA+ males, (4) w elavGAL4 dtorsin\textsuperscript{K013/Y}; UAS-htorsinA ΔE/+ males, (5) w elavGAL4 dtorsin\textsuperscript{K013/Y}; UAS-htorsinA, UAS-htorsinA ΔE/+ males. The locations of GTPCH (Pu-RA: 45 kDa, Pu-RC: 43 kDa) and actin (42 kDa) are indicated. Thirty mg of proteins were loaded in each lane. (B) Adult head extracts were analyzed by western blots. The membrane was probed with rabbit anti-human torsinA and reprobed with rabbit anti-actin antibodies. The genotypes are: (1) w elavGAL4 dtorsin\textsuperscript{K013/Y} (dtorsin-null) males, (2) w elavGAL4 dtorsin\textsuperscript{K013/Y}; UAS-htorsinA+ males, (3) w elavGAL4 dtorsin\textsuperscript{K013/Y}; UAS-htorsinA ΔE/+ males, (4) w elavGAL4 dtorsin\textsuperscript{K013/Y}; UAS-htorsinA+/+; UAS-htorsinA ΔE/+ males. The locations of human torsinA (37 kDa) (double bands) and actin (42 kDa) are indicated. Thirty mg of proteins were loaded in each lane.
was unable to rescue GTPCH protein levels when expressed alone in dtorsin\textsuperscript{KO13} adult males (Fig. 5.2A, lane 4 compared to lane 2). In contrast, neuronal expression of wild type human torsinA alone (Fig. 5.2B, lane 2) strongly rescued both isoforms of GTPCH in dtorsin-null males (compare Fig. 5.2A, lanes 2 and 3). Severe reduction of GTPCH was observed in dtorsin\textsuperscript{KO13} adult males expressing human torsinA and human torsinA\textDelta E together (Fig. 5.2A, lane 5 compared to lane 3), even though the expression of torsinA\textDelta E with the wild type form does not diminish the total level of human torsinA expressed in fly neurons (Fig. 5.2B, lane 4; supplementary material Fig. S3B).

We obtained comparable results using brain extracts from third instar larvae of the corresponding genotypes (Fig. 5.3; supplementary material Figs S4, S5) as those of adult head extracts (Fig. 5.2; supplementary material Fig. S3). That is, the htorsinA\textDelta E transgene fails to rescue either isoform of brain GTPCH, both of which are affected by complete knockout of the dtorsin gene (Fig. 5.3, lane 3 and 4; supplementary material Fig. S4, columns 3, 4). Expression of wild type htorsinA (Fig. 5.3, lane 5; supplementary material Fig. S4, column 5) rescues expression of both isoforms (Pu-RA and Pu-RC) of GTPCH expression with Pu-RA rescue slightly more effectively than Pu-RC. The basis for this slight difference is unclear at this time. Nevertheless, these results confirm that wild type human torsinA is capable of rescuing neuronal expression of Drosophila GTPCH and demonstrate that the human torsinA\textDelta E, when co-expressed with the wild type human transgene, dominantly suppresses GTPCH protein levels in both larval and adult brains without negatively affecting the expression of wild type human torsinA.
Figure 5.3. Neuronal expression of human torsinA ΔE has a dominant-negative effect on GTPCH protein levels in larval brains. Larval brain extracts were analyzed by western blots. The membrane was probed with rabbit anti-GTPCH (upper panel) and reprobed with mouse anti-syntaxin (lower panel). The genotypes are: (1) Pu^{Z22+} (Pu null mutation) males, (2) Canton S-B (wild type) males, (3) y w dtorsin^{KO13/Y} (dtorsin-null) males, (4) w elavGAL4 dtorsin^{KO13/Y}; UAS-htorsinA ΔE/+ males, (5) w elavGAL4 dtorsin^{KO13/Y}; UAS-htorsinA/+ males, (6) w elavGAL4 dtorsin^{KO13/Y}; UAS-htorsinA, UAS-htorsinA ΔE/+ males. Two isoforms of GTPCH (Pu-RA: 45 kDa, Pu-RC: 43 kDa) are expressed in the brain of wild type flies. Both were reduced in Pu^{Z22+} heterozygotes, indicating that both of these proteins are encoded by the Pu gene (Mackay et al., 1985). In addition, both isoforms are severely reduced in the dtorsin^{KO13} null hemizygous brain extracts. The htorsinA ΔE transgene is unable to rescue GTPCH expression in the dtorsin-null background, while the wild type htorsinA transgene strongly rescued the both isoforms of GTPCH in larval brains. The presence of the htorsinA ΔE transgene prevents wild type htorsinA rescue of GTPCH in the dtorsin-null background. Anti-syntaxin was employed as a loading control. Twenty mg of total brain proteins were loaded in each lane.
**Human torsinA ΔE dominantly reduces BH4 and dopamine level**

Tyrosine hydroxylase is the rate-limiting enzyme in dopamine synthesis (Friggi-Grelin et al., 2003) and its activity is limited by the availability of the BH4 cofactor (Kumer and Vrana, 1996). In flies and mammals, activity of GTPCH, the first enzyme in the BH4 biosynthesis pathway, controls the intracellular concentration of the cofactor (Kumer and Vrana, 1996; Krishnakumar et al., 2000; Thony et al., 2000). Thus, dopamine pools are subject to regulation by protein levels and catalytic activity of GTPCH. We have previously reported that there is a significant reduction of GTPCH activity and dopamine levels in larval and adult head of heterozygous dtorsinKO13/+ and dtorsinKO78/+ females (Wakabayashi-Ito et al., 2011). To investigate whether expression of human torsinA ΔE could also reduce the dopamine pool level, we measured BH4 levels and dopamine levels in extracts from brains of wild type male larvae expressing wild type human torsinA, dtorsinKO13 male larvae expressing human torsinA ΔE, and dtorsinKO13 male larvae expressing both human torsinA and human torsinA ΔE (Fig. 5.4A,B). The level of BH4 in dtorsinKO13 male brains was significantly lower (0.09860.007 ng/brain, n=53 replicate samples, each sample575 brains, p<0.001) (Fig. 5.4A, column 2) compared to wild type brains (0.30060.010, n=53 replications) (Fig. 5.4A, column 1). The BH4 level in dtorsinKO13 male brains expressing wild type human torsinA (0.34160.009, n=53, p<0.001) (Fig. 5.4A, column 3) was significantly higher compared to those in dtorsinKO13 male brains (Fig. 5.4A, column 2). Neuronal expression of human torsinA ΔE further decreased BH4 levels (0.01860.002, n=53, p<0.01) (Fig. 5.4A, column 4 compared to column 2). Co-expression of human torsinA ΔE with wild type human torsinA blocked the rescue by human torsinA (0.03060.004, n=53, p<0.001) (Fig. 5.4A, column 5 compared to column 3).
Similarly, the level of dopamine in dtorsin\textsuperscript{KO13} male larval brains was significantly lower (0.02160.002 ng/brain, n53, p,0.001) (Fig. 5.4B, column 2) as compared to wild type brains (0.06260.002, n53) (Fig. 5.4B, column 1). The dopamine level in dtorsin\textsuperscript{KO13} male brains expressing wild type human torsinA (0.07360.001, n53, p,0.001) (Fig. 5.4B, column 3) was significantly higher compared to that in dtorsin\textsuperscript{KO13} male brains (Fig. 5.4B, column 2). Neuronal expression of human torsinA\(\Delta\)E in dtorsin\textsuperscript{KO13} males further decreased dopamine levels (0.01160.001, n53, p,0.01) (Fig. 5.4B, column 4 compared to column 2). Co-expression of human torsinA\(\Delta\)E with wild type human torsinA dominantly blocked the rescue of dopamine levels by wild type torsinA (0.007460.0008, n53, p,0.001) (Fig. 5.4B, column 5 compared to column 3).

DtorsinA\(\Delta\)E dominantly inhibits larval locomotion  Dtorsin protein has conserved amino acids E306/D307, compared to E302/E303 in human torsinA (supplementary material Fig. S1). To determine whether Dtorsin with either DE306 or DD307 deleted would have a similar dominant-negative activity on the wild type Dtorsin protein as observed for the human torsinA\(\Delta\)E302/303 mutation, we made two deletion mutant constructs of the dtorsin cDNA in E306 (UAS-dtorsin\(\Delta\)E) and D307 (UAS-dtorsin\(\Delta\)D) and expressed them with the elavGAL4 driver. Although pan-neuronal expression of wild type Dtorsin did not affect larval locomotion in wild type Drosophila (peristaltic frequency 53.061.5, n58, not significant) (Fig. 5.5, column 2) compared to wild type (53.061.8, n59) (Fig. 5.5, column 1), wild type male larvae expressing Dtorsin\(\Delta\)E exhibited a significant locomotion deficit (38.762.5, n523, p50.002) (Fig. 5.5, column 3). Male larvae co-expressing Dtorsin\(\Delta\)E and wild type Dtorsin also exhibited a locomotion deficit (38.5462.8, n515, p50.0012) (Fig. 5.5, column 4 compared to column 1) similar to the deficit caused by
expression of Dtorsin ΔE only (column 3).

Figure 5.4. Neuronal expression of human torsinA ΔE has a dominant-negative effect on BH4 and dopamine levels. (A) Effect of neural expression of human torsinA ΔE on BH4 pools in larval brains (nanograms per brain). BH4 was extracted from third instar brains and separated and quantified by HPLC. The genotypes of larvae were: (1) y w/Y males, (2) w elavGAL4 dtorsinKO13/Y males, (3) w elavGAL4 dtorsinKO13/Y; UAS-htorsinA/+ males, (4) w elavGAL4 dtorsinKO13/Y; UAS-htorsinA ΔE/+ males, (5) w elavGAL4 dtorsinKO13/Y; UAS-htorsinA, UAS-htorsinA ΔE/+ males. (B) Effect of neural expression of human torsinA ΔE on dopamine pools in larval brains (nanograms per brain). Monoamines were extracted from third instar brains and dopamine was separated and quantified by HPLC. The genotypes of larvae were: (1) y w/Y (wild type) males, (2) w elavGAL4 dtorsinKO13/Y (dtorsin-null) males, (3) w elavGAL4 dtorsinKO13/Y;
UAS-htorsinA/+ males, (4) w elavGAL4 dtorsinKO13/Y; UAS-htorsinA ΔE/+ males, (5) w elavGAL4 dtorsinKO13/Y; UAS-htorsinA, UAS-htorsinA ΔE/+ males. Results are expressed as the mean±S.E.M. ***p<0.0001. (C) Peristaltic frequencies were counted for the wandering stage third instar larvae. The genotypes are: (1) w elavGAL4/Y males without dopamine supplementation (n=12), (2) w elavGAL4/Y males with 20 mM dopamine supplementation (n=11), (3) w elavGAL4 dtorsinKO13/Y males without dopamine supplementation (n=11), (4) w elavGAL4 dtorsinKO13/Y males with 20 mM dopamine supplementation (n=11), (5) w elavGAL4 dtorsinKO13/Y; UAS-dtorsinΔE(#12)(II)/+; UAS-dtorsin(A11)(III)/+ males without dopamine supplementation (n=11), (6) w elavGAL4 dtorsinKO13/Y; UAS-dtorsin ΔE(#12)(II)/+; UAS-dtorsin(A11)(III)/+ males with 20 mM dopamine supplementation (n=58), (7) w elavGAL4 dtorsinKO13/Y; UAS-dtorsinA, UAS-htorsinA ΔE/+ males without dopamine supplementation (n=14), (8) w elavGAL4 dtorsinKO13/Y; UAS-dtorsinA, UAS-htorsinA ΔE/+ males with 20 mM dopamine supplementation (n=55), (9) w elavGAL4/Y; GTPCH (Pu)-RNAi (v107296) males without dopamine supplementation (n=11), (10) w elavGAL4/Y; GTPCH (Pu)-RNAi (v107296) males with 20 mM dopamine supplementation (n=510), (11) w elavGAL4 dtorsinKO13/Y; GTPCH (Pu)-RNAi (v107296) males without dopamine supplementation (n=11), (12) w elavGAL4 dtorsinKO13/Y; GTPCH (Pu)-RNAi (v107296) males with 20 mM dopamine supplementation (n=510). Results are mean S.E.M. ***p<0.0001, very significant difference between without and with 20 mM dopamine supplementation.

Mutant male larvae (dtorsinKO13) expressing wild type Dtorsin showed much improved larval locomotion. We tested two independent transgenic lines expressing wild type Dtorsin. Expression of a second chromosome transgene, UAS-dtorsin(B5) in male dtorsinKO13 larvae resulted in a peristaltic frequency of 50.5±2.5, n=20, p<0.001 (Fig. 5.5, column 6), while expression of another transgene UAS-dtorsin(A11), on the third chromosome, rescued the peristaltic frequency to 48.2±1.1, n=9, p<0.0001 (Fig. 5.5, column 7) compared to the dtorsin-null (dtorsinKO13) males (Fig. 5.5, column 5). The presence of two copies of UAS-dtorsin transgenes (B5 and A11) together in the dtorsin-null background did not elevate locomotion further (peristaltic frequency: 50.2±1.2, n=23, p<0.0001) (Fig. 5.5, column 8).

In striking contrast to the rescuing effect of wild type Dtorsin expression, Dtorsin ΔE expression in male dtorsinKO13 larvae failed to rescue the locomotion deficit (22.9±2.8, n=13, not significant)
with a slight reduction of peristaltic rate (Fig. 5.5, column 9), relative to dtorsin\textsuperscript{KO13} males (Fig. 5.5, column 5). Similarly, mutant males co-expressing Dtorsin ΔE and wild type Dtorsin transgenes exhibited a locomotion deficit that was not significantly different from that of the dtorsin\textsuperscript{KO13} larvae (UAS-dtorsin(A11) and UAS-dtorsin ΔE(#12); 32.464.2, n=57, not significant) (Fig. 5.5, column 11); UAS-dtorsin(B5) and UAS- dtorsin ΔE(#21): 29.963.6, n=514, not significant) (Fig. 5.5, column 12).

Interestingly, pan-neuronal expression of Dtorsin ΔD in dtorsin\textsuperscript{KO13} males rescued the larval mobility (45.262.9, n=511, \( p<0.0001 \)) (Fig. 5.5, column 10 compared to column 5). Similarly, co-expression of Dtorsin ΔD with the wild type Dtorsin in dtorsin\textsuperscript{KO13} males had no effect on locomotion (peristaltic frequency: 49.463.2, n=520) (Fig. 5.5, column 13). These results indicate that E302/303 of human torsinA protein and E306 of Drosophila Dtorsin protein are functionally similar and that deletion of these glutamates both cause reduced locomotion in Drosophila larvae, presumably due to the same functional abnormality, while Dtorsin ΔD appears similar to wild type Dtorsin.

**Dtorsin ΔD dominantly suppresses GTPCH expression**

These studies described above demonstrate a striking similarity in the dominant inhibition of larval locomotion by Dtorsin ΔD and human torsinA ΔE. Since we found that human torsinA ΔE dominantly inhibited GTPCH protein expression, we next examined the protein levels of GTPCH in adult male heads expressing wild type Dtorsin and Dtorsin ΔD in the dtorsin-null background (Fig. 5.6). The expression of endogenous GTPCH in the dtorsin\textsuperscript{KO13} mutant line and in the dtorsin\textsuperscript{KO13} elavGAL4 transgene line revealed similar patterns of reduced GTPCH
expression of both RA and RC isoforms (Fig. 5.6, lane 2 and 3). Dtorsin expressed in dtorsin\textsuperscript{KO13} males, under the control of elavGAL4, rescued GTPCH expression substantially (Fig. 5.6, lane 4, compared to lane 3; supplementary material Fig. S6 column 4, compared to column 3).

Dtorsin ΔD expressed in dtorsin\textsuperscript{KO13} neurons failed to affect the GTPCH protein level (Fig. 5.6, lane 5, compared to lane 3; supplementary material Fig. S6, column 5, compared to column 3). Severe reduction of GTPCH was also observed in adult males co-expressingDtorsin and Dtorsin ΔD in dtorsin\textsuperscript{KO13} (Fig. 5.6, lane 6, compared to lane 4; supplementary material Fig. S6, column 6, compared to column 4). These results demonstrate that Dtorsin ΔD and human torsinA ΔE have indistinguishable effects on the expression of GTPCH protein in Drosophila brains, both dominantly inhibiting GTPCH expression. In contrast, Dtorsin ΔD expressed in dtorsin\textsuperscript{KO13} neurons moderately rescued GTPCH protein level (supplementary material Fig. S7), consistent with the results of larval locomotion assays (Fig. 5.5).

*The mobility defect of larvae expressing either human torsinA ΔE or Dtorsin ΔD can be rescued by dopamine supplementation*

In Drosophila, ingestion of dopamine increases dopamine pools in the fly head, though in mammals peripheral dopamine does not enter the brain (Chaudhuri et al., 2007). We previously showed that the locomotor deficit phenotype in dtorsin\textsuperscript{KO13} mutant male was partially rescued by dopamine supplementation to the larval growth medium, but not by serotonin or octopamine (Wakabayashi-Ito et al., 2011). Since we observed in the current study a very similar reduction of dopamine levels in larval brains expressing human torsinA ΔE, we hypothesized that dopamine supplementation to the larval growth medium could also restore the locomotion defect of larvae expressing human torsinA ΔE (or Drosophila Dtorsin ΔD). To test this hypothesis, we
added 20 mM dopamine in the food of larvae with different dtorsin genotypes (Fig. 5.4C). Dopamine supplementation had no effect on the locomotion of wild type (elavGAL4/Y) larvae (56.361.0, n=511, p=0.562) (Fig. 5.4C, column 2) compared to the larvae of the wild type without dopamine (55.461.0, n=512) (Fig. 5.4C, column 1).

**Figure 5.5. Neuronal expression of Drosophila Dtorsin ΔE has a dominant-negative effect on larval locomotion.** Peristaltic frequencies were counted for the wandering stage third instar larvae of the genotype: (1) w elavGAL4/Y (wild type) male (n=59), (2) w elavGAL4/Y; UAS-dtorsin(A11)(III)+ male (n=58), (3) w elavGAL4/Y; UAS-dtorsin ΔE(#12)(II)+ male (n=523), (4) w elavGAL4/Y; UAS-dtorsin ΔE(#12)(II)+; UAS-dtorsin(A11)(III)+ male (n=515), (5) w elavGAL4 dtorsin<sub>KO13</sub>/Y (dtorsin-null) male (n=548), (6) w elavGAL4 dtorsin<sub>KO13</sub>/Y; UAS-dtorsin(A11)(III)+ male (n=520), (7) w elavGAL4 dtorsin<sub>KO13</sub>/Y; UAS- dtorsin(A11)(III)+ male (n=59), (8) w elavGAL4 dtorsin<sub>KO13</sub>/Y; UAS-dtorsin(B5)(II)+; UAS-dtorsin(A11)(III)+ male (n=523), (9) w elavGAL4 dtorsin<sub>KO13</sub>/Y; UAS-dtorsin ΔE(#12)(II)+ male (n=513), (10) w
elavGAL4 dtorsin\textsuperscript{KO13}/Y; UAS-dtorsin ΔD/+ male (n511), (11) w elavGAL4 dtorsin\textsuperscript{KO13}/Y; UAS-dtorsin(A11)(III)/+; UAS-dtorsin ΔE(#12)(II)/+ male (n57), (12) w elavGAL4 dtorsin\textsuperscript{KO13}/Y; UAS-dtorsin(B5)(II)/+; UAS-dtorsin ΔE(#21)(III)/+ male (n514), (13) w elavGAL4 dtorsin\textsuperscript{KO13}/Y; UAS-dtorsin(B5)(II)/+; UAS-dtorsin ΔD/+ male (n520). Results are expressed as the means S.E.M. ***p,0.0001, **p,0.001.

In contrast, dopamine supplementation substantially rescued the locomotion defect of dtorsin\textsuperscript{KO13} larvae (49.36±1.8, n511, p<0.0001) (Fig. 5.4C, column 4) compared to the larvae of the same genotype without dopamine (27.76±2.8, n511) (Fig. 5.4C, column 3) confirming our previous results (Wakabayashi-Ito et al., 2011). Dopamine supplementation also rescued the locomotion defect of dtorsin\textsuperscript{KO13} larvae expressing Dtorsin ΔD and wild type Dtorsin (51.46±2.6, n58, p<0.0001) (Fig. 5.4C, column 6) compared to the larvae of the same genotype without dopamine (25.96±2.5, n511) (Fig. 5.4C, column 5). Similarly, dopamine supplementation also substantially rescued the locomotion defect of dtorsin\textsuperscript{KO13} larvae expressing human torsinA ΔE and wild type torsinA (52.26±3.6, n55, p<0.0001) (Fig. 5.4C, column 8) compared to the larvae of the same genotype without dopamine (25.36±2.8, n514) (Fig. 5.4C, column 7). These results demonstrate that locomotor defects caused by the pan-neuronal expression of human torsinA ΔE or Dtorsin ΔD, measured by our larval locomotion assay, can be substantially rescued by dopamine supplementation. As a control, knockdown of GTPCH (Pu) mRNA expression levels was accomplished by neuronal expression of GTPCH RNAi, a short-hairpin specific for GTPCH (Pu) gene (Fig. 5.7), which was accompanied by a moderate reduction of larval locomotion (38.76±1.1, n511) (Fig. 5.4C, column 9). Dopamine supplementation almost completely rescued locomotion defect of wild type larvae expressing GTPCH RNAi (57.56±1.0, n510, p<0.0001) (Fig. 5.4C, column 10). Dopamine supplementation also substantially rescued the locomotion defect of dtorsin\textsuperscript{KO13} larvae expressing GTPCH RNAi (42.36±1.7, n510, p<0.0001) (Fig. 5.4C, column 12).
compared to the larvae of the same genotype without dopamine (24.463.1, n=511) (Fig. 5.4C, column 11).

The expression level of GTPCH is regulated at the post-transcriptional level

Having found that Dtorsin/torsinA regulates GTPCH protein, we next tested whether torsin regulates GTPCH expression at the transcriptional or post-transcriptional level. In order to analyze these alternative possibilities, we prepared total RNA from adult brains and analyzed GTPCH (Pu) mRNA levels by quantitative RT-PCR (qRT-PCR). The relative amount of GTPCH mRNA was determined by normalizing to mRNA for the housekeeping gene RpL32 (rp49) as an internal control (see Materials and Methods). A significant increase, rather than reduction of GTPCH mRNA levels was observed in the brains of elavGAL4 dtorsinKO13/Y (4.5260.20, n=53, p,<0.0001) (Fig. 5.7, column 2) compared to those in wild type: elavGAL4/Y (1.00, n=53) (Fig. 5.7, column 1). Expression of wild type human torsinA (2.5760.24, n=53, p=0.0007) (Fig. 5.7, column 3) substantially increased GTPCH mRNA levels, while mutant human torsinA ΔE (1.4860.23, n=53, p=0.534, not significant) (Fig. 5.7, column 4) had no effect on GTPCH mRNA levels. Neuronal expression of GTPCH RNAi substantially reduced GTPCH RNA levels (0.2760.01, n=53, p=0.0001) (Fig. 5.7, column 5), validating our quantification of GTPCH mRNA by qRT-PCR. There were some variations in the relative abundance of GTPCH mRNA, but the reason is not clear at this moment. These results, however, indicate that dtorsin/torsin mutant brains do not have decreased levels of GTPCH mRNA and therefore have a defect in GTPCH expression at the post-transcriptional level.
DISCUSSION

Drosophila has a single torsin-related gene, dtorsin (Torsin), with 31.9% amino acid identity to human torsinA (supplementary material Fig. S1). dtorsin-null animals have reduced locomotion at the third instar larval stage and reduced pigmentation in the adult stage (Wakabayashi-Ito et al., 2021).

Figure 5.6. Drosophila dtorsin-null mutation does not reduce GTPCH mRNA levels. Total RNA was extracted from adult heads. Relative amount of GTPCH RNA was calculated compared to the internal control, RpL32 (rp49) RNA in each sample. The genotypes are: (1) w elavGAL4/Y males (n=53), (2) w elavGAL4 dtorsin^{Ko13}/Y (dtorsin-null) males (n=53), (3) w elavGAL4/Y; UAS-htorA/+ males (n=53), (4) w elavGAL4/Y; UAS-htorA ΔE/+ males (n=53), and (5) w elavGAL4/Y; GTPCH (Pu)-RNAi (v107296) males (n=53). Results are expressed as the means±S.E.M. ***p<0.0001, **p<0.001.
The dopamine levels and GTPCH activity/protein levels are severely reduced in dtorsin- null animals, suggesting GTPCH deficiency is responsible for dopamine depletion since TH protein is unaffected in the mutant (Wakabayashi-Ito et al., 2011). The pan-neuronal expression of wild type Drosophila Dtorsin or human torsinA rescued the locomotion defect in dtorsin-null larvae and adults, suggesting that human torsinA and Drosophila Dtorsin are functionally conserved (Figs 5.1, 5.5) (Wakabayashi-Ito et al., 2011). Pan-neuronal expression of human torsinA ΔE protein alone did not rescue the locomotion defect, or the depletion of GTPCH protein, BH4, and dopamine (Figs 5.1–5.4) in dtorsin-null larvae and adults, demonstrating that human torsinA ΔE protein is inactive. Further, co-expression of human wild type torsinA and torsinA ΔE did not rescue the defects (Figs 5.1–5.4), demonstrating a dominant-negative effect of torsinA ΔE on wild type torsinA activity. These results, for the first time, clearly show that torsinA ΔE inhibits wild type torsinA activity in neurons, resulting in reduced locomotion and dopamine levels in Drosophila.

Drosophila Dtorsin has similar types of amino acids E306- D307 compared to human torsinA E302-E303 in the conserved location near the C terminal region of the protein (supplementary material Fig. S1). Dtorsin ΔD306, when expressed in neurons, had a similar dominant-negative effect on locomotion and GTPCH protein levels as human torsinA ΔE (Figs 5.5, 5.6), while Dtorsin ΔD307 was still active as it could rescue the dtorsin-null locomotion defect as well as GTPCH protein expression, and had no inhibitory effect on wild type Dtorsin (Fig. 5.5; supplementary material Fig. S7). Furthermore, neuronal expression of Dtorsin ΔD306 inhibited locomotion of wild type larvae, demonstrating a dominant- negative effect on the wild type protein. The phenotypes caused by neuronal expression of human torsinA ΔE or Drosophila
DtorsinΔD are indistinguishable from those of dtorsin-null larvae or adults, resulting in co-reduction of locomotion, dopamine levels, and GTPCH protein levels. These results strongly support the hypothesis that torsinA ΔE acts as a dominant-negative molecule that suppresses the wild type protein activity (Breakefield et al., 2001).

We have demonstrated that dtorsin-null larvae and DtorsinΔD (or human torsinA ΔE) expressing larvae have very similar phenotypes, resulting in the severely decreased level of GTPCH. Rates of dopamine synthesis depend on the activity of TH, which in turn depends on the amount of BH₄ produced by GTPCH (O’Donnell et al., 1989; Thony et al., 2000). Severe reduction of GTPCH protein levels results in a shortage of BH₄ and decreased activity of TH, thereby leading to decreased dopamine pool levels in brains of dtorsin-null animals (Wakabayashi-Ito et al., 2011), as well as in brains expressing torsinA ΔE (Fig. 5.4A,B). This defect, however, is unlikely to be the only defect in the dopamine signal transduction system in dtorsin-null or torsinA ΔE-expressing animals. Although feeding dopamine could partially rescue the locomotion defect in our assay in dtorsin-null larvae (Wakabayashi-Ito et al., 2011) or in DtorsinΔD (or human torsinA ΔE)-expressing larvae (Fig. 5.4C), very few larvae of dtorsin-null or torsinA ΔE-expressing animals survived until the late third instar larval stage (data not shown). Early lethality could be the result of earlier developmental requirements for dopamine since strong loss-of-function mutations in the TH-encoding gene cause embryonic lethality in Drosophila (Neckameyer and White, 1993). Alternatively, Dtorsin may be affecting other neurotransmitter signaling systems directly or indirectly through dysfunction in dopaminergic circuitry. In the case of DYT1 dystonia patients, L-dopa is not therapeutic, suggesting that dopamine cannot compensate for defects resulting from mutant torsinA (Breakefield et al.,
2008). Recent publications in mouse DYT1 model systems demonstrated defective dopamine D2 receptor signaling in the striatal cholinergic neurons (Sciamanna et al., 2009; Sciamanna et al., 2011; Sciamanna et al., 2012). The lack of responsiveness of DYT1 patients to L-dopa treatment would be expected if the dopamine D2 receptor signaling or other component of the dopaminergic system is defective in addition to defects in dopamine synthesis.

Translational control of localized mRNA is a common mechanism for regulating protein expression in specific subdomains of a cell, in processes such as body axis formation, asymmetric cell division and synaptic plasticity (St Johnston, 2005; Holt and Bullock, 2009; Medioni et al., 2012). These localized mRNAs are often transported in large ribonucleoprotein particles (RNPs) or RNA granules (Kiebler and DesGroseillers, 2000; Kiebler and Bassell, 2006; Holt and Bullock, 2009; Medioni et al., 2012). We have recently shown that dtorsin is involved in export of large RNPs out of nuclei on the way to the neuromuscular junction (Jokhi et al., 2013). Here, we have shown that the mRNA levels of GTPCH/Punch gene were not significantly decreased in dtorsin-null adult brains (Fig. 5.7), suggesting that the regulation of GTPCH/Punch expression is at the post-transcriptional level. This is consistent with a model in which GTPCH mRNA is transported through the nuclear membrane as a part of a large RNP complex whose transport depends on Dtorsin. If this hypothesis is correct, the Dtorsin protein could regulate the nuclear export and subsequent transport of large RNP complexes with subsequent compromise of the translation of the GTPCH mRNA. This nuclear export of mRNAs within RNPs could explain the mechanism by which torsin regulates expression of multiple proteins such as GTPCH and dopamine D2 receptor at the same time and thereby modulate synaptic plasticity (Sciamanna et al., 2012). Further testing of this hypothesis will be very important for understanding the molecular function of torsin proteins and the pathophysiology of DYT1 disease in human
patients. The Drosophila system with its abundant genetic tools provides us an excellent model system to probe this hypothesis.

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REFERENCES


CHAPTER SIX
FINAL DISCUSSION AND FUTURE DIRECTIONS

In Chapter Two, I presented a comprehensive study across three distinct yet conserved model systems investigating whether modifying critical regulators of the insulin signaling and glucose metabolic pathways are able to alter age-dependent \textit{alpha-synuclein} proteotoxicity. Together, our findings present a highly-conserved mechanism in which DAF-16/FOXO mediates a complex regulation of metabolism, dopamine homeostasis and synuclein-induced oxidative damage. Specifically, I found that knocking down the insulin receptor substrate (IRS)/\textit{chico} resulted in the rescue of age-dependent \textit{\alpha-syn} toxicity. Flies expressing the \textit{chico} mutation were significantly protected against \textit{\alpha-syn}- induced dopaminergic neuron damage and degeneration (Fig. 2.1). In aged flies expressing mutant \textit{Pgi}, an enzyme critical to glucose metabolism, we found dramatically exacerbated neurodegeneration and disruptions in dopamine regulation. These flies exhibited significantly heightened dopamine turnover and neuroinflammation, further worsening the toxicity of \textit{\alpha-syn}. The foundational nature of these results establish a mechanistic platform with which to investigate the molecular cross-talk between cellular metabolism and dopamine regulation in the context of neurodegenerative disease models.

Moving forward, the examination of upstream molecular signals, particularly those involved in insulin/insulin-like signaling, is vital to our understanding of the downstream consequences on aging and cellular response to proteotoxicity. One such pathway is the PI3K/Akt signaling
cascade. Directly downstream of the insulin receptor, the PI3K/Akt pathway is critical for cell survival and longevity. Initiated by the cellular stress response, JNK and PTEN inhibit the PI3K/Akt pathway, arresting cell growth and metabolism and inducing a pro-apoptotic response within the cell. Both pathways are suggested to play major roles in Parkinson’s disease pathology.

This pathway has been strongly linked to diabetes in human (Pandey and Nichols, 2011) and rodents (Musselman et al., 2011) and interestingly, clinical studies done on drug-naïve PD patients found elevated levels of IGF-1 in both the serum and cerebrospinal fluid compared with drug-naive controls (Baker and Thummel, 2007; Knight et al., 2014), prompting many to look towards the PI3K/Akt pathway as a potential link between these two diseases. While the IIS pathway, particularly the PI3K/Akt pathway, has been well-researched in regards to aging and stress resistance, no studies have investigated how specific components of this pathway expressed in varying PD backgrounds affect dopamine regulation or dopaminergic neurodegeneration. To test this, we could use the Drosophila GAL4/UAS system to overexpress or knockdown IRS, PI3K, AKT, JNK and PTEN spatially in dopaminergic neurons within a Parkinson’s disease genetic background, such as α-syn mutants, Parkin, or DJ-1α. These lines can then be implemented in age-related studies such as lifespan, mobility, and neurodegeneration. The PI3K/AKT pathway can be inhibited pharmacologically using LY294002 or Wortmannin, reversible and irreversible inhibitors of PI3K, respectively. To validate the ability of these chemicals to inhibit PI3K activity in Drosophila, flies fed these inhibitors can be assayed using immunoblotting to determine the levels of phosphorylated versus
Figure 6.1. Overview of the insulin-mediated PI3K/AKT signaling cascade. Downstream of the insulin receptor, the PI3K/AKT pathway is critical for cell survival and longevity. Initiated by cellular stress response, JNK and PTEN inhibit the PI3K/AKT pathway, arresting cell growth and metabolism and inducing a pro-apoptotic response within the cell. Both pathways are suggested to play major roles in PD pathology. Abbreviations: InR, insulin receptor; IRS, insulin receptor substrate; LR, ligand-binding receptor; JAK, janus kinase 2; STAT, signal transducer and activator of transcription; PI3K, phosphoinositide-3-kinase; AKT, v-akt murine thymoma viral oncogene homolog 1; mTOR, mammalian target of rapamycin; S6K, S6 kinase; CR, cytokine receptor; JNK, c-Jun N-terminal kinase; PTEN, phosphatase and tensin homolog; ROS, reactive oxygen species.
non-phosphorylated AKT, the direct function of PI3K. Components of the pathway that exhibit the most significant results in age-dependent studies can then be used to investigate relationships between downstream pathways, including cell survival, apoptosis and metabolism. Immunoblotting can be performed to assess the activation of NO-mediated neuroinflammation, caspase signaling and the formation of superoxides, to determine potential regulators of downstream dopamine homeostasis and stress response pathways. Importantly, our data has also shown for the first time that *Drosophila* expressing human *alpha-synuclein* in dopaminergic neurons fed either glucose or an inhibitor of glucose metabolism (2-deoxyglucose) experience significant changes in climbing ability (Fig. 2-5F), an indication of the neurochemical loss of dopamine signaling. These results can be strengthened by investigating the effects of glucose or insulin feeding on the transgenic lines knocking down PI3K/AKT pathway proteins or in PD genotypic backgrounds to assess activity levels of dopamine regulatory components, such as TH, Catsup and VMAT. This work offers valuable insight into the impact of insulin signaling and glucose metabolism on dopamine regulation and neuron survival, and provides a foundation for the use of these conserved models, such as *Drosophila melanogaster*, in evaluating these IIS-like pathways in Parkinson’s disease pathology.

In Chapter Four, we provide strong evidence for the ability of Catecholamines-up to modulate *alpha-syn*- induced dopaminergic toxicity through the coordinated regulation of dopamine production and VMAT-mediated dopamine transmission. Though the exact mechanisms underlining the sensitivity of dopaminergic neurons in PD pathology remain to be understood, current evidence strongly suggests the highly-reactive nature of dopamine molecules results in the exacerbation of oxidative stress associated with PD pathology. Our work contributes to the process by which dopamine is regulated under both normal conditions and under *alpha-syn*- induced neurotoxicity.
Figure 6.2. BH4 pathway and its role in the biosynthesis of dopamine and nitric oxide through distinctly-different mechanisms. Punch, encoding for GTP cyclohydrolase, is required for the synthesis of tetrahyrobiopterin (BH4) by converting guanosine triphosphate (GTP) to dihydronopterin triphosphate (H2NPPP). BH4 then acts as a necessary cofactor with tyrosine hydroxylase (TH) in the synthesis of dopamine (DA) in the neuron or it acts with nitric oxide synthase (NOS) in the conversion of arginine to citrulline and nitric oxide (NO), a critical cell-signaling molecule, especially in the activation of the innate immune response.

Our work clearly supports the importance of Catsup and VMAT functioning closely to regulate dopamine synthesis and transmission, particularly in a PD-like genetic background. Through the use of heterozygous Catsup mutant lines and VMAT inhibitor reserpine, we could continue this work by evaluating several different avenues. One such direction is to consider the impact of Catsup knockdown on BH4 production and activity. BH4, in addition to acting as a necessary cofactor for TH activity, is required in the production of nitric oxide, which as discussed in Chapter Three, is a critical cellular signaling molecule involved in activation of the inflammatory immune response.
Our lab has previously shown that BH₄ levels are significantly increased in heterozygous loss-of-function Catsup mutants (Chaudhuri et al., 2007), yet whether this increased BH₄ production translates into increased inflammation has yet to be tested using this model. We have found that Catsup mutant flies do not present an increase in NO production under typical conditions, and in fact reduce NO levels in the α-syn background (data not shown). This is likely due, however, to the role of Catsup in reducing cellular stress via acting on VMAT to alleviate dopamine metabolism. However, as Catsup is involved in regulating BH₄ production, dopamine synthesis and dopamine synaptic transmission, it is not definitively known which Catsup-mediated effects are responsible for alleviating cellular stress or neurodegeneration. It is possible, for example, that the increased BH₄ levels present in Catsup mutant flies (Chaudhuri et al., 2007) aid in the activation of the nitric oxide synthase (NOS)-mediated inflammatory response network. It is worth noting that the chronic activation of mammalian microglia in neurodegenerative diseases has been shown to cause accelerated neuron loss and disease pathology (McGeer et al., 1988; Langston et al., 1999; Imamura et al., 2003). During this sustained neuroinflammation, neuron loss is further exacerbated when the production of NOS and proinflammatory cytokines is stimulated and leads to the damage to healthy neurons (Dawson et al., 1993, Boka et al., 1994, McCoy et al., 2006). Therefore, a thorough analysis of the consequences of knocking-down Catsup must be explored within Drosophila and other model systems. To explore this potential effect in Drosophila, I propose evaluating the changes in the protein levels of nitric oxide synthase in response to knocking down Catsup, as well as using immunohistochemistry to label and observe hemocyte activation in response to oxidative challenge in Catsup mutants. I would then inhibit NOS function using L-NAME and determine if the Catsup mutant phenotypes are altered in the absence of NOS function to determine whether the rescuing affects of Catsup
knockdown are dependent on its role in modulating NOS activity through BH₄ production.

In addition, the increase in VMAT and synaptic activity due to \textit{Catsup} knockdown also results in an increase in time-in-motion, even in 3-5 day old male flies (Fig. 4-7 B, D). This elevated mobility is thought to result from the heightened levels of dopamine into the synapse. This hypothesis can be tested by investigating the activity of dopamine transporter, or DAT, which is responsible for the reuptake of dopamine back into the pre-synaptic neuron, as well as the binding of dopamine to the receptors on the post-synaptic neuron. These receptors can be inhibited pharmacologically or overexpressed using transgenic \textit{Drosophila} lines. An increase in DAT and in post-synaptic neuron receptors would indicate an influx of exocytosis of dopamine into the synapse. This can be evaluated using behavioral assays to measure sleep and mobility, which have been shown to be altered in DAT mutants (Kume et al., 2005; Pfeiffenberger and Allada, 2012; Kayser et al., 2014; Kong et al., 2010, Hamilton et al., 2013) or using \textit{in vivo} voltametric studies (Makos et al., 2009).

As proposed previously in this chapter, exploring the regulation of the PI3K/AKT pathway could shed meaningful insight into the communication between the IIS pathway and dopamine regulation. By knocking down the PI3K/AKT pathway via chemical inhibitors or UAS-RNAi lines, we could assess the downstream consequences of insulin signaling on Catsup, TH and VMAT function. It is important to determine whether any affect on these proteins occurs at the transcriptional, translational or enzymatic activity levels. To evaluate this, we could inhibit the PI3K/AKT pathway using chemical inhibitors mentioned above, or through the use of Gal80ts \textit{Drosophila} lines, which allow for the temporal expression or suppression of specific UAS targets, and use RT-PCR to measure transcript levels and immunoblotting to determine protein levels. To assess changes in activity, we can test TH using TH activity assays and Catsup activity
by testing for the activation and function GTPCH and TH, proteins Catsup has been shown to
inhibit. VMAT activity can be measured by HPLC analysis of DOPAC/DA levels (Chaudhuri et
al., 2007), the production of toxic dopamine metabolites which accumulate when VMAT
function is reduced, and also using mobility assays as a readout of dopamine synaptic release.

Throughout this report, we have contributed robust supporting evidence for the future elucidation
of dopamine-related neurotoxicity in PD pathology and dopamine-regulation. The work in this
report resulted in a wide range of novel findings contributing to our understanding of dopamine
within multiple regulatory and disease pathways, as well as the utility of *Drosophila
melanogaster* as a model for examining the underlying genetic and molecular interactions
associated with these conditions.

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