[Forensic Science Theses](#)   [Chemistry Department](#)

12-2014

Suppression of Neurodegenerative Symptoms via Suppressor of levy Mutation in Drosophila melanogaster

William Kaputa
State University of New York Buffalo State, wkaputa2@gmail.com

Advisor
Joonyeong Kim, Ph.D., Associate Professor of Chemistry
First Reader
Satpal Singh, Ph.D., Associate Professor of Pharmacology and Toxicology, State University of New York at Buffalo
Second Reader
M. Scott Goodman, Ph.D., Chair and Associate Professor of Chemistry
Third Reader
Amy McMillan, Ph.D., Associate Professor of Biology
Department Chair
M. Scott Goodman, Ph.D., Chair and Associate Professor of Chemistry

To learn more about the Chemistry Department and its educational programs, research, and resources, go to http://chemistry.buffalostate.edu/forensic-science-ms-5.

Suppression of Neurodegenerative Symptoms via *Suppressor of levy* Mutation in *Drosophila melanogaster*

by

William Kaputa

An Abstract of a Thesis
in Forensic Science

Submitted in Partial Fulfillment
of the Requirements for the Degree of

Master of Science

December 2014

Buffalo State
State University of New York

Abstract

Suppression of Neurodegenerative Symptoms via *Suppressor of levy* Mutation in *Drosophila melanogaster*

Mutation of the *levy* gene on chromosome 2 of *Drosophila melanogaster* has previously been shown to cause temperature-induced paralysis and neurodegeneration. The *Suppressor of levy* mutation, *Su(levy),* also on chromosome 2, modulates the effects of the *levy* mutation and partly rescues the wild-type phenotype. The goal of this research was to determine if *Su(levy)* mutation alleviates the effects of neurodegeneration caused by mutations associated with Alzheimer's and Parkinson's diseases in humans. The mutant genes used were *hLRRK2*, α-*Synuclein*, *DJ1-α*, *Pink*, *Parkin*, and *Aβ-42*. To determine this, flies carrying the above-mentioned mutant genes were crossed with flies carrying *Su(levy)* to produce progeny carrying both mutations. The lifespan and locomotor abilities of these progeny were then tested and compared to control and mutant flies. The flies were also tested for their response to environmental toxins introduced to their diet. Our results show that in many cases the *Suppressor of levy* mutation plays a considerable role in alleviating neurodegenerative symptoms in *Drosophila*. Flies carrying *Su(levy)* show a significant improvement in lifespan, climbing ability, resistance to paralysis, and resistance to environmental toxins.

Buffalo State
State University of New York
Department of Chemistry

Suppression of Neurodegenerative Symptoms via *Suppressor of levy* Mutation in *Drosophila melanogaster*

A Thesis in
Forensic Science

by

William Kaputa

Submitted in Partial Fulfillment
of the Requirements for the Degree of

Master of Science

December 2014

Joonyeong Kim, Ph.D.
Associate Professor of Chemistry
Chairperson of the Committee
Thesis Co-Advisor

Satpal Singh, Ph.D.
Associate Professor of Pharmacology and Toxicology,
State University of New York at Buffalo
Thesis Co-Advisor

M. Scott Goodman, Ph.D.
Chair and Associate Professor of the Chemistry Department

Kevin Railey, Ph.D.
Associate Provost and Dean of the Graduate School

THESIS COMMITTEE

M. Scott Goodman, Ph.D.
Chair and Associate Professor of the Chemistry Department

Jinseok Heo, Ph.D.
Assistant Professor of Chemistry

Joonyeong Kim, Ph.D.
Associate Professor of Chemistry

Amy McMillan, Ph.D.
Associate Professor of Biology

Satpal Singh, Ph.D.
Associate Professor of Pharmacology and Toxicology
State University of New York at Buffalo

Acknowledgements

There are many people whom I would like to thank for their help and guidance on this project. Without their ongoing support, I never would have been able to complete my thesis or maintained my sanity. Thanks to Dr. Satpal Singh, for your constant advice, guidance, and wisdom about life, and the world of *Drosophila*. Thank you to Andrew Cotronea, for indoctrinating me into the world of fruit flies, training me in the lab, and being a constant source of help and friendship along the way. To Alexandra Tibil, for all the help with fly maintenance, testing, data collection, and profound discussions about life. To Bill Bannister, for making all the food to keep my flies alive, and for your companionship during long hours of research. To my family members, for providing love, support, and guidance in all aspects of my life. Finally, to my amazing wife Jillian, for being loving and supportive throughout this entire process, and dealing with my many late nights in the fly lab.

Table of Contents

Table of Abbreviations

α-SYN =   Alpha synuclein

αβ-42 =   Alpha beta 42

AD   =   Alzheimer's Disease

APP   =   Amyloid precursor protein

APPL =   Amyloid precursor protein-like

COX   =   Cytochrome C oxidase

FAD   =   Familial Alzheimer's disease

FPD   =   Familial Parkinson's disease

FTD   =   Fronto-temporal dimentia

GAL-4 =   Galactosidase 4

LRRK =   Leucine-rich repeat kinase

MND   =   Motor neuron disease

ND   =   Neurodegeneration

NFT   =   Neurofibrillary tangles

PARK =   Parkin

PD   =   Parkinson's disease

PINK1 =   PTEN-induced kinase

Su(levy) =   Suppressor of levy

UAS   =   Upstream activating sequence

List of Figures and Tables

I. Introduction

1.1 Human neurodegenerative disorders

Human neurodegenerative conditions are characterized by progressive loss of neurons usually leading to cognitive, physical, and behavioral defects that often end in death [1]. The onset of such diseases is usually multifactorial, resulting from a combination of genetic mutations coupled with environmental factors [2]. Neurodegenerative disorders may be classified based on the type of neurons affected (such as dopaminergic neurons, motor neurons, etc.), the type of pathology exhibited (such as movement disorders, memory loss, dementias, etc.), and by the origin of the disease (genetically inherited, environmental, etc.) [3]. Many human neurodegenerative disorders are late onset and affect only aging members of the population. Most common among these are Alzheimer's disease (AD), Parkinson's disease (PD), and Fronto-temporal dementia (FTD). Evidence shows that neurodegenerative diseases may be passed on in a familial manner from parent to child, but over 90% of cases are sporadic and not related to familial inheritance [4,5]. There does not seem to be a racial or ethnic bias toward inheriting this family of diseases, and the single most abundant risk factor seems to be age. Although there is widespread occurrence of neurodegenerative disease in the United States, the actual mechanisms underlying their function is not well understood.

Human neurodegenerative diseases unmistakably are devastating to their victims both physically and psychologically. What often goes unrecognized are the emotional and financial burdens carried by the families and loved ones of the victims. A conservative estimate holds that in the next 15 years, over 8 million Americans will develop Alzheimer's disease, and yearly costs are already in the hundreds of billions [6]. This does not even take into account the income lost by caregivers who need to leave their jobs to provide 24-hour care for family members—a

cost estimated to be at 220 billion dollars yearly [6]. As of yet the therapeutic treatments are still lacking and what few drugs that are on the market are very limited in their effectiveness. This is due in large part to a lack of understanding of the actual biochemical mechanisms behind such diseases. Significant research is currently being done on such model organisms as fruit flies and mice in order to elucidate these mechanisms and hopefully improve upon current treatments.

On a cellular level, most neurodegenerative diseases are characterized by a buildup of amyloid protein plaques and neurofibrillary tangles in portions of the brain [7]. The physiological implications of this are typically motor or cognitive impairment, caused by the degradation of, or improper functioning of neurons; most commonly dopamine-related or dopaminergic neurons [1]. The phenotypic effects may manifest themselves in memory loss (Alzheimer's), impaired motor coordination (Parkinson's), and speech and emotional impairment (FTD) among other things.

Besides AD and PD, there are dozens of other lesser-known neurological disorders prevalent in the human population. These include Amyotrophic Lateral Sclerosis (ALS), Motor Neuron diseases, Trinucleotide Repeat disease, Leigh Syndrome, Huntington's disease, and Fragile X Syndrome, to name a few [8]. They exhibit a wide array of symptoms including mental retardation, ataxia, muscle atrophy, loss of balance, tremors, and behavioral abnormalities [3]. Although an exhaustive overview of the mechanisms behind these diseases would be extremely worthwhile, for the purposes of this paper, AD and PD will be the neurological disorders of the greatest focus.

1.2 Alzheimer's Disease

AD is the most prevalent neurodegenerative disorder in the United States, affecting roughly 1% of the population over 65 and roughly 30% of the population over 85 [9]. It is considered a disease of old age, and as such, as the average life expectancies continue to rise in this country, the physical, emotional, and financial burdens placed on patients and families will reach epidemic proportions. Currently, it is estimated that 5.2 million Americans have AD. With the discovery of causative environmental agents in addition to genetic factors, it is currently projected that by the year 2050, this number will rise to over 14 million. It is the 6th leading cause of death in this country, and it is estimated that it cost the nation $203 billion in the year 2013 [9].

The significant majority of AD cases (95%) are idiopathic or sporadic, meaning that there is no genetic component to them [4]. Though occurring much less frequently (about 5% of the total cases), hereditary or familial cases of AD have yielded substantial insight into the mechanism behind its formation. These cases are designated as Familial Alzheimer's Disease, or FAD. These familial cases of AD can provide insight into the mechanisms behind Alzheimer's, and as such, they have been the target of the majority of research. Currently, three "causative" gene defects have been identified in human early onset AD (early onset being characterized as ☐ 60 years) [10]. These include Aβ, APP, and the PSN family; mutations that have been shown to contribute to plaques and neurofibrillary tangles [11]. It is these familial inheritance cases that will provide the most insight into the actual pathways involved in neurodegenerative disorders.

Symptomatically, AD first manifests itself with short-term memory loss, escalating to loss of "executive" functions, and in worst case scenarios, full-on dementia [8]. The brain of Alzheimer's patients is characterized by the significant degeneration of the cortex and

hippocampus; regions associated with learning and memory [8]. Additionally, there are two classic features seen upon histological sectioning of an AD brain; extracellular β-amyloid plaques [12] and intracellular neurofibrillary tangles (NFTs) [13]. Essentially, β-amyloid plaques are protein deposits that accumulate in the brains of AD patients. The β-amyloid plaques are rich in Aβ40 and Aβ42 peptides, with Aβ42 thought to be the more deleterious of the two. They are formed by the proteolytic processing of β-amyloid precursor protein (APP), which will be discussed in greater detail later. NFTs are essentially little knots and tangles that occur in central nervous system neurons. These NFTs have been found to be rich in TAU, a protein associated with microtubule formation [14]. Curiously, these protein deposits can be found in different diseases, such as PD or MND, but in various different neurons. As a result, the identification of these characteristic buildups has not yielded much in the realm of a mechanistic understanding of how they are related to disease.

1.3 Parkinson's Disease

Parkinson's disease is the most prevalent neurodegenerative movement disorder, with 4-6 million cases diagnosed worldwide [11]. PD is characterized by tremors, rigidity, slow movements, and loss of balance. PD has an average onset age of 70, although there are a fair amount of cases (4%) that are considered early onset, occurring before age 50 [11]. The mean amount of time from diagnosis to death is 15 years, although several other factors may be at play [15]. Since PD is a progressive neurodegenerative condition, it is often difficult to determine the actual cause of death. Much like with AD, the vast majority of PD cases are idiopathic, most likely caused by a variety of environmental or genetic factors. There is no apparent racial or ethnic risk factors, with age being the only consistent culprit. Inherited or familial forms of the

disease account for roughly 10% of all the cases but show very similar features [16]. Though few in number, these cases yield a wealth of information pertaining to genetic inheritance of PD. Several loci have been implicated in the pathogenesis of Parkinson's, most notably α-*synuclein*. These specific gene mutations will be discussed in detail in the section on Materials and Methods.

As with AD, there are some characteristic features associated with PD as it pertains to the nervous system. The most notable pathological change is the progressive breakdown of dopaminergic neurons in the substanta nigra and locus cereoulus of the brain; both regions of the midbrain associated with movement [13]. This loss of neuronal functioning is the direct cause of movement disorders observed in PD. Another feature common in PD is the accumulation of Lewy bodies, which are essentially abnormal aggregations of proteins that accumulate in the nervous tissue. In the case of PD, these Lewy bodies are principally comprised of the protein α-*synuclein* [17]. As well as Lewy bodies, there are two other characteristics commonly associated with PD; oxidative damage, and mitochondrial dysfunction [18]. Though these are not necessarily causative agents, they are common occurrences associated with this disorder.

For the bulk of neurodegenerative diseases, the molecular mechanisms behind the disease are not well understood. There are currently a lack of effective treatments, and a complete absence of preventative measures for the disorders. The most commonly prescribed drugs are *L-*Dopa and Acetylcholinesterase inhibitors, for PD and AD, respectively [19]. They are reactive in nature, however, and provide only symptomatic relief without addressing the underlying cause of the disease. As such, an understanding of how these disorders work on a molecular level is necessary to develop new and novel treatments for them.

1.4 The role of Mitochondrial dysfunction in Neurodegenerative Disorders

In recent years, there has been increasing evidence for the role of mitochondrial involvement in neurodegenerative disorders [20]. Many studies have shown a correlation between ND and impaired electron transport chain function, particularly the function of iron-containing cytochromes associated with movement over the mitochondrial membrane [20,21]. As a result of this dysfunction, there is an increase in the amount of free radicals, which are harmful to the proper functioning of a cell.

Increasingly, evidence has pointed to oxidative stress, and in particular to reactive oxygen species (ROS), as a culprit for these mitochondrial dysfunctions. In common terms, oxidative stress refers to a cell's increased production of oxidizing species, such as hydrogen peroxide, H_2O_2, or superoxide O_2^- [21]. These species are more commonly referred to as free radicals, or atoms containing unpaired electrons. They are known for wandering the cell and covalently bonding with anything they can find, very often forming dimers with other free radicals. These radicals are naturally produced as a byproduct of respiration, and in moderation are, in fact, harmless. However, when generated in high quantities, they can be extremely detrimental to the proper functioning of cells. They have been implicated in everything from deformation of ion channels in the membrane, to inducing apoptosis, to disrupting DNA transcription by inhibiting critical transcription factors [21].

In properly functioning mitochondria, excess free radicals are scavenged up by enzymes, such as peroxidase. However, when ROS build up, they interact with membrane lipids and proteins, altering their conformations and ultimately disrupting their function. In the context of AD, disruption of the Aβ peptide causes neurofibrillary tangles that are the hallmark of the disease [22].

1.5 Drosophila as a model organism

The common fruit fly, *Drosophila melanogaster,* has been used for a century as a model organism for laboratory study, particularly in genetics [23]. Thomas Hunt Morgan, the scientist responsible for much of the pioneering work on *Drosophila* in the early 1900s, was first attracted to the fact that they have a reasonably short life cycle (10 days at 25°C), and produce a substantial number of progeny (upwards of 100 eggs per female) [23]. This allows large-scale testing and high throughput analysis to be simple and performed in a short amount of time, yielding significant amounts of data [23]. The lifespan is typically 40-120 days but can be easily manipulated by altering conditions such as temperature, food source, and amount of stress [24]. Relative to other lab organisms such as the mouse or rat, *Drosophila* can yield a wealth of information in a much shorter amount of time. Also, due to the very cooperative nature of *Drosophila* researchers, there are an abundance of online information and databases available, with several avenues for obtaining various strains of *Drosophila*. As a result, it is extremely easy and cheap to obtain *Drosophila,* making it a very attractive choice for study.

Ostensibly the fruit fly looks fairly primitive in physiology and behavior. However, it actually demonstrates complex behavior, including such things as learning and memory, and has a rather sophisticated brain [25]. The genetic makeup, development, anatomy, and behavior of *Drosophila* has been very well characterized over the last 100 years, allowing for extensive study of molecular and biological processes [26]. The fruit fly has 13,600 genes in its genome, or roughly half of the 27,000 genes that comprise the human genome [27]. These are housed in four pairs of chromosomes, with the first chromosome acting as the sex-determiner much like in humans. Several years of research have contributed to the multitude of genetic "tools" that allow

for *Drosophila* study, including such things as transgenic flies, GAL-4 constructs, P elements, and differential gene expression [28, 29].

Finally, and perhaps most importantly, *Drosophila* has proven to be a great organism for modeling human neurodegenerative conditions [28]. It has been shown that roughly 75 % of the molecular pathways in humans that are implicated in disease show a great deal of sequence conservation in *Drosophila* at the protein level [30]. Not only disease-associated genes, but many molecular pathways including cell signaling and the regulation of cell cycle and growth are conserved across the species [31]. In layman's terms, the genetic sequences that code for certain proteins in *Drosophila* are very similar to those that exist in *Homo sapiens*. Consequently, disease models in *Drosophila* can be very useful in obtaining information on the biochemical level as to the mechanisms behind human ND disorders [8].

1.6 Genetic tools available for Drosophila work

As mentioned above, *Drosophila* makes for a great model organism due to the availability of several genetic tools and techniques. One of the fundamental aims in the study of neurodegeneration is to identify the underlying molecular pathways that cause the neurodegeneration. This allows for the development of targeted treatments, specific to the problem at hand. *Drosophila* provides a great medium for performing genetic studies aimed at understanding these mechanisms.

One of the most common practices used to identify disease pathways is the over-expression or under-expression of a human disease gene in *Drosophila*. This can be done with both wild type genes and mutant human genes. The location and timing of gene expression can be controlled by UAS-GAL 4 system, which will be discussed in greater detail later. In short,

this system allows for the controlled expression of a given gene (responder) in a specific tissue (driver) of choice, allowing the researcher to control the expression of the gene [32]. There are some drawbacks to this approach. The GAL-4 protein itself can cause neurodegeneration if it is present in excess, and the degree of severity of a given neurodegenerative phenotype may not be commensurate with the amount of protein expressed.

Another technique commonly used is gene knockouts and knockins. This allows a given *Drosophila* gene to be 'turned off' or 'turned on,' and the resultant phenotype to be analyzed. Inactiviation can be achieved through means such as DNA methylation, interfering RNAs, or mutational inactivations. Gene overexpression is performed (typically through a GAL-4 system) in order to study gain-of-function scenarios. Once a gene's expression is altered, the progeny can be screened for the presence of neurodegenerative phenotypes [32].

Once a given phenotype has been identified, it is often followed by a screen for enhancers and suppressors. This is often done by inducing mutations through the feeding of ethyl methane sulfonate (EMS) and propagating any flies that show worsening of the mutant phenotype (enhancer) or rescue of the wild-type phenotype (suppressor). This is exactly what occurred in the case of *Suppressor of levy*. Once the *levy* gene mutation was identified, mutations were induced using EMS, and progeny that did not show neurodegeneration were isolated and propagated. The focus of this study, *Supressor of levy* mutation, was recognized as rescuing wild-type phenotypes in neurodegenerative *levy* flies. As a result, this gene became the basis for our research.

II. Background

2.1 The levy mutation

The basis for this research started with the identification of the *levy* mutation in the lab of

Satpal Singh, PhD at the University at Buffalo. The mutation was identified by using a forward

genetics approach using temperature-induced paralysis as the basis for identification. Mutations

were induced by using ethyl methane sulfonate as described by Ashburner [32]. Flies were then

exposed to 38°C for a period of 5 minutes, which led to paralysis of certain mutant flies, which

were then propagated. One of these mutations were designated as *levy*. Deficiency mapping and

P elements were then used to localize the mutation, which was subsequently sequenced [33].

The *levy* mutation was mapped to the right arm of chromosome 2. The mutated gene

corresponded to the gene CG17280 as designated in the flybase database [34]. It codes for

subunit VIa of the cytochrome C oxidase, a protein complex critical in the normal functioning of

mitochondrial biochemical pathways [33]. Mitochondrial misfunction has been linked to

neurodegeneration and several neurodegenerative diseases, including Parkinson's and

Alzheimer's. In addition to temperature-induced paralysis, the *levy* mutation causes

encephalomyopathic effects, reduced ATP generation, reduced lifespan, and reduced motor

recovery after mechanically-induced shock (bang-sensitivity) [33].

Figure 1 shows the reduced lifespan characteristic of *levy* flies. The percentage of

surviving flies grown at 29°C is shown as a function of time. Wild-type flies (CS) lived to a

median age of 28 days. *Levy* transformants (T) were generated by integrating the wild-type copy

of the *levy* gene into the *levy* mutant genome using germline transformation. The transformants

showed a similar lifespan to wild-type flies. The *levy* mutants (*levy*) and transformant controls

(Tc) both showed a median lifespan of roughly 8 days, which is a 70 % decrease in median lifespan [33].

As mentioned above, flies exhibiting neurodegeneration show a reduced ability to recover from mechanically induced shock, a phenomenon termed "bang-sensitivity." This is measured quantitatively by vortexing a vial of flies on high speed for ten seconds which paralyzes the flies. The length of time it takes for the flies to stand and begin moving again is then measured. **Figure 2** displays the bang-sensitivity of *levy* flies when compared to wild-type flies. The percentage of flies paralyzed is displayed as a function of time. Notice that twenty-day old *levy* flies (20d levy) and twenty-day old transformant controls (20d Tc) took significantly longer to recover than ten-day old *levy* flies (10d levy) and ten-day old transformant controls (10d Tc). Two-day old flies did not show any significant bang-sensitivity [33].

Upon histological cross-sectioning, the brain of a *levy* mutant fly shows a characteristic "swiss-cheese" effect commonplace in spongiform neurodegeneration [33]. **Figure 3** shows a wild-type fruit fly brain (A) in cross-section as compared to a *levy* fly brain (B). The holes in the *levy* brain are characteristic of neuron cell degradation and death [33].

Lifespan analysis in *levy* flies
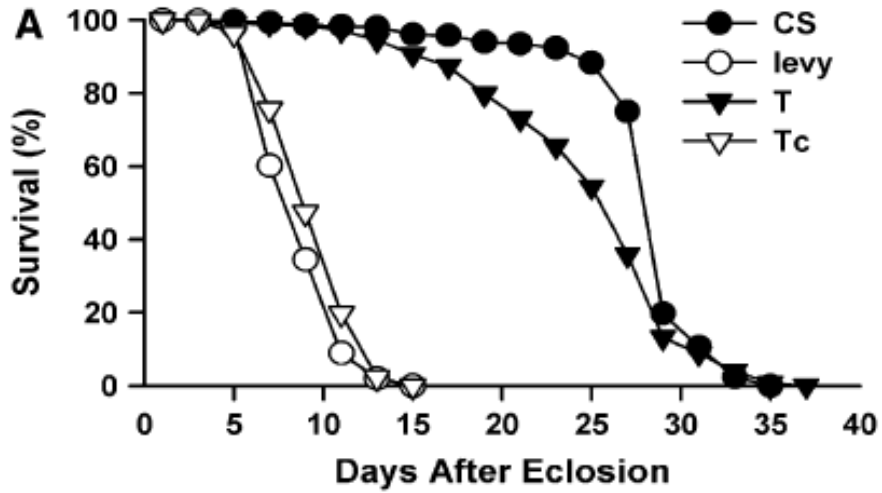
Figure 1: Lifespan of *levy* and wild-type flies. *Levy* flies (levy) and tranformant controls (Tc) show a significantly shorter median lifespan than wild-type flies (CS) and transformants (T) when grown under the same conditions [33].

Bang-sensitivity in *levy* flies
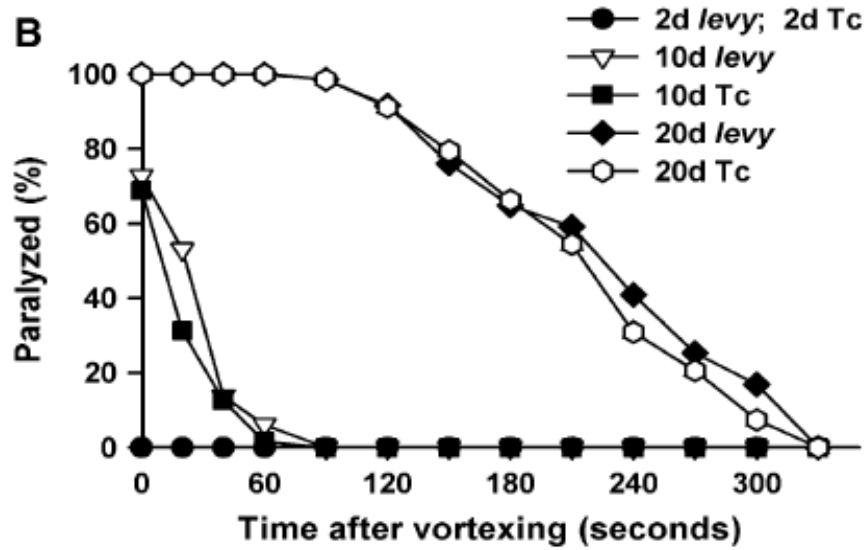
Figure 2: Bang-sensitivity of *levy* flies and transformant controls. Flies showed age-dependent increases in sensitivity to mechanical shock as induced by vortexing [33].
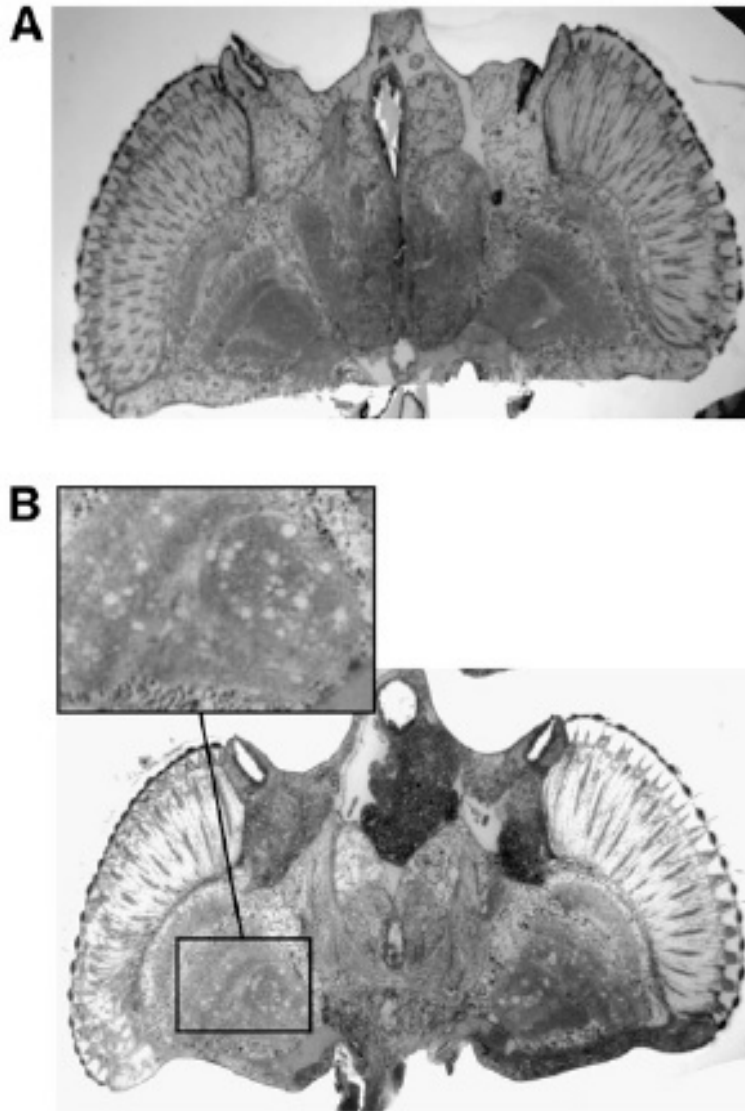
Figure 3: Cross-section of wild-type brain (A) compared to that of a *levy* fly brain (B). The small white holes in the *levy* brain are the result of neurodegeneration in the brain, a condition which leads to reduced lifespan and locomotor abilities [33].

2.2 Suppressor of levy mutation

Subsequent to the isolation of the *levy* gene, a mutation was discovered that suppressed the effects of *levy* and partially rescued a wild-type phenotype; that is, certain flies with the *levy* mutation did not exhibit the same neurodegenerative symptoms that the rest of the *levy* flies did. Something in these exceptions was preventing or suppressing the symptoms associated with the *levy* mutants. Accordingly, this mutation was called *Suppressor of levy*, notated *Su(levy)*, and was subsequently mapped to the 2nd chromosome as well. This suppressor mutation was induced in the same fashion as *levy*, by feeding ethyl methane sulfonate to *levy* flies and screening for rescue upon exposure to 38°C. A complementation test was used to verify that it was in fact a novel and unique gene that was rescuing the wild-type phenotype, and not some mutation in the *levy* gene itself. These *Suppressor of levy* flies were propagated and balanced with *CyO* such that they would produce a stable line. One of the cornerstones of our research focuses on the following question: Since this *Suppressor* mutation rescues a wild-type phenotype in *levy* flies, might it also rescue wild-type phenotype in flies with other ND-inducing mutations?

2.3 Selected genes of interest

There are a number of genes that have been identified as either contributing to and/or being causative agents of familial PD. As is often the case, many of them have orthologs in the *Drosophila* genome. **Table 1** shows the most commonly identified culprits in familial PD [3]. With the exception of α-*synuclein*, these genes all have orthologs in the *Drosophila* genome, making them very accessible for study. **Table 2** shows some of the gene families associated with AD [3]. Our research focused primarily on five genes associated with PD; namely α-*synuclein*, *DJ 1-α, LRRK2, Pink1, and Parkin*, as well as one gene associated with AD; namely *Aβ-42*.

Table 1: Parkinson's disease-associated genes [3]

Gene/Protein	Fly Ortholog	Protein Function
Alpha-synuclein	None	Pre-synaptic protein
Parkin	CG10523	E3 ubiquitin ligase
UCH-L1	Uch/CG4265	E3 ubiquitin hydrolase/ligase
PINK1	Pink1/CG4265	Mitochondrial kinase
DJ-1	DJ-1a/CG6646 DJ-1b/CG1349	Redox sensor/Chaperone
LRRK2	lrrk2/CG5483	Kinase/GTPase
HtrA2	HtrA2/CG8486	Mitochondrial pro-apoptotic protease
GBA	CG33090	Lysosomal enzyme
POLG	tamas/CG8987	Mitochondrial DNA polymerase
Tau	tau/CG31057	Microtubule stabilization

Table 2: Alzheimer's disease-associated genes [3]

Gene/Protein	Fly Ortholog	Protein Function
APP	Appl/7727	Pre-synaptic protein
PSN-1/2	dPs/CG18803	Gamma-secretase activity
Tau	tau/CG31057	Microtubule stabilization
APOe4	None	Lipid/cholesterol metabolism

2.3a: LRRK-2

LRRK2 stands for leucine-rich repeat kinase, which is a gene associated with kinase production and GTP enzymatic function [35]. It is inherited in an autosomal dominant fashion, and has a well-conserved ortholog in *Drosophila* denoted *LRRK2* (CG5483). *LRRK2* mutations are the most common cause of Familial Parkinson's Disease (FPD), accounting for nearly 40 % of FPD cases in certain populations [35]. The actual *in vivo* role of wild-type *LRRK2* proteins is unknown at this point. Interestingly enough, overexpression of wild-type *LRRK2* in *Drosophila* leads to cell death *in vitro*, most noticeably in dopaminergic neurons [35].

In terms of lifespan, overexpression of *LRRK2* has no significant effect [35]. Interestingly enough, overexpression actually increased the fecundity of female flies, a surprising result given its seemingly deleterious nature in the brain. Locomotor ability varied with age of mutants as well as the particular *LRRK2* mutation. In some cases, climbing was improved relative to the control, whereas in some cases it showed a significant decrease. An external phenotype consistent with all *LRRK2* mutants seems to be eye defects. Particularly in males, *LRRK2* flies show black lesions on the eyes, loss of pigmentation, and architectural defects that are particularly noticeable under electron microscope [35].

Overall, overexpression of *LRRK2* increased the rate of dopaminergic cell death [35]. Finally, *LRRK2* mutants have been shown to interact with other recessive PD genes, including *pink* and *parkin*.

2.3b: DJ 1-α

The DJ-1 gene in humans has been identified as an inherited PD gene, and its protein has been linked with chaperone function and protection from oxidative stress [36]. It has two orthologs in flies: DJ1-α (CG6646) and DJ1-ß (CG1349), each of the coded proteins sharing about 70% similarity with the human DJ1 protein.

Much information has been learned about *DJ1-α* from doing double knockout experiments. Essentially, this involves deleting both the DJ1-α and the DJ1-ß genes from the DNA sequence so that protein production is blocked at the transcriptional level. Interestingly enough, in *Drosophila*, double knockout mutants of *DJ1-α* and *DJ1-ß* are both viable and fertile, and don't show any noticeable phenotypic defects. Likewise, the double mutants show lifespans, fertility, viability, and dopaminergic neuron functionality all similar to what is found in wild-type flies. As such, there appears to be no overt phenotypic defects in flies lacking the *DJ1* function [37]. However, these flies show significantly enhanced sensitivity to oxidative stress [37].

2.3c: α-Synuclein

Alpha-synuclein is a dominantly inherited gene that codes for a pre-synaptic neuronal protein of unknown function [38]. It has been shown that mutations in *α-synuclein* lead to neurodegeneration in familial PD cases. In particular, *α-syn* mutations cause loss of dopamine neurons, formation of Lewy bodies, and locomotor dysfunction [38]. There is no *α-syn* gene endogenous to flies. As such, animal models associated with *α-syn* mutations use human *α-syn* to generate transgenic Drosophila mutants through germline transformation. These transgenic flies

are thought to adequately model Parkinson's symptoms that are seen in humans with *α-syn* mutations [37].

The expression of wild-type human *α-syn* in flies leads to normal development and functioning of neurons, histological appearance, and fly viability and behavior [38]. It is the expression of mutant A30P and A53T α-*syn* (α-*syn* proteins associated with PD) that shows symptoms of neurodegeneration. The degeneration and disappearance of dopaminergic neurons occurs in 30-60 day old flies expressing either A30P, or more notably, A53T α-*syn*. The only significant phenotypic differences in mutant flies that is observable with light microscopy lies in the retina of the eye. Overexpression of human α-*syn* in the eye leads to progressive retinal degeneration that is noticeable after 10 days, and is very distinct after 30 days [38]. This deviation in eye structure can be observed as a manifestation called the pseudopupil [39]. In terms of behavioral assays, one of the most telling features of mutant *α-syn* is their significantly decreased climbing ability. Compared to wild-type flies, all mutant strains of *α-syn* show markedly decreased ability to climb, particularly as they get older. This dysfunction is indicative of degeneration of dopaminergic neurons and formation of Lewy bodies in the cortex [38].

2.3d: Pink & Parkin

Both *Parkin* and *Pink1* are recessively inherited genes that code for a ubiquitin protein ligase and a mitochondrial kinase, respectively [3]. They are endogenous to both humans and *Drosophila* and show a significant degree of sequence similarity between the species. *Parkin* is shown to code for ubiquitin ligase proteins, used in several cellular functions, particularly conferring substrate specificity in ubiquitination pathways [40]. *Pink1* codes for a mitochondrial kinase that is critical in the successful functioning of mitochondrial transport chains [41]. Both

Pink1 and *Parkin* mutant flies show remarkable similarity in terms of their phenotypes and behavior, and will therefore be discussed in concert.

A fair amount of work has been done on *parkin* and *pink1* null alleles. A null allele is essentially a mutation whereby the gene of interest has either been completely removed, or the protein that it codes for has been deactivated in some fashion. It has been shown that flies with null alleles for *parkin* and *pink* are viable, but exhibit several symptoms, both physical and behavioral, associated with neurodegeneration. First and foremost, the lifespan of null mutants is significantly reduced. Wild-type flies showed a median lifespan of 39 days and a maximum age of 75 days at 25°C, whereas null mutants showed a median lifespan of 27 days, with a maximum age of 50 days. *Parkin* mutants also show a downturned wing phenotype in both the homozygous and the heterozygous state. This phenotype is not fully penetrant, but does show up in roughly 40 % of newborn flies, and in 70% of flies by age 10 days. As a result of this wing condition, many *parkin* flies show defects in flight and climbing ability. This has been verified by expressing *parkin* in mesodermal tissue using a GAL-4 system. As a result, both flight and climbing abilities returned to wild-type status, showing that *parkin* is required for normal functioning of the musculature. Finally, *parkin* mutants show a distinct alteration of normal structure in dopaminergic neurons, upon histological examination. This is a finding consistent with other PD mutants, including *α-syn*. *LRRK2*, and *DJ1-α.*

2.3e: Aβ-42

The first gene that was identified as being a causative agent of AD is the gene coding for β-amyloid precursor protein, or APP, located on chromosome 21 [22]. Several different APP mutations have been characterized, all leading to the classic β-amyloid protein deposits,

specifically *Aβ-42*, found in the brain of AD patients. These aggregations of *Aβ* have been found

to be toxic to neurons, and ultimately lead to neuronal cell death [10]. According to what has

been termed the 'amyloid hypothesis,' accumulation of the Aβ 42 peptide initiates the pathogenic

cascade in AD, including Tau hyperphosphorylation, aberrant cellular signaling, and ultimately,

cell death [41]. Efforts to identify the underlying process of protein deposition in the brain have

been aimed at categorizing the pathways involved in the movement of intracellular APP [43].

Defects in APP lead to several AD symptoms, including plaque deposition, defective axon

transport, lack of synaptic plasticity, and vacuolization of the brain [3].

The ortholog in *Drosophila* is notated *APPL*, or APP-like gene, and is expressed in all

neuronal tissue. Knock-out mutation of *APPL* in flies does not cause the same neurodegenerative

phenotype found in humans. This is most likely because there is a lack of homology between

human *APP* and fly *APPL* in the *Aβ* region, and as a result *Aβ* deposition does not occur in the

flies. Therefore, *Drosophila* models use the GAL-4 system to overexpress human forms of *Aβ* in

desired regions [3]. Although flies do not produce *Aβ42* naturally, overexpression of human APP

results in *Aβ42* production and neuronal cell death [44].

2.4 The UAS-GAL-4 Driver system

As mentioned above, the last 20 years has seen an explosion of powerful genetic tools

being used with *Drosophila*. Perhaps one of the most ubiquitous techniques is the bipartite

UAS/GAL 4 system used for targeted expression of genes in specific tissues.

GAL4 is a gene that is endogenous to common yeast, *S. cerevisae*. It codes for a protein

that is 881 amino acids long whose transcription is induced by galactose. The gene has been

fairly well characterized due to many studies done on transcriptional activation in yeast. GAL4

has been shown to be a transcriptional activator in yeast by binding upstream of the promoter site, much like an enhancer would in animal cells. These upstream sites are now commonly called Upstream Activating Sequences, or UAS. The beauty of this system is that the GAL4 gene will work in multiple different settings, regardless of the organism, as long as the UAS is present, and perhaps even more importantly, it seems to have no harmful effects on the organism.

This is a very useful system particularly when dealing with genes with toxic or deleterious effects. For example, a harmful gene can be maintained in a silent, untranscribed state via a UAS in a parental fly line. Upon crossing with a line carrying a GAL4 driver for a particular tissue type, the progeny will express the UAS-linked gene in that target tissue only. For example, a UAS is inserted into a male fly upstream of a gene denoted 'X,' which codes for a protein producing red pigment. A GAL4 driver is inserted into a female fly with a gene denoted 'Y,' which is expressed only in thoracic bristles. When these two parental lines are mated, the progeny will express both genes simultaneously. That is, the GAL4 driver will stimulate the production of red pigment from gene X (the responder) in the thoracic bristles.

This can also be useful to induce the transcription of a deleterious responder gene in a region of interest. The resultant cell death or dysfunction can elucidate the role of the cells and the effect of the cell death on the organism as a whole. It may even shed light on the mechanisms behind how certain diseases or disorders work [45].

In this research, we use an APPL-GAL4 driver to induce activation of gene mutations associated with PD and AD via Upstream Activating Sequences. *APPL* stands for "APP-like" and is a *Drosophila* ortholog of the human *APP* gene, which stands for B-amyloid precursor protein. It is a gene that codes for a protein that functions pre-synaptically in neurons. The GAL4/UAS system is being used in this scenario to express proteins from various PD-related

and AD-related genes in the neurons. As such, the APPL-GAL4 driver needs to be combined with UAS-tagged PD and AD genes via selective crossing of parent lines. The subsequent progeny should show neurodegeneration as a result of these mutations being expressed in the CNS. Subsequently, the *Su(levy)* mutation can be crossed to determine if it shows rescue of wild-type behavior in the PD and AD flies.

2.5 The Goal of our research

The purpose of this research is to determine if the *Suppressor of levy* gene alleviates neurodegenerative phenotypes in fly mutants other than *levy*; namely, *Parkin*, *Pink1*, *DJ1-α*, *α-synuclein*, *LRRK2,* and *Aβ42*. These strains have all been shown to exhibit symptoms of neurodegeneration in one form or another. Reduced locomotor ability, including flight and climbing defects, are shown in *Parkin*, *Pink*, *α-Syn*, *LRRK2*, and *Aβ-42* flies. Reduced longevity is shown in *Parkin*, *Pink*, and *Aβ-42* flies. Increased sensitivity to environmental toxins is shown in *DJ 1* flies [3]. It is the goal of this project to determine if combining *Su(levy)* with flies carrying these mutations will lessen these symptoms and rescue a wild-type phenotype. If this proves to be the case, further study into the mechanisms behind the rescue will become necessary, as there may be significant implications for the understanding of neurodegeneration in a human model.

A future goal of this research is to identify and sequence the *Suppressor of levy* mutation, and to gain insight into the mechanisms behind its function. A mechanistic understanding of how *Su(levy)* alleviates neurodegenerative symptoms has therapeutic ramifications, potentially yielding information about the underlying functioning of human neurodegenerative diseases. Clearly, the absence of a gene product (or perhaps the production of a dysfunctional gene

product) coded for by the *Su(levy)* mutation is playing some role in alleviating the neurodegenerative symptoms in *levy* fruit flies. If a drug that mimics the role of the *Su(levy)* mutation (by blocking or otherwise inhibiting the gene product) could be produced pharmacologically, it may prove to be a promising step forward to the treatment of neurodegenerative disorders in humans.

III. Materials and Methods

3.1 Fly strains and rearing:

      All flies used during this research were raised on a standard cornmeal medium with the exception of *APPL-GAL 4* flies. They were raised on Carolina® instant Drosophila medium. All flies were grown and aged at 25°C with the exception of flies being used for lifespan analysis. They were aged at 29°C. In some instances, it was necessary to transfer flies to 18°C for a period of time for the purposes of virgin isolation. Canton Special (*CS*) flies were used as the wild-type for all experiments. The mutant strains *levy* and *Su(levy)* were provided by the lab of Satpal Singh, PhD, University at Buffalo Department of Toxicology and Pharmacology. The mutant AD and PD flies were obtained from the lab of Shermali Gunawardena, PhD, University at Buffalo Department of Biological Sciences. All crosses were performed in bottles at 25°C, with females and males being used in a roughly 2:1 ratio, respectively. **Table 3** lists information about all of the strains of flies used for these experiments and **Table 4** lists the specific AD and PD strains used for our research. The leftmost column of **Table 3** provides the abbreviated notation for each strain. The middle column provides the complete genotype, and the rightmost column provides any specific phenotypic markers that were used for identification. Fly genotypes are written using specific notation (See **Figure 4**). Each pair of chromosomes is written using horizontal lines separated by semicolons, with the 1st chromosome being the sex chromosome (the Y is designated with a small diagonal hashmark). Since the 4th chromosome in *Drosophila* contains very little coding DNA, it is often omitted for the sake of convenience. Any mutations are written above or below the respective chromosomes, and the first letters of the dominant mutations are typically capitalized. Where multiple mutations are present on the same

chromosome, they are separated by a space. A cross between a female fly and a male fly is separated by an "x" symbol.

$$\frac{\text{w1118}}{\qquad} \ ; \ \frac{\qquad}{\qquad} \ ; \ \frac{\qquad}{\qquad} \quad \text{X} \quad \frac{\text{w1118}}{\text{w1118}} \ ; \ \frac{\text{Pin}}{\qquad} \ ; \ \frac{\qquad}{\qquad}$$

Figure 4: The above notation depicts a cross between a male, white-eyed fly (w1118) and a female white-eyed fly carrying a dominant Pin mutation on the second chromosome

Table 3: Fly strains used for genetic crosses

| Fly Strain notation	Complete Genotype (Female notation used for convenience)	Phenotypic Markers
CS	$\dfrac{}{} \; ; \; \dfrac{}{} \; ; \; \dfrac{}{}$	External phenotype is wild-type
APPL-GAL 4	$\dfrac{\text{Appl-Gal-4}}{\text{Appl-Gal-4}} \; ; \; \dfrac{\text{Cyo Tb Antp}}{\text{Pin}} \; ; \; \dfrac{}{}$	Curly wings, red eyes, tubby larva, additional bristles on thorax, x-shaped bristles along dorsal thorax
ISH	$\dfrac{}{} \; ; \; \dfrac{\text{Su(levy) L}^1\text{ Pin}}{\text{CyO}} \; ; \; \dfrac{}{}$	Lobed eyes, x-shaped bristles along dorsal thorax, curly wings
LEVY1	$\dfrac{}{} \; ; \; \dfrac{\text{levy}}{\text{levy}} \; ; \; \dfrac{}{}$	External phenotype is wild-type
UAS-DJ1-α	$\dfrac{}{} \; ; \; \dfrac{\text{UAS-DJ1-}\alpha}{\text{UAS-DJ1-}\alpha} \; ; \; \dfrac{}{}$	External phenotype is wild-type
UAS-LRRK2	$\dfrac{}{} \; ; \; \dfrac{}{} \; ; \; \dfrac{\text{UAS-hLRRK2}}{\text{UAS-hLRRK2}}$	External phenotype is wild-type
UAS-PARKIN	$\dfrac{}{} \; ; \; \dfrac{}{} \; ; \; \dfrac{\text{UAS-PARKIN}}{\text{UAS-PARKIN}}$	External phenotype is wild-type
UAS-PINK	$\dfrac{}{} \; ; \; \dfrac{\text{UAS-PINK}}{\text{CyO}} \; ; \; \dfrac{}{}$	Curly wings
UAS-αSYN	$\dfrac{}{} \; ; \; \dfrac{\text{UAS-}\alpha\text{Syn}}{\text{UAS-}\alpha\text{Syn}} \; ; \; \dfrac{}{}$	External phenotype is wild-type
FLY #3 (Unnamed)	$\dfrac{\text{Appl-Gal 4}}{\text{Appl-Gal 4}} \; ; \; \dfrac{\text{CyO Tb Antp}}{\text{Su(levy) L}^1\text{ Pin}} \; ; \; \dfrac{}{}$	Curly wings, tubby larva, extra bristles on thorax, red eyes, lobed eyes, x-shaped bristles on dorsal thorax

Table 4: Specific AD and PD genes used for this research.

| Gene/Protein	Fly Homolog	Associated disease	Protein function
APP	Appl/CG7727	AD	Pre-synaptic protein
Alpha-synuclein	N/A	PD	Pre-synaptic protein
Parkin	parkin/CG10523	PD	E3 ubiquitin ligase
PINK1	pink1/CG4523	PD	Mitochondrial kinase
DJ-1	dJ-1α/CG6646	PD	Redox sensor/Chaperone
LRRK2	Lrrk2/CG5483	PD	Kinase/GTPase

3.2 Crosses

In order to test the effects of the *Su(levy)* on *Drosophila* neurodegeneration, it was necessary to perform crosses in order to combine flies carrying *Su(levy)* with flies carrying specific AD and PD mutations. This was done by crossing females containing APPL-GAL 4 driver as well as *Su(levy)* mutation with males containing a specific responder controlled by an upstream activating sequence. The resulting progeny were selected for flies carrying APPL-GAL-4 driver and the UAS-linked responder gene. In this case, the responder is expressed in neuronal tissue due to the neuronal expression of APPL-GAL-4 driver. Since multiple AD and PD mutations were used, it was necessary to generate a stable fly carrying both the driver and the *Suppressor* mutation, so that this fly could be mated to mutants and produce the desired progeny within one generation. The crosses utilized to obtain this stable line are shown in **Figure 5**.
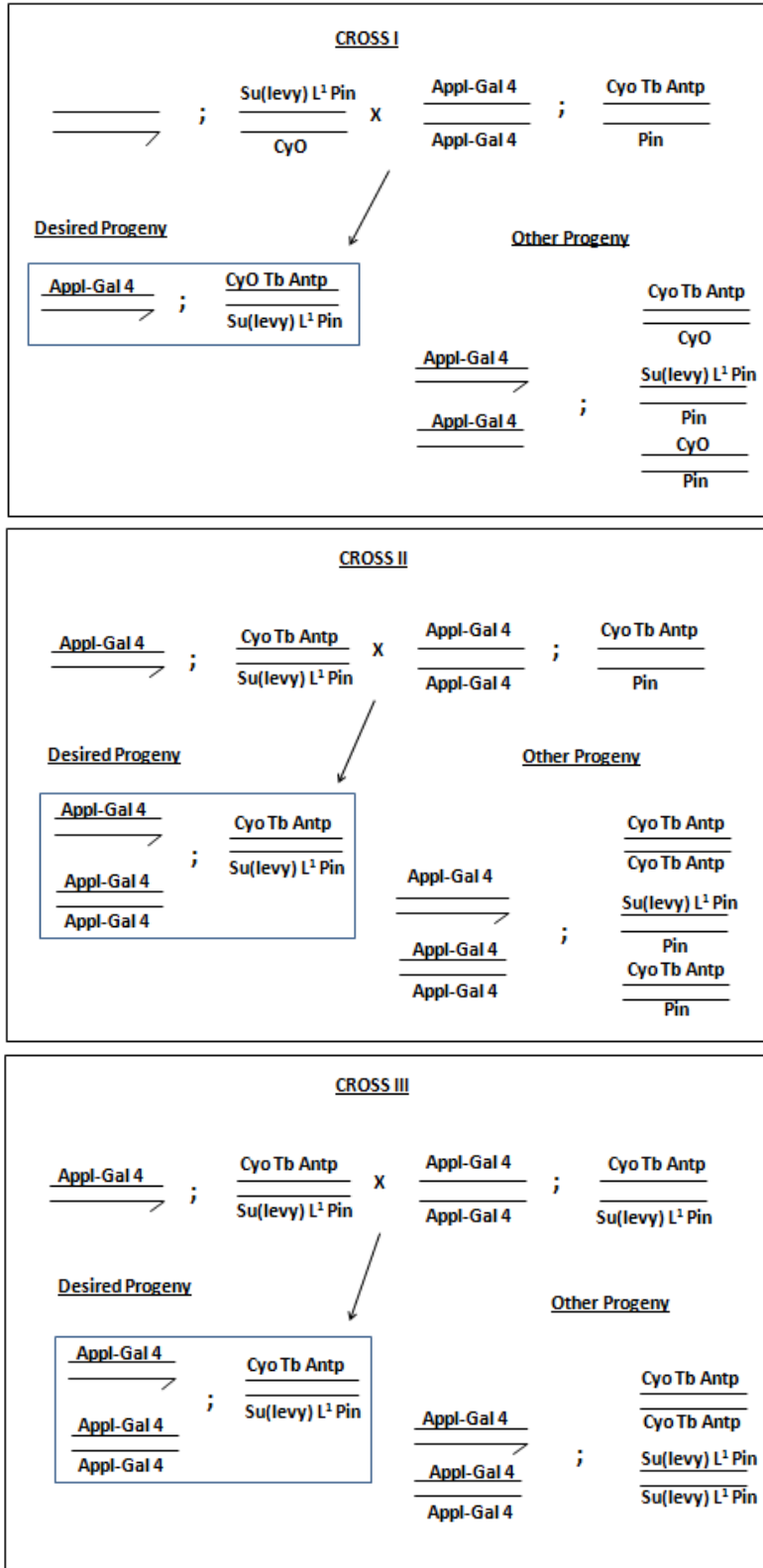
Figure 5: Crosses used to obtain a stable heterozygous *Su(levy)* fly stock. The parental setup is shown at the top of each cross, with the selected progeny boxed in on the left, and the other progeny shown along the right.

Once a stable line containing the APPL-GAL-4 driver and the *Suppressor of levy* mutation was established, virgins from this line were mated to males carrying AD and PD mutations males and progeny were screened for the desired phenotypes. The flies collected were those that contained both the *Su(levy)* mutation as well as the respective AD or PD mutation along with the APPL-GAL-4 driver. An example of one of these crosses is shown in **Figure 6**.
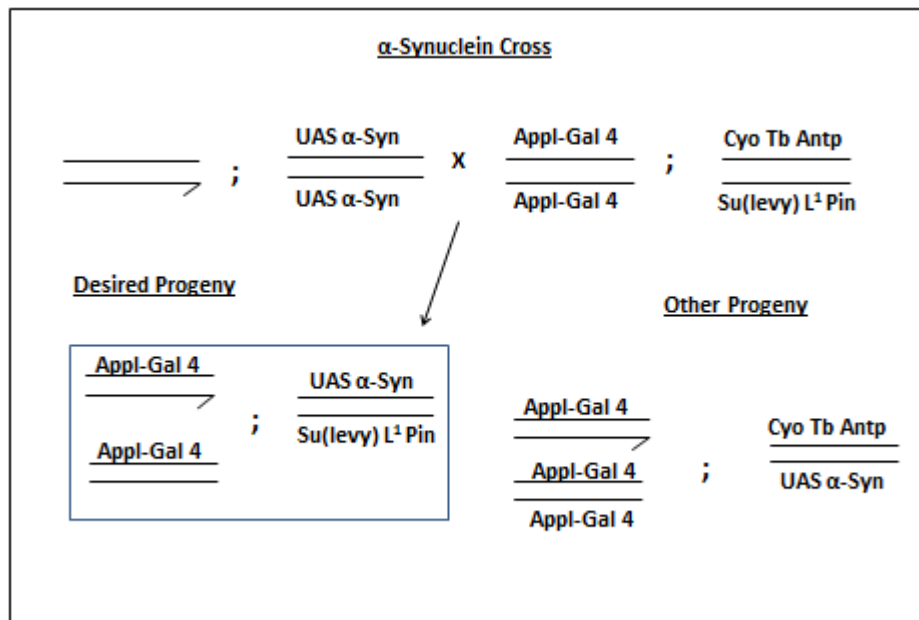
Figure 6: An example of a cross to obtain flies carrying both *Su(levy)* mutation as well as a ND mutation. The parental cross is shown at the top. All progeny of this cross carry the GAL-4 driver. The desired progeny carry the GAL-4 driver, as well as the responder gene (in this case *α-Syn*, and *Su(levy)*. The other progeny carry the GAL-4 driver and the responder gene, but not *Su(levy)*.

In order to ensure the validity of the paternity for a given cross, it was necessary to isolate virgin flies of the genotype of interest. This was performed by emptying vials of all adults and collecting any newly eclosed females hatchlings within 8 hours. These females were isolated into a separate vial and aged for 7 days. After 7 days, the vial was screened for the presence of eggs and/or larvae. Once virginity was ensured (lack of eggs or larvae present), the flies were added to bottles with the males of interest. Female virgins were also collected by isolating and separating flies during the pupal stage. Late-stage pupa were collected with a brush and segregated into Eppindorf tubes capped with a moist cotton ball. Upon eclosion, females were used for crosses.

3.3 Assays

Mechanical and behavioral assays were chosen based on a variety of factors. The primary drivers for our research assays were the previously published literature and the availability of fly stocks and equipment in our lab. Since the hallmarks of neurodegeneration in animals are motor dysfunction, learning & memory impairment, and shortened lifespan, related assays were conducted. In most cases, protocols were borrowed from established literature with minor variations to suit the purpose of our experiments.

Lifespan analysis: Flies used for lifespan analysis were raised at 25ºC and aged at 29 ºC . Newly eclosed males and females were segregated and placed in standard vials, 20 to a vial. Flies were counted each day. Vials were changed every 2-3 days and dead flies were counted and removed at time of transfer. The percent of surviving flies were graphed as a function of time. Flies that escaped during transfer or that died as a result of some other mitigating factor (getting stuck in the food) were not counted toward the lifespan data.

Bang-sensitivity assay: Bang sensitivity was performed on flies aged at 25ºC. Twenty-day old flies were placed in empty plastic vials in groups of 10 and vortexed on high for 10 seconds. This simulates mechanical trauma to the fly, and in many cases causes a fly to become paralyzed, or unable to move. After paralysis, the flies eventually regain motor function and begin moving. The length of time it took for flies to stand and walk around was recorded. The percentage of flies paralyzed was graphed as a function of time.

Temperature-induced paralysis assay: Paralysis was performed at 38ºC. Flies were placed in clean, empty plastic vials, 10 to a vial. Vials were submerged in 38ºC water bath and the time taken by flies to paralyze was recorded. In this assay, paralysis was defined as the time when the fly took its last step and fell over onto its back. The percentage of flies paralyzed was graphed as a function of time.

Locomotor assay: The climbing ability of flies is a direct indicator of motor skills and coordination. To test for climbing ability, 7-day old flies were placed ten to a vial, and a secondary vial was inverted and taped over the first vial. A line was drawn 8 cm up from the bottom of the first vial. Flies were allowed 10 minutes to acclimate to the surroundings. Flies were gently tapped to the bottom of the vial and the number of flies crossing the line after 10 seconds was recorded. This was repeated five times with each grouping of ten flies, and the data were recorded as a percentage of successful flies.

Drug sensitivity assay: Since many forms of human AD and PD are linked to environmental factors, it seemed worthwhile to test the ability of *Su(levy)* to suppress

neurodegenerative symptoms associated with exposure to environmental toxins. As such, we decided to compare the lifespan of both wild-type and *Suppressor* flies that were raised on normal food versus those that were raised on food laced with 500 µM Rotenone® (See **Figure 7**). Rotenone® is a chemical that is widespread in pesticides, and has been linked to symptoms of PD in animals. All flies were grown at 25° C, and all other conditions were kept constant. We wanted to determine the phenotypic effects of exposure to this chemical, and whether or not *Suppressor* alleviated any of these symptoms.
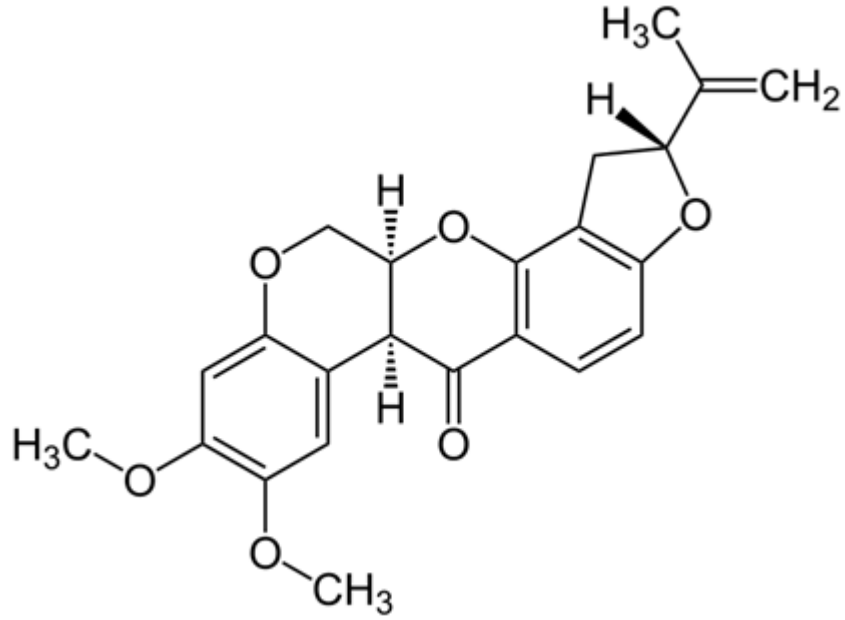
Figure 7: Rotenone®, chemical formula $C_{23}H_{22}O_6$, is a chemical commonly used in pesticides that has been linked to environmental forms of Parkinson's disease

IV. Results

Lifespan Analysis: Lifespan assays were run as separate replicates and the data were pooled and graphed as an overall survivorship percentage. **Figure 8** shows the survivorship curves for wild-type, *Su(levy), levy*, and *Su(levy) w/ levy*. The median lifespan at 29°C for CS, *Su(levy)*, *levy,* and *Su(levy) w/levy* was 26 days, 34 days, 9 days, and 12 days, respectively. *Su(levy)* mutation increased the median lifespan of *levy* flies and wild-type flies by 33% and 31%, respectively.

Figure 9 and **Figure 10** show the survivorship curves for each of the six strains of flies carrying neurodegenerative mutations, as well as those strains of flies carrying the respective mutation alongside the *Su(levy)* mutation. In four out of the six cases, *Su(levy)* increased the median lifespan by a significant margin. In one case (*Pink*), *Su(levy)* decreased the lifespan by a significant margin, and in one case (*Parkin*), *Su(levy)* decreased lifespan, but not by a statistically significant amount.

Table 5 shows the median lifespan for each of the six strains mentioned above, as well as the changes in median lifespan when carrying the *Su(levy)* mutation. *α-Syn, DJ 1-α, LRRK2,* and *αβ-42* flies each showed increases in lifespan of 22%, 32%, 23%, and 56%, respectively, when carrying the *Su(levy)* mutation. *Pink* and *Parkin* flies showed decreases in lifespan of 24% and 7% respectively, when carrying the *Su(levy)* mutation.

A student's T-test was run to determine if there was any statistically significant difference between the lifespan of the flies carrying the mutant gene and their counterparts carrying the mutant gene as well as *Su(levy).* The lifespan data of each fly tested for both strains was pooled and the average taken. The standard deviation was then calculated and a P-value of < 0.05 was considered to be statistically significant (95 % confidence interval). As a result, the

lifespan curves of CS, *levy*, *α-Syn*, *DJ 1-α*, *Pink*, *LRRK*, and *αβ-42* were statistically significantly

different from their *Su(levy)* counterparts. Based on these criteria, *Parkin* did not show

statistically significant differences in lifespan curves (P value > 0.05).
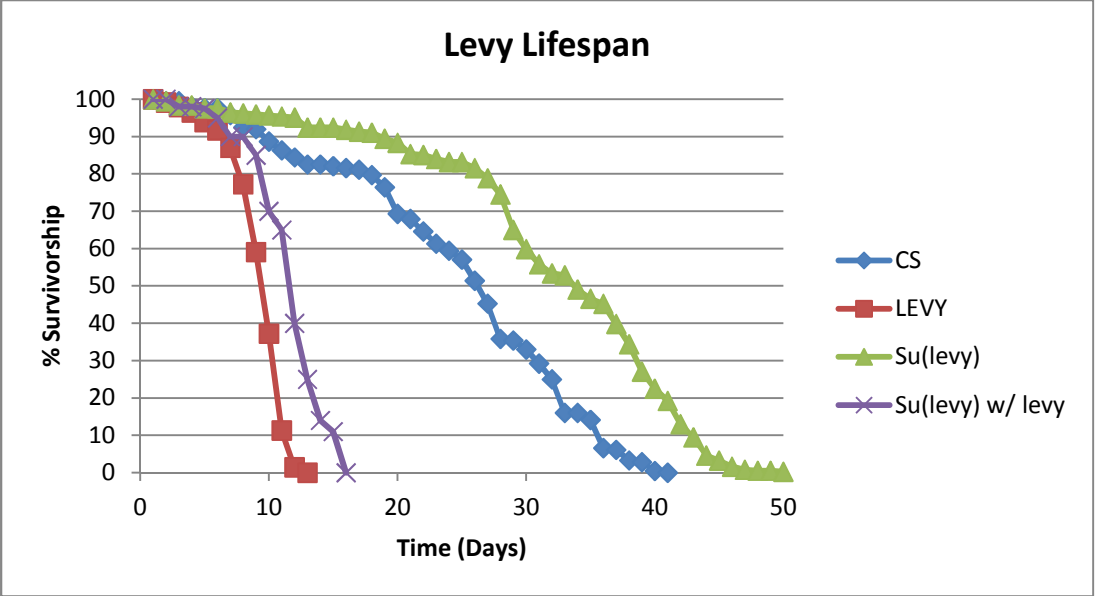
Levy Lifespan

Fly Strain	N value	Median Lifespan (Days)
CS	212	26
Su(levy)	279	34
levy	419	9
Su(levy) w/ levy	135	12

Figure 8: Survivorship curves for CS, *levy*, *Su(levy),* and *Su(levy) w/ levy* flies. The *Suppressor* mutation increased the median lifespan of wild-type flies as well as that of *levy* flies exhibiting neurodegeneration.
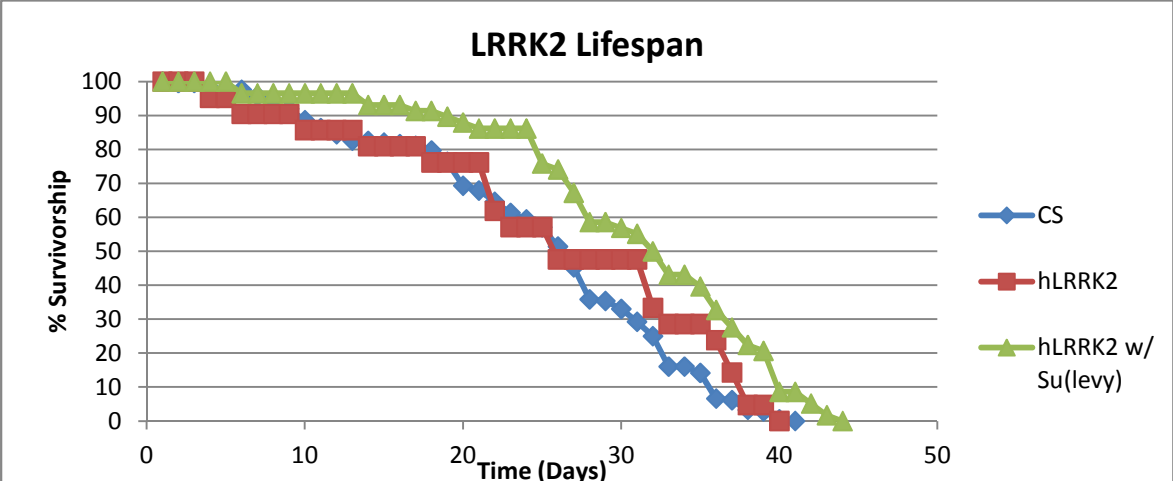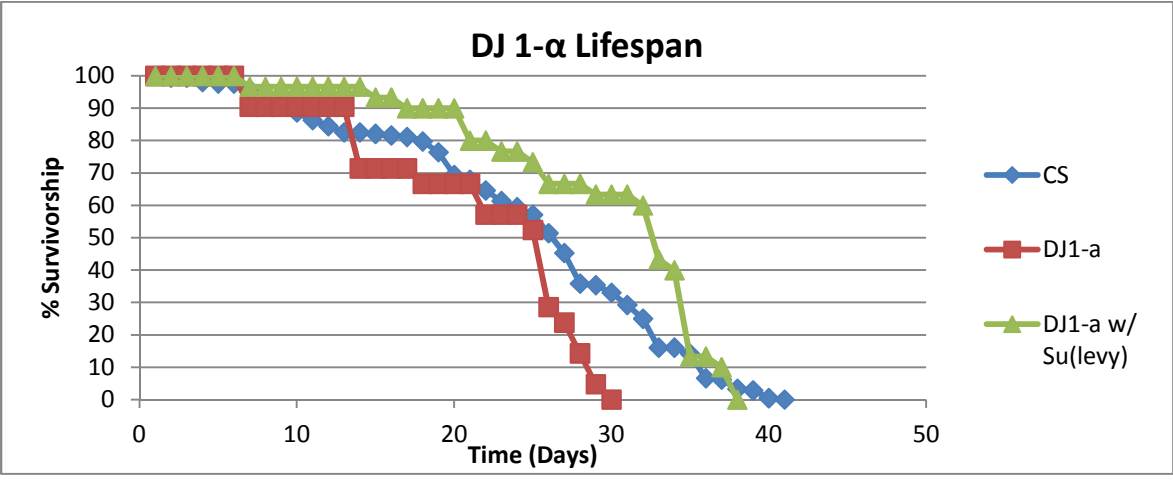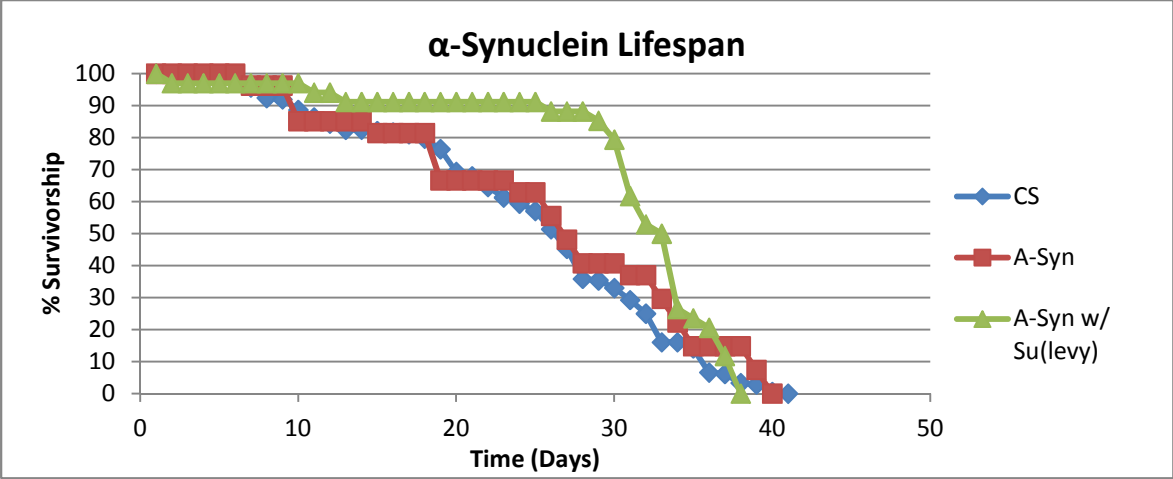
**Figure 9:** Survivorship curves for CS, *α-syn, α-syn w/ Su(levy), DJ 1-α, DJ 1-α w/ Su(levy), LRRK2,* and *LRRK2 w/ Su(levy)*
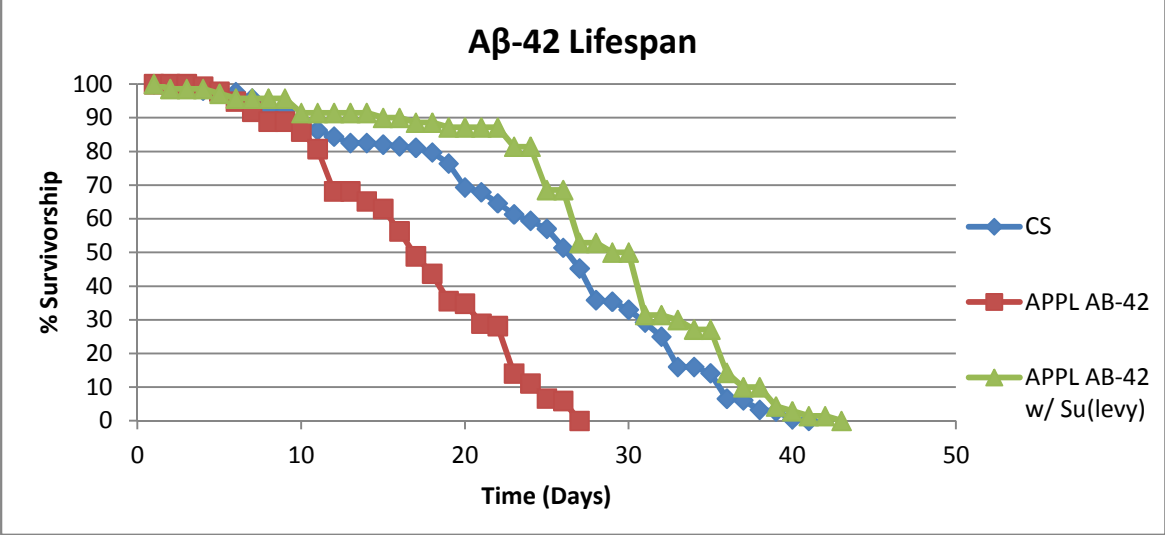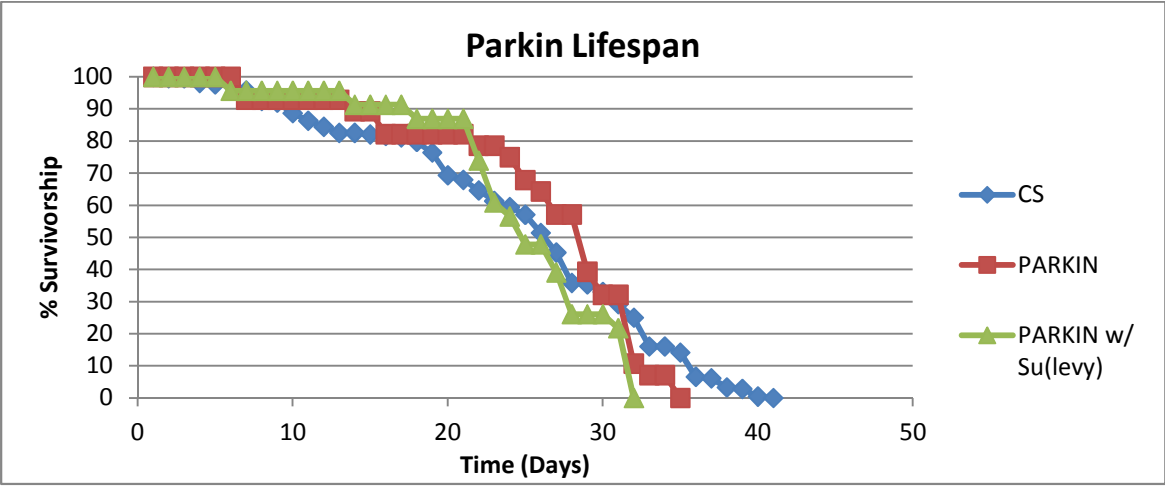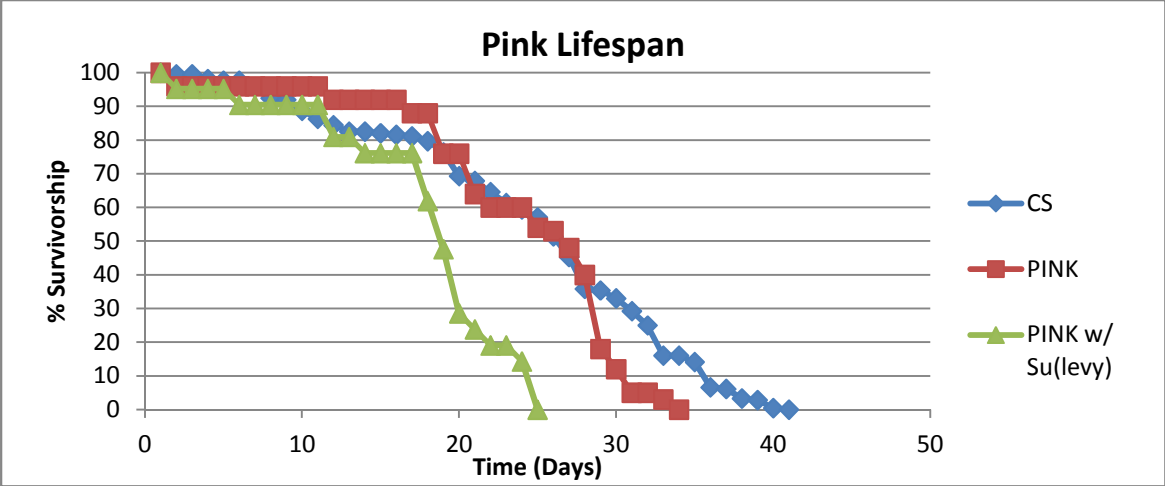
Figure 10: Survivorship curves for CS, *Pink, Pink w/ Su(levy), Parkin, Parkin w/ Su(levy), αβ-42, and αβ-42 w/ Su(levy)*

Table 5: Median lifespans and percent change in lifespan for mutant fly strains and mutant fly strains w/ *Su(levy)* mutation

Fly Strain	N value	Median Lifespan	Percent Change
α-Syn	77	27	
α-Syn w/ Su(levy)	84	33	22%
DJ 1-α	71	25	
DJ 1-α w/ Su(levy)	80	33	32%
LRRK2	71	26	
LRRK2 w/ Su(levy)	108	32	23%
Pink	75	25	
Pink w/ Su(levy)	71	19	(24%)
Parkin	78	26	
Parkin w/ Su(levy)	73	24	(7%)
αβ-42	120	18	
αβ-42 w/ Su(levy)	98	28	56%

Bang Sensitivity Assay: Neither CS flies nor *Su(levy)* flies showed any paralysis as a result of mechanical shock. They resumed normal flight and climbing behavior immediately after vortexing. *Levy* flies showed temporary paralysis as a result of vortexing. *Levy* flies took a median time of 6 seconds to recover from mechanical trauma and resume flight and climbing behavior. The shortest paralysis time was 2 seconds and the longest paralysis time was 50 seconds. **Figure 11** shows the bang-sensitivity of levy, CS, and *Su(levy)* flies, with the percent paralyzed graphed as a function of time.
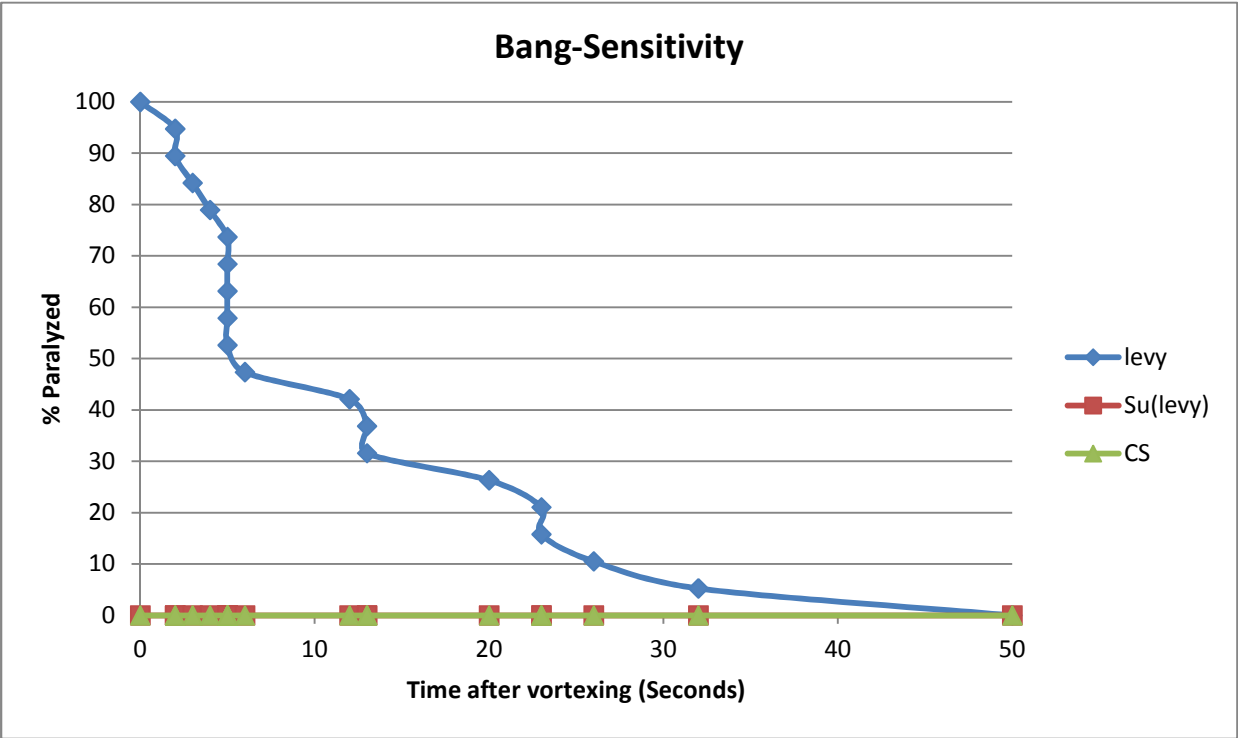
Figure 11: Bang sensitivity of *levy* flies compared to wild-type flies. *Levy* flies took a median time of 6 seconds to recover from mechanical shock. Wild-type and *Su(levy)* flies showed no paralysis as a result of mechanical shock.

Temperature-Induced Paralysis: When exposed to 38°C, all strains of flies tested eventually became paralyzed. Paralysis was defined in this case as the point in time when the fly took its last step and fell over. The number of flies still standing was plotted as a function of time for each strain. **Figure 12** displays the paralysis curves for wild-type, *levy*, *Su(levy),* and *Su(levy) w/ levy*, as well as the number of flies tested in each strain, and their median paralysis time. The median paralysis times for wild-type, *levy*, *Su(levy),* and *Su(levy) w/ levy* were 1254, 1447, 186, and 285 seconds, respectively.

Figures 13 and Figure 14 display the data for each of the AD and PD mutant fly strains tested. Each graph displays the wild-type fly as a control, as well as the flies carrying a neurodegenerative mutation, and finally those flies carrying the same neurodegenerative mutation as well as *Su(levy).* **Table 6** displays the number of flies tested in each strain, the median paralysis times for each strain, and the percent change in median paralysis times.

A student's T-test was used to determine if there was any statistically significant difference in the length of time it took for different strains of flies to paralyze. Each strain of fly carrying a mutation was compared to their counterparts carrying that mutation as well as *Su(levy).* The paralysis time for each fly was logged, and the average and standard deviation were determined. A P value of < 0.05 was considered to be statistically significant (95% confidence interval). The paralysis curves were found to be statistically different for each of the fly strains tested and their respective *Su(levy)* counterparts.
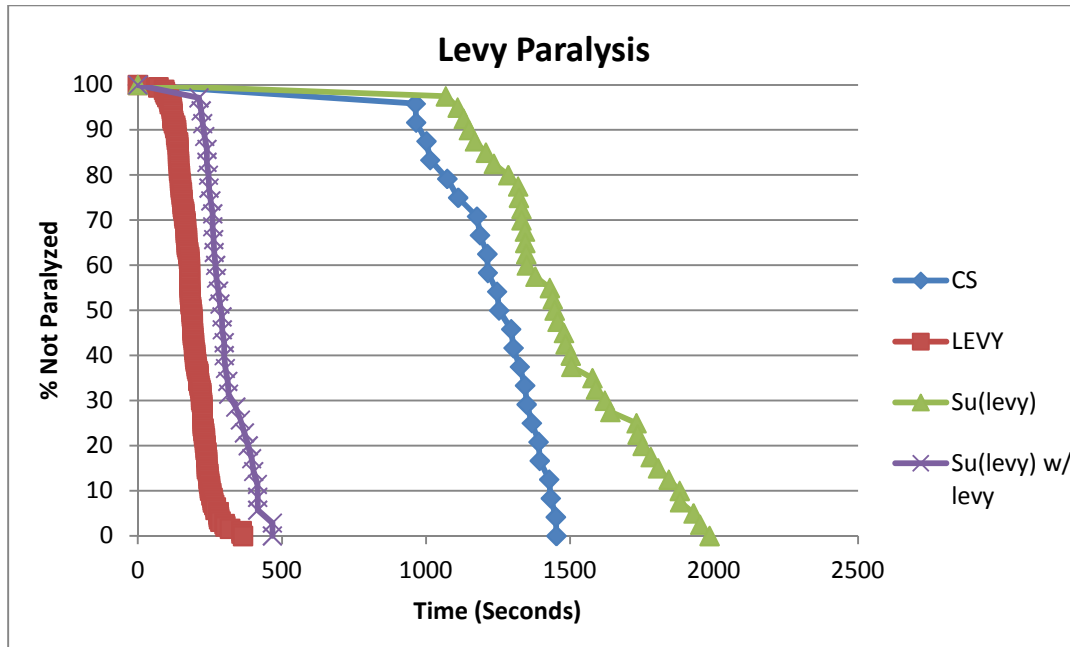
Levy Paralysis

Fly Strain	**N value**	**Median Paralysis Time (Seconds)**
CS	50	1254
Su(levy)	38	1447
levy	190	186
Su(levy) w/ levy	36	285

Figure 12: Temperature-induced paralysis for CS, *Su(levy)*, *levy*, and *Su(levy) w/ levy*. The presence of *Su(levy)* increased the resistance of the flies to paralysis causing it to take significantly longer for them to paralyze at 38˚C.
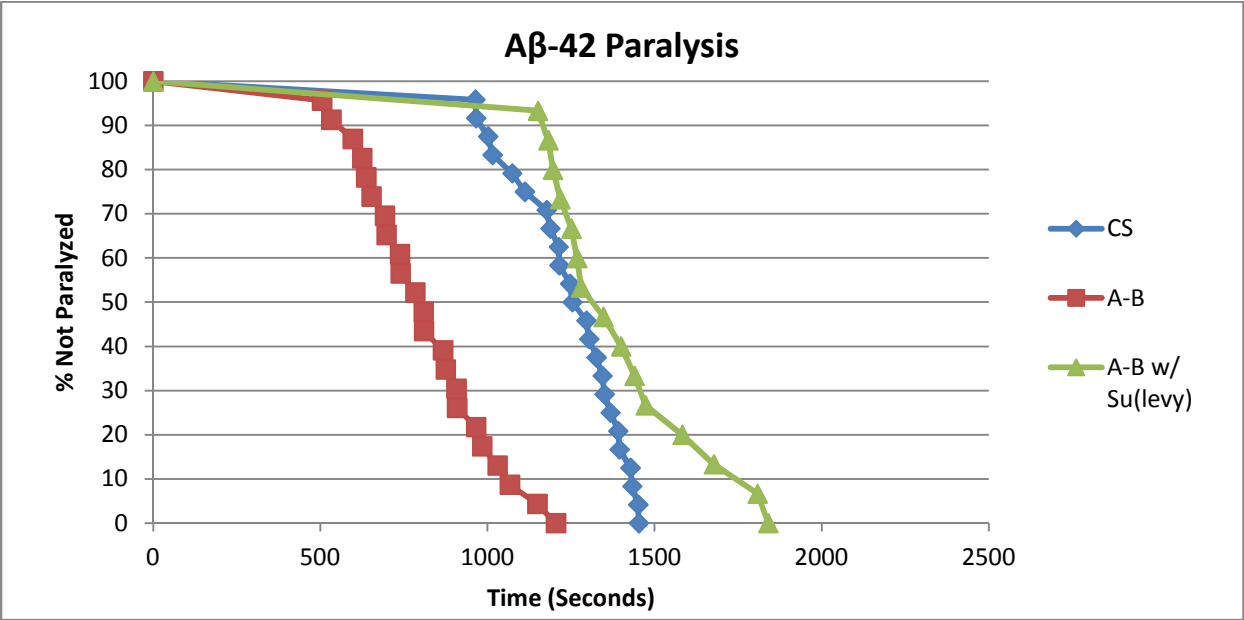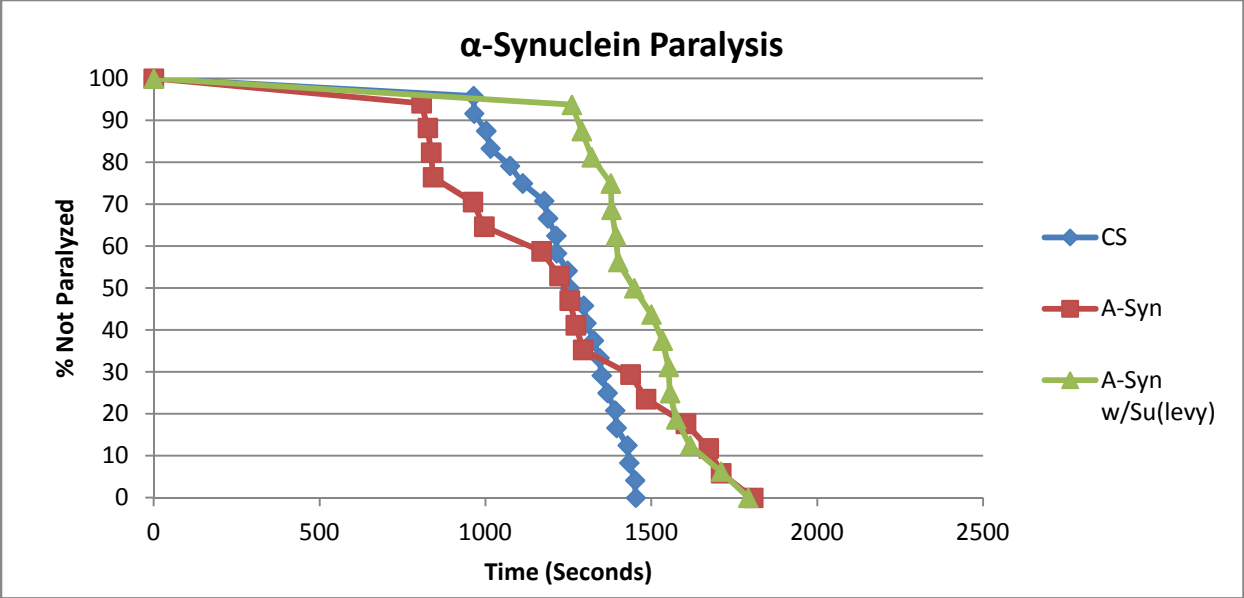
Figure 13: Temperature-induced paralysis data for *α-Synuclein* and *Aβ-42* flies, and their respective counterparts carrying *Su(levy)* mutation.
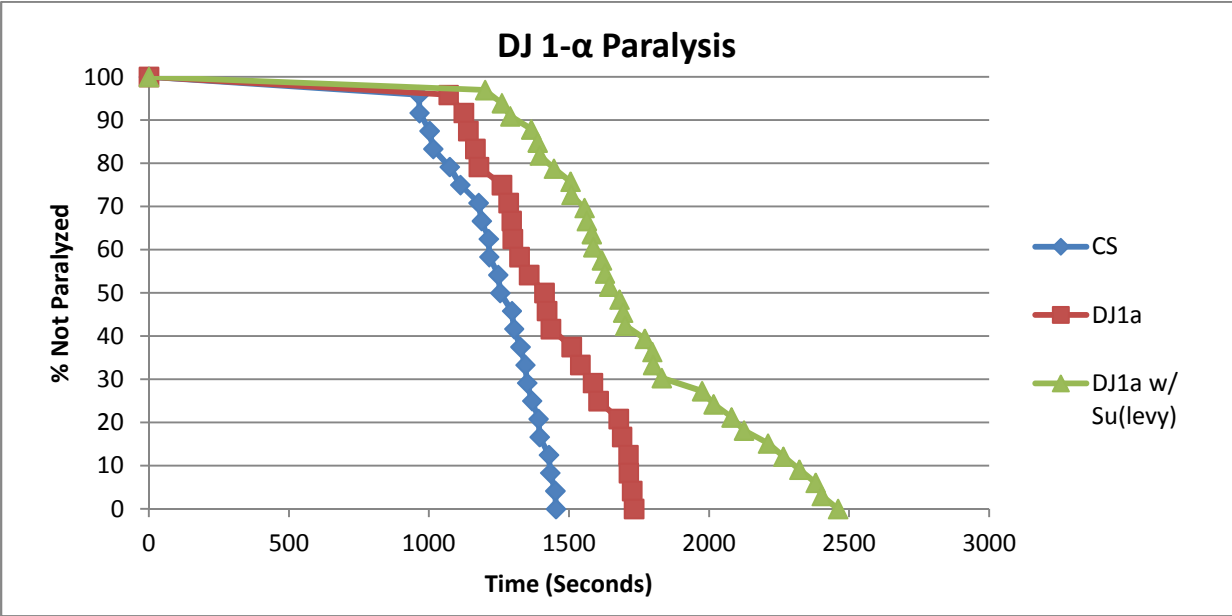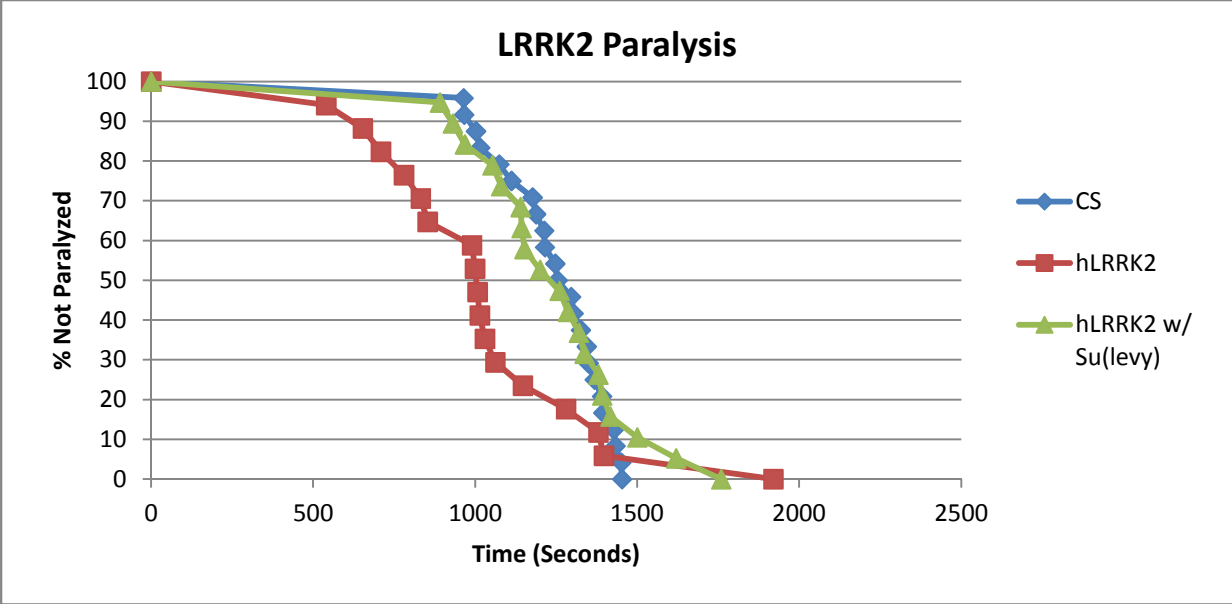
Figure 14: Temperature-induced paralysis data for *LRRK2* and *DJ 1-α* flies, and their respective counterparts carrying *Su(levy)* mutation

Table 6: Median paralysis times for flies carrying neurodegenerative mutations

Fly Strain	**N value**	**Median Time o Paralysis (Seconds)**	**Percent Change**
CS	50	1254	
α-Syn	18	1223	
α-Syn w/ Su(levy)	16	1448	18%
Aβ-42	24	784	
Aβ-42 w/ Su(levy)	19	1280	39%
LRRK2	22	999	
LRRK2 w/ Su(levy)	23	1200	20%
DJ 1-α	26	1412	
DJ 1-α w/ Su(levy)	35	1643	16%

Locomotor Assay (Climbing Assay): Each strain of fly tested showed an increase in climbing ability when carrying the *Su(levy)* mutation with the degree of significance varying for each strain. **Figure 15** displays the rate of climbing success for each strain of fly tested. The error bars show the variance associated with each test. There is a substantial amount of variance associated with this assay, and as a result the usefulness of these data is brought into question. For example, the average success rate of *DJ 1-α* flies is 75%, but the range of their success is from 61% to 90%. The fact that *DJ 1-α w/ Su(levy)* has an average success rate of 81% appears to show an overall improvement, but the significant amount of variance makes it hard to speculate.
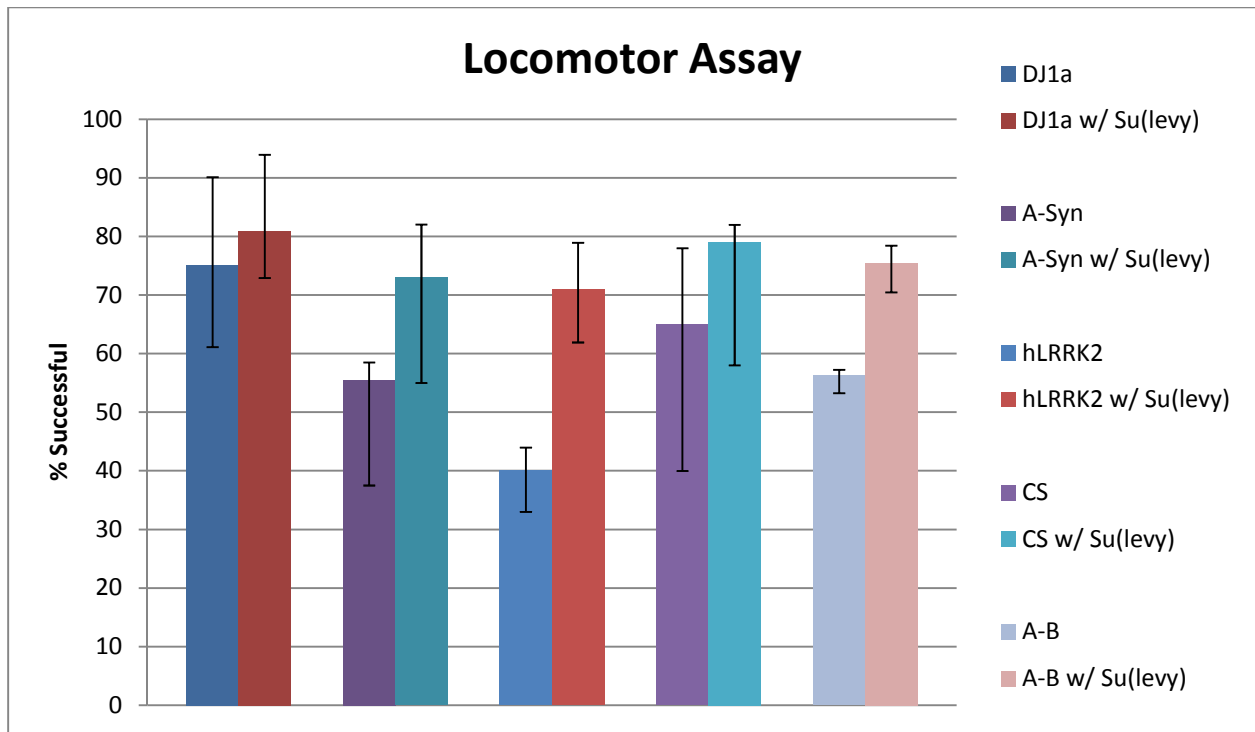
Locomotor Assay

Strain	N Value
DJ 1-α	567
DJ 1-α w/ *Su(levy)*	850
α-Syn	290
α-Syn w/ *Su(levy)*	686
hLRRK2	150
hLRRK2 w/ *Su(levy)*	282
CS	165
CS w/ *Su(levy)*	179
Aβ	56
Aβ w/ *Su(levy)*	75

Figure 15: *Su(levy)* flies showed better locomotor ability than their counterparts without *Su(levy),* but there is a high degree of variance.

Drug Sensitivity Assay: Regardless of the strain, flies reared on food laced with Rotenone® had a shorter median lifespan than flies reared on regular cornstarch medium. The Rotenone® seemed to have a less deleterious effect on *Su(levy)* flies, as their lifespan was slightly less affected by the drug. *Su(levy)* flies had a median lifespan decrease from 32 days to 26 days, or 19% decrease. Wild-type flies had a median lifespan decrease from 25 days to 14 days, or 44% decrease. There was a slightly greater degree of variance with this lifespan assay as compared with others, with the CS control fly showing the greatest degree of variance. **Figure 16** displays the median lifespan for wild-type and *Su(levy)* flies raised on regular food medium and those raised on medium laced with Rotenone®.
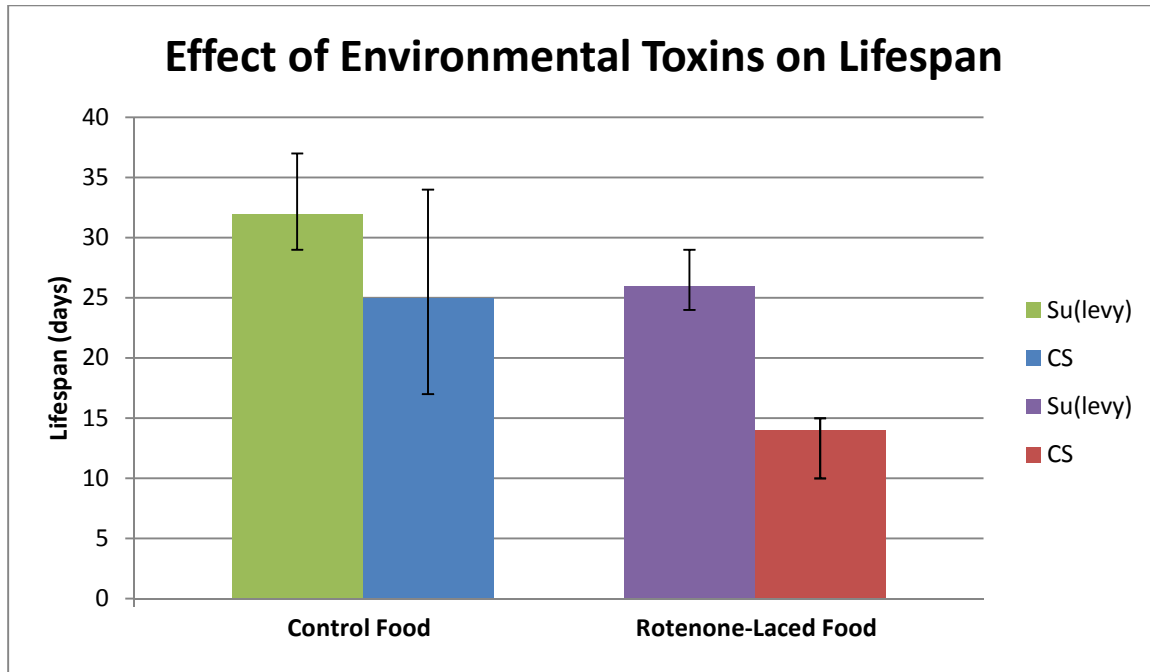
Figure 16: Lifespan of both wild-type and *Su(levy)* flies raised on regular food versus food laced with Rotenone®. While both flies showed a decrease in median lifespan, the effect was lessened in flies carrying *Su(levy)*.

V. Discussion

5.1 Suppressor of levy increases lifespan, locomotor function, resistance to mechanical shock, resistance to temperature-induced paralysis, and resistance to environmental toxins

All fly progeny containing *Su(levy)* mutation showed an increased median lifespan when compared to their wild-type or mutant counterpart, with the exception of *Pink* and *Parkin* (see **Table 5**). The degree of change varied with the specific mutation, but it is interesting to note that in the cases of *α-Syn, DJ 1-α*, and *LRRK2*, the median lifespan w/ *Su(levy)* was roughly 33 days in each case. It is worth noting that in the case of *Pink* and *Parkin*, the flies containing the *Su(levy)* mutation actually showed a decrease in median lifespan. In these cases, the protein produced by *Su(levy)* may play a deleterious role in neuronal cells. While this data in itself does not support the rescue role of *Su(levy)* demonstrated with other strains of flies, it does lend a degree of credence and validity to the experimental setup. For one thing, it demonstrates that there is not some other variable factor that is actually leading to the changes in lifespan. If every fly strain containing *Su(levy)* tested showed an increase in lifespan, one could not be sure that it was the mutation itself causing the change, as opposed to some other factor in the experimental design such as the GAL-4 construct, or the UAS sequence itself.

These changes represent significant increases to the lifespan of *Drosophila*. The average increase in lifespan across fly strains was roughly 30%. Extrapolated to a human model, it is the equivalent of a person's longevity increasing from 60 years to 78 years. Since both AD and PD are characterized by reduced lifespan, these results are very promising.

A major hallmark of neurodegenerative disorders in humans is a decrease in motor activity and coordination. This manifests itself in *Drosophila* via a decreased ability to climb the walls of a vial. When crossed with *Su(levy),* all PD and AD mutants showed a markedly better ability to climb than their counterpart flies that only contained the ND mutation. This provides

evidence for the notion that *Su(levy)* is somehow relieving motor dysfunction associated with ND. All fly progeny containing *Su(levy)* mutation showed improved climbing ability when compared to their mutant or wild-type counterpart. There was substantial statistical variance in this assay (see Figure 15), making it difficult to say just how much of a role *Su(levy)* was playing. It is interesting to note that all flies had a success rate of between 71-81% when carrying the *Suppressor* mutation, regardless of the AD or PD gene that they carried. It would seem that *Su(levy)* rescues locomotor function to somewhat of a "baseline" without regard for how dysfunctional the locomotor ability is to begin with. Further research is necessary to verify this hypothesis.

When exposed to an increase in temperature, flies expressing AD and PD genes show a greater tendency to become paralyzed more quickly than wild-type flies. However, progeny expressing a ND gene as well as *Su(levy)* show improved resistance to paralysis than their counterparts expressing only the ND gene. Since temperature-induced paralysis is a direct measure of motor function, the fact that the results are analogous to the results of the locomotor test is promising. This indicates that perhaps *Suppressor* is acting in a similar fashion in both cases.

It is difficult to speculate on how *Su(levy)* may alleviate temperature-induced paralysis. It is known that *levy* flies show reduced COX activity. However, the pathway from reduced COX activity to neurodegeneration is not known. *Su(levy)* most probably functions in this pathway or an interacting pathway, and it is important to identify the suppressor gene to determine how exactly it is operating.

Environmental toxins have been linked to neurological disorders in humans [47]. As mentioned above, much current research suggests mitochondrial dysfunction as a major culprit in idiopathic cases of AD and PD. Environmental toxins have been shown to generate the afore-mentioned reactive oxygen species (ROS), which interfere with proper mitochondrial function. In humans, these environmental toxins include things commonly found in pesticides, herbicides, and fungicides; two of the most common culprits being Benomyl and Rotenone®.

In *Drosophila*, as in humans, there are a variety of toxins linked to motor dysfunction, reduced lifespan, and coordination deficits. Research has shown strong correlation between exposure to toxins and reduced lifespan and locomotor ability. The most well-studied toxins in Drosophila are Rotenone, Paraquat, and hydrogen peroxide [47].

Our results showed that *Suppressor of levy* mutation reduced the effects of Rotenone® on *Drosophila* lifespan, when compared to wild-type flies. Again, this is very promising data, with the implication being that the presence of *Suppressor of levy* is reducing the impact of said environmental toxin on the nervous system of *Drosophila*. The fact that *Su(levy)* flies that had been fed Rotenone showed a markedly smaller reduction in lifespan when compared to CS flies that had been fed Rotenone is promising. The common thread between many of these rescue-type behaviors seems to be the role of biochemical pathways in the mitochondria.

5.2 Suppressor of levy may play a role in biochemical pathways in the mitochondria

The underlying biochemistry behind the action of *Su(levy)* has not yet been investigated, so it is difficult to draw conclusions about its mechanisms of action. However, based on what is known about *levy*, and the data that has been accumulated, it is worthwhile to speculate. It has long been known that oxidative stress associated with mitochondrial function can plays a key

role in human neurodegenerative conditions [21]. Specifically, reactive oxygen species tend to interact with electron transport chains and damage membrane proteins, which subsequently accumulate and crosslink to other proteins, inhibiting biochemical function [46]. This oxidative stress significantly reduces the overall life of the cell [46].

Previous work on the *levy* mutation showed that flies carrying the *levy* gene had markedly reduced cytochrome c oxidase (COX) activity [33]. It was suggested that this reduction in COX activity was not due to a decrease in overall number of mitochondria, but rather a reduction in the enzymatic activity of the cytochrome itself [33]. This subsequently leads to the neurodegeneration prevalent in *levy* flies. It is unknown whether the *Su(levy)* mutation is coding for a dysfunctional gene product, or whether it produces no gene product at all. However, based on the behavior of *Su(levy)* flies, one could speculate that it is this lack of functional gene product that inhibits the oxidative action of free radicals in the mitochondria.

It is worthwhile to note that a characteristic feature of ND in human patients is accumulation of amyloid protein aggregates and neurofibrillary tangles that occur through the aforementioned oxidative stress [46]. Also, the content of carbonyl groups in the brain of human AD patients is significantly higher than those in similar-aged controls. It is very likely that *Su(levy)* is blocking the accumulation and buildup of these aggregates either by a) specifically interacting with ROS and blocking their oxidative ability, or b) preventing the accumulation of tangles and peptides once the oxidatively damaged proteins have been formed. A worthwhile assay would be to perform a carbonyl assay on *levy* and *Su(levy)* flies to determine if there is a significant difference in accumulation, which may lend credence to this method of action.

5.3 Suppressor of levy may play a general protective role in Drosophila

Our preliminary data supports the notion that *Suppressor of levy* plays a significant role in alleviating neurodegenerative symptoms in *Drosophila*. For most of the flies tested, *Su(levy)* increased lifespan, reduced motor deficit, and protected from paralysis to varying degrees. This leads to the possibility that it is not a gene-specific suppressor, but rather might act in a non-specific manner to mitigate neurodegeneration. *Suppressor of levy* should be tested with other mutant strains of flies exhibiting ND to determine if this trend holds true.

VI. Summary and Future Work

6.1 Identification and sequencing of Suppressor gene

In order to gain any understanding of the underlying mechanisms behind how *Su(levy)* relieves ND, the gene must be identified and subsequently sequenced. Previous work in the lab has already identified some candidate genes for this mutation. The next logical step would be to sequence these gene regions from a *Su(levy)* fly, a *levy* fly, and a *CS* fly, to determine the specific regions of dissimilarity. Once the *Su(levy)* gene is identified, it would open the door to pursue experiments that will allow a much greater understanding of the how the gene is working.

6.2 Potential therapeutic implications

The ultimate goal of this research is to identify the underlying mechanisms behind human AD and PD. Once these mechanisms are known, improved therapeutic treatments can be pursued. If the dysfunctional gene product (or lack of product altogether) coded for by the *Su(levy)* gene mutation is identified as inhibiting neurodegenerative symptoms in a *Drosophila* model, it may have analogous behavior in a mammalian model. From a pharmacological perspective, it may be possible to synthesize a drug that mimics this behavior, with potential treatment applications in humans. Continued research on this gene may uncover therapeutic implications for human neurodegenerative conditions.

VII. References

[1] Beal, M.F., Lang, A.E., Ludolph, A.C. *Neurodegener. Dis.* UK: Cambridge University Press, 2005.

[2] Lessing, D., Bonini, N.M. (2009), *Nat. Rev. Genet.* 10, 359-370.

[3] Hirth, F. (2010), *CNS Neurol. Disord.* 9, 504-523.

[4] Goedert, M., Spillantini, M. G. (2006), *Science* 314, 777–781.

[5] Hardy, J. (2006), *Neuron* 52, 3-13.

[6] Lang, A. E. (2010), *Nat. Med.* 16, 1223-26.

[7] Bilen, J., Bonini, N. (2005), *Annu. Rev. Genet.* 39, 153-71.

[8] Rincon-Limas, D., Jensen, K., Fernandez-Funez, P. (2012), *Curr. Pharm. Design.* 18, 1108-22.

[9] Alzheimer's Association. http://www.alz.org/ (Accessed April, 2014).

[10] Tanzi, R., et al. (1996), *Neurobiol. Dis.* 3, 159-68.

[11] Lee, A. J., Hardy, J., Revesz, T. (2009), *Lancet* 373, 2055-66.

[12] Goguel, V., et al. (2001), *J. Neurosci.* 3, 1032-37.

[13] Braak H., Del Tredici K. (2008), *Neurology* 70, 1916-25.

[14] Chan, H.Y., Bonini, N. (2000), *Cell. Death. Differ.* 7, 1075-80.

[15] Farrer, M. J. (2006), *Nat. Rev. Genet.* 7, 303-18.

[16] Betarbet, R., Sherer, T. B., Greenamyre, J. T. (2002), *Bioessays* 24, 308-18.

[17] Spillantini, M. G., Schmidt, M. L., Lee, V. M., Trojanowski, J. Q., Jakes, R., Goedert, M. (1997), *Nature* 388, 839-40.

[18] Dauer, W., Przedborski, S. (2003), *Neuron* 39, 889-909.

[19] Schapira, A. H., Emre, M., Jenner, P., Poewe, W. (2009), *Eur. J. Neurol*. 16, 982-89.

[20] Abou-Sleiman, P., Muqit, M., Wood, N. (2006), *Nat. Rev. Neurosci.* 7, 207-19.

[21] Trushina, E., McMurray, C. T. (2007), *Neuroscience* 145, 1233-48.

[22] Finelli, E., Kelkar, A., Ho-Juhn, S., Yang, H., Konsalkaki, M. (2004), *Mol. Cell. Neurosci.* 26, 365-75.

[23] Bellen, H. J., Tong, C., Tsuda, H. (2010), *Nat. Rev. Neurosci.* 11, 514-22.

[24] Piper, M. D., Skorupa, D., Partridge, L. (2005), *Exp. Gerontol.* 40, 857-62.

[25] McGuire, S. E., Deshazer, M., Davis, R. L. (2005), *Prog. Neurobiol.* 76, 328-47.

[26] Nichols, C. D. (2006), *Pharmacol. Ther.* 112, 677-700.

[27] Adams, M. D., et al. (2000), *Science* 287, 2185-95.

[28] Rubin, G. M., Spradling, A. C. (1982), *Science* 218, 348-53.

[29] Brand, A. H., Perrimon, N. (1993), *Development* 118, 401-15.

[30] Bier, E. (2005), *Nat. Rev. Genet.* 6, 9-23.

[31] Edgar, B. A., Lehner, C. F. (1996), *Science* 274, 1646-52.

[32] Ashburner, *Drosophila, a laboratory handbook*. Cold Spring Harbor Laboratory, 1989.

[33] Liu, W., et al. (2007), *Genetics* 176, 937-46.

[34] Flybase. http://flybase.org

[35] Venderova, K., et al. (2009), *Hum. Mol. Genet.* 18, 4390-4404.

[36] Mitsumoto, A., Nakagawa, Y. (2001), *Free Radic. Res.* 35, 885-93.

[37] Muellner, M., Whitworth, A., Armstrong-Gold, C., Rizzu, P., Heutink, P., Wes, P., Pallanck, L., Bonini, N. (2005), *Curr Biol* 15, 1572-77.

[38] Feany, M., Bender, W. (2000), *Nature* 404, 394-98.

[39] Franceschini, N., Kirschfield, K., Minke, B. (1981), *Science* 213, 1264-67.

[40] Greene, J., Whitworth, A., Kuo, I., Andrews, L., Feany, M., Pallanck, L. (2003), *Genetics* 100, 4078-83.

[41] Park, J., et al. (2006), *Nature* 441, 1157-61.

[42] Crews, L., Masliah, E. (2010), *Hum. Mol. Genet.* 19, 12-20.

[43] Crowther, D., Page, R., Chandraratna, D., Lomas, D. (2006), *Method. Enzymol.* 412, 235-43.

[44] Greeve, I., Kretzschmar, D., Tschape, J. A., et al. (2004), *J. Neurosci*. 24, 3899-3906.

[45] Duffy, J. B. (2002), *Genesis* 34, 1-15.

[46] Calabrese, V., Raffaele, L., Tonon, C., D'Agata, V., Sapienza, M., Scapagnini, G., Mangiameli, A., Pennisi, A., Stella, G., Butterfield, A. (2005), *J. Neurosci*. 233, 145-162.

[47] Coulom, H., Birman, S. (2004), *J. Neurosci*. 24, 10993-10998.