Interactions among models of Amyotrophic Lateral Sclerosis and Parkinson Disease in *Drosophila melanogaster*

by

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Abstract

Amyotrophic Lateral Sclerosis (ALS) is a neurodegenerative disease characterized by the progressive loss of upper and lower motor neurons. In contrast, Parkinson Disease (PD) is a neurodegenerative disease characterized by the loss of dopaminergic neurons within the *substantia nigra* region of the brain. Recently, a number of genes have been identified to be involved in the progression of these diseases, this study focuses upon four ALS-related genes: *TARDBP(TBPH), p62/SQSTM1(Ref(2)P), TBK1(IK2),* and *VCP(TER94)*. These genes have been linked to the autophagy pathway and its sub-type, mitophagy, which have been suggested to play substantial roles in the progression of ALS and PD. Employing the Drosophila model organism, I have investigated the consequences of the altered expression of these ALS-related genes in combination with modified PD gene activities in an attempt to discover potential interactions and similarities between the biological basis of neurodegenerative diseases and aging. Notable observations show that the inhibition of *TBPH* in the motor neurons leads to a reduction in longevity and locomotor ability, and, in complementary experiments, inhibition of *TBPH* in the developing neuron-rich Drosophila eye reduces the ommatidia and interommatidial bristle counts. The overexpression of *IK2* in the motor neurons reduces longevity and locomotor ability. The inhibition of *Ref(2)P* in the motor neurons, as well as in the dopaminergic neurons, increases median lifespan, slightly, while severely reducing locomotor ability, which may suggest a compensational relationship between longevity and motor function. The inhibition of *Ref(2)P* and *parkin* in the *ddc-Gal4*-expressing neurons provided an increase in lifespan, while resulting in a reduction in locomotor ability. Investigation of

the inhibition of *TER94* provided variable results, however I have shown that inhibition of *TER94* in the dopaminergic neurons can reduce longevity and locomotor ability over time. The co-inhibition of *TER94* and *parkin* has shown a marked reduction in lifespan (by ~30%) and a reduction in locomotor ability. In contrast, the inhibition of *TER94* in combination with the expression of *alpha-synuclein* has shown an large increase in lifespan (by ~28%), with an accompanying reduction in locomotor ability over time. Investigation of these candidate genes and their role in a model of human disease progression has provided some clues to our understanding of ALS and PD.

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List of Abbreviations

arm – armadillo

ALS – Amyotrophic Lateral Sclerosis

ATG8 – Autophagy-related protein 8

CCD1 – Coiled coil domain 1

Caz -Cabeza

Clu – Clueless

ddc – Dopa Decarboxylase

dsRNA – double stranded Ribonucleic Acid

elav – embryonic lethal, abnormal vision

ERAD – Endoplasmic-reticulum-associated protein degradation

FUS – Fused in Sarcoma

GPCRs – G-protein-coupled Receptors

IK2 – IkappaB Kinase-like 2

IKKs – IkappaB Kinases

Lac Z – ß-galactosidase

LC3 – Light Chain 3

LRRK2 – Leucine Rich Repeat Kinase 2

mRNA – messenger Ribonucleic acid

NIH – National Institute of Health

OPTN – Optineurin

PARK7 – Parkinsonism Associated Deglycase

PBI – Phox and Bem1p

PD – Parkinson Disease

p62/SQSTM1 – Sequestosome 1

PINK1 – PTEN-induced kinase 1

RNAi – Ribonucleic Acid Interference

R1PK1 - Receptor-interacting serine/threonine-protein kinase 1

RISC – RNA-inducing silencing complex

RRM – RNA Recognition Motif

SCNA – Alpha synuclein

SE – Standard Error

SEM – Standard Error of the Mean

siRNA – small interfering Ribonucleic Acid

SOD1 – Superoxide dismutase 1

TARDBP – TAR DNA Binding Protein

TBK1 – TANK Binding Kinase 1

TH – Tyrosine Hydroxylase

UAS – Upstream Activation Sequence

UBA – Ubiquitin-Associated domain

UPS – Ubiquitin proteasome system

VAPB – VAMP Associated Protein B

VCP – Valosin Containing Protein

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Chapter 1 – Introduction And Overview

1.1 Purpose

The neurodegenerative diseases, Amyotrophic Lateral Sclerosis (ALS) and Parkinson Disease (PD), have a high prevalence in our society. Despite being very common, much has been left unclear with regards to the pathogenesis of the diseases. The research presented herein focuses on four ALS-related genes: *TARDBP(TBPH), p62/SQSTM1(Ref(2)P), TBK1(IK2),* and *VCP(TER94)*. The aim of this study is that through the manipulation of the expression of a select group of ALS and PD genes, there is the great potential to create extremely versatile models of these diseases through *Drosophila melanogaster*. These models have the potential to develop a better understanding of how these genes influence the longevity and locomotor ability of the organism. Specifically, the relationship between mitochondrial function and the consequences that ALS and PD genes may have upon this organelle is of great emphasis in this study.

1.2 Amyotrophic Lateral Sclerosis

The neurodegenerative disease Amyotrophic Lateral Sclerosis (ALS) is one of the most common adult-onset motor neuron diseases. Characterized by the progressive loss of upper and lower motor neurons of the spinal cord, brain stem, and motor cortex, ALS progression eventually leads to muscle weakness and atrophy (Scotter, Chen, & Shaw, 2015). To date, at least three major ALS-associated genes have been identified, along with several other, less prominent ALS-linked genes that have been associated with the

disease. The major genes include *superoxide dismutase 1* (*SOD1*), *Fused in Sarcoma* (*FUS*) and *TAR DNA Binding Protein* (*TARDBP*), and the others include *Sequestosome-1* (*p62/SQSTM1)*, *Optineurin* (*OPTN), TANK-binding kinase 1* (*TBK1)*, *VAMP-associated protein B* (*VAPB*), and *Valosin Containing Protein* (*VCP)* (Andersen & Al-Chalabi, 2011; Nguyen, Thombre, & Wang, 2018). For the most part, these genes are linked to the cellular process of autophagy and its mitochondrial-directed sub-type mitophagy, both of which have been suggested to play substantial roles in the progression of neurodegenerative diseases such as ALS. As age increases, the prevalence of ALS continues to grow, with a median age of onset of 55 years (Pasinelli & Brown, 2006), and patients are typically surviving for three to five years after diagnosis (Schmolck, Mosnik, & Schulz, 2007). In general it is believed widely that genetics can explain only a fraction of many ALS cases, as the majority seem to arise sporadically, to suggest that approximately 10% of cases to have a familial basis. Most research involving ALS pathogenesis has been centred around mutations in the *SOD1* gene. However, the focus has recently shifted to other ALS-associated genes and their protein products, such as the DNA/RNA-binding protein TDP-43, encoded by the *TARDBP* gene, along with the recent discovery of *TBK1* as an ALS gene (Lagier-Tourenne & Cleveland, 2009; Oakes, Davies, & Collins, 2017). There is currently no cure and few treatments for this motor neuron disease. Focusing research upon a number of ALS-linked genes opens the possibility of the development of treatments and therapies.

1.3 Parkinson Disease

Parkinson Disease (PD) is a common neurogenerative disease with a prevalence of 4 to 5% of the population over the age of 85 (Trinh et al., 2014). Similar to ALS, the majority of PD cases are considered to be sporadic, while the remaining ~10% seem to have a familial basis (Eriksen, Wszolek, & Petrucelli, 2005). The central pathology of PD involves cell loss within the *substantia nigra*, a basal structure located in the human midbrain. When compared to unaffected individuals, this brain region has been shown to have a 50 to 70% loss of neurons by the time of the individuals death (Cheng, Ulane, & Burke, 2011). The progression of this disease results in individuals that exhibit a resting tremor, muscular rigour, posture instability, and bradykinesia. The primary PD genes include *alpha-synuclein* (*SCNA*), *parkin* and *PINK1*, along with others such as *leucine-rich repeat kinase 2* (*LRRK2*) and *Parkinsonism associated deglycase* (*PARK7*). Similar to ALS, several PD genes, including *parkin* and *PINK1*, have vital roles in mitophagy. The alpha-synuclein protein is essential in PD, as it is highly expressed in the neurons and is involved in a range of neurodegenerative disorders, including Alzheimer disease, the accumulation of this protein is toxic to human neurons (Polymeropoulos et al., 1997). A well-known indicator of PD is the presence of alpha-synuclein-immunoreactive inclusions, also referred to as Lewy bodies, comprised of proteins that are responsible for proteolysis and the degradation of proteins. These inclusions are known to reside in various locations such as the *substantia nigra, hypothalamus, cerebral cortex, locus ceruleus, nucleus basalis*, cranial nerve motor nuclei and the central and peripheral divisions of the autonomic nervous system (Polymeropoulos et al., 1997). Mutations in

the *alpha-synuclein* gene, as well as mutations in parkin and PINK1, two proteins essential in mitophagy, are known to result in the progression of PD (Evans & Holzbaur, 2018). As PD has no cure although some treatments exist that provide some temporary relief, the cellular and molecular pathways governing disease progression must be investigated further.

1.4 Mitochondria and Neurodegenerative Disease

Mitochondria are cytoplasmic organelles that are responsible for the maintenance of homeostasis, carrying out vital cellular functions such as ATP production, redox signalling and programmed cell death (Franz, Kevei, & Hoppe, 2015; Reddy, 2009). The quality and quantity of mitochondria are kept at equilibrium due to regulated biogenesis and the process of mitophagy, a form of selective autophagy responsible for the degradation of damaged and dysfunctional mitochondria (Franz et al., 2015; Oakes et al., 2017). In particular, mitophagy is a vital process in neurons as these cells seem to be more vulnerable to mitochondrial dysfunction than other cell types (Rodolfo, Campello, & Cecconi, 2018). Evidence has shown that compromised mitophagy can be a strong factor in the progression of PD, as a functional decline in mitophagy within the dopaminergic neurons has been identified as a characteristic of PD (Bingol & Sheng, 2016). The PD genes, *PINK1* and *parkin*, encode protein components essential to regulation of mitophagy: PINK1-parkin-mediated mitophagy being a widely investigated mechanism. In this pathway, PINK1 and parkin, among others, act together to mark, degrade and clear damaged mitochondria (Ashrafi, Schlehe, LaVoie, & Schwarz, 2014;

Whitworth & Pallanck, 2017). Parkinson Disease has been reported to arise when mutations occur in the *PINK1* and *parkin* genes to impair mitophagy (Heo, Ordureau, Paulo, Rinehart, & Harper, 2015). However, compromised mitophagy has been linked to cases of ALS as well as PD. A reduction in mitophagy function, specifically within the motor neurons, in combination with an increase in dysfunctional mitochondria has been linked to degeneration of ALS neuromuscular junctions (NMJs) and ALS neuropathy (Rogers et al., 2017). Processes such as mitophagy, are essential in the homeostasis of many cell types, without proper clearance of damaged organelles, the perfect scenario for rapid disease pathogenesis is created. The desire for a more complete understanding of the link between mitochondrial dysfunction and neurodegenerative disease provides a strong motive to conduct further research into this relationship.

1.5 Mitochondria and Age

Impairment, coupled with abnormalities of the mitochondria, represent a major factor in both aging and age-related diseases, particularly neurodegenerative diseases. However, causal factors for many age-related neurodegenerative diseases, such as PD and ALS, are unknown (Reddy, 2009). Many studies have been conducted to investigate the link between the nervous system, age, autophagy and mitophagy, but many unanswered questions still remain. Compromised autophagy in conjunction with an increasing age further contributes to disease (Martinez-lopez, Athonvarangkul, & Singh, 2015). Abnormal macroautophagic processes, such as mitophagy, has been reported to associate with age in many systems, such as *Saccharomyces cerevisiae* and *Caenorhabditis elegans*

(Hansen & Rubinsztein, 2018). Based upon such observations, many studies have linked macroautophagy to the quality control of the mitochondrial population and suggests that the mechanisms that contribute to the continuous loss of the responsible processes of control strongly influence the aging process. (Hansen & Rubinsztein, 2018; Martinez-lopez et al., 2015). Advanced age is considered to be a significant risk factor in the development of neurodegenerative disease and is known to be an essential contributor in both genetic and sporadic forms of the disorders. The mechanisms that are behind the interaction between aging and genetic predispositions to contribute to neurodegeneration are still unclear (Xu et al., 2018). However, the aging process is linked to a reduction of the physiological functions within tissues. Therefore, understanding the roles that autophagy may play in specific tissue types, such as nervous system and muscle tissues, with an emphasis upon aging is extremely important (Hansen & Rubinsztein, 2018). As the nervous system and muscle are essential tissue types in neurodegenerative disease, the mechanisms responsible must be further investigated.

1.6 *Drosophila melanogaster* as a Model Organism

Drosophila melanogaster has been a standard model organism in many scientific laboratories throughout the world, in particular to those who study models of human diseases, for over a hundred years. The popularity of this model organism stems from the many advantages that the organism offers that other model organisms lack. These benefits include short lifespan, which allows for the quick production of data. A rapid generation time of ten to twelve days, to provide a large amount of offspring and allow experiments

to be conducted in a relatively short period; as well as they are inexpensive to maintain and require little resources (Jeibmann & Paulus, 2009). However, the most essential advantage that the Drosophila system offers may be the similarity to humans in regards to many biological, physiological and neurological properties. Using the fly as a model organism is particularly important in those interested in studying human disease, as according to genomic studies, approximately 75% of human disease-causing genes having a functional homologue in *Drosophila melanogaster* (Pandey & Nichols, 2011). By investigating the consequences of altered expression of such disease-causing genes in the fly, we may gain a better understanding of the mechanisms that contribute to disease progression.

Many genetic techniques can be carried out in Drosophila that allows for gene manipulation and provides researchers with the ability to understand biological processes in non-human species. A commonly used method for tissue-specific expression of a gene in Drosophila is the transcription activation system known as the UAS/Gal4 system, a method of genetic manipulation in which gene expression can be studied in model organisms. The two key parts of this system are a transactivator gene, *Gal4*, and a effector gene, containing the upstream activation sequence (UAS). The *Gal4* gene encodes the yeast transcription activator protein *Gal4*, while the UAS sequence is an enhancer which *Gal4* binds to in order to activate the transcription of a gene. It is when the *Gal4* portion binds to the UAS enhancer sequences located in the DNA that the transgene can be expressed in a time/tissue-dependent manner, allowing for the Drosophila homologue of

the gene to be inserted in various tissues of the fly, such as the motor neurons (Barwell et al., 2017; Jeibmann & Paulus, 2009). The UAS/Gal4 system is a critical molecular tool in the investigation of genes in the fly, allowing us to study human disease.

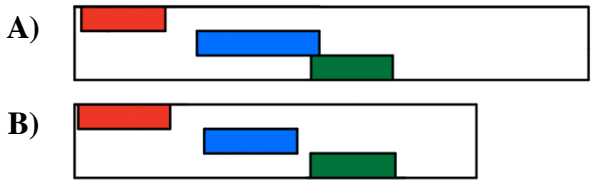

Figure 1.1: Comparison of *Homo sapiens* TDP-43 protein (A) and *Drosophila melanogaster* TBPH protein (B) with conserved domains. Highlighted are the N-terminal domain (red), the RRM1 domain (blue) and the RRM2 domain (green).

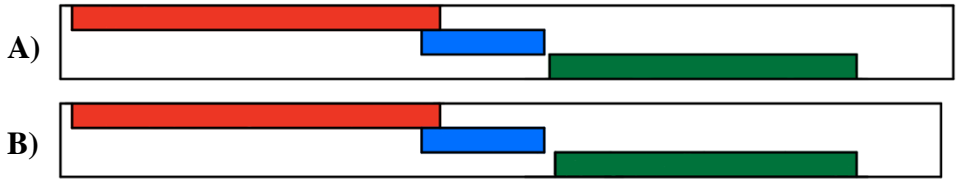

Figure 1.2: Comparison of *Homo sapiens* TBK1 protein (A) and *Drosophila melanogaster* IK2 protein (B) with conserved domains. Highlighted are the P kinase domain (red), the ULD domain (blue) and the CCD domain (green).

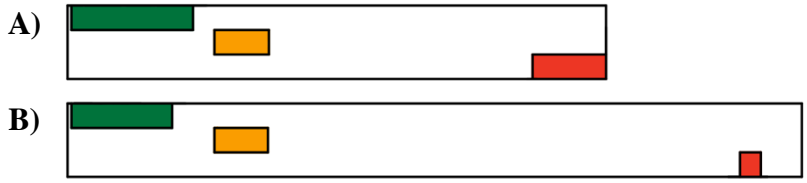

Figure 1.3: Comparison of *Homo sapiens* p62/SQSTM1 protein (A) and *Drosophila melanogaster* Ref(2)P protein (B) with conserved domains. Highlighted are the PB1 domain (green), the ZZ domain (yellow) and the UBA domain (red).

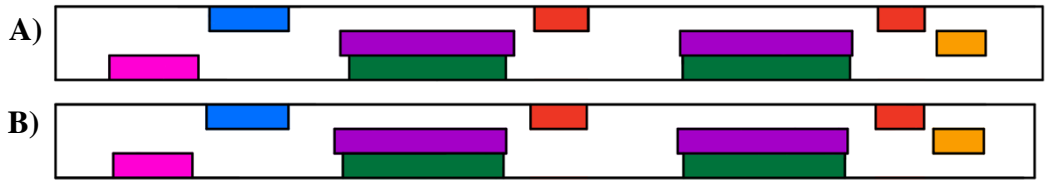

Figure 1.4: Comparison of *Homo sapiens* VCP protein (A) and *Drosophila melanogaster* TER94 protein (B) with conserved domains. Highlighted are the CDC48 domain (blue), the AAA ATPase domain (purple), the ATPase AAA domain (green), the Lid domain (red), the C-terminal domain (yellow) and the CDC N-terminal sub-domain (pink).

1.7 Research Goals

While *TARDBP*, *TBK1*, *p62/SQSTM1* and *VCP* have been suggested as candidate genes in the development of neurodegenerative diseases, their role in disease progression has not been fully characterized. This study aims to study the effects of genes directly related to ALS and PD and to study biological outcomes of the altered expression of such significant genes that associate and interact. This study aims to examine the effects of altered ALS gene expression in combination with altered PD gene activity and to investigate the consequences of altered ALS gene expression in the dopaminergic neurons. By manipulating gene expression of selected genes in *D. melanogaster*, researchers may gain insight into how their alteration, both the overexpression and inhibition, impacts Drosophila lifespan, motor functions, and, in complementary experiments, the developing compound eye.

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Chapter 2 – Methodology

2.1 *Drosophila melanogaster* stocks and culture

Drosophila melanogaster was maintained on a standard media comprised of 65 g/L cornmeal, 50 ml/L fancy grade molasses, 10 g/L yeast and 5.5 g/L agar which was then treated with 2.5 ml propionic acid and 5 ml of 0.1 g/ml methylparaben. This mixture was then allowed to solidify at the bottom of vials and stored in 4 to 6º C until use. Stocks were stored at room temperature (~ 21º C) due to limited incubator space.

2.2 Longevity Assay

Critical class male progeny were collected daily and placed in vials with fresh medium. A sample size of 300 males was collected in total and stored at 25º C for the duration of the experiment. The flies were scored every two days to examine if any death had occurred. A fly was considered dead when no movement was observed. Males were transferred onto fresh media every four days to obtain a healthy environment. The data was analyzed using the Graphpad Prism 8 software (Graphpad Software Inc.) with a comparison of the survival curves analyzed by the Log-rank (Mantel-Cox) test. Significance was determined at 95%, at a P-value less than or equal to 0.05 with Bonferroni correction.

2.3 Locomotive Assay

Critical class male progeny were collected within a 24-hour time period for a sample size of 70 male progeny. Critical class males were maintained in vials with ten

flies per vial, stored at 25º C, and placed on new medium once per week throughout the experiment. The climbing ability of critical class flies was carried out to examined the motor function of the flies over time. The analysis began one week after collection and then every seven days after until flies had a minimum climbing score for two consecutive weeks, or less than ten flies remained alive. Climbing ability was analyzed at the same time each week for consistency. For each genotype, the climbing ability of five cohorts was analyzed. For each cohort of 10 flies, ten trials were then carried out, which resulted in a total of 500 trials per genotype per week. To score the climbing ability of the flies a 30 cm glass tube with a 1.5 cm diameter was used which was marked with five 2 cm sections starting from the bottom with the remainder of the glass tube left as a buffer zone (Todd & Staveley, 2004). The flies were scored based on the height that was reached on the tube after a ten second time period. A climbing index was then calculated as Climbing index = Σ nm/N, where n represents the number of flies at a given level, m is the score of the level (between 1 and 5) and N is the total number of flies climbed in that trial. The data was analyzed using the Graphpad Prism 8 software (Graphpad Software Inc.) Using this software, a nonlinear regression curve was produced with a 95% confidence interval, with the slope of each curve representing the rate of decline in climbing ability and the Y-intercept representing the initial climbing ability. The curves were considered to be significantly different if $P < 0.05$.

2.4 Scanning Electron Microscopy of the *Drosophila* eye

The *GMR-Gal4* transgenic line was used, which allowed expression of the paternally contributed transgenes in the eye of the fly. Critical class male progeny from each cross was collected, aged for 3 to 5 days post eclosion, and frozen at -80 °C, in order to sacrifice and preserve the flies before being placed on SEM studs. Studs were placed in a desiccator for at least 48 hours to dry. Using either the Mineral Liberation Analyzer FEI 650F or the FEI Quanta 400 Scanning Electron Microscope, ten different eye images for each genotype were taken to visualise the left eye of the fly. The ten images were selected based on the quality and clarity of the image. Images were then examined using the National Institutes of Health (NIH) ImageJ software for the extent of eye development, counting both the number of ommatidia and bristles, this data was then analyzed using Graphpad Prism 8 (Graphpad Software Inc.)

Chapter 3 – Modelling Amyotrophic Lateral Sclerosis in *Drosophila melanogaster* Through Alteration of *TBPH*

3.1 Introduction

Amyotrophic Lateral Sclerosis (ALS) is a progressive neurodegenerative disease characterized by the loss of upper and lower motor neurons within the spinal cord, brain stem and motor cortex. Eventually, this gradual deterioration of motor neurons results in muscle weakness and atrophy (Scotter, Chen, & Shaw, 2015). Although the progression of ALS remains poorly understood; there are a number of candidate genes of great interest that can contribute to the development of the disease. One of the major ALS-assoicated genes, *TARDBP*, encodes the multifunctional DNA/RNA binding protein TDP-43, known as *TBPH* in Drosophila. The TDP-43 protein is located primarily in the nucleus of neurons, glial cells, and muscle cells but can shuttle to the cytoplasm up to a level of 30% (Mackenzie & Rademakers, 2008; Neumann et al., 2007). In the nucleus, TDP-43 regulates RNA splicing and modulates microRNA biogenesis (Scotter et al., 2015). While in the cytoplasm of neurons, TDP-43 is a critical component of dendritic and somatodendritic RNA transport granules, and exhibits a key role in neuronal plasticity through the regulation of local protein synthesis in dendrites (Scotter et al., 2015; Xu, 2012). Events that can cause the TDP-43 protein to move into the cytoplasm are highly stress-related and include oxidative stress, endoplasmic reticulum stress, and heat stress (Buratti & Baralle, 2008). Once in the cytoplasm, TDP-43 can incorporate into stress granules that possibly then lead to its pathological transformation. These abnormal TDP-43 cytoplasmic aggregates have multiple modifications, including

hyperphosphorylation, ubiquitination and cleaving, preventing TDP-43 from crossing the nuclear membrane (Gasset-rosa et al., 2019; Buratti & Baralle, 2008). This TDP-43 shuttling event has been suggested to be a critical component in disease pathogenesis (Lu et al., 2016), as abnormal cytoplasmic aggregates have been reported within the neurons and glia in over 90% of ALS cases (Ling, Polymenidou, & Cleveland, 2013; Gasset-Rosa et al., 2019). However, this shuttling event is not the only factor to consider in TDP-43 pathology. The interactions and roles which TDP-43 has in the mitochondria are important to consider, as mitochondrial damage and impaired mitophagy are known to be associated with many neurodegenerative diseases. TDP-43 is known to be associated with the mitochondria and mitochondrial defects, with TDP-43 pathology becoming an emerging topic (Davis et al., 2018; Gautam, Xie, Kocak, & Ozdinler, 2019). Studies have demonstrated mitochondrial impairment by TDP-43 to be an early event, resulting in cell death (Wang et al., 2019). There are many unanswered questions involving the specific role that TDP-43 plays in ALS pathogenesis; however, it is clear that the loss-of-function of TDP-43 in the nucleus is a substantial factor in disease progression (Ling et al., 2013; Van Deerlin et al., 2008). A further investigation must occur to understand the cellular mechanisms that TDP-43 plays in disease.

The *TARDBP* gene has been highly conserved throughout evolutionary history. A comparison of human TDP-43 and *D. melanogaster TBPH* proteins reveals the TDP-43 N-terminal domain as a highly conserved domain, as well as two highly conserved motifs, RNA recognition motif 1 (RRM1) and RNA recognition motif 2 (RRM2). The degree of

evolutionary conservation suggests a highly conserved function for TDP-43. Thus exploration of the effects of altered *TARDBP* expression in *D. melanogaster* may provide insights into the pathology of ALS in humans. In this study, inhibition of *TBPH* in *D. melanogaster* are used to mimic human conditions, examining the effects on lifespan and locomotor ability. The overexpression of *TBPH* was also examined to investigate the biological impacts on *D. melanogaster*.

3.2 Materials and Methods

3.2.1 *Drosophila melanogaster* stocks and culture

All Drosophila stocks were obtained from the Bloomington Drosophila Stock Center at Indiana University (IN, USA). See Table 1.1 for list of genotypes used. See Chapter 2, section 2.1 for detail of *D. melanogaster* stocks and culture.

3.2.2 Longevity Assay

The survival of *D. melanogaster* was analyzed to examine the median lifespan of experimental flies in comparison to control flies. See Chapter 2, section 2.2 for full longevity assay methods.

3.2.3 Locomotive Assay

The motor function of *D. melanogaster* was analyzed to examine the locomotor function over time of experimental flies in comparison to control flies. See Chapter 2

section 2.3 for full locomotive assay methods.

3.2.4 Scanning Electron Microscopy of the *Drosophila* eye

Eye analysis of *D. melanogaster* was used to determine the effects of gene manipulation. See Chapter 2 section 2.4 for full methods on eye experiments.

Table 1.1: Genotypes and location of expression patterns used in the analysis of altered expression of *TBPH*.

Abbreviated Genotype	Location of Expression	Insertion Chromosome	Reference
Control Lines			
UAS-lacZ	---	2	Brand et al., 1993
Transgene Lines			
GMR-Gal4	Eye	2	Freeman, 1996
arm-Gal4	Ubiquitous	2	Sanson et al., 1996
elav-Gal4	Pan neuronal	1	Lin & Goodman, 1994
TH-Gal4	Dopaminergic neuron	3	Inamdar et al., 2014
ddc-Gal4$^{HL4.3D}$	Dopaminergic and serotonergic neuron	2	Li et al., 2000
ddc-Gal4$^{HL4.36}$	Dopaminergic and serotonergic neuron	3	Li et al., 2000
D42-Gal4	Motor neuron	3	Parkes et al., 1998
Responder Lines			
UAS-TBPH^{EY10530}	---	2	Bellen et al., 2011
UAS-TBPH-RNAi^{HMS05194}	---	3	Perkins et al., 2015
UAS-TBPH-RNAi^{HMS01932}	---	2	Perkins et al., 2015

3.3 Results

3.3.1 Inhibition of *TBPH* decreases median lifespan and climbing ability

An investigation of how the inhibition of *TBPH* impacts the lifespan and climbing ability of *D. melanogaster* has shown that one of the two inhibition transgenes has a significantly more significant impact than the other. Several *TBPH* inhibition lines were selected to investigate the consequences of both to the fly. The *TBPH* RNAi transgene, *UAS-TBPH-RNAi*^{HMS01932}**,** has been shown to significantly reduce median lifespan when expressed with the ubiquitous transgene *arm-Gal4* (Figure 2.1) and the pan-neuronal transgene *elav-Gal4* (Figure 2.2) when compared to the control (*UAS-lacZ*). When expressed through the *elav-Gal4* transgene, inhibition of *TBPH* via *UAS-TBPH-RNAi*^{HMS01932} resulted in extremely poor viability of critical class males. The inhibition of *TBPH*, via *UAS-TBPH-RNAi*^{HMS01932}, significantly reduced median lifespan (Figure 2.3a), and climbing ability over time (Figure 2.3b) when expressed with the motor neuron-specific transgene *D42-Gal4*. When expressed through the dopaminergic neuron-specific transgene *TH-Gal4*, inhibition of *TBPH* significantly decreased median lifespan (Figure 2.4a), but not climbing ability (Figure 2.4b). The *TBPH* inhibition through the neuron-specific transgene *ddc-Gal4*$^{HL4.3D}$ greatly reduced median lifespan (Figure 2.5a) and climbing ability over time (Figure 2.5b). However, when expressed through the neuron-specific transgene *ddc-Gal4*$^{HL4.36}$, inhibition of *TBPH* did not provide any statistically significant changes in median lifespan (Figure 2.6a), or to climbing ability over time (Figure 2.6b) when compared to the control (*UAS-lacZ*).

3.3.2 Inhibition of *TBPH* decreases ommatidia and bristle count

The developmental pattern of the Drosophila compound eye is a highly regulated and specific event with each eye being comprised of approximately 800 ommatidia under standard development. Once the pattern of ommatidia are laid down, the bristles are generated later as a form of protection for the ommatidia. Impairment in this process may result in characteristic phenotypes as a consequence, such as changes in the number of ommatidia and bristles. To investigate such phenotypic changes in *D. melanogaster*, the eye-specific transgene *GMR-Gal4* has been used. The *TBPH* inhibition, through the directed expression of *UAS-TBPH-RNAi*^{HMS01932}, when expressed in the developing eye of *D. melanogaster* has shown to significantly reduce the quantity of ommatidia (Figure 2.8a) and interommatidial bristles (Figure 2.8b) when compared to the control (*UAS-lacZ*).

3.3.3 Overexpression of *TBPH* decreases median lifespan and climbing ability

When *TBPH* is overexpressed through the action of the pan-neuronal transgene, *elav-Gal4* median lifespan is significantly reduced (Figure 2.2). When *TBPH* was overexpressed through the dopaminergic neuron-specific transgene *TH-Gal4,* median lifespan (Figure 2.4a) and climbing ability over time (Figure 2.4b) are reduced. As well, when *TBPH* is overexpressed through the motor neuron-specific transgene *D42-Gal4*, median lifespan (Figure 2.3a) and climbing ability over time (Figure 2.3b) are significantly reduced when compared to the control (*UAS-lacZ*).

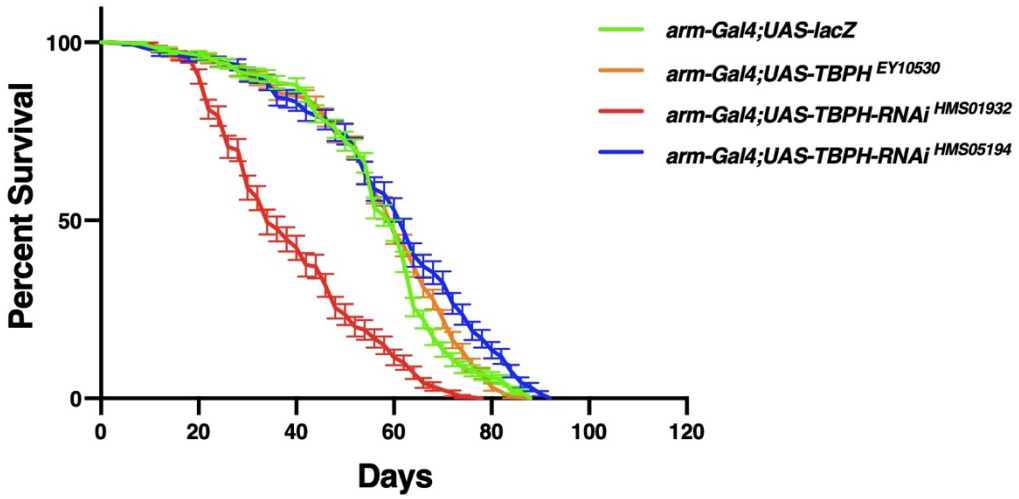

Figure 2.1: Altered expression of *TBPH* directed through the *arm-Gal4* transgene affects lifespan. Longevity is depicted by percent survival. Significance is $P < 0.05$ using the log-rank test with Bonferroni correction. Error bars represent standard error of the mean. Genotypes are as follows: *arm-Gal4;UAS-lacZ* (n=284), *arm-Gal4;UAS-TBPH*^{EY10530} (n=256), *arm-Gal4;UAS-TBPH-RNAi*^{HMS01932} (n=208), *arm-Gal4;UAS-TBPH-RNAi*^{HMS05194} (n=221).

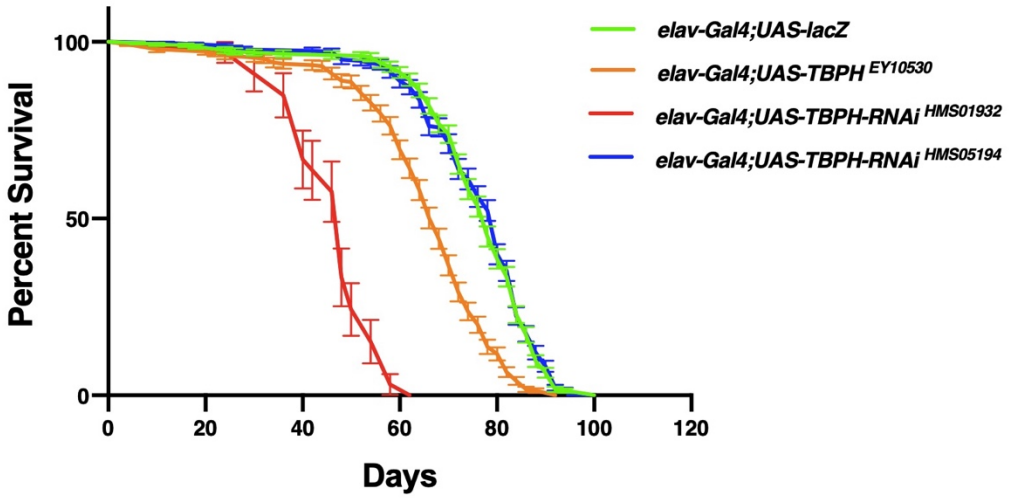

Figure 2.2: Altered expression of *TBPH* directed through the *elav-Gal4* transgene affects lifespan. Longevity is depicted by percent survival. Significance is $P < 0.05$ using the log-rank test with Bonferroni correction. Error bars represent standard error of the mean. Genotypes are as follows: *elav-Gal4;UAS-lacZ* (n=298), *elav-Gal4;UAS-TBPH*^{EY10530} (n=291), *elav-Gal4;UAS-TBPH-RNAi*^{HMS01932} (n=33), *elav-Gal4;UAS-TBPH-RNAi*^{HMS05194} (n=281).

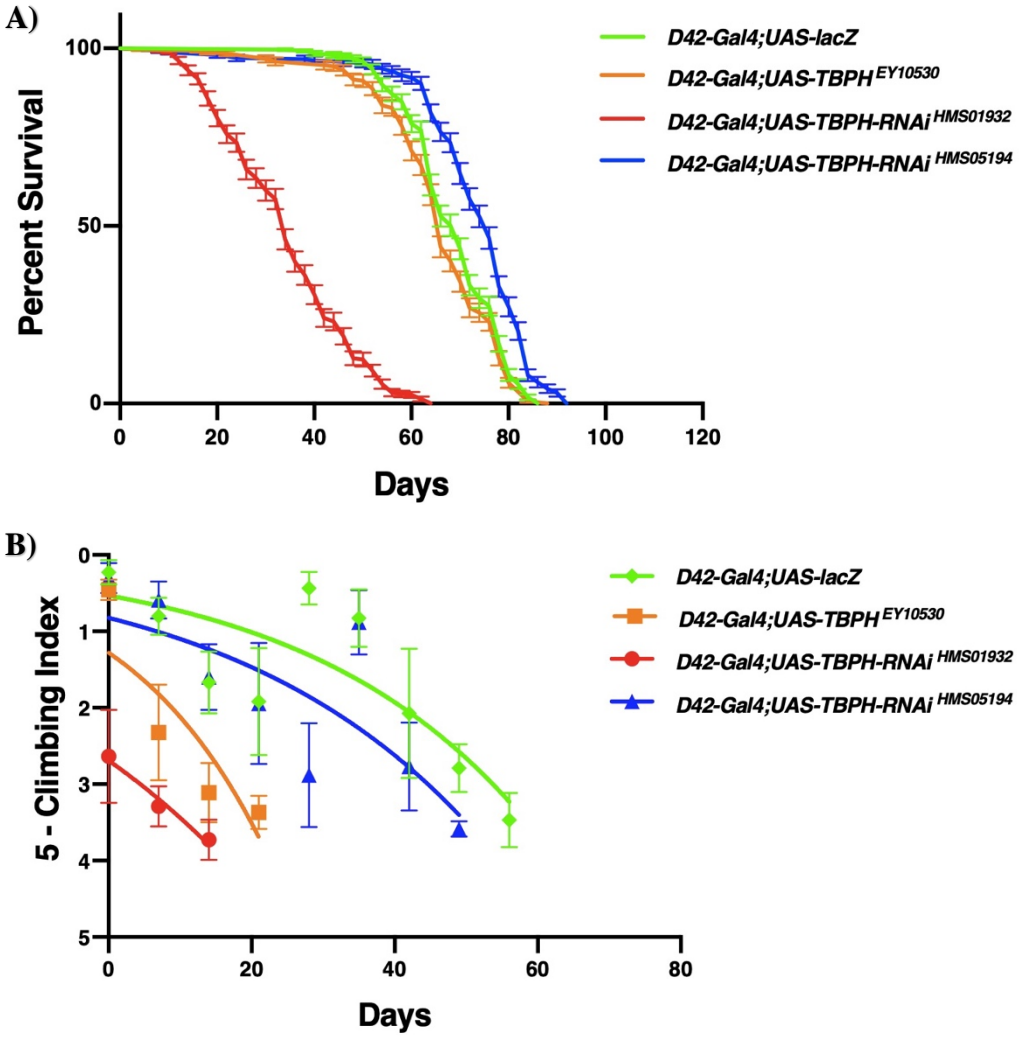

Figure 2.3: Altered expression of *TBPH* directed through the *D42-Gal4* transgene affects longevity and climbing ability. **A:** Longevity assay of *Drosophila melanogaster* males displaying altered *TBPH* expression in the motor neurons. Longevity is depicted by percent survival. Significance is $P < 0.05$ using the log-rank test with Bonferroni correction. Error bars represent standard error of the mean. Genotypes are as follows: *D42-Gal4;UAS-lacZ* (n=273), *D42-Gal4;UAS-TBPH*^{EY10530} (n=274), *D42-Gal4;UAS-TBPH-RNAi*^{HMS01932} (n=290), *D42-Gal4;UAS-TBPH-RNAi*^{HMS05194} (n=264). **B:** Locomotor assay of *D. melanogaster* males displaying altered *TBPH* expression in the motor neurons. Climbing ability was determined by a nonlinear curve fit (CI=95%). Error bars indicate standard error of the mean.

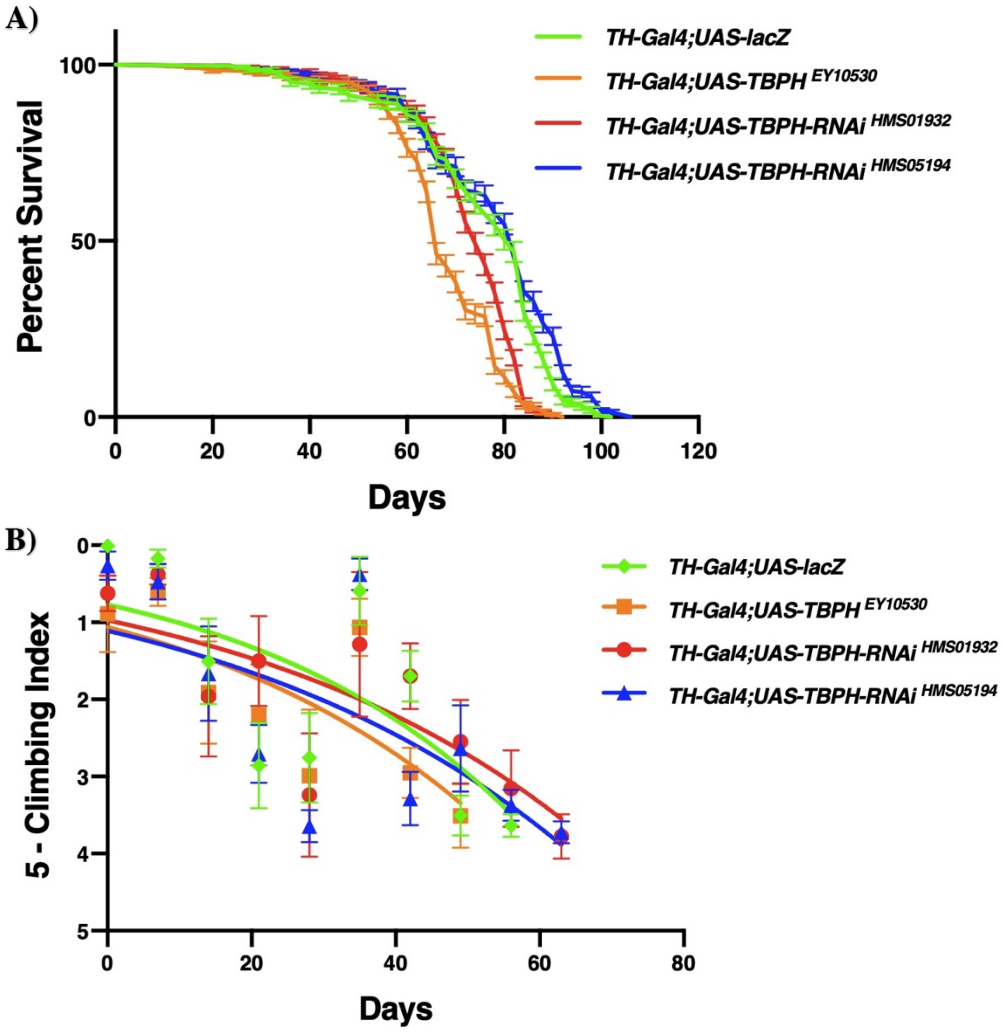

Figure 2.4: Altered expression of *TBPH* directed through the *TH-Gal4* transgene affects longevity and climbing ability. **A:** Longevity assay of *Drosophila melanogaster* males displaying altered *TBPH* expression in the dopaminergic neurons. Longevity is depicted by percent survival. Significance is $P < 0.05$ using the log-rank test with Bonferroni correction. Error bars represent standard error of the mean. Genotypes are as follows: *TH-Gal4;UAS-lacZ* (n=290), *TH-Gal4;UAS-TBPH*^{EY10530} (n=263), *TH-Gal4;UAS-TBPH-RNAi*^{HMS01932} (n=280), *TH-Gal4;UAS-TBPH-RNAi*^{HMS05194} (n=283). **B:** Locomotor assay of *D. melanogaster* males displaying altered *TBPH* expression in the dopaminergic neurons. Climbing ability was determined by a nonlinear curve fit (CI=95%). Error bars indicate standard error of the mean.

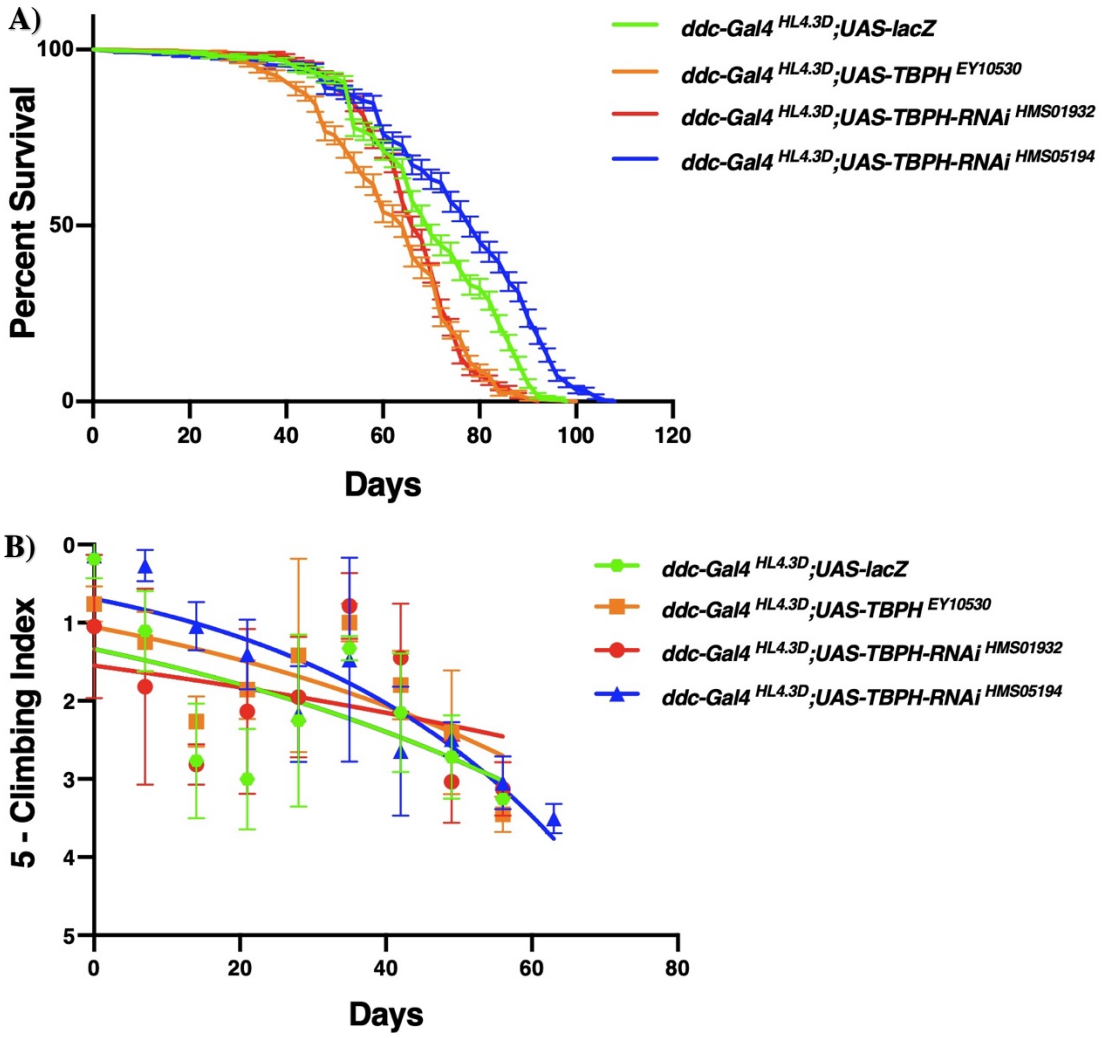

Figure 2.5: Altered expression of *TBPH* directed through the *ddc-Gal4*$^{HL4.3D}$ transgene affects longevity and climbing ability. **A:** Longevity assay of *Drosophila melanogaster* males displaying altered *TBPH* expression in the neurons. Longevity is depicted by percent survival. Significance is $P < 0.05$ using the log-rank test with Bonferroni correction. Error bars represent standard error of the mean. Genotypes are as follows: *ddc-Gal4*$^{HL4.3D}$*;UAS-lacZ* (n=293), *ddc-Gal4*$^{HL4.3D}$*;UAS-TBPH*^{EY10530} (n=271), *ddc-Gal4*$^{HL4.3D}$*;UAS-TBPH-RNAi*^{HMS01932} (n=298), *ddc-Gal4*$^{HL4.3D}$*;UAS-TBPH-RNAi*^{HMS05194} (n=296). **B:** Locomotor assay of *D. melanogaster* males displaying altered *TBPH* expression in the neurons. Climbing ability was determined by a nonlinear curve fit (CI=95%). Error bars indicate standard error of the mean

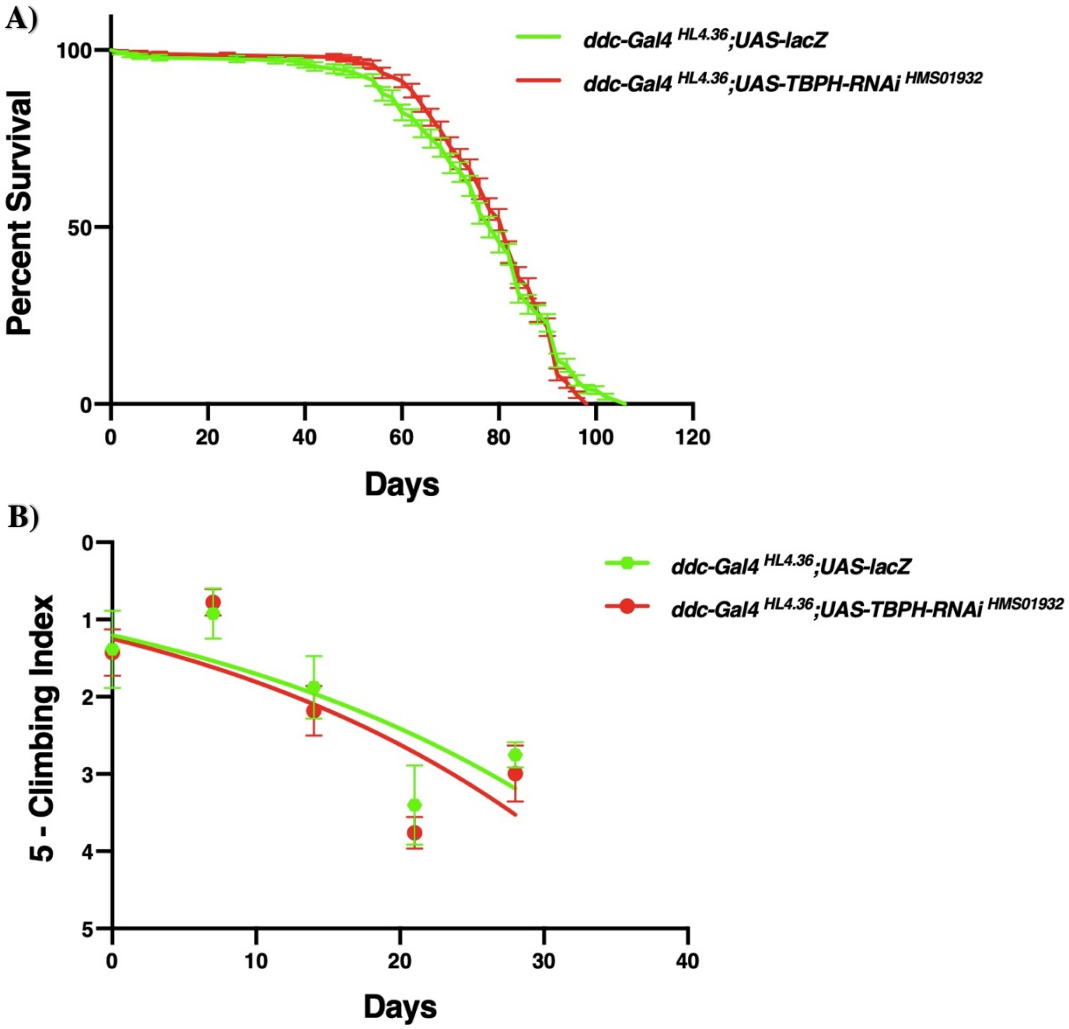

Figure 2.6: Altered expression of *TBPH* directed through the *ddc-Gal4*$^{HL4.36}$ transgene does not affect longevity and climbing ability. **A:** Longevity assay of *Drosophila melanogaster* males displaying altered *TBPH* expression in the neurons. Longevity is depicted by percent survival. Significance is $P < 0.05$ using the log-rank test with Bonferroni correction. Error bars represent standard error of the mean. Genotypes are as follows: *ddc-Gal4*$^{HL4.36}$*;UAS-lacZ* (n=284), *ddc-Gal4*$^{HL4.36}$*;UAS-TBPH-RNAi*^{HMS01932} (n=263). **B:** Locomotor assay of *D. melanogaster* males displaying altered *TBPH* expression in the neurons. Climbing ability was determined by a nonlinear curve fit (CI=95%). Error bars indicate standard error of the mean.

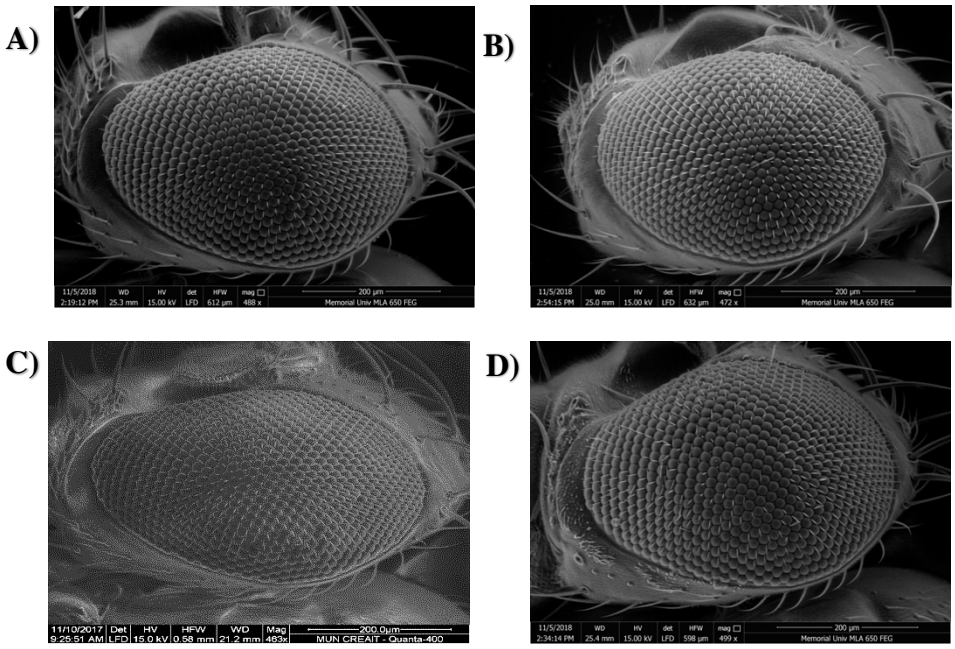

Figure 2.7: Directed alteration of the *TBPH* gene expression using eye-specific transgene *GMR-Gal4* in Drosophila. Scanning electron micrographs of A: *GMR-Gal4;UAS-lacZ*, B: *GMR-Gal4;UAS-TBPH*^{EY10530}, C: *GMR-Gal4;UAS-TBPH-RNAi*^{HMS05194}, D: *GMR-Gal4;UAS-TBPH-RNAi*^{HMS01932}

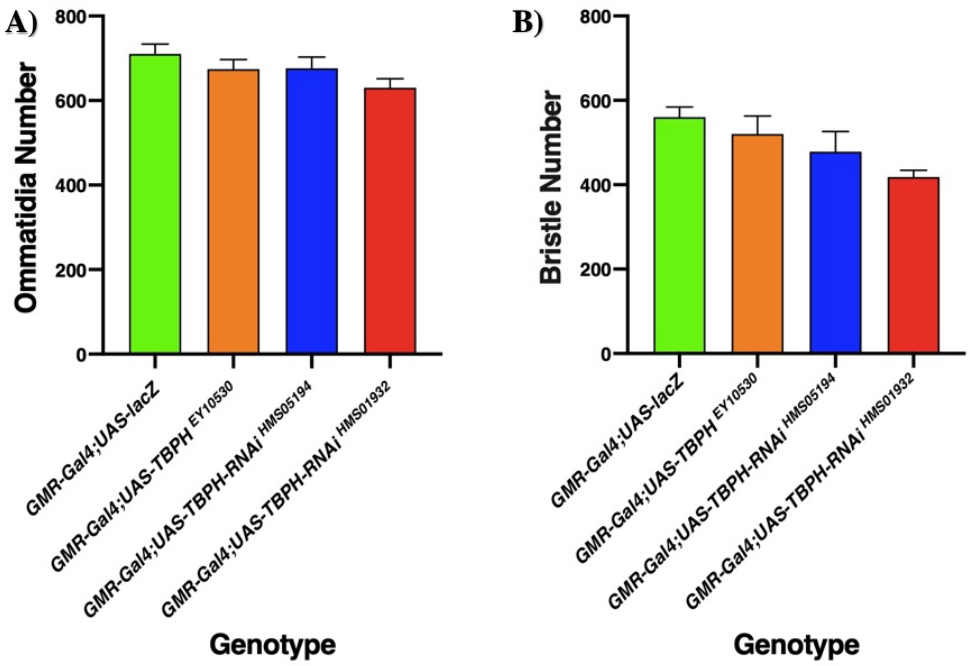

Figure 2.8: Biometric analysis of Drosophila compound eye under direct eye expression of *TBPH* through the *GMR-Gal4* transgene. Inhibition of *TBPH* through *UAS-TBPH-RNAi*^{HMS01932} caused a significant decrease in both (A) ommatidia number and (B) bristle number. Error bars represent standard deviation.

3.4 Discussion

The development and progression of ALS remains unclear and the desire to reach a greater understanding of this provides the incentive for an ongoing field of scientific research. Of great importance, as the prevalence of the disease continues to increase, the search for this understanding may lead to the development of treatments and therapies. Altered expression of *TBPH* produced a significant effect upon the longevity, locomotor function and neurodevelopment in *D. melanogaster*. Critical class flies that inhibited function through the expression of *UAS-TBPH-RNAi*^{HMS01932} display a reduction in median lifespan and of locomotor function over time. This reduction in longevity caused by the loss-of-function of *TBPH* suggests that the function of this gene product may play a substantial role in cell survival and death. Specifically, when inhibition of *TBPH* is accomplished through the activity of the pan-neuronal transgene *elav-Gal4*, viability is significantly reduced, and survival to adulthood is extremely poor. Certainly, the reduced rate of eclosion may be due to the toxic effects produced by of the loss of *TBPH* function in Drosophila. The most significant reduction in median lifespan and motor function is seen when *TBPH* function is inhibited in the motor neuron, through the *D42-Gal4* transgene. This significant reduction in median lifespan and motor ability when *TBPH* expression is altered corresponds with the characteristic loss of motor neurons associated with ALS, making the inhibition of *TBPH* in the motor neuron a promising model of neurodegenerative disease. Studies have observed the impacts that TDP-43 has in the motor neuron, demonstrating the severe impact that loss-of-function has to aspects such as microglia morphology (Spiller et al., 2018), axon degeneration, and TDP-43 median

splicing repression (Donde et al., 2019). In regards to Drosophila, loss-of-function of *TBPH* in the motor neurons has been shown to affect the neuromuscular junctions, displaying anatomical defects such as a reduction in axonal branches and synaptic boutons located within the muscles (Feiguin et al., 2009). The role that TDP-43 has in the motor neuron appears to be essential in at least certain forms of ALS pathology, with the TDP-43 shuttling event and functions in the mitochondria being a critical factor in ALS development and progression.

The development of the compound eye of Drosophila is a highly regulated and specifically controlled process. Critical class flies that expressed the loss-of-function of *UAS-TBPH-RNAi*^{HMS01932} displayed a significant reduction in the quantity of both the ommatidia and interommatidial bristles. Often, these characteristic phenotypes are due to an impairment during the development of the Drosophila eye and may reveal a specific defect during neurodevelopment. Regarding the expression of TDP-43 in the Drosophila eye, one study has shown through whole-genome microarrays, highly upregulated and downregulated genes from a family of G-protein coupled receptors (GPCRs), the methuselah family, known to be involved in regulating adult lifespan. Other genes identified in this study played a role in regulating the mitochondria as well as oxidative cellular processes (Zhan, Hanson, Kim, Tare, & Tibbetts, 2013). As *TBPH* inhibition compromised the phenotype of the Drosophila eye insights from the neurodegenerative process may be made. The inhibition of *TBPH* appears to mimic the suggested pathology of ALS and thus making this a promising model of neurodegenerative disease.

The overexpression of *TBPH* influenced critical class flies only when expressed through a subset of the specific *Gal4* transgenes. When overexpressed through the pan-neuronal directing transgene *elav-Gal4*, flies displayed a reduction in median lifespan. Overexpression of *TBPH* through the dopaminergic neuron-specific transgene *TH-Gal4* and the neuron-specific transgene *ddc-Gal4*$^{HL4.3D}$ displayed a significant reduction in median lifespan. Locomotor ability was not altered by *TBPH* overexpression when expressed through either *TH-Gal4* or *ddc-Gal4*$^{HL4.3D}$ transgenes. However, locomotor function was significantly reduced when *TBPH* was overexpressed through the activity of the motor neuron-specific transgene *D42-Gal4,* but median lifespan was minimally impacted. Previous studies have shown that the overexpression of TDP-43 has the ability to induce neurotoxicity as well as early death in the fly (Li et al., 2010; Voigt et al., 2010; Zhan et al., 2013). However, some have suggested that the degeneration caused by the overexpression of TDP-43 is not through the "classical" programmed cell death pathways (Hanson, Kim, Wassarman, & Tibbetts, 2010; Zhan et al., 2013). A further investigation into the consequences of the overexpression of TDP-43 is required to fully understands its role(s) in neurodegenerative disease and healthy aging.

3.5 References

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Chapter 4 – Modelling Amyotrophic Lateral Sclerosis in *Drosophila melanogaster* Through Alteration of *IK2*

4.1 Introduction

The *TANK-binding kinase 1* (*TBK1*) is one of the most recently identified genes that contributes to inherited forms of ALS. *TBK1*, also known as NAK or T2K, encodes a multifunctional serine/threonine kinase that belongs to the IkappaB kinase-like 2 (IK2) family of kinases. This group of related proteins has been demonstrated to act as essential regulators in the responses regulating inflammation, immunity, apoptosis and autophagy (Heo, Ordureau, Paulo, Rinehart, & Harper, 2015; Larabi et al., 2013; Lazarou et al., 2015). In the cell, the location of *TBK1* is in the cytoplasm, cytosol, nucleoplasm and the endosome membrane, where it has significant roles in both the immune system and autophagy (Ahmad, Zhang, Casanova, & Sancho-Shimizu, 2016; Oakes, Davies, & Collins, 2017). With regards to the role that *TBK1* has in the process of autophagy, *TBK1* is activated through the phosphorylation of the receptor proteins that control autophagy. These autophagic receptors include sequestosome 1, also known as p62/SQSTM1, and optineurin (OPTN) (Nguyen, Thombre, & Wang, 2018; Richter et al., 2016). These autophagy-related receptors play a role related to their association with autophagy-related proteins, which can recognize both specific and non-specific cargo, that are then to be degraded then recycled via the activity of the lysosomes (Ahmad et al., 2016; Oakes et al., 2017). The ability of autophagy-related proteins to recognize specific cargo is through the activity of p62/SQSTM1 and OPTN and the ability of proteins to bind to ubiquitin residues on the cargo to be targeted. Once bound, this enables the process by which the

ubiquitinated protein product to be sent for degradation (Ahmad et al., 2016; Oakes et al., 2017). As proteins such as p62/SQSTM1 and OPTN have critical roles in autophagy processes, it is vital to explore their role of these in the development of neurodegenerative disease.

Studies have shown that mutations in *TBK1* result in the impairment of autophagy, which in turn contributes to the formation of protein aggregates and thus disease pathology. At least a partial loss of *TBK1* has been reported to result in the progression of ALS and frontotemporal dementia (FTD) (Oakes et al., 2017). Recent studies have shown that loss of *TBK1* results in both embryonic death and neurodegeneration, as a result of activated *Receptor-interacting serine/threonine-protein kinase 1(RIPK1*), an enzyme that is a crucial regulator in cell survival and death. Specifically, *RIPK1* binds to the *TBK1* substrate OPTN and functions in various cellular pathways involved in cell survival and death (Ito et al., 2016; Xu et al., 2018; Yu & Cleveland, 2018). This activated *RIPK1* is caused by a mutation in *TBK1*, with the inhibition of *RIPK1* shown to prevent *TBK1* knockdown, embryonic lethality and neurodegeneration (Xu et al., 2018; Yu & Cleveland, 2018). Therefore, for both healthy development and to reduce the risk of neurodegeneration, *RIPK1* kinase activity must be inhibited (Xu et al., 2018; Yu & Cleveland, 2018). As *TBK1* is a newly identified gene, and much remains unknown about the role of it in neurogenerative disease, thus making it an excellent candidate gene to research. However, not only mutations in *TBK1* result in impaired autophagy but loss of function mutations in p62/SQSTM1 and OPTN, two autophagy receptors for *TBK1* have been reported to cause abnormal autophagy (Ahmad et al., 2016; Oakes et al., 2017).

Therefore, it is essential to investigate the functions of *TBK1* and its autophagy receptors, and to manipulate their expression in a selected set of neurons.

The *TBK1* gene and the *IK2* family of genes have been highly conserved throughout evolutionary history. A comparison of human TBK1 and *D. melanogaster* IK2 proteins reveals three highly conserved domains: 1) the P kinase domain; 2) a ubiquitin-like domain; and 3) the coiled-coil domain 1 (CCD1). The degree of evolutionary conservation suggests a highly conserved function for *TBK1*/*IK2*. Thus exploration of the effects of altered *IK2* expression in *D. melanogaster* should be expected to provide insights into the pathology of ALS in humans. In this study, both the overexpression and inhibition of *IK2* in *D. melanogaster* is used to mimic human conditions, examining the effects on lifespan and locomotor ability.

4.2 Materials and Methods

4.2.1 *Drosophila melanogaster* stocks and culture

All Drosophila stocks were obtained from the Bloomington Drosophila Stock Center at Indiana University (IN, USA). See Table 2.1 for list of genotypes used. See Chapter 2, section 2.1 for detail of *D. melanogaster* stocks and culture.

3.2.2 Longevity Assay

The survival of *D. melanogaster* was analyzed to examine the median lifespan of experimental flies in comparison to control flies. See Chapter 2, section 2.2 for full longevity assay methods.

2.2.3 Locomotive Assay

The motor function of *D. melanogaster* was analyzed to examine the locomotor function over time of experimental flies in comparison to control flies. See Chapter 2, section 2.3 for full locomotive assay methods.

2.2.4 Scanning Electron Microscopy of the *Drosophila* eye

Eye analysis of *D. melanogaster* was used to determine the effects of gene manipulation. See Chapter 2, section 2.4 for full methods on eye experiments.

Table 2.1: Genotypes and location of expression patterns used in the analysis of altered expression of *IK2*.

Abbreviated Genotype	Location of Expression	Insertion Chromosome	Reference
Control Lines			
UAS-lacZ	---	2	Brand et al., 1993
Driver Lines			
GMR-Gal4	Eye	2	Freeman, 1996
arm-Gal4	Ubiquitous	2	Sanson et al., 1996
elav-Gal4	Pan neuronal	1	Lin & Goodman, 1994
TH-Gal4	Dopaminergic neuron	3	Inamdar et al., 2014
ddc-Gal4$^{HL4.3D}$	Dopaminergic and serotonergic neuron	2	Li et al., 2000
ddc-Gal4$^{HL4.36}$	Dopaminergic and serotonergic neuron	3	Li et al., 2000
D42-Gal4	Motor neuron	3	Parkes et al., 1998
Responder Lines			
UAS-IK2^{EY09774}	---	2	Bellen et al., 2004
UAS-IK2-RNAi^{HMS01188}	---	3	Perkins et al., 2015
UAS-IK2-RNAi^{GL00160}	---	3	Perkins et al., 2015

4.3 Results

4.3.1 Overexpression of *IK2* decreases median lifespan and climbing ability over time

When *IK2* is overexpressed *D. melanogaster* median lifespan, and climbing ability over time is greatly influenced. Overexpression of *IK2* significantly reduces median lifespan when expressed through the ubiquitous transgene *arm-Gal4* (Figure 3.1) and the pan-neuronal transgene *elav-Gal4* (Figure 3.2). *IK2* overexpression through the motor neuron-specific transgene *D42-Gal4* significantly reduced median lifespan (Figure 3.3a) and climbing ability over time (Figure 3.3b) compared to the control (*UAS-lacZ*). *IK2* overexpression through the dopaminergic neuron-specific transgene *TH-Gal4* significantly reduced median lifespan (Figure 3.4a). However, the climbing ability of *D. melanogaster* was not influenced (Figure 3.4b). Notably, it is worth mentioning that *IK2* overexpression through *TH-Gal4* provided very poor viability of critical class males. When *IK2* is overexpressed through the neuron-specific transgenes *ddc-Gal4*$^{HL4.3D}$ and *ddc-Gal4*$^{HL4.36}$ no critical class male progeny survived to adulthood.

4.3.2 Inhibition of *IK2* increases median lifespan and decreases climbing ability in the motor neuron over time

An investigation of the consequences of inhibition of *IK2* upon the median lifespan and climbing function of *D. melanogaster* was carried out using two *IK2* inhibition transgenes, *UAS-IK2-RNAi*^{HMS01188} and *UAS-IK2-RNAi*^{GL00160}. Two *IK2* inhibition lines were selected to investigate the consequences of both on the fly. Both *IK2* RNAi transgenes increased median lifespan when expressed through the

ubiquitous transgene *arm-Gal4* (Figure 3.1) and the motor neuron-specific transgene *D42-Gal4* (Figure 3.3a), while decreasing median lifespan when expressed through the pan-neuronal transgene *elav-Gal4* (Figure 3.2) when compared to the control (*UAS-lacZ*). When expressed through the dopaminergic neuron-specific transgene *TH-Gal4* (Figure 3.4a), as well as the neuron-specific transgene *ddc-Gal4*$^{HL4.3D}$ (Figure 3.5a), the *IK2* RNAi transgene *UAS-IK2-RNAi*^{GL00160} resulted in an increased median lifespan, while the *IK2* RNAi transgene *UAS-IK2-RNAi*^{HMS01188} lead to a reduction in median lifespan. Both *IK2* inhibition transgenes impacted climbing ability over time when expressed through the motor neuron-specific transgene *D42-Gal4* (Figure 3.3b), leading to a significant reduction in motor function. Inhibition of *IK2* through the dopaminergic neuron-specific transgene *TH-Gal4* did not significantly alter climbing ability over time (Figure 3.4b) when compared to the control (*UAS-lacZ*). Expression of the *IK2* inhibition line *UAS-IK2-RNAi*^{HMS01188} through the neuron-specific transgene *ddc-Gal4*$^{HL4.3D}$ did not significantly influence climbing ability over time (Figure 3.5b); however, the inhibition line *UAS-IK2-RNAi*^{GL00160} lead to a significant reduction in climbing ability over time (Figure 3.5b) when compared to the control (*UAS-lacZ*).

4.3.3 Inhibition of *IK2* decreases ommatidia and bristle count

The developmental pattern of the Drosophila compound eye is a highly regulated and specific event. Once the ommatidia are laid down, the bristles come next as a form of protection for the ommatidia. Impairment in this process may result in characteristic phenotypes as a consequence, such as changes in the number of ommatidia and bristles. The eye-specific transgene *GMR-Gal4* is used to investigate such phenotypic changes in

the compound eye of *D. melanogaster.* Expression of the *IK2* inhibition transgene *UAS-IK2-RNAi*^{HMS01188} in the developing eye of *D. melanogaster* has shown to significantly reduce the number of ommatidia (Figure 3.7a) and interommatidial bristles (Figure 3.7b) when compared to the control (*UAS-lacZ*).

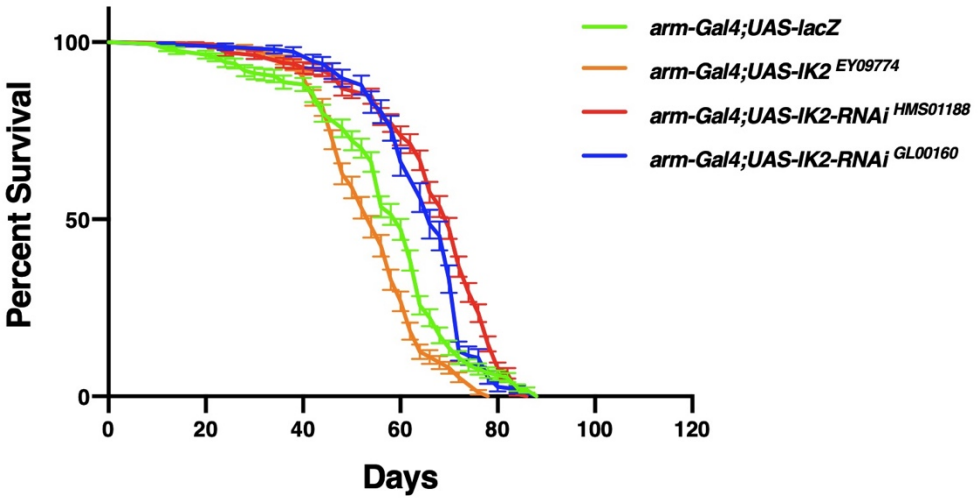

Figure 3.1: Altered expression of *IK2* directed through the *arm-Gal4* transgene affects lifespan. Longevity is depicted by percent survival. Significance is $P < 0.05$ using the log-rank test with Bonferroni correction. Error bars represent standard error of the mean. Genotypes are as follows: *arm-Gal4;UAS-lacZ* (n=284), *arm-Gal4;UAS-IK2*^{EY09774} (n=261), *arm-Gal4;UAS-IK2-RNAi*^{HMS01188} (n=276), *arm-Gal4;UAS-IK2 RNAi*^{GL00160}(n=148).

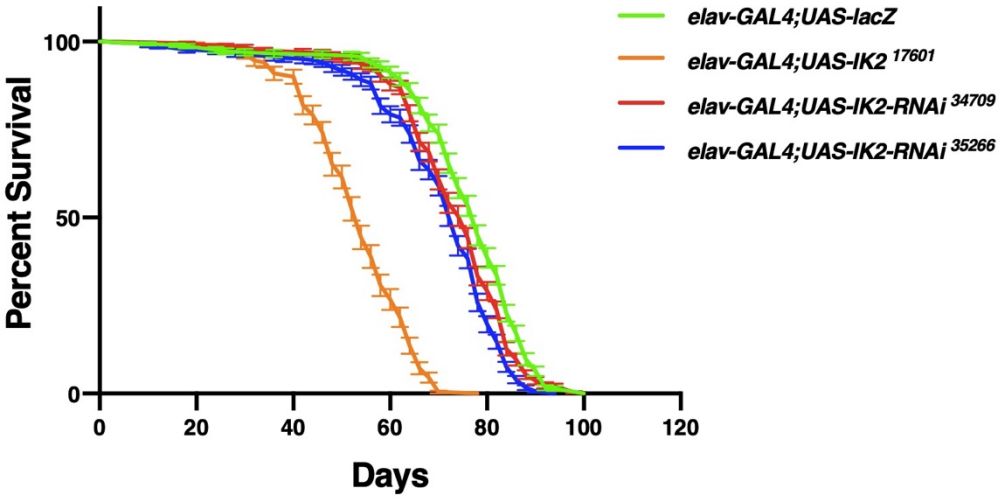

Figure 3.2: Altered expression of *IK2* directed through the *elav-Gal4* transgene affects lifespan. Longevity is depicted by percent survival. Significance is $P < 0.05$ using the log-rank test with Bonferroni correction. Error bars represent standard error of the mean. Genotypes are as follows: *elav-Gal4;UAS-lacZ* (n=298), *elav-Gal4;UAS-IK2*^{EY09774} (n=221), *elav-Gal4;UAS-IK2-RNAi*^{HMS01188} (n=299), *elav-Gal4;UAS-IK2 RNAi*^{GL00160}(n=305).

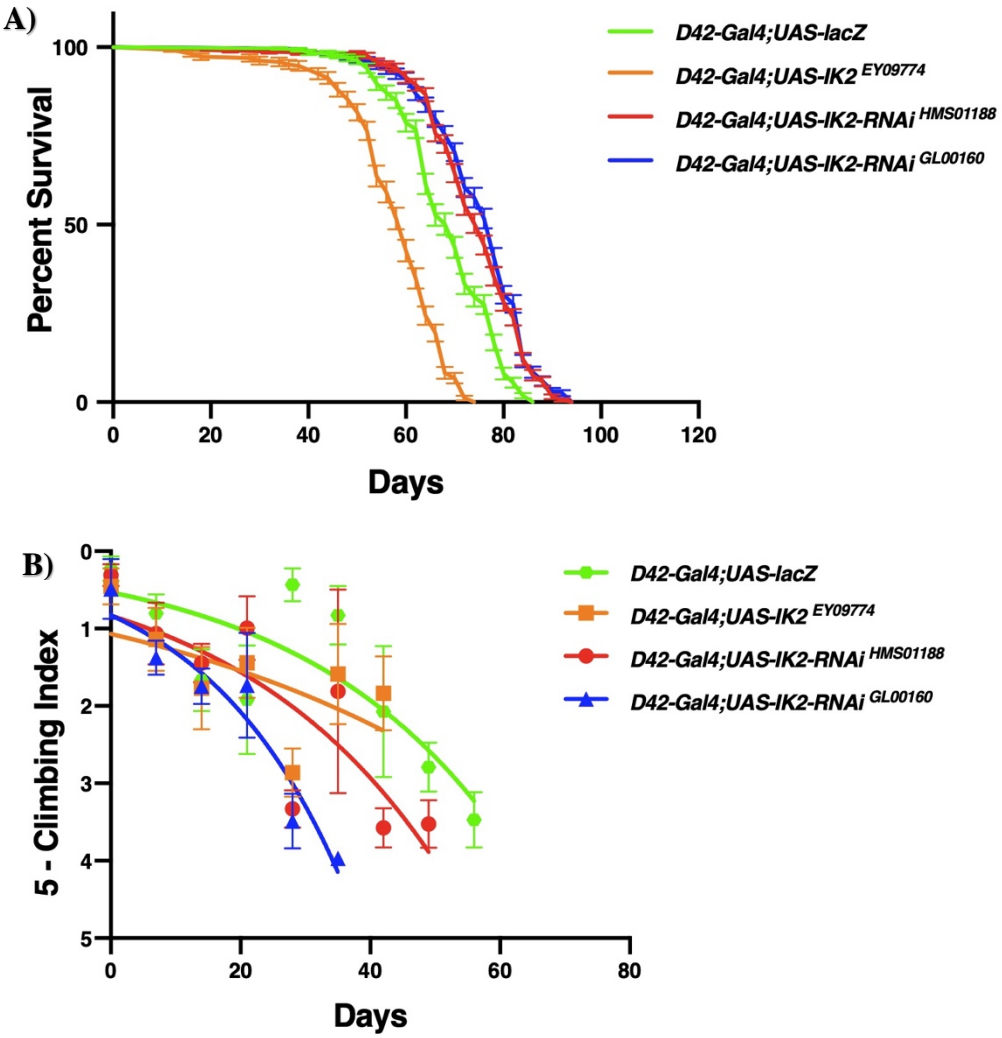

Figure 3.3: Altered expression of *IK2* directed through the *D42-Gal4* transgene affects longevity and climbing ability. **A:** Longevity assay of *Drosophila melanogaster* males displaying altered *IK2* expression in the motor neurons. Longevity is depicted by percent survival. Significance is $P < 0.05$ using the log-rank test with Bonferroni correction. Error bars represent standard error of the mean. Genotypes are as follows: *D42-Gal4;UAS-lacZ* (n=273), *D42-Gal4;UAS-IK2*^{EY09774} (n=267), *D42-Gal4;UAS-IK2-RNAi*^{HMS01188} (n=330), *D42-Gal4;UAS-IK2 RNAi*^{GL00160}(n=321). **B:** Locomotor assay of *D. melanogaster* males displaying altered *IK2* expression in the motor neurons. Climbing ability was determined by a nonlinear curve fit (CI=95%). Error bars indicate standard error of the mean.

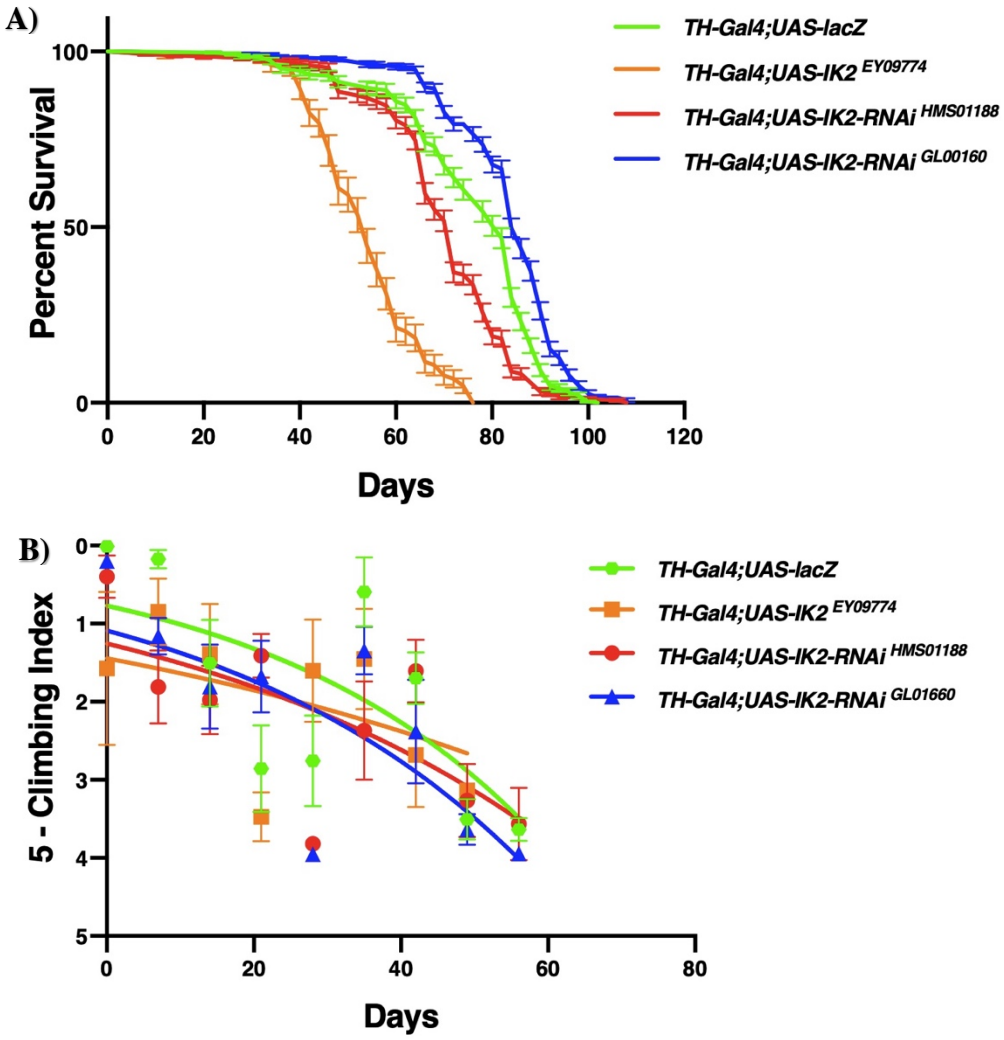

Figure 3.4: Altered expression of *IK2* directed through the *TH-Gal4* transgene affects longevity and climbing ability. **A:** Longevity assay of *Drosophila melanogaster* males displaying altered *IK2* expression in the dopaminergic neurons. Longevity is depicted by percent survival. Significance is $P < 0.05$ using the log-rank test with Bonferroni correction. Error bars represent standard error of the mean. Genotypes are as follows: *TH-Gal4;UAS-lacZ* (n=290), *TH-Gal4;UAS-IK2*^{EY09774} (n=103), *TH-Gal4;UAS-IK2-RNAi*^{HMS01188} (n=280), *TH-Gal4;UAS-IK2 RNAi*^{GL00160}(n=324). **B:** Locomotor assay of *D. melanogaster* males displaying altered *IK2* expression in the dopaminergic neurons. Climbing ability was determined by a nonlinear curve fit (CI=95%). Error bars indicate standard error of the mean.

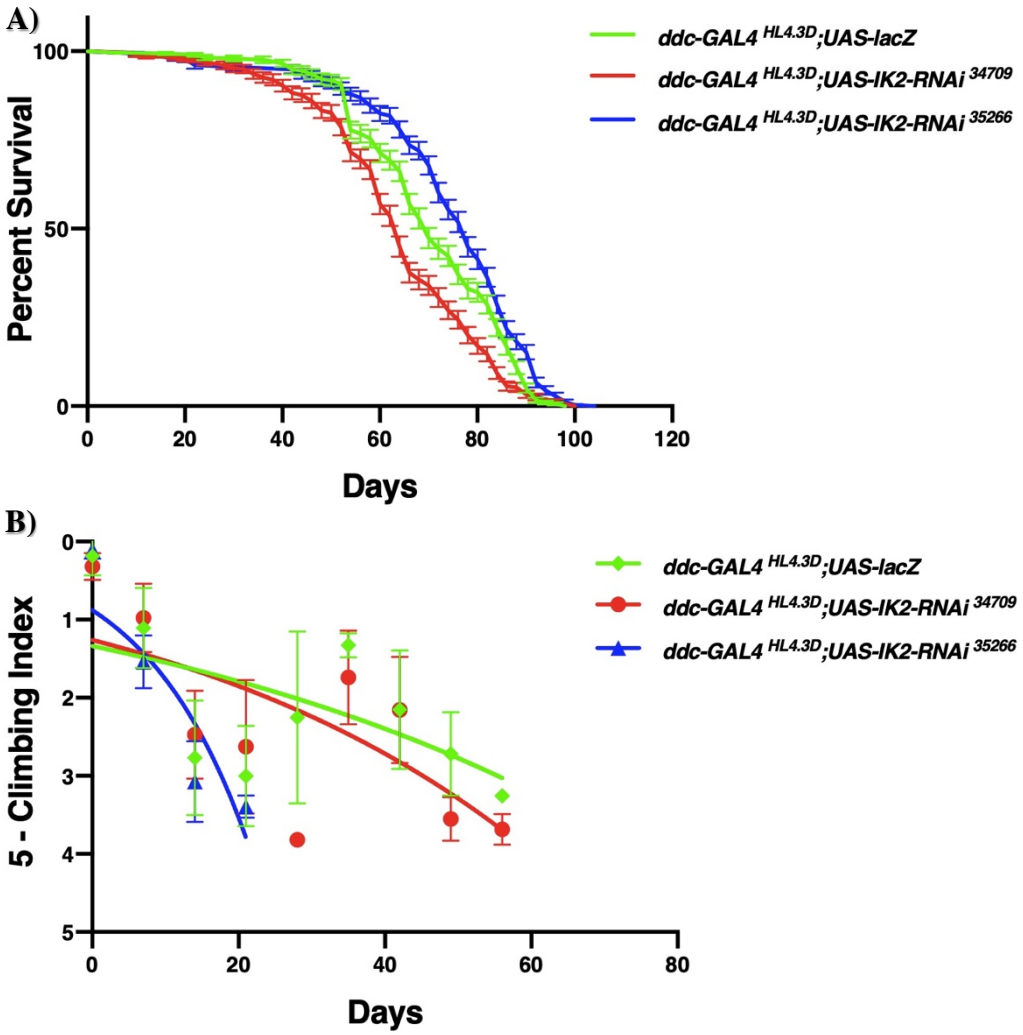

Figure 3.5: Altered expression of *IK2* directed through the *ddc-Gal4*$^{HL4.3D}$ transgene affects longevity and climbing ability. **A:** Longevity assay of *Drosophila melanogaster* males displaying altered *IK2* expression in the neurons. Longevity is depicted by percent survival. Significance is $P < 0.05$ using the log-rank test with Bonferroni correction. Error bars represent standard error of the mean. Genotypes are as follows: *ddc-Gal4*$^{HL4.3D}$*;UAS-lacZ* (n=293), *ddc-Gal4*$^{HL4.3D}$*;UAS-IK2-RNAi*^{HMS01188} (n=300), *ddc-Gal4*$^{HL4.3D}$*;UAS-IK2 RNAi*^{GL00160}(n=314). **B:** Locomotor assay of *D. melanogaster* males displaying altered *IK2* expression in the neurons. Climbing ability was determined by a nonlinear curve fit (CI=95%). Error bars indicate standard error of the mean.

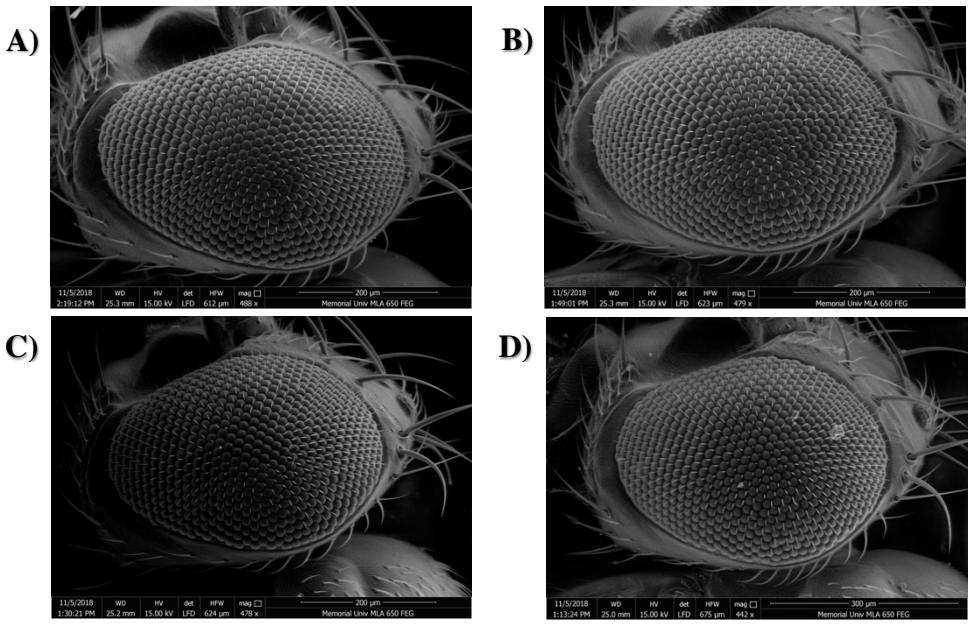

Figure 3.6: Directed *IK2* gene expression using eye-specific transgene *GMR-Gal4* in Drosophila. Scanning electron micrographs of A: *GMR-Gal4;UAS-lacZ*, B: *GMR-Gal4;IK2^{EY09774}*, C: *GMR-Gal4;IK2-RNAi^{HMS01188}*, D: *GMR-Gal4;UAS-IK2-RNAi^{GL00160}*

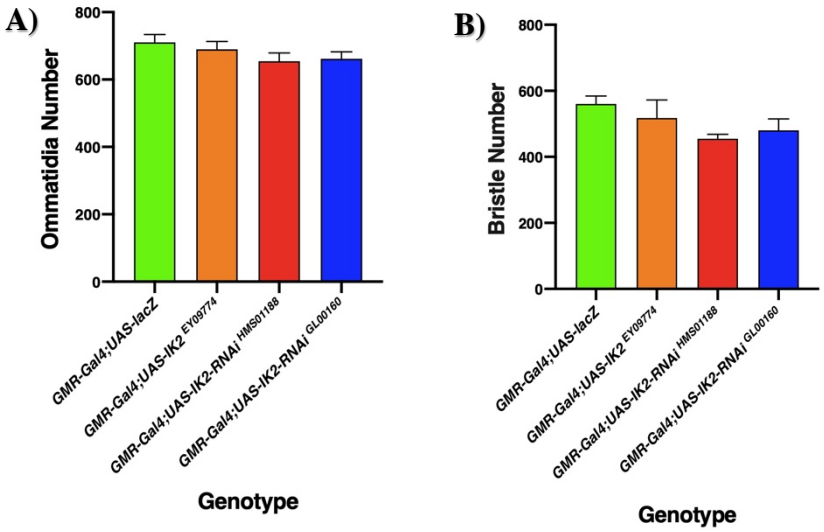

Figure 3.7: Biometric analysis of Drosophila compound eye under direct eye expression of *IK2* though the *GMR-Gal4* transgene. Inhibition of *IK2* causes significant decrease in (B) bristle number. Error bars represent standard deviation.

4.4 Discussion

Understanding the development and the progression of neurodegenerative diseases such as ALS and PD is a critical goal of modern research. As a recently discovered ALS gene, *TBK1,* there is much left unknown with regards to its functions and roles in neurodegeneration, making it an excellent candidate gene to investigate in model organisms. Human *TBK1* is a serine/threonine-protein kinase from the *IkappaB kinases* (*IKKs*) family (Heo et al., 2015). A member of the *IKK* family and the Drosophila homologue of *TBK1*, *IK2* has been shown to influence longevity of the organism, locomotor function and neuronal development when manipulated. Critical class flies overexpressing *IK2* displayed reduced median lifespan when directed by selected *Gal4* transgenes, with a considerable reduction seen when *IK2* was overexpressed through the motor neuron-specific transgene *D42-Gal4* and the dopaminergic neuron-specific transgene *TH-Gal4*. Interestingly, when *IK2* was overexpressed through the activity of *TH-Gal4*, the viability of critical class males was poor. This lethality seen by the gain-of-function of *IK2* suggests that normal function is essential to provide viability. Attempts were made to examine the effects of the overexpression of *IK2* through the neuron-specific transgenes *ddc-Gal4*$^{HL4.3D}$ and *ddc-Gal4*$^{HL4.36}$; however critical class males did not survive to adulthood. The reduction in lifespan, as well as the multiple cases of impaired viability, suggest that *IK2* plays a substantial role in cellular pathways that govern cell survival and death. The overexpression of *IK2* greatly influenced motor ability throughout life. Overexpression of *IK2* directed through the motor neuron-specific transgene *D42-Gal4*, and the dopaminergic neuron-specific transgene *TH-Gal4* leads to a phenotype that displayed a minimal reduction in climbing ability overtime, at the least.

The overexpression of *IK2* appears to mimic aspects of the pathology of ALS. As ALS is a motor neuron disease, the reduction in median lifespan and a minimal decline in motor function observed when *IK2* is overexpressed through the activity of the motor neuron-specific transgene *D42-Gal4* produces an imperfect model of neurodegenerative disease.

Much is still left unclear with regards to the mechanisms by which alteration to the activity of *TBK1* contributes to the progression of neurodegenerative disease. However, studies have shown the importance of *IK2* in Drosophila with regards to the process of dendrite pruning, a highly controlled procedure required for the development of neurons (Lee, Jan, & Jan, 2009). During neurodevelopment, specific Drosophila sensory neurons undergo a "pruning" of the dendrite metamorphosis before the time that adult dendrites generate. Throughout this process, the individual Drosophila undergo extensive neuronal remodeling where the *IK2* gene, among others, play vital roles (Lee et al., 2009; Lin et al., 2015). The reduced viability observed when *IK2* has been overexpressed through the activity of certain transgenes may be partially explained, at the least, by impaired neuronal development. Furthermore, the protein product of *IK2* is a component in the organization of the cytoskeleton for mRNA localization during oogenesis (Lee et al., 2009; Shapiro & Andreson, 2006), where the IK2 protein along with Spindle-F proteins form a complex to play a substantial role during oogenesis in the regulation of cytoskeleton dynamics (Dubin-bar et al., 2008). Further investigations into the altered expression of *TBK1* is required to more fully understand the role of this key gene in neurodegeneration.

The effects of the inhibition of *IK2* seem to produce varied results. Both *IK2* inhibition experimental transgenes significantly increased lifespan when directed by the ubiquitous transgene *arm-Gal4*, while the same inhibition lines decreased lifespan when expressed through the pan-neuronal transgene *elav-Gal4*. Interestingly, when inhibited through the motor neuron-specific transgene *D42-Gal4* both RNAi transgenes increased median lifespan, while reducing locomotor function over time. When the two *IK2* inhibition transgenes are expressed under control of the dopaminergic neuron-specific transgene *TH-Gal4* and the neuron-specific transgene *ddc-Gal4*$^{HL4.3D}$ is when distinct differences are observed. When expressed through *TH-Gal4* and *ddc-Gal4*$^{HL4.3D}$, the *IK2* RNAi transgene, *UAS-IK2-RNAi*^{HMS01188} results in a much more reduced median lifespan, while when expressed through these same transgenes, the other *IK2* inhibition transgene, *UAS-IK2-RNAi*^{GL00160}, leads to a significant increase in median lifespan. Climbing ability was reduced for both forms of *IK2* inhibition when expressed through all transgenes. The varied results of these experiments may suggest that one of the two *IK2* inhibition transgenes used may have another, off-target hit to result in the median lifespan and locomotor function of the fly altered, while the other IK2 inhibition transgene may be functioning, as usual, causing actual inhibition of *IK2*. However, not only does *IK2* inhibition impact median lifespan and climbing ability, but also the development of the Drosophila compound eye. Critical class flies that expressed inhibition of *IK2* through *UAS-IK2-RNAi*^{HMS01188} displayed a significant reduction in the quantity of both the ommatidia and bristle counts. These characteristic phenotypes are often due to an impairment in the development of the Drosophila eye, and thus may suggest an impact on

neurodevelopment. As human *TBK1* is a relatively new ALS gene, more experiments should be done to better understand the role of the IK2 family members in neurodegeneration.

4.5 References

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Chapter 5 - Modelling Human Neurodegenerative Disease Through Alteration of *p62/Ref(2)P* in *Drosophila melanogaster*

5.1 Introduction

The elucidation of the cellular mechanisms that are altered during the progression of neurodegenerative diseases, such as ALS and Parkinson Disease, is an ongoing subject of current research. The protein, sequestosome1, which is also known as p62 (p62/SQSTM1), has been suggested to be a potential contributor to in the pathogenesis of a number of neurodegenerative diseases (Ma, Attarwala, & Xie, 2019). The p62/SQSTM1 protein is a multifunctional scaffold/adaptor protein encoded by the *p62/SQSTM1* gene (Bartolome, Esteras, Martin-requero, & Boutoleau, 2017). Alternatively designated as "Refractory to Sigma P" (*Ref(2)P*) in Drosophila, p62/SQSTM1 is involved in various aspects of selective autophagy - such as mitophagy, the ubiquitin-proteasome system (UPS) and in some signal transduction pathways (Bitto et al., 2014). The p62/SQSTM1 protein is localized throughout the cell in the cytoplasm, in the cytosol, and the endoplasmic reticulum, among other places such as autophagosomes, aggresomes, and autolysosomes, as this protein functions during the process of autophagy (Liu et al., 2016; Matsumoto, Shimogori, Hattori, & Nukina, 2015). The role which p62/SQSTM1 has in autophagy is critical as it seems to regulates the removal of protein aggregates and damaged organelles through the activities of several of its many functional domains (Bitto et al., 2014). Structurally the p62/SQSTM1 protein has many functional domains, several of which are essential to autophagic activities. These domains include 1) the Phox and Bem1 (PB1) domain; 2) the LC3 interacting region (LIR) domain; and 3) the UBA

domain at the C-terminus (Liang & Guan, 2017). During autophagy, the LIR domain and the UBA domains of p62/SQSTM1 have vital roles in the pathway (Hou et al., 2019; Johansen & Lamark, 2011; Ma, Attarwala, & Xie, 2019), as p62/SQSTM1 promotes autophagic degradation by binding to LC3 via its LIR region (Pankiv et al., 2007). The PB1 and the UBA domains function together to form protein aggregates (Bartlett et al., 2011), and are known to be critical for mitochondrial clustering (Pimenta De Castro et al., 2013). The UBA domain recognizes ubiquitinated protein aggregates, while the PB1 domain sequesters these into inclusion bodies (Lee, Weihl, Lee, & Weihl, 2017). Other p62/SQSTM1 domains include the ZZ-type zinc finger domain, a domain capable of binding with the p38 mitogen-activated protein kinase, the TB domain, PEST sequences made up of the amino acids proline, glutamate, serine and threonine, and the KIR domain (Liang & Guan, 2017). As p62/SQSTM1 has multiple functional domains with various roles, investigating the *p62/SQSTM1* gene in neurodegenerative disease may be of great advantage.

In humans, p62/SQSTM1 can act as an indicator of autophagic flux as the protein accumulates when autophagy is inhibited. Similarly, it has been demonstrated that *Ref(2)P* behaves in the same manner, with accumulation of *Ref(2)P* levels in Drosophila when autophagy is genetically inhibited (Devorkin & Gorski, 2014). However, not only does *p62/SQSTM1* function in quality control, but it is known to have roles in age-related diseases. Studies with a mouse model have shown that *p62/SQSTM1* expression decreases as age increases, with protein knockdown reducing the lifespan and displaying premature signs of aging (Bitto et al., 2014). Mutations in *p62/SQSTM1* are known to be associated

with ALS and frontotemporal dementia (FTD) (Bartolome et al., 2017). However, *p62/SQSTM1* is not only associated with ALS and FTD, but it is known to be associated with some forms of Parkinson Disease through a defined role in mitophagy (Narendra, Kane, Hauser, Fearnley, & Youle, 2010; Yamada et al., 2019). Where during PINK1-parkin-mediated mitophagy, p62/SQSTM1 among other adaptor proteins are recruited to damaged mitochondria (Xiao et al., 2017). Studies in Drosophila have demonstrated that loss-of-function of *Ref(2)P* results in poor locomotor function related to mitochondrial dysfunction and accumulation of mitochondrial DNA (Pimenta De Castro et al., 2013; Pimenta De Castro et al., 2012). Whereas in humans, knockdown of *p62/SQSTM1* has shown to increase in both oxidative stress and mitochondrial damage and dysfunction (Bartolome et al., 2017; Bitto et al., 2014). Recent work has shown that *p62/SQSTM1* interacts with LRRK2, a PD-associated protein kinase as an autophagic receptor (Park, Han, Choi, Kim, & Park, 2016). Through the investigation of *p62/SQSTM1* as a candidate gene we may gain further insight into our understanding of *p62/SQSTM1* function in both aging and neurodegenerative disease.

The *p62/SQSTM1* gene has been highly conserved throughout evolutionary history and a comparison of human and *D. melanogaster* p62/SQSTM1 proteins reveals three highly conserved domains, 1) the Phox and Bem1p (PB1) domain; 2) a zinc finger, ZZ type domain, as well as, 3) a ubiquitin associated domain (UBA). The degree of evolutionary conservation suggests a highly conserved function for *p62*/*SQSTM1*. Thus exploration of the effects of altered *p62/SQSTM1* expression in *D. melanogaster* may provide insights into the pathology of ALS in humans. In this study, the inhibition

of *p62/SQSTM1* in *D. melanogaster* is used to mimic human conditions, examining the effects on lifespan and locomotor ability.

5.2 Materials and Methods

5.2.1 *Drosophila melanogaster* stocks and culture

All Drosophila stocks were obtained from the Bloomington Drosophila Stock Center at Indiana University (IN, USA). See Table 3.1 for list of genotypes used. See Chapter 2, section 2.1 for detail of *D. melanogaster* stocks and culture.

5.2.2 Longevity Assay

The survival of *D. melanogaster* was analyzed to examine the median lifespan of experimental flies in comparison to control flies. See Chapter 2, section 2.2 for full longevity assay methods.

5.2.3 Locomotive Assay

The motor function of *D. melanogaster* was analyzed to examine the locomotor function over time of experimental flies in comparison to control flies. See Chapter 2, section 2.3 for full locomotive assay methods.

5.2.4 Scanning Electron Microscopy of the *Drosophila* eye

Eye analysis of *D. melanogaster* was used to determine the effects of gene manipulation. See Chapter 2, section 2.4 for full methods on eye experiments.

Table 3.1: Genotypes and location of expression patterns used in the analysis of altered expression of *Ref(2)P*.

Abbreviated Genotype	Location of Expression	Insertion Chromosome	Reference
Control Lines			
UAS-lacZ	---	2	Brand et al, 1993
Driver Lines			
GMR-Gal4	Eye	2	Freeman, 1996
arm-Gal4	Ubiquitous	2	Sanson et al., 1996
elav-Gal4	Pan neuronal	1	Lin & Goodman, 1994
TH-Gal4	Dopaminergic neuron	3	Inamdar et al., 2014
ddc-Gal4$^{HL4.3D}$	Dopaminergic and serotonergic neuron	2	Li et al., 2000
ddc-Gal4$^{HL4.36}$	Dopaminergic and serotonergic neuron	3	Li et al., 2000
D42-Gal4	Motor neuron	3	Parkes et al., 1998
Responder Lines			
UAS-Ref(2)P-RNAi^{HMS00551}	---	3	Perkins et al., 2015
UAS-Ref(2)P-RNAi^{HMS00938}	---	3	Perkins et al., 2015

5.3 Results

5.3.1 Inhibition of *Ref(2)P* increases median lifespan and decreases climbing ability

An investigation of the consequences of the inhibition of *Ref(2)P* upon the lifespan and climbing function over time of *D. melanogaster* was carried out using two *Ref(2)P* inhibition transgenes, *UAS-Ref(2)P-RNAi* ^{HMS00551} and *UAS-Ref(2)P-RNAi*^{HMS00938}. Two *Ref(2)P* inhibition lines were selected to investigate the consequences of both on the fly. By examining *Ref(2)P* in *D. melanogaster* it was revealed that loss of function increased median lifespan. Expression of both *Ref(2)P* inhibition transgenes provided a significantly longer lifespan when expressed with the ubiquitous transgene *arm-Gal4* (Figure 4.1) and the pan-neuronal transgene *elav-Gal4* (Figure 4.2). Median lifespan was significantly increased when *Ref(2)P* was inhibited through the activity of the motor neuron-specific transgene *D42-Gal4* (Figure 4.3a), and the dopaminergic neuron-specific transgene *TH-Gal4* (Figure 4.4a) when compared to the control (*UAS-lacZ*). When expressed through the neuron-specific transgenes *ddc-GAL4*$^{HL4.3D}$ (Figure 4.5a) and *ddc-GAL4*$^{HL4.36}$ (Figure 4.6a) inhibition of *Ref(2)P* resulted in a significant increase in median lifespan. Despite the significant influence upon longevity, inhibition of *Ref(2)P* decreases climbing ability over time in *D. melanogaster*. When expressed in the motor neuron-specific transgene *D42-Gal4* (Figure 4.3b), the dopaminergic neuron-specific transgene *TH-Gal4* (Figure 4.4b), the inhibition of *Ref(2)P* through *UAS-Ref(2)P-RNAi*^{HMS00938} significantly reduce climbing ability when compared to the control (*UAS-lacZ*). When expressed through the neuron-specific transgene *ddc-Gal4*$^{HLD.3D}$ both

Ref(2)P inhibition transgenes led to a significant decrease in climbing ability (Figure 4.5b). Expression of *Ref(2)P* inhibition via *UAS-Ref(2)P-RNAi*^{HMS00938} through the neuronal-specific transgene *ddc-GAL4*$^{HL4.36}$ (Figure 4.6b) significantly reduced climbing ability when compared to the control (*UAS-lacZ)*.

5.3.2 Inhibition of *Ref(2)P* decreases ommatidia and bristle number

The developmental pattern of the Drosophila compound eye is a highly regulated and specific event. Once the ommatidia are laid down, the bristles come next as a form of protection for the ommatidia. Impairment in this process may result in characteristic phenotypes as a consequence, such as changes in the number of ommatidia and bristles. The eye-specific transgene *GMR-Gal4* is used to investigate such phenotypic changes in the compound eye of *D. melanogaster.* Inhibition of *Ref(2)P* in the developing Drosophila eye results in a reduction in ommatidia number (Figure 4.7a) and interommatidial bristle number (Figure 4.7b) when compared to the control (*UAS-lacZ*).

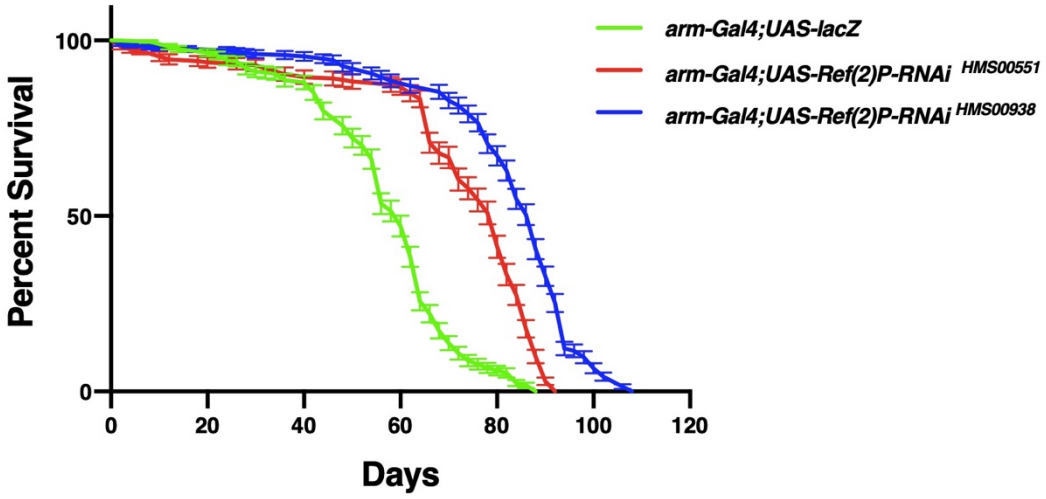

Figure 4.1: Altered expression of *Ref(2)P* directed through the *arm-Gal4* transgene affects lifespan. Longevity is depicted by percent survival. Significance is $P < 0.05$ using the log-rank test with Bonferroni correction. Error bars represent standard error of the mean. Genotypes are as follows: *arm-Gal4;UAS-lacZ* (n=284), *arm-Gal4;UAS-Ref(2)P-RNAi*^{HMS00551} (n=240), *arm-Gal4;UAS-Ref(2)P-RNAi*^{HMS00938} (n=286).

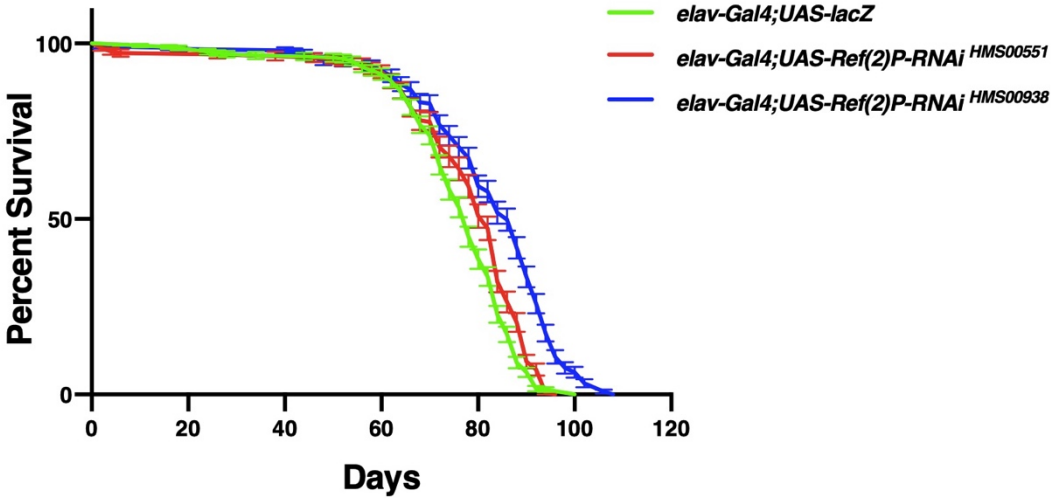

Figure 4.2: Altered expression of *Ref(2)P* directed through the *elav-Gal4* transgene affects lifespan. Longevity is depicted by percent survival. Significance is $P < 0.05$ using the log-rank test with Bonferroni correction. Error bars represent standard error of the mean. Genotypes are as follows: *elav-Gal4;UAS-lacZ* (n=298), *elav-Gal4;UAS-Ref(2)P-RNAi*^{HMS00551} (n=224), *elav-Gal4;UAS-Ref(2)P-RNAi*^{HMS00938} (n=251).

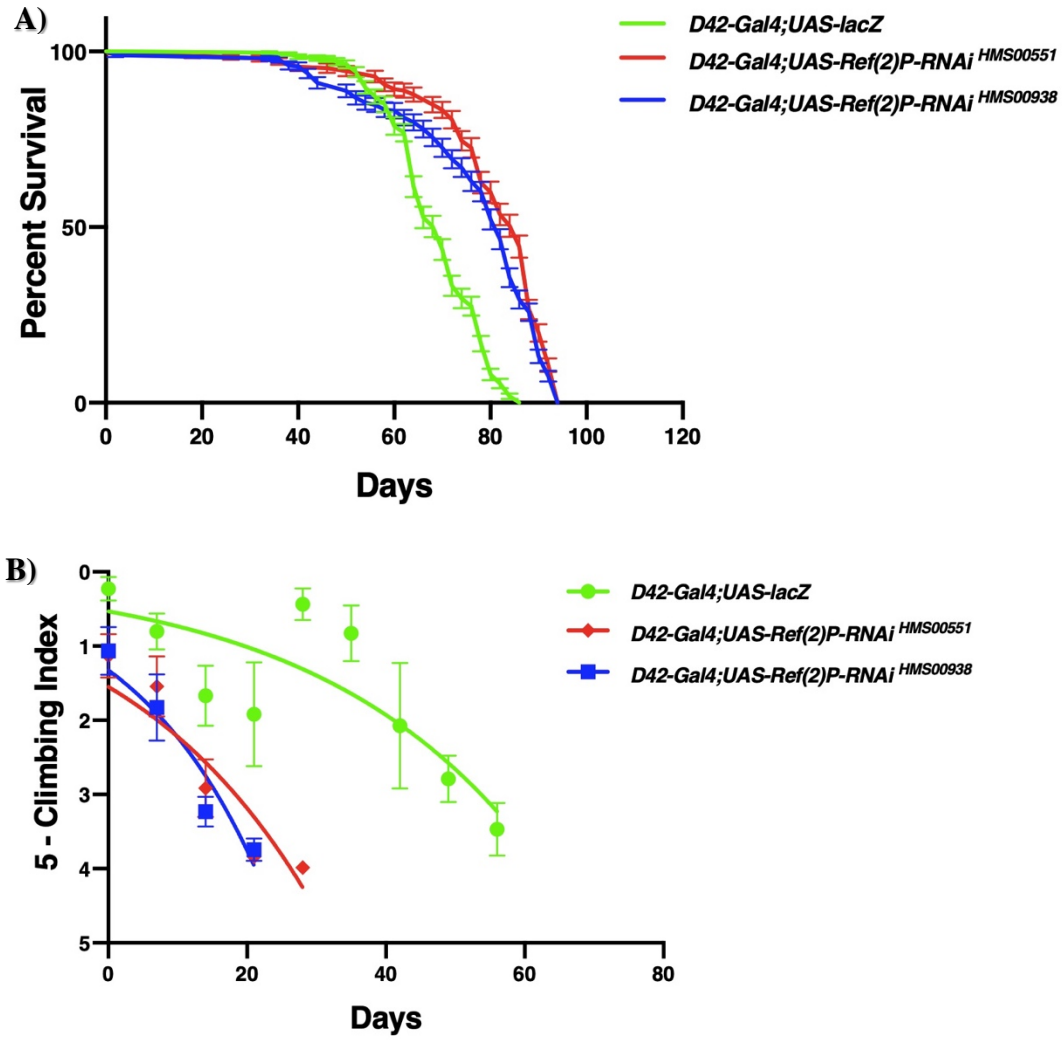

Figure 4.3: Altered expression of *Ref(2)P* directed through the *D42-Gal4* transgene affects longevity and climbing ability. **A:** Longevity assay of *Drosophila melanogaster* males displaying altered *Ref(2)P* expression in the motor neurons. Longevity is depicted by percent survival. Significance is $P < 0.05$ using the log-rank test with Bonferroni correction. Error bars represent standard error of the mean. Genotypes are as follows: *D42-Gal4;UAS-lacZ* (n=273), *D42-Gal4;UAS-Ref(2)P-RNAi*^{HMS00551} (n=252), *D42-Gal4;UAS-Ref(2)P-RNAi*^{HMS00938} (n=303). **B:** Locomotor assay of *D. melanogaster* males displaying altered *Ref(2)P* expression in the motor neurons. Climbing ability was determined by a nonlinear curve fit (CI=95%). Error bars indicate standard error of the mean.

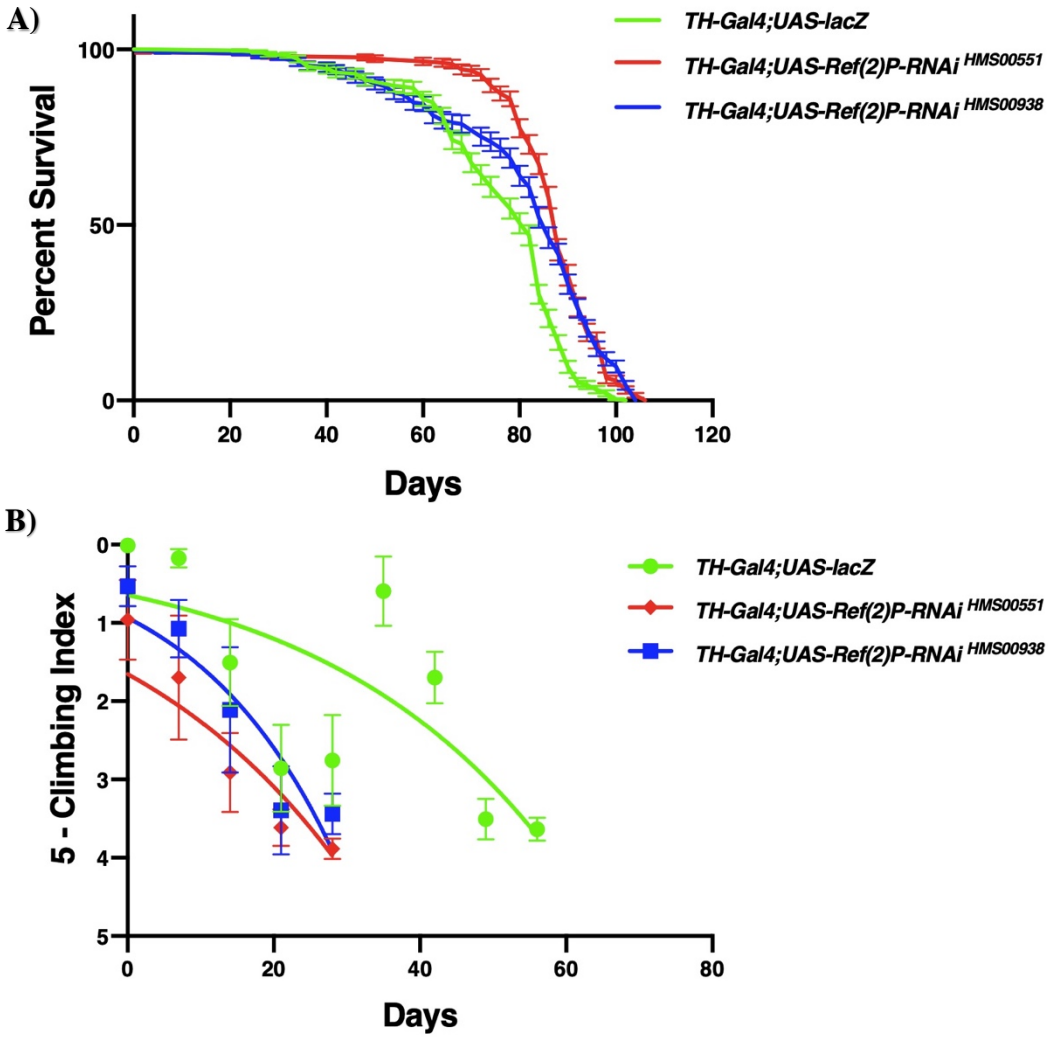

Figure 4.4: Altered expression of *Ref(2)P* through the *TH-Gal4* transgene affects longevity and climbing ability. **A:** Longevity assay of *Drosophila melanogaster* males displaying altered *Ref(2)P* expression in the dopaminergic neurons. Longevity is depicted by percent survival. Significance is $P < 0.05$ using the log-rank test with Bonferroni correction. Error bars represent standard error of the mean. Genotypes are as follows: *TH-Gal4;UAS-lacZ* (n=291), *TH-Gal4;UAS-Ref(2)P-RNAi*^{HMS00551} (n=263), *TH-Gal4;UAS-Ref(2)P-RNAi*^{HMS00938} (n=278). **B:** Locomotor assay of *D. melanogaster* males displaying altered *Ref(2)P* expression in the dopaminergic neurons. Climbing ability was determined by a nonlinear curve fit (CI=95%). Error bars indicate standard error of the mean.

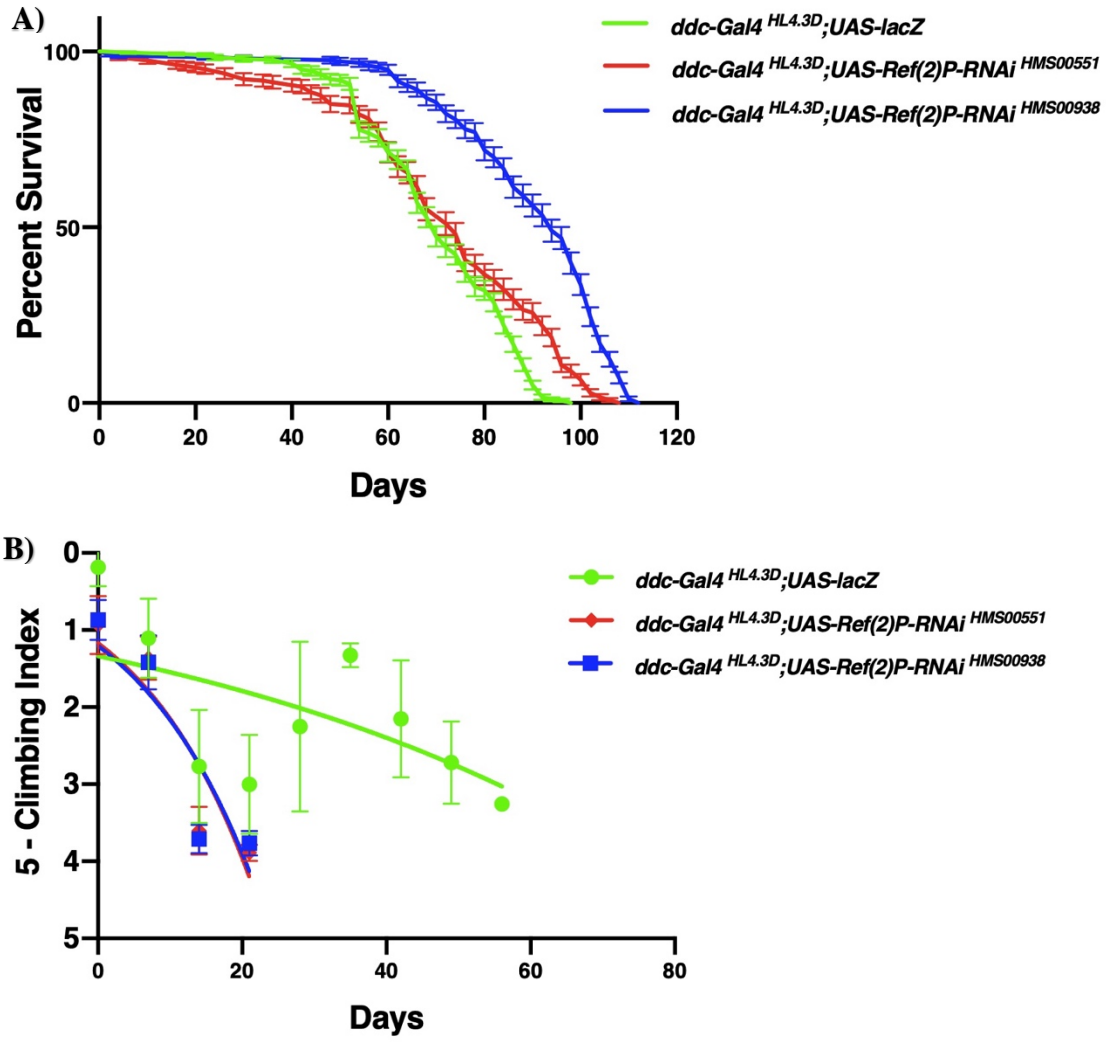

Figure 4.5: Altered expression of *Ref(2)P* directed through the *ddc-Gal4$^{HL4.3D}$* transgene affects longevity and climbing ability. **A:** Longevity assay of *Drosophila melanogaster* males displaying altered *Ref(2)P* expression in the motor neurons. Longevity is depicted by percent survival. Significance is $P < 0.05$ using the log-rank test with Bonferroni correction. Error bars represent standard error of the mean. Genotypes are as follows: *ddc-Gal4$^{HL4.3D}$;UAS-lacZ* (n=293), *ddc-Gal4$^{HL4.3D}$;UAS-Ref(2)P-RNAi^{HMS00551}* (n=241), *ddc-Gal4$^{HL4.3D}$;UAS-Ref(2)P-RNAi^{HMS00938}* (n=249). **B:** Locomotor assay of *D. melanogaster* males displaying altered *Ref(2)P* expression in the motor neurons. Climbing ability was determined by a nonlinear curve fit (CI=95%). Error bars indicate standard error of the mean.

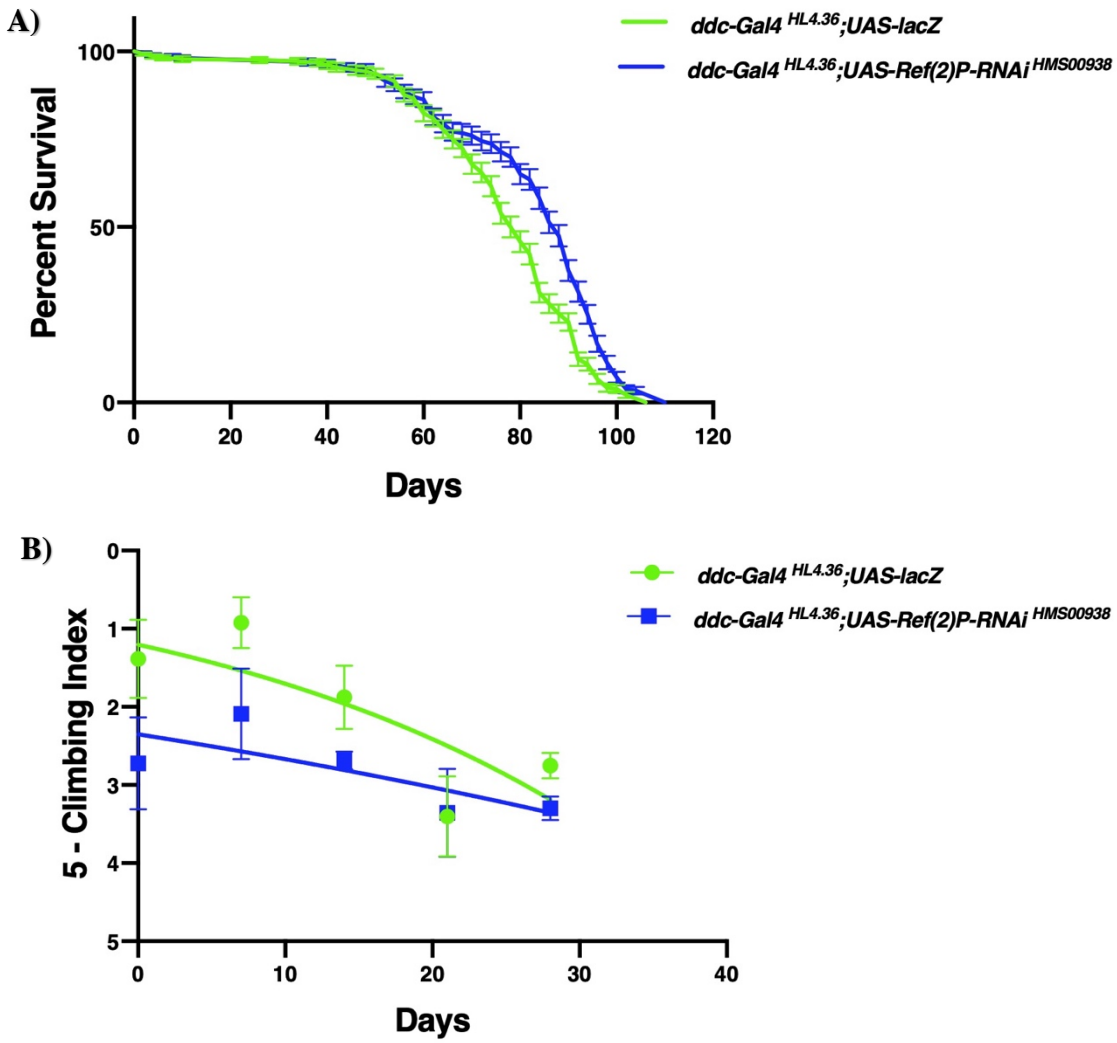

Figure 4.6: Altered expression of *Ref(2)P* directed through the *ddc-Gal4*$^{HL4.36}$ transgene affects longevity and climbing ability. **A:** Longevity assay of *Drosophila melanogaster* males displaying altered *Ref(2)P* expression in the motor neurons. Longevity is depicted by percent survival. Significance is $P < 0.05$ using the log-rank test with Bonferroni correction. Error bars represent standard error of the mean. Genotypes are as follows: *ddc-Gal4*$^{HL4.36}$*;UAS-lacZ* (n=284), *ddc-Gal4*$^{HL4.36}$*;UAS-Ref(2)P-RNAi*^{HMS00938} (n=263). **B:** Locomotor assay of *D. melanogaster* males displaying altered *Ref(2)P* expression in the motor neurons. Climbing ability was determined by a nonlinear curve fit (CI=95%). Error bars indicate standard error of the mean.

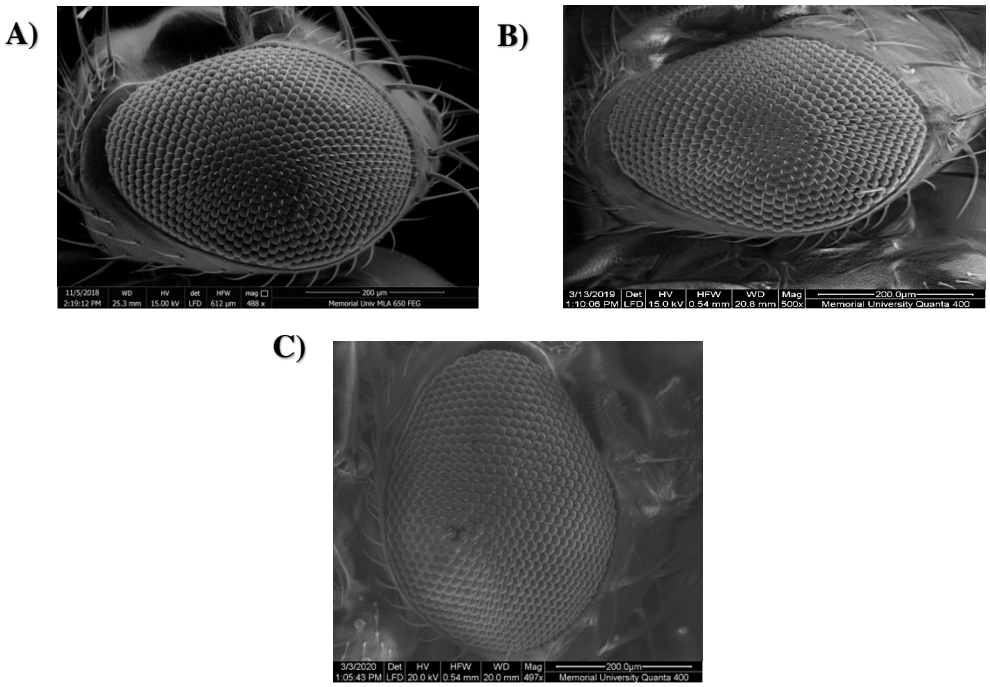

Figure 4.7: Directed *Ref(2)P* gene expression using eye-specific transgene *GMR-Gal4* in Drosophila. Scanning electron micrographs of A: *GMR-Gal4;UAS-lacZ*, B: *GMR-Gal4;UAS-Ref(2)P-RNAi*^{HMS00551}, C: *GMR-Gal4;UAS-Ref(2)P-RNAi*^{HMS00938}

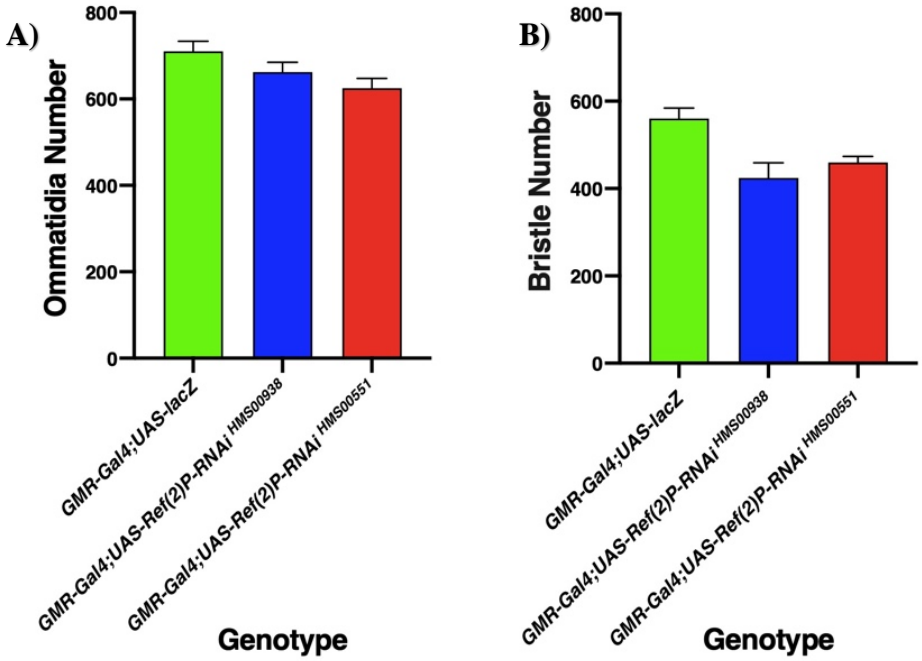

Figure 4.8: Biometric analysis of Drosophila compound eye under direct eye expression of *Ref(2)P* though the *GMR-Gal4* transgene. Inhibition of *Ref(2)P* causes significant decrease in (A) ommatidia number and (B) bristle number. Error bars represent standard deviation**.**

5.4 Discussion

At the very least, the progression and pathogenesis of many neurodegenerative diseases are influenced by an impairment of cellular quality control, through conserved processes such as autophagy and the ubiquitin-proteasome system (UPS). As human *p62/SQSTM1* encoded product is intimately involved in the autophagy process, with mutations known to be linked to neurodegenerative diseases (Bartolome et al., 2017; Narendra et al., 2010; Yamanda et al., 2019), investigation of this candidate gene may be quite informative. Altered expression of the Drosophila homologue, *Ref(2)P* has shown to affect median lifespan of the organism, locomotor function and neuronal development. Critical class flies, that express an inhibitory *Ref(2)P* transgene, exhibit a significant increase in aspects of longevity while displaying a significant reduction in motor function over time. Critical class males that cause inhibition of *Ref(2)P* in the developing compound eye displayed a significant reduction in ommatidia and bristle numbers. Such characteristic phenotypes are often due to an impairment in the development of the largely neuronally comprised Drosophila eye, and thus may suggest an impact on neurodevelopment. Unfortunately, a line that could readily lead to the overexpression of *Ref(2)P* was not readily available.

Inhibition of *Ref(2)P* expression through the motor neuron-specific transgene *D42-Gal4* has shown a significant increase in median lifespan while greatly reducing climbing ability throughout time. The increase in lifespan accompanied by a sharp decrease in motor function over time may be interpreted as trade-off, where the slight increase in longevity is a type of compensation for a severe decline in motor skills. This

significant reduction in motor ability and increase in lifespan when *Ref(2)P* e*xp*ression is altered corresponds with the characteristic loss of motor neurons associated with ALS, making the inhibition of *Ref(2)P* in the motor neuron an imperfect model of neurodegenerative disease. Similarly, expression of *Ref(2)P* inhibition though the direction of the dopaminergic neuron-specific transgene *TH-Gal4* has shown a significant, but minimal increase in median lifespan with a severe decline in climbing ability. Similar to the inhibition of *Ref(2)P* in the motor neurons, this increase in lifespan accompanied by a sharp decrease in motor function over time seen when *Ref(2)P* is inhibited in the dopaminergic neurons may also be interpreted as a type of compensation for a severe decline in motor skills. This significant reduction in motor ability and minimal increase in lifespan seen when *Ref(2)P* expression is altered, corresponds with the characteristic loss of dopaminergic neurons associated with Parkinson Disease, making the inhibition of *Ref(2)P* in the motor neuron an promising model of neurodegenerative disease.

The inhibition of *Ref(2)P* expression through the activities of the neuron-specific transgene *ddc-Gal4*$^{HL4.3D}$ significantly increased median lifespan, while displaying a severe decrease in climbing ability. Expressing *Ref(2)P* inhibition via *UAS-Ref(2)P-RNAi*^{HMS00938} through the neuron-specific transgene *ddc-Gal4*$^{HL4.36}$ has shown a significant increase in longevity, while significantly reducing climbing ability. This significant reduction in motor ability and increase in lifespan seen when *Ref(2)P* expression is altered may explained as compensational relationship, and seen as an imperfect model of neurodegenerative disease. Furthermore, the significant increase in

lifespan across all transgenes when *Ref(2)P* is inhibited suggests that it plays a substantial role in cell survival and death. As human *p62/SQSTM1* has critical functions throughout the cell, specifically in autophagy and mitophagy, impairment and dysfunction of *p62/SQSTM1* can be detrimental, with the loss-of-function of *p62/SQSTM1* leading to an increase in oxidative stress and mitochondria damage (Bitto et al., 2014). The importance of functional *p62/SQSTM1* in mitochondrial dynamics is evident in Drosophila as impairment of *Ref(2)P* reveals mitochondrial dysfunction, resulting in a decline in motor function (Pimenta De Castro et al., 2013). As the *p62/SQSTM1* gene is a crucial player in the development and progression of many neurodegenerative diseases, including ALS, FTD and PD, along with having multiple functions within various cell pathways, a further investigation of the *p62/SQSTM1* gene is desired.

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Chapter 6 - Modelling Human Neurodegenerative Disease Through Alteration of *VCP/TER94* in *Drosophila melanogaster*

6.1 Introduction

As neurodegenerative diseases are prevalent in society and influence the lives of many individuals, the molecular pathways and cellular processes that are involved in disease pathogenesis must be more fully understood. Many neurodegenerative diseases, such as ALS and PD, have a number of genes linked to their development and progression of these diseases. Aside from the well-characterized disease-causing genes, there are several genes linked to disease pathology. *Valosin-containing protein* (*VCP*), known as *TER94* in Drosophila, is an ALS-related gene which encodes the enzyme Valosin-Containing Protein, an essential AAA+ ATPase. In the cell, *VCP* is ubiquitously expressed in the endoplasmic reticulum, mitochondria and nucleus, with diverse functions in processes such as mitophagy, autophagy, UPS (Guo et al., 2016; Ludtmann et al., 2017; Nguyen, Thombre, & Wang, 2018), as well as in ER-associated protein degradation and DNA repair (Guo et al., 2016; Nguyen et al., 2018). During mitophagy, *VCP* is required for mitochondrial outer membrane protein turnover (Tanaka et al., 2010), and is a direct component in the PINK1/parkin-mediated process of mitophagy (Kim et al., 2013; Tanaka et al., 2010). In autophagy, *VCP* is heavily involved in the initiation phase and in the maturation of autophagosomes (Ju et al., 2009). An absence of *VCP* has been known to disturb both the aggregation of misfolded proteins, referred to as an aggreosome, along with the degradation of proteins (Ju et al., 2009). The human

VCP gene has interactions with two biochemical markers of autophagy, LC3 and p62/SQSTM1 (Y. Wang et al., 2011; Yeo & Yu, 2016), where mouse models expressing mutant *VCP* demonstrated an accumulation of both LC3 and p62/SQSTM1 (Yeo & Yu, 2016). The various functions and cellular processes which *VCP* is involved suggest that this may be an excellent candidate gene to study neurodegenerative disease.

Not only is the *VCP* gene associated with ALS, it is associated with many other diseases. *VCP* is known to be connected to early on-set Paget disease, FTD (Ludtmann et al., 2017; Mori et al., 2013), and more recently in PD (Mori et al., 2013). Through whole-exome sequencing, mutations in the *VCP* gene have been linked to patients with familial ALS (Johnson et al., 2011), with mutations in *VCP* accounting for approximately 1 to 2% of familial ALS cases, demonstrating that *VCP* mutations can result in impaired autophagy (Nguyen et al., 2018). Dominant pathogenic mutations of *VCP*, result in changes within the N-domain or within the ATPase domains, that severely reduce mitochondrial function. (T. Wang et al., 2016). Similar to human *VCP*, the protein product of *TER94* has associations with various select proteins in Drosophila, such as *Cabeza* (*Cas*), the Drosophila orthologue of the significant ALS gene *FUS*, where it functions as a modulator of motor neuron degeneration (Azuma et al., 2014). Consistent with *VCP* in humans, Drosophila *TER94* regulates the Notch signalling pathway, which is critical in tissue development and homeostasis. Impairment of Notch signaling has been known to lead to various diseases, particularly neurodegenerative diseases (Li, Liu, & Zhang, 2019). Furthermore, *TER94* interacts with Drosophila *clueless* (*clu*) through PINK1/Parkin-dependent mitophagy, where *clu* functions with *VCP* and *parkin* to

degrade and promote the clearance of dysfunctional mitochondria (T. Wang et al., 2016). As *VCP* has strong roles in the autophagy processes, particularly in the initiation stages, impairment in this gene along with its protein products, can have detrimental impacts on such pathways. Although previous work has been conducted on the role of *VCP* in degeneration, much is still unclear. The mechanisms by which mutations in *VCP* contribute to disease progression is an area of research that must be further investigated.

The *VCP* gene has been highly conserved throughout evolutionary history. A comparison of human and *D. melanogaster VCP* proteins reveals six highly conserved domains: 1) the CDC48 N-terminal sub-domain; 2) the CDC48 domain 2; 3) the AAA+ ATPase domain; 4) the ATPase AAA type core domain; 5) the AAA ATPase AAA+ lid domain; and 6) the Vps4 oligomerization C-terminal domain. The degree of evolutionary conservation suggests a highly conserved function for the product of the *VCP* gene. Thus exploration of the effects of altered *VCP* expression in *D. melanogaster* promises to provide insights into the pathology of ALS in humans. In this study, overexpression and inhibition of *VCP* in *D. melanogaster* are used to mimic human conditions, examining the effects on lifespan and locomotor ability.

6.2 Materials and Methods

6.2.1 *Drosophila melanogaster* stocks and culture

All Drosophila stocks were obtained from the Bloomington Drosophila Stock Center at Indiana University (IN, USA). See Table 4.1 for list of genotypes used. See Chapter 2, section 2.1 for detail of *D. melanogaster* stocks and culture.

6.2.2 Longevity Assay

The survival of *D. melanogaster* was analyzed to examine the median lifespan of experimental flies in comparison to control flies. See Chapter 2, section 2.2 for full longevity assay methods.

6.2.3 Locomotive Assay

The motor function of *D. melanogaster* was analyzed to examine the locomotor function over time of experimental flies in comparison to control flies. See Chapter 2, section 2.3 for full locomotive assay methods.

6.2.4 Scanning Electron Microscopy of the *Drosophila* eye

Eye analysis of *D. melanogaster* was used to determine the effects of gene manipulation. See Chapter 2, section 2.4 for full methods on eye experiments.

Table 4.1: Genotypes and location of expression patterns used in the analysis of altered expression of *TER94*.

Abbreviated Genotype	Location of Expression	Insertion Chromosome	Reference
Control Lines			
UAS-lacZ	---	2	Brand et al, 1993
Driver Lines			
GMR-Gal4	Eye	2	Freeman, 1996
arm-Gal4	Ubiquitous	2	Sanson et al., 1996
elav-Gal4	Pan neuronal	1	Lin & Goodman, 1994
TH-Gal4	Dopaminergic neuron	3	Inamdar et al., 2014
ddc-Gal4$^{HL4.3D}$	Dopaminergic and serotonergic neuron	2	Li et al., 2000
ddc-Gal4$^{HL4.36}$	Dopaminergic and serotonergic neuron	3	Li et al., 2000
D42-Gal4	Motor neuron	3	Parkes et al., 1998
Responder Lines			
UAS-TER94^{EY03486}	---	2	Bellen et al., 2004
UAS-TER94-RNAi^{GS00593}	---	2	Perkins et al., 2015
UAS-TER94-RNAi^{GL00448}	---	3	Perkins et al., 2015
UAS-TER94-RNAi^{JF03402}	---	3	Perkins et al., 2015
UAS-TER94-RNAi^{HMS00656}	---	3	Perkins et al., 2015

6.3 Results

6.3.1 Overexpression of *TER94* influences median lifespan and climbing ability dependent on the expression pattern of the transgene

An investigation of the extent that the overexpression of *TER94* influences the lifespan and climbing ability of *D. melanogaster* have shown that overexpression alters median lifespan and climbing depending on the expression of the transgene. When expressed through both the ubiquitous transgene *arm-Gal4* **(**Figure 5.1) and the pan-neuronal transgene *elav-Gal4* (Figure 5.2) overexpression of *TER94* significantly increases median lifespan when compared to the control (*UAS-lacZ*). When *TER94* is overexpressed through the motor neuron-specific transgene, *D42-Gal4*, median lifespan significantly increased (Figure 5.3a), while climbing ability was reduced over time (Figure 5.3b) when compared to the control (*UAS-lacZ*). When *TER94* is overexpressed through the dopaminergic neuron-specific transgene *TH-Gal4*, median lifespan was significantly reduced (Figure 5.4a), while climbing ability over time was not greatly changed (Figure 5.4b) when compared to the control (*UAS-lacZ*). When *TER94* is overexpressed through the neuron-specific transgene, $ddc\text{-}Gal4^{HL4.3D}$, median lifespan (Figure 5.5a) and climbing ability over time (Figure 5.5b) were significantly reduced when compared to the control (*UAS-lacZ*).

6.3.2 Inhibition of *TER94* decreases climbing ability but median lifespan is influenced dependent on the expression of the transgene

An investigation of the inhibition of *TER94* found that the lifespan and climbing ability of *D. melanogaster* was shown to be differentially altered. Four *TER94* inhibition

lines were selected to investigate the consequences of each on the fly. When expressed through the activity of *arm-Gal4* (Figure 5.1) and *elav-Gal4* (Figure 5.2), *TER94* inhibition via *UAS-TER94-RNAi*^{GS00593} significantly increased median lifespan when compared to the control (*UAS-lacZ*). When expressed through *arm-Gal4* and *elav-Gal4*, *TER94* inhibition through both, *UAS-TER94-RNAi*^{HMS00656} and *UAS-TER94-RNAi*^{GL00448}, significantly decreased median lifespan. Inhibition of *TER94* through *UAS-TER94-RNAi*^{JF03402} resulted in no critical class male progeny. When *TER94* was inhibited via *UAS-TER94-RNAi*^{GS00593} through the motor neuron-specific transgene *D42-Gal4*, median lifespan was not significantly altered (Figure 5.3a), however climbing ability over time was reduced (Figure 5.3b) when compared to the control (*UAS-lacZ*). When *TER94* was inhibited via both *UAS-TER94-RNAi*^{HMS00656} and *UAS-TER94-RNAi*^{JF03402} through *D42-Gal4* median lifespan was significantly increased, while *TER94* inhibition through *UAS-TER94-RNAi*^{GL00448} significantly reduced lifespan. Climbing ability was significantly reduced in these *TER94* inhibition lines (Figure 5.3b) when compared to the control (*UAS-lacZ*). When *TER94* was inhibited via *UAS-TER94-RNAi*^{GS00593} through the dopaminergic neuron-specific transgene *TH-Gal4*, median lifespan was not altered, while significantly reducing climbing ability. Inhibition of *TER94* via *UAS-TER94-RNAi*^{HMS00656} and *UAS-TER94-RNAi*^{GL00448} through *TH-Gal4* significantly reduced median lifespan (Figure 5.4a) and climbing ability over time (Figure 5.4b) when compared to the control (*UAS-lacZ*). Inhibition of *TER94* through *UAS-TER94-RNAi*^{JF03402} did not provide critical class male progeny when expressed through *TH-Gal4*. When *TER94* was inhibited via *UAS-TER94-RNAi*^{GS00593} and *UAS-TER94-RNAi*^{HMS00656} through the neuron-specific transgene *ddc-Gal4*$^{HL4.3D}$, median lifespan was significantly

increased (Figure 5.5a) while climbing ability was significantly reduced over time (Figure 5.5b) when compared to the control (*UAS-lacZ*). When *TER94* was inhibited via *UAS-TER94-RNAi*^{GL00448} through *ddc-Gal4*$^{HL4.3D}$ lifespan was not influenced (Figure 5.5a), however climbing ability was reduced (Figure 5.5b). The inhibition of *TER94* through *UAS-TER94-RNAi*^{JF03402} did not provide any critical class male progeny when expressed through *ddc-Gal4*$^{HL4.3D}$. Lastly, *TER94* inhibition via *UAS-TER94-RNAi*^{HMS00656} and *UAS-TER94-RNAi*^{GL00448} through the neuron-specific transgene *ddc-Gal4*$^{HL4.36}$, significantly increased median lifespan (Figure 5.6a) while climbing ability was significantly reduced over time (Figure 5.6b). Interestingly, when *TER94* was inhibited via *UAS-TER94-RNAi*^{JF03402} through *ddc-Gal4*$^{HL4.36}$, critical class male progeny were viable. However, median lifespan (Figure 5.6a) and climbing ability over time (Figure 5.6b) were significantly reduced when compared to the control (*UAS-lacZ*).

6.3.3 Overexpression and inhibition of *TER94* decrease ommatidia and bristle number

The developmental pattern of the Drosophila compound eye is a highly regulated and specific event with each eye being comprised of approximately 800 ommatidia under healthy development. Impairment in this process may result in characteristic phenotypes as a consequence, such as changes in the number of ommatidia and bristles. To investigate such phenotypic changes in *D. melanogaster*, the eye-specific transgene *GMR-Gal4* was used. Altered *TER94* expression underwent experimentation to investigate potential neurodevelopmental defects. Overexpression of *TER94* shown a significant decrease in ommatidia number (Figure 5.8a) and interommatidial bristle

number (Figure 5.8b). While all *TER94* inhibitory transgenes resulted in a significant decrease in bristle number when compared to the control (*UAS-lacZ*). Except for the *TER94* inhibition transgene *UAS-TER94-RNAi*^{GL00448}, all other loss-of-function genotypes result in a significant decrease in ommatidia number when compared to the control *UAS-lacZ* (Figure 5.8a).

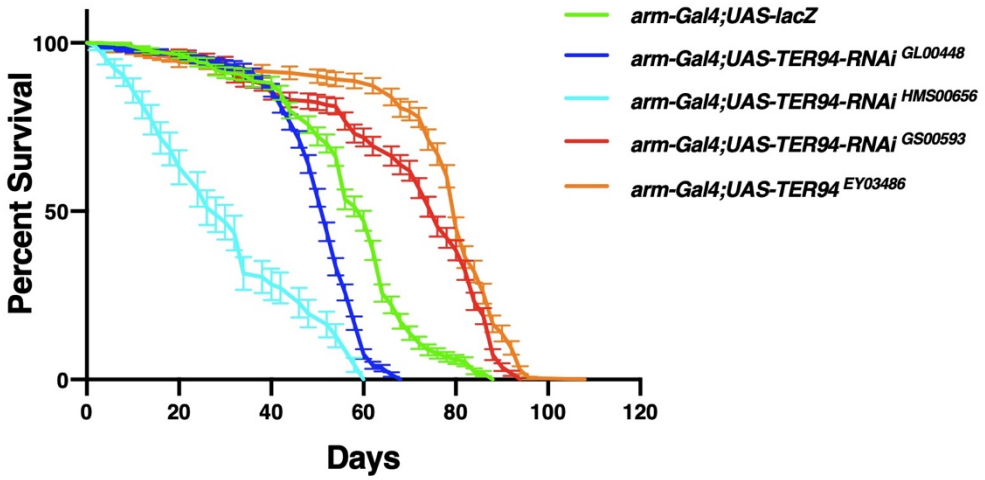

Figure 5.1: Altered expression of *TER94* directed through the *arm-Gal4* transgene affects lifespan. Longevity is depicted by percent survival. Significance is $P < 0.05$ using the log-rank test with Bonferroni correction. Error bars represent standard error of the mean. Genotypes are as follows: *arm-Gal4;UAS-lacZ* (n=284), *arm-Gal4;UAS-TER94-RNAi*^{GL00448} (n=328), *arm-Gal4;UAS-TER94-RNAi*^{HMS00656} (n=92), *arm-Gal4;UAS-TER94-RNAi*^{GS00593} (n=304), *arm-Gal4;UAS-TER94*^{EY03486} (n=212).

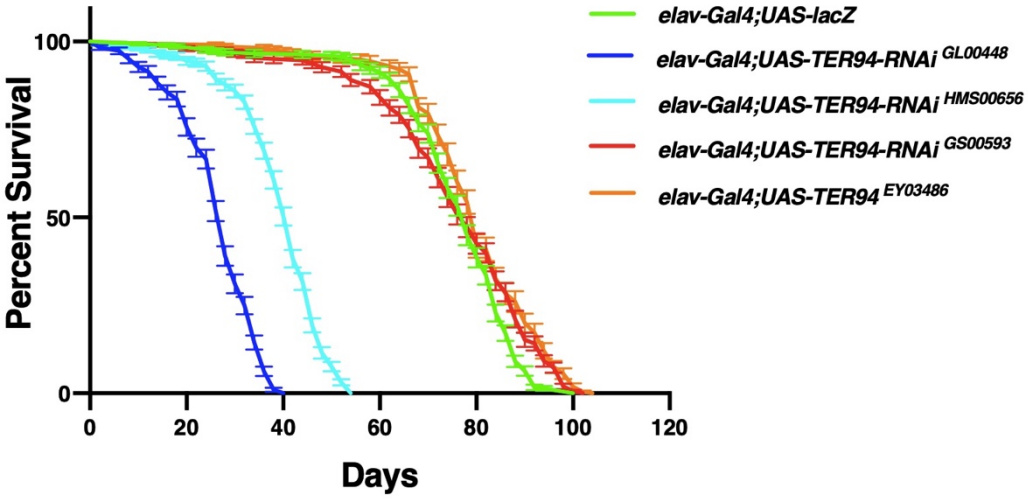

Figure 5.2: Altered expression of *TER94* directed through the *elav-Gal4* transgene affects lifespan. Longevity is depicted by percent survival. Significance is $P < 0.05$ using the log-rank test with Bonferroni correction. Error bars represent standard error of the mean. Genotypes are as follows: *elav-Gal4;UAS-lacZ* (n=298), *elav-Gal4;UAS-TER94-RNAi*^{GL00448} (n=305), *elav-Gal4;UAS-TER94-RNAi*^{HMS00656} (n=384), *elav-Gal4;UAS-TER94-RNAi*^{GS00593} (n=303), *elav-Gal4;UAS-TER94*^{EY03486} (n=215).

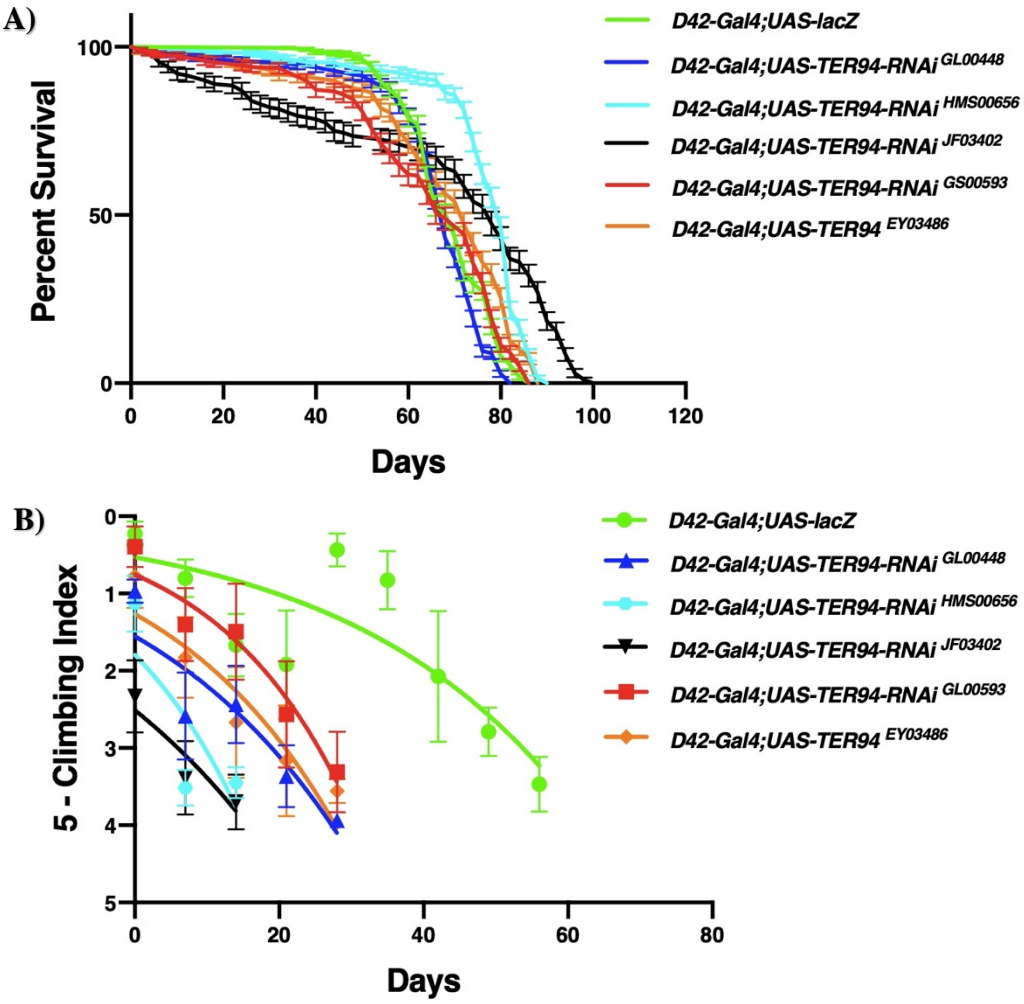

Figure 5.3: Altered expression of *TER94* directed through the *D42-Gal4* transgene affects longevity and climbing ability. **A:** Longevity assay of *Drosophila melanogaster* males displaying altered *TER94* expression in the motor neurons. Longevity is depicted by percent survival. Significance is $P < 0.05$ using the log-rank test with Bonferroni correction. Error bars represent standard error of the mean. Genotypes are as follows: *D42-Gal4;UAS-lacZ* (n=273), *D42-Gal4;UAS-TER94-RNAi*^{GL00448} (n=281), *D42-Gal4;UAS-TER94-RNAi*^{HMS00656} (n=272), *D42-Gal4;UAS-TER94-RNAi*^{JF03402} (n=200), *D42-Gal4;UAS-TER94-RNAi*^{GS00593} (n=219), *D42-Gal4;UAS-TER94*^{EY03486} (n=220). **B:** Locomotor assay of *D. melanogaster* males displaying altered *TER94* expression in the motor neurons. Climbing ability was determined by a nonlinear curve fit (CI=95%). Error bars indicate standard error of the mean

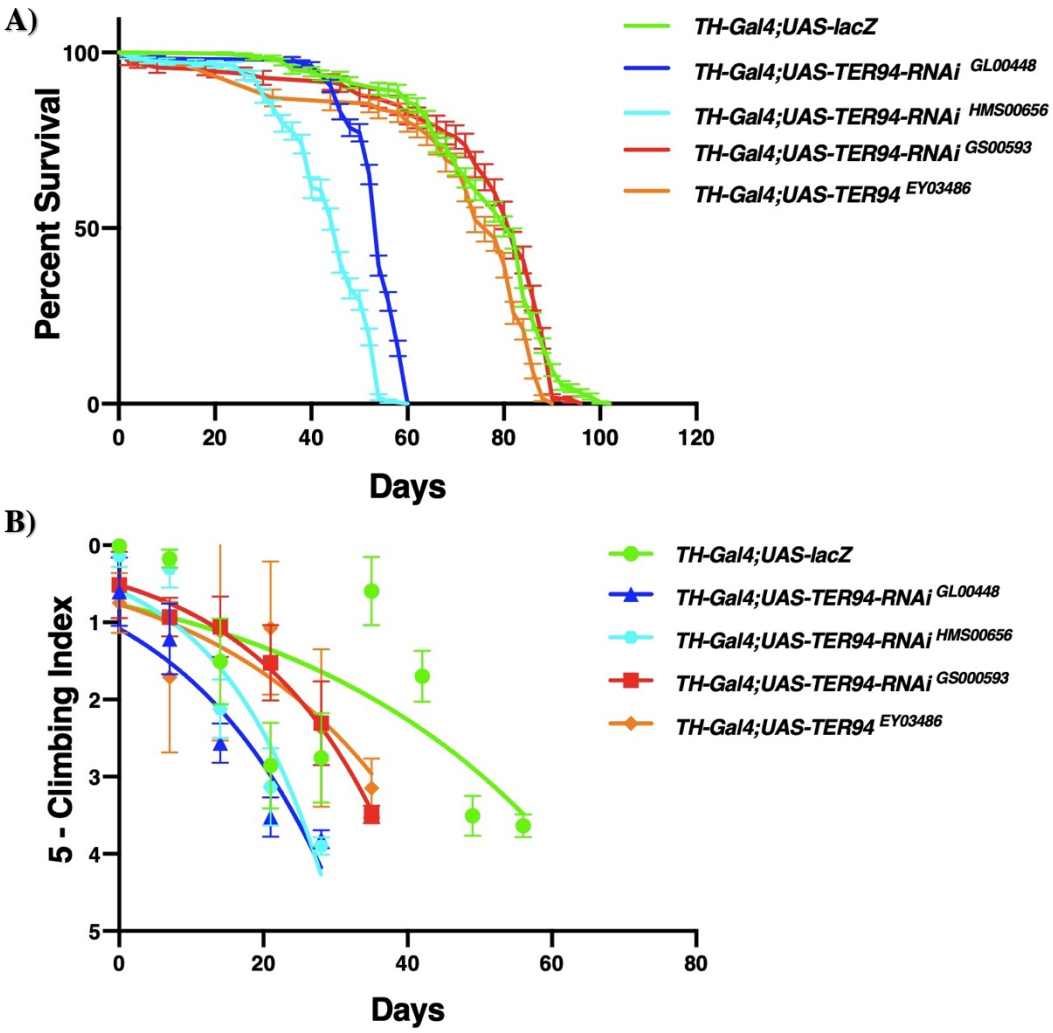

Figure 5.4: Altered expression of *TER94* directed through the *TH-Gal4* transgene affects longevity and climbing ability. **A:** Longevity assay of *Drosophila melanogaster* males displaying altered *TER94* expression in the dopaminergic neurons. Longevity is depicted by percent survival. Significance is $P < 0.05$ using the log-rank test with Bonferroni correction. Error bars represent standard error of the mean. Genotypes are as follows: *TH-Gal4;UAS-lacZ* (n=291), *TH-Gal4;UAS-TER94-RNAi*^{GL00448} (n=285), *TH-Gal4;UAS-TER94-RNAi*^{HMS00656} (n=268), *TH-Gal4;UAS-TER94-RNAi*^{GS00593} (n=166), *TH-Gal4;UAS-TER94*^{EY03486} (n=193). **B:** Locomotor assay of *D. melanogaster* males displaying altered *TER94* expression in the dopaminergic neurons. Climbing ability was determined by a nonlinear curve fit (CI=95%). Error bars indicate standard error of the mean.

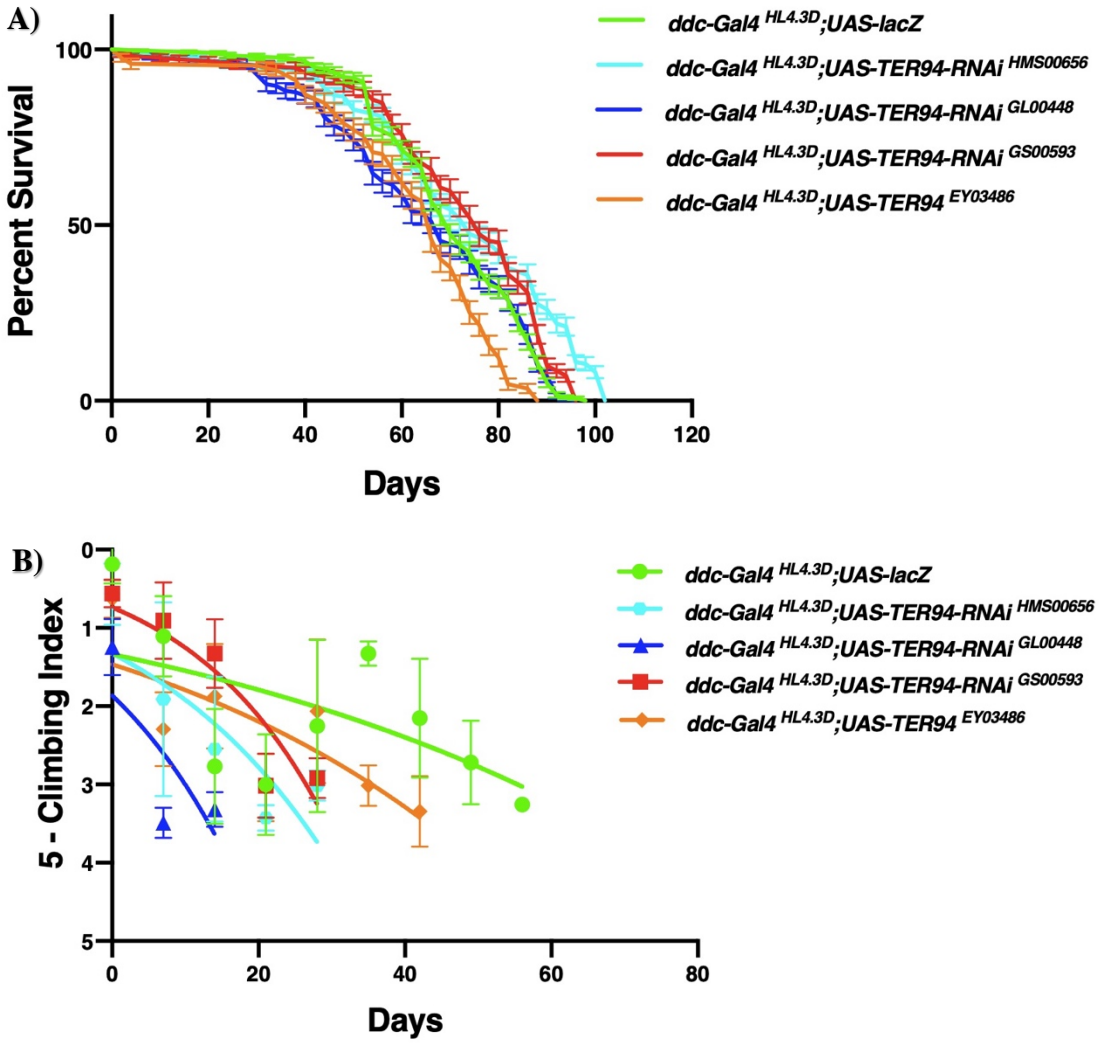

Figure 5.5: Altered expression of *TER94* directed through the *ddc-Gal4$^{HL4.3D}$* transgene affects longevity and climbing ability. **A:** Longevity assay of *Drosophila melanogaster* males displaying altered *TER94* expression in the neurons. Longevity is depicted by percent survival. Significance is $P < 0.05$ using the log-rank test with Bonferroni correction. Error bars represent standard error of the mean. Genotypes are as follows: *ddc-Gal4$^{HL4.3D}$;UAS-lacZ* (n=293), *ddc-Gal4$^{HL4.3D}$;UAS-TER94-RNAi^{GL00448}* (n=213), *ddc-Gal4$^{HL4.3D}$;UAS-TER94-RNAi^{HMS00656}* (n=246), *ddc-Gal4$^{HL4.3D}$;UAS-TER94-RNAi^{GS00593}* (n=211), *ddc-Gal4$^{HL4.3D}$;UAS-TER94^{EY03486}* (n=171). **B:** Locomotor assay of *D. melanogaster* males displaying altered *TER94* expression in the neurons. Climbing ability was determined by a nonlinear curve fit (CI=95%). Error bars indicate standard error of the mean.

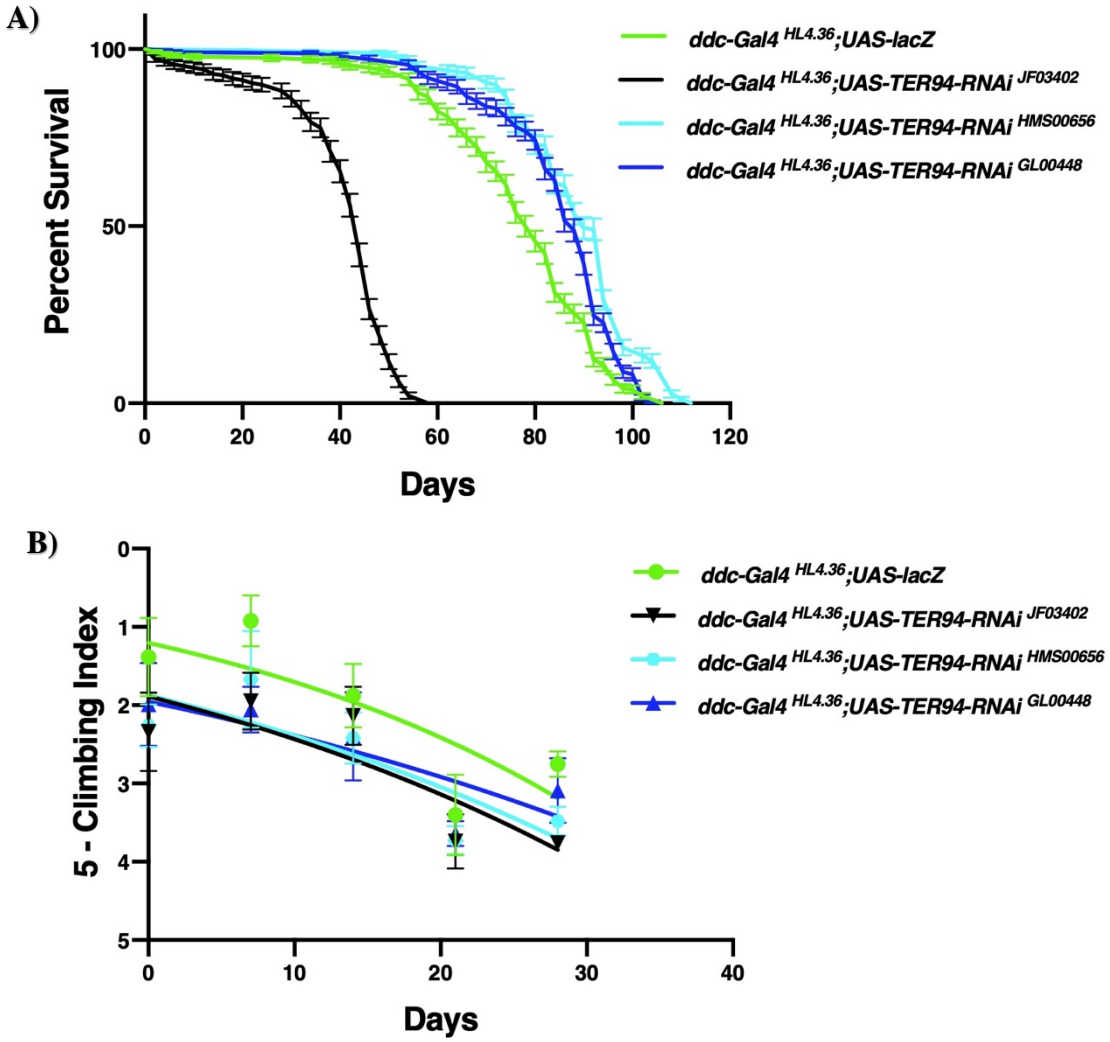

Figure 5.6: Altered expression of *TER94* directed through the *ddc-Gal4*$^{HL4.36}$ transgene affects longevity and climbing ability. **A:** Longevity assay of *Drosophila melanogaster* males displaying altered *TER94* expression in the neurons. Longevity is depicted by percent survival. Significance is $P < 0.05$ using the log-rank test with Bonferroni correction. Error bars represent standard error of the mean. Genotypes are as follows: *ddc-Gal4*$^{HL4.36}$*;UAS-lacZ* (n=284), *ddc-Gal4*$^{HL4.36}$*;UAS-TER94-RNAi*^{JF03042} (n=229), *ddc-Gal4*$^{HL4.36}$*;UAS-TER94-RNAi*^{HMS00656} (n=246), *ddc-Gal4*$^{HL4.36}$*;UAS-TER94-RNAi*^{GL00448} (n=213). **B:** Locomotor assay of *D. melanogaster* males displaying altered *TER94* expression in the neurons. Climbing ability was determined by a nonlinear curve fit (CI=95%). Error bars indicate standard error of the mean.

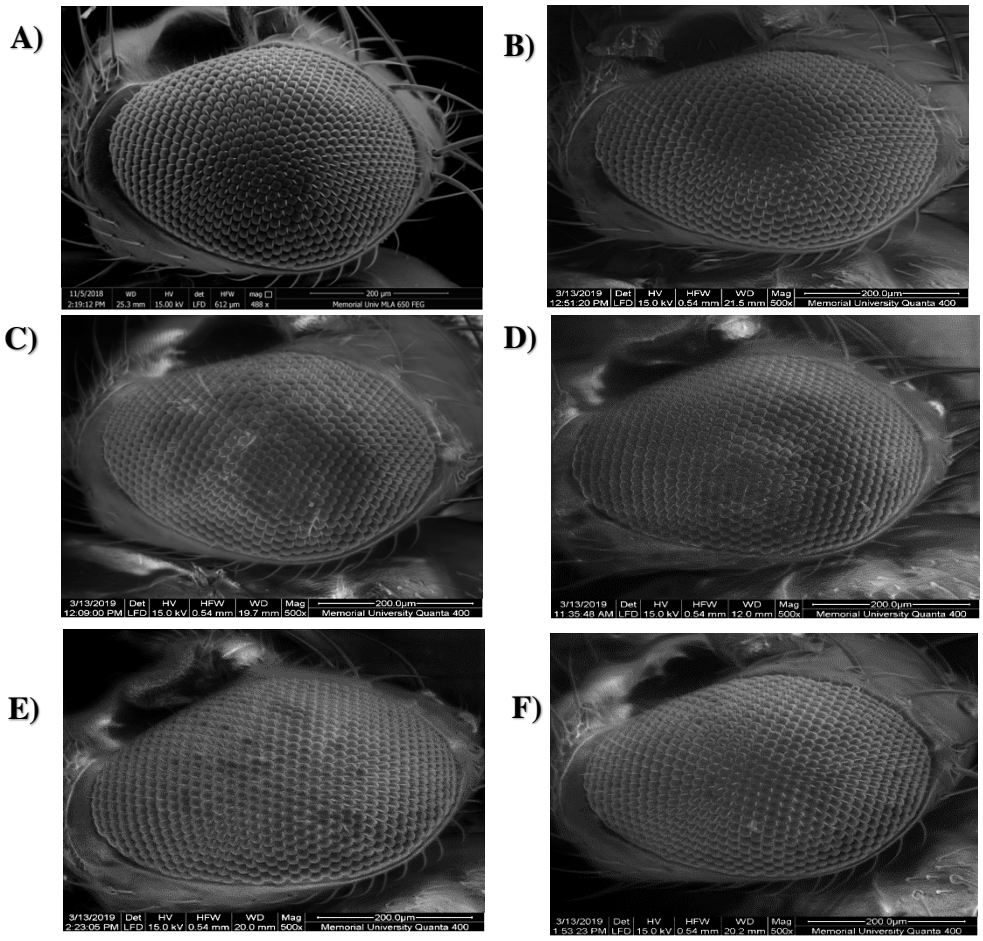

Figure 5.7: Directed *TER94* gene expression using the eye-specific transgene *GMR-Gal4* in Drosophila. Scanning electron micrographs of A: *GMR-Gal4;UAS-lacZ*, B: *GMR-Gal4;UAS-TER94*^{EY03486}, C: *GMR-Gal4;UAS-TER94-RNAi*^{JF03402}, D: *GMR-Gal4;UAS-TER94-RNAi*^{HMS00656}, E: *GMR-Gal4;UAS-TER94-RNAi*^{GL00448}, F: *GMR-Gal4;UAS-TER94-RNAi*^{GS00593}

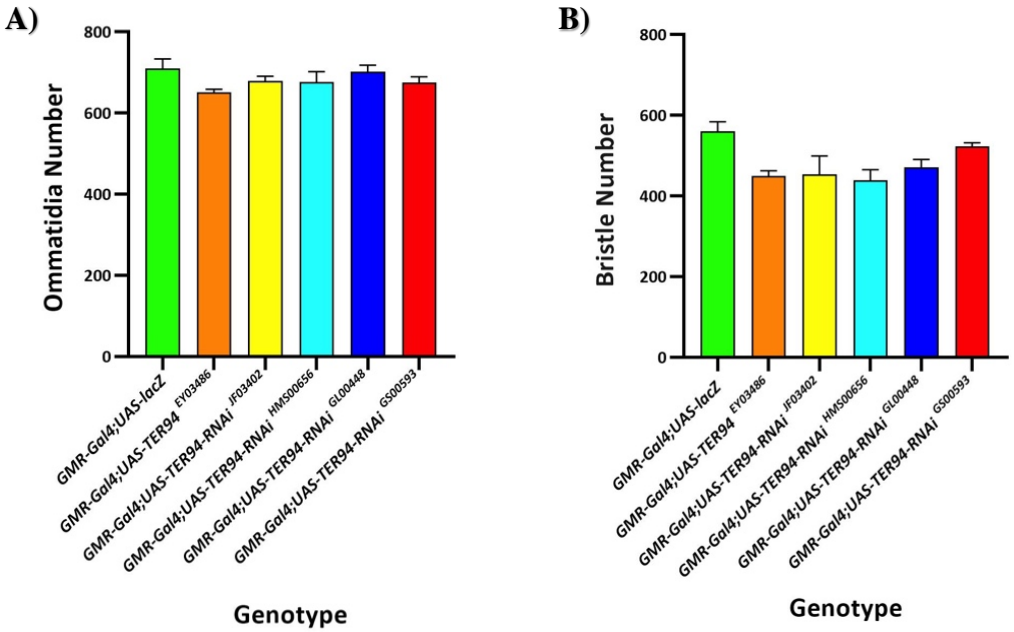

Figure 5.8: Biometric analysis of Drosophila compound eye under direct eye expression of T*ER94* though the *GMR-Gal4* transgene. Overexpression of *TER94* causes significant decrease in (A) ommatidia number. Both overexpression and inhibition of *TER94* cause a significant decrease in (B) bristle number. Error bars represent standard deviation.

6.4 Discussion

In order to gain a fuller knowledge on the pathogenies of many neurodegenerative diseases, it is essential to examine not only the effects of major disease-causing genes, but the effects of less prominent disease-causing genes. Altered expression of *TER94* influenced Drosophila longevity, locomotor function and neuronal development, dependent upon the investigated pattern of expression. When *TER94* was overexpressed through the ubiquitous transgene *arm-Gal4*, and the pan-neuronal transgene *elav-Gal4*, critical class males displayed a significant increase in median lifespan. Overexpression of *TER94* though the motor neuron-specific transgene *D42-Gal4* resulted in a slight increase in median lifespan. Whereas the overexpression of *TER94* through the dopaminergic neuron-specific transgene *TH-Gal4* and through the neuron-specific transgene *ddc-Gal4*$^{HL4.3D}$ decreased median lifespan. Overexpression of *TER94* reduced climbing ability when expressed through *D42-Gal4* and *ddc-Gal4*$^{HL4.3D}$. The slight increase in median lifespan accompanied by the decline in motor skills over time when *TER94* is overexpressed through the motor neuron-specific transgene *D42-Gal4* seems to generate an imperfect model of neurodegenerative disease. As ALS is a motor neuron disease characterized by the loss of motor neurons, a decline in both longevity and motor function would appear to mimic the suggested pathology of ALS. However, the minimal increase in lifespan seen when *TER94* is overexpressed in the motor neuron may suggest a delicate balance between longevity and motor function, where the slight increase in longevity is a reaction to the severe decline in motor skills. Critical class males overexpressing *TER94* in the developing compound eye displayed a significant reduction

in ommatidia and bristle numbers. Such characteristic phenotypes are often due to an impairment in the development of the Drosophila eye, and thus may suggest a substantial role in neurodevelopment.

Critical class males expressing transgenes that lead to the inhibition of *TER94* provided many inconclusive results. Expression of *TER94* inhibition through *UAS-TER94-RNAi*^{GS00593} displayed a significant increase in lifespan through the ubiquitous transgene *arm-Gal4* and the neuron-specific transgene *ddc-Gal4*$^{HL4.3D}$. While when expressed through the pan-neuronal transgene *elav-Gal4*, the motor neuron-specific transgene *D42-Gal4,* and the dopaminergic neuron-specific transgene *TH-Gal4*, lifespan was not changed. Inhibition of *TER94* through expression of *UAS-TER94-RNAi*^{HMS00656} and *UAS-TER94-RNAi*^{GL00448} produced varied results. When expressed through the transgenes *arm-Gal4*, *elav-Gal4*, and *TH-Gal4*, both *TER94* inhibitory transgenes reduced median lifespan. While when expressed through *ddc-Gal4*$^{HL4.36}$, both *TER94* inhibitory transgenes increased median lifespan. Inhibition of *TER94* via *UAS-TER94-RNAi*^{GL00448} through *D42-Gal4* and *ddc-Gal4*$^{HL4.3D}$ displayed a reduction in median lifespan, while *TER94* inhibition through *UAS-TER94-RNAi*^{HMS00656} displayed a significant increase in median lifespan through these transgenes. Climbing ability was significantly reduced in all cases of *TER94* inhibition across all transgenes investigated. From the varied results of this experiment several successful models of human neurodegeneration can be found. The reduction in lifespan and motor ability seen when *TER94* is inhibited through *TH-Gal4* corresponds with the characteristic loss of dopaminergic neurons associated with PD—making the inhibition of *TER94* in the dopaminergic neurons a promising model of

neurodegenerative disease. While the reduction in median lifespan and motor function seen when *TER94* is inhibited through *D42-Gal4* corresponds with the characteristic loss of motor neurons associated with ALS – making the inhibition of *TER94* in the motor neurons a promising model of neurodegenerative disease.

Interestingly, *TER94* inhibition through *UAS-TER94-RNAi*^{JF03402} provided critical class males when expressed through the transgenes *D42-Gal4* and *ddc-Gal4*$^{HL4.36}$ but not when expressed through other transgenes investigated. When expressed through *D42-Gal4*, this *TER94* inhibitory transgene produced an increase in median lifespan, while the climbing ability over time was reduced in a significant way. When expressed through *ddc-Gal4*$^{HL4.36}$, both median lifespan and climbing ability over the reduced life of the critical class flies were significantly reduced. As this particular *TER94-RNAi* transgene was not viable when expressed under the control of other *Gal4* transgenes, and therefore, in other subsets of tissues, this may suggest that *TER94* has a significant role in governing cell survival and viability in some tissues. As the human VCP protein is known to function in many cellular processes including autophagy, mitophagy and UPS (Guo et al., 2016; Ludtmann et al., 2017; Nguyen, Thombre, & Wang, 2018), it is difficult to interpret the results of this study. Furthermore, as the *VCP* gene is a crucial component in various diseases, such as ALS and PD (Mori et al., 2013), a further investigation into the exact mechanisms of *TER94* is required to understand its role fully. However, it can be said that *TER94* is a strong participant in the development of some neurodegenerative diseases, such as ALS and PD.

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Chapter 7 – Investigation of The Consequences of Combined Altered ALS And PD Gene Expression Activities in *Drosophila melanogaster*

7.1 Introduction

The neurodegenerative diseases Amyotrophic Lateral Sclerosis and Parkinson Disease are two movement disorders with substantial defects in proteostasis (Bosco, LaVoie, Petsko, & Ringe, 2011). Similarities between ALS and PD include the histopathological hallmarks of each disease, which typically are Lewy bodies in PD and Bunina bodies in ALS (Bosco et al., 2011; Yang & Choi, 2013), along with that current estimation that approximately 10% of cases have a known familial basis, with the majority thought to be sporadic in origin (Bosco et al., 2011). Other commonalities shared between ALS and PD are found within the cellular processes and pathways which govern disease progression, such as mitophagy. Specifically, it is the PINK1-parkin mitophagy pathway that is known to interact to at least some extent with major and minor ALS-related genes. The major PD protein *alpha-synuclein* is known to have connections to multiple ALS-associated genes. Through further investigation of these potential connections, it may reveal links and gain a better understanding of the two diseases based on the cellular processes and pathways that contribute to disease progression.

With regards to the major ALS gene *TARDBP*, its protein product, TDP-43 is reported to target long intron-containing pre-mRNA of *parkin* in humans (Sun et al., 2018). Spinal cord samples taken from the autopsies of patients diagnosed with the sporadic form of ALS have shown that neurons containing TDP-43 protein inclusions

display reduced levels of the parkin protein (Sun et al., 2018). Studies have demonstrated impaired regulation of both *PINK1* and *parkin* by the loss-of-function of TDP-43, to suggest that this mis-regulation may result in TDP-43-dependent proteinopathy (Lagier-Tourenne et al., 2012; Sun et al., 2018). Specifically, the overexpression of TDP-43 in Drosophila has been shown to result in a decrease of *parkin* levels, which alters the turnover of *PINK1* to cause an increase of the fraction of PINK1 protein that has had the amino terminal mitochondrial-localization peptide cleaved away in the cytosol (Sun et al., 2018). The TDP-43 protein interacts with the alpha-synuclein protein, as they are known to coexist within Lewy bodies. The overexpression of TDP-43 in mice results in a moderate loss of cortical neurons (Tian et al., 2011), however, the combination of TDP-43 overexpression and the presence of mutant forms of alpha-synuclein can lead to dopaminergic neurodegeneration. The loss of dopaminergic neurons from this combination is more severe than the consequences of increased expression of TDP-43, to suggest that these two proteins can play a cooperative role in neurodegeneration (Tian et al., 2011). The connections and roles that abnormal TDP-43, *PINK1, parkin* and alpha-synuclein have on the cell may be indicators of a causal link between aspects of ALS and of PD.

The ALS gene *TBK1* is known to interact with PINK1-parkin pathway as this mechanism promotes *TBK1* activation (Heo, Ordureau, Paulo, Rinehart, & Harper, 2015). Once *TBK1* is activated, it then promotes the phosphorylation of its autophagy adaptors, p62/SQSTM1, OPTN and NDP52, which associate with autophagy ATG8 proteins through an LC3 interacting region motif (Heo et al., 2015). Among other proteins,

p62/SQSTM1 is recruited to the mitochondria by parkin and participates in the aggregation of damaged mitochondria. However, it has been suggested that p62/SQSTM1 may only have a role in the progression of aggregation and not in the process of mitophagy directly (Bitto et al., 2014; Narendra, Kane, Hauser, Fearnley, & Youle, 2010). Although this may be the case, *p62/SQSTM1* is an excellent candidate gene to study due to an involvement in cellular homeostasis.

Not only does the *TBK1* gene interact with the PINK1-parkin pathway, its protein product interacts with the alpha-synuclein protein, where it has been demonstrated that alpha-synuclein fibrils in microglial cells have roles in the induction of autophagy by recruiting both TBK1 and OPTN to damaged sites in the microglial cell (Bussi et al., 2018). The genetic interaction that TBK1 and OPTN have is of great interest to study, as to date, there is no obvious Drosophila homologue of the *OPTN* gene, and such experiments have to be delayed until, and if, a gene with similar duties is identified in Drosophila. Despite this, there are functional homologues of another major autophagy adaptor, p62/SQSTM1 which can be explored. Regarding known connections between p62/SQSTM1 and *alpha-synuclein*, it is known that alpha-synuclein protein inclusions can act as ideal targets for p62-dependent autophagy and that a p62/SQSTM1-deficiency enhances *alpha-synuclein* pathology (Tanji et al., 2015; Watanabe et al., 2012). As both TBK1 and p62/SQSTM1 appear to be highly influenced by PD gene activities, it would be beneficial to explore the extents of these connections.

The interactions that the *VCP* gene product has with the PINK1-parkin pathway may provide a great deal of insight, as this cooperation seems to function to directly identify damaged mitochondria for degradation (Ashrafi, Schlehe, LaVoie, & Schwarz, 2014; Kim et al., 2013; Tanaka et al., 2010). Studies have been conducted on wild-type *VCP* and its interactions with this pathway, and with *VCP* mutants, which demonstrated that loss of *VCP* functions impair the PINK1-Parkin pathway (Kim et al., 2013). The Drosophila homologue of *VCP*, *TER94* interacts with the *clueless* (*clu*) through PINK1/Parkin-dependent mitophagy, whereas *clu* functions with *VCP* and *parkin* to degrade and promote the clearance of dysfunctional mitochondria (T. Wang et al., 2016). As VCP has an active involvement with the process of PINK1-parkin mitophagy, and is a known ALS-related gene, it would be beneficial to study this ALS-associated gene in terms of a potential relationship to PD.

7.2 Materials and Methods

7.2.1 *Drosophila melanogaster* stocks and culture

All Drosophila stocks were obtained from the Bloomington Drosophila Stock Center at Indiana University (IN, USA), with the exception of the *Gal4* lines *w;ddc-Gal4$^{HL4.3D}$/CyO;UAS-parkin-RNAi/TM3* (*ddc-Gal4$^{HL4.3D}$;UAS-parkin-RNAi*) and *w;ddc-Gal4$^{HL4.36}$/Tm3 iso1;UAS-alpha-synuclein/CyO* (*ddc-Gal4$^{HL4.36}$;UAS-alpha-synucelin*) which were created in the Staveley Laboratory, Memorial University of Newfoundland

(St. John's, Canada). See Table 5.1 for list of genotypes used. See, Chapter 2, section 2.1 for detail of *D. melanogaster* stocks and culture.

7.2.2 Longevity Assay

The survival of *D. melanogaster* was analyzed to examine the median lifespan of experimental flies in comparison to control flies. See Chapter 2, section 2.2 for full longevity assay methods.

7.2.3 Locomotive Assay

The motor function of *D. melanogaster* was analyzed to examine the locomotor function over time of experimental flies in comparison to control flies. See Chapter 2, section 2.3 for full locomotive assay methods.

Table 5.1: Genotypes and location of expression patterns used in the analysis of altered expression of ALS and PD gene activity.

Abbreviated Genotype	Location of Expression	Insertion Chromosome	Reference
Control Lines			
UAS-lacZ	---	2	Brand et al, 1993
Recombinant Driver Lines			
ddc-Gal4$^{HL4.3D}$;UAS-parkin-RNAi	Neuron	2	Staveley, Unpublished
ddc-Gal4$^{HL4.36}$;UAS-alpha-synuclein	Neuron	3	Staveley, Unpublished
Responder Lines			
UAS-TBPH-RNAi^{HMS01932}	---	2	Perkins et al., 2015
UAS-IK2^{EY09774}	---	2	Bellen et al., 2004
UAS-Ref(2)P-RNAi^{HMS00938}	---	2	Perkins et al., 2015
UAS-TER94-RNAi^{GL00448}	---	3	Perkins et al., 2015
UAS-TER94-RNAi^{JF03402}	---	3	Perkins et al., 2015
UAS-TER94-RNAi^{HMS00656}	---	3	Perkins et al., 2015

7.3 Results

7.3.3 Inhibition of *TBPH* and the expression of *alpha-synuclein* significantly increases median lifespan but does not alter climbing ability

When expressed through the neuron-specific transgene *ddc-Gal4*$^{HL4.36}$, located on insertion chromosome 3, *TBPH* inhibition through *UAS-TBPH-RNAi*^{HMS01932} did not show significant changes in median lifespan or climbing ability over time when compared to the control *UAS-lacZ* [see Chapter 3, section 3.3.1] and seen in [Figure 2.6]. However, the expression of *alpha-synuclein*, in addition to this, significantly reduced median lifespan (Figure 6.1a), while leaving climbing ability not significantly challenged (Figure 6.1b)

7.3.4 Inhibition of *TBPH* and *parkin* significantly increases lifespan but does not alter lifetime climbing ability

When expressed through the neuron-specific transgene *ddc-Gal4*$^{HL4.3D}$, located on insertion chromosome 2, *TBPH* inhibition via *UAS-TBPH-RNAi*^{HMS01932} showed a significant reduction in lifespan and climbing ability when compared to the control *UAS-lacZ* [see Chapter 3, section 3.3.1; Figure 2.5]. The inhibition of *parkin*, in addition to the above, results in a significant reduction in median lifespan (Figure 6.2a) and climbing ability over time (Figure 6.2b). However, this reduction seems to be far greater than without the inhibition of *parkin*.

7.3.4 Inhibition of *Ref(2)P* and the expression of *alpha-synuclein* increases lifespan and reduces climbing ability

When expressed through the neuron-specific transgene *ddc-Gal4*$^{HL4.36}$, located on insertion chromosome 3, *Ref(2)P* inhibition through *UAS-Ref(2)P-RNAi*^{HMS00938} significantly increased lifespan, while significantly decreasing climbing ability when compared to the control *UAS-lacZ* [see Chapter 5, section 5.3.1; Figure 4.6]. The expression of *alpha-synuclein*, in addition to this, lead to a significant increase in median lifespan (Figure 6.3a), and decrease in climbing ability (Figure 6.3b).

7.3.5 Inhibition of *Ref(2)P* and *parkin* increases lifespan and reduces climbing ability

When expressed through the neuron-specific transgene *ddc-Gal4*$^{HL4.3D}$, located on insertion chromosome 2, *Ref(2)P* inhibition via *UAS-Ref(2)P-RNAi*^{HMS00938} showed a significant increase in lifespan and decreased climbing ability when compared to the control *UAS-lacZ* [see Chapter 5, section 5.3.1; Figure 4.5]. The inhibition of *parkin*, in addition to this, also results in a significantly increased lifespan (Figure 6.2a), and reduced climbing ability (Figure 6.2b), however, this increase in lifespan is not as significant than without the inhibition of *parkin*.

7.3.6 Inhibition of *TER94* influences both lifespan and climbing ability when expressed with *alpha-synuclein* or *parkin*

When expressed through the neuron-specific transgene *ddc-Gal4*$^{HL4.36}$, located on insertion chromosome 3, *TER94* inhibition via *UAS-TER94-RNAi*^{HMS00656} and *UAS-*

TER94-RNAi^{GL00448} significantly increased median lifespan. In contrast, *TER94* inhibition though *UAS-TER94-RNAi*^{JF03402} significantly decreased lifespan when compared to the control *UAS-lacZ* [see Chapter 6, section 6.3.2; Figure 5.6]. All inhibition lines significantly reduce climbing ability. The expression of *alpha-synuclein*, in addition to this, results in a significant decrease in median lifespan when *TER94* inhibition was through *UAS-TER94-RNAi*^{JF03402} and *UAS-TER94-RNAi*^{GL00448}. A significant increase in lifespan is seen with *TER94* inhibition through *UAS-TER94-RNAi*^{HMS00656} (by ~28%) (Figure 6.5a). All inhibition lines result in a significant decrease in climbing ability (Figure 6.5b).

7.3.7 Inhibition of *TER94* influences both lifespan and climbing ability when expressed with *alpha-synuclein* or *parkin*

When expressed through the neuron-specific transgene *ddc-Gal4*$^{HL4.3D}$, located on insertion chromosome 2, *TER94* inhibition via *UAS-TER94-RNAi*^{GL00448} did not significantly impact lifespan but significantly decreased climbing ability compared to the control *UAS-lacZ* [see Chapter 6, section 6.3.2; Figure 5.5]. When *parkin* is inhibited, in addition to this, median lifespan was significantly reduced by (~30%) (Figure 6.6a), while also significantly reducing climbing ability (Figure 6.6b).

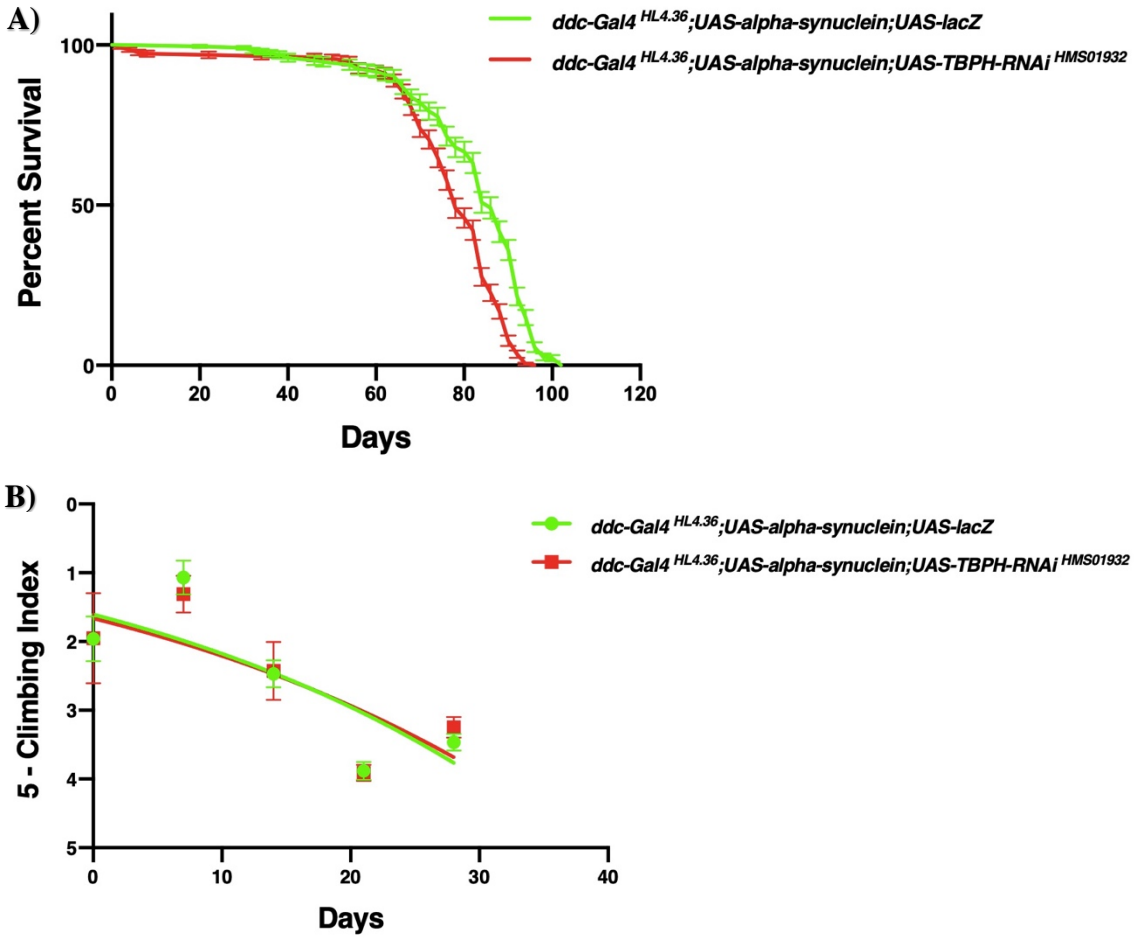

Figure 6.1: Altered expression of *TBPH* and the expression of *alpha-synuclein* directed through the *ddc-Gal4*$^{HL4.36}$ transgene does not affects longevity and climbing ability. **A:** Longevity assay of *Drosophila melanogaster* males displaying the expression of *alpha-synuclein* and the altered *TBPH* expression in the neurons. Longevity is depicted by percent survival. Significance is $P < 0.05$ using the log-rank test with Bonferroni correction. Error bars represent standard error of the mean. Genotypes are as follows: *ddc-Gal4*$^{HL4.36}$*;UAS-alpha-synuclein;UAS-lacZ* (n=228), *ddc-Gal4*$^{HL4.36}$*;UAS-alpha-synuclein;UAS-TBPH-RNAi*^{HMS01932} (n=261). **B:** Locomotor assay of *D. melanogaster* males displaying the expression of *alpha-synuclein* and the altered *TBPH* expression in the neurons. Climbing ability was determined by a nonlinear curve fit (CI=95%). Error bars indicate standard error of the mean.

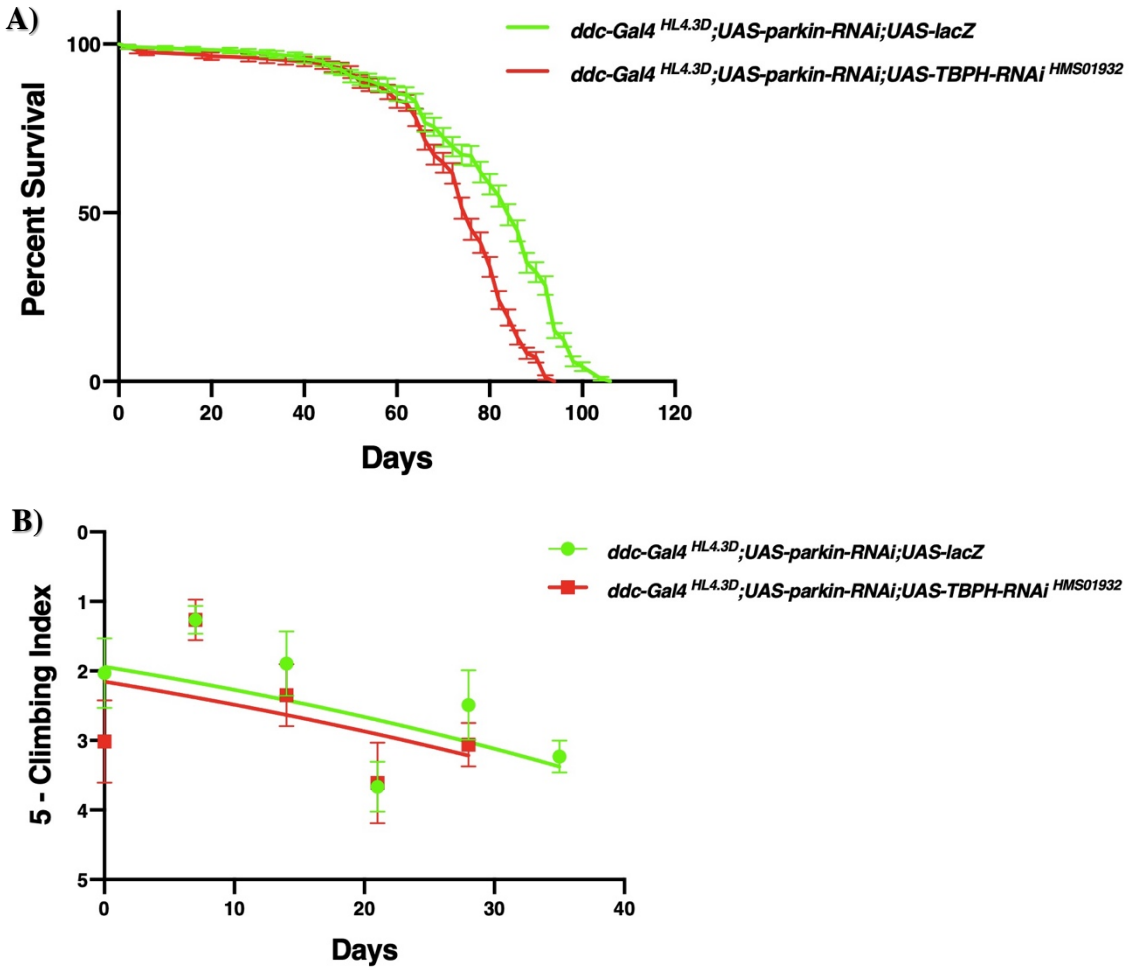

Figure 6.2 : Altered expression of *TBPH* and *parkin* directed through the *ddc-Gal4$^{HL4.3D}$* transgene does not affects longevity and climbing ability. **A:** Longevity assay of *Drosophila melanogaster* males displaying altered *TBPH* and *parkin* expression in the neurons. Longevity is depicted by percent survival. Significance is $P < 0.05$ using the log-rank test with Bonferroni correction. Error bars represent standard error of the mean. Genotypes are as follows: *ddc-Gal4$^{HL4.3D}$;UAS-parkin-RNAi;UAS-lacZ* (n=253), *ddc-Gal4$^{HL4.3D}$;UAS-alpha-synuclein;UAS-TBPH-RNAi^{HMS01932}* (n=253). **B:** Locomotor assay of *D. melanogaster* males altered *TBPH* and *parkin* expression in the neurons. Climbing ability was determined by a nonlinear curve fit (CI=95%). Error bars indicate standard error of the mean.

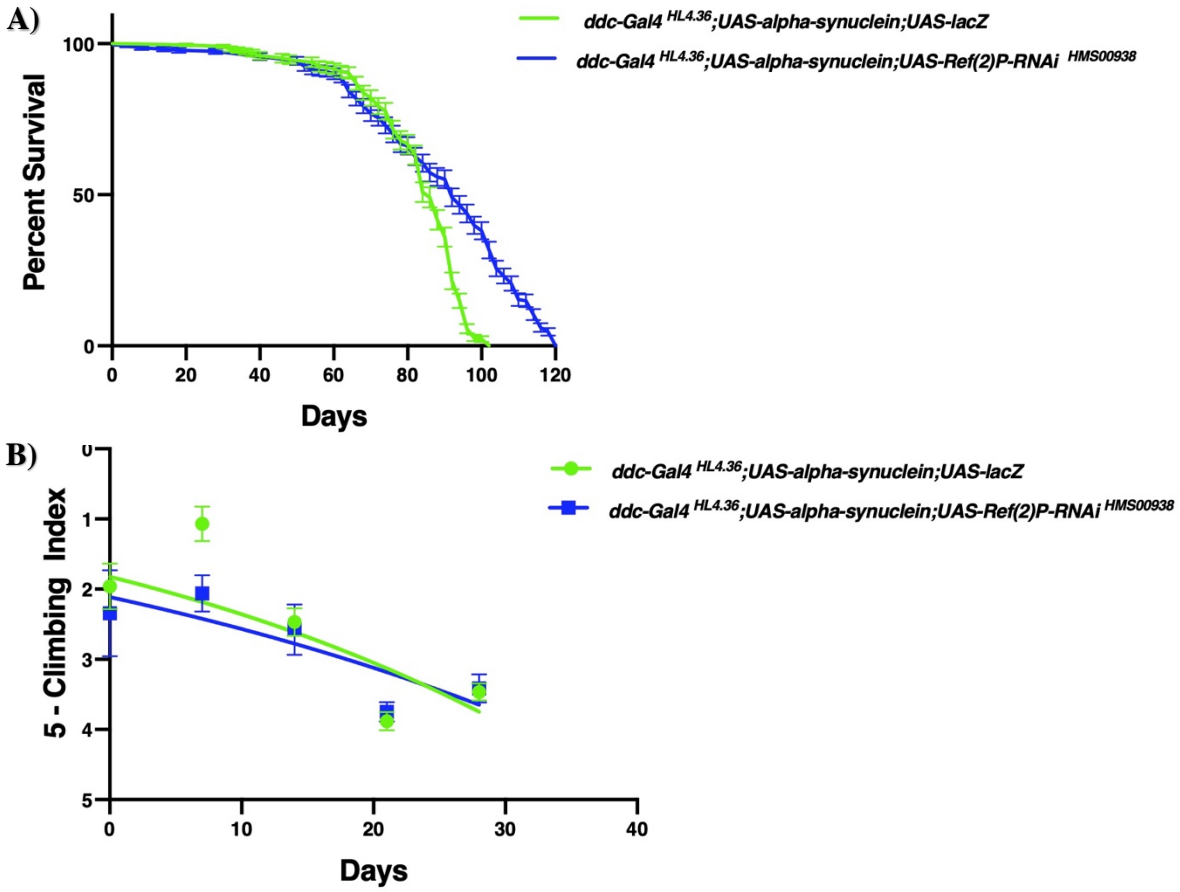

Figure 6.3 : Altered expression of *Ref(2)P* and the expression of *alpha-synuclein* directed through the *ddc-Gal4*$^{HL4.36}$ transgene affects longevity and climbing ability. **A:** Longevity assay of *Drosophila melanogaster* males displaying the expression of *alpha-synuclein* and the altered *Ref(2)P* expression in the neurons. Longevity is depicted by percent survival. Significance is $P < 0.05$ using the log-rank test with Bonferroni correction. Error bars represent standard error of the mean. Genotypes are as follows: *ddc-Gal4*$^{HL4.36}$*;UAS-alpha-synuclein;UAS-lacZ* (n=228), *ddc-Gal4*$^{HL4.36}$*;UAS-alpha-synuclein;UAS-Ref(2)P-RNAi*^{HMS00938} (n=281). **B:** Locomotor assay of *D. melanogaster* males displaying the expression of *alpha-synuclein* and the altered *Ref(2)P* expression in the neurons. Climbing ability was determined by a nonlinear curve fit (CI=95%). Error bars indicate standard error of the mean.

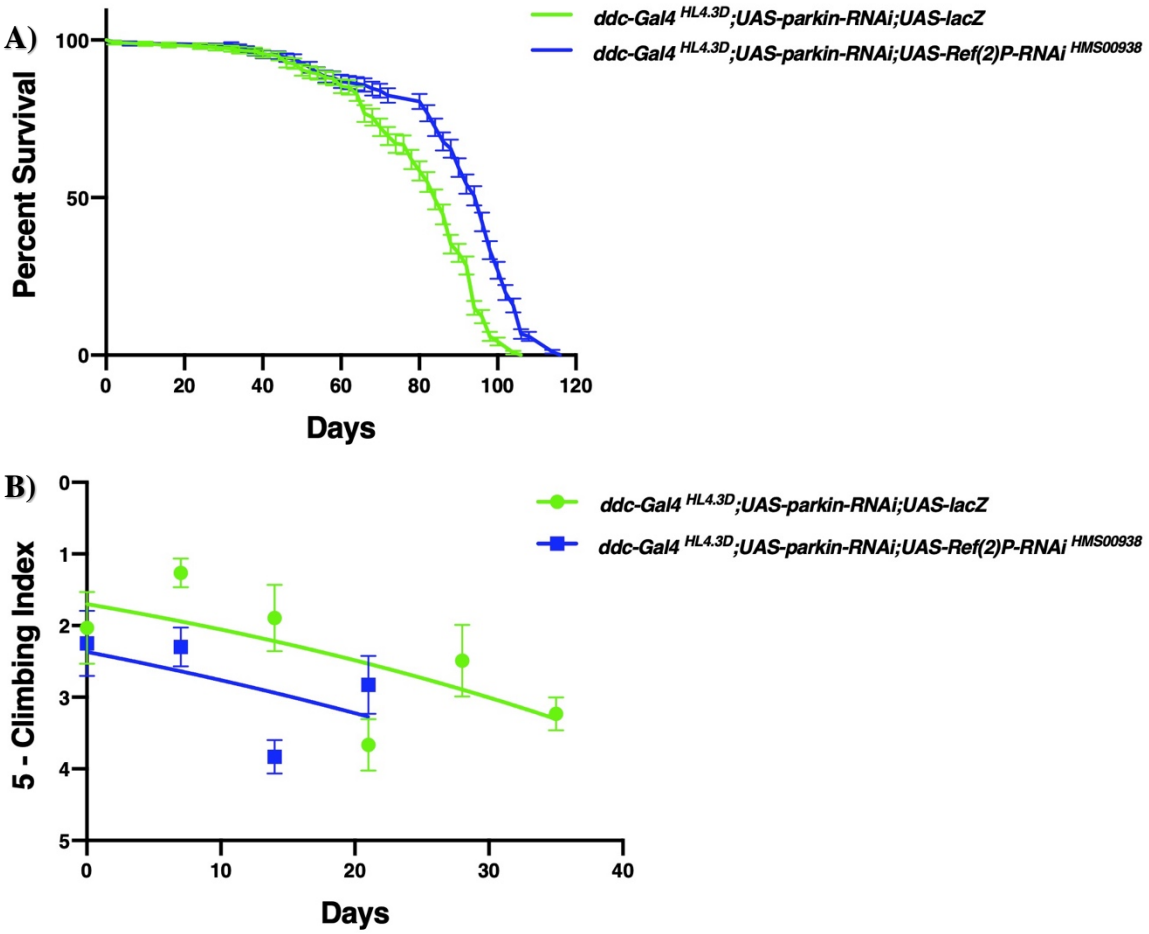

Figure 6.4: Altered expression of *Ref(2)P* and *parkin* directed through the *ddc-Gal4*$^{HL4.3D}$ transgene affects longevity and climbing ability. **A:** Longevity assay of *Drosophila melanogaster* males displaying altered *Ref(2)P* and *parkin* expression in the neurons. Longevity is depicted by percent survival. Significance is $P < 0.05$ using the log-rank test with Bonferroni correction. Error bars represent standard error of the mean. Genotypes are as follows: *ddc-Gal4*$^{HL4.3D}$*;UAS-parkin-RNAi;UAS-lacZ* (n=253), *ddc-Gal4*$^{HL4.3D}$*;UAS-parkin-RNAi;UAS-Ref(2)P-RNAi*^{HMS00938} (n=267). **B:** Locomotor assay of *D. melanogaster* males altered *Ref(2)P* and *parkin* expression in the neurons. Climbing ability was determined by a nonlinear curve fit (CI=95%). Error bars indicate standard error of the mean.

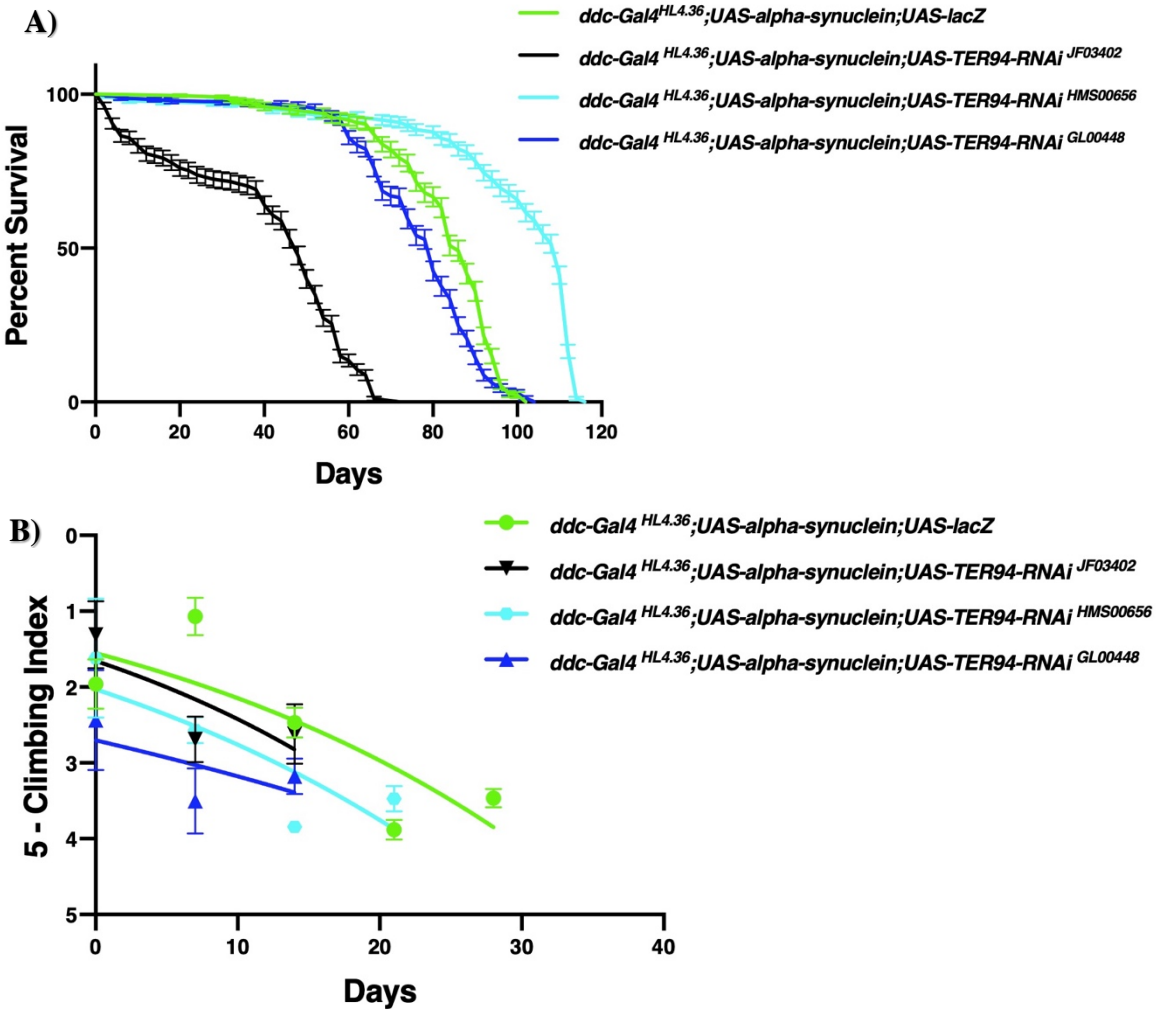

Figure 6.5: Altered expression of *TER94* and the expression of *alpha-synuclein* directed through the *ddc-Gal4*$^{HL4.36}$ transgene affects longevity and climbing ability. **A:** Longevity assay of *Drosophila melanogaster* males displaying the expression of *alpha-synuclein* and the altered *TER94* expression in the neurons. Longevity is depicted by percent survival. Significance is $P < 0.05$ using the log-rank test with Bonferroni correction. Error bars represent standard error of the mean. Genotypes are as follows: *ddc-Gal4*$^{HL4.36}$*;UAS-alpha-synuclein;UAS-lacZ* (n=228), *ddc-Gal4*$^{HL4.36}$*;UAS-alpha-synuclein;UAS-TER94-RNAi*^{JF03402} (n=275), *ddc-Gal4*$^{HL4.36}$*;UAS-alpha-synuclein;UAS-TER94-RNAi*^{HMS00656} (n=286), *ddc-Gal4*$^{HL4.36}$*;UAS-alpha-synuclein;UAS-TER94-RNAi*^{GL00448} (n=242). **B:** Locomotor assay of *D. melanogaster* males displaying the expression of *alpha-synuclein* and the altered *TER94* expression in the neurons. Climbing ability was determined by a nonlinear curve fit (CI=95%). Error bars indicate standard error of the mean.

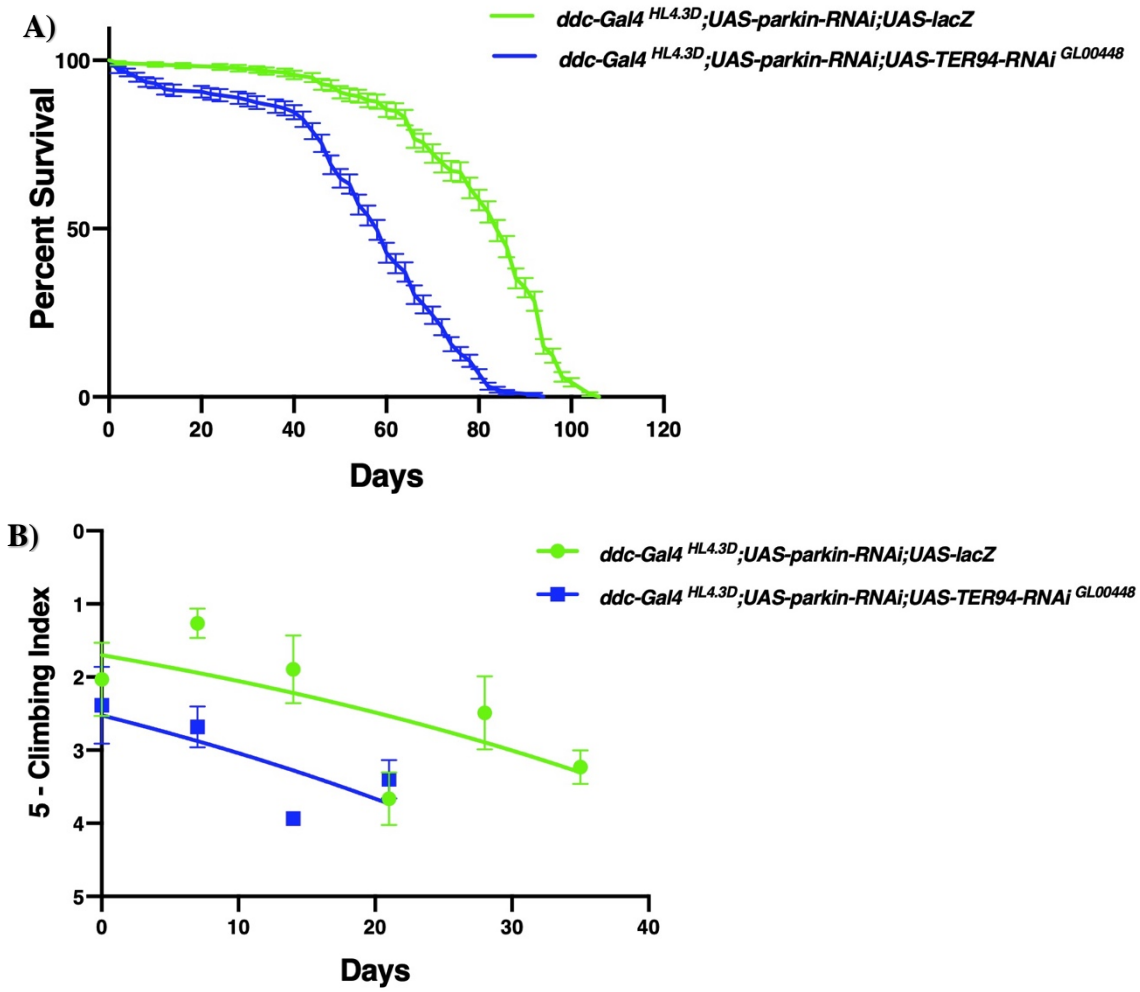

Figure 6.6: Altered expression of *TER94* and *parkin* directed through the *ddc-Gal4*$^{HL4.3D}$ transgene affects longevity and climbing ability. **A:** Longevity assay of *Drosophila melanogaster* males displaying altered *TER94* and *parkin* expression in the neurons. Longevity is depicted by percent survival. Significance is $P < 0.05$ using the log-rank test with Bonferroni correction. Error bars represent standard error of the mean. Genotypes are as follows: *ddc-Gal4*$^{HL4.3D}$*;UAS-parkin-RNAi;UAS-lacZ* (n=253), *ddc-Gal4*$^{HL4.3D}$*;UAS-parkin-RNAi;UAS-TER94-RNAi*^{GL00448} (n=280). **B:** Locomotor assay of *D. melanogaster* males altered *TER94* and *parkin* expression in the neurons. Climbing ability was determined by a nonlinear curve fit (CI=95%). Error bars indicate standard error of the mean.

7.4 Discussion

D. melanogaster was used as a model to examine the consequences of the altered combined of ALS-related and PD-related gene expression. To explore this interaction several longevity and locomotor function throughout lifespan experiments were conducted. When directed through the neuron-specific transgene *ddc-Gal4*$^{HL4.36}$, inhibition of *TBPH* via the *UAS-TBPH-RNAi*^{HMS01932} transgene did not influence median lifespan or climbing ability [see Chapter 3, section 3.3.1]. However, when *alpha-synuclein* is expressed in addition to the inhibition of *TBPH*, the median lifespan was significantly increased with no great changes in climbing ability. The large increase in lifespan that is obtained when *alpha-synuclein* is expressed in the *ddc-Gal4*-expressing neural tissues suggests that the interaction between *TBPH* and *alpha-synuclein* is important.

Furthermore, the TDP-43 protein is known to interact with the parkin protein of the PINK1/parkin pathway. When expressed through the neuron-specific transgene *ddc-Gal4*$^{HL4.3D}$ the inhibition of the Drosophila homologue, *TBPH* through *UAS-TBPH-RNAi*^{HMS01932} reduced both lifespan and climbing ability [see Chapter 3, section 3.3.1]. When *parkin* was inhibited in addition to this, lifespan and climbing ability were also reduced. However, the reduction in lifespan, as well as climbing ability, displayed when both *TBPH* and *parkin* were inhibited was more severe than the inhibition of *TBPH* without altering *parkin*, to suggest that the interaction the two proteins share is essential to cell survival. As human *TDP-43* is known to reduce *parkin* levels in the neuron and impair the regulation of both *parkin* and *PINK1* (Sun et al., 2018), the results of this

experiment seem to support this finding, with the influence upon Drosophila being a reduction in lifespan and locomotor functions. The inhibition of *TBPH* and *parkin* in the neuron appears to mimic the suggested pathology of ALS and PD, making it a promising model of neurodegenerative disease.

Human *TBK1* and *p62/SQSTM1* are two ALS-related genes that have their activation dependent upon the PINK1/parkin pathway. Gene interaction experiments were attempted between the overexpression of Drosophila *IK2,* the fly equivalent of *TBK1*, and the addition of *alpha-synuclein* through the neuron-specific transgene *ddc-Gal4*$^{HL4.36}$. Experiments were attempted between the overexpression of *IK2* and inhibition of *parkin* through the neuron-specific transgene *ddc-Gal4*$^{HL4.3D}$; however critical class males were not obtained for either experiment, once again suggesting that *IK2* has essential roles in viability. Gene interactions experiments between Drosophila *Ref(2)P*, the fly equivalent of *p62/SQSTM1*, with both *alpha-synuclein* and *parkin* were conducted to examine the impacts to the fly. When expressed through the neuron-specific transgene *ddc-Gal4*$^{HL4.36}$, the inhibition of *Ref(2)P* through *UAS-Ref(2)P-RNAi*^{HMS00938} increased median lifespan, while decreased climbing ability [see Chapter 5, section 5.3.1]. When *alpha-synuclein* was expressed in addition to this, median lifespan was increased, however climbing ability was not impacted. Despite this increase in lifespan displayed when *alpha synuclein* is expressed concurrently with the inhibition of *Ref(2)P*, this increase in very minimal. As a reduction in human p62/SQSTM1 is known to enhance the pathology of *alpha-synuclein*, this may support the small reduction in lifespan seen when *alpha-synuclein* was expressed with a loss-of-function of *Ref(2)P*.

During PINK1-parkin-mediated mitophagy, the human protein p62/SQSTM1 is recruited by *parkin* to the mitochondria, where it acts to function in the aggregation of damaged mitochondria (Bitto et al., 2014; Narendra et al., 2010). Genetic interaction experiments were conducted to observe the consequences of the impairment of *p62/SQSTM1* and *parkin* have upon the median lifespan and the locomotor function. When expressed through the neuron-specific transgene *ddc-Gal4*$^{HL4.3D}$, inhibition of Drosophila *Ref(2)P* via *UAS-Ref(2)P-RNAi*^{HMS00938} increased lifespan but decreased climbing ability over the life of the flies [see Chapter 5, section 5.3.1]. The inhibition of *parkin* in combination with the loss of function of *Ref(2)P*, increased median lifespan, while climbing ability was reduced in a manner similar to the simple loss of *Ref(2)P* function. The results of gene interaction experiments between *Ref(2)P* and *parkin* genes suggest a strong connection; however, a further investigation must be done to understand their roles thoroughly.

As the VCP protein directly functions to mark mitochondria for destruction and aid in the clearance, it appears to be a key component in the PINK1-parkin mitophagy pathway (Ashrafi, Schlehe, LaVoie, & Schwarz, 2014; Kim et al., 2013). Gene interaction experiments between Drosophila *TER94*, the fly equivalent of *VCP*, and altered PD gene activities has revealed complex results when *TER94* is expressed through the neuron-specific transgene *ddc-Gal4*$^{HL4.36}$. Inhibition of *TER94* through *UAS-TER94-RNAi*^{JF03402} provided a significant decrease in both lifespan and climbing ability, while the inhibition of *TER94* through the directed expression of *UAS-TER94-RNAi*^{HMS00656} and *UAS-TER94-RNAi*^{GL00448} gave an increase in lifespan coupled with a significant lifetime reduction in

climbing ability [see Chapter 6, section 6.3.2]. When *alpha-synuclein* was expressed in addition to the loss of *TER94* function, *TER94* inhibition through *UAS-TER94-RNAi*^{JF03402} continued to display a similar reduction in lifespan and climbing ability. In contrast, *TER94* inhibition through *UAS-TER94-RNAi*^{GL0044} resulted in a significant decrease in lifespan. Interestingly, *TER94* inhibition through *UAS-TER94-RNAi*^{HMS00656} gave a significant increase in lifespan when *alpha-synuclein* was co-expressed. As *TER94* inhibition through *UAS-TER94-RNAi*^{GL00448} alone resulted in an increased lifespan, but the addition of *alpha-synuclein* lead to a reduction in lifespan, this suggests that *alpha-synuclein* interacts with the *TER94* protein to some extent. The inhibition of *TER94* through *UAS-TER94-RNAi*^{HMS00656} increased lifespan; however, the addition of *alpha-synuclein* lead to an even greater increase in lifespan by approximately 28%. This major increase in longevity seen by the inhibition of *TER94* and expression of *alpha-synuclein* to suggest a strong connection between the two gene activities and a clear synergistic effect where the combined effects of *TER94* inhibition and the expression of *alpha-synuclein* are greater than either alteration in isolation.

Human *VCP* has been suggested to play critical roles in mitophagy as it has been demonstrated to be a component of the PINK1/parkin pathway. As both mutant and wild-type *VCP* are known to have great influence upon aspects of the PINK1/parkin pathway (Kim et al., 2013), which may result in impairment of this pathways, genetic interactions between the Drosophila *TER94* and *parkin* genes were conducted. When expressed through the neuron-specific transgene *ddc-Gal4*$^{HL4.3D,}$ the inhibition of *TER94* through the directed expression of *UAS-TER94-RNAi*^{GL00448} did not alter median lifespan but did

reduce climbing ability over time [see Chapter 6, section 6.3.2]. The combined inhibition of both *parkin* and *TER94* resulted in a major reduction in median lifespan by approximately 30% along with the reduction of climbing ability. The considerable reduction in longevity observed when *parkin* has been inhibited further suggests a significant functional connection between *TER94* and *parkin*. This substantial decrease in lifespan and decline in motor function appears to mimic aspects of the pathology of ALS and PD, thus reinforcing the promise of this combination of altered gene expression as a model of neurodegenerative disease. However, further investigation into *TER94* and *parkin* is desired to better understand the potential roles of these genes.

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Chapter 8 – Summary

8.1 Summary

The use of model organisms such as *D. melanogaster* provides a simple but powerful way to investigate the biological consequences of altered gene expression. As altered *TBPH* is a hallmark of degenerating neurons, specifically the motor neurons, the reduction in median lifespan and locomotor function observed in *D. melanogaster* when levels of TBPH activity were inhibited in the motor neurons is an attempt to mimic the related pathologies observed in ALS patients (Feiguin et al., 2009; Zhan, Hanson, Kim, Tare, & Tibbetts, 2013). Critical class flies that expressed the loss-of-function of *TBPH* in the motor neurons presented with a reduction in median lifespan and motor ability over time, to provide a model of the neurodegenerative disease [see Chapter 3, Figure 2.3]. The investigation of interactions between *TBPH* and *alpha-synuclein* and with *parkin* provided unremarkable results as the additional expression of *alpha-synuclein* nor the inhibition of *parkin* in combination with *TBPH* inhibition did not strongly influence *D. melanogaster* [see Chapter 7, Figure 6.1-6.2]. A more in-depth exploration into the cellular mechanisms by which *TBPH* expression is altered in a diseased state, eventually may lead to novel treatment options for ALS patients.

Investigating the consequences of altered *IK2* expression gave either a lethal phenotype or lack of viability when *IK2* was overexpressed, while *IK2* inhibition was produced variable outcomes. Previously, it was known that *IK2* plays a vital role in developing neurons, with functions in dendrite pruning and neuronal re-modelling (Lee,

Jan, & Jan, 2009; Lin et al., 2015). The reduction in lifespan and locomotor function produced in *D. melanogaster* when *IK2* levels were increased in the motor neuron in an attempt to mimic the effects seen in ALS patients. The overexpression of *IK2* in the motor neuron can provide an imperfect model of neurodegenerative disease [see Chapter 4, Figure 3.3]; however, the process by which *IK2* expression is altered must be further investigated.

The high level of conservation and similarity between human *p62/SQSTM1* and Drosophila *Ref(2)P* allows investigation of the effects of altered *Ref(2)P* expression in Drosophila neurons is of particular interest (Devorkin & Gorski, 2014).The increase in median lifespan and reduction in motor ability displayed in *D. melanogaster* when *Ref(2)P* levels are inhibited in the neurons, motor neurons as well as dopaminergic neurons, can provide a range of models of neurodegenerative disease. However, the loss of *Ref(2)P* function led to an increase in median lifespan accompanied by a severe reduction in motor skills may be functioning as a type of compensation, where the slight increase in longevity is the consequence for a severe decline in motor skills [see Chapter 5, Figures 4.3 to 4.5]. The role that *Ref(2)P* plays in autophagy is substantial, with protein levels as well as protein aggregates accumulate when autophagy is impaired (Bartlett et al., 2011; Devorkin & Gorski, 2014). The accretion of protein aggregates when the processes of autophagy are non-functional well may be thought of as a signature characteristic of a number of neurodegenerative diseases.

The investigation of potential synergies between *Ref(2)P* and *alpha-synuclein* did not produce striking results: the directed expression of *alpha-synuclein* in combination with the inhibition of *Ref(2)P* did not show a strong interactive relationship upon the phenotypes of median lifespan or health-span in *D. melanogaster* [see Chapter 7, Figure 6.3]. However, the phenotypes observed *in D. melanogaster* when both *Ref(2)P* and *parkin* are inhibited through RNAi have shown that the inhibition of this combination of genes results in a significant increase in median lifespan with a notable reduction in lifetime motor function [see Chapter 7, Figure 6.4]. However, the increase in median lifespan obtained with the inhibition of *Ref(2)P* without alteration in *parkin* is more substantial than the inhibition of *parkin* without concurrent changes to *Ref(2)P* expression. Further investigation into the cellular mechanisms dependent upon *Ref(2)P* and *parkin* is required to better understand this biological connection.

Investigation of the consequences of altered *TER94* expression provided a number of divergent results which were dependent upon both the *Gal4* transgene used and the *TER94* and *TER94-RNAi* transgenes selected. As *TER94* has been demonstrated to process multiple roles in the cell, interact with various proteins and functioning in several cellular pathways, it is difficult to understand the extent of its influence through these experiments. The small increase in median lifespan and the sharp decline in locomotor function was observed in *D. melanogaster* when *TER94* levels were increased in the *D42-Gal4*-expressing motor neurons is an attempt to mimic the effects seen in ALS patients. Despite *TER94* overexpression providing a slight increase in lifespan, this may be functioning as an indirect consequence, where the slight increase in longevity may be a

compensation mechanism for a severe decline in motor skills. The minimal increase in lifespan and a sharp decline in motor ability observed in *D. melanogaster* when *TER94* was overexpressed in the motor neuron provides an imperfect model of neurodegenerative disease [see Chapter 6, Figure 5.3]. Moreover, the reduction in median lifespan and motor ability generated when *TER94* expression is inhibited in the dopaminergic neurons corresponds with the characteristic loss of dopaminergic neurons associated with PD, to suggest strongly that the inhibition of *TER94* in the dopaminergic neuron a promising model of neurodegenerative disease [see Chapter 6, Figure 5.4].

The investigation of interactions between *TER94* and *alpha-synuclein* resulted in a great increase in lifespan (~28%) and reduction in motor ability in *D. melanogaster* when *TER94* levels were inhibited, and *alpha-synuclein* was co-expressed, to suggests the potential of a synergistic interaction between the two [see Chapter 7, Figure 6.5]. On the other hand, the major reduction in median lifespan (~30%) and motor ability observed when both *TER94* and *parkin* levels were reduced is a promising model of neurodegenerative disease [see Chapter 7, Figure 6.6], as *TER94* is thought to be a component of the PINK1/parkin mitophagy mechanisms, with an important function in the destruction and clearance of damaged mitochondria (Ashrafi et al., 2014; Kim et al., 2013). A more in-depth understanding of *TER94* functions in the cell is required to broaden our knowledge of ALS and PD pathogenesis.

Neurodegenerative diseases have become very prevalent in the aging population of today, with a significant impact upon the lives of many individuals. Such diseases

greatly influence the lifespan, health and overall quality of life of those affected. By understanding the molecular pathways and cellular processes that are involved in disease pathogenesis, it may then be possible to create more advanced treatments, therapeutics to improve the longevity and health of these patients, and, eventually, a series of treatments to contribute to an eventual cure. The implication of the disease-related genes in this study may be of clinical significance, as altered gene expression affects longevity and motor function in *Drosophila melanogaster*.

8.2 Future Directions

The work of this study lays down a solid foundation of the biological consequences that altered autophagic gene activity has on *Drosophila melanogaster* survival and motor function. However, despite this there is continued work that is still to come from this study. Future research should investigate other ALS-related genes, such as *FUS*, which is known to act in conjunction with TDP-43, as well as genes from the Atg protein family, such as LC3/Atg8. Other experiments could look into the role of diet in disease, as diet is known to have a huge influence in our health. Studies have suggested the role of a high glucose diet as a form of treatment for ALS as it has been observed to protect nerve cells from pathological protein aggregates, such as TDP-43 aggregates, that result in neuronal death (Manzo et al., 2019). Furthermore, experiments investigating the interactions of *TER94* with other genes involved in the mitophagy process such as *Ref(2)P*, *clueless* and *parkin* would be of great interest.

8.3 References

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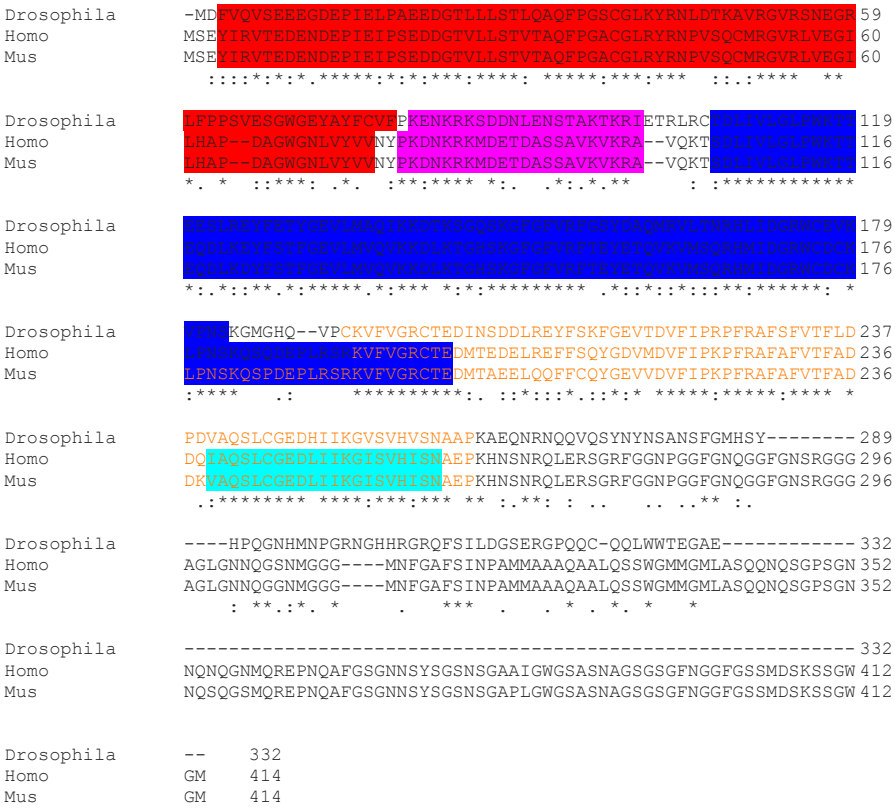
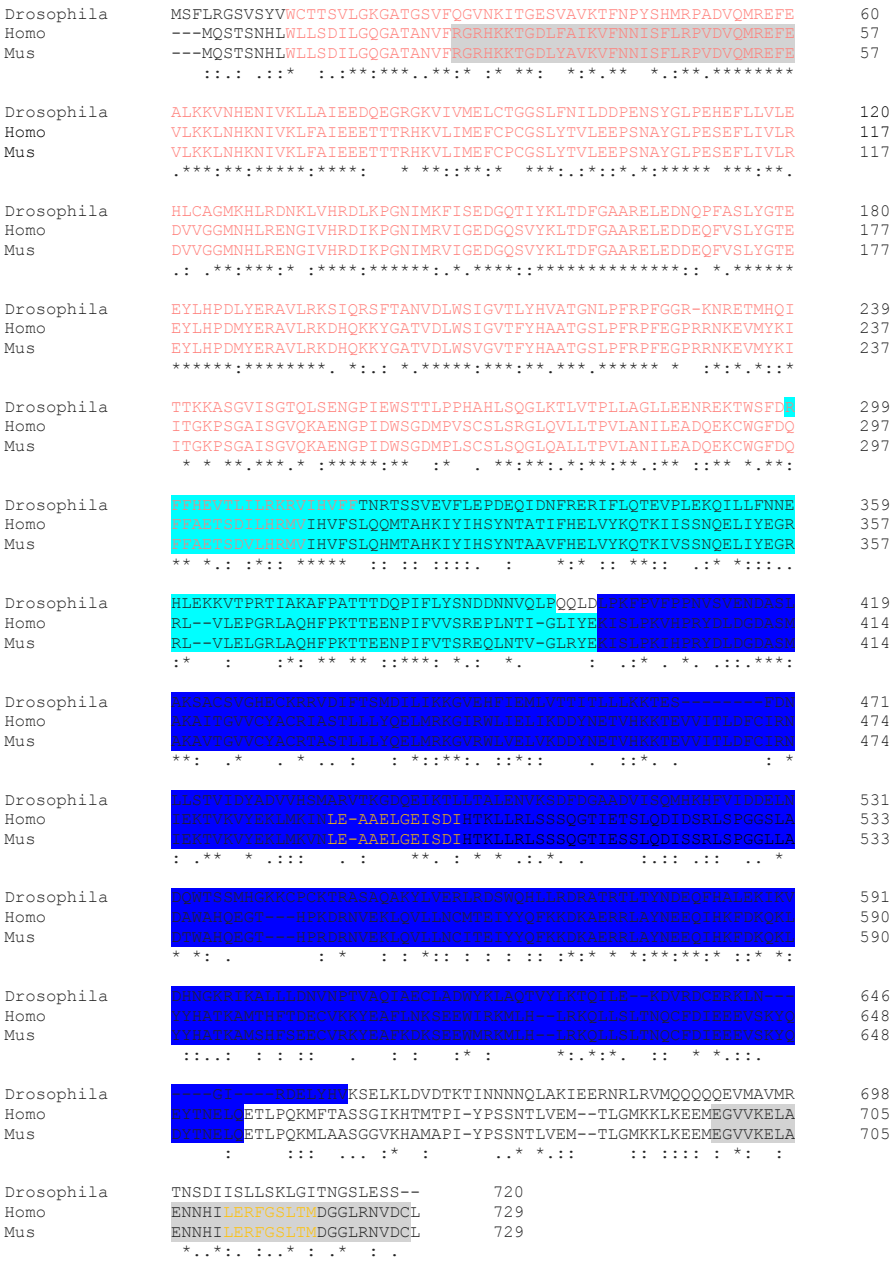
Appendix A – Supplemental Data for Chapter 1

A1. Protein sequence alignment of *Drosophila melanogaster TBPH* and *Homo sapiens* TDP-43. Alignment performed in Clustral Omega.



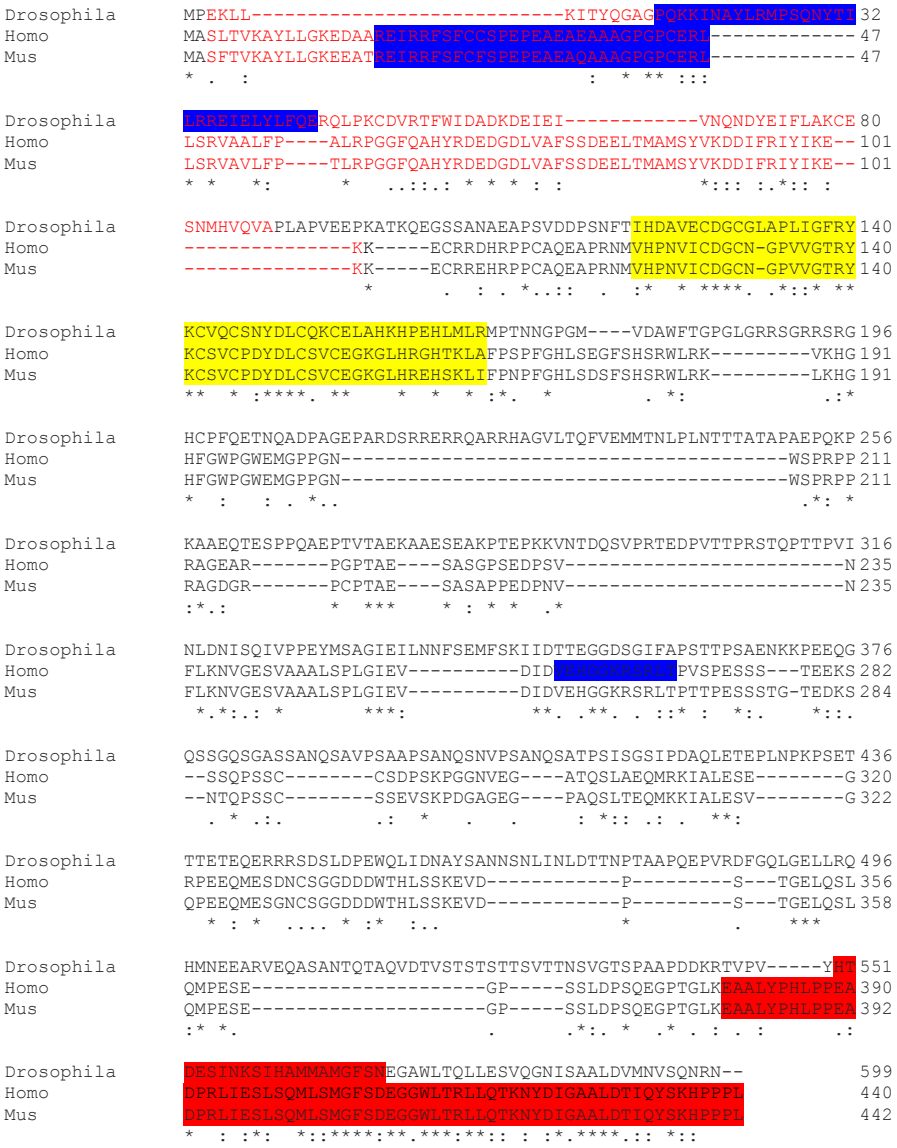
Highlighted are the TAR DNA binding protein 43, N-terminal domain (red), RNA recognition motif 1 (dark blue), RNA recognition motif 2 (orange text), Nuclear localization sequence (magenta) and Nuclear export signal (turquoise). "*" indicates amino acids that are identical in all sequences within the alignment, ":" indicates conserved substitutions, and "." indicates semiconserved substitutions. Protein sequences for *Homo sapiens* (accession number NP_031401.1), *Drosophila melanogaster* (accession number NM_058051), and *Mus musculus* (accession number sp | Q921F2.1) were retrieved from NCBI.

A2. Protein sequence alignment of *Drosophila melanogaster IK2* and *Homo sapiens* TBK1. Alignment performed in Clustral Omega



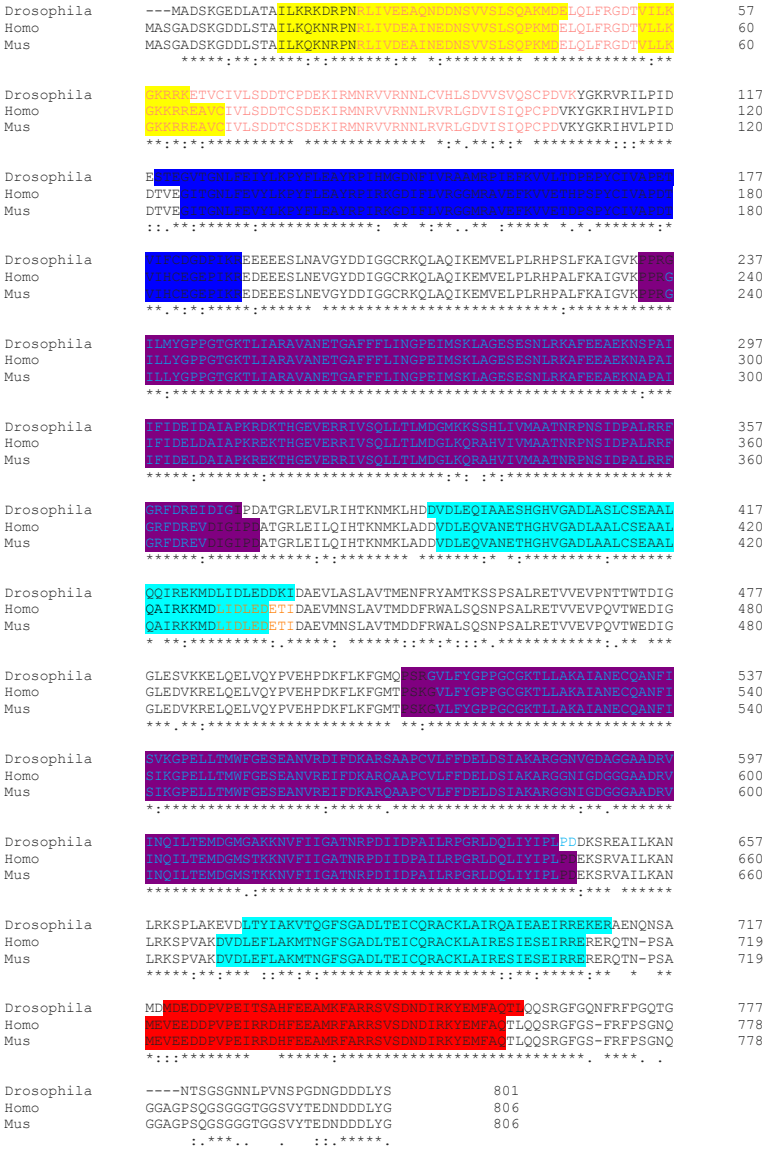
Highlighted are the Pkinase domain (red text), Ubiquitin-like domain (turquoise), Coiled coil domain 1 (dark blue), Nuclear localization signal (grey) and Nuclear export signal (orange text). "*" indicates amino acids that are identical in all sequences within the alignment, ":" indicates conserved substitutions, and "." indicates semiconserved substitutions. Protein sequences for *Homo sapiens* (accession number AAF05989.1), *Drosophila melanogaster* (accession number AAF53911.2), and *Mus musculus* (accession number AAF05990.1) were retrieved from NCBI.

A3. Protein sequence alignment of *Drosophila melanogaster Ref(2)P* and *Homo sapiens p62*. Alignment performed in Clustral Omega



Highlighted are the PB1 domain (red text), Zinc finger, ZZ type domain (yellow), Ubiquitin associated domain (red) and Nuclear localization signal (dark blue). "*" indicates amino acids that are identical in all sequences within the alignment, ":" indicates conserved substitutions, and "." indicates semiconserved substitutions. Protein sequences for *Homo sapiens* (accession number AAH17222.1), *Drosophila melanogaster* (accession number sp | P14199.2), and *Mus musculus* (accession number sp | Q64337.1) were retrieved from NCBI.

A4. Protein sequence alignment of *Drosophila melanogaster TER94* and *Homo sapiens VCP*. Alignment performed in Clustral Omega



Highlighted are the CDC48 N-terminal subdomain (pink text), CDC48 domain 2 (dark blue), AAA+ ATPase domain (dark purple), ATPase AAA type core domain (blue text), AAA ATPase AAAA+ lid domain (turquoise), Vps4 oligomerization C-terminal domain (red), Nuclear localization signal (yellow) and Nuclear export signal (orange text). "*" indicates amino acids that are identical in all sequences within the alignment, ":" indicates conserved substitutions, and "." indicates semiconserved substitutions. Protein sequences for *Homo sapiens* (accession number AAI21795.1), *Drosophila melanogaster* (accession number AAF58863.1), and *Mus musculus* (accession number AAH43053.1) were retrieved from NCBI.

Appendix B – Supplemental Data for Chapter 3

Table B1. Completed list of genotypes used in the analysis of altered expression of *TBPH*.

Genotype	Abbreviation	Reference
Control Lines		
w; P{UAS-lacZ.B}meltBg4-1-2	*UAS-lacZ*	Brand et al, 1993
Driver Lines		
w; GMR-Gal4^{12}	*GMR-Gal4*	Freeman, 1996
w[*]; P{w[+mW.hs]=GAL4-arm.S}11	*arm-Gal4*	Sanson et al., 1996
P{w[+mW.hs]=GawB}elav[C155]	*elav-Gal4*	Lin & Goodman, 1994
w[*]; P{w[+mC]=ple-GAL4.F}3	*TH-Gal4*	Inamdar et al., 2014
w[1118]; P{w[=mC]=Ddc-Gal4.L}4.3D	*ddc-Gal4$^{HL4.3D}$*	Li et al., 2000
w;[1118]; P{w[+mC]=Ddc-GAL4.L}Lmpt[4.36]	*ddc-Gal4$^{HL4.36}$*	Li et al., 2000
w[*];P{w[+mW.hs]=GawB}D42	*D42-Gal4*	Parkes et al.,1998
Responder Lines		
y[1] w[67c23]; P{w[=mC] y[+mDint2]=EPgy2}TBPH [EY10530]	*UAS-TBPH^{EY10530}*	Bellen et al., 2011
y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HMS05194}attP2	*UAS-TBPH-RNAi^{HMS05194}*	Perkins et al., 2015
y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HMS01932}attP40	*UAS-TBPH-RNAi^{HMS01932}*	Perkins et al, 2015

Table B2. Log-rank statistical analysis of fly longevity with altered ubiquitous expression of *TBPH* through the *arm-Gal4* transgene.

Genotype	Number of flies	Median survival (days)	Bonferroni corrected P-value	Chi-Square value	Significant
arm-Gal4;UAS-lacZ	284	60	N/A	N/A	N/A
arm-Gal4;UAS-TBPH-RNAi^{HMS05194}	221	62	0.0001	13.20	Yes (↑)
arm-Gal4;UAS-TBPH-RNAi^{HMS01932}	208	34	<0.0001	122.9	Yes (↓)
arm-Gal4;UAS-TBPH^{EY10530}	256	60	0.1215	0.8218	No

Table B3. Log-rank statistical analysis of fly longevity with altered expression of *TBPH* through the *elav-Gal4* transgene.

Genotype	Number of flies	Median survival (days)	Bonferroni corrected P-value	Chi-Square value	Significant
elav-Gal4;UAS-lacZ	298	78	N/A	N/A	N/A
elav-Gal4;UAS-TBPH-RNAi^{HMS05194}	281	80	0.2278	0.1654	No
elav-Gal4;UAS-TBPH-RNAi^{HMS01932}	33	48	<0.0001	294.5	Yes (↓)
elav-Gal4;UAS-TBPH^{EY10530}	291	68	<0.0001	106.8	Yes (↓)

Table B4. Log-rank statistical analysis of fly longevity with altered expression of *TBPH* through the *D42-Gal4* transgene.

Genotype	Number of flies	Median survival (days)	Bonferroni corrected P-value	Chi-Square value	Significant
D42-Gal4;UAS-lacZ	273	70	N/A	N/A	N/A
D42-Gal4;UAS-TBPH-RNAi^{HMS05194}	264	76	<0.0001	46.24	Yes (↑)
D42-Gal4;UAS-TBPH-RNAi^{HMS01932}	290	34	<0.0001	529.2	Yes (↓)
D42-Gal4;UAS-TBPH^{EY10530}	274	66	0.0279	2.989	Yes (↓)

Table B5. Statistical analysis of locomotor ability with altered expression of *TBPH* through the *D42-Gal4* transgene.

Genotype	Slope (k)	Standard Error	95% Confidence Interval	P-value	Significant
D42-Gal4;UAS-lacZ	0.03222	0.005270	0.02153 – 0.04509	N/A	N/A
D42-Gal4;UAS-TBPH-RNAi^{HMS05194}	0.02898	0.005633	0.01846 – 0.04088	0.0172	Yes (↓)
D42-Gal4;UAS-TBPH-RNAi^{HMS01932}	0.02456	0.006742	0.0098555-0.03938	<0.0001	Yes (↓)
D42-Gal4;UAS-TBPH^{EY10530}	0.05049	0.009656	0.03294 – 0.07035	<0.0001	Yes (↓)

Table B6. Log-rank statistical analysis of fly longevity with altered expression of *TBPH* through the *ddc-Gal4$^{HL4.3D}$* transgene.

Genotype	Number of flies	Median survival (days)	Bonferroni corrected P-value	Chi-Square value	Significant
ddc-Gal4$^{HL4.3D}$;UAS-lacZ	293	70	N/A	N/A	N/A
ddc-Gal4$^{HL4.3D}$;UAS-TBPH-RNAi^{HMS05194}	296	78	<0.0001	43.32	Yes (↑)
ddc-Gal4$^{HL4.3D}$;UAS-TBPH-RNAi^{HMS01932}	298	66	<0.0001	34.26	Yes (↓)
ddc-Gal4$^{HL4.3D}$;UAS-TBPH^{EY10530}	271	64	<0.0001	43.93	No

Table B7. Statistical analysis of locomotor ability altered expression of *TBPH* through the *ddc-Gal4$^{HL4.3D}$* transgene.

Genotype	Slope (k)	Standard Error	95% Confidence Interval	P-value	Significant
ddc-Gal4$^{HL4.3D}$;UAS-lacZ	0.01459	0.004440	0.006237 – 0.02363	N/A	N/A
ddc-Gal4$^{HL4.3D}$;UAS-TBPH-RNAi^{HMS05194}	0.02686	0.003317	0.02088 – 0.03345	0.0165	Yes (↑)
ddc-Gal4$^{HL4.3D}$;UAS-TBPH-RNAi^{HMS01932}	0.008222	0.004739	-0.001686 – 0.01875	0.5289	No
ddc-Gal4$^{HL4.3D}$;UAS-TBPH^{EY10530}	0.01680	0.004170	0.008046 – 0.02633	0.2774	No

Table B8. Log-rank statistical analysis of fly longevity with altered expression of *TBPH* through the *TH-Gal4* transgene.

Genotype	Number of flies	Median survival (days)	Bonferroni corrected P-value	Chi-Square value	Significant
TH-Gal4;UAS-lacZ	290	82	N/A	N/A	N/A
TH-Gal4;UAS-TBPH-RNAi^{HMS05194}	283	84	0.0003	10.60	Yes (↑)
TH-Gal4;UAS-TBPH-RNAi^{HMS01932}	280	74	<0.0001	58.51	Yes (↓)
TH-Gal4;UAS-TBPH^{EY10530}	263	66	<0.0001	108.1	Yes (↓)

Table B9. Statistical analysis of locomotor ability with altered expression of *TBPH* through the *TH-Gal4* transgene.

Genotype	Slope (k)	Standard Error	95% Confidence Interval	P-value	Significant
TH-Gal4;UAS-lacZ	0.02694	0.005922	0.01597 – 0.03987	N/A	N/A
TH-Gal4;UAS-TBPH-RNAi^{HMS05194}	0.01991	0.004162	0.01226 – 0.02832	0.4692	No
TH-Gal4;UAS-TBPH-RNAi^{HMS01932}	0.02053	0.004042	0.01279 – 0.02905	0.6299	No
TH-Gal4;UAS-TBPH^{EY10530}	0.02341	0.004877	0.01407 – 0.03375	0.2223	No

Table B10. Log-rank statistical analysis of fly longevity with altered neuronal expression of *TBPH* through the *ddc-Gal4$^{HL4.36}$* transgene.

Genotype	Number of flies	Median survival (days)	P-value	Chi-Square value	Significant
ddc-Gal4$^{HL4.36}$;UAS-lacZ	284	79	N/A	N/A	N/A
ddc-Gal4$^{HL4.36}$;UAS-TBPH-RNAi^{HMS01932}	263	82	0.1986	0.2797	No

Table B11. Statistical analysis of locomotor ability with directed neuronal expression with *TBPH* through the *ddc-Gal4$^{HL4.36}$* transgene.

Genotype	Slope (k)	Standard Error	95% Confidence Interval	P-value	Significant
ddc-Gal4$^{HL4.36}$;UAS-lacZ	0.03476	0.007093	0.02126 – 0.04966	N/A	N/A
ddc-Gal4$^{HL4.36}$;UAS-TBPH-RNAi^{HMS01932}	0.03709	0.007215	0.02357 – 0.05204	0.6115	No

Table B12. Summary of ommatidia number when *TBPH* expression in manipulated in the compound eye through the *GMR-Gal4* transgene.

Genotype	Sample Size (n)	Mean	P-value	Significant
GMR-Gal4;UAS-lacZ	10	710.3	N/A	N/A
GMR-Gal4;UAS-TBPH^{EY10530}	10	674.5	0.0027	Yes (↓)
GMR-Gal4;UAS-TBPH-RNAi ^{HMS05194}	10	676.1	0.0071	Yes (↓)
GMR-Gal4;UAS-TBPH-RNAi^{HMS01932}	10	630.4	<0.0001	Yes (↓)

Table B13. Summary of bristle number when *TBPH* expression in manipulated in the compound eye through the *GMR-Gal4* transgene.

Genotype	Sample Size (n)	Mean	P-value	Significant
GMR-Gal4;UAS-lacZ	10	560.1	N/A	N/A
GMR-Gal4;UAS-TBPH^{EY10530}	10	520.2	0.0196	Yes (↓)
GMR-Gal4;UAS-TBPH-RNAi ^{HMS05194}	10	478.6	0.0001	Yes (↓)
GMR-Gal4;UAS-TBPH-RNAi^{HMS01932}	10	418.5	<0.0001	Yes (↓)

Appendix C – Supplemental Data for Chapter 4

Table C1. Completed list of genotypes used in the analysis of altered expression of *IK2*.

Genotype	Abbreviation	Reference
Control Lines		
w; P{UAS-lacZ.B}meltBg4-1-2	*UAS-lacZ*	Brand et al, 1993
Driver Lines		
w; GMR-Gal4^{12}	*GMR-Gal4*	Freeman, 1996
w[*]; P{w[+mW.hs]=GAL4-arm.S}11	*arm-Gal4*	Sanson et al., 1996
P{w[+mW.hs]=GawB}elav[C155]	*elav-Gal4*	Lin & Goodman, 1994
w[*]; P{w[+mC]=ple-GAL4.F}3	*TH-Gal4*	Inamdar et al., 2014
w[1118]; P{w[=mC]=Ddc-Gal4.L}4.3D	*ddc-Gal4$^{HL4.3D}$*	Li et al., 2000
w;[1118]; P{w[+mC]=Ddc-GAL4.L}Lmpt[4.36]	*ddc-Gal4$^{HL4.36}$*	Li et al., 2000
w[*];P{w[+mW.hs]=GawB}D42	*D42-Gal4*	Parkes et al.,1998
Responder Lines		
y[1] w[67c23]; P{w[=mC] y[+mDint2]=EPgy2}CG31678[EY09774]	*UAS-IK2^{EY09774}*	Bellen et al., 2004
y[1] sc[*] v[1]; P{y[+t7.7] v[+t1.8] = TRiP.HMS01188}attP2	*UAS-IK2-RNAi^{HMS01188}*	Perkins et al., 2015
y[1] sc[*] v[1]; P{y[+t7.7] v[+t1.8] = TRiP.GL00160}attP2	*UAS-IK2-RNAi^{GL00160}*	Perkins et al, 2015

Table C2. Log-rank statistical analysis of fly longevity with altered ubiquitous expression with *IK2* through the *arm-Gal4* transgene.

Genotype	Number of flies	Median survival (days)	Bonferroni corrected P-value	Chi-Square value	Significant
arm-Gal4;UAS-lacZ	284	60	N/A	N/A	N/A
arm-Gal4;UAS-IK2-RNAi^{GL00160}	148	66	<0.0001	18.61	Yes (↑)
arm-Gal4;UAS-IK2-RNAi^{HMS01188}	276	70	<0.0001	50.00	Yes (↑)
arm-Gal4;UAS-IK2^{EY09774}	261	54	<0.0001	23.98	Yes (↓)

Table C3. Log-rank statistical analysis of fly longevity with altered expression of *IK2* through the *elav-Gal4* transgene.

Genotype	Number of flies	Median survival (days)	P-value	Chi-Square value	Significant
elav-Gal4;UAS-lacZ	298	78	N/A	N/A	N/A
elav-Gal4;UAS-IK2-RNAi^{GL00160}	305	74	<0.0001	41.05	Yes (↓)
elav-Gal4;UAS-IK2-RNAi^{HMS01188}	299	76	0.0005	9.930	Yes (↓)
elav-Gal4;UAS-IK2^{EY09774}	221	54	<0.0001	435.7	Yes (↓)

Table C4. Log-rank statistical analysis of fly longevity with altered expression of *IK2* through the *D42-Gal4* transgene.

Genotype	Number of flies	Median survival (days)	Bonferroni corrected P-value	Chi-Square value	Significant
D42-Gal4;UAS-lacZ	273	70	N/A	N/A	N/A
D42-Gal4;UAS-IK2-RNAi^{GL00160}	321	78	<0.0001	75.58	Yes (↑)
D42-Gal4;UAS-IK2-RNAi^{HMS01188}	330	76	<0.0001	59.05	Yes (↑)
D42-Gal4;UAS-IK2^{EY09774}	267	60	<0.0001	162.0	Yes (↓)

Table C5. Statistical analysis of locomotor ability with altered expression of *IK2* through the *D42-Gal4* transgene.

Genotype	Slope (k)	Standard Error	95% Confidence Interval	P-value	Significant
D42-Gal4;UAS-lacZ	0.03222	0.005270	0.02153 – 0.04509	N/A	N/A
D42-Gal4;UAS-IK2-RNAi^{GL00160}	0.04611	0.004191	0.03809 – 0.05493	<0.0001	Yes (↓)
D42-Gal4;UAS-IK2-RNAi^{HMS01188}	0.03143	0.004853	0.02259 – 0.04146	0.0001	Yes (↓)
D42-Gal4;UAS-IK2^{EY09774}	0.01844	0.006784	0.006088 – 0.0317	0.0417	Yes (↓)

Table C6. Log-rank statistical analysis of fly longevity with altered expression of *IK2* through the *ddc-Gal4$^{HL4.3D}$* transgene.

Genotype	Number of flies	Median survival (days)	Bonferroni corrected P-value	Chi-Square value	Significant
ddc-Gal4$^{HL4.3D}$;UAS-lacZ	293	70	N/A	N/A	N/A
ddc-Gal4$^{HL4.3D}$;UAS-IK2-RNAi^{GL00160}	314	78	<0.0001	17.17	Yes (↑)
ddc-Gal4$^{HL4.3D}$;UAS-IK2-RNAi^{HMS01188}	300	64	<0.0001	17.70	Yes (↓)

Table C7. Statistical analysis of locomotor ability with altered expression of *IK2* through the *ddc-Gal4$^{HL4.3D}$* transgene.

Genotype	Slope (k)	Standard Error	95% Confidence Interval	P-value	Significant
ddc-Gal4$^{HL4.3D}$;UAS-lacZ	0.01459	0.004440	0.006237 – 0.02363	N/A	N/A
ddc-Gal4$^{HL4.3D}$;UAS-IK2-RNAi^{GL00160}	0.06960	0.01194	0.04872 – 0.09401	<0.0001	Yes (↓)
ddc-Gal4$^{HL4.3D}$;UAS-IK2-RNAi^{HMS01188}	0.01919	0.004019	0.01164 – 0.02746	0.4194	No

Table C8. Log-rank statistical analysis of fly longevity with altered dopaminergic neuron expression with *IK2* through the *TH-Gal4* transgene.

Genotype	Number of flies	Median survival (days)	Bonferroni corrected P-value	Chi-Square value	Significant
TH-Gal4;UAS-lacZ	290	82	N/A	N/A	N/A
TH-Gal4;UAS-IK2-RNAi^{GL00160}	324	84	<0.0001	48.53	Yes (↑)
TH-Gal4;UAS-IK2-RNAi ^{HMS01188}	280	72	<0.0001	34.01	Yes (↓)
TH-Gal4;UAS-IK2^{EY09774}	103	54	<0.0001	231.7	Yes (↓)

Table C9. Statistical analysis of locomotor ability with altered dopaminergic neuron expression with *IK2* through the *TH-Gal4* transgene.

Genotype	Slope (k)	Standard Error	95% Confidence Interval	P-value	Significant
TH-Gal4;UAS-lacZ	0.02694	0.005922	0.01597 – 0.03987	N/A	N/A
TH-Gal4;UAS-IK2-RNAi^{GL00160}	0.02329	0.004447	0.01500 – 0.03234	0.1592	No
TH-Gal4;UAS-IK2-RNAi^{HMS01188}	0.01837	0.003970	0.01072 – 0.02651	0.1536	No
TH-Gal4;UAS-IK2^{EY09774}	0.01248	0.007808	-0.003747 – 0.03021	0.2191	No

Table C10. Summary of ommatidia number when *IK2* expression in manipulated in the compound eye though the *GMR-Gal4* transgene.

Genotype	Sample Size (n)	Mean	P-value	Significant
GMR-Gal4;UAS-lacZ	10	710.3	N/A	N/A
GMR-Gal4;UAS-IK2^{EY09774}	10	689.5	0.0618	No
GMR-Gal4;UAS-IK2-RNAi^{HMS01188}	10	654.3	<0.0001	Yes (↓)
GMR-Gal4;UAS-IK2-RNAi^{GL00160}	10	661.6	0.0001	Yes (↓)

Table C11. Summary of bristle number when *IK2* expression in manipulated in the compound eye though the *GMR-Gal4* transgene.

Genotype	Sample Size (n)	Mean	P-value	Significant
GMR-Gal4;UAS-lacZ	10	560.1	N/A	N/A
GMR-Gal4;UAS-IK2^{EY09774}	10	516.8	0.0355	Yes (↓)
GMR-Gal4;UAS-IK2-RNAi^{HMS01188}	10	455.5	<0.0001	Yes (↓)
GMR-Gal4;UAS-IK2-RNAi^{GL00160}	10	480.2	<0.0001	Yes (↓)

Appendix D – Supplemental Data for Chapter 5

Table D1. Completed list of genotypes used in the analysis of altered expression of *Ref(2)P*.

Genotype	Abbreviation	Reference
Control Lines		
w; P{UAS-lacZ.B}meltBg4-1-2	*UAS-lacZ*	Brand et al, 1993
Driver Lines		
w; GMR-Gal4^{12}	*GMR-Gal4*	Freeman, 1996
w[*]; P{w[+mW.hs]=GAL4-arm.S}11	*arm-Gal4*	Sanson et al., 1996
P{w[+mW.hs]=GawB}elav[C155]	*elav-Gal4*	Lin & Goodman, 1994
w[*]; P{w[+mC]=ple-GAL4.F}3	*TH-Gal4*	Inamdar et al., 2014
w[1118]; P{w[=mC]=Ddc-Gal4.L}4.3D	*ddc-Gal4$^{HL4.3D}$*	Li et al., 2000
w;[1118]; P{w[+mC]=Ddc-GAL4.L}Lmpt[4.36]	*ddc-Gal4$^{HL4.36}$*	Li et al., 2000
w[*];P{w[+mW.hs]=GawB}D42	*D42-Gal4*	Parkes et al., 1998
Responder Lines		
y[1] sc[*] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HMS00551}attP2	*UAS-Ref(2)P-RNAi^{HMS00551}*	Perkins et al., 2015
y[1] sc[*] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HMS00938}attP2	*UAS-Ref(2)P-RNAi^{HMS00938}*	Perkins et al., 2015

Table D2. Log-rank statistical analysis of fly longevity with altered expression of *Ref(2)P* through the *arm-Gal4* transgene.

Genotype	Number of flies	Median survival (days)	Bonferroni corrected P-value	Chi-Square value	Significant
arm-Gal4;UAS-lacZ	284	60	N/A	N/A	N/A
arm-Gal4;UAS-Ref(2)P-RNAi^{HMS00938}	286	88	<0.0001	384.1	Yes (↑)
arm-Gal4;UAS-Ref(2)P-RNAi^{HMS00551}	240	80	<0.0001	173.0	Yes (↑)

Table D3. Log-rank statistical analysis of fly longevity with altered expression of *Ref(2)P* through the *elav-Gal4* transgene.

Genotype	Number of flies	Median survival (days)	Bonferroni corrected P-value	Chi-Square value	Significant
elav-Gal4;UAS-lacZ	298	78	N/A	N/A	N/A
elav-Gal4;UAS-Ref(2)P-RNAi^{HMS00938}	251	86	<0.0001	77.91	Yes (↑)
elav-Gal4;UAS-Ref(2)P-RNAi^{HMS00551}	224	82	0.0009	9.758	Yes (↑)

Table D4. Log-rank statistical analysis of fly longevity with altered expression of *Ref(2)P* though the *D42-Gal4* transgene.

Genotype	Number of flies	Median survival (days)	Bonferroni corrected P-value	Chi-Square value	Significant
D42-Gal4;UAS-lacZ	273	70	N/A	N/A	N/A
D42-Gal4;UAS-Ref(2)P-RNAi^{HMS00938}	303	82	<0.0001	146.8	Yes (↑)
D42-Gal4;UAS-Ref(2)P-RNAi^{HMS00551}	252	86	<0.0001	210.4	Yes (↑)

Table D5. Statistical analysis of locomotor ability with altered expression of *Ref(2)P* though the *D42-Gal4* transgene.

Genotype	Slope (k)	Standard Error	95% Confidence Interval	P-value	Significant
D42-Gal4;UAS-lacZ	0.03222	0.005270	0.02153 – 0.04509	N/A	N/A
D42-Gal4;UAS-Ref(2)P-RNAi^{HMS00938}	0.05212	0.005511	0.04145 – 0.06364	0.0421	Yes (↓)
D42-Gal4;UAS-Ref(2)P-RNAi^{HMS00551}	0.03964	0.004145	0.03179 – 0.04801	0.3181	No

Table D6. Log-rank statistical analysis of fly longevity with altered expression of *Ref(2)P* though the *TH-Gal4* transgene.

Genotype	Number of flies	Median survival (days)	Bonferroni corrected P-value	Chi-Square value	Significant
TH-Gal4;UAS-lacZ	290	82	N/A	N/A	N/A
TH-Gal4;UAS-Ref(2)P-RNAi^{HMS00938}	278	86	<0.0001	48.56	Yes (↑)
TH-Gal4;UAS-Ref(2)P-RNAi^{HMS00551}	263	88	<0.0001	88.21	Yes (↑)

Table D7. Statistical analysis of locomotor ability with altered expression of *Ref(2)P* though the *TH-Gal4* transgene.

Genotype	Slope (k)	Standard Error	95% Confidence Interval	P-value	Significant
TH-Gal4;UAS-lacZ	0.02694	0.005922	0.01597 – 0.03987	N/A	N/A
TH-Gal4;UAS-Ref(2)P-RNAi^{HMS00938}	0.05106	0.007466	0.03756 – 0.06621	0.0261	Yes (↓)
TH-Gal4;UAS-Ref(2)P-RNAi^{HMS00551}	0.03898	0.005173	0.02927 – 0.04949	0.1740	No

Table D8. Log-rank statistical analysis of fly longevity with altered expression of *Ref(2)P* through the *ddc-Gal4$^{HL4.3D}$* transgene.

Genotype	Number of flies	Median survival (days)	Bonferroni corrected P-value	Chi-Square value	Significant
ddc-Gal4$^{HL4.3D}$;UAS-lacZ	293	70	N/A	N/A	N/A
ddc-Gal4$^{HL4.3D}$;UAS-Ref(2)P-RNAi^{HMS00938}	249	94	<0.0001	220.0	Yes (↑)
ddc-Gal4$^{HL4.3D}$;UAS-Ref(2)P-RNAi^{HMS00551}	241	74	<0.0001	22.92	Yes (↑)

Table D9. Statistical analysis of locomotor ability with directed neuronal expression of *Ref(2)P* through the *ddc-Gal4$^{HL4.3D}$* transgene.

Genotype	Slope (k)	Standard Error	95% Confidence Interval	P-value	Significant
ddc-Gal4$^{HL4.3D}$;UAS-lacZ	0.01459	0.004440	0.006237 – 0.02363	N/A	N/A
ddc-Gal4$^{HL4.3D}$;UAS-Ref(2)P-RNAi^{HMS00938}	0.05879	0.008876	0.04246 – 0.07724	0.0002	Yes (↓)
ddc-Gal4$^{HL4.3D}$;UAS-Ref(2)P-RNAi^{HMS00551}	0.06091	0.008299	0.04544 – 0.07834	<0.0001	Yes (↓)

Table D10. Log-rank statistical analysis of fly longevity with altered expression of *Ref(2)P* though the *ddc-Gal4$^{HL4.36}$* transgene.

Genotype	Number of flies	Median survival (days)	Bonferroni corrected P-value	Chi-Square value	Significant
ddc-Gal4$^{HL4.36}$;UAS-lacZ	284	79	N/A	N/A	N/A
ddc-Gal4$^{HL4.36}$;UAS-Ref(2)P-RNAi^{HMS00938}	263	88	<0.0001	27.49	Yes (↑)

Table D11. Statistical analysis of locomotor ability with altered expression of *Ref(2)P* though the *ddc-Gal4$^{HL4.36}$* transgene

Genotype	Slope (k)	Standard Error	95% Confidence Interval	P-value	Significant
ddc-Gal4$^{HL4.36}$;UAS-lacZ	0.03476	0.007093	0.02126-0.04966	N/A	N/A
ddc-Gal4$^{HL4.36}$;UAS-Ref(2)P-RNAi^{HMS00938}	0.01270	0.003820	0.004738 – 0.02086	<0.0001	Yes (↓)

Table D12. Summary of ommatidia number when *Ref(2)P* expression in manipulated in the compound eye though the *GMR-Gal4* transgene.

Genotype	Sample Size (n)	Mean	P-value	Significant
GMR-Gal4;UAS-lacZ	10	710.3	N/A	N/A
GMR-Gal4;UAS-Ref(2)P-RNAi^{HMS00938}	10	662.4	0.0002	Yes (↓)
GMR-Gal4;UAS-Ref(2)P-RNAi^{HMS00551}	10	624.8	<0.0001	Yes (↓)

Table D13. Summary of ommatidia number when *Ref(2)P* expression in manipulated in the compound eye though the *GMR-Gal4* transgene.

Genotype	Sample Size (n)	Mean	P-value	Significant
GMR-Gal4;UAS-lacZ	10	560.1	N/A	N/A
GMR-Gal4;UAS-Ref(2)P-RNAi^{HMS00938}	10	424.0	<0.0001	Yes (↓)
GMR-Gal4;UAS-Ref(2)P-RNAi^{HMS00551}	10	459.9	<0.0001	Yes (↓)

Appendix E – Supplemental Data for Chapter 6

Table E1. Completed list of genotypes used in the analysis of altered expression of *TER94*.

Genotype	Abbreviation	Reference
Control Lines		
w; P{UAS-lacZ.B}meltBg4-1-2	*UAS-lacZ*	Brand et al, 1993
Driver Lines		
w; GMR-Gal4^{12}	*GMR-Gal4*	Freeman, 1996
w[*]; P{w[+mW.hs]=GAL4-arm.S}11	*arm-Gal4*	Sanson et al., 1996
P{w[+mW.hs]=GawB}elav[C155]	*elav-Gal4*	Lin & Goodman, 1994
w[*]; P{w[+mC]=ple-GAL4.F}3	*TH-Gal4*	Inamdar et al., 2014
w[1118]; P{w[=mC]=Ddc-Gal4.L}4.3D	*ddc-Gal4$^{HL4.3D}$*	Li et al., 2000
w;[1118]; P{w[+mC]=Ddc-GAL4.L}Lmpt[4.36]	*ddc-Gal4$^{HL4.36}$*	Li et al., 2000
w[*];P{w[+mW.hs]=GawB}D42	*D42-Gal4*	Parkes et al., 1998
Responder Lines		
y1w67c23; P{EPgy2}TER94EY03486/CyO	*UAS-TER94^{EY03486}*	Bellen et al., 2004
y1sc*v1; P{TKO.GS00593}attP40	*UAS-TER94-RNAi^{GS00593}*	Perkins et al., 2015
y[1] sc[*] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.GL00448}attP2	*UAS-TER94-RNAi^{GL00448}*	Perkins et al., 2015
y1 v1; P{TRiP.JF03402}attP2	*UAS-TER94-RNAi^{JF03402}*	Perkins et al., 2015
y1 v1; P{TRiP.HMS00656}attP2	*UAS-TER94-RNAi^{HMS00656}*	Perkins et al., 2015

Table E2. Log-rank statistical analysis of fly longevity with altered expression of *TER94* through the *arm-Gal4* transgene.

Genotype	Number of flies	Median survival (days)	Bonferroni corrected P-value	Chi-Square value	Significant
arm-Gal4;UAS-lacZ	284	60	N/A	N/A	N/A
arm-Gal4;UAS-TER94^{EY03486}	212	80	<0.0001	211.7	Yes (↑)
arm-Gal4;UAS-TER94-RNAi^{HMS00656}	92	28	<0.0001	185.2	Yes (↓)
arm-Gal4;UAS-TER94-RNAi^{GL00448}	328	52	<0.0001	114.6	Yes (↓)
arm-Gal4;UAS-TER94-RNAi^{GS00593}	271	76	<0.0001	124.2	Yes (↑)

Table E3. Log-rank statistical analysis of fly longevity with altered expression of *TER94* through the *elav-Gal4* transgene.

Genotype	Number of flies	Median survival (days)	Bonferroni corrected P-value	Chi-Square value	Significant
elav-Gal4;UAS-lacZ	298	78	N/A	N/A	N/A
elav-Gal4;UAS-TER94^{EY03486}	215	80	<0.0001	19.83	Yes (↑)
elav-Gal4;UAS-TER94-RNAi^{HMS00656}	384	42	<0.0001	656.0	Yes (↓)
elav-Gal4;UAS-TER94-RNAi^{GL00448}	305	28	<0.0001	638.9	Yes (↓)
elav-Gal4;UAS-TER94-RNAi^{GS00593}	303	78	0.04277	1.873	No

Table E4. Log-rank statistical analysis of fly longevity with altered expression of *TER94* through the *D42-Gal4* transgene.

Genotype	Number of flies	Median survival (days)	Bonferroni corrected P-value	Chi-Square value	Significant
D42-Gal4;UAS-lacZ	238	70	N/A	N/A	N/A
D42-Gal4;UAS-TER94^{EY03486}	220	72	<0.0001	14.24	Yes (↑)
D42-Gal4;UAS-TER94-RNAi^{HMS00656}	272	80	<0.0001	131.1	Yes (↑)
D42-Gal4;UAS-TER94-RNAi^{GL00448}	281	68	0.0003	10.42	Yes (↓)
D42-Gal4;UAS-TER94-RNAi^{GS00593}	219	68	0.1819	0.1210	No
D42-Gal4;UAS-TER94-RNAi^{JF03402}	200	78	<0.0001	65.03	Yes (↑)

Table E5. Statistical analysis of locomotor ability with altered expression of *TER94* through the *D42-Gal4* transgene.

Genotype	Slope (k)	Standard Error	95% Confidence Interval	P-value	Significant
D42-Gal4;UAS-lacZ	0.03222	0.005270	0.02153 – 0.04509	N/A	N/A
D42-Gal4;UAS-TER94^{EY03486}	0.04146	0.006718	0.02868 – 0.05498	<0.0001	Yes (↓)
D42-Gal4;UAS-TER94-RNAi^{HMS00656}	0.05289	0.013999	0.02644 - 0.08283	<0.0001	Yes (↓)
D42-Gal4;UAS-TER94-RNAi^{GL00448}	0.03467	0.004751	0.02542 - 0.04453	<0.0001	Yes (↓)
D42-Gal4;UAS-TER94-RNAi^{GS00593}	0.05414	0.007627	0.03976 – 0.07059	<0.0001	Yes (↓)
D42-Gal4;UAS-TER94-RNAi^{JF03402}	0.02989	0.006989	0.01538 – 0.04502	<0.0001	Yes (↓)

Table E6. Log-rank statistical analysis of fly longevity with altered expression of *TER94* though the *TH-Gal4* transgene.

Genotype	Number of flies	Median survival (days)	Bonferroni corrected P-value	Chi-Square value	Significant
TH-Gal4;UAS-lacZ	291	82	N/A	N/A	N/A
TH-Gal4;UAS-TER94^{EY03486}	193	76	<0.0001	22.44	Yes (↓)
TH-Gal4;UAS-TER94-RNAi^{HMS00656}	268	46	<0.0001	493.2	Yes (↓)
TH-Gal4;UAS-TER94-RNAi^{GL00448}	285	54	<0.0001	438.8	Yes (↓)
TH-Gal4;UAS-TER94-RNAi^{GS00593}	166	82	0.2330	0.0072	No

Table E7. Statistical analysis of locomotor ability with altered expression of *TER94* though the *TH-Gal4* transgene.

Genotype	Slope (k)	Standard Error	95% Confidence Interval	P-value	Significant
TH-Gal4;UAS-lacZ	0.02694	0.005922	0.01597 – 0.03987	N/A	N/A
TH-Gal4;UAS-TER94^{EY03486}	0.03874	0.01053	0.01750 – 0.06594	0.1025	No
TH-Gal4;UAS-TER94-RNAi^{HMS00656}	0.07050	0.008695	0.05546 – 0.08791	<0.0001	Yes (↓)
TH-Gal4;UAS-TER94-RNAi^{GL00448}	0.04828	0.005441	0.03830 – 0.05920	<0.0001	Yes (↓)
TH-Gal4;UAS-TER94-RNAi^{GS00593}	0.05430	0.004538	0.04532 – 0.06439	0.0005	Yes (↓)

Table E8. Log-rank statistical analysis of fly longevity with altered expression of *TER94* though the *ddc-Gal4$^{HL4.3D}$* transgene.

Genotype	Number of flies	Median survival (days)	Bonferroni corrected P-value	Chi-Square value	Significant
ddc-Gal4$^{HL4.3D}$;UAS-lacZ	293	70	N/A	N/A	N/A
ddc-Gal4$^{HL4.3D}$;UAS-TER94^{EY03486}	171	66	<0.0001	32.06	Yes (↓)
ddc-Gal4$^{HL4.3D}$;UAS-TER94-RNAi^{HMS00656}	246	74	<0.0001	29.60	Yes (↑)
ddc-Gal4$^{HL4.3D}$;UAS-TER94-RNAi^{GL00448}	213	68	0.0807	0.9766	No
ddc-Gal4$^{HL4.3D}$;UAS-TER94-RNAi^{GS00593}	211	76	0.0002	10.94	Yes (↑)

Table E9. Statistical analysis of locomotor ability with altered expression of *TER94* though the *ddc-Gal4$^{HL4.3D}$* transgene.

Genotype	Slope (k)	Standard Error	95% Confidence Interval	P-value	Significant
ddc-Gal4$^{HL4.3D}$;UAS-lacZ	0.01459	0.004440	0.006237-0.02363	N/A	N/A
ddc-Gal4$^{HL4.3D}$;UAS-TER94^{EY03486}	0.02019	0.004323	0.01191-0.02901	0.0267	Yes (↓)
ddc-Gal4$^{HL4.3D}$;UAS-TER94-RNAi^{HMS00656}	0.03701	0.009203	0.02029-0.05545	0.0010	Yes (↓)
ddc-Gal4$^{HL4.3D}$;UAS-TER94-RNAi^{GL00448}	0.04776	0.01334	0.02228 – 0.07618	<0.0001	Yes (↓)
ddc-Gal4$^{HL4.3D}$;UAS-TER94-RNAi^{GS00593}	0.05297	0.007462	0.03939 – 0.06944	0.0007	Yes (↓)

Table E10. Log-rank statistical analysis of fly longevity with altered expression of *TER94* though the *ddc-Gal4$^{HL4.36}$* transgene.

Genotype	Number of flies	Median survival (days)	Bonferroni corrected P-value	Chi-Square value	Significant
ddc-Gal4$^{HL4.36}$;UAS-lacZ	284	79	N/A	N/A	N/A
ddc-Gal4$^{HL4.36}$;UAS-TER94-RNAi^{HMS00656}	267	90	<0.0001	93.27	Yes (↑)
ddc-Gal4$^{HL4.36}$;UAS-TER94-RNAi^{GL00448}	246	88	<0.0001	26.41	Yes (↑)
ddc-Gal4$^{HL4.36}$;UAS-TER94-RNAi^{JF03402}	229	44	<0.0001	521.1	Yes (↓)

Table E11. Statistical analysis of locomotor ability with altered expression of *TER94* though the *ddc-Gal4$^{HL4.36}$* transgene.

Genotype	Slope (k)	Standard Error	95% Confidence Interval	P-value	Significant
ddc-Gal4$^{HL4.36}$;UAS-lacZ	0.03476	0.007093	0.02126 – 0.04966	N/A	N/A
ddc-Gal4$^{HL4.36}$;UAS-TER94-RNAi^{HMS00656}	0.02436	0.004306	0.01561 – 0.03355	0.0026	Yes (↓)
ddc-Gal4$^{HL4.36}$;UAS-TER94-RNAi^{GL00448}	0.01999	0.004225	0.01155 – 0.02876	0.0045	Yes (↓)
ddc-Gal4$^{HL4.36}$;UAS-TER94-RNAi^{JF03402}	0.02542	0.004192	0.01674-0.03456	0.0008	Yes (↓)

Table E12. Summary of ommatidia number when *TER94* expression in manipulated in the compound eye though the *GMR-Gal4* transgene.

Genotype	Sample Size (n)	Mean	P-value	Significant
GMR-Gal4;UAS-lacZ	10	710.3	N/A	N/A
GMR-Gal4;UAS-TER94^{EY03486}	10	651.7	<0.0001	Yes (↓)
GMR-Gal4;UAS-TER94-RNAi^{JF03402}	10	678.9	0.0016	Yes (↓)
GMR-Gal4;UAS-TER94-RNAi^{HMS00656}	10	677.1	0.0069	Yes (↓)
GMR-Gal4;UAS-TER94-RNAi^{GL00448}	10	701.4	0.3422	No
GMR-Gal4;UAS-TER94-RNAi^{GS00593}	10	675.1	0.0008	Yes (↓)

Table E13. Summary of bristle number when *TER94* expression in manipulated in the compound eye though the *GMR-Gal4* transgene.

Genotype	Sample Size (n)	Mean	P-value	Significant
GMR-Gal4;UAS-lacZ	10	560.1	N/A	N/A
GMR-Gal4;UAS-TER94^{EY03486}	10	450.6	<0.0001	Yes (↓)
GMR-Gal4;UAS-TER94-RNAi^{JF03402}	10	453.8	<0.0001	Yes (↓)
GMR-Gal4;UAS-TER94-RNAi^{HMS00656}	10	438.8	<0.0001	Yes (↓)
GMR-Gal4;UAS-TER94-RNAi^{GL00448}	10	470.8	<0.0001	Yes (↓)
GMR-Gal4;UAS-TER94-RNAi^{GS00593}	10	523.2	0.0003	Yes (↓)

Appendix F – Supplemental Data for Chapter 7

Table F1. Completed list of genotypes used in the analysis of altered expression ALS-related genes with altered PD gene activity.

Genotype	Abbreviation	Reference
Control Lines		
w; P{UAS-lacZ.B}meltBg4-1-2	*UAS-lacZ*	Brand et al, 1993
Driver Lines		
w; ddc-Gal4/CyO; UAS-parkin-RNAi/TM3	*ddc-Gal4$^{HL4.3D}$;UAS-parkin-RNAi*	Staveley, Unpublished
w;ddc-Gal4$^{HL4.36}$/Tm3 iso1; UAS-alpha-synuclein/CyO	*ddc-Gal4$^{HL4.36}$;UAS-alpha-synucelin*	Staveley, Unpublished
Responder Lines		
y[1] sc[*] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.GL00448}attP2	*UAS-TER94-RNAi^{GL00448}*	Perkins et al., 2015
y1 v1; P{TRiP.JF03402}attP2	*UAS-TER94-RNAi^{JF03402}*	Perkins et al., 2015
y1 v1; P{TRiP.HMS00656}attP2	*UAS-TER94-RNAi^{HMS00656}*	Perkins et al.,2015
y[1] sc[*] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HMS00938}attP2	*UAS-Ref(2)P-RNAi^{HMS00938}*	Perkins et al., 2015
y[1] w[67c23]; P{w[=mC] y[+mDint2]=EPgy2}CG31678[EY09774]	*UAS-IK2^{EY09774}*	Bellen et al., 2004
y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HMS01932}attP40	*UAS-TBPH-RNAi^{HMS01932}*	Perkins et al, 2015

Table F2. Log-rank statistical analysis of fly longevity with altered expression of *TBPH* through the *ddc-Gal4$^{HL4.36}$;UAS-alpha-synuclein* transgene.

Genotype	Number of flies	Median survival (days)	Bonferroni corrected P-value	Chi-Square value	Significant
ddc-Gal4$^{HL4.36}$;UAS-alpha-synuclein;UAS-lacZ	228	86	N/A	N/A	N/A
ddc-Gal4$^{HL4.36}$;UAS-alpha-synuclein;UAS-TBPH-RNAi^{HMS01932}	261	78	<0.0001	57.88	Yes (↓)

Table F3. Statistical analysis of locomotor ability with altered expression of *TBPH* through the *ddc-Gal4$^{HL4.36}$;UAS-alpha-synuclein* transgene.

Genotype	Slope (k)	Standard Error	95% Confidence Interval	P-value	Significant
ddc-Gal4$^{HL4.36}$;UAS-alpha-synuclein;UAS-lacZ	0.03241	0.005495	0.02162 – 0.04402	N/A	N/A
ddc-Gal4$^{HL4.36}$;UAS-alpha-synuclein;UAS-TBPH-RNAi^{HMS01932}	0.02840	0.005691	0.01725 – 0.04035	0.8661	No

Table F4. Log-rank statistical analysis of fly longevity with altered expression of *TBPH* through the *ddc-Gal4$^{HL4.3D}$;UAS-parkin-RNAi* transgene.

Genotype	Number of flies	Median survival (days)	Bonferroni corrected P-value	Chi-Square value	Significant
ddc-Gal4$^{HL4.3D}$;UAS-parkin-RNAi;UAS-lacZ	253	84	N/A	N/A	N/A
ddc-Gal4$^{HL4.3D}$;UAS-parkin-RNAi;UAS-TBPH-RNAi^{HMS01932}	253	76	<0.0001	73.86	Yes (↓)

Table F5. Statistical analysis of locomotor ability with altered expression of *TBPH* through the *ddc-Gal4$^{HL4.3D}$;UAS-parkin-RNAi* transgene.

Genotype	Slope (k)	Standard Error	95% Confidence Interval	P-value	Significant
ddc-Gal4$^{HL4.3D}$;UAS-parkin-RNAi;UAS-lacZ	0.01900	0.004663	0.009792 – 0.02875	N/A	N/A
ddc-Gal4$^{HL4.3D}$;UAS-parkin-RNAi;UAS-TBPH-RNAi^{HMS01932}	0.01433	0.006658	0.0001933 – 0.02912	0.1922	No

Table F6. Log-rank statistical analysis of fly longevity with altered neuronal expression of *Ref(2)P* though the *ddc-Gal4$^{HL4.36}$;UAS-alpha-synuclein* transgene.

Genotype	Number of flies	Median survival (days)	Bonferroni corrected P-value	Chi-Square value	Significant
ddc-Gal4$^{HL4.36}$;UAS-alpha-synuclein;UAS-lacZ	228	86	N/A	N/A	N/A
ddc-Gal4$^{HL4.36}$;UAS-alpha-synucelin;UAS-Ref(2)P-RNAi^{HMS00938}	281	92	<0.0001	67.27	Yes (↑)

Table F7. Statistical analysis of locomotor ability with altered neuronal expression of *Ref(2)P* though the *ddc-Gal4$^{HL4.36}$;UAS-alpha-synuclein* transgene.

Genotype	Slope (k)	Standard Error	95% Confidence Interval	P-value	Significant
ddc-Gal4$^{HL4.36}$;UAS-alpha-synuclein;UAS-lacZ	0.03241	0.005495	0.02162 – 0.04402	N/A	N/A
ddc-Gal4$^{HL4.36}$;UAS-alpha-synuclein;UAS-Ref(2)P-RNAi^{HMS00938}	0.01951	0.003581	0.01222 – 0.02703	0.0761	No

Table F8. Log-rank statistical analysis of fly longevity with altered neuronal expression of *Ref(2)P* though the *ddc-Gal4$^{HL4.3D}$; UAS-parkin-RNAi* transgene.

Genotype	Number of flies	Median survival (days)	Bonferroni corrected P-value	Chi-Square value	Significant
ddc-Gal4$^{HL4.3D}$;UAS-parkin-RNAi;UAS-lacZ	253	84	N/A	N/A	N/A
ddc-Gal4$^{HL4.3D}$;UAS-parkin-RNAi;UAS-Ref(2)P-RNAi^{HMS00938}	267	96	<0.0001	77.54	Yes (↑)

Table F9. Statistical analysis of locomotor ability with altered expression of *Ref(2)P* though the *ddc-Gal4$^{HL4.3D}$; UAS-parkin-RNAi* transgene.

Genotype	Slope (k)	Standard Error	95% Confidence Interval	P-value	Significant
ddc-Gal4$^{HL4.3D}$;UAS-parkin-RNAi;UAS-lacZ	0.01900	0.004663	0.009792 – 0.02875	N/A	N/A
ddc-Gal4$^{HL4.3D}$;UAS-parkin-RNAi;UAS-Ref(2)P-RNAi^{HMS00938}	0.01541	0.006710	0.002026 – 0.02922	0.0064	Yes (↓)

Table F10. Log-rank statistical analysis of fly longevity with altered expression of *TER94* though the *ddc-Gal4$^{HL4.36}$;UAS-alpha-synuclein* transgene.

Genotype	Number of flies	Median survival (days)	Bonferroni corrected P-value	Chi-Square value	Significant
ddc-Gal4$^{HL4.36}$;UAS-alpha-synuclein;UAS-lacZ	228	86	N/A	N/A	N/A
ddc-Gal4$^{HL4.36}$;UAS-alpha-synuclein;UAS-TER94-RNAi^{JF03402}	275	48	<0.0001	442.6	Yes (↓)
ddc-Gal4$^{HL4.36}$;UAS-alpha-synuclein;UAS-TER94-RNAi^{HMS00656}	286	110	<0.0001	260.2	Yes (↑)
ddc-Gal4$^{HL4.36}$;UAS-alpha-synuclein;UAS-TER94-RNAi^{GL00448}	242	80	<0.0001	21.55	Yes (↓)

Table F11. Statistical analysis of locomotor ability with altered expression of *TER94* though the *ddc-Gal4$^{HL4.36}$;UAS-alpha-synuclein* transgene.

Genotype	Slope (k)	Standard Error	95% Confidence Interval	P-value	Significant
ddc-Gal4$^{HL4.36}$;UAS-alpha-synuclein;UAS-lacZ	0.03241	0.005495	0.02162 – 0.04402	N/A	N/A
ddc-Gal4$^{HL4.36}$;UAS-alpha-synuclein;UAS-TER94-RNAi^{JF03402}	0.03813	0.01171	0.01498 – 0.06325	0.4190	No
ddc-Gal4$^{HL4.36}$;UAS-alpha-synuclein;UAS-TER94-RNAi^{HMS00656}	0.03076	0.006437	0.01830 – 0.04395	0.0064	Yes (↓)
ddc-Gal4$^{HL4.36}$;UAS-alpha-synuclein;UAS-TER94-RNAi^{GL00448}	0.01619	0.008684	-0.001745 – 0.03464	0.0001	Yes (↓)

Table F12. Log-rank statistical analysis of fly longevity with altered expression of *TER94* through the *ddc-Gal4$^{HL4.3D}$; UAS-parkin-RNAi* transgene.

Genotype	Number of flies	Median survival (days)	Bonferroni corrected P-value	Chi-Square value	Significant
ddc-Gal4$^{HL4.3D}$;UAS-parkin-RNAi;UAS-lacZ	253	84	N/A	N/A	N/A
ddc-Gal4$^{HL4.3D}$;UAS-parkin-RNAi;UAS-TER94-RNAi^{GL00448}	280	58	<0.0001	230.60	Yes (↓)

Table F13. Statistical analysis of locomotor ability with altered expression of *TER94* through the *ddc-Gal4$^{HL4.3D}$; UAS-parkin-RNAi* transgene.

Genotype	Slope (k)	Standard Error	95% Confidence Interval	P-value	Significant
ddc-Gal4$^{HL4.3D}$;UAS-parkin-RNAi;UAS-lacZ	0.01900	0.004663	0.009792 – 0.02875	N/A	N/A
ddc-Gal4$^{HL4.3D}$;UAS-parkin-RNAi;UAS-TER94-RNAi^{GL00448}	0.01865	0.004787	0.009020 – 0.02854	<0.0001	Yes (↓)