Calcium-independent Phospholipase $A_2\beta$ is Vital in Neurons and Possibly Other Tissues to Inhibit Age-Related Motor Degeneration

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Abstract

By 2020, nearly one million Americans will be living with Parkinson's disease (PD), and nearly ten million worldwide. This number outstrips every other age-related neurodegenerative disease save for Alzheimer disease (AD). The underlying cause of PD remains elusive, as does a medical remedy. Several genes have been linked to the disease, such as *PLA2G6* (also known as *iPLA$_2$β, PARK14*, and *iPLA2-VIA*), a calcium-independent phospholipase A2 (iPLA$_2$), which has been implicated in two forms of PD: Dystonia-parkinsonism (DP) and autosomal recessive early-onset parkinsonism (AREP). *PLA2G6* has also been associated with Infantile neuroaxonal dystrophy (INAD) and atypical neuroaxonal dystrophy (aNAD). The model organism *Drosophila melanogaster* contains one homolog for *PLA2G6*. Previous studies have established knockout *PLA2G6 Drosophila* mutants showing locomotor defects, decreased lifespan, and increased sensitivity to oxidative stress – all symptoms of PD and other *PLA2G6*-associated neurodegeneration (PLAN) diseases. To better understand the mechanism and cell types by which *PLA2G6* works, I used the *GAL4-UAS* and RNAi systems to generate two fly lines: one with *PLA2G6* knockdown in neuronal tissue and one with ubiquitous knockdown. I found that the while *PLA2G6* was required for neuronal upkeep to prevent age-related locomotor decline, neuronal tissue alone does not explain the full extent of the disease pathology. Based on these findings, a more universal approach should be taken when dealing with *PLA2G6* related diseases. I believe that this information will be insightful and useful for future therapeutic approaches combating PLAN diseases.

Introduction

This year, 60,000 Americans will be diagnosed with Parkinson's disease (*Statistics*, 2019). Simple tasks such as sipping coffee, walking up a flight of stairs, and speaking to loved ones will become insurmountable challenges for these people. They will join the almost one million Americans who live and struggle with this debilitating disease. Resting tremors, rigid muscles, slowed movements, and impaired speech will result from the gradual loss of motor control. A hallmark of the disease is progressive deterioration of dopaminergic neurons in the substantia

nigra pars compacta (SNpc) portion of the mid-brain. The molecular causes of dopaminergic cell death, however, are still unclear. Even after decades and billions of dollars' worth of research, a cure to the disease has alluded the medical community (Chai & Lim, 2013).

In recent years, several single-gene mutations have been linked to instances of inherited familial PD. These inherited cases, however, are rare. Most cases of PD are sporadic in nature, meaning they stem from a mix of environmental and genetic factors (Chai & Lim, 2013; Modi *et al*., 2016). While the sporadic and familial forms of PD appear to differ from one another, the two are pathologically and clinically identical. This commonality supports the idea that mechanistically the same underlying issues are occurring in both forms of the disease. An analysis of inherited PD, therefore, can offer insights into the molecular mechanisms behind the more frequent sporadic PD.

One such gene, which when mutated leads to PD, is *iPLA2β,* a calcium-independent phospholipase. Phospholipases A_2 (PLA_2) are a group of enzymes that catalyze the transformation of glycerophospholipids to fatty acids and lysophospholipids through the cleavage of the sn-2 ester. They have been implicated in a variety of functions, and there are currently more than 30 types of PLA_2 known (Schaloske, 2006; Ramanadham *et al*., 2015). A subset of PLA_2 enzymes, known as calcium-independent phospholipases or $iPLA_2$, perform their catalytic function independently of Ca^{2+}. Of the 9 enzymes in the $iPLA_2$ family, the most researched of the group is the enzyme encoded by *iPLA$_2$β* (synonyms: *PLA2G6, iPLA2-VIA, GVIA PLA2, iPLA2-β, PNPLA9, PARK14*). In humans, the *PLA2G6* gene encodes $iPLA_2$β, is found on chromosome 22, and has broad expression throughout the body (*PLA2G6 Phospholipase,* 2019).

PLAN diseases

Several diseases have been linked to mutations in *PLA2G6*. Collectively these diseases are referred to as *PLA2G6*-associated neurodegeneration (PLAN) diseases, and include: infantile neuroaxonal dystrophy (INAD), atypical neuroaxonal dystrophy (aNAD) (Khateeb *et al*. 2006; Morgan *et al*., 2006), Dystonia-Parkinsonism (DP) (Paizen-Ruiz *et al*., 2009), and autosomal recessive early-onset Parkinsonism (AREP) (Gregory *et al*., 2008). INAD is an autosomal recessive disease which commences from six months to three years of age. Children with INAD

have severely limited motor and neurological function, retardation, visual issues, and death before age 10. In aNAD, onset of the disease is later and progresses more slowly (Khateeb *et al*. 2006; Kurian *et al*. 2011). *PLA2G6* related PD usually sets in during one's adult years, and patients display a slew of neurodegenerative symptoms including, but not limited to, tremors, hypokinesia, gait disturbance, dystonia, dysarthria, rapid cognitive decline, and neuropsychiatric changes (*NBIA*, 2019). In the past decade, much research has been done to better understand these diseases.

Animal models

In 2008, two independent teams established knockout (KO) *PLA2G6* mouse models (Shinzawa *et al.*, 2008; Malik *et al*., 2008). Both studies found that the mutated mice had motor impairment by age 1, and death by age 2. At 15 weeks, spheroids and vacuoles were found throughout the central and peripheral nervous system. There was axonal degeneration in the central and peripheral nervous system, as well as cerebral atrophy. Similar issues are seen in PLAN patients (Shinzawa *et al.*, 2008; Malik *et al.*, 2008; Beck *et al.*, 2011; Sumi-Akamura *et al.*, 2015).

Interestingly, in a subsequent study, it was found that mice with point mutations, as opposed to complete KO of *PLA2G6*, had earlier onset of the disease, more rapid progression, and lethality occurred at an earlier age. These phenotypes align with INAD, while the null mutant phenotypes seem to align with aNAD (Wada *et al.*, 2009; Sumi-Akamaru *et al.*, 2015). In a 2018 study, a knockin (KI) mouse model of early-onset PARK14 was generated from a homozygous point mutation in *PLA2G6* (D33Y1). This model phenocopied early-onset PD and showed that mice containing a specific homozygous point mutation had early neurodegeneration in the SNpc due to mitochondrial dysfunction, mitophagy impairment, transcriptional abnormality, and ER stress (Chiu *et al.*, 2018).

While mice models are useful, the fruit fly, *Drosophila melanogaster*, has proven to be an apt model organism for studying human diseases, specifically neurodegenerative ones. Cheap costs, a short lifecycle, and high functional conservation between fruit flies and vertebrates have made *Drosophila* a fundamental model organism for studying human biology. In addition, numerous tools and systems have been developed to help manipulate and analyze the fly genome. Two systems which I employed were the *GAL4-UAS* and the RNAi systems.

GAL4-UAS and RNAi systems

The *GAL4-UAS* system (Caygil & Brand, 2006) uses GAL4, a transcriptional activator from yeast, which binds to an upstream activating sequence (UAS). Any gene downstream of the UAS is transcribed in the presence of GAL4. *Drosophila* has no endogenous UAS or GAL4, but both can be inserted into the fly genome. The system is bipartite: one fly line containing GAL4 is crossed with another fly line containing a UAS element. The resulting progeny contain both the GAL4 and UAS genes (Fig. 1).

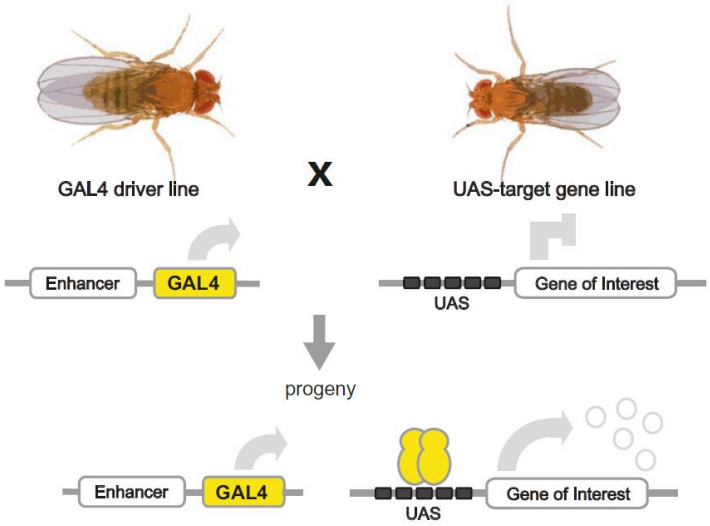


Figure 1. Schematic of *GAL4-UAS* system. Separate GAL4 driver (under the control of a tissue-specific enhancer) and UAS-target lines (driving expression of the gene of interest) are crossed to one another. The progeny produces the gene of interest, but the original driver and target line do not. Image adapted from Caygil & Brand, 2006.

The GAL4 system can be spatially and developmentally specific depending on the genomic locus into which the GAL4 gene is inserted. For instance, when GAL4 is inserted downstream of the endogenous *elav* gene promoter, a gene which is transcribed only in neuronal tissues, GAL4 will be selectively transcribed in neuronal tissue. Today there are numerous fly stock centers containing thousands of different GAL4 driver lines with specific spatial, as well as developmental, transcription patterns.

RNA interference, or RNAi, is a nucleotide sequence-specific tool which mediates mRNA degradation (Fire *et al.*, 1998). Used in conjunction with the *GAL4-UAS* system, RNAi can selectively knock down gene function in a single cell type (Roignant *et al.*, 2003). In my study, I used a fly line containing an *iPLA$_2\beta$* specific RNAi transgene downstream of UAS. When crossed with a GAL4 fly line, *iPLA$_2\beta$* was selectively knocked down in the progeny.

Drosophila model

One ortholog for *iPLA$_2\beta$* has been found in *Drosophila*: CG6718 (*iPLA2*-VIA). Fly models with partial or complete loss of iPLA$_2$β function mutations were reported by Malhorta *et al.* (2009), Kinghorn *et al.* (2015), Lin *et al.* (2018), Iliadi *et al.* (2018), and Schonbrun *et al.* (publication in progress). Such fly lines had a reduced lifespan and severe locomotor defects, both symptoms of PLAN patients (Fig. 2).

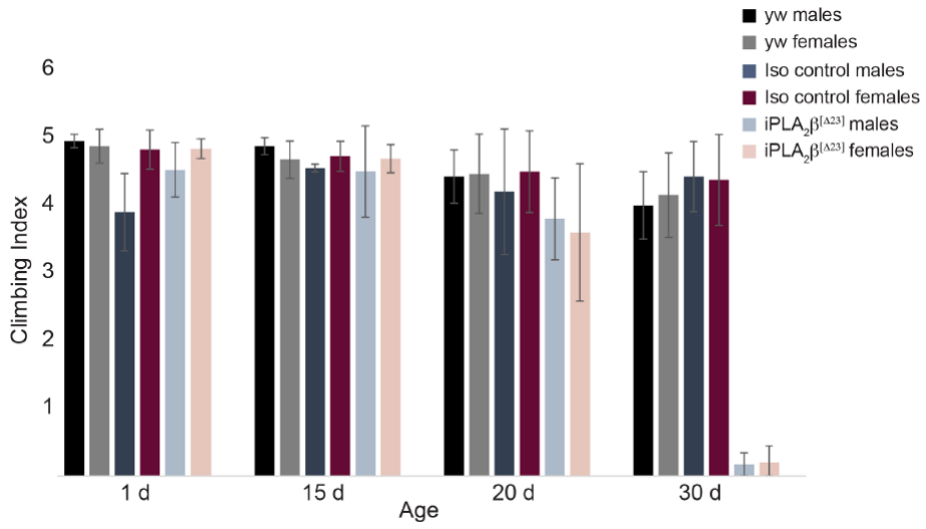


Figure 2. *iPLA$_2\beta$* knockout mutants from Schonbrun *et al.* Both male and female mutants show rapid locomotor decline after 20 days compared to isogenic control. Climbing index measures the climbing ability of the flies (explained more in-depth below). Image adapted from Schonbrun *et al.*, publication in progress.

As the *PLA2G6* gene is associated with locomotor decline, the prime suspects for its locus of action were muscle and neuronal tissue. Based on this, Schonbrun *et al.* compared the effects

of *iPLA$_2\beta$* knockdown in muscle tissue to knockdown in neuronal tissue. They observed severe climbing defects in flies with neuronal knockdown, but little to no impact in the muscle knockdown line. Unfortunately, the study done by Schonbrun *et al.* was limited in size and control groups, and the flies were not tested throughout their lives to observe progressive motor decline. Other studies used similar neuronal knockdown procedures to observe the spatial effects of *iPLA$_2\beta$* (Kinghorn *et al.*, 2015; Illiadi *et al.*, 2018). These observations have led to the belief that neuronal damage is the main culprit behind the locomotor decline and early mortality seen in animal models, and human patients, with mutated *iPLA$_2\beta$*. In my study, however, I show that this theory is an oversimplification of the PLAN pathology.

I generated two fly lines: one with knockdown of *iPLA$_2\beta$* in neuronal tissue and another with ubiquitous knockdown. While both lines showed locomotor defects, the line with ubiquitous knockdown showed more severe and earlier locomotor defects. This points to the possibility that *iPLA$_2\beta$* plays a larger role, outside of neurodegeneration, which contributes to locomotor defects and early mortality. These finding will hopefully be insightful for future research on PLAN diseases, including PD, and subsequent therapeutic approaches. By using the *GAL4-UAS* system in conjunction with RNAi, I observed that while neuronal tissue plays a large role in locomotor defects, it is not the only piece of the puzzle.

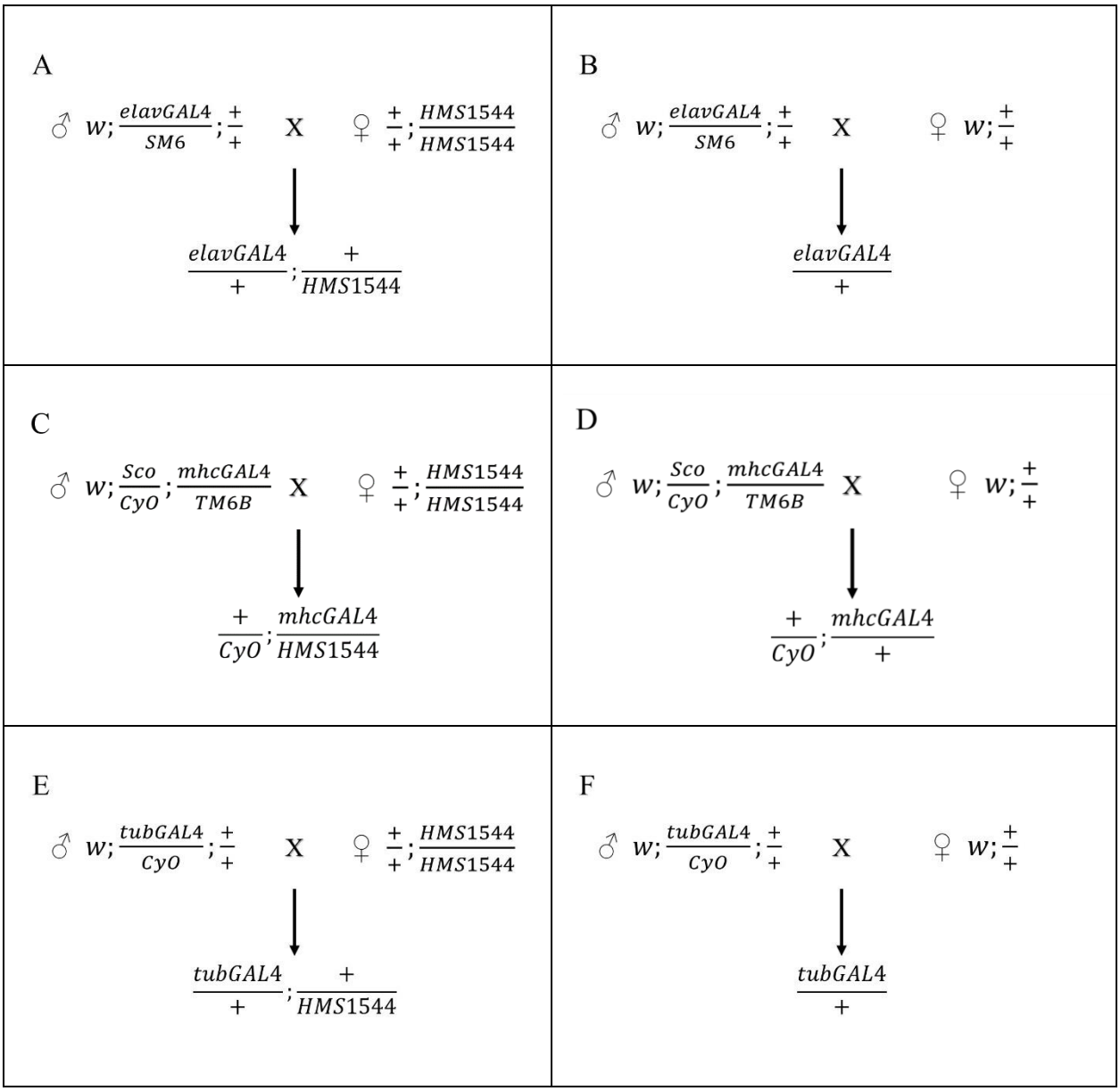
Material and Methods

Drosophila strains and husbandry

HMS01544, *mhc-GAL4*, *tubulin-GAL4*, and w^{1118} were from Bloomington Drosophila Stock Center. *Elav-GAL4* was a gift from J. Treisman. All flies were kept at 26.0 ± 0.2 ˚C and standard media was used for culturing flies: 3.83% molasses, 1.58% yeast, and 3.83% corn meal supplemented with 0.11% methyl paraben and 0.38% propionic acid as mold inhibitors.

Fly crosses

Five fly lines were used to set seven crosses constituting the parental (P) generation. The lines were: *elav-GAL4* (neuronal driver), *mhc-GAL4* (muscle driver), *tub-GAL4* (ubiquitous driver), *HMS01544* (*UAS-RNAi* for *iPLA$_2\beta$*), and w^{1118} (wild type). Each of the three GAL4 lines were crossed to both *HMS1544* and w^{1118} for a total of six crosses. The seventh cross was *HMS1544* to w^{1118}. Virgin females were used for crosses containing w^{1118}, and virgin *HMS1544* females were used for the remaining three crosses. Fig. 3 shows these seven crosses.



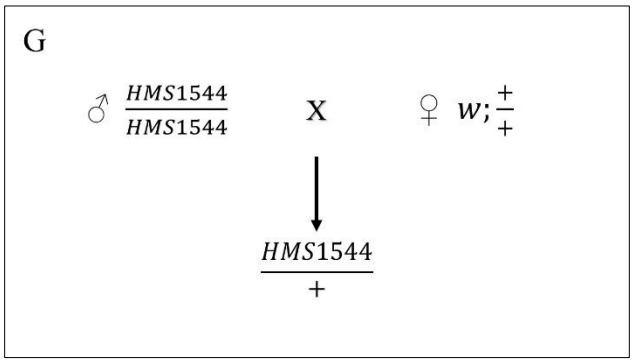


Figure 3. Seven crosses performed. Only the desired progenies are written. Male fly genotype on left side and female on right side of each both . The progenies were selected against based on balancer markers. Both SM6 (Cross A and B) and CyO (Cross E and F) are curly winged phenotypes. Therefore, curly flies were selected against for Cross A, B, E, and F. TM6B (Cross C and D) is humeral phenotype; curly and non-humeral were, therefore, selected for in Cross C and D.

F1 collection

For each cross, two vials were set with a total of 10-15 flies per vial. Each P vial was passed every two days. After ~10 days, the F1 generation flies were sorted and collected over CO_2. All crosses, other than *HMS1544* crossed to w^{1118}, had balancers to select against (Fig. 3). For crosses containing *elav-GAL4* and *tub-GAL4,* curly wings were selected against (Cross A, B, E, and F). In crosses containing *mhc-GAL4* there were two balancers. *Mhc-GAL4* was balanced on the third chromosome with humeral, so humeral was selected against. The second chromosome contained scutoid/curly (Sco/CyO), and scutoid masks the humeral phenotype. Therefore, progeny containing scutoid could not be used as it was unclear whether the *mhc-GAL4* gene was present or not. For the *mhc-GAL4* crosses, therefore, curly wings were selected for and humeral was selected against. In the w^{1118} to *HMS1544* cross all progeny were collected. Both male and female progeny were collected and kept in separate vials with ~10-15 flies per vial. F1 vials were passed 1-2 times a week.

Climbing assays

Loss of locomotor ability, especially with aging, is a common pathology in PLAN patients. To relate this symptom in *Drosophila,* climbing assays were conducted to analyze the age-dependent effect of *PLA2G6* knockdown. Assays, which utilize a fly's negative geotaxis system, were conducted at varying ages: 10, 20, 30, 35, and 40 days. Progeny of w^{1118} crossed to *HMS1544* were tested up to 70 days as a comparison for wild type climbing ability. For tests, 8-15 flies were transferred into a new food vial, and an empty vial was secured atop it with scotch tape. The flies were tapped to the bottom and given 20 seconds to climb into the upper vial. The process was repeated four more times in succession, for a total of five tests. Each fly to reach the top half of the apparatus per trial was given a point. The Climbing Index (CI) for each assay was determined based on the sum of the number of flies that climbed into the upper vial over the five 20 s trials, divided by the number of flies in the group (5 being the best climbing ability, 0 being the worst). For each condition, at least three groups were averaged to derive the mean CI. For 10 d assays, the flies were transferred using CO_2 and then given time to recover before performing the assay. For all other age groups, flies were transferred without CO_2.

Statistical analysis

T-tests and their resulting P values were used to compare *GAL4>RNAi* CI to their genetic background control lines carrying *GAL4* alone. *p < 0.05, **p < 0.01, ***p < 0.005, ****p < 0.0005. Statistical analysis by unpaired t-test. Error bars represent standard deviation.

Results

Whole-body knockdown of *iPLA$_2\beta$* causes severe climbing defects

The *tub-GAL4* driver was used as a positive control to observe the effects of ubiquitous knockdown of *iPLA$_2\beta$.* This cross verified the effectiveness of the *GAL4>RNAi* system in knocking down iPLA$_2$β production. *Tub-GAL4* alone was used a genetic background control to ensure that there were no issues with the *tub-GAL4* fly line.

Even at 10 d, both male and female *tub>RNAi* flies had lower climbing indexes than their control counterparts; only male climbing, however, was statistically significant (male: p=0.0045; female: p=0.14) (Fig. 4). This trend continued at 20 d: *tub>RNAi* male climbing was severely diminished, dropping to a CI of almost 2 ($p=1.2\ (10^{-7})$), while female CI stayed above 4 (p=0.03). Both the female and male controls had a CI close to 5 at 20 d. By 30 d the climbing issues for both male and female flies were severe, and only ~35% of male flies survived. Although background control flies' CI decreased slightly, both male and female CI stayed above 4. Statistical comparison of control to RNAi confirmed that by 30 d the climbing ability of the RNAi flies was severely diminished (male: $p=9.5\ (10^{-9})$; female: $p=2.6\ (10^{-6})$). By 35 d all male mutant flies had died, and female flies had severe climbing defects ($p=1.5\ (10^{-4})$). All RNAi flies were dead by 40 d, while both male and female background control flies continued to have high CI.

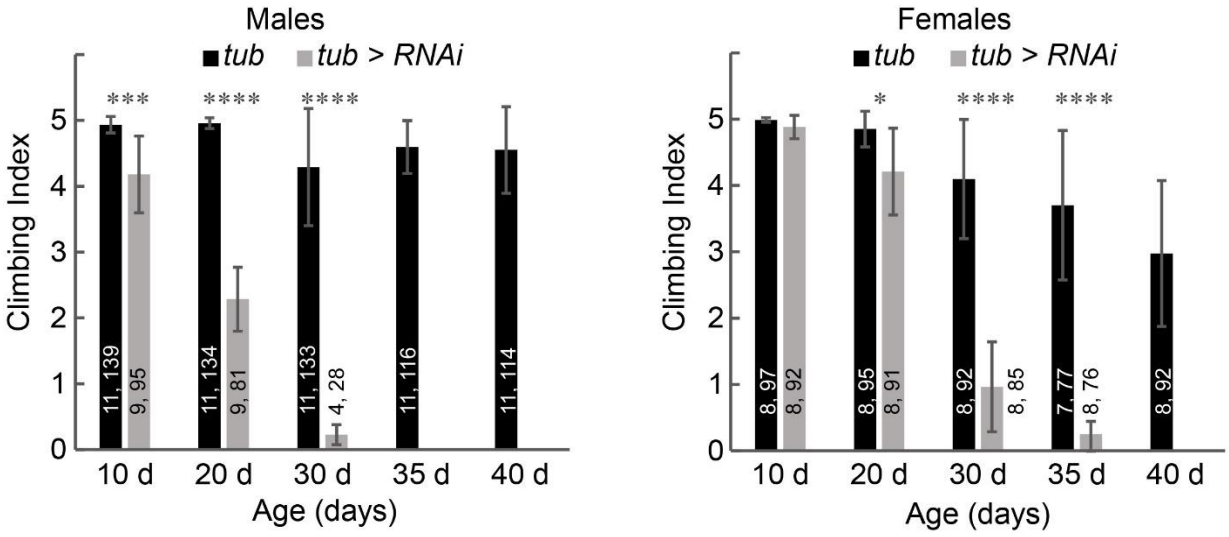


Figure 4. Male and Female *tub>RNAi* CI. The lower number in the bar line signifies the number of vials tested, and the upper number signifies the total number of flies tested. Error bars show standards deviation.

Neuronal specific knockdown of *iPLA$_2\beta$* causes climbing defects in aged male flies

Elav-GAL4 is a neuronal driver, and *elav>RNAi*, therefore, had selective knockdown of *iPLA$_2\beta$* in neuronal tissue. *Elav-GAL4* alone flies were used as a genetic background control to ensure that there were no issues with the *elav-GAL4* line.

While both male and female *elav>RNAi* flies decreased in climbing ability over time, when comparing their CI to the CI of controls, only male flies at 40 d showed a strong statistically significant difference ($p=2.0\ (10^{-4})$) (Fig. 5).

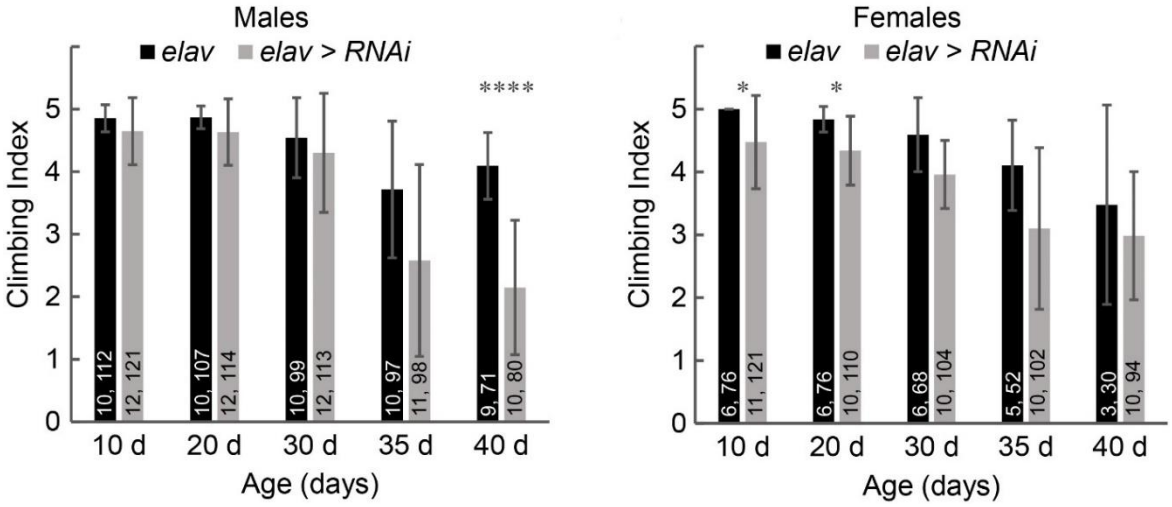


Figure 5. Male and Female *elav>RNAi* CI. The lower number in the bar line signifies the number of vials tested, and the higher numbers signifies the number of total flies tested. Error bars show standards deviation.

Control flies show minimal climbing defects until 60 days

Wild-type flies were crossed to the UAS-RNAi line to ensure that the RNAi line alone does not induce age-related climbing defects without GAL4. Up to 35 d, both male and female *wt>RNAi* had climbing indexes above 4 (Fig. 6). From 40 to 60 d both CI stayed relatively high between 3 and 4, with only females dropping below 3 at 60 d. By 70 d both male and female flies had CI below 1, again with females slightly lower than males.

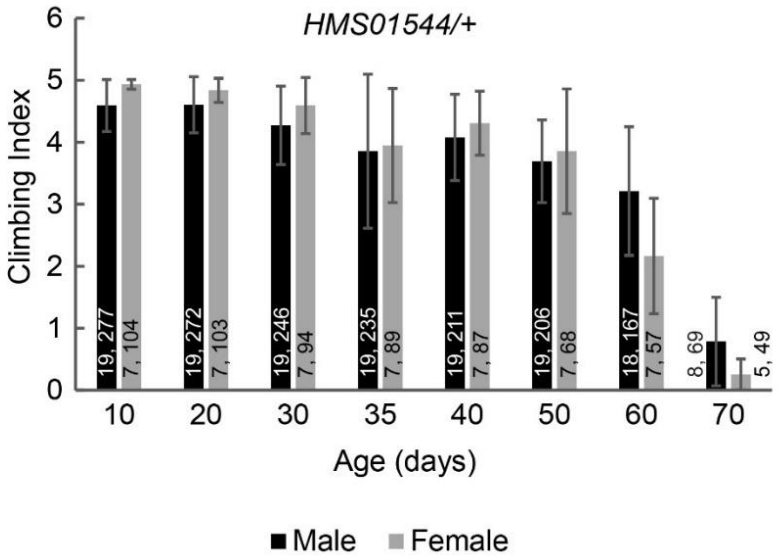


Figure 6. Male and Female *wt>RNAi*. The lower number in the bar line signifies the number of vials tested, and the higher numbers signifies the number of total flies tested. Error bars show standards deviation.

Muscle-specific GAL4 driver line was found to be faulty

Mhc-GAL4, which is a muscle-specific driver, was used to test the effects of *iPLA$_2\beta$* knockdown in muscle tissue. *Mhc-GAL4* alone was used as a genetic background control to ensure there were no issues with the *mhc-GAL4* driver line. At 10 d there seemed to be major climbing defects in male *mhc>RNAi* (Fig. 7). It was determined that it was not the knockdown of *iPLA$_2\beta$* in muscle tissue however, which was causing the climbing defects. Rather the *mhc-GAL4* line was faulty. *Mhc>wt* had a low CI even at 10 d, for both male and female flies, which points to the fact that it was the *mhc-GAL4* line that was responsible for the climbing defects and not the knockdown of *iPLA$_2\beta$.*

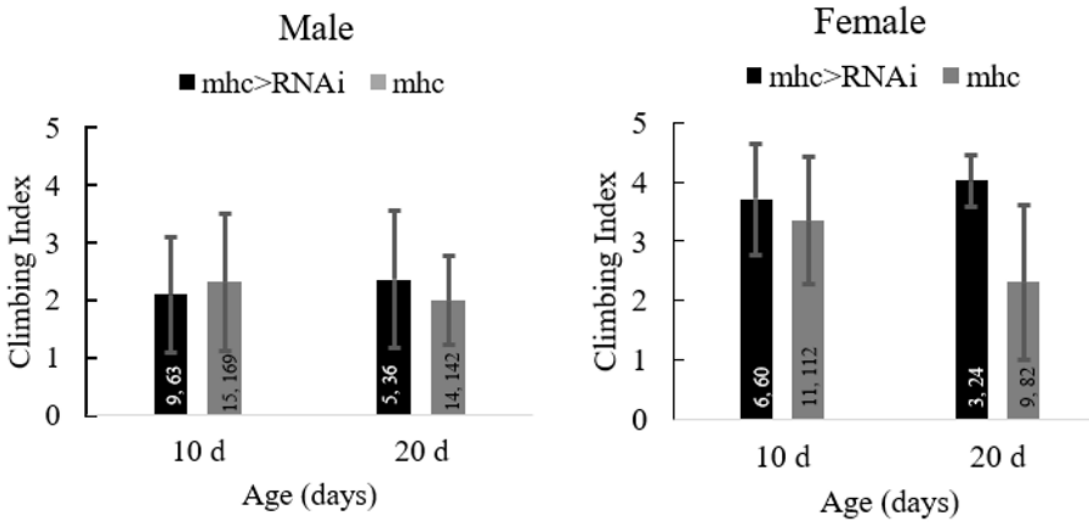


Figure 7. ***Male and Female mhc>RNAi CI.*** The lower number in the bar line signifies the number of vials tested, and the higher numbers signifies the number of total flies tested. Error bars show standards deviation.

Discussion

Male vs. female climbing ability

Throughout the study an interesting phenomenon was observed: male RNAi flies, when compared to female RNAi flies, had more severe and earlier climbing defects. This was observed in both *elav>RNAi* as well as *tub>RNAi.* T-tests confirmed this difference between male and female *tub>RNAi* flies which had a $p<0.05$ for all aged assays (Appendix 1). T-tests comparing male and female *elav>RNAi* however only had $p<0.1$ for 40-day assays (Appendix 1). Interestingly, the opposite was observed in the *wt>RNAi*, as well as both *elav>wt* and *tub>wt* background controls, with males having a higher CI than females. These results fit with the literature which has shown that RNAi usually works better in males (Kaya-Copur & Schnorrer, 2016). It is unclear whether this is because of the higher RNAi efficiency or GAL4 activity; either way, it is important to control for sex with experiments using RNAi and GAL4, something which previous studies of *PLA2G6* in *Drosophila* labs had not done (for example, see Illiadi *et al*., 2018).

Pan-neuronal and ubiquitous knockdown

A hallmark of PD, as well as the other PLAN diseases, is age-related motor decline. Climbing assays are used to model this motor dysfunction in *Drosophila*, which have a negative geotaxis system that senesces with age (Grotewiel *et al*., 2005; Illiadi *et al*., 2016). In previous studies, it had been shown that *iPLA$_2\beta$* knockout mutants had severe climbing defects by 30 d (Iliadi *et al*., 2018; Lin *et al*., 2018; Schonbrun *et al*., publication in progress). Muscle and neuronal cell death were the primary suspects for the motor dysfunction, as iPLA$_2$β has been connected to neuronal protection and mitochondrial maintenance (Kinghorn *et al*., 2015; Sum-Akamaru *et al*., 2015). I set out to test both tissue types with tissue specific GAL4 drivers in conjunction with RNAi knockdown of *iPLA$_2\beta$*. Unfortunately, the muscle-specific GAL4 line was found to be faulty, as the background control line had climbing issues as early as 10 d. The *mhc>RNAi* portion of the experiment, therefore, had to be discarded. While the underlying issues with the *mhc-GAL4* line are unclear, one theory is that the presence of multiple balancers caused the flies to be unhealthy (*Bloomington Drosophila Stock Center*, 2019).

The pan-neuronal *elav-GAL4* line contained only one balancer and there seemed to be no problems with this fly line; *elav>wt* climbed well throughout the assays. The results from the *elav>RNAi* cross showed that *iPLA$_2\beta$* plays a vital role in neuronal cells' integrity. *Elav>RNAi* consistently had a lower CI than its background control, and while only male 40 d flies had a $p<0.05$, the CI of both the males and females were consistently lower than their background control and decreased over time (Fig. 5). These results confirm what had previously been observed by Kinghorn *et al*. (2015), Iliadi *et al*. (2018), and Schonbrun *et al*. (publication in progress). This behavior, of progressive neurodegeneration, aligns with PLAN symptoms, specifically PD where locomotor issues arise later in life.

In previous studies of *iPLA$_2\beta$* there was a focus on neuronal tissue. My study of *tub>RNAi*, however, points to the possibility that a more whole-body outlook must be taken. Had neuronal damage been the sole cause of the climbing defects, the *tub>RNAi* should have phenocopied *elav>RNAi*. *Tub>RNAi* flies however, had more severe as well as earlier climbing defects. This points to the conclusion that climbing defects resulting from mutations in the *iPLA$_2\beta$* gene cannot be solely attributed to neuronal damage. This also explains why the climbing ability of

elav>RNAi knockdown flies was not as severe as mutant knockout flies in previous studies (Iliadi, *et al*. 2018; Schonbrun *et al*., publication in progress). *Elav>RNAi* is neuronal specific while knockout mutant flies, as well as humans with PLAN diseases, have mutations throughout their bodies. Another possible explanation for this disparity between *elav>RNAi* and knockout lines is that knockout lines contain no iPLA$_2$β protein while knockdown lines contain small amounts of protein from mRNA that evaded RNAi degradation. This, however, would not explain the difference between *tub>RNAi* and *elav>RNAi* as both are knockdown fly lines.

This line of reasoning assumes that the *tub-GAL4* driver is of equal strength as the *elav-GAL4* driver; a fact that is not necessarily true. While *elav-GAL4* driver is a known neuronal-specific driver, there are no studies comparing the strength of the *tub-GAL4* driver to the *elav-GAL4* driver (Kaya-Copur & Schnorrer, 2016). Yet even if the *tub* driver is stronger than the *elav* driver, the discrepancy between the two crosses seems too large to be explained only by the strength of the driver. The age discrepancy particularly implies that there is some other aspect of the *tub>RNAi* line which is not explained by the driver strength. More likely, iPLA$_2$β plays an upkeep role in other parts of the fly body outside the neurons that affects motor function.

While knockout *PLA2G6* mutants represent inherited PD, a small portion of overall PD cases, the process occurring in the cells that lead to PD is the same as in the more common sporadic PD. Therefore, an analysis of *PLA2G6* mutant cells can be extremely useful in understanding the mechanisms of sporadic PD. For instance, the mechanisms of cell death resulting from mutations in *PLA2G6* are still being studied and debated by researchers. Many publications have posited that the resulting pathology from mutated *iPLA$_2$β* is the consequence of a defect in phospholipid metabolism and the subsequent inability of cells to perform cardiolipin remodeling and upkeep of the mitochondrial membrane (Beck *et al.*, 2011; Kinghorn *et al.*, 2015). Lin *et al.* (2018) and Schonburn *et al.* (publication in progress) however, showed that phospholipid levels remain unchanged in flies with mutated *iPLA$_2$β.* Lin *et al.* claimed that iPLA$_2$β plays a vital role in retromer function, sphingolipid metabolism, and vesicle trafficking; it is the breakdown of these cellular functions which causes the subsequent pathologies of PLAN patients. My results from *tub>RNAi* flies imply that cell death is occurring in additional parts of the body other than the neurons. This is an important finding as these locations can be pinpointed and then used as a

proxy to observe mechanisms of cell death. This information will hopefully be helpful in designing future therapeutic treatments for PLAN patients.

References

Beck G, Kato S, Mochizuki H. 2015. Neuroaxonal dystrophy in PLA2G6 knockout mice - Sumi-Akamaru - 2015 - Neuropathology - Wiley Online Library. Neuropathology. https://onlinelibrary.wiley.com/doi/full/10.1111/neup.12202

Bloomington Drosophila Stock Center. https://bdsc.indiana.edu/stocks/balancers/balancer_intro.html

Caygill EE, Brand AH. 2016. Erratum to: The GAL4 System: A Versatile System for the Manipulation and Analysis of Gene Expression. Methods in Molecular Biology Drosophila

Chai C, Lim K-L. 2013. Genetic insights into sporadic Parkinson's disease pathogenesis. Current genomics. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3924245/

Chiu C-C, Lu C-S, Weng Y-H, Chen Y-L, Huang Y-Z, et al. 2018. PARK14 (D331Y) PLA2G6 Causes Early-Onset Degeneration of Substantia Nigra Dopaminergic Neurons by Inducing Mitochondrial Dysfunction, Er Stress, Mitophagy Impairment and Transcriptional Dysregulation in a Knockin Mouse Model. *Molecular Neurobiology*. 56(6):3835–53

Forsell PKAL, Kennedy BP, Claesson H-E. 1999. The human calcium-independent phospholipase A2 gene. Multiple enzymes with distinct properties from a single gene. European Journal of Biochemistry. 262(2):575–85

Gregory A, Westaway SK, Holm IE, Kotzbauer PT, Hogarth P, *et al*. 2008. Neurodegeneration associated with genetic defects in phospholipase A2. Neurology. https://n.neurology.org/content/71/18/1402.long

Gregory A. 2017. *PLA2G6-Associated Neurodegeneration*. GeneReviews® [Internet]. https://www.ncbi.nlm.nih.gov/books/NBK1675/

Grotewiel MS, Martin I, Bhandari P, Cook-Wiens E. 2005. Functional senescence in Drosophila melanogaster. Ageing Research Reviews. 4(3):372–97

Hewitt, V.l., and A.j. Whitworth. “Mechanisms of Parkinson's Disease.” Current Topics in Developmental Biology Fly Models of Human Diseases, 2017, pp. 173–200., doi:10.1016/bs.ctdb.2016.07.005.

Iliadi KG, Gluscencova OB, Boulianne GL. 2016. Psychomotor Behavior: A Practical Approach in Drosophila. Frontiers in Psychiatry. 7:

Iliadi KG, Gluscencova OB, Iliadi N, Boulianne GL. 2018. Mutations in the Drosophila homolog of human PLA2G6 give rise to age-dependent loss of psychomotor activity and neurodegeneration. Nature News. https://www.nature.com/articles/s41598-018-21343-8

Kurian MA, Hayflick SJ. 2013. Pantothenate Kinase-Associated Neurodegeneration (Pkan) and PLA2G6-Associated Neurodegeneration (Plan). International Review of Neurobiology Metal Related Neurodegenerative Disease, pp. 49–71

Morgan NV, Westaway SK, Morton JEV, Gregory A, Gissen P, *et al*. 2006. PLA2G6 , encoding a phospholipase A 2 , is mutated in neurodegenerative disorders with high brain iron. Nature News. https://www.nature.com/articles/ng1826

Murakami M, Taketomi Y, Miki Y, Sato H, Hirabayashi T, Yamamoto K. 2011. Recent progress in phospholipase A2 research: From cells to animals to humans. Progress in Lipid Research. 50(2):152–92

PLA2G6 phospholipase A2 group VI [Homo sapiens (human)] - Gene - NCBI. Current neurology and neuroscience reports. https://www.ncbi.nlm.nih.gov/gene/8398#variation

Kaya-Çopur A, Schnorrer F. 2016. A Guide to Genome-Wide In Vivo RNAi Applications in Drosophila. Methods in Molecular Biology Drosophila, pp. 117–43

Khateeb S, Flusser H, Ofir R, Shelef I, Narkis G, et al. 2006. PLA2G6 mutation underlies infantile neuroaxonal dystrophy. American journal of human genetics. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1698558/

Kinghorn KJ, Castillo-Quan JI, Bartolome F, Angelova PR, Li L, et al. 2015. Loss of PLA2G6 leads to elevated mitochondrial lipid peroxidation and mitochondrial dysfunction. Brain : a journal of neurology. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4559908/

Lands WE. 2000. Stories about acyl chains. Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids. 1483(1):1–14

Malhotra A, Edelman-Novemsky I, Xu Y, Plesken H, Ma J, et al. 2009. Role of calcium-independent phospholipase A2 in the pathogenesis of Barth syndrome. Proceedings of the National Academy of Sciences. 106(7):2337–41

Malik I, Turk J, Mancuso DJ, Montier L, Wohltmann M, et al. 2008. Disrupted membrane homeostasis and accumulation of ubiquitinated proteins in a mouse model of infantile neuroaxonal dystrophy caused by PLA2G6 mutations. The American journal of pathology. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2312364/

Modi P, Mohamad A, Phom L, Zevelou, Koza, et al. 2016. Understanding Pathophysiology of Sporadic Parkinson's Disease in Drosophila Model: Potential Opportunities and Notable Limitations. IntechOpen. https://www.intechopen.com/books/challenges-in-parkinson-s-disease/understanding-pathophysiology-of-sporadic-parkinson-s-disease-in-drosophila-model-potential-opportun

NBIA. http://nbiacure.org/learn/nbia-disorders/plan/dystonia-parkinsonism/

Paisan-Ruiz, C., Bhatia, K.P., Li, A., Hernandez, D., Davis, M., Wood, N.W., Hardy, J., Houlden, H., Singleton, A., and Schneider, S.A. 2009. Characterization of PLA2G6 as a locus for dystonia-parkinsonism. Ann. Neurol. 65, 19–23.

Phospholipase PLA2G6, a Parkinsonism-Associated Gene, Affects Vps26 and Vps35, Retromer Function, and Ceramide Levels, Similar to α-Synuclein Gain. 2018. Cell Metabolism. https://www.sciencedirect.com/science/article/pii/S1550413118303267?via=ihub

Ramanadham S, Ali T, Ashley JW, Bone RN, Hancock WD, Lei X. 2015. Calcium-independent phospholipases A2and their roles in biological processes and diseases. Journal of Lipid Research. 56(9):1643–68

Renne MF, Bao X, Smet CHD, Anton I. P. M. De Kroon. Lipid Acyl Chain Remodeling in Yeast - Mike F. Renne, Xue Bao, Cedric H. De Smet, Anton I. P. M. De Kroon, 2015. SAGE Journals.

https://journals.sagepub.com/doi/full/10.4137/LPI.S31780?url_ver=Z39.88-2003&rfr_id=ori:rid:crossref.org&rfr_dat=cr_pub=pubmed
Roignant J-Y. 2003. Absence of transitive and systemic pathways allows cell-specific and isoform-specific RNAi in Drosophila. Rna. 9(3):299–308
Schaloske RH, Dennis EA. 2006. The phospholipase A2 superfamily and its group numbering system. Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids. 1761(11):1246–59
Shinzawa K, Sumi H, Ikawa M, Matsuoka Y, Okabe M, et al. 2008. Neuroaxonal Dystrophy Caused by Group VIA Phospholipase A2 Deficiency in Mice: A Model of Human Neurodegenerative Disease. Journal of Neuroscience. https://www.jneurosci.org/content/28/9/2212.long
“Statistics.” Parkinson's Foundation, 13 June 2019, www.parkinson.org/Understanding-Parkinsons/Statistics.
Sun GY, Xu J, Jensen MD, Simonyi A. 2003. Phospholipase A2in the central nervous system. Journal of Lipid Research. 45(2):205–13
Sumi-Akamaru H, Beck G, Kato S, Mochizuki H. 2015. Neuroaxonal dystrophy inPLA2G6knockout mice. Neuropathology. 35(3):289–302
van Meer, G., Voelker, D.R., and Feigenson, G.W. (2008). Membrane lipids: where they are and how they behave. Nat Rev Mol Cell Biol 9, 112-124.
Wada H, Yasuda T, Miura I, Watabe K, Sawa C, et al. 2009. Establishment of an Improved Mouse Model for Infantile Neuroaxonal Dystrophy That Shows Early Disease Onset and Bears a Point Mutation in Pla2g6. The American Journal of Pathology. 175(6):2257–63
Yamashita A, Hayashi Y, Nemoto-Sasaki Y, Ito M, Oka S, et al. 2014. Acyltransferases and transacylases that determine the fatty acid composition of glycerolipids and the metabolism of bioactive lipid mediators in mammalian cells and model organisms. Progress in Lipid Research. 53:18–81
Ye C, Shen Z, Greenberg ML. 2014. Cardiolipin remodeling: a regulatory hub for modulating cardiolipin metabolism and function. Journal of Bioenergetics and Biomembranes. 48(2):113–23

Appendix

Appendix 1. P values comparing Male and Female *elav>RNAi* and *tub>RNAi.*

elav>RNAi	
Day	**P Value**
10	0.219188
20	0.733774
30	0.302194
35	0.407566
40	0.091545

tub>RNAi	
Day	**P Value**
10	0.006780081
20	1.29007E-05
30	0.018071517