# Effects of RNAi knockdown of the gene for iPLA2β in muscles and neurons of *Drosophila Melanogaster* and rescue of climbing phenotype

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## Abstract

Parkinson's disease (PD) is a neurodegenerative disorder in which neurons deteriorate and die in the brain (Elkouzi, 2020). PD affects many areas of life including memory, work, sleep, and exercise. There is currently no cure for a disease that affects more than ten million people worldwide (Marras, 2018). Numerous genes have been implicated in PD, including *PARK2*, *PINK1*, and *LRRK2*, among others (Klein, 2012). *PLA2G6* is a gene that creates the protein iPLA2β. This gene has been implicated in PD and is a homologue to the gene *CG6718* in the model organism *Drosophila melanogaster* (*D. melanogaster*) (UniProt, 2020). Studying this gene in *D. melanogaster* allows researchers to understand more about PD in humans. I performed two experiments to investigate some of the biological pathways that *CG6718* affects in flies. The first experiment attempted to determine whether RNAi knockdown of *CG6718* in muscles caused a PD-like phenotype in flies. The results were inconclusive due to unforeseen effects in some of the fly lines. The second experiment prevented the onset of the PD-like symptoms in flies through the use of a complementary DNA (cDNA) transgene to rescue flies carrying a deletion in *CG6718*. This experiment was successful, demonstrating that a tagged transgene can rescue *CG6718* mutants.

## Introduction

### Parkinson's disease and iPLA2β

First described in 1817 by James Parkinson, PD is neurodegenerative disease that affects the basal ganglia, including the substantia nigra region, of the brain (Goetz, 2011). The risk of PD increases in those that have relatives with PD, men, and people over the age of 60. Symptoms for PD include tremors, impaired muscle control, loss of posture and balance (Parkinson's disease - Symptoms and causes, 2020). Close to one million people currently have PD in the United States, more than the combined number of people with similarly debilitating diseases like multiple sclerosis, muscular dystrophy, and ALS (Parkinson's Foundation - Statistics, 2018). A number of different genes have been found to be involved in PD, making it imperative for researchers to discover all they can about each gene and how each contributes to this debilitating disease.

*PLA2G6* is a PD gene (also known as *PARK14)*. It produces the protein iPLA2β, also known as calcium independent phospholipase A2 beta. Phospholipids are the primary molecules that compose the cellular membrane in cells. Their most basic molecular form is a "head" containing a phosphate group and two "tails" composed of fatty acids. There are two main types of phospholipids: sphingolipids and glycerophospholipids. Glycerophospholipids are the standard form of phospholipid. They contain two fatty acids tails, a polar head group and a phosphate group bonded to glycerol (Structural Lipids in Membranes, Chapter 9; Christie, 2020). A cell can hydrolyze a phospholipid using a phospholipase in order to facilitate membrane repair and digestion of old phospholipids, or to regulate phospholipid signaling (Wilton, 2008; Yao and Bamji-Mirza, 2020). Phospholipases are enzymes grouped into four different families, A, B, C, and D. Phospholipase A2 (PLA2) is a subfamily of A. It cleaves the phospholipid at the *sn-2* position to form a fatty acid and a lysolipid. (Burke and Dennis, 2009). iPLA2β is involved in numerous different signaling pathways and affects cell differentiation, mitochondrial integrity, cell membrane remodeling, cell growth, and cell death (Ramanadham et al. 2015, Hooks and Cummings, 2008).

iPLA2β has been implicated in neurodegeneration (called *PLA2G6*-associated neurodegeneration, PLAN), which can take the form of infantile neuroaxonal dystrophy (INAD), atypical neuroaxonal dystrophy (atypical NAD), and *PLA2G6*-related dystonia-parkinsonism (Parkinson's Disease), as well as schizophrenia and Alzheimer's disease in humans (Gregory et. al, 2017; PLA2G6-Associated Neurodegeneration, 2018). Additionally, reduced bone density, vascular smooth muscle degeneration, and sperm motility as well as neuronal and mitochondrial degeneration have been found in iPLA2β-null mice (Ramanadham et al. 2015; Turk et al. 2018).

## *Drosophila melanogaster* as a model organism

Different species, from yeast to mice, have a similar genetic makeup to humans. Using these organisms, which have shorter lifespans, we can examine multiple generations in a short amount of time. Furthermore, they are easy to study in a laboratory, allowing researchers to learn more about human genetics. One of these so-called "model organisms," *Drosophila melanogaster*, has a genome that is 60% homologous to a human genome and shares 75% of

its disease genes with human disease genes (Panday and Nichols, 2011, Mirzoyan et al., 2019). The *PLA2G6* gene in humans is conserved in *D. melanogaster* as the gene *CG6718.* Studying this gene allows researchers a greater understanding of this gene's effects in humans. This gene has been known to cause Parkinson's disease-like symptoms in null *D. melanogaster* including early death, reduced climbing ability, and locomotor deficits (Kinghorn et al., 2015; Illadi et al., 2018, Schonbrun, 2021). However, it is unclear what cellular effects and biochemical pathways cause these symptoms to develop.

## *GAL4-UAS* system

The *GAL4-UAS* system is the most commonly used method for manipulating fly genetics. A *GAL4* transcriptional activator gene from yeast along with a *Drosophila* promotor and tissue-specific enhancer is added to the DNA of a fly. An upstream activating sequence (*UAS*) along with a gene of interest is added to the DNA of another fly. When the two flies mate, the *GAL4* gene creates the GAL4 protein, which binds to the *UAS* and causes the transcription of a researcher's gene of choice (Toh and Bang, 2014; Nadar et al., 2017). In this way, the *GAL4-UAS* system is tissue-specific, allowing for targeted gene activation. To knockdown a gene instead of inducing gene transcription, the *GAL4-UAS* system can be used to activate RNA interference (RNAi) by expressing short interfering RNAs (siRNA) that destroy messenger RNA (mRNA) for a specific target protein (Bosch et al., 2016, Blake et al., 2018).

For my first experiment, I used the *GAL4-UAS* system to knock down iPLA2β using RNAi in *D. melanogaster*'s muscles, using the *mhc-GAL4* driver (Steinhauer Grantome, 2018). In my second experiment, I used the *GAL4-UAS* system to express an iPLA2β complementary DNA (cDNA) transgene in all cells using the ubiquitously expressed *tub-GAL4*.

## Current Research

Flies with a global knockdown of iPLA2β were found to exhibit a PD-like phenotype, phenocopying the defects seen in *CG6718* loss of function mutants (Kinghorn *et al.* 2015, Iliadi *et al.* 2018, and Schonbrun et al., 2021). However, it was unclear if these defects were due to neuron deterioration alone or a combination of degeneration in neurons as well as

other tissues. The *mhc-GAL4* driver was used for muscle specific iPLA2β knockdown to test if muscles are sensitive to the loss of iPLA2β in my experiment. Our first set of results was inconclusive, so we cleaned the *GAL4* stock with better balancer chromosomes and repeated the experiment, including positive and negative controls to ensure that the *mhc-GAL4* was not causing other changes in the flies.

Previous experiments in the Steinhauer lab had suggested that an HA-tagged wild-type iPLA2β cDNA transgene could rescue the reduced lifespan and climbing ability in *D. melanogaster* lacking endogenous iPLA2β, called $CG6718^{\Delta 23}$ mutants (Schonbrun, 2021). Repeating the experiment allowed for a statistical analysis of the rescue and confirmation of the results.

## Materials and Methods

## Climbing assay and analysis

For both experiments, flies were raised in standard media in fly vials in a 26ºC incubator. Flies were tested every 10, 20, and 30 days (only negative controls survived to 40 days) for the knockdown of iPLA2β in muscles and for 15, 20, and 30 days for the rescue of the $CG6718^{\Delta 23}$ mutant. Flies were tested using climbing assays (Fig. 1). A climbing index was assigned to each fly vial based on the average number of flies that managed to climb into the topmost vial over a period of twenty seconds for each of five runs per vial.

Figure 1. Standard climbing assay. Two vials taped together. Flies are tapped to the bottom of the food vial and timed as they climb up. Flies have inherent negative geotaxis behavior, which causes them to climb or fly in an upwards direction.

After 30 days of testing for each fly vial, the data were compiled into bar graphs, and t-tests were used to determine the significance of each fly line's change in climbing ability over time. For the unpaired t-tests, a p value below 0.050 was considered significant.

## Knockdown of iPLA2β in muscles

To create the *mhc-GAL4* stock, parent (P) flies were crossed to form two F1 generation fly progeny. The F1 generation progeny were then self-crossed to form a cleaner *GAL4* driver stock, with fewer background mutations (Fig. 2). All crosses were performed in the $w^{1118}$ white-eyed background. The second chromosome markers *Sco/SM6* were removed by crossing to flies carrying wild-type second chromosomes. On the third chromosome, *mhc-GAL4*/*TM6B* was crossed with *TM3,Sb/TM6B*. In the F1 progeny, *mhc-GAL4*/*TM3,Sb* or *mhc-GAL4*/*TM6B* flies were selected. The *mhc-GAL4*/*TM6B* stock was ultimately chosen for the experiment because *TM6B* was a stronger balancer. F1 generation flies were self-crossed to generate the clean the fly stock.

$$\frac{Sco}{Sm6};\frac{mhc-Gal4}{TM6B}\ x\ \frac{+}{+};\frac{TM3Sb}{TM6B}\rightarrow\frac{+}{Sm6};\frac{mhc-Gal4}{TM6B}\ and\ \frac{+}{Sm6};\frac{mhc-Gal4}{TM3Sb}$$

$$\frac{+}{Sm6};\frac{mhc-Gal4}{TM6B}\ x\ \frac{+}{Sm6};\frac{mhc-Gal4}{TM6B}\rightarrow\frac{+}{+};\frac{mhc-Gal4}{TM6B}$$

Figure 2. Parent and F1 generation crosses. Cleaning the fly stock was an attempt to remove confounding genetic variables.

The F2 generation was crossed to flies with *UAS-iPLA2β-RNAi* to produce the experimental line of flies (Fig. 3).

$$\frac{+}{+};\frac{mhc-Gal4}{TM6B}\ x\ \frac{+}{+};\frac{UAS-iPLA2\beta-RNAi}{UAS-iPLA2\beta-RNAi}\rightarrow\frac{+}{+};\frac{mhc-GAL4}{UAS-iPLA2\beta-RNAi}$$

Figure 3. Crosses to produce experimental fly line

Two positive control fly genotypes were bred. The positive control crosses were *mhc-GAL4*/*TM6B* crossed with *UAS-pink1-RNAi* and *UAS-parkin-RNAi* (Fig 4). *pink1* and *parkin* are two well-known genes whose loss produces PD like symptoms in both humans and flies. Crossing the *mhc-GAL4* line to *RNAi* that knocks down these genes was intended to demonstrate that this *GAL4* driver could be used to produce PD-like symptoms in flies.

$$\frac{+}{Sm6};\frac{mhc-Gal4}{TM6B}\ x\ \frac{+}{Sm6};\frac{UAS-pink1-RNAi}{UAS-pink1-RNAi}\rightarrow\frac{+}{Sm6};\frac{mhc-GAL4}{UAS-pink1-RNAi}$$

$$\frac{+}{Sm6};\frac{mhc-Gal4}{TM6B}\ x\ \frac{+}{Sm6};\frac{UAS-parkin-RNAi}{TM3\ Sb}\rightarrow\frac{+}{Sm6};\frac{mhc-GAL4}{UAS-parkin-RNAi}$$

Figure 4. Crosses to produce positive control fly lines

Three negative controls were bred. The first two negative controls crossed $w^{1118}$ flies with *parkin-RNAi* and *pink1-RNAi*. These two controls were meant to ensure the *pink1-RNAi* and

*parkin-RNAi* were not causing any effects by themselves without activation by *mhc-GAL4*. The final negative control crossed *mhc-GAL4* with a chromosome marked with the *Drop* mutation (Fig. 5). This control was meant to show that *mhc-GAL4* was not causing any climbing or age-related defects on its own.

$$\frac{+}{Sm6};\frac{w^{1118}}{w^{118}}\ x\ \frac{+}{Sm6};\frac{UAS-pink1-RNAi}{UAS-pink1-RNAi}\rightarrow\frac{+}{Sm6};\frac{UAS-pink1-RNA}{w^{1118}}$$

$$\frac{+}{Sm6};\frac{w^{1118}}{w^{1118}}\ x\ \frac{+}{Sm6};\frac{UAS-parkin-RNAi}{TM3\ Sb}\rightarrow\frac{+}{Sm6};\frac{UAS-parkin-RNA}{w^{1118}}$$

$$\frac{+}{Sm6};\frac{mhc-Gal4}{TM6B}\ x\ \frac{+}{Sm6};\frac{Drop}{TM3\ Sb}\rightarrow\frac{+}{Sm6};\frac{mhc-GAL4}{Drop}$$

Figure 5. Crosses to produce negative control fly lines

## Rescue of *CG6718*

Two crosses were made. The first cross was experimental and crossed *tub-GAL4/SM6* and *UAS-iPLA2β/SM6* on chromosome two and *CG6718$^{\Delta 23}$/TM6B* and *CG6718$^{\Delta 23}$/TM6B* on chromosome three (Fig. 6). F1 flies carrying both *tub-GAL4* and *UAS-iPLA2β* were selected by their straight wings and orange eyes, in contrast to flies carrying only *tub-GAL4*, with yellow eyes and curly wings. For the control cross, *tub-GAL4/SM6* was crossed with *Sco/SM6* on chromosome two and *CG6718$^{\Delta 23}$/TM6B* crossed with *CG6718$^{\Delta 23}$/TM6B* on chromosome three. Flies with *tub-GAL4* (yellow eyes) were selected, easily distinguished from flies with white eyes that lacked the *tub-GAL4*.

$$\frac{tub-Gal4}{Sm6};\frac{\Delta 23}{TM6B}\ x\ \frac{UAS-iPLA2\beta}{Sm6};\frac{\Delta 23}{TM6B}\rightarrow\frac{tub-Gal4}{UAS-iPLA2\beta};\frac{\Delta 23}{\Delta 23}$$

$$\frac{tub-Gal4}{Sm6};\frac{\Delta 23}{TM6B}\ x\ \frac{Sco}{Sm6};\frac{\Delta 23}{TM6B}\rightarrow\frac{tub-Gal4}{Sm6};\frac{\Delta 23}{\Delta 23}$$

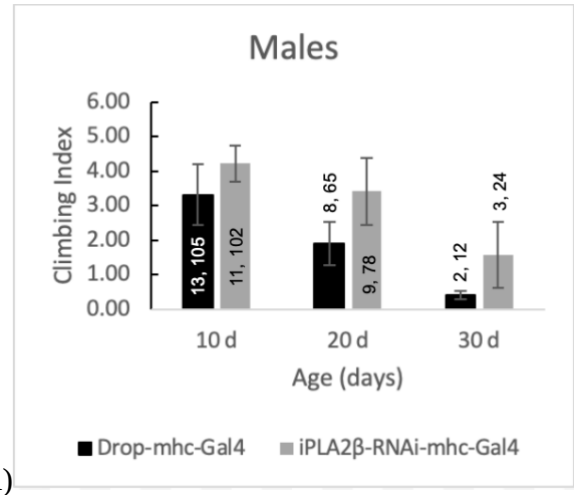
Figure 6. Crosses for experimental and control fly lines respectively.

## Results

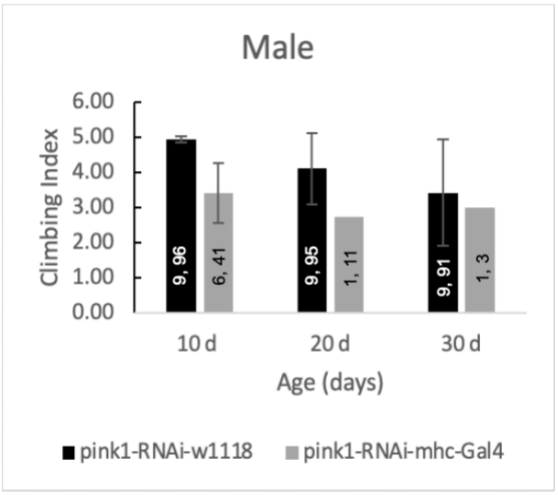
## Knockdown of iPLA2β in muscles

The males of the experimental cross (*mhc-GAL4>UAS-iPLA2β-RNAi*) exhibited reduced life span and impaired climbing ability over time, like $CG6718^{\Delta 23}$ mutant flies (Fig. 7A, gray bars). However, the experimental females did not exhibit any noticeable PD-like phenotype (Fig. 7D, gray bars). The positive control flies (*mhc-GAL4>UAS-pink1-RNAi* and *mhc-GAL4>parkin-RNAi*) (Fig. 7B,C,E, and F, grey bars) showed reduced climbing and died earlier than their respective negative controls (*pink1-RNAi-*$w^{1118}$ and *parkin-RNAi-*$w^{1118}$)(Fig. 7B,C,E, and F, black bars). These negative control flies had a typical lifespan and strong climbing ability throughout the forty days they were tested. However, the negative control flies for *mhc-GAL4* (*Drop-mhc-GAL4*) showed both reduced lifespan and climbing ability over time (Fig. 7A and D, black bars). T-tests were used to compare the experimental flies to *Drop-mhc-GAL4*, the *mhc-GAL4>Pink1-RNAi* to *Pink1-RNAi-*$w^{1118}$, and *mhc-GAL4>parkin-RNAi* to *parkin-RNAi-*$w^{1118}$ for both sexes (Fig. 8).

A)




B)



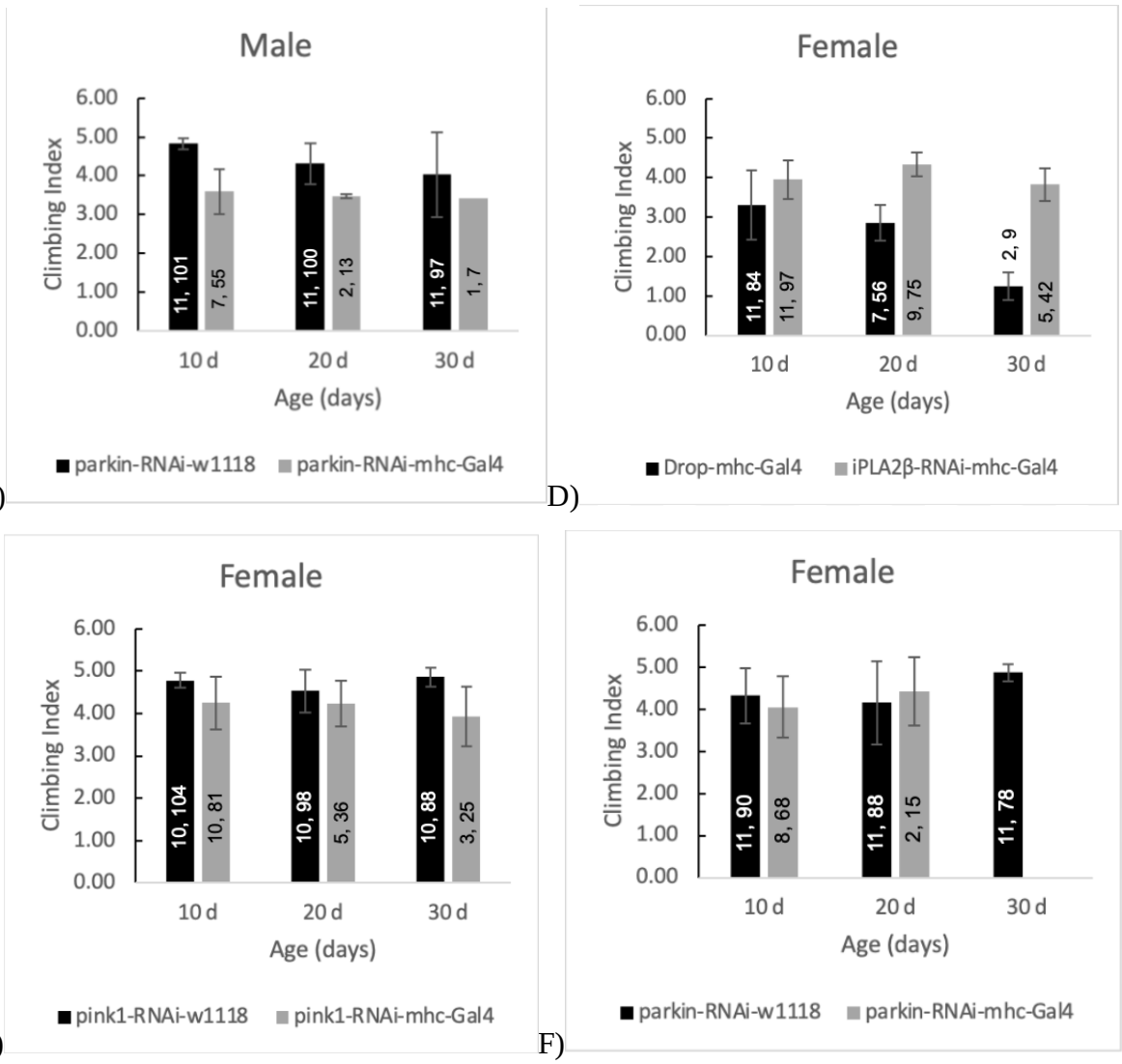



Figure 7. Results of knockdown experiment. The lower number on the bar represents the number of vials tested for each fly line and the upper number indicates the total number of flies tested. Surprisingly, male and female controls carrying *Drop-mhc-GAL4* showed a decrease in climbing compared to *mhc-GAL4>UAS-iPLA2β-RNAi* (A, D). Male and female *mhc-GAL4>pink1-RNAi* (B, E) and male *mhc-GAL4>parkin-RNAi* (C) showed reduced climbing ability compared to controls (*pink1-RNAi-w$^{1118}$* and *parkin-RNAi-w$^{1118}$*), while female *mhc-GAL4>parkin-RNAi* did not (F). By 30 days, all female *mhc-GAL4>parkin-RNAi* had died. See Figure 8 for statistical analysis.

| *mhc-GAL4>iPLA2β-RNAi* **vs** *Drop-mhc-GAL4* | | |
|---|---|---|
| **Age (days)** | **p-value** | |
| | **Male** | **Female** |
| 10 | 0.0062 | 0.050 |
| 20 | 0.0017 | $2.5 \times 10^{-5}$ |
| 30 | 0.17 | 0.0098 |

| *mhc-GAL4>pink1-RNAi* **vs** *pink1-RNAi-w$^{1118}$* | | |
|---|---|---|
| **Age (days)** | **p-value** | |
| | **Male** | **Female** |
| 10 | 0.0071 | 0.015 |
| 20 | ---- | 0.19 |
| 30 | ---- | 0.15 |

| *mhc-GAL4>parkin-RNAi* **vs** *parkin-RNAi-w$^{1118}$* | | |
|---|---|---|
| **Age (days)** | **p-value** | |
| | **Male** | **Female** |
| 10 | 0.0011 | 0.42 |
| 20 | 0.00030 | 0.79 |
| 30 | 0.0098 | ---- |

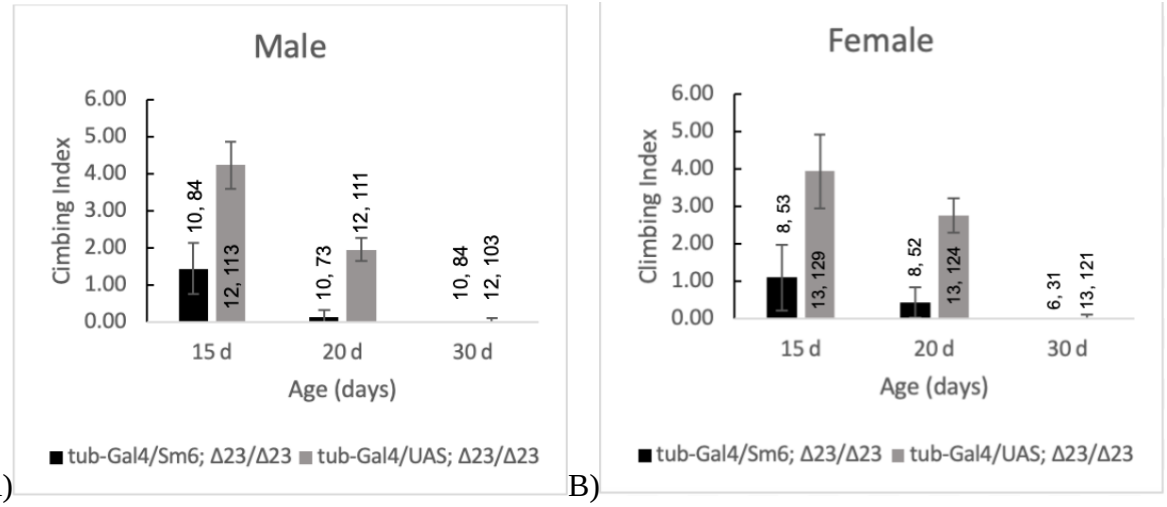
Figure 8. p-values for all flies in iPLA2β knockdown experiment. Cells with empty values are due to sample sizes of 0 or 1, meaning that t-tests could not be performed. Highlighted p-values indicate significance.

## Rescue of *CG6718*

The males and females of the experimental line (*tub-GAL4/UAS-iPLA2β; Δ23/Δ23*) showed a normal lifespan and no significant reduction in climbing ability after 15 days. After 30 days the experimental flies showed a slight reduction in climbing ability.

The males and females of the control line (*tub-GAL4/SM6; Δ23/Δ23*) showed a significantly

reduced life span and climbing ability by 15 days. After 20 days most of the flies had stopped climbing completely. By 30 days the majority of the flies were alive, but could not climb (Fig. 9A,B). T-tests were performed between *tub-GAL4/UAS; Δ23/Δ23* and *tub-GAL4/Sm6; Δ23/Δ23* for both sexes (Fig. 10).




A) B)

Figure 9. Results of rescue experiment. The lower number on the bar represents the number of vials tested for each fly line and the upper number stands for the total number of flies tested. Male and female *tub-GAL4/UAS-iPLA2β; Δ23/Δ23* showed a significantly improved ability to climb compared to *tub-GAL4/SM6; Δ23/Δ23*.

| *tub-GAL4/UAS; Δ23/Δ23* **vs** *tub-GAL4/Sm6; Δ23/Δ23* | | |
|---|---|---|
| **Age (days)** | **p-value** | |
| | **Male** | **Female** |
| 15 | $5.9 \times 10^{-10}$ | $3.7 \times 10^{-6}$ |
| 20 | $8.3 \times 10^{-13}$ | $1.4 \times 10^{-9}$ |
| 30 | 0.045 | 0.099 |

Figure 10. p-values for flies in rescue experiment. With the exception of *tub-GAL4/UAS; Δ23/Δ23* **vs** *tub-GAL4/Sm6; Δ23/Δ23* females at 30 days, all other T-tests have p-values of <0.050. Highlighted p-values indicate significance. This shows that the results of the experiment can be accepted and are significant.

## Discussion

## Knockdown of iPLA2β in muscles

All three negative controls were expected to exhibit normal lifespan and climbing ability. Surprisingly, only the *pink1-RNAi-w$^{1118}$* and *parkin-RNAi-w$^{1118}$* exhibited these phenotypes, whereas *Drop-mhc-GAL4* flies showed reduced lifespan and climbing ability. The exception were the female *mhc-GAL4>parkin-RNAi* flies which died within 30 days and showed that the knockdown worked, albeit not significantly compared to the control. Overall, this mimics PD-like phenotypes in the flies and suggests that the *mhc-GAL4* driver affects locomotor activity and aging on its own. The observation that *Drop-mhc-GAL4* flies had more severe defects than *mhc-GAL4>iPLA2β* may suggest also that *mhc-GAL4* is very sensitive to genetic interactions from background alleles, which prevents the use of this genotype as a standard of comparison. The small sample sizes after 20 days may also have contributed to the wide variability in the results. Thus, while the experimental male flies carrying *mhc-GAL4* knockdown of iPLA2β showed reduced climbing with age, the results are inconclusive as to whether an iPLA2β muscle knockdown can cause reduced lifespan and climbing ability in *D. melanogaster*.

## Rescue of *CG6718*

Both the experimental male and female flies climbed successfully and had significantly longer life spans than the control male and female flies. The experimental male and female flies with the transgene had a reduced climbing ability and life span compared to wild-type flies which can live for 2-3 months (Sun et al., 2015). However, this was expected due to the flies being kept at 26ºC. The hotter environment caused them to age faster and made it harder for them to climb. It also is possible that the transgene rescued the climbing phenotype and lifespan of the flies but was not a complete rescue. The control flies, both male and female, had a low climbing index by day 15. 74% of experimental male flies and 93% of female experimental flies were alive after 35 days, while no control male flies and only 32% of female control flies were alive after 35 days (Fig. 11)

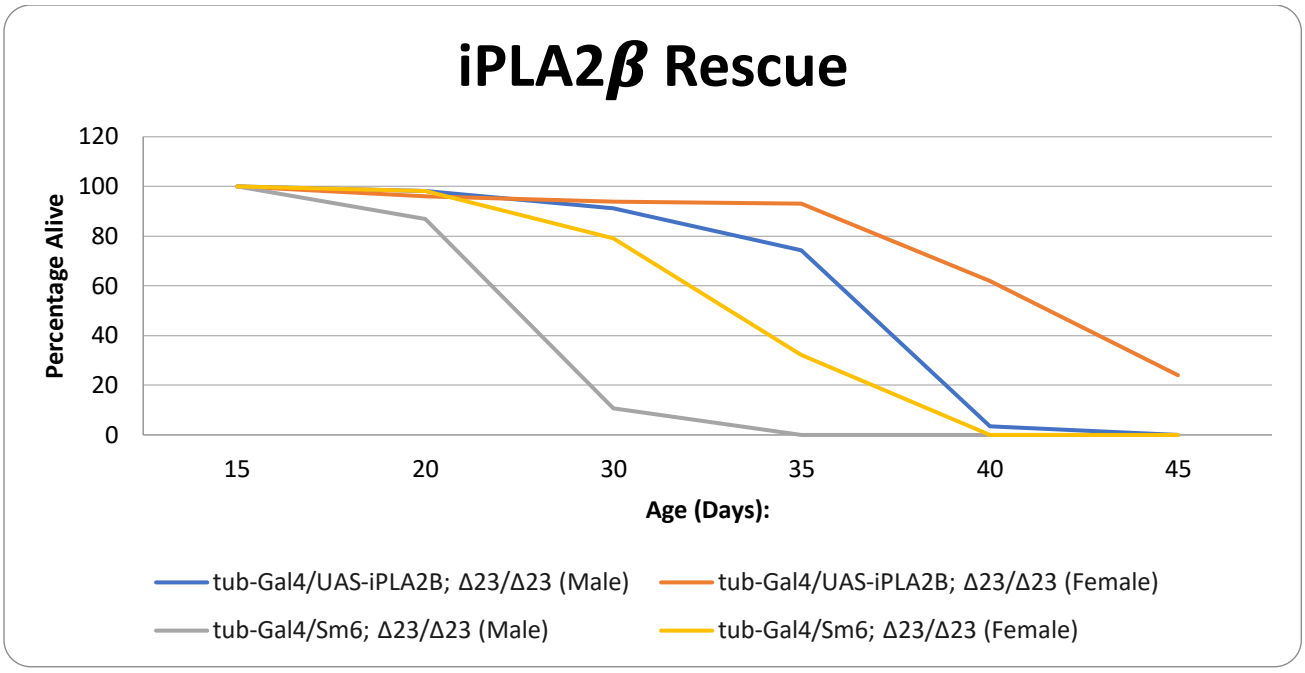



Figure 11. Lifespan data for male and female experimental and control fly lines.

## Conclusion

The data from the knockdown of iPLA2β in muscles was inconclusive due to confounding effects of the *mhc-GAL4* driver alone. It was hypothesized that the *mhc-GAL4* driver line was impacting climbing ability and lifespan. Therefore, it could not be shown that a knockdown of PD genes in muscles of flies was responsible for this effect. However, further experiments in the Steinhauer lab showed that with a different muscle specific *GAL4* driver, knockdown of iPLA2β did cause reduced climbing ability and lifespan (Fig. 12) (Schonbrun, 2021). We are therefore able to conclude that knockdown of *iPLA2β* affects other tissues along with nervous system when it comes to aging health. Degeneration of other cell types in addition to neurons may contribute to the severity of diseases that result from its loss in humans as well as *D. melanogaster*. Finally, the new muscle specific *GAL4* driver can cause PD phenotypes in flies when knocking down other PD related genes as well, as seen in the case of *pink1* knockdown (Fig. 12).

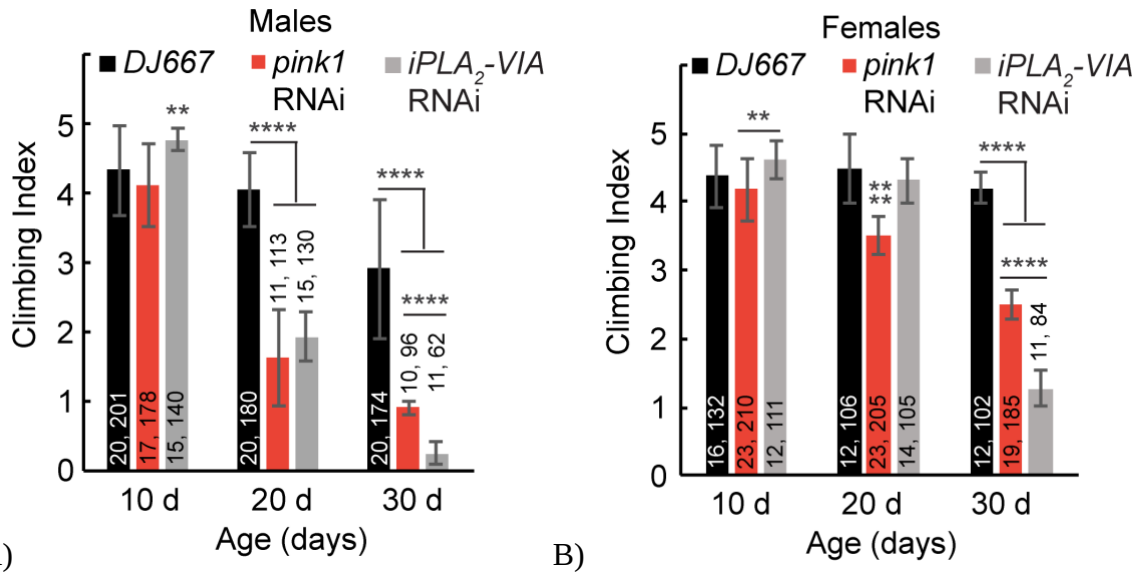



Figure 12. Use of a different muscle specific *GAL4* driver (*DJ667-GAL4*) effectively knocked down iPLA2β in flies and showed reduced climbing ability (gray). *DJ667-GAL4* knockdown of *pink1* also reduced climbing ability (pink bars) in both males (A) and females (B).

As previous research has shown, rescuing iPLA2β using a transgene and a *GAL4* driver can improve climbing ability (Steinhauer lab, unpublished) and increase lifespan (Iliadi et. al, 2018). My experiment added further evidence that the climbing phenotype and lifespan could be rescued. The success of the rescue experiment demonstrates that the *CG6718* gene is necessary and sufficient to prevent loss of locomotor activity with age. It will be important in the future to investigate whether expression of wild-type iPLA2β in neurons or muscle only can rescue the PD-like symptoms. These experiments can be conducted easily using the same tools described here and could inform development of treatments for humans with mutations in this gene.

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