PLA2G6 Associated Neurodegeneration Modeled in *Drosophila melanogaster*

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Abstract

PLA2G6 has been implicated as a locus for several neurodegenerative diseases. The *PLA2G6* protein product is a phospholipase involved in phospholipid remodeling in cell membranes, regulating apoptosis, and cell signaling. A knockdown of the *Drosophila melanogaster* ortholog to *PLA2G6 – CG6718 –* results in phenotypes that model age-related neurodegenerative disease development in humans. Through the *GAL4*-UAS system, tissue specific knockdown of *CG6718* can be achieved to determine in which cell types the *PLA2G6* protein product is essential to maintain neuronal health with aging. Here, a climbing assay measuring the negative geotaxis response in *Drosophila melanogaster* with and without an RNAi knockdown in tyraminergic and octopaminergic neurons was performed to analyze whether *PLA2G6* expressed in this neuronal subtype maintains health with aging. A statistically significant difference between the experimental control flies was observed at 20 days of age, suggesting that *PLA2G6* is required in these neurons to prevent neurodegeneration during aging.

Introduction

Neurodegenerative Diseases

Neurodegenerative diseases, such as Parkinson’s and Alzheimer's, are marked by characteristic phenotypes that can develop with aging. For example, Parkinson's disease is characterized by bradykinesia (slowness of movement), along with muscular rigidity, resting tremors, or disturbance to one’s gait or posture. The reduction of dopaminergic neurons in the substantia nigra and the presence of subcellular aggregates called Lewy bodies are the primary histopathological features of Parkinson’s disease (PD) [1].

In 2017, an estimated 1 million Americans suffered from PD [2]. This number is expected to increase by 20% in the year 2030. In the U.S., the economic burden of PD in 2017 was estimated to be $51.9 billion. Current treatment options for PD patients are limited, the treatments do not alter the disease course, and are often associated with significant side effects [3].

While the death of dopaminergic neurons in the substantia nigra is the primary cause of the motor symptoms that characterize PD, other cell types have also been implicated in PD, leading to a variety of other symptoms including sleep disturbance, anosmia, gastrointestinal dysfunction, hallucinations, and dementia. Neurodegenerative diseases are largely sporadic, lacking a genetic cause [4]. For example, one study on Parkinson's disease estimated that 95% of cases were sporadic [5]. Nevertheless, a mutation in the gene coding for α-Synuclein, a protein found to be the primary constituent of Lewy bodies, in an Italian family in which many family members suffered from Parkinson’s disease, highlighted the possibility that genetic factors could in some cases explain the onset of neurodegenerative disease [6]. Since this discovery, genome wide studies have identified numerous loci that are associated with neurodegenerative diseases. Several loci have been linked to familial PD. These loci have accordingly been dubbed *PARK* genes [7]. In some cases, these *PARK* loci have also been implicated in sporadic PD, suggesting a common etiology between inherited and sporadic forms as well as a genetic component even in sporadic cases [8]. The research presented here centers around *PARK14,* also known as *PLA2G6.*

PLA2G6

PLA2G6 was implicated as a locus in inherited infantile neuroaxonal dystrophy, a neurodegenerative disease in children characterized by motor and sensory impairment, by pedigree analysis of affected families [9]. Mutations in the *PARK14* gene have also been linked

to adult-onset inherited parkinsonism [10]. The *PLA2G6* gene has more than one protein product of different lengths. Transcript variant 1, for example, is an 806 amino acid long dimeric enzyme which catalyzes the hydrolysis of glycerophospholipids, and it is thought to be involved in phospholipid remodeling in cell membranes, regulating apoptosis, and cell signaling (figure 1) [9,11].

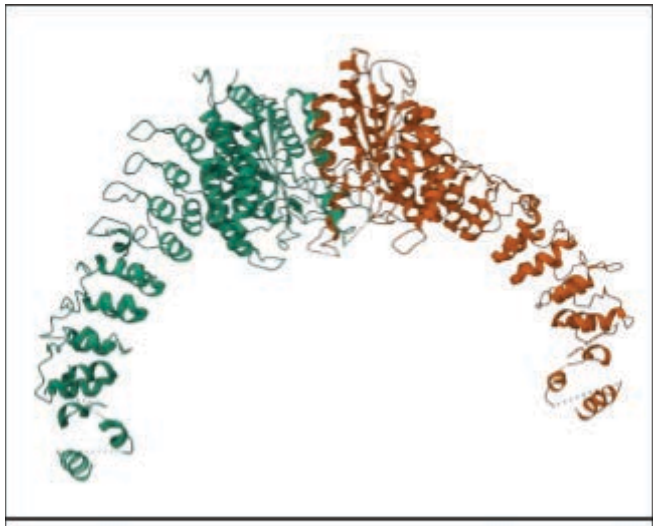


Figure 1: The *PLA2G6* protein product, a dimeric phospholipase is shown [33].

The *PLA2G6* protein product is an A_2 phospholipase, and it attacks the ester bond of the glycerophospholipid, a type of membrane lipid, at C-2 as shown in figure 2. The protein product may have additional uncharacterized biochemical activities.

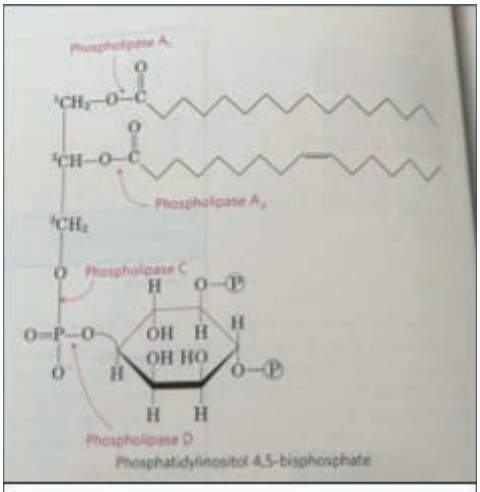


Figure 2: Substrate site of phospholipases [12].

To further investigate the role of mutations in the *PLA2G6* gene in neuronal degeneration, *Drosophila melanogaster* were employed as a model organism [13,14,15,16]. As seen in an NCBI-Blast comparison, human *PLA2G6* and the *Drosophila melanogaster* ortholog – *CG6718* – are highly conserved throughout the entire sequence, with especially conserved sequence in their phospholipase domains [17] (figure 3).

Figure 3: Multiple sequence alignment between the amino acid sequence of *CG6718* (NP_648366.2) and *PLA2G6* (NP_003551.2) protein products. The active site region (559-725 aa and 486-652 aa respectively) is shown. The asterisks below the two residues indicate total sequence overlaps, a colon (:) indicates the residues are different but are similar in charge and/or polarity, a period (.) indicates loose similarity in charge and/or polarity, and no symbol indicates unsimilarity in charge and/or polarity. The color of each residue differentiates amino acids with a given charge and polarity (ex: red indicates aa with hydrophobic side chains). The phospholipase active sites display strong consensus [18].

Various techniques have been employed to induce null or hypomorphic mutations in the *Drosophila CG6718* gene. As observed in the negative geotaxis assay, described below, *Drosophila melanogaster* carrying a null mutation of the *CG6718* gene were observed to have locomotor decline and other symptoms of neurodegenerative disease, like humans with mutations in this gene. Some studies have argued that neurodegeneration resulting from *PLA2G6* mutation is attributable to altered lipid composition [14,16]. This attribution is supported by the fact that flies which were fed certain fatty acids were rescued from *CG6718* mutant induced neurodegenerative, and these mutant flies were also rescued through gain of function mutations to other lipid metabolism genes. However, the locomotor decline and other symptoms of neurodegeneration were rescued by a transgene carrying a serine-to-alanine mutation in the catalytic residue, a well characterized mutation that disrupts catalytic activity[13]. This suggests that the important functional aspects needed for *PLA2G6* to preserve neuronal survival are independent of the protein product's phospholipase activity [13]. The *PLA2G6* protein product has 8-9 ankyrin repeats, which serve as protein-protein interaction domains [20]. This protein is known to have several proteins with which it interacts, as well as other as yet unidentified binding partners. Thus, its relevant biochemical activity may reside in these uncharacterized interactions. There is still much that is unknown about how this protein protects neurons from age-related degeneration. The mechanism underlying how a mutated *PLA2G6* gene protein product contributes to neurodegeneration is an area of current research.

Figure 4: Protein-protein interaction network of iPLA2-VIA. Interaction network has 7 nodes and 8 edges. The iPLA2-VIA connectivity degree value in this network is 6 [21].

GAL4-UAS and RNAi systems

The function of a gene can be determined by observing the change in phenotype that results from a mutation in that gene. Similarly, the function of the gene can be determined by observing the phenotype of the organism in which the gene product is targeted, without altering the gene itself. One method to phenocopy a mutation of the gene itself is to prevent the translation of the gene's mRNA into amino acids through interference RNA.

Specific small strands of RNA, microRNAs, are transcribed in higher eukaryotes, including fruit flies, that mediate in silencing several genes [22]. After transcription of these noncoding regions, which are comprised of internally complementary sequences, the miRNA is processed by endoribonucleases and RNA helicase, forming a mature miRNA. In the cytoplasm, this mature miRNA associates with an RNA-induced silencing protein complex (RISC). If the miRNA-RISC complex hybridizes with mRNA, this mRNA is cleaved [22] (figure 5).

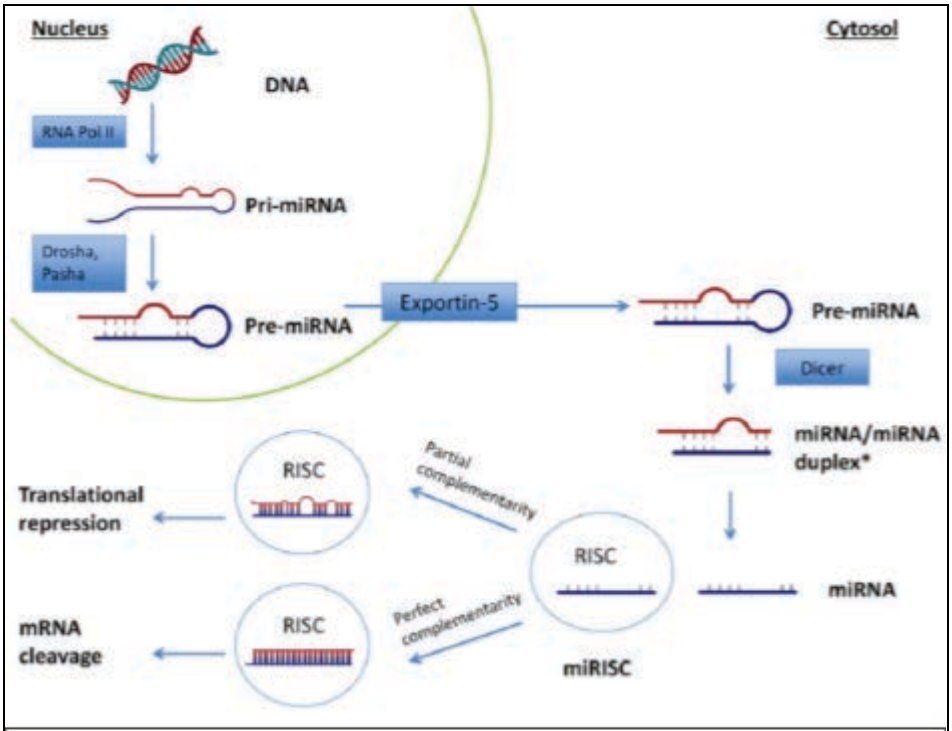


Figure 5: The RNAi pathway is depicted with noncoding DNA being transcribed, forming a hairpin loop shaped pri-mRNA structure. After endonuclease activity, the pre-miRNA structure is exported to the cytosol. An miRNA duplex is formed after the pre-miRNA is subject to yet another endonuclease. After the duplex is separated, the now single stranded miRNA associates with the RISC protein complex. Based on the degree of overlap between exposed miRNA nucleotides and those of a given mRNA, the mRNA will either be degraded or will be unable to be translated [23].

Researchers can induce this interference RNA pathway against specific mRNA by making a transgenic organism carrying a complementary dsRNA[24]. The expression of the RNAi can be limited to specific tissues via the *GAL4*-UAS system.

In yeast, the *GAL4* gene encodes a transcription factor that increases the expression level of several enzymes in the galactose-glucose pathway. In order for the Gal4 protein to upregulate expression of a gene, the protein must bind upstream of a gene on a regulatory sequence - the upstream activation sequence or UAS [25]. The *GAL4* gene and its UAS can be exogenously engineered into an organism, with a desired gene or noncoding region downstream from the UAS. If the *GAL4* gene is transposed into *Drosophila melanogaster* downstream from a

tissue-specific promoter, then the Gal4 protein will only be transcribed in the specified tissues [26]. Separately, internally complementary sequences can be transposed downstream from a UAS, yielding an miRNA which will knockdown a particular mRNA sequence [27]. Thus, by mating one *Drosophila melanogaster* stock containing the *GAL4* gene alongside a tissue specific promoter with another *Drosophila melanogaster* stock contain the UAS followed by a sequence yielding a desired miRNA, the knockdown of a particular mRNA sequence can be achieved in a tissue specific manner (figure 6).

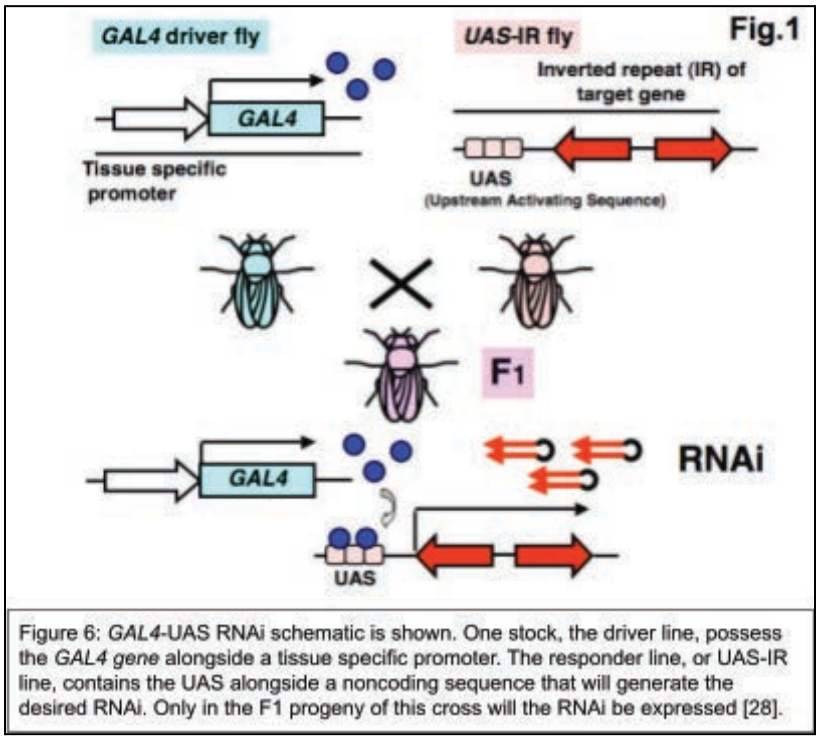


Figure 6: *GAL4*-UAS RNAi schematic is shown. One stock, the driver line, possess the *GAL4 gene* alongside a tissue specific promoter. The responder line, or UAS-IR line, contains the UAS alongside a noncoding sequence that will generate the desired RNAi. Only in the F1 progeny of this cross will the RNAi be expressed [28].

Previous research has shown that a knockout of *CG6718* leads to neurodegeneration and the knockdown of the protein pan-neuronally phenocopies neurodegeneration [13]. To better understand the neural pathway leading to neurodegeneration, *GAL4*-UAS RNAi knockdown of the *CG6718* gene was performed in a neuron specific manner.

Negative Geotaxis Assay

With the ability to knockdown a single gene in the *GAL4*-UAS RNAi system, changes in neural function related to the function of a protein product can similarly be detected [29]. For example, changes in behavioral patterns, such as reduced walking speed with age or observing increased cell death with age in whole-mount fly brains with *CG6718* knockdowns relative to control *Drosophila,* suggest that mutation of *PLA2G6* contributes to age-related neurodegeneration [17].

One widely used assay to distinguish between flies with different behavioral patterns is the negative geotaxis assay. Flies naturally display negative geotaxis, which is easily observed in their climbing away from the ground when tapped to the bottom of a vial. The negative geotaxis response is observed to deteriorate with age [30]. Age-related neurodegeneration would be indicated if flies with *CG6718* knockdowns displayed progressively diminishing climbing ability that was statistically worse compared to the climbing ability of control flies.

For each of the neuron specific knockdowns, a negative geotaxis climbing assay was performed to test for a statistically significant difference between the control flies and the flies expressing the RNAi induced knockdown. Here, *GAL4* driven RNAi expression will only be induced by the cis regulatory elements of the *tdc2* gene, which encodes an enzyme involved in the biosynthesis of octopamine needed for octopaminergic and tyraminergic neurons to determine whether the *PLA2G6* protein product functions to maintain aging health in octopaminergic and tyraminergic neurons.

Methods and Materials

Drosophila Stocks and Maintenance

Tdc2-GAL4, *HMS1544*, *yw*, and *Valium 10* stocks were from Bloomington Drosophila Stock Center. The *tdc2-GAL4* stock expresses the Gal4 protein specifically in octopaminergic and tyraminergic neurons. *HMS1544* (experimental) contains the UAS for the Gal 4 protein followed by the nucleotide sequence yielding RNAi against *CG6718.* The *yw* (control 1) stock lacks the UAS sequence. The *Valium 10* (control 2) stock contained the UAS for the Gal4 protein but was not followed by a sequence yielding RNAi. Instead, it expressed GFP.

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 [31]. Male F1s from the experimental, control 1, and control 2 crosses were regularly passed to new fly vials to ensure a healthy environment, approximately every 5 days.

Isolating Virgins

- 20 female virgins were collected from the control 1, control 2, and experimental stocks (*yw*, *Valium 10*, and *HMS1544* respectively) to ensure progeny were from the set up cross

1. Stocks of *yw, Valium 10* and *HMS1544* flies were incubated in fly vials containing standard media at 25.0˚C
2. Flies were anesthetized on a CO_2 pad, and female virgins were identified by their large white bodies and black meconium
3. Confirmed virgin females were stored in a designated vial and suspected virgin females were stored in a separate vial
4. Virgin vials were quarantined for 5 days to ensure that no larvae were present in the vial

Setting Up Crosses

- Control 1 F1 flies were the progeny of crosses between *tdc2-Gal4* males with *yw* females. Control 2 F1 flies were the progeny of crosses between *tdc2-Gal4* males with *Valium 10* females. Experimental F1 flies were the progeny of crosses between *tdc2GAL4* males with *HMS1544* female virgin flies respectively. A given cross vial was comprised of 4 males *tdc2GAL4* males and 6-8 *yw*, *Valium 10* or *HMS1544* female virgin flies

1. 3 control 1 cross vials containing 4 *tdc2GAL4* males and ~7 yw female virgin flies were assembled. 3 control 2 cross vials containing 4 *tdc2GAL4* males and ~7 *Valium 10* female virgin flies were assembled. 3 experimental cross vials containing 4 *tdc2GAL4* males and ~7 *HMS1544* female virgin flies were assembled.
2. The cross vials were all incubated in fly vials at 26.0˚C
3. The parental generation flies were moved to a new fly vial 5 days after the most recent cross was made. This step was repeated to yield a total of >100 F1 males from the control 1, control 2, and experimental crosses
4. >100 F1 males from the experimental, control 1, and control 2 crosses were gathered into vials based on the date of eclosion. No more than 13 flies were housed in a single vial. These groups of 13 were numbered, and the flies in each group were kept together throughout the experiment
5. Every 5 days, the experimental and control F1 males were passed to new food vials

Climbing Assays

- At days 10, 20, and 30 following eclosing, the climbing indices of the flies were measured in a negative geotaxis assay.

1. On days 10, 20, and 30, after passing the groups of control 1, control 2, and experimental F1 flies to new food vials, an empty fly vial was taped over the food vial
2. A mark was made on the bottom vial, indicating the point 6 cm above the top of the fly food
3. The flies were tapped down onto the food, and the number of flies that climbed above the 6 cm mark within 20 seconds following the tapping were counted. For each group of flies, the climbing assay was run a total of 5 times.
4. Climbing index (CI) for each group was calculated as the sum of flies to successfully reach above the 6 cm mark within 20 seconds in 5 trials divided by the number of flies in the group. Max CI = 5.
5. The sets of climbing assays were completed for each group until more than 100 F1 control and experimental flies had been tested.

Statistical analysis

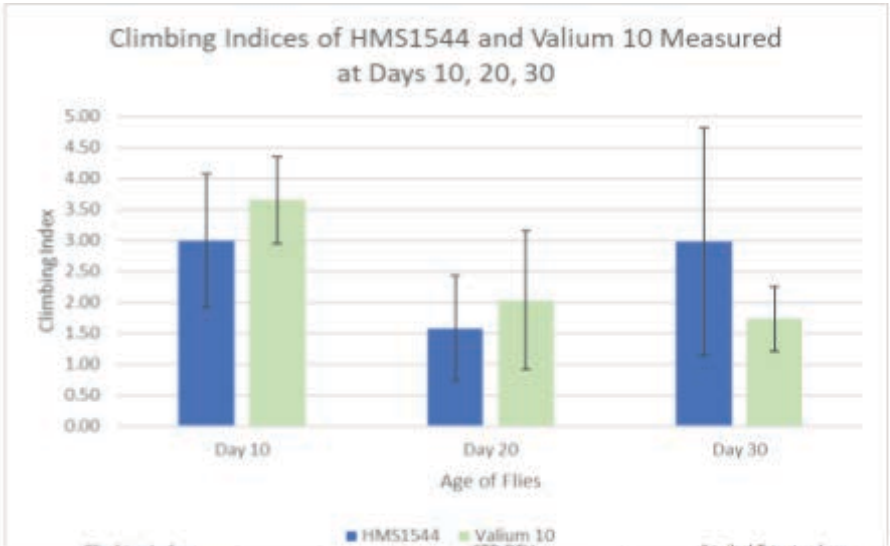
- Climbing indices for all groups of each condition were averaged and subjected to statistical analysis. Given the average climbing index at each age for the control 1, control 2, and experimental F1, an assessment was made for whether differences in the climbing indices were statistically significant. Moreover, T-tests were calculated to assess whether differences in season of testing (fall vs. spring), and different researchers performing the climbing assays (me vs. my lab partners) were statistically significant.

1. The average climbing index for each group between the 5 trials was calculated.
2. The climbing index for all groups for each condition were averaged, and the standard deviation of the averages was calculated. All climbing index values are written in the appendix.

3. A T-test p value was calculated for day 10, 20, and 30 trials using the excel T-test function. The T-test calculation was for a 2-tailed T-test for two samples displaying heteroscedasticity.
4. T-tests were calculated to assess whether differences in season of testing and different researchers performing the climbing assays were statistically significant

Results

In the Fall, climbing assays for 102 control 2 F1s and for 123 experimental F1s were conducted at days 10, 20, and 30. A bar graph with the average climbing indices is shown in Fig 7, with the bars representing +/- 1 standard deviation. The t-test p values are shown comparing the climbing index values measured in figure 7 at days 10, 20, and 30.



Climbing Index

	HMS1544	Valium 10
Day 10	2.990	3.650
Day 20	1.580	2.030
Day 30	2.980	1.730

STD DEV

	Day 10	Day 20	Day 30
Valium 10	0.703	1.12	0.52
HMS1544	1.083	0.843	1.843

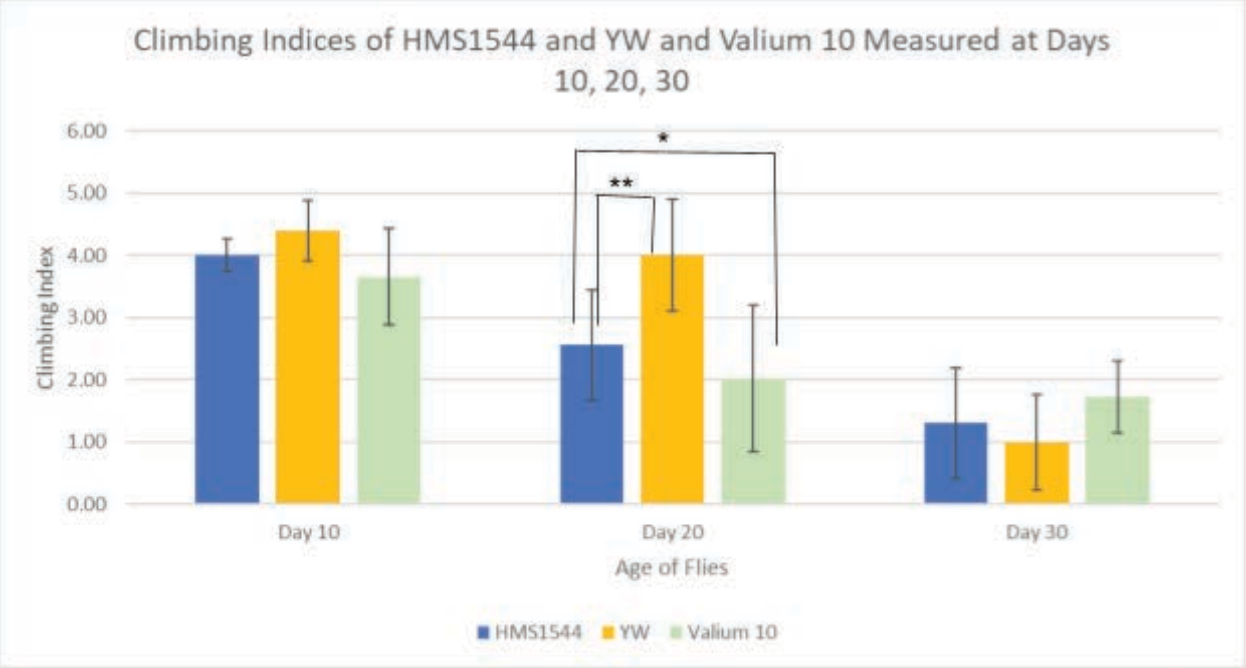
2 tailed T-test values

Day 10 t Test:	0.136
Day 20 t Test:	0.366
Day 30 t Test:	0.083

Figure 7: The climbing indices for experimental (indicated in this figure as *HMS1544*) and control 2 flies (indicated in this figure as *Valium 10*) at days 10, 20, and 30 are graphed. The 2 tailed T-test values are also shown. Statistical significance is indicated when appropriate by asterisks (*: p<0.05, **: p<0.01, ***: p<0.001).

In the Spring, the experiment was repeated using an additional control genotype. Climbing assays for 106 control 1 F1s, 103 control 2 F1s, and 96 experimental F1s were

conducted at 10, 20, and 30 days after eclosion. A bar graph with the average climbing indices is shown in Fig 8, with the bars representing +/- 1 standard deviation. The t-test p values are shown comparing the climbing index values measured in figures 8 at days 10, 20, and 30.



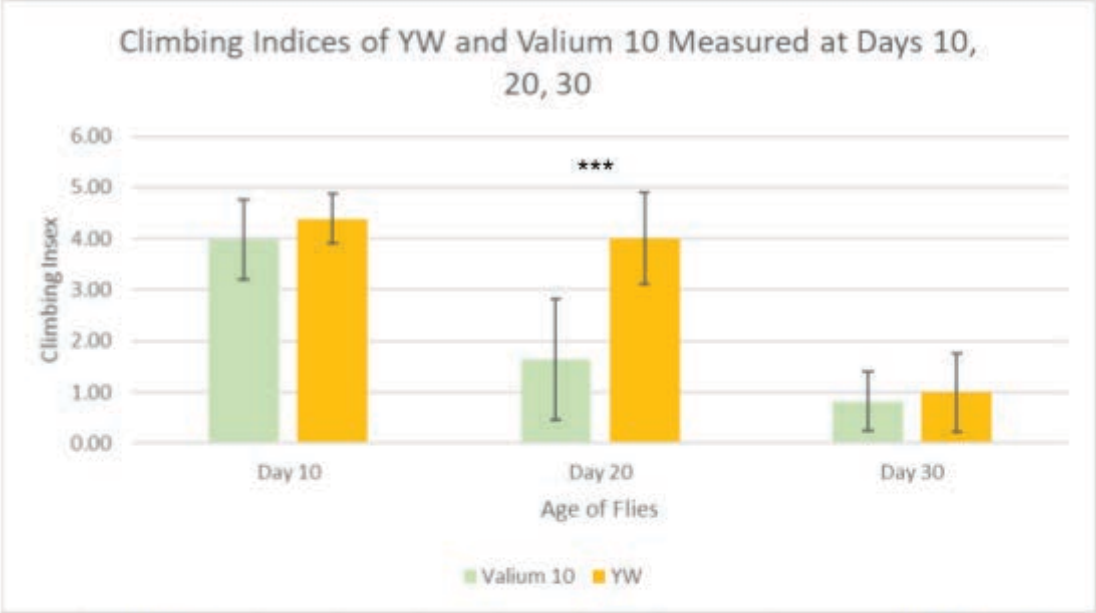
Climbing Index	HMS1544	YW	Valium 10
Day 10	4.000	4.390	3.650
Day 20	2.559	3.999	2.030
Day 30	1.313	0.993	1.730

STD DEV	Day 10	Day 20	Day 30
YW	0.486	0.897	0.764
HMS1544	0.256	0.881	0.887
Valium 10	0.776	1.175	0.575

Climbing Assay T-Test	Exp vs. YW	Exp vs. Val 10
Day 10 t Test:	0.0569	0.9509
Day 20 t Test:	0.0019	0.049
Day 30 t Test:	0.3992	0.1614

Figure 8: The climbing indices for experimental (indicated in this figure as HMS1544), control 1 flies (indicated in this figure as YW), and control 2 flies (Valium 10) at days 10, 20, and 30 are graphed. The 2 tailed T-test values are also shown. Statistical significance is indicated when appropriate by asterisks (*: $p<0.05$, **: $p<0.01$, ***: $p<0.001$).

Given concerns about the suitability of control 2 as a control, the climbing indices of control 1 and control 2 measured in the Spring are depicted in Figure 9, with the bars representing +/- 1 standard deviation. The t-test p values are shown comparing the climbing index values measured in figure 9 at days 10, 20, and 30.



	Climbing Index	
	Valium 10	YW
Day 10	3.983	4.390
Day 20	1.643	3.999
Day 30	0.822	0.993

	STD DEV		
	Day 10	Day 20	Day 30
YW	0.486	0.897	0.764
Valium 10	0.776	1.175	0.575

2 tailed T-test values	
Day 10 t Test:	0.1875
Day 20 t Test:	0.00003
Day 30 t Test:	0.5772

Figure 9: The climbing indices for control 1 (indicated in the figure as YW) and control 2 flies (indicated in the figure as Valium 10) at days 10, 20, and 30 are graphed. The 2 tailed T-test values are also shown. Statistical significance is indicated when appropriate by asterisks (*: $p<0.05$, **: $p<0.01$, ***: $p<0.001$).

To assess whether there was a statistically significant difference between climbing assay results between the testing conditions in the Fall and Spring, a T-test was performed comparing climbing indices that I performed on control 2 F1 of the same age in the Fall and the Spring as well as for experimental F1 (figure 10).

Climbing Index	Fall	Spring
Experimental F1 Day 10	2.9923	4.0002
Control 2 F1 Day 20	2.0270	1.3338

Std. Dev	Fall	Spring
Experimental F1 Day 10	1.0836	0.2556
Control 2 F1 Day 20	1.1958	1.1966

T-test Fall vs. Spring	
Experimental F1 Day 10	0.0171
Control 2 F1 Day 20	0.2367

Figure 10: Climbing indices that were performed by the same examiner on the same sampling group were compared between the fall and spring results to test for statistical significance. Data points were limited to compare climbing indices from the same genotype that I had performed in both the Fall and Spring. A box highlighted in green indicates statistical significance ($p<0.05$)

To assess whether there was a statistically significant difference between climbing indices when measured by different examiners, a T-test was performed comparing the climbing indices I

performed to another examiner who assisted in measuring the climbing indices during the same season for the same sample group (figure 11).

Climbing Index	Ari	Lab Partner
Control 1 F1 Day 10	4.391	4.387
Control 2 Day 20	1.334	2.569
Control 1 F1 Day 30	0.555	1.432

Std. Dev	Ari	Lab Partner
Control 1 F1 Day 10	0.471	0.624
Control 2 Day 20	1.197	0.168
Control 1 F1 Day 30	0.282	0.866

T-test Ari vs. lab partner	
Control 1 F1 Day 10	0.994
Control 2 Day 20	0.015
Control 1 F1 Day 30	0.086

Figure 11: Climbing indices that were performed by different lab members on the same fly sample group at the same age were compared to test for statistical significance via a 2-tailed T-test. Where applicable, statistical significance is indicated by a shaded box. Data points were limited to compare climbing indices performed during the same season and on the same group genotype.

Discussion

As seen in the T-test values from figure 8, a statistically significant difference was observed between the experimental and control 1 (*yw)* F1 sample groups at day 20 ($p=0.0019$), suggesting that knockdown of *PLA2G6* in octopaminergic and tyraminergic neurons alone leads to neurodegenerative motor symptoms. This indicates that this group of neurons, or the analogous epinerphrinergic and norepinephrinergic neurons in humans, are an important group of neurons to investigate in neurodegenerative diseases with *PLA2G6* mutations.

The poor climbing for all groups at all timepoints in the Fall suggested that our experimental conditions were suboptimal. Nevertheless, the control stock *Valium 10* still performed poorly in the Spring as compared to the *yw* control at day 20 (figure 9). This may suggest that the *Valium 10* line carries a background mutation that perturbs its climbing. As seen in the T-test values from figure 7, no statistically significant difference was observed between the experimental and control 2 F1 sample groups. Yet, the lack of a statistically significant difference might be attributable to the climbing index values for the control 2 F1 sample group being particularly low. It is expected that day 10 control flies would have a climbing index value between 4 and 5 (actual climbing index value was 3.65). More surprisingly, the experimental

group (CI: 2.98) significantly outperformed the control 2 group (CI: 1.73) in the day 30 climbing assay. These two observations raise questions about the utility of the control 2 F1 sample group as a viable control. Accordingly, the control 1 F1 were used in the next iteration of the experiment during the Spring. The additional control genotype, *yw*, performed better in the climbing assay than the original control, *Valium 10*, at days 10 and 20, and demonstrated a statistically significant difference in climbing as compared to the experimental group at day 20. However, by 30 days, the *yw* control also performed poorly, yielding no difference between controls and experimentals at 30 days.

Additionally, it is worth noting that no statistically significant difference between the control 1 and experimental populations was observed at day 30 ($p=0.399$), with both groups showing poor climbing ability. Careful inspection of the culture vials revealed bacterial growth on top of the media. This bacterial growth was attributed to not sustaining the media at a rolling boil during preparation. Due to this confound, experimental conditions in the Spring were not entirely optimal.

As seen in figure 10 and 11, mixed evidence was found for whether there was a statistically significant difference between Fall and Spring testing conditions as well as between different lab members conducting the climbing assay. Further investigation of these questions is in order.

Conclusion

A statistically significant difference between the *HMS1544 x tdc2Gal4* and *yw* x *tdc2Gal4* control flies was observed, suggesting that knockdown of *PLA2G6* in octopaminergic and tyraminergic neurons leads to symptoms of neurodegeneration. This is consistent with prior results from the Steinhauer lab and implicates these neurons, as well as their human counterparts

the epinephrinergic and norepinephrinergic neurons, in *PLA2G6* associated neurodegeneration [32]. Nevertheless, no statistically significant difference was observed between the experimental and control groups at day 30. Further iterations of this climbing assay are required to make a decisive claim as to any difference between these two sample populations.

A statistically significant difference was found between the *yw* x *tdc2Gal4* control and the *Valium 10* x *tdc2Gal4* control 2 flies. While no statistically significant difference was observed between these sample populations at day 30, the poor climbing ability of the *Valium 10* control flies suggests that this stock is not suitable for a control.

Considerations for Future Research

The presence of bacterial growth on the fly media was suggested as an explanation for the *yw* flies' reduced climbing ability. Further exploration of this suggestion might entail growing the bacteria on sterile agar, isolating a colony, running PCR using generic bacterial primers, sending off for Sanger sequencing, and identifying the bacteria via the NCBI BLAST feature. It would be interesting to explore whether this bacterial strain has been associated with a depreciation in climbing indices in other studies.

The statistical analysis in this paper to test for statistically significant changes in experimental set up, such as who performed the climbing assay and what time of year the climbing assay was performed, could be made more robust by adding information to the climbing index log sheet that the lab members filled out. Filling out the time of day, the temperature in the room, the amount of time the flies had been left to rest outside of the incubator before conducting the assay would provide a wealth of data necessary to ascertain the repeatability of a given climbing assay trial with these varying conditions. Moreover, it would be worthwhile to explore a method of achieving a uniform manner of tapping down the flies with

consistent force. Testing for a statistically significant difference between the lab partners performing the assay attempted to assess whether inconsistencies in the force used in tapping down the flies were statistically significant. Additionally, as observed in the appendix section below, some groups of flies did not climb in any of the trials. A standardized method for selecting F1s to be tested as well as identifying outliers is in order.

References

The content of this paper drew heavily from the previous research of Dr. Steinhauer's lab. The collection of data referenced in this paper was shared between Eliezer Heller, Samuel Intrator, and myself.

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Appendix

Fall Semester Testing Data

Experimental Climbing Assays - DAY 10

Group #	N	Trail 1	Trail 2	Trail 3	Trail 4	Trail 5	Sum Trial 1-5	Climbing Index	
1	11	5	4	5	4	6	24	2.182	
2	11	2	2	2	2	3	11	1.000	
3	12	4	2	6	5	5	22	1.833	
4	11	9	8	8	10	9	44	4.000	
5	14	9	10	11	7	9	46	3.286	
6	15	12	14	13	14	13	66	4.400	
7	15	10	10	11	10	10	51	3.400	
8	15	12	13	13	13	11	62	4.133	
9	10	8	4	5	6	5	28	2.800	
10	9	4	6	6	6	4	26	2.889	
Total:	123						Cumalative (Avg, Std. dev):	2.992	1.084

Experimental Climbing Assays - DAY 20

Group #	N	Trail 1	Trail 2	Trail 3	Trail 4	Trail 5	Sum Trial 1-5	Climbing Index	
1	7	0,0	0,0	0,0	0,0	0,0	0	0.000	
2	13	6	5	6	6	6	29	2.231	
3	13	7	5	8	6	6	32	2.462	
4	6	1	1	3	4	3	12	2.000	
5	10	6	5	5	5	6	27	2.700	
6	13	5	3	4	2	3	17	1.308	
7	13	8	5	4	3	3	23	1.769	
8	13	1	1	2	2	1	7	0.538	
9	11	2	4	3	3	4	16	1.455	
10	9	3	1	3	3	2	12	1.333	
N=	108						Cumalative (Avg,	1.580	0.843

Experimental Climbing Assays - DAY 30

Group #	N	Trail 1	Trail 2	Trail 3	Trail 4	Trail 5	Sum Trial 1-5	Climbing Index	
1	7	0	0	0	0	0	0	0.000	
2	11	2	2	2	1	2	9	0.818	
3	11	4	4	4	4	4	20	1.818	
4	11	5	6	4	3	3	21	1.909	
5	13	11	12	11	11	13	58	4.462	
6	13	13	13	13	11	13	63	4.846	
7	13	11	12	9	10	13	55	4.231	
8	13	13	12	10	12	13	60	4.615	
9	13	12	12	9	9	11	53	4.077	
	105						Cumalative (Avg, Std. dev):	2.975	1.843

Control 2 Climbing Assays - DAY 10

Group #	N	Trail 1	Trail 2	Trail 3	Trail 4	Trail 5	Sum Trial 1-5	Climbing Index	
1	12	5	4	8	7	7	31	2.583	
2	12	9	7	7	9	9	41	3.417	
3	12	10	11	11	12	11	55	4.583	
7*	13	8	6	8	9	8	39	3.000	
8	12	5	7	7	8	8	35	2.917	
9	8	5	6	7	6	6	30	3.750	
10	12	10	10	11	10	11	52	4.333	
11	12	11	9	9	8	11	48	4.000	
12	9	6	9	9	7	7	38	4.222	
Total	102						Cumulative (Avg, Std. dev):	3.645	0.703

* groups 4, 5, and 6 were not counted due to the assay having been tested with N>13

Control Climbing Assays - DAY 20

Group #	N	Trail 1	Trail 2	Trail 3	Trail 4	Trail 5	Sum Trial 1-5	Climbing Index	
1	12	0	2	3	3	4	12	1.000	
2	12	0	4	1	1	2	8	0.667	
3	11	5	3	3	6	1	18	1.636	
4	11	7	2	5	2	3	19	1.727	
5	11	4	1	2	2	3	12	1.091	
6	12	3	5	5	4	2	19	1.583	
7	11	9	9	8	9	9	44	4.000	
8	13	5	9	9	8	7	38	2.923	
9	13	10	10	9	9	9	47	3.615	
N=	106						Cumulative:	2.027	1.196

Control Climbing Assays - DAY 30

Group #	N	Trail 1	Trail 2	Trail 3	Trail 4	Trail 5	Sum Trial 1-5	Climbing Index	
1	10	3	2	2	3	3	13	1.300	
2	12	3	1	3	3	3	13	1.083	
3	13	4	6	4	7	8	29	2.231	
4	11	2	3	5	4	4	18	1.636	
5	13	5	6	5	6	5	27	2.077	
6	13	3	2	4	3	4	16	1.231	
7	13	3	5	4	5	5	22	1.692	
8	13	5	6	9	9	6	35	2.692	
9	12	3	3	3	5	6	20	1.667	
	110						Cumulative:	1.734	0.520

Spring Semester Testing Data

Experimental Climbing Assays - DAY 10 HMS1544

Group #	Date collected	10 d climbing	Time of Testing	Temperature in Room [C]	N	Trail 1	Trail 2	Trail 3	Trail 4	Trail 5	Sum Trial 1-5	Climbing Index	Who performed climbing assay
1	2/18	2/28	1:20 PM	21	11	8	9	10	10	11	48	4.364	Ari
2	2/21	3/2	8:10 PM	21.5	11	9	9	10	9	10	47	4.273	Ari
3	2/21	3/2	8:20	21.5	13	12	10	8	9	11	50	3.846	Ari
4	2/21	3/2	8:32	21.5	13	9	10	10	9	11	49	3.769	Ari
5	2/21	3/2	8:42	21.5	12	10	9	8	9	10	46	3.833	Ari
6	2/21	3/2	9:00 PM	21.5	12	9	8	9	9	9	44	3.667	Ari
7	2/21	3/2		21.5	n/a *escaped? n=4								
8	2/21	3/2	9:05	21.5	12	11	11	8	10	9	49	4.083	Ari
9	2/21	3/2	9:13	21.5	12	11	9	10	11	9	50	4.167	Ari
10	3/1	3/11											
				Total:	96				Cumalative (Avg, Std. dev):	4.000	0.256		

Experimental Climbing Assays - DAY 20 HMS1544

Group #	Date collected	20 d climbing	Time of Testing	Temperature in Room [C]	N	Trail 1	Trail 2	Trail 3	Trail 4	Trail 5	Sum Trial 1-5	Climbing Index	Who performed climbing assay
1	2/18	3/10	12:36	22	8	2	3	3	3	4	15	1.875	Sammy
2	2/21	3/13	20:39	22	9	8	8	8	6	8	38	4.222	Eliezer
3	2/21	3/13	20:42	22	12	5	6	10	8	8	37	3.083	Eliezer
4	2/21	3/13	20:46	22	13	7	9	8	9	10	43	3.308	Eliezer
5	2/21	3/13	20:50	22	11	3	6	6	7	5	27	2.455	Eliezer
6	2/21	3/13	20:53	22	12	5	4	4	4	5	22	1.833	Eliezer
7	2/21	3/13		22									Eliezer
8	2/21	3/13	20:57	22	12	4	8	7	6	7	32	2.667	Eliezer
9	2/21	3/13	20:59	22	11	5	6	8	5	7	31	2.818	Eliezer
10	3/1	3/21	16:47	20	13	3	3	3	2	3	14	1.077	ari
11	3/1	3/21	4:55 PM	20	12	5	5	6	5	6	27	2.250	ari
		Total:			113				Cumalative (Avg, Std. dev):	2.559	0.881		

Experimental Climbing Assays - DAY 30 **HMS1544**

*bolded done on 3/24

Group #	Date collected	30 d climbing	Time of Testing	Temperature in Room [C]	N	Trail 1	Trail 2	Trail 3	Trail 4	Trail 5	Sum Trial 1-5	Climbing Index	Who performed climbing assay
1	2/18	3/20	18:57	21	8	0	0	0	2	0	2	0.250	Sammy
2	2/21	3/23	17:46	20	7	5	4	4	4	5	22	3.143	ari
3	2/21	3/23	17:56	20	11	3	4	2	5	5	19	1.727	ari
4	2/21	**3/23**	start 11am	21	13	5	5	4	6	5	25	1.923	Eliezer
5	2/21	**3/23**		21	11	2	1	0	2	2	7	0.636	Eliezer
6	2/21	**3/23**		21	11	0	4	0	2	0	6	0.545	Eliezer
7	2/21	**3/23**			X						0		Eliezer
8	2/21	**3/23**		21	11	4	1	2	2	2	11	1.000	Eliezer
9	2/21	**3/23**	end 11:30am	21	10	1	3	1	2	2	9	0.900	Eliezer
10	3/1	3/31	14:21	22	13	1	2	3	2	4	12	0.923	sammy
11	3/1	3/31	14:30	22	12	7	5	5	3	5	25	2.083	sammy
		Total:			107				Cumalati	1.313	0.887		

Control **YW** Climbing Assays - DAY 10

Group #	Date collected	10 d climbing	Time of Testing	Temperature in Room [C]	N	Trail 1	Trail 2	Trail 3	Trail 4	Trail 5	Sum Trial 1-5	Climbing Index	Who performed climbing assay
1	2/18	2/28	21:30	21.5	11	9	10	10	10	11	50	4.545	Eliezer
2	2/18	2/28	21:33	21.5	12	12	12	12	12	11	59	4.917	Eliezer
3	2/18	2/28	21:37	21.5	10	7	7	8	8	7	37	3.700	Eliezer
4	2/18	2/28	21:44	21.5									
5	2/21	3/2	21:30	21.5	12	12	12	12	12	12	60	5.000	Ari
6	2/21	3/2	21:35	21.5	11	8	10	9	10	11	48	4.364	ari
7	2/21	3/2	21:42	21.5	12	7	11	10	11	11	50	4.167	ari
8	2/21	3/2	21:46	21.5	13	12	12	12	12	12	60	4.615	Ari
9	2/21	3/2	21:52	21.5	12	10	10	11	12	12	55	4.583	Ari
10	2/23	3/5	19:52	20.8	13	9	8	9	11	10	47	3.615	ari
		Total			106						Cumalative:	4.390	0.486

Control **YW** Climbing Assays - DAY 20

*4 + *9= There was a mixup between groups 4 and 9 where they were combined despite having different hatch dates

Group #	Date collected	20 d climbing	Time of Testing	Temperature in Room [C]	N	Trail 1	Trail 2	Trail 3	Trail 4	Trail 5	Sum Trial 1-5	Climbing Index	Who performed climbing assay
1	2/18	3/10	12:56	22	9	8	8	9	8	9	42	4.667	Sammy
2	2/18	3/10	13:08	22	10	9	10	10	10	10	49	4.900	Sammy
3	2/18	3/10	13:25	22	10	5	6	8	8	8	35	3.500	Sammy
~~*4~~	~~2/18~~	~~3/10~~	~~X~~	~~X~~	~~X~~	~~X~~	~~X~~	~~X~~	~~X~~	~~X~~	x	x	
5	2/21	3/13	21:19	22	12	12	12	12	12	12	60	5.000	Ari
6	2/21	3/13	21:24	22	11	9	9	10	10	11	49	4.455	ari
7	2/21	**3/13**	21:20	22	10	6	5	4	3	5	23	2.300	Eliezer
8	2/21	**3/13**	21:24	22	12	8	8	8	5	6	35	2.917	Eliezer
~~*9~~	~~2/24~~	~~3/13~~	~~X~~	~~X~~	~~X~~	~~X~~	~~X~~	~~X~~	~~X~~	~~X~~	x	x	
10	2/23	3/15	13:32	22	9	6	8	6	6	6	32	3.556	Ari
11	3/1	3/21	17:08	20	13	8	13	12	12	13	58	4.462	ari
12	3/2	3/21	17:01	20	13	11	10	12	10	12	55	4.231	ari
		Total			109						Cumulative:	3.999	0.897

Control **YW** Climbing Assays - DAY 30

Group #	Date collected	30 d climbing	Time of Testing	Temperature in Room [C]	N	Trail 1	Trail 2	Trail 3	Trail 4	Trail 5	Sum Trial 1-5	Climbing Index	Who performed climbing assay
1	2/18	3/20	19:21	21	9	6	5	4	6	4	25	2.778	Sammy
2	2/18	3/20	19:31	21	10	1	0	1	1	1	4	0.400	sammy
3	2/18	3/20	19:40	21	9	2	2	3	3	3	13	1.444	sammy
~~*4~~	*	x	x	x	x	x	x	x	x	x	x	x	
5	2/21	3/23	18:06	20	9	0	2	2	3	2	9	1.000	ari
6	2/21	3/23	18:13	20	10	1	0	2	1	1	5	0.500	ari
7	2/21	3/23	18:23	20	8	0	0	1	3	1	5	0.625	ari
8	2/21	3/23	18:34	20	11	0	0	1	1	1	3	0.273	ari
~~*9~~	*	x	x	x	x	x	x	x	x	x	x	x	
10	2/23	3/25	11:23	21	8	1	1	0	1	0	3	0.375	ari
11	3/1	3/31	15:03	22	13	0	4	6	6	3	19	1.462	sammy
12	3/1	3/31	15:10	22	13	3	1	2	4	4	14	1.077	sammy
		Total			100							0.993	0.764

Control **Valium 10** Climbing Assays **-** DAY 10

* = Lost 1 fly in 10d climbing, new N= N-1

Group #	Date collected	10 d climbing	Time of Testing	Temperature in Room [C]	N	Trail 1	Trail 2	Trail 3	Trail 4	Trail 5	Sum Trial 1-5	Climbing Index	Who performed climbing assay
*1	2/18	2/28	22:25	22	7	7	6	7	7	6	33	4.714	Sammy
*2	2/18	2/28	22:35	22	10	5	7	6	7	8	33	3.300	Sammy
*3	2/18	2/28	22:45	22	9	6	7	7	7	7	34	3.778	Sammy
4	2/21	3/3	12:30	22	12	12	12	11	11	12	58	4.833	Steinhauer
5	2/21	3/3	12:36	22	11	11	11	11	10	11	54	4.909	Steinhauer
6	2/21	3/3	12:42	22	11	3	10	9	10	10	42	3.818	Steinhauer
7	2/21	3/3	12:48	22	12	9	10	7	12	10	48	4.000	Steinhauer
8	2/21	3/3	12:55	22	9	9	8	8	8	9	42	4.667	Steinhauer
9	2/22	3/5	19:32	20.8	11	6	5	6	8	7	32	2.909	ari
11	2/22	3/5	19:45	20.8	11	7	5	7	7	6	32	2.909	ari
12	2/23												
				Total	103						Cumalative:	3.984	0.776

Control **Valium 10** Climbing Assays - DAY 20

*1+2 were combined after the climbing assay

Group #	Date collected	20 d climbing	Time of Testing	Temperature in Room [C]	N	Trail 1	Trail 2	Trail 3	Trail 4	Trail 5	Sum Trial 1-5	Climbing Index	Who performed climbing assay
1	2/18	3/10	13:36	22	6	3	3	3	4	3	16	2.667	Sammy
2	2/18	3/10	13:45	22	6	3	4	4	2	3	16	2.667	Sammy
3	2/18	3/10	13:54	22	8	2	3	3	6	5	19	2.375	Sammy
4	2/21	3/13	20:50	22	12	2	1	2	0	0	5	0.417	Ari
5	2/21	3/13	20:56	22	10	4	3	3	5	4	19	1.900	Ari
6	2/21	3/13	21:01	22	11	0	0	1	0	1	2	0.182	Ari
7	2/21	3/13	21:08	22	10	0	2	2	3	1	8	0.800	Ari
8	2/21	3/13	21:14	22	8	2	1	2	3	1	9	1.125	Ari
9	2/22	3/14	13:41	22	11	9	7	6	8	10	40	3.636	ari
x	x	x	x	x	x	x	x	x	x	x	x	x	
11	2/23	3/15	13:47	22	9	5	3	7	5	5	25	2.778	ari
12	3/1	3/21	17:22	20	12	0	0	2	5	3	10	0.833	ari
13	3/1	3/21	17:15	20	12	0	2	0	0	2	4	0.333	ari
		Total			91						Cumulative:	1.643	1.175

Control **Valium 10** Climbing Assays - DAY 30

Group #	Date collected	30 d climbing	Time of Testing	Temperature in Room [C]	N	Trail 1	Trail 2	Trail 3	Trail 4	Trail 5	Sum Trial 1-5	Climbing Index	Who performed climbing assay
1	2/18	3/20	19:59	21	12	4	4	4	3	3	18	1.500	sammy
2	2/18	3/20	Group 1 and 2 have been combined			~~x~~	~~x~~	~~x~~	~~x~~	~~x~~	~~x~~	~~x~~	
3	2/18	3/20	19:09	21	6	0	0	0	0	0	0	0.000	sammy
4	2/21	3/23	18:01	20	11	1	1	0	0	0	2	0.182	sammy
5	2/21	3/23	18:11	20	8	0	2	1	0	2	5	0.625	sammy
6	2/21	3/23	18:18	20	10	0	0	0	0	0	0	0.000	sammy
7	2/21	3/23	18:26	20	8	3	4	1	1	1	10	1.250	sammy
8	2/21	3/23	x	combined with 9	x	x					x	x	
9	2/22	3/24	10:34	21	12	3	5	2	3	3	16	1.333	ari
x	x	x	x	x	x	x	x	x	x	x	x	x	
11	2/23	3/24	11:14	21	8	2	2	2	3	0	9	1.125	ari
12	3/1	3/31	14:45	22	7	2	2	2	0	1	7	1.000	sammy
13	3/1	3/31	14:56	22	10	3	1	1	2	5	12	1.200	sammy
		Total			92							0.822	0.575

Data for Analysis of Climbing Assay by Fall versus Spring Semester

Exp 10 Spring	Exp 10 Fall
4.364	2.182
4.273	1.000
3.846	1.833
3.769	4.000
3.833	3.286
3.667	4.400
4.083	3.400
4.167	4.133
	2.800
	2.889

Val 10 Fall	Val 10 Spring
1.000	0.417
0.667	1.900
1.636	0.182
1.727	0.800
1.091	1.125
1.583	3.636
4.000	2.778
2.923	0.833
3.615	0.333

Data for Analysis of Climbing Assay by Lab Partner

YW Day 10 Ari	YW Day 10 Eliezer
5.000	4.545
4.364	4.917
4.167	3.700
4.615	
4.583	
3.615	

Valium 10 Ari	Valium 10 sammy
0.417	2.667
1.900	2.667
0.182	2.375
0.800	
1.125	
3.636	
2.778	
0.833	
0.333	

YW Day 30 Ari	YW Day 30 Sammy
1.000	2.778
0.500	0.400
0.625	1.444
0.273	1.462
0.375	1.077