# Effect of Downregulation of cdk5 and p35 by RNAi on Neurodegeneration in the Adult CNS of *Drosophila melanogaster*.

By:
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A Senior Comprehensive Project in Partial Fulfillment of the Requirements for a Bachelor of Science Degree from Allegheny College

***I hereby recognize and pledge to fulfill my responsibilities as defined in the Honor Code and to maintain the integrity of both myself and the College community as a whole.***

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Pledge

Approval by the Senior Thesis Committee:

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**Abstract**

Cyclin dependent kinase 5 (cdk5) is a serine/threonine directed kinase that requires association with a protein regulator, p35, for proper localization and activity. Cdk5 has been shown to be important in central nervous system development, maintenance of synapses within the central nervous system, and nervous system plasticity. Recently, cdk5 deregulation has been implicated in neurodegenerative diseases including Alzheimer's Disease and amyotrophic lateral sclerosis (ALS). Previous experiments have shown that *Drosophila melanogaster* mutants lacking p35 show a shortened life span and decreased performance in a wall climb assay, both of which are consistent with neurodegeneration. The current experiment sought to further elucidate the role of cdk5 and p35 by eliminating the possibility of the adult neurodegenerative phenotype being the result of developmental miswiring, as well as provide histological evidence of neurodegeneration occurring within both cdk5 and p35 *Drosophila* mutants. With the use of RNAi, we were able to downregulate cdk5 or p35 activity within the adult central nervous system gradually, which eliminated any possibility for developmental miswiring due to a lack of cdk5/p35 during development. While experimental strains confirmed motor deficits previously seen in the wall climb assay, it was not found that the strains had a shortened lifespan. Furthermore, histological analysis of these strains showed no signs of neurodegeneration. Therefore, the results indicate a vital role of cdk5/p35 in preserving motor function within the adult central nervous system, however, the downregulation of cdk5/p35 in adults does not seem to be involved in neurodegeneration.

**Background**

Cyclin dependent kinase 5 (cdk5) is a member of the serine/threonine cyclin dependent kinase family (Dhavan and Tsai 2001)**.** Kinases, in general, are important regulators of biological processes through their phosphorylation of protein substrates and other biological targets. More specifically, the serine/threonine cyclin dependent kinases are typically associated with cell cycle progression, especially in the regulation of cell differentiation and mitosis (Cruz and Tsai 2004)**.** However, cdk5 is a unique member of this family of kinases and has yet to have a discovered role in cell cycle progression. Rather, it is associated with the regulation of neuronal migration in the developing central nervous system, phosphorylation of many substrates involved in cell adhesion and motility, synaptic plasticity, and dopamine signaling. Recently, a possible role of cdk5 in neurodegeneration has been described as a result of deregulation of this kinase (Dhavan and Tsai 2001).

Cdk5 was discovered in 1990, aided by it's sharing of 60% sequence homology to cdk2. Cdk5 has been shown to be important in many cellular functions other than cell cycle progression. Cdk5 is a proline directed kinase, meaning that it phosphorylates a serine or threonine immediately upstream of a proline. Also, cdk5 prefers one of three basic residues (lysine, histidine, or arginine) at the + 3 position (Figure 1). Like other cyclin dependent kinases, cdk5 has no enzymatic activity until it is associated with a regulatory partner. In typical cdk's, the regulatory partner is a cyclin; however, cdk5 has two identified regulatory partners that do not assume a cyclin fold prior to association with cdk5 (Dhavan and Tsai 2001). p35 and p39, the two regulatory partners of cdk5,

only assume a cyclin like fold upon association with cdk5 as predicted by computer modeling and mutagenesis experiments (Smith and Tsai 2002).

**(S/T)-P-**X**-(K/H/R)**

*Figure 1*. Proline directed kinase activity of cdk5. Cdk5 phosphorylates a serine or threonine (S/T) upstream of a proline (P) in the +1 position and a basic residue, lysine, histidine, arginine (K/H/R) in the +3 position. X is equal to any amino acid (Dhavan and Tsai 2001).

Due to their required association with regulatory partners, cdk's typically are found spatially and temporally in the same cells and areas as their regulatory partners. In the case of cdk5, p35 and p39 tend to be highly concentrated at the cell periphery, especially growth cones, synapses, and cytoskeleton and membrane fractions. Therefore, as expected, cdk5 is typically localized to the same areas of post-mitotic cells, especially in the nervous system (Tsai et. al. 1994). This leads many to hypothesize that the normal substrates of cdk5 are transmembrane and membrane associated proteins (Dhavan and Tsai 2001).

While cdk5 is constitutively expressed in areas where p35 or p39 are active, its regulatory partners are not expressed ubiquitously. Rather, p35 and p39 are unstable compounds with a half-life of only 20-30 minutes. This short half life has been shown to be the result of a negative feedback loop between cdk5 and p35. In short, p35 is phosphorylated by cdk5 which leads to its degradation. This was shown by experiments which illustrated that p35 mutants lacking phosphorylation sites normally targeted by cdk5 have a significantly longer half life. p35 expression seems to be directed by the ERK (extracellular signaling regulated kinase) pathway as well as by levels of BDNF (brain derived neurotropic growth factor) in post-mitotic neurons (Figure 2). Regulation

of p35 expression, as shown by the negative feedback loop with cdk5, as well as the requirement that p35 and/or p39 associate with cdk5 in order for it to become active, demonstrates that cdk5 activity within cells is tightly regulated, especially in post-mitotic neurons (Dhavan and Tsai 2001).

ERK→NGF→ERG1→p35→cdk5

*Figure 2.* ERK Signaling pathway. ERK activates Nerve Growth Factor (NGF) which then activates Early Growth Response 1 (ERG1). ERG1 then binds to the promoter of p35 which increases its expression and helps activate cdk5 activity. Once activated, cdk5 phosphorylates p35 in order to increase its degradation and provide a negative feedback loop for cdk5 activity.

One of cdk5's major roles is regulating the migration and development of the central nervous system. Mice that are deficient in p35 or cdk5 show a lack of glial dependant migration and instead use 'branch migration,' which doesn't allow for proper inside out layering of the neocortex. Cdk5 phosphorylates many proteins during development that are important in microtubule structure and stability of glial cells which aids in the migration of neurons in the developing cortex (Cruz and Tsai 2004). Without cdk5 or its activator p35, these mice show defective inside out layering of the cortex as well as dendrites that project randomly, rather than radially, out towards the brain's surface (Smith and Tsai 2002). Null mutants for cdk5, or both its activators (p35 and p39), are not viable and typically die before birth or within 12 hours after birth (Ohshima et. al. 1996).

Cdk5 also has a major effect on synaptic transmission in the adult nervous system. It is critical for the formation and maintenance of the neuromuscular junction. Neuroregulin directly controls acetylcholine receptor expression and activates the cdk5/p35 complex which in turn phosphorylates ErbB and allows for ErbB endocytosis

and increased AchR expression (Smith and Tsai 2002). Cdk5 is also implicated in dopamine signaling through its conversion of DARPP-32 into an inhibitor of protein kinase A. Normally, DARPP-32 conveys DA signaling through its inhibition of protein phosphate 1 and activation of protein kinase A. By phosphorylation of DARPP-32, cdk5 is able to reduce DA signaling by indirectly inhibiting protein kinase A, an activator of dopamine signaling (Smith and Tsai 2002). Also, cdk5 can regulate ion channels and NMDA receptors at synapses, as well as the expression of voltage dependent $Ca^{2+}$ ion channels (Cruz and Tsai 2004).

Disregulation of cdk5 activity has recently been linked to neurodegeneration. Perhaps the most fundamental disregulation of cdk5 is through the proteolytic cleavage of p35 to p25 by the calcium dependent protease, calpain (Dhavan and Tsai 2001). Normally, p35 contains an N-terminus myristoylation signal motif which is highly conserved in mammalian p35 homologues and in *Xenopus* and *C. Elegans* (Figure 3). This signal motif is required for subcellular localization towards the cell periphery. During the proteolytic cleavage of p35 to p25, this signal motif is lost resulting in localization of p25 in the nucleus and perinuclear regions of the cell. This results in the sequestering of cdk5 away from the cell periphery and directs it to different protein substrates due to the fact that the regulatory partners of cdk5 control its subcellular localization (Patrick et. al. 1999). Also, p25 displays a 5 fold longer half life than p35, which has a half life of 20-30 minutes. The redirection of cdk5 from normal cellular substrates and the accumulation of p25 has been shown to correlate with neurite retraction, microtubule collapse, as well as the loss of phosphorylation of normal cdk5

targets including synapsin, syntaxin, and DARPP-32, all of which may lead to neuronal dysfunction (Patrick et. al. 1999)**.**

| | |
|---|---|
| P35 (Human) | M**G**TVLSLS-PSY |
| P35.1 (*Xenopus)* | M**G**TVLSLS-PGS |
| P39 (Human) | M**G**TVLSLS-PAS |
| *C. Elegans* | M**G**ANLTSPLPHH |

*Figure 3.* Conservation of myristoylation signal motifs in p35 homologues. Myristoylation signal motifs typically contain glycine residues (G) and are critical to membrane targeting (Patrick et. al. 1999).

Brain tissue of sporadic Alzheimer's disease patients has shown higher levels of p25, cdk5, and calpain. It is proposed that the cleavage of p35 to p25 redirects cdk5 to phosphorylate the Tau protein, eventually leading to hyperphosphorylation of Tau, disassociation of Tau from microtubule structures, the formation of neurofibrillary tangles (NFT), and disruption and collapse of microtubule structure in neurons, a pathological hallmark of Alzheimer's (Dhavan and Tsai 2001). It is proposed that Tau hyperphosphorylation is the toxic component which makes amyloid beta (Aβ) plaques toxic as inhibition of cdk5 activity blocks Aβ toxicity without preventing its accumulation (Dhavan and Tsai 2001). Transgenic mice with a mutation for both p25 formation and human Tau show increased NFT's in the brainstem as compared to transgenic lines only expressing mutant human Tau protein. Also, it was shown that p25 expressed in the postnatal forebrain was enough to drive NFT formation without a mutation for deregulated human tau. These mice also showed massive brain atrophy and neuronal loss in the cortex and hippocampus, which are the major brain areas involved in taupathies such as Alzheimer's and other frontotemporal dementias (Cruz and Tsai 2004). Lastly, elevated levels of p25 are not found in brain tissue of Alzheimer patients in the end stage of the disease, suggesting further that p25 may be an early upstream regulator

of Tau pathology progression and may serve as the link between plaque/tangle toxicity pathologies. However, more research must be done on this idea as there are many other possibilities as to why p25 is not found in end-stage Alzheimer's patients (Dhavan and Tsai 2001, Cruz and Tsai 2004).

Cdk5 deregulation is also implicated in amyotrophic lateral sclerosis (ALS). Motor neurons expressing mutant SOD-1, a gene related to familial cases of the disease, are more susceptible to glutamate mediated cell death and disruption of intracellular levels of $Ca^{2+}$. This intracellular calcium causes a cleavage of p35 to p25, causing an elevation of p25 in end stage ALS patients. SOD1 mutant mice show mislocalization and hyperactivation of cdk5 as well as a disruption of cdk5 activity in cell adhesion and synaptic activity. Therefore, there may be both a toxic gain of function of the p25/cdk5 substrate as well as a toxic loss of function of the p35/cdk5 complex, resulting in motor neuron degeneration (Figure 4) (Patzke and Tsai 2002).

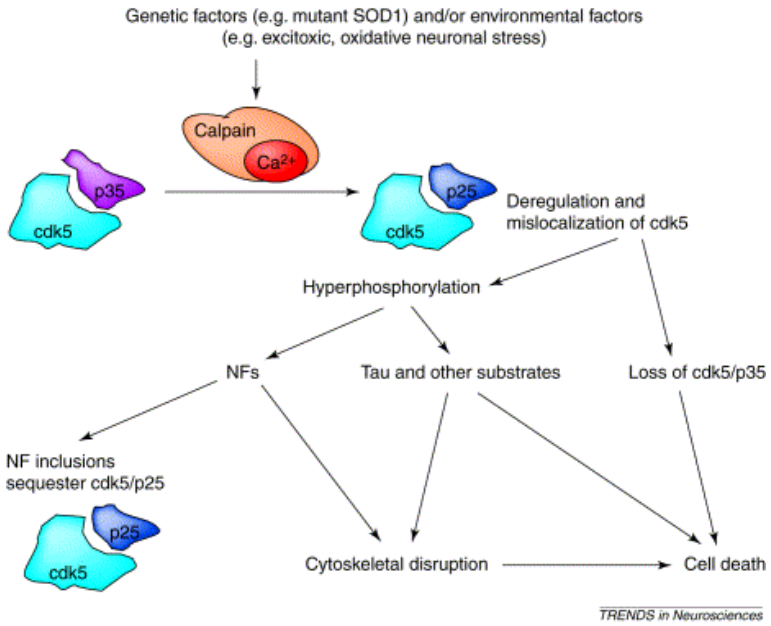



*Figure 4.* Cdk5's role in the pathogenesis of ALS. Changes in intracellular $Ca^{2+}$ levels cause activation of the protease calpain which generates p25 via cleavage of p35. p25 induces deregulation and mislocalization of cdk5 which causes hyperphosphorylation of NFs, tau, and other substrates and leads to cytoskeletal disruption and motor neuron cell death. Furthermore, the loss of cdk5/p35 activity may also contribute to disease pathogenesis. NF accumulation can also act as a 'phosphorylation sink' to sequester cdk5/p25, alleviating some of the hyperphosphorylation of cdk5 substrates (Patzke and Tsai 2002).

Although many studies have focused on the hyperactivity of cdk5 in relation to neurodegeneration, many hypothesize that a knockout or downregulation of cdk5 activity may also cause cell death. Neruofilaments (NF's) are among the most extensively phosphorylated proteins in the adult central nervous system. This phosphorylation stabilizes the NF network in the axon and aids in axonal transport and conduction velocity down the axon (Wild et. al. 2007). Cdk5 is suggested to be the main kinase involved in proper phosphorylation of NF's. Cdk5 null mutant mice showed motor neurons with increased cytoplasmic vacuoles in the ventral horn of the spinal cord but not in the dorsal root ganglion. Therefore, it is hypothesized that these NF are specific to CNS motor neurons. Neurons in the brainstem of these animals showed increased

immunoreactivity of NF protein in the cell body which leads many to speculate that there are transport defects of NFs in cdk5 null mutants (Ohshima et. al. 1996). Neurons of cdk5 null mutants and p35/p39 double null mutants also showed ballooned axon hillocks, as well as eccentric nuclei in motor neurons. Chromatolytic changes in these neurons were associated with aberrant accumulation of phosphorylated NF's in the cell soma, which are normally relegated to the axon via phosphorylation by cdk5. Phosphorylated NF accumulation is especially prevalent in large motor neuron cell bodies in the cervical and upper thoratic spinal cord, but not in the lower thoratic or lumbar regions. It is hypothesized that stress due to lack of phosphorylation of NF by cdk5 leads other substrates to phosphorylate NF's, leading to hyperphosphorylation of NF's and an increase in NF tangles in motor neurons in these mutant animals. Also, it may be that the stress caused by the accumulation of NF leads to the cleavage of p35 to p25 due to oxidative and other cell stressors that result from the accumulation of NF's in the soma (Ko et. al. 2001).

A recent publication found that p35 null *Drosophila* mutants were viable but displayed defective axon guidance and age dependent behavior defects, as well as a reduced lifespan. p35 is the only known activator of cdk5 in *Drosophila,* as a homologue for p39 has yet to be found. Although these mutants showed the same motor activity as control groups at the onset of testing, they progressively became immobile with age, showing a close phenotype to neurodegeneration. Also, these mutants had a reduced lifespan as compared to controls in a life span assay (Connell-Crowley et. al. 2007).

Due to the fact that the experiments in *Drosophila* described above demonstrate that p35 mutant motor function was nearly normal at eclosion and declined with

advancing age, including periods of rigidity and premature death, one can speculate that this phenotype may represent some required adult function of cdk5 and its activator p35 for the maintenance of neuronal function, especially in motor neurons. However, no histological experiments were ever performed to determine whether or not this phenotype was the result of developmental miswiring, as it has been shown that cdk5 and p35 are essential during development, or whether it truly did represent an adult neurodegenerative phenotype. Therefore, I hypothesize that using RNAi constructs in *Drosophila* to knockdown cdk5 and/or p35 activity after eclosion will result in a phenotype of progressive loss of motor function, reduced life span, and neurodegeration (Connell-Crowley et. al. 2007). Due to the essential role of cdk5 in the maintenance of synaptic transmission and cytoskeletal structure and neurofilament transport, it is reasonable to suggest that knockdown of this gene during adulthood would result in neurodegeneration. Due to the extensive genetic tools available in the *Drosophila* system, it is possible to express RNAi only during adulthood which would eliminate the possibility of neuronal miswiring during development and focus the study on the role of this kinase and its activator in the adult CNS. This study serves the purpose of further advancing understanding of the role of cdk5 in the adult CNS, as well as supporting the current belief that it is only hyperactivity of cdk5 that results in neurodegeneration. The histology will also serve as a means to detect whether or not previous studies using p35 and cdk5 null mutants in *Drosophila* actually showed neurodegeneration and not developmental miswiring.

## Materials and Methods

*Transgenic Flies and Crosses*

Transgenic flies containing the RNAi constructs were ordered from the Vienna Drosophila RNAi Center. Three transgenic lines were used in the experiments, two of which were different constructs for the cdk5 gene and one construct was for RNAi interference of p35. These transgenic lines were then crossed with a Gal-4 linked system, which will express the RNAi, and increase its expression over time, specifically in the central nervous system during adulthood. All of the offspring of the crosses between transgenic RNAi lines and the Gal-4 transgenic lines were experimental genotypes.

Table 1

*Transgenic line genotypes*

| Stock Code | Genotype | Use |
|---|---|---|
| 35855 | $W^{118}$; 34855-$w^{+}$; +/+ | RNAi interference of cdk5 |
| 35856 | $W^{118}$; 34856-$w^{+}$; +/+ | RNAi interference of cdk5 |
| 34990 | $W^{118}$; 34990-$w^{+}$; +/+ | RNAi interference of p35 |
| 8177 | $W^{118}$; PGAL4-$w^{+}$; +/+ | Progressive increase in expression of RNAi constructs in Adult CNS |

* $w^{+}$ symbolizes the gene mini white which is a typical carrier gene used in *Drosophila*
* each (;) separates individual chromosomes, if there is no ( /), the chromosome is homologous for the allele shown

*Analysis of Gene Expression*

RT-PCR followed by 2% agarose gel electrophoresis was used to quantify the amount of gene expression in adult flies at different time points throughout their life course. Three sets of 5 vials were set up for each cross in order to allow enough offspring for quantification at different time points. After being given a week to mate, the parentals were discarded and vials were watched closely until there was a significant eclosion of adult flies (at least 5 flies per vial). Males were then separated for analysis. Three vials of 18-20 male experimental flies were set up for each cross and one vial was used for each time point. RNA was extracted using the RNAqueous kit (#AM1912, Ambion). PCR analysis was carried out using the procedures outlined in the RETROscript (#AM1710, Ambion) instruction manual for reverse transcription for RT-PCR (days 1, 20, or 40 of adulthood) and analyzed for levels of cdk5 or p35. Primers used for reverse transcription are shown in Table 2. RNA underwent 50 cycles of denaturation for one minute at 96°C followed by annealing at 46°C for 30 seconds and extension at 72°C for 30 seconds in order to allow viewing of the p35 band after gel electrophoresis.

Table 2

*Cdk5 and p35 primers for RT-PCR*

| Primer | Sequence |
|---|---|
| Cdk5 fwd | 5’-GGC AAT ACA TCT GGT TTC CAA-3’ |
| Cdk5 rev | 5’-AGT GCT GCG GTA AGT GTC TTT-3’ |
| p35 fwd | 5’-ATT TCC GGA TGA AAT GAT GG-3’ |
| p35 rev | 5-TTT GAT TTG AAC GAT TGT GA-3’ |

*Behavioral and Lifespan Assay*

Flies were collected in the same manner as those collected for gene expression analysis. The only difference was that only 20-25 male flies were needed per cross and therefore only five vials were needed per cross (3 experimental crosses + control). Upon collection of 20 adult males of the same age for each cross, males were put into a vial which was changed every two to three days until all flies were dead. The wall climb assay was carried out by transferring those flies to be tested (1 vial at a time) into an empty glass vial with a line drawn 7 cm from the bottom. Flies were then tapped to the bottom of the vial and the number of flies successful at climbing over the line in 10 seconds was counted. This was repeated three times, and the average percent of successful flies was calculated. This test was carried out every 5 days until all the flies were dead and/or unable to successfully complete the test. The number of flies alive in each vial was determined on days 0,3,5,8,10,13,15,etc. until all the flies were dead so that the number of living flies was known prior to each day of testing in order to adjust the population number for the calculation of percent successful (Connell-Crowley et. al. 2007). The entire protocol was repeated four times, each time using 23-25 flies for each strain.

*Histology-Whole Mount Procedure*

For whole mount immunostaining of adult brains, flies were collected in the same manner as described previously for gene expression analysis. Three vials of approximately 20 flies were set aside, one vial for each time point (Day 0, Day 20, Day 40). On the appropriate day of maturation, the flies were anesthetized with CO2 and threaded into a fly collar(#48-100, FlyStuff.com). Brains were then dissected within a

fixative containing 3.7% formaldehyde in 1X phosphate buffer solution. As brains were removed, they were placed into fixative in a well on a 9 well glass plate and allowed to fix at room temperature for 35 minutes. After the first fixation step, the fixative was removed and a new fixative was added [3.7% formaldehyde, 1X phosphate buffer, 0.5% Triton X100 (1xPBTr)] for an additional 35 minutes. Brains were then rinsed and washed three times for 45 minutes with 1xPBTr prior to the addition of a blocking solution (0.5% bovine serum albumin, 5% normal goat serum, 0.5% Triton X100 in 1X PBS) for 2 hours. Following blocking, the solution was removed and an anti-synaptotagmin antibody (#3H2 2D7) provided by Hybridoma Bank (dilution 1:120 in blocking solution) was added for at least 6 hours at room temperature or overnight at 4°C. Brains were then rinsed 3 times for 45 minutes with 1XPBTr prior to the addition of Alexa Fluor® 680 goat anti-mouse IgG (H+L) highly cross-adsorbed secondary antibody (#A-21058, Molecular Probes, Inc; 1:100 dilution in blocking solution). Brains were incubated in a dark humid chamber for 4 hours at room temperature and then washed 3 times again with 1XPBTr, being careful to protect samples from light exposure. Brains were then mounted in an anti-fade solution (1,4-phenylenediamine and glycerol in an aqueous buffer solution), given two hours to settle, and then cover slipped. Evidence for neurodegeneration was based on whether or not the brains showed a decrease in cell density. Cell density was determined by counting of cells in a controlled region of the tissue.

*Histology-Hematoxylin and Eosin Staining of Sectioned Tissue*

Flies were collected in the same manner as described for the whole mount protocol, except only 5 flies were needed for each strain for each time point (Day 0, Day

20, Day 40). Heads were removed from the body and the proboscis removed prior to being placed in freshly prepared Carnoy's fixative. Tissue was then taken to the M.J. Palladino lab at the University of Pittsburgh where they were washed in 70% ethanol and processed into paraffin using standard histological procedures. 5 µm frontal sections were then obtained from the heads. Tissue was then stained with hematoxylin and eosin and examined under a light microscope. Neurodegeneration was scored on the basis of vacuole appearance in the neural tissue (Palladino, Bower, Kreber, & Ganetzky 2003).

**Results**

*Gene Expression Analysis*

Genetic analysis of both cdk5 and p35 expression in experimental and control strains by gel electrophoresis did not show a noticeable difference in band intensity over time. Therefore, a reduction in gene expression over time within experimental strains as compared to controls of either of the two genes was not seen (Figure 5, next page).

Due to the fact that no initial difference was found in gene expression, it was hypothesized that this may be the result of there being too much template in the reaction mixtures. With too much template, replication may cause the band intensity to "plateau," which may have been the reason for there being no difference in gene expression between control and experimental strains. As a follow up experiment, Day 0 and Day 37 reaction mixtures of the experimental strains 8177*856 (cdk5 RNAi) and 8177*990 (p35 RNAi) from the initial PCR experiment were run against reaction mixtures of the same strain aged 37 days with only 2 µL of sample. While the first gel analyzed (Figure 6A, page 17) lacked a band in the initial 8177*856 D37 reaction mixture lane, as well as weak bands in each of the 8177*990 lanes, the second gel ran with the same reaction mixtures

(Figure 6B, page 17) displayed equal band intensities for the 5μL and 2 μL reaction mixtures for both experimental strains, suggesting that a “plateau effect” was not the cause for the lack of difference between band intensities over time seen in Figure 5 (next page).

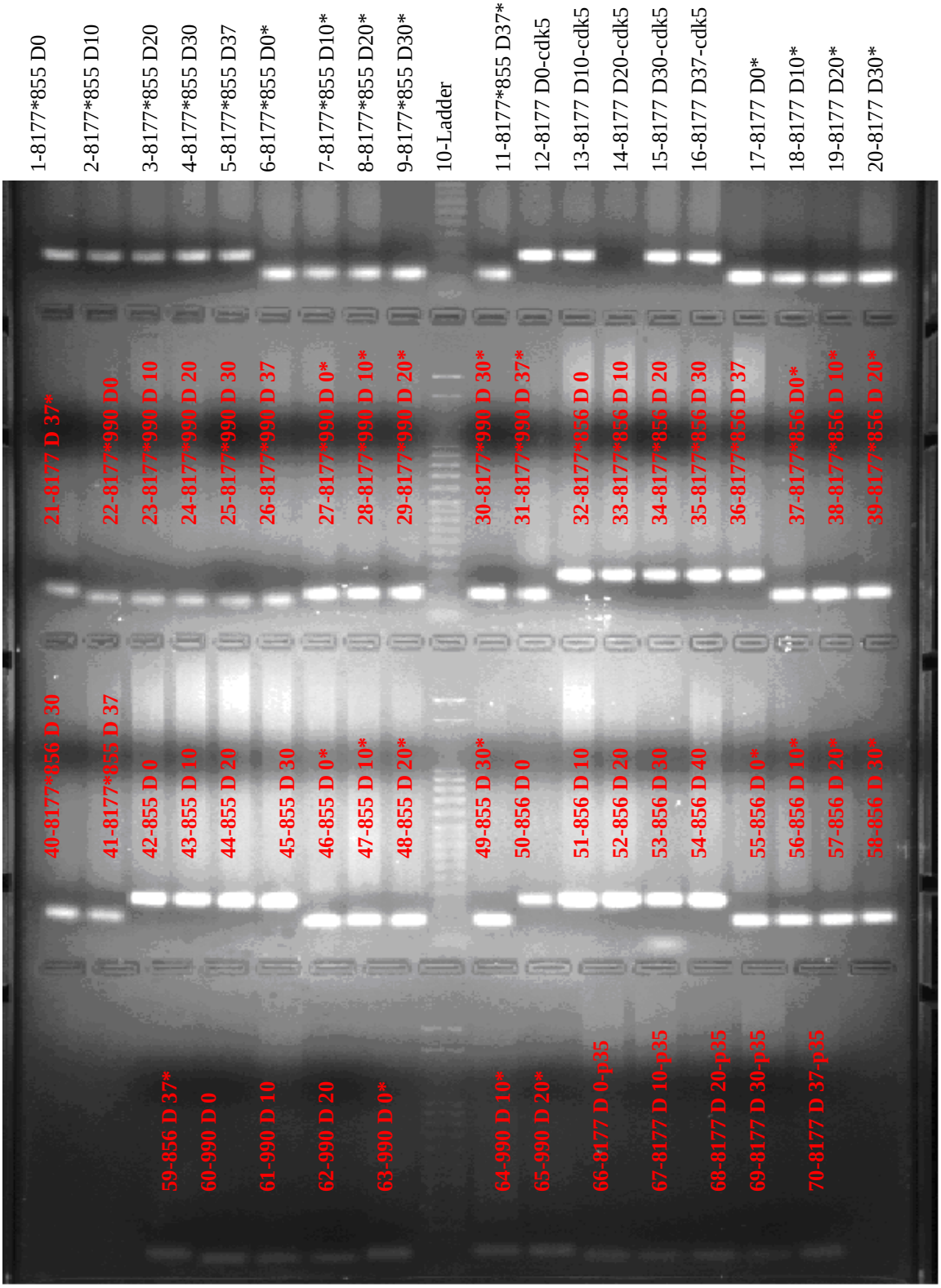



*Figure 5.* Analysis of cdk5 or p35 expression in experimental and control strains. 5 µL of template of each strain was placed into a PCR reaction mixture that contained primers for either cdk5 (any strain containing 855 or 856), p35 (any strain containing 990), or actin (all strains). For strain 8177, three reaction mixtures were prepared, containing primers for either cdk5, p35, or actin. Cdk5 has a molecular weight of 58 kDa while p35 weighs approximately 35 kDa and actin approximately 42 kDa.* denotes reaction mixtures prepared with an actin primer.

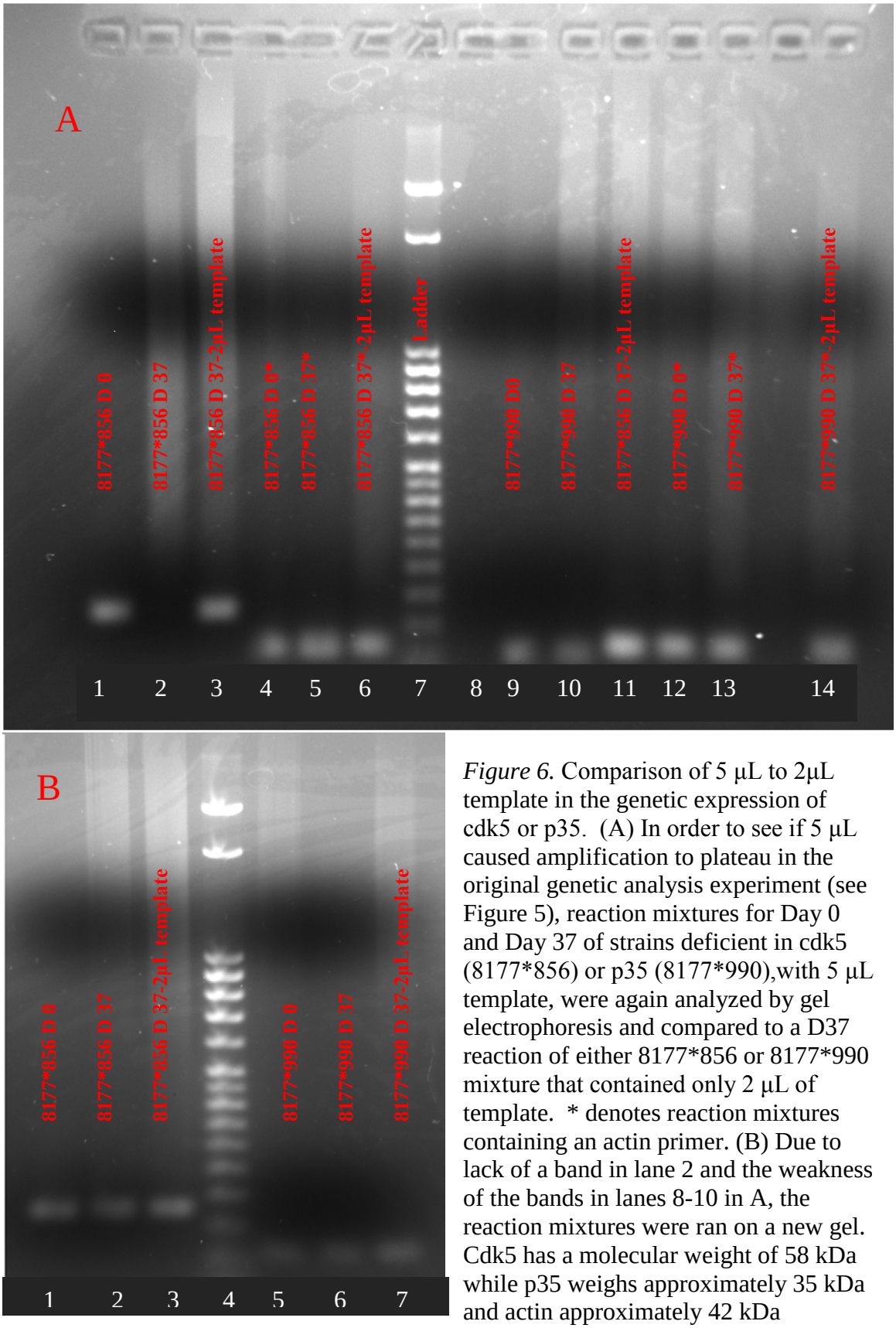



*Figure 6.* Comparison of 5 µL to 2µL template in the genetic expression of cdk5 or p35. (A) In order to see if 5 µL caused amplification to plateau in the original genetic analysis experiment (see Figure 5), reaction mixtures for Day 0 and Day 37 of strains deficient in cdk5 (8177*856) or p35 (8177*990),with 5 µL template, were again analyzed by gel electrophoresis and compared to a D37 reaction of either 8177*856 or 8177*990 mixture that contained only 2 µL of template. * denotes reaction mixtures containing an actin primer. (B) Due to lack of a band in lane 2 and the weakness of the bands in lanes 8-10 in A, the reaction mixtures were ran on a new gel. Cdk5 has a molecular weight of 58 kDa while p35 weighs approximately 35 kDa and actin approximately 42 kDa

*Life Span Assay*

There was no significant difference between strains in respect to life span, $F(6,72)=1.312$, $p>.05$. All strains showed some flies living beyond 47 days, while experimental strain 8177*855 ($M=51.00$, $SD=10.520$) showed an average greater than 50% survival beyond 47 days (Figure 7).

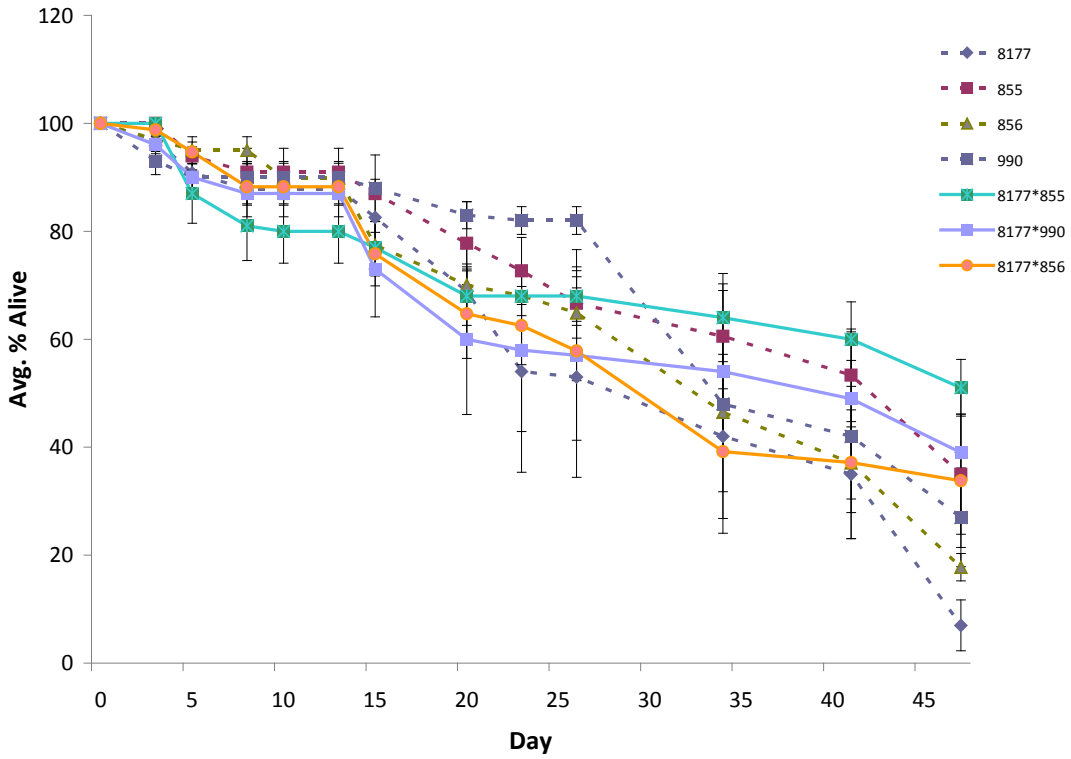



*Figure 7.* Life Span Assay. Experimental strains were followed over a period of 50 days and analyzed for the percent alive at each time point. There was no significant difference between strains in lifespan (F=1.312, p>.05). Error bars represent standard error.

*Wall Climb Assay*

Despite the lack of difference between experimental and control strains in respect to lifespan, there was a significant difference between strains on their performance in the wall climb assay, $F(6,36)=4.244$, $p<.05$ (Figure 8). More specifically, strains 8177*855 ($M$= 67.151, $SD$=26.084) and 8177*990 ($M$=64.906, $SD$=24.509) were significantly different from strain 855 ($M$=79.532, $SD$=15.911), Tukey's HSD, p<.05. All experimental strains (8177*856; $M$= 69.663, $SD$=24.512) were significantly different from strain 856($M$=81.843, $SD$=16.170), Tukey's HSD, $p<.05$. However, no significant difference was found between any of the experimental strains and the control strains 990 ($M$=74.665, $SD$=20.168) and 8177 ($M$=71.549, $SD$=17.133).

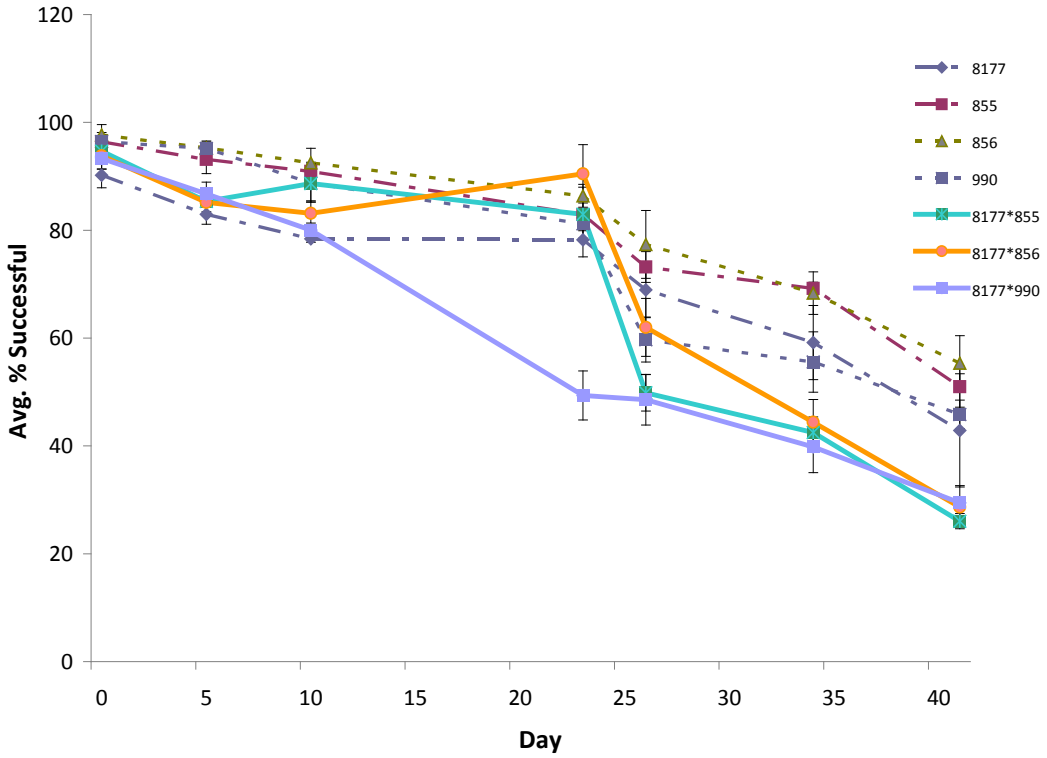



*Figure 8.* Percent successful at wall climb assay over time. As seen above there was a significant difference in experimental strain performance as compared to control strains. Strains 8177*855 and 8177*990 were significantly different from strain 855 and all experimental strains 8177*856 were significantly different from strain 856. Error bars represent standard error.

*Whole Mount Histology*

The whole mount histological portion of the experiment did not provide results suitable for determining whether or not neurodegeneration was taking place in any of the experimental strains. While immunostaining of the adult brains was successful, the brains were not all oriented in the same plane on the slide which made consistent analysis of fluorescence density between strains difficult. Perhaps more importantly, some of the brains did not seem to stain as well as others, which led to some pictures to appear too faint to analyze visually (data not shown). Last, the final Day 40 time point was missing due to an infestation of mites into some of the vials used for analysis, as well as a problem in staining that caused some of the brains to appear black (data not shown). Figure 9 (below) provides a view of some of the more successful stains in the whole mount method.

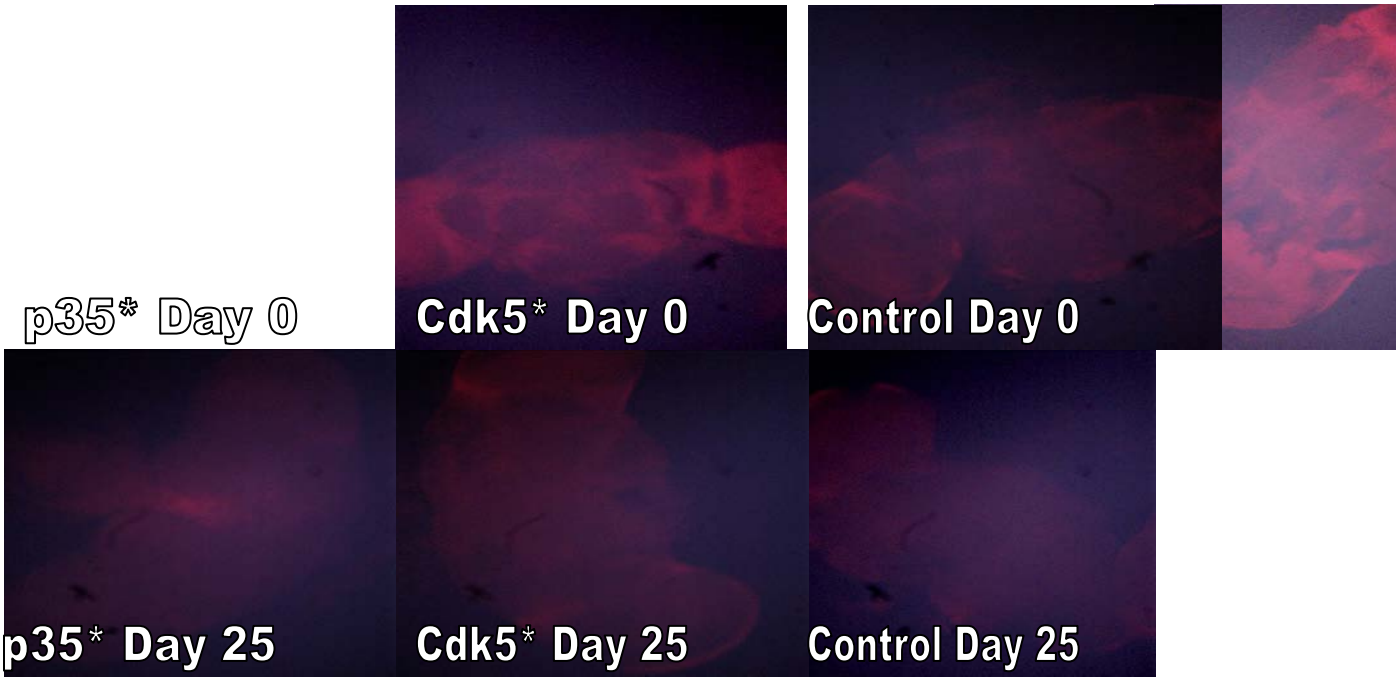



*Figure 9.* Whole-mount histology. As seen above, variations in intensity of staining (seemingly procedural variations) as well as differences in orientation of the brains due to difficulties keeping brains aligned while mounting makes it difficult to form any reliable conclusions.

*Hematoxylin and Eosin Staining of Sectioned Tissue*

Due to the fact that the whole mount method has been known to be hard to quantify, we deemed it necessary to obtain sectioned material in order to determine whether or not neurodegeneration was taking place in any of the experimental strains. As seen in Figure 10, there are no stark differences between tissue from control strains compared to the three experimental strains over time. None of the samples showed any signs of extensive vacuolization that would be consistent with neurodegeneration. Therefore, the progressive loss within the central nervous system of cdk5 or p35 throughout adulthood does not seem to result in neurodegeneration in *Drosophila melanogaster*. While it may appear that the 8177*855, D40 picture shows some signs of vacuolization, it was determined that this was most likely due to damage to the tissue during sectioning. This conclusion was based on the fact that all other samples of this strain at day 40 showed extensive damage due to sectioning. The section displayed was still used as this was the only sample that seemed to hold enough integrity to be able to view the sectioned brain.

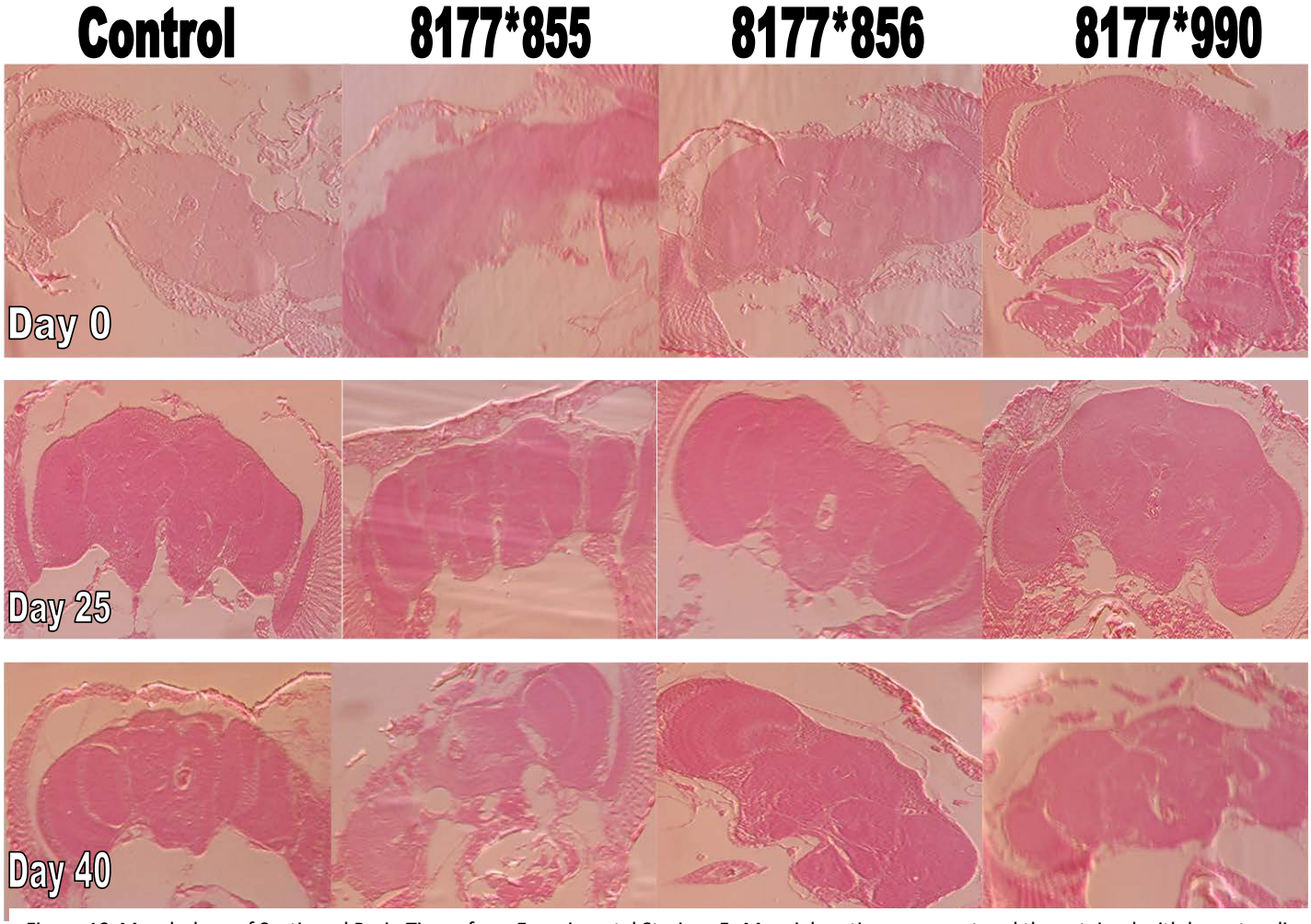



*Figure 10.* Morphology of Sectioned Brain Tissue from Experimental Strains. 5µM serial sections were cut and then stained with hematoxylin and eosin to detect signs of neurodegeneration (scored by signs of vacuolization). As seen above, there were no major gross changes in brain morphology in experimental strains that would support neurodegneration. Note: While 8177*855 D40 does appear to have vacuolization, it is suspected that this is due to damage during sectioning rather than degeneration.

**Discussion**

Accumulating evidence has supported a role for cdk5 and its regulator, p35, in the regulation of neurotransmission and synaptic function (Cheung, Fu and Ip 2006). Furthermore, recent studies have indicated that deregulation of cdk5 may contribute to the pathology of neurodegenerative diseases such as Alzheimer's disease and ALS (Dhavan and Tsai 2001). Most recently, *Drosophila* mutants lacking the cdk5 activator, p35, showed age dependent behavioral defects in a wall climb assay and demonstrated a shortened lifespan that seemed to be consistent with a neurodegenerative phenotype (Connell-Crowley et. al. 2007). The current study sought to confirm whether the behavioral deficits and shortened life span were in fact the result of neurodegeneration. By looking at the downregulation of cdk5 or p35 within the adult central nervous system over time using RNAi, this experiment eliminated the possibility of developmental miswiring of the nervous system (due to a lack of cdk5), and focused on the adult function of cdk5 and its regulator, p35.

The first part of the experiment focused on whether or not our genetic construct to downregulate cdk5 or p35 was effective. As seen in Figures 5 and 6, it does not appear that cdk5 nor p35 expression decreases over time in any of the experimental strains. However, this result could be due to the fact that rather than only analyzing nervous tissue (where the transgene was acting), we were analyzing tissue from the entire organism. The fact that cdk5 and p35 is found throughout the rest of the organism, despite its primary role in the nervous system, could result in any difference in nervous tissue expression over time being overshadowed by overall levels in the entire organism. It was concluded that rather than try to repeat the experiment with only nervous tissue, it

would be more effective to study the transgene by way of phenotypic and histological differences between strains. It would take too much time and resources to collect enough nervous tissue from each strain to analyze by means of PCR and gel electrophoresis, and therefore, we believed the results of the behavioral and histological studies would provide enough evidence to confirm that the transgene was in fact working.

While previous studies showed p35 null mutants had a decreased lifespan, with null mutants reaching 50% survival after only 25 days, the current study did not reflect this result (Connell Crowley et. al. 2007). As seen in Figure 7, experimental strains with decreased expression of cdk5 or p35 over time did not differ significantly from control strain in respect to lifespan. Furthermore, all strains reached 50% survival between days 30-35, which was comparable to the wild type strains in previous studies (Connell Crowley et. al. 2007).

Despite the lack of a shortened life span in experimental strains, there was a significant difference found in their performance on a wall climb assay as compared to control strains (Figure 8). Strains deficient in cdk5 or p35 (8177*855, 8177*856, and 8177*990) were only 50% successful at the assay between days 25-30, while control strains reached a 50% success rate (8177), or were nearing it, by day 41 of testing. This result confirms the phenotype seen in previous studies, with null mutants for cdk5 or p35 showing behavioral defects in the wall climb assay, reaching 50% success between 21 and 28 days of testing (Connell Crowley et. al. 2007). However, because this study only interfered with cdk5 or p35 levels in the adult central nervous system rather than disrupting either of the genes during development, this result shows a vital adult role for

cdk5 and its regulator p35 in the adult central nervous system that seems to involve motor function.

Analysis of sectioned brain tissue from experimental strains reveals no signs of neurodegeneration over time due to the decrease in expression of cdk5 or p35 over time (Figure 10). The experimental strains showed similar morphology to controls and lacked any true signs of vacuolization. While the Day 40 section from 8177*855 did seem to show a different gross morphology than controls, it was determined that this was due to tissue damage during sectioning (Figure 10). This conclusion was based on the fact that all slides for 8177*855 showed extensive tissue damage and the image used in Figure 10 was the most intact found on any of the slides. Hematoxylin and eosin staining of adult *Drosophila* nervous tissue is thought to be the most reliable form of nervous system histology and therefore, it was not deemed necessary to try and analyze the samples obtained by the whole-mount method (Palladino et. al. 2003). Furthermore, the whole-mount method produced images that were difficult to analyze reliably for a number of reasons. The brains are in slightly different orientations and demonstrate different degrees of fluorescence, not believed to be due to differences in cell density after analyzing the results of the samples stained with hematoxylin and eosin.

The results of this experiment do not seem to support a role for the downregulation of adult cdk5 or p35 in neurodegeneration. While further work needs to be done to clarify the exact mode of deregulation of cdk5 that implicates it in neurodegenerative diseases, research seems to be indicating that cdk5 plays a major role in neurodegeneration when its activity becomes aberrant. Many neurodegenerative diseases have neurofibrillary tangles as a pathological hallmark. Cdk5 has been shown to

associate itself with these tangles *in vivo* and phosphorylates substrates such as Tau, especially the paired helical Tau filaments characteristic of those tangles found in Alzheimer's patients (Dhavan and Tsai 2001). Furthermore, neurotoxic insults created by excess glutamate or reactive oxygen species (ROS) can induce the cleaving of p35 to p25 by means of excess intracellular $Ca^{2+}$, which in turn activates the protease calpain. Activation of the p25/cdk5 complex can then cause an onset of aberrant activity due to p25's ability to mislocalize cdk5 and cause it to target different substrates, including the Tau protein and those neurofilament inclusions found in motor neurons of ALS patients (Patrick et. al. 1999). This evidence seems to support the fact that in order for cdk5 activity to become detrimental and play a role in neurodegeneration, its activity must become aberrant and mislocalized, seemingly by the conversion of the p35 activator to p25.

Despite a lack of neurodegeneration, experimental strains with a downregulation of cdk5 or p35 did show motor deficits in the wall climb assay. Because this confirmed results from a previous experiment, this result does indicate a vital role for the p35/cdk5 complex in the adult central nervous system (Connell Crowley et. al. 2007). Because the deficit is found in motor function, it is reasonable to assume that p35/cdk5 plays a vital role at the neuromuscular junction (NMJ). Previous studies have shown that cdk5 is indispensible for neuregulin-mediated maintenance of the NMJ due to the fact that cdk5 is responsible for phosphorylating and activating the neuregulin receptor which allows for downstream mitogen-activated protein kinase (MAPK) signaling to induce acetylcholine receptor expression (Cheung et. al. 2006). Furthermore, larvae lacking cdk5 show a slight enlargement of their NMJ coupled with smaller boutons (Kisser et. al.

2009). The smaller boutons may reflect a lack of specialized synaptic connections which may result in less efficient signaling at the NMJ in experimental strains deficient in p35/cdk5. Furthermore, enlargement of the NMJ may be an artifact of a compensatory mechanism, as more connections are needed due to the connections being less elaborate and effective (as the boutons are smaller). Cdk5 has also been shown to be required for dispersion of acetylcholine receptor (AchR) clusters (Cheung et. al. 2006). Dispersion of AchR clusters is essential for concentration of AchR beneath nerve terminals which allows for efficient transmission of signals from motor neurons to muscle cells (Sugiyama et. al. 1997). Therefore, due to its critical role in maintaining the NMJ, it is not surprising that the experimental strains with decreased cdk5 or p35 expression over time showed decreased motor function and had a lower success rate in the wall climb assay as compared to controls.

Despite the fact that a motor deficit was found within the experimental strains used in this experiment, repeating the behavioral test may be beneficial in order to establish a significant difference between the experimental strains and the two other controls, 990 and 8177. Furthermore, repeating the life span assay may also prove beneficial as it was found that after the experiment, some of the stock vials had an infestation of mites that may or may not have infested any of the vials used for the life span assay. While it seems that the genetic construct used to downregulate cdk5 and p35 worked, due to deficits seen in the behavioral test, an experiment that analyzed the amount of cdk5 or p35 within nervous tissue specifically over time in these strains may be beneficial in order to make the results of this experiment more reliable. Lastly, experiments downregulating cdk5 or p35 within the central nervous system using

different genetic constructs to either disrupt expression entirely during adulthood (rather than progressively over time), or in specific regions of the CNS may help further clarify the role of cdk5 and p35 within the *Drosophila* adult central nervous system.

Further work must be done in order to fully elucidate the role of p35/cdk5 within the central nervous system, as well as understand the consequences of its deregulation. While histology was done in this experiment on the gross brain morphology, it would be interesting to look at morphology of the NMJ within the experimental strains as compared to controls. This type of histology would elucidate any changes that have occurred at the NMJ and may help explain the motor deficits seen in strains deficient in cdk5 or p35. In addition to this, it would be interesting to perform electrophysiology at the NMJ and record any changes in signal transduction between motor neurons and muscle cells as a result of the lack of cdk5 or p35. Furthermore, there are a variety of behavioral tests that can be used in *Drosophila* to test other functions of the central nervous system, such as memory and learning, that may help determine if cdk5 or p35 deficits have any other effects on the CNS.

In conclusion, it seems that cdk5 and p35 serve a vital role in preserving motor function within the adult central nervous system. However, despite previous implications of the loss of cdk5 or p35 causing a decreased lifespan and possible neurodegeneration in *Drosophila melanogaster*, the results of this experiment suggest that previous results may have been due to developmental miswiring (due to the vital role of cdk5 in development), or some other artifact of the constructs used, rather than neurodegeneration itself. Therefore, it seems that cdk5 and p35 serve a vital role in the *Drosophila* adult central nervous system, in respect to motor function, but more work must be done to elucidate

other roles of the protein complex within the adult central nervous system, as well as determine the exact role of cdk5 and p35 in neurodegeneration.

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