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DENDRITE REGENERATION MECHANISMS AFTER DAMAGE

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ABSTRACT

While axon regeneration requires a kinase cascade, the mechanism for dendritic regeneration is independent of this pathway and remains unknown. Kinases regulate many different stress responses, but it is not definitively known which kinases, if any, play a role in dendrite regeneration. When axons are damaged, glial cells play a role in clearance of axonal debris, but it is unknown whether glial cells are involved in dendrite regeneration after damage. I hypothesize that a kinase and a signal from glial cells are both required for dendrite regeneration after injury. In order to test the role of kinases and related signaling molecules in dendrite regeneration, these genes were knocked down using RNAi. To injure the sensory neurons or glial cells, I used a UV pulse laser. I monitored dendrite regeneration 24 hours post-injury, and classified the tested neuron as having the same, a reduced or complete lack of dendrite regeneration in comparison with the control. I have tested 35 kinases and related signaling molecules thus far, and none appear to play a role in the dendrite regeneration pathway. In the future, I will continue to screen for proteins involved in dendrite regeneration, and continue to explore if glial cells are involved in dendrite regeneration. Once we identify the first regulator of dendrite regeneration through this screen, we will make predictions based on the known functions and interactions with other candidate proteins.

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Introduction

Neuronal Response to Injury

A neuronal cell is the major functional subunit of the nervous system. Though neurons can take on a variety of different shapes, every neuron is composed of at least four main parts: dendrites, a soma (the cell body), an axon, and synaptic terminals (Figure 1). Many of the functional features of a neuron can be ascertained by observing its structural features. Dendrites, often with many elaborate branches extending over a broader width than the soma, are responsible for receiving information from other neurons or the external environment. Dendrites can be thought of as the *input* center, where information from several different sources is received by the neuron. The soma of a neuron takes on many of the functional features of a typical somatic cell; it holds the nucleus, where genomic DNA is held; and it is the major site of gene expression and protein synthesis. An axon is a long cable-like structure that sends a signal in the form of electrochemical potentials down towards the end of the neuron towards the synaptic terminals. The synaptic terminals are the *output* of the cell, where neurotransmitter release occurs and the electrochemical signal is transmitted to another neuron, gland, or muscle.

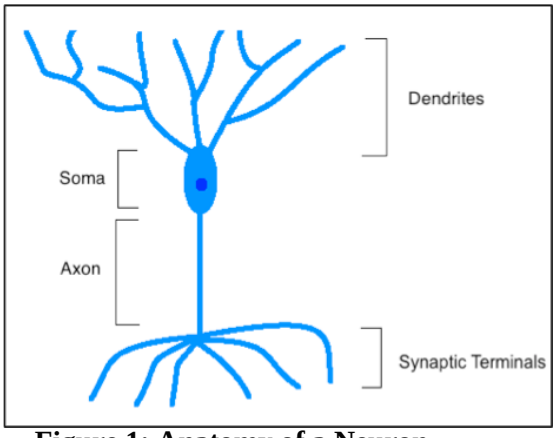

Figure 1: Anatomy of a Neuron

Each part of a neuron has a functional requirement that contributes to normal nervous system function. What happens when one of the parts of a neuron is damaged? A typical somatic cell may divide or choose to undergo apoptosis in response to stress. Unlike other somatic cells, neurons do not undergo cell proliferation. If neurons are damaged, they either choose a path to regenerate and repair the area that has been damaged, or undergo apoptosis. Since neurons are delegated the daunting task to last a lifetime, it makes sense that they have extensive systems in place to respond to damaged axons and dendrites. Understanding the mechanisms of neuronal response to damage is clinically relevant, as dendrites and axons are damaged during ischemic stroke, traumatic brain injury, or seizures (Gao and Chen, 2011; Murphy et al., 2008; Zeng et al., 2007).

When damaged, axons undergo a process known as Wallerian degeneration followed by subsequent regeneration. After axonal injury and Wallerian degeneration, Draper on glial cell membranes is activated through phosphorylation by Shark-Src42A (Ziegenfuss et al., 2008). Glial cells help to clear axonal debris. Perforation of the neuronal cell membrane of the axon creates an increase in intracellular calcium that travels towards the cell body. Calcium release triggers the pathway for axonal regeneration by activation of adenylyl cyclase and subsequently the cAMP-PKA pathway (Appenzeller and Palmer, 1972; Carlsen, 1982; Ghosh-Roy et al., 2010). The activated PKA helps to promote axonal outgrowth (Ayaz et al., 2008). At the same time, axon injury inhibits activity of the protein hiw, whose normal function is to downregulate levels of DLK (Xiong et al., 2010). Dual-leucine zipper kinase (DLK) is a mitogen-activated protein kinase kinase kinase (MAPKKK) that is activated after axonal injury, which subsequently activates the Jun N-terminal kinase (JNK) and the transcription factor FOS (Gallo and Johnson, 2002). Enhanced JNK/FOS signaling corresponds with an increased production of

profilin, a cytoskeleton-interacting protein (Chen and Chisholm, 2011; Gallo and Johnson, 2002; Wang and Jin, 2011). An increase in DLK/JNK/Fos signaling is necessary for axonal regrowth of damaged axons (Xiong et al., 2010).

Like axons, dendrites undergo degeneration and regenerate in response to injury. While axonal regeneration mechanisms have been elucidated, dendrite regeneration mechanisms remain largely unknown. While dendrites and axons both undergo degeneration followed by subsequent regeneration, the methods by which the neurons accomplish these tasks are distinctive. When dendrites of sensory neurons in *Drosophila* are damaged, epithelial cells clear dendritic debris (Han et al., 2014). Dendrite regeneration is a process that occurs independent of the DLK pathway that axons utilize for regeneration after damage (Stone et al., 2014). We are aware that dendrite regeneration is a transcription-dependent process (Figure 2), but the molecular pathway for dendrite regeneration is unknown.

Our lab has previously tested many proteins for their role in dendrite regeneration, including those involved in dendrite growth and stress responses. None of these candidates have been found to play a role in dendrite regeneration. As such, we are using a broad interference RNA (RNAi) screen to determine what proteins may regulate the dendrite regeneration response to injury. In the experiments described herein, I will explore the mechanisms of dendrite regeneration by performing a broad genetic screen as well as exploring glial cell response to dendrite injury. My hypothesis is that a kinase or related signaling molecule and a signal from glial cells are both required for dendrite regeneration after damage (Figure 3).

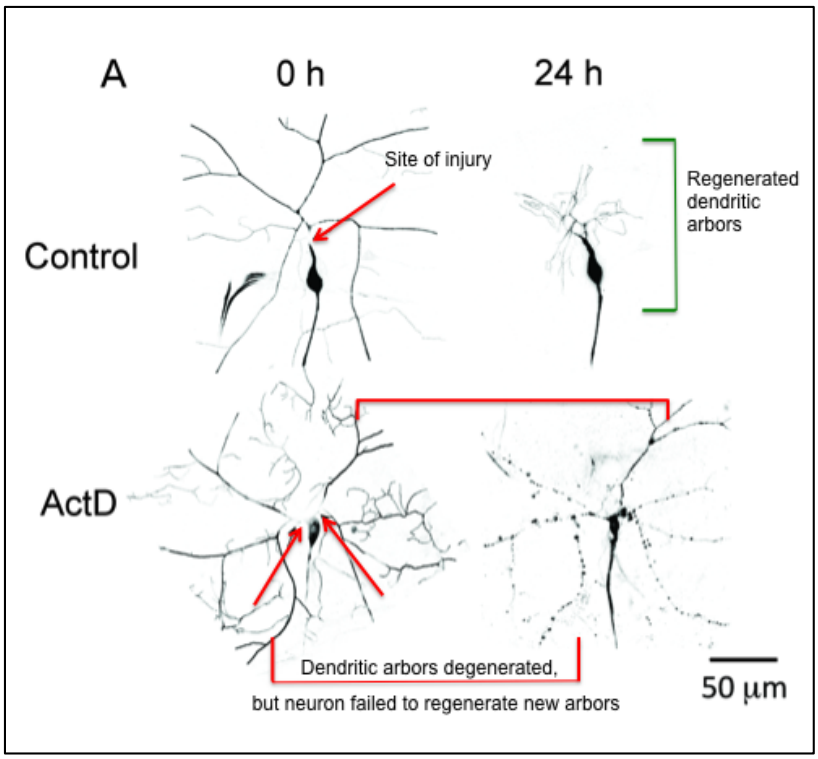

Figure 2: Dendrite regeneration is a transcription-dependent process. Sites of injury are marked with red arrows. Class IV sensory neuron after dendrotomy is able to regenerate dendrites after 24 hours, as indicated with the green bracket. In a larvae treated with actinomycin D, an inhibitor of transcription, dendrite degeneration was able to proceed but the neuron was unable to regenerate new dendritic arbors after 24 hours, as indicated with red brackets. Image courtesy of Richard Albertson.

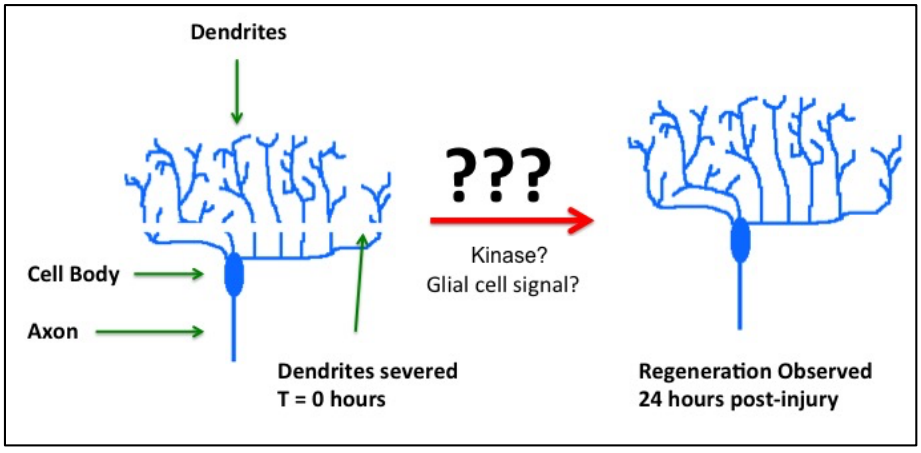

Figure 3: Dendrites regenerate after damage. I hypothesize that a glial cell response and a kinase or related signaling molecule are required for dendrite regeneration after damage.

Kinases in Dendrite Regeneration

Kinases are enzymes that catalyze phosphorylation of their substrates. Phosphorylation is the most common form of post-translational modification. Kinases are ubiquitously represented throughout molecular pathways, including many involved in regulation of normal cell functioning or stress responses. For instance, in higher eukaryotes, p-TEFb kinase plays a role in a method of gene regulation known as promoter proximal pausing. Shortly after RNA polymerase II has begun elongation (at ~20-60 nucleotides downstream of transcription start site), pausing factors DSIF and NELF bind to the RNA polymerase II, preventing elongation from continuing. When p-TEFb kinase (heterodimer of CDK9 and cyclin T) phosphorylates the DSIF-NELF complex, NELF is released, allowing for elongation by RNA Polymerase II to resume. One benefit of this regulation system is that it allows for rapid transcription of a particular gene; chromatin remodeling has already occurred and transcription machinery is already present. Thus, it would be reasonable to suspect that many stress responses utilize promoter proximal pausing to allow for more rapid production of mRNA transcripts (Adelman and Lis, 2012). The p-TEFb kinase is an example of one of hundreds of kinases. As there are so many kinases encoded in the genome, it makes sense to perform a broad genetic screen to quickly determine if a kinase plays a role in dendrite regeneration after damage; a broad genetic screen provides a quick snapshot of what proteins may or may not be involved in a certain process. Due to the ubiquitous nature of kinases in molecular pathways, I suspect that a kinase may be required for dendrite regeneration after damage.

Glial Cells in Dendrite Regeneration

Glial cells are critical for maintaining proper function of the nervous system and are distinct from neurons, as they lack axons and dendrites. Though glial cells do not play a direct role in nerve signal propagation, glial cells add myelination to axons, which increases the conduction velocity of an action potential. Glial cells also play a role in maintaining an appropriate chemical environment for generating action potentials (Jessen, 2004). Interestingly, glial cells have been shown to have both beneficial and detrimental effects on the health of a neuron. When axons are damaged, PI3K signaling and Draper in glial cells regulate glial cell responsiveness to dendrite injury (Doherty et al., 2014). Clearance of degenerated axons promotes outgrowth of new axons. On the other hand, glial cells can release cytokines that lead to inflammation (Guo et al., 2007). I hypothesize that when dendrites are damaged, glial cells send a signal to the cell body in order to trigger dendrite regeneration.

Drosophila as a Model Organism for Neural Injury

As with any model system, there are distinct disadvantages and advantages to using *Drosophila* as a model organism to study neural injury. One important distinction between vertebrate and invertebrate neurons is the direction of growing microtubules; while both vertebrate and invertebrate neurons both have plus-end-out microtubule polarity in the axons, invertebrate *Drosophila* neurons have minus-end out polarity in dendrites rather than mixed polarity as seen in vertebrate neurons (Stone et al., 2008). Sodium voltage-gated channels evolutionary diverged at the level of vertebrates and invertebrates, and so many of the functions of voltage-gated sodium channels are different between *Drosophila* and humans, for instance

(Goldin, 2002). Though these disadvantages exist, invertebrates such as *Drosophila* and *C. elegans* provide distinct advantages for studying the nervous system and injury responses. The size of *Drosophila* does not dictate its nervous system complexity – *Drosophila* have 12,000-15,000 genes, some of which help to encode proteins for its 100,000 neurons. Most importantly, use of *Drosophila* as a model organism provides an excellent way to study neural response to injury *in vivo*. Not to mention, the short life cycle (14 days), well-established mutant and RNAi line availability, and relative ease of maintenance make *Drosophila* a convenient model organism for research.

Drosophila have several distinctive subclasses of sensory neurons (Bodmer and Jan, 1987). Our laboratory mainly studies class I and class IV dendritic arborization (da) neurons (Figure 4A and 4B). While class I neurons have comb-like dendrites, class IV neurons have dendritic arbors that extend in all directions around the cell body. As I am observing dendritic regeneration, it makes sense to focus on neurons that display expansive dendritic arbors. Thus, the experiments described herein utilize fluorescent microscopy techniques in order to study dendrite response to injury in class IV da neurons in *Drosophila*. Using sensory neurons for this experiment as opposed to neurons of the central nervous system serves a practical purpose, as sensory neurons are close to the epidermal layer of the larvae, providing easiest access to the neurons for imaging and damage.

In these experiments, I use a UV pulse laser in order to induce damage to glial cells and dendrites. Though functional, this assay is relatively difficult, as there are several opportunities for the *Drosophila* larvae to die over the time course of the experiment. I have taken advantage of a new potential way to induce damage to dendrites by making transgenic *Drosophila* expressing two *C. elegans* genes, *osm-9* and *ocr-4*. In *C. elegans*, OSM-9 and OCR-4 together

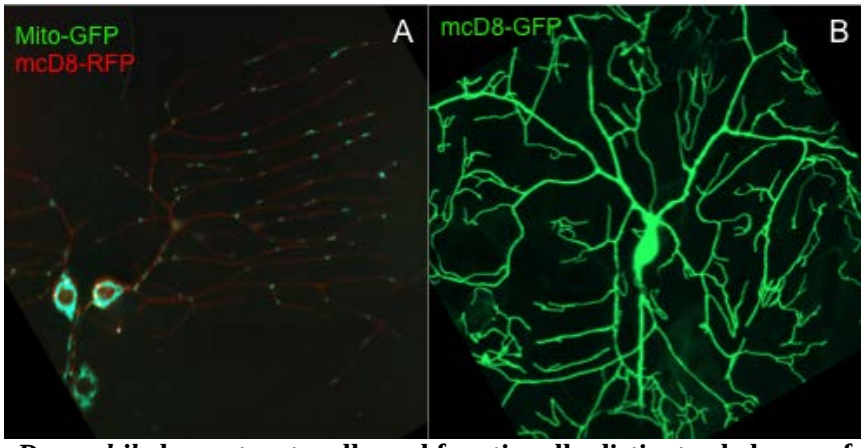

Figure 4: *Drosophila* have structurally and functionally distinct subclasses of sensory neurons. In Figure A, Class I dendrites have comb-like dendrites, and are proprioceptive sensory neurons (Grueber et al., 2007; Hughes and Thomas, 2007). Class IV neurons (B) have broad dendritic arborization patterns are nociceptive and help to protect the larvae from parasitoid wasps (Hwang et al., 2007).

form a heterotetramer in a subset of neurons. When exposed to nicotinamide, nicotinamide binds the *osm-9* and *ocr-4* channel, the channel opens, and allows for an excess of calcium ions to pass (Upadhyay et al., 2016). The influx of calcium can lead to excitotoxicity; excessive intracellular calcium concentrations activate calcium-dependent proteases, which in turn destroy the components of the neuron required for survival (Bano and Nicotera, 2007). By cloning these two *C. elegans* genes into a *Drosophila* plasmid vector and making transgenic *Drosophila*, we aim to be able to damage the dendrites of the sensory by adding nicotinamide to the food instead of using a UV laser to induce damage.

Kinases are ubiquitously found in molecular pathways, and glial cells often play a supporting role in the nervous system. Therefore, I have chosen to study dendrite regeneration in the context of kinases and glial cells. By making use of two different damage assays and testing kinases and glial cells for their roles in dendrite regeneration, I ultimately aim to uncover the dendrite regeneration pathway.

Methodology

GAL4/UAS System

I used a GAL4/UAS (upstream activating sequence) system in order to visualize class IV sensory neurons and glial cells (Figure 5). GAL4, a yeast transcription factor, was inserted downstream of an enhancer that is present specifically in sensory neurons or peripheral glial cells. UAS is activated in response to the GAL4 transcription factor binding (Brand and Perrimon, 1993). Most lines have a fluorescently-tagged protein (green fluorescent protein, GFP, or red fluorescent protein, RFP) under control of the UAS, and so the fluorescent proteins were expressed exclusively in either sensory neurons or the glial cells (Henry et al., 2012). Pickpocket (PPK) was used to drive GAL4 expression for class IV sensory neurons, whereas repo was used for glial cells. Balancer chromosomes such TM6 or Cyo function to prevent homologous recombination. Balancer chromosomes have a visible marker, in order to differentiate flies that have the transgene from flies that do not, and are homozygous lethal, so the fly never obtains a copy of TM6 or Cyo on both chromosomes.

Fluorescent Imaging and Confocal Microscopy

In each experiment, third-instar larvae were mounted onto a 3% agarose slide with the dorsal side of the larvae facing upwards. Larvae were rinsed with PBS buffer and were imaged with a combination of two different microscopes - Olympus FV1000 microscope using Fluoview Software or and LSM510 microscope using the LSM510 software. A table with information on

the various fluorophores used their corresponding absorbance and emission wavelength is depicted in the table below (Table 1). Images were compiled using ImageJ software.

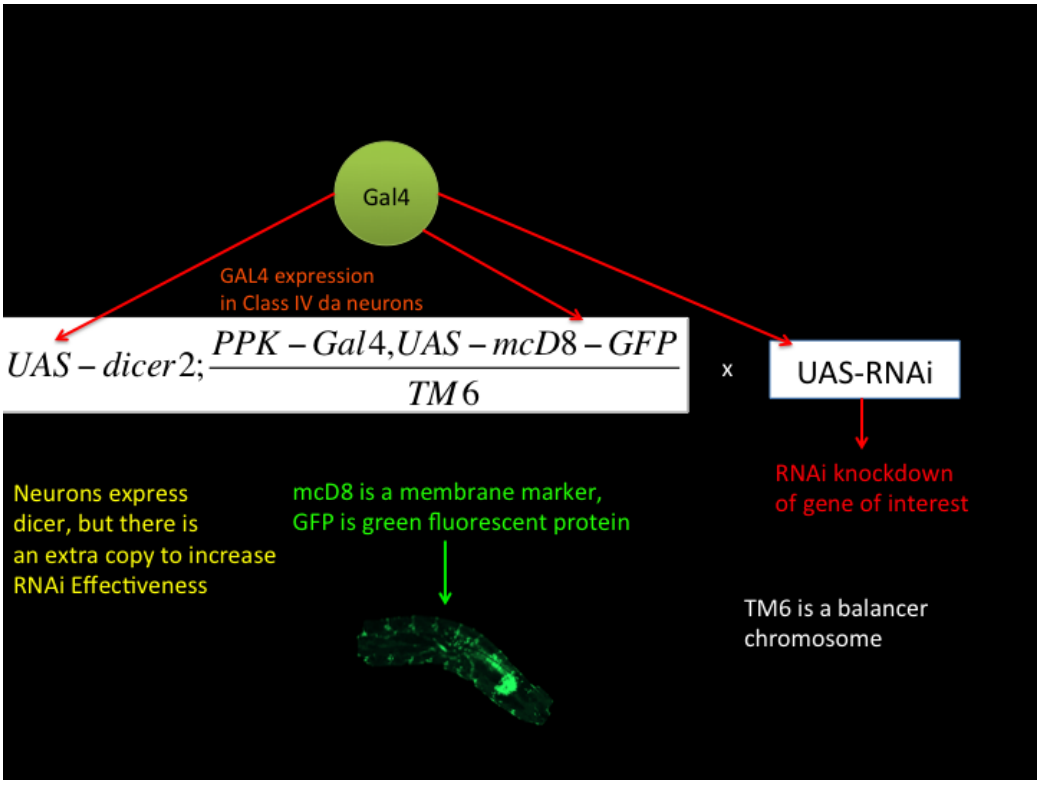

Figure 5: Example of a GAL4/UAS System. Gal4 transcription factor is placed downstream from a promoter of a protein (PPK) that is expressed in Class IV sensory neurons. Gal4 binds to UAS sequences and activates the dicer2, green-fluorescent protein tagged mcD8, and RNAi of the candidate being tested. As PPK is only expressed in sensory neurons, expression of fluorescent protein, RNAi and dicer2 is only expressed in class IV sensory neurons.

Fluorophore	Fly Lines	Absorbance peak λ (nm)	Emission peak λ (nm)
GFP	mcD8-GFP	488	507
tdTomato	PPK-tdtomato	554	581
dsRed	Red-stinger	558	583

Table 1: Fluorophores used with corresponding absorbance and emission peaks.

Dendrite Regeneration Screen

The tester line for the dendrite regeneration screen was $UAS - dicer2; \frac{PPK-Gal4,UAS-mcD8-GFP}{TM6}$. RNAi knockdown of the gene of interest was induced by mating the tester line with flies that have a UAS-RNAi sequence (Figure 5 and Table 2). As a control, I performed RNAi knockdown of γ-Tubulin 37C. γ-Tubulin 37C is only expressed in early embryogenesis, making it an ideal choice for a control (Tavosanis, 1997). In experiments where dendrites were damaged, I used a UV pulse laser to sever all of the primary dendrites from the neuron (Figure 6). The UV pulse laser allows for accurate targeting of dendrites; our lab has performed experiments where axons were cut without damaging the glial cell membrane that wraps the axon.

After imaging, I placed the larvae on a cap with food and stored the larvae for 24 hours at 25ºC. After 24 hours, the larvae were imaged once again to observe presence of dendrite regeneration. Results were qualitative in nature, and so each RNAi tested was placed in a category of either “did regenerate” or “did not regenerate.” However, there was also attention to note RNAi candidates which produced significantly reduced dendrite regeneration. For each RNAi candidate, 8-12 larvae were tested. If I observed dendrite regeneration for a particular RNAi candidate, then I concluded that the tested protein likely is not involved in dendrite regeneration. If regeneration did not occur or there was significantly reduced dendrite regeneration present, then I concluded that particular kinase might be involved in the dendrite regeneration pathway.

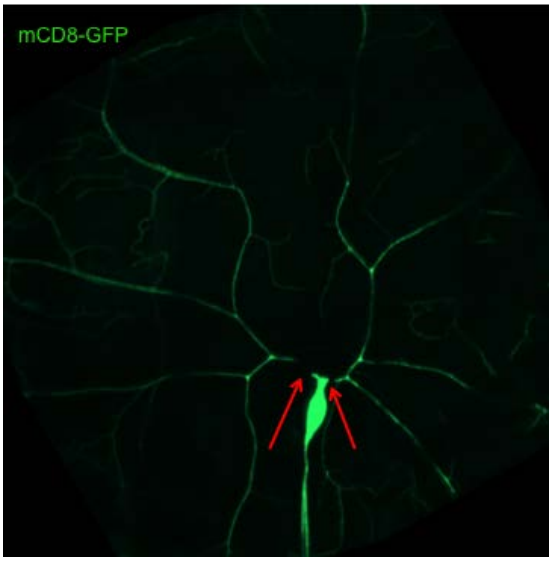

Figure 6: Inducing Dendrite Injury. Primary dendrites of sensory neurons were cut using UV pulse laser. Red arrows indicate site of dendrite injury.

Table 2: RNAi Lines for Dendrite Regeneration Screen

Stock Number	Gene Name	Stock Number	Gene Name
VDRC 25271	γ-Tubulin 37C	BL 26292	CG17528
VDRC 4138	Cut	VDRC 108937	Dpak1
BL 32932	Cp1	VDRC 47507	Hep
VDRC 992	ILR	VDRC 101517	msn
VDRC 100296	PI3K	VDRC 100717	SNF-related
VDRC 106642	Ras	VDRC 106980	Stat92E
VDRC 7005	sgg	VDRC 25528	MEKK
VDRC 104548	varicose	VDRC 106449	MLK2
VDRC 35252	src-64B	VDRC 105773	p38C
VDRC 26805	BcDNA	VDRC 39131	Spock
VDRC 106573	Anon-71Aa	VDRC 110765	Takl1
BL 31263	Tak1	VDRC 108356	LKB1
VDRC 103748	IK2	VDRC 107083	ALK
VDRC 104782	Ric	VDRC 101624	skittles
VDRC 52553	par-1	VDRC 33516	slpr
BL 25786	CKI-alpha	VDRC 32249	cdc2rk
VDRC 104793	caki	VDRC 103413	Cdk7
VDRC 25343	dlimk	VDRC 103561	cdk9

Glial Cells and Dendrite Regeneration

In order to visualize glial cells, I observed progeny from the experimental cross $\frac{UAS-redstinger}{Cyo};\frac{UAS-mCD8-GFP}{TM6} \times \frac{PPK-tdtomato}{Cyo};\frac{repo-Gal4}{TM6}$ (Figure 7). The PPK-tdtomato has RFP directly fused to the PPK protein. UAS-redstinger allowed me to visualize the nuclei of the glial cells in order to induce glial cell ablation. I used the UV pulse laser to induce glial cell ablation by targeting the nuclei. I induced ablation to all accessible glial cells that surrounded the class IV neuron. After glial cell ablation, I placed the larvae on a cap with food and stored the larvae for 24 hours at 25ºC. After 24 hours, I cut off the primary dendrites of the class IV sensory neuron, and then checked for regeneration 24 hours later. Similar procedures were used to classify dendrite regeneration as used in the dendrite regeneration screen.

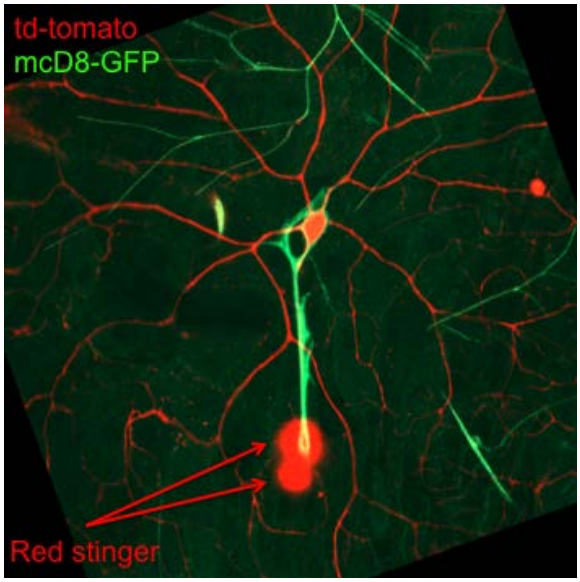

Figure 7: Fluorescent Glial Cells and Sensory Neurons. Glial cell membranes are labeled with mcD8-GFP, the glial cell nuclei are labeled with red stinger, and the class IV sensory neurons are labeled with td-tomato.

Molecular Cloning

osm-9 (2811 bp) and *ocr-4* (2271 bp) were cloned into a pUAST *Drosophila* vector from plasmids containing *osm-9* and *ocr-4* cDNA. *osm-9* was amplified using the forward primer (5’ – TGGGAATTCGTTAACAGATCTGCCACCATGGGCGGTGGAAGT – 3’) and the reverse primer (5’ – TGTTAGCAGACTTCCTCTGCCCTCTCCACTGCCTTCGCTTTTGTC ATTTGT – 3’). *ocr-4* was amplified using the forward primer (5’ – AGAGGAAGTCTGCT AACATGCGGTGACGTCGAGGAGAATATGGGTAATGCATCTAGTGCT – 3’) and the reverse primer (5’ – TCGAGCGCGGCCGCAAGATCTTTAGCCGGTAGGAATAATCAACTT – 3’). In order to have bicistronic expression of *osm-9* and *ocr-4*, a viral 2A peptide sequence (5’ – GGCAGTGGAGAGGGCAGAGGAAGTCTGCTAACATGCGGTGACGTCGAGGAGAAT – 3’) was built into the primer sequences of *osm-9* and *ocr-4*. The restriction enzyme BglII (cleaves **A|G**ATCT sequence) was used in order to linearize the pUAST plasmid. BglII restriction sites were also maintained in the primer sequence, to be able to confirm presence of *osm-9*, viral 2A, and *ocr-4* inserts.

In-Fusion cloning was used in order to generate the plasmid construct. In-Fusion differs from classical molecular cloning in that it solely requires 15 base pairs of homology between the inserted sequences and the vector (Figure 8). The plasmid construct was amplified by transforming and culturing Stellar chemically competent *E. coli* cells onto an LB agar plate with ampicillin. Transformants were checked by colony PCR and plasmid was isolated and purified using a miniprep protocol. Plasmid digestions were performed to verify plasmid product. The plasmid was then further amplified and purified using a midiprep protocol and sent to be injected into *Drosophila* to generate a transgenic fly line expressing these genes.

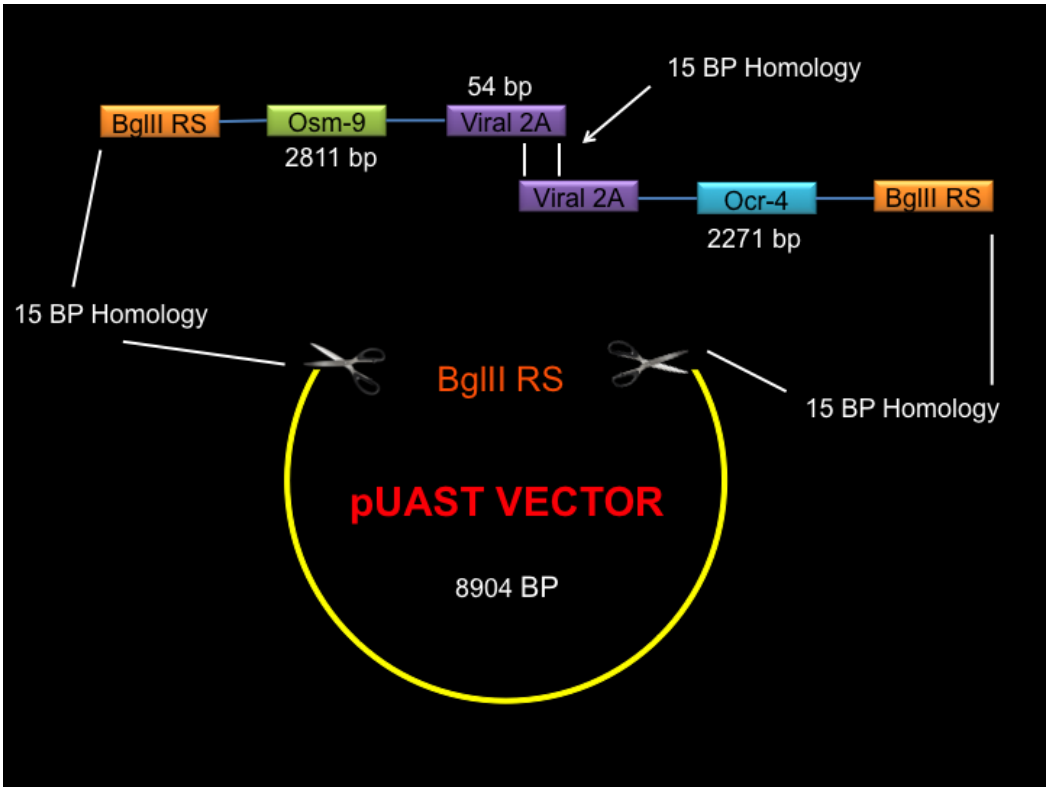

Figure 8: In-Fusion Cloning Design. In-Fusion cloning requires 15 base pairs of homology between the digested vector and the inserts. In the case of multiple inserts, there is also a required 15 base pairs between the inserts. Vector was digested using BglII. Three different genes, osm-9 (2811 bp), the viral 2A sequence (54 bp) and ocr-4 (2271 bp) were inserted into the pUAST sequence. pUAST has an ampicillin resistance gene so as to allow for selection of bacteria that have been transformed with this plasmid. The final product should be 14,052 bp (with addition of Kozak Sequence and an additional BglII site).

Results

Sensory Neurons in Control Group Exhibit Rapid Regeneration After Damage

Our laboratory has developed methods to study neuronal responses in both class I and class IV sensory neurons. In order to study dendrite injury responses, we study class IV sensory neurons instead of class I neurons, as regenerating dendrites in class I neurons exhibit periaxonal growth after damage (Stone et al., 2014). The directed growth of the regenerating dendrites in class I neurons is in the direction of the axon instead of towards the epithelial layer, where they normally exist (Kim et al., 2012). When class IV neurons have their dendrites damaged, there are broad dendritic regeneration patterns that extend in all directions.(Stone et al., 2014). Thus, to test to see if dendritic arbors regenerated after damage, I cut each of the primary dendrites from a class IV neuronal cell body using a UV pulse laser (Figure 6).

Larvae were maintained at 25ºC for 24 hours, at which point they were screened to see if dendrites regenerated. I chose γ-Tubulin 37C RNAi as a control for this screen, as γ-Tubulin 37C is only expressed during early embryogenesis (Tavosanis et al., 1997). As such, we would expect for dendritic arbors to regenerate after damage with γ-Tubulin 37C RNAi knockdown. This was the observed result, as when primary dendrites of class IV dendrites were severed using a UV pulse laser, neurons regenerated dendritic arbors 24 hours later in the γ-tubulin 37C RNAi control (Figure 9). Dendritic arbors do not take on the exact same arborization as the neuron prior to injury. Rather, the regenerating dendrites will eventually regenerate to span approximately the same area as the uninjured neuron.

For each candidate RNAi, approximately ten larvae were tested for dendrite regeneration. After the primary dendrites were severed with the UV pulse laser, dendrite regeneration was

observed 24 hours later and compared with the γ-tubulin 37C control. Thirty-five different proteins were tested for their role in dendrite regeneration. Candidates were compared with the γ-tubulin 37C control RNAi to see if there was regeneration, significantly reduced regeneration, or a complete lack of regeneration. I used a binary system to classify the tested proteins. If a sample showed regenerated dendritic arbors like that of the γ-Tubulin 37C control, I classified the neuron as "regenerated." If the sample showed significantly reduced or a lack of dendrite regeneration, I classified the neuron as "did not regenerate."

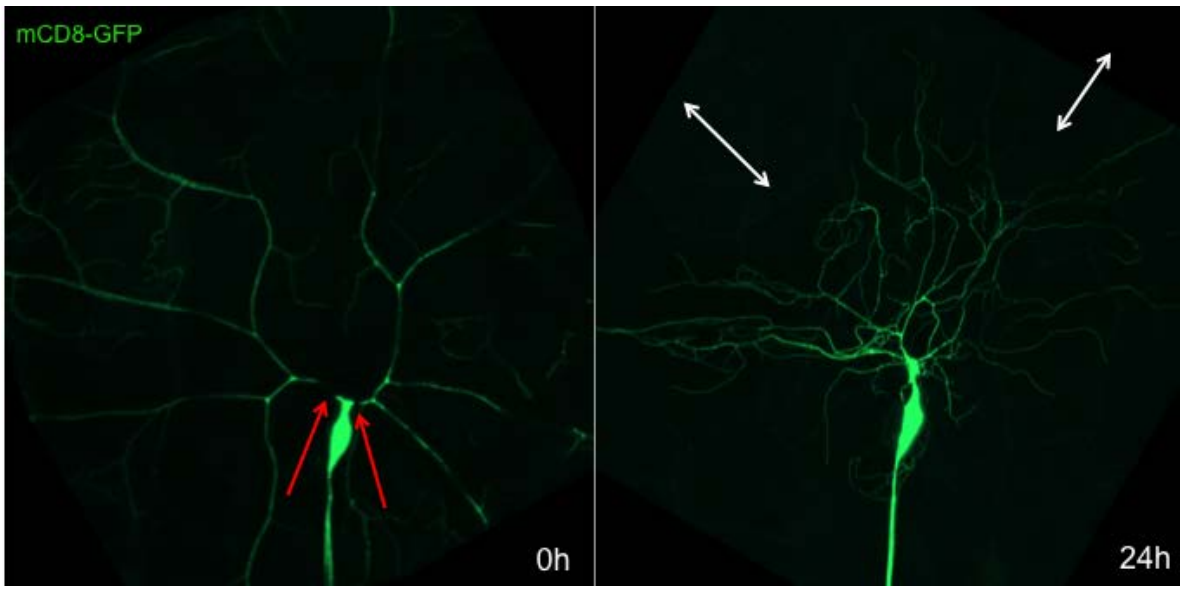

Figure 9: Dendrites show rapid regeneration in γ-tubulin 37C control knockdown. At time t = 0h, primary dendrites were cut from the cell body, as indicated with the red arrows. After 24 hours, dendrites have undergone degeneration and show regeneration of dendritic arbors. Dendritic arbors do not yet span the same area as the uninjured neuron, as depicted with the white arrows.

Neurons with RNAi Knockdown of Dendritic Pruning and Regrowth Factors Show Dendrite Regeneration

Drosophila neurons have mechanisms to prune and regrow new dendrites during metamorphosis. Ecdysone, Brm, and CBP function to prune dendrites and allow for neurons to

exhibit different dendrite morphologies during the pupal stage (Kirilly et al., 2011). Ecdysone is a steroid hormone that interacts with epigenetic factors, primarily a Brahma-containing chromatin remodeler (Brm) and a CREB-binding protein (CBP) in Drosophila to prune excessive or unnecessary dendrite formation (Kirilly et al., 2011). IK2 is a microtubule severing protein that also helps to regulate pruning of dendrites during metamorphosis (Lee et al., 2009). The regrowth of pruned dendrites is controlled partly through the use of the Cut transcription factor and *Cysteine proteinase-1* (Cp1). Cp1 functions to control production of the Cut transcription factor, and Cp1 has been shown to be indispensible for the regrowth of pruned dendrites (Lyons et al., 2014).

Because of their role in dendritic pruning and regrowth during metamorphosis, I was interested in testing IK2, Cut and Cp1 to see if they would play a role in dendrite regeneration. I hypothesized that dendrites would be unable to regenerate when levels of these proteins were reduced. Upon testing these candidates, I found that dendrite regeneration was able to occur in knockdown of IK2, Cut, and Cp1 (Figure 10).

Neurons with RNAi Knockdown of RTKs and Downstream Effectors Show Dendrite Regeneration

Receptor Tyrosine Kinases (RTKs) are receptors that bind to growth factors or hormones, leading to cross-phosphorylation of the receptor on tyrosine residues. RTKs have a rather diverse set of downstream targets that vary considerably by cell-type. Ultimately, RTKs lead to activation of factors that monitor cell development, growth and survival. For instance, in *Drosophila*, inhibition of the ILR/PI3K/TOR pathway by E3 ligase contributes to dendritic pruning (Wong et al., 2013), suggesting that this pathway contributes to processes that allow for

normal cell function and maintenance. I decided to test two RTKs, ALK and an insulin-like receptor (ILR). Ras (a GTPase), Ric (a ras GTPase), Dpak1, and PI3K are each proteins that are eventually activated downstream from initial RTK signaling. I suspected that neurons with RNAi knockdown of these genes would lead to a reduction in dendrite regeneration after damage in comparison with the γ-tubulin 37C control. Knockdown of the RTK's (Figure 11) or the earlier downstream effectors of the RTK signaling pathway (Figure 12) did not show a significant reduction in dendrite regeneration in comparison with the γ-tubulin 37C control.

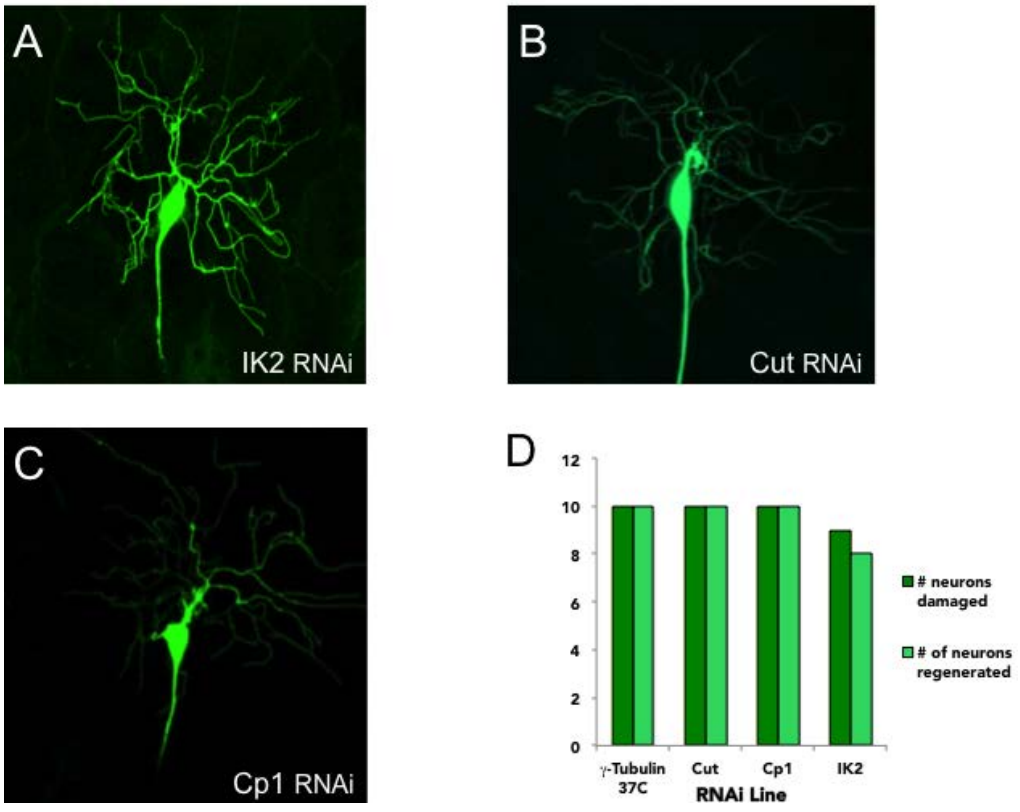

Figure 10: Dendrite regeneration occurs in knockdown of dendritic pruning and regrowth factors. RNAi knockdown of IK2 (A), Cut (B), and Cp1 (C) did not prevent dendrite regeneration from occuring to a similar extent as the γ-tubulin 37C control. Almost all of the neurons tested in these knockdowns showed dendrite regeneration (D).

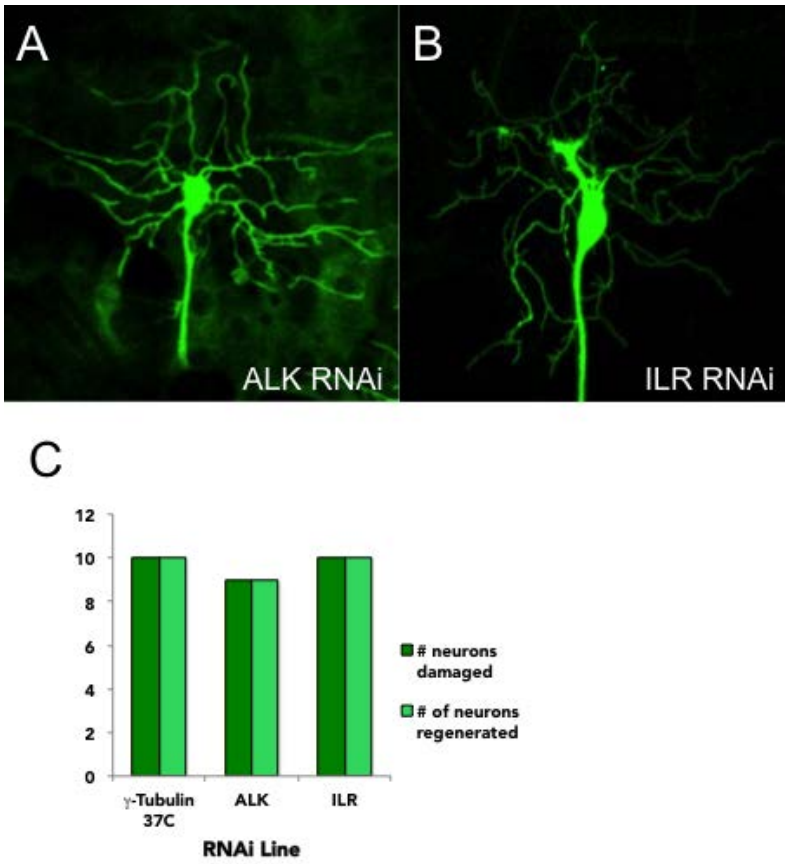

Figure 11: Dendrite regeneration occurs in knockdown of two RTKs. RNAi knockdown of ALK (A) and ILR (B) did not prevent or reduce dendrite regeneration. There is no significant reduction in the number of neurons that displayed dendrite regeneration out of the total number of neurons tested between the control and the tested groups (C).

Neurons with RNAi Knockdown of MAPKKKs Show Dendrite Regeneration

Mitogen-activated protein kinase kinase kinase (MAPKKK) cascades are commonly used downstream of initial RTK signaling to activate cellular responses. MAPKKKs activate MAPKKs via phosphorylation, and these activated MAPKKs activate MAPKs. In the axon regeneration pathway, dual-leucine zipper kinase (DLK) is a mitogen-activated protein kinase kinase kinase (MAPKKK) that is activated after axonal injury, which subsequently activates the

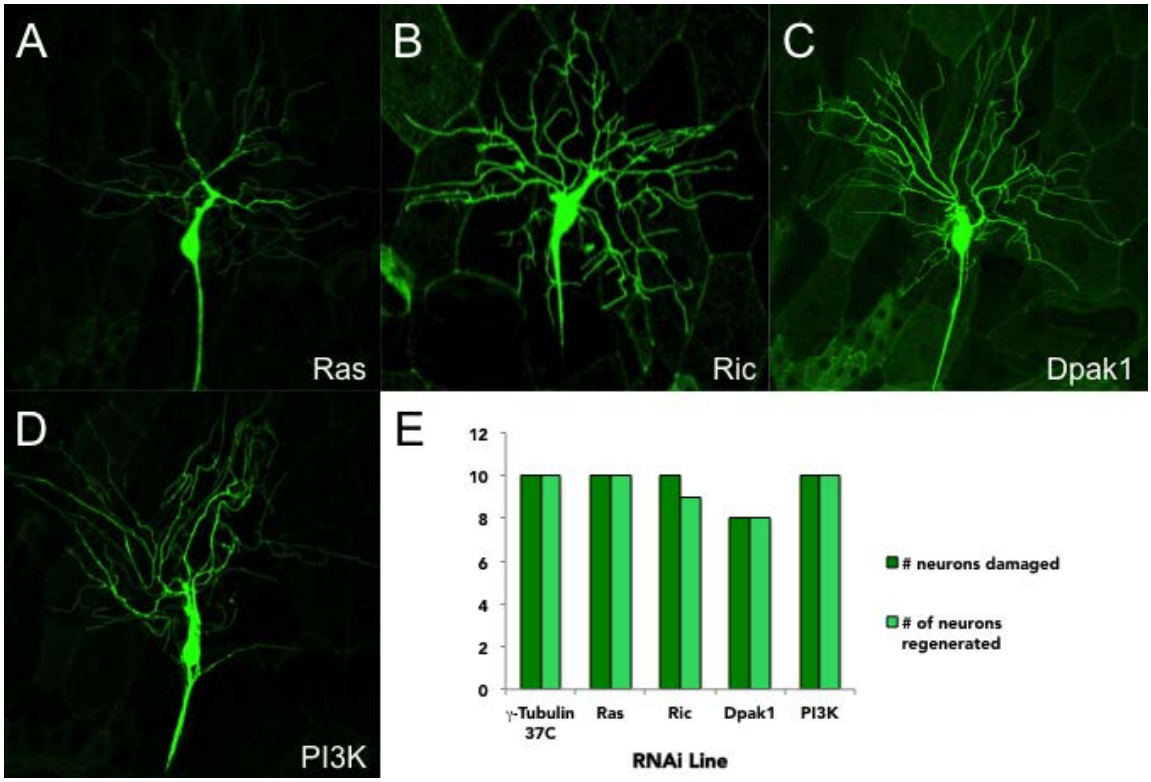

Figure 12: Dendrite regeneration occurs in knockdown of several proteins in RTK signaling pathway. RNAi knockdown of Ras (A), Ric (B), Dpak (C), or PI3K does not produce a significant reduction in dendrite regeneraiton after damage. There is no significant reduction in the number of neurons that displayed dendrite regeneration out of the total number of neurons tested between the control and the tested groups (D).

Jun N-terminal kinase (JNK, MAPK) and the transcription factor FOS (Gallo and Johnson, 2002). An increase in DLK/JNK/Fos signaling is necessary for axonal regrowth of damaged axons (Xiong et al., 2010). Though dendrite regeneration uses a pathway that is independent of DLK (Stone et al., 2014), I decided to test other members in the MAP kinase protein subfamilies – slpr, p38C, Hep, msn, MEKK, takl1, tak1, and MLK2 – to see if they would have an affect on dendrite regeneration (Figures 13 and 14). By targeting proteins of the RTK pathway that ultimately act to affect responses such as cell growth, I predicted that I would see reduced or complete lack of dendrite regeneration after damage. Dendrites were able to regenerate in

neurons with RNAi knockdown of these MAP kinases (Figure 13 and 14). Interestingly, however, msn appeared to produce regenerated dendrites with complex arbors (Figure 13D).

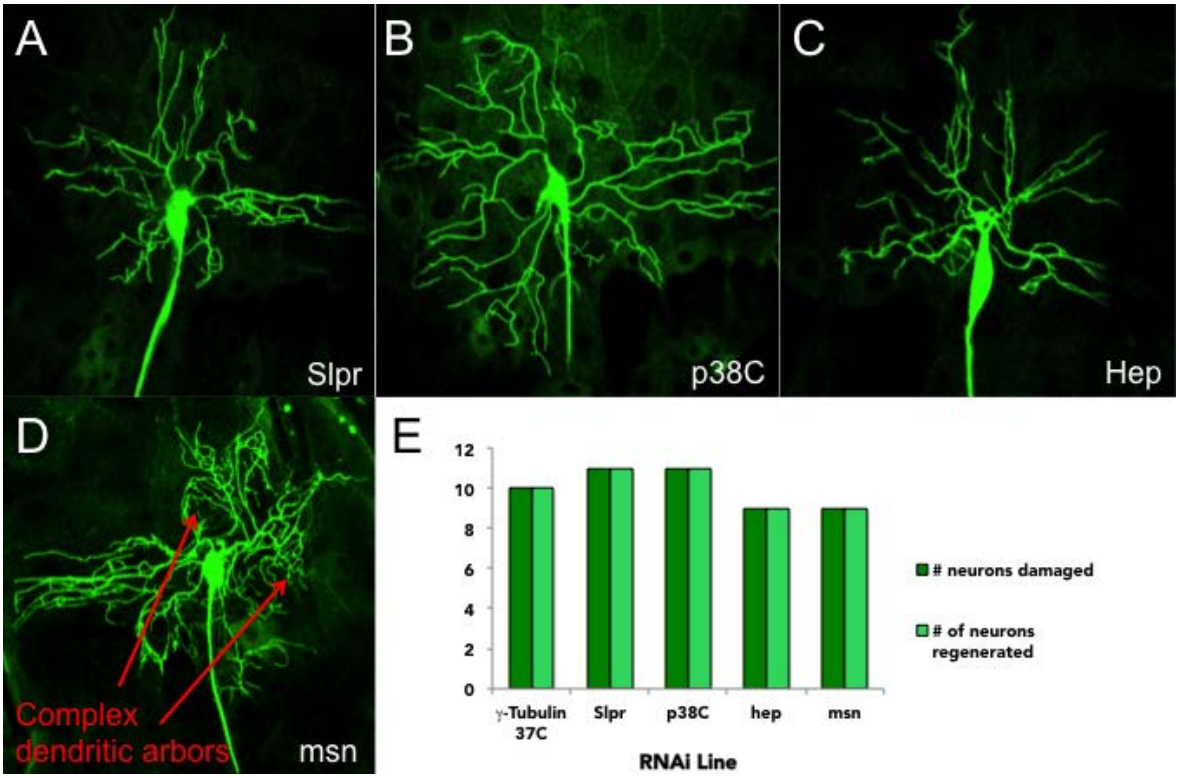

Figure 13: Neurons with RNAi knockdown of various MAP kinases showed dendrite regeneration after damage. Slpr (A), p38C (B), Hep (C), and msn (D) were knocked down with RNAi to see if they play a role in dendrite regeneration. Dendrites were able to regenerate in all tested proteins (E). Interestingly, reduced levels of msn seems to produce complex dendritic arbors 24 hours after damage, as indicated with the red arrows.

Neurons with RNAi Knockdown of Calcium-calmodulin Dependent Kinases Show Dendrite Regeneration

Intracellular calcium levels are typically low in comparison with the extracellular matrix. Thus, it makes sense that perforation of the neuronal cell membrane of the axon creates an increase in intracellular calcium that travels towards the cell body. This calcium then triggers the

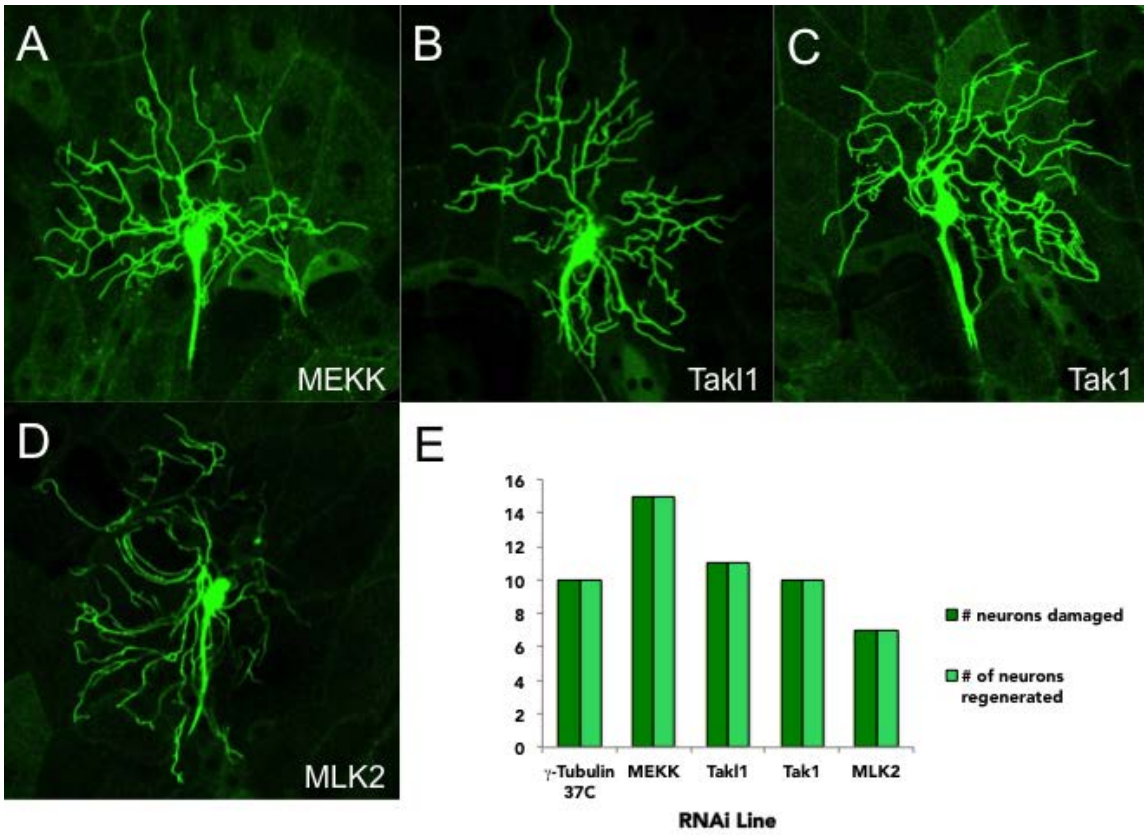

Figure 14: Neurons with RNAi knockdown of various MAP kinases showed dendrite regeneration after damage (continued). MEKK (A), Takl1(B), Tak1 (C) and MLK2 (D) were tested for their roles in dendrite regeneration, and dendrites were able to regenerate in all cases (E).

pathway for axonal regeneration by activation of cAMP and PKA. Calcium-calmodulin dependent kinases (CAMKs) are another type of calcium-dependent protein. CAMKs, by their name, require calcium for activation. Upon activation, CAMKs can phosphorylate cAMP response element-binding proteins (CREBs), and phosphorylated CREBs act as transcription factors. Activated CAMKs ultimately lead to regulation of gene expression through CREBs. It would be reasonable to suspect that a gene for dendritic growth is activated in part by this calcium-dependent CAMK signaling, as a perforation in the cell membrane at the site of dendrite injury could lead to an influx of calcium to activate this gene expression system. Thus, I decided

to test several different CAMKs – LKB1, an SNF-related kinase, par-1, caki, and CG17528 – with the prediction that one of these proteins may lead to a reduction or complete lack of dendrite regeneration (Figure 15 and 16). I also decided to test SPoCK (Figure 16), a secretory pathway calcium ATPase pump that functions to maintain calcium levels in the cytosol (Van Baelen et al., 2004). If this pump were not present in a neuron that has had dendrite damage, I would suspect that the high intracellular calcium concentration from dendrite injury would not be as well maintained, and that there would be reduced dendrite regeneration. Despite RNAi knockdown of the CAMKs and SPoCK, dendrites were able to regenerate in response to damage.

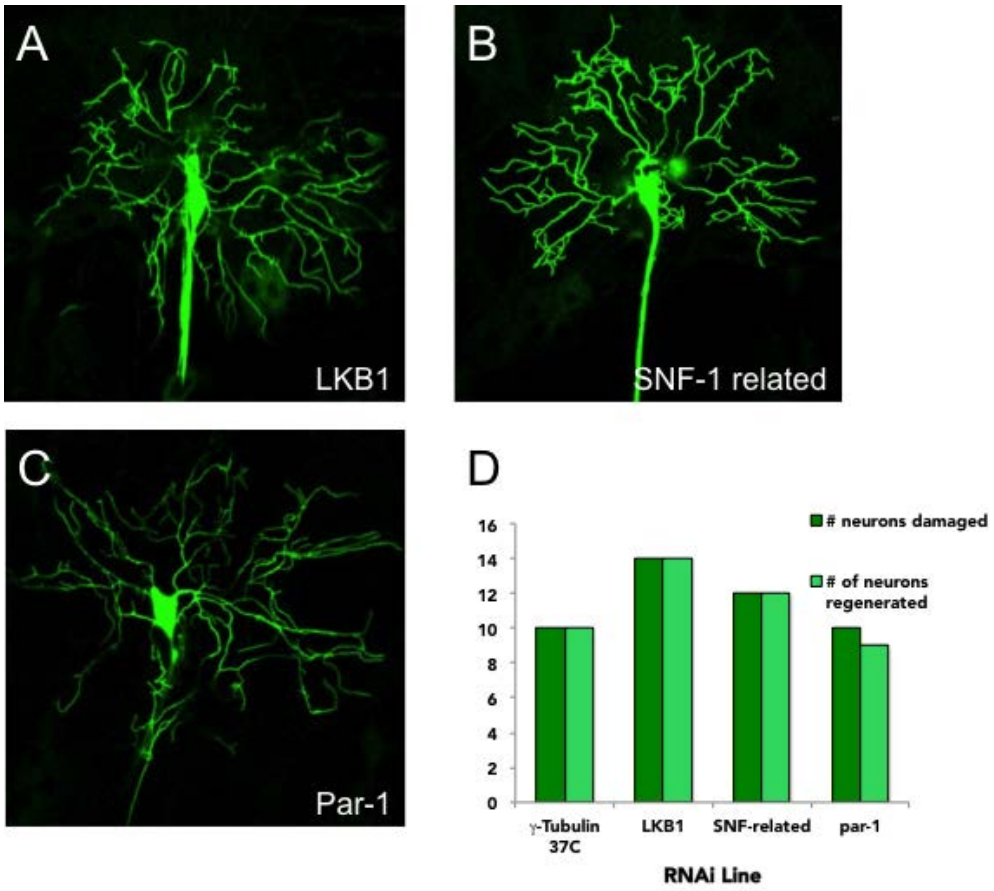

Figure 15: Neurons with RNAi knockdown of various calcium-calmodulin dependent kinases showed dendrite regeneration after damage. LKB1 (A), SNF-1 related (B), and Par-1 (C) were tested for their roles in dendrite regeneraiton using RNAi knockdown, and dendrites were able to regenerate in all cases (D).

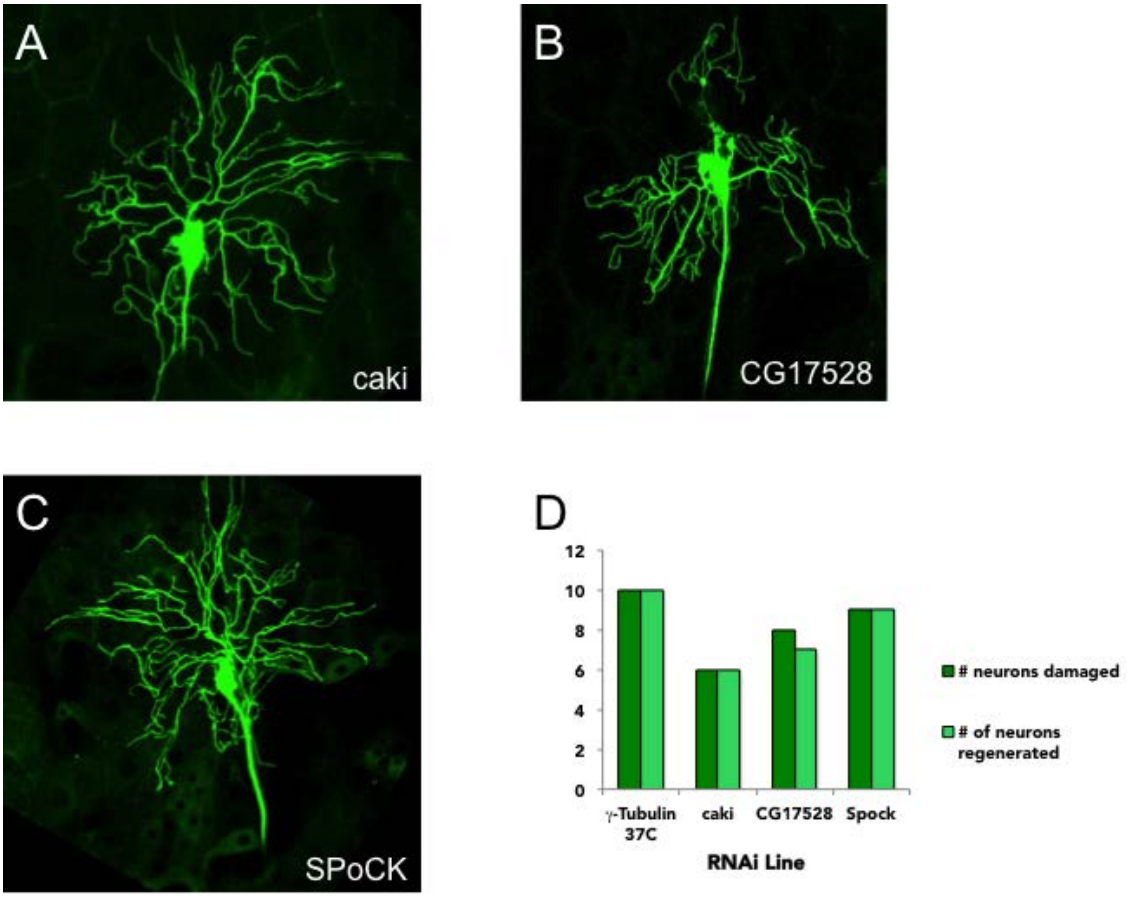

Figure 16: Neurons with RNAi knockdown of various calcium dependent kinases showed dendrite regeneration after damage. RNAi knockdown of two CAMKs – Caki (A) and CG17528 – (B) in sensory neurons did not show significantly reduced dendrite regeneration (D). SPoCK is a calcium ATPase that was tested for dendrite regeneration (C), and this also did not produce a significant reduction in dendrite regeneration (D).

Neurons with RNAi Knockdown of CDKs Show Dendrite Regeneration

Cyclin-dependent kinases (CDKs) are known most notably for their regulation of the cell cycle. Some CDKs also have a role in regulation of gene expression; cdk9 and cyclin T make up the p-TEFb kinase, which removes transcription pausing factors DSIF and NELF, allowing for the transcription of RNA polymerase to continue on a gene of interest (Adelman and Lis, 2012). In mammals, cdk7 has been shown to directly regulate mRNA synthesis to manage cellular homeostasis (Kelso et al., 2014). Due to the role of many of these proteins

required for normal cell function, I decided to test cdc2rk, cdk7, and cdk9 for dendrite regeneration. There was no significant reduction in dendrite regeneration in neurons with cdc2rk, cdk7, or cdk9 knockdown (Figure 17).

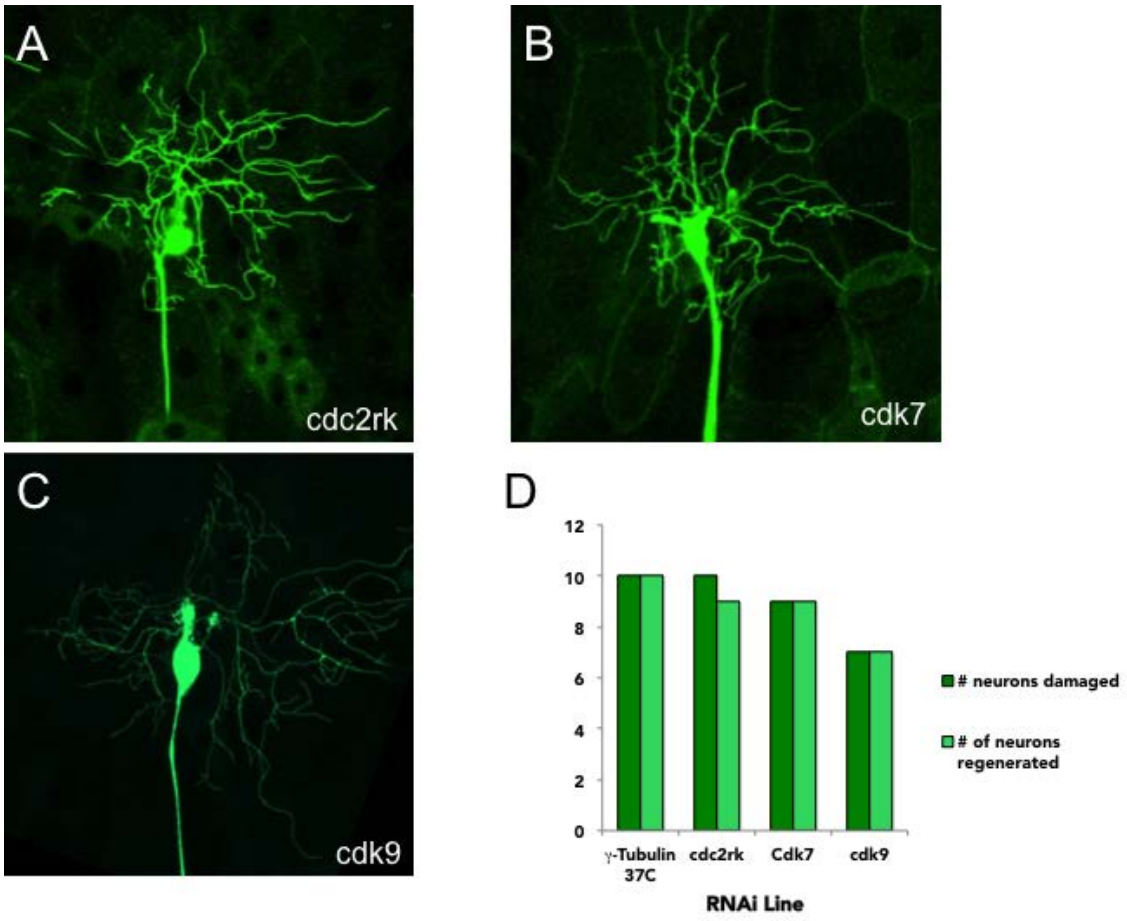

Figure 17: Neurons with RNAi knockdown of various cyclin-dependent kinases showed dendrite regeneration after damage. Cdc2rk (A), cdk7 (B), and cdk9 (C) were tested for their roles in dendrite regeneration using RNAi knockdown, and dendrites were able to regenerate in almost all tested neurons (D).

Neurons with RNAi Knockdown of Microtubule Proteins Show Dendrite Regeneration

In *Drosophila*, dendrites have minus-end out microtubules, meaning that the direction of polymerizing microtubules is oriented from the tips of the dendrites towards the cell body (Stone

et al., 2008). Functionally speaking, this means that vesicular transport of protein cargo from the cell body towards the dendrites utilizes a dynein motor. When axons are damaged, there is an upregulation of growing microtubules, and a dendrite is converted from minus-end out to a plus-end out. The plus-end out dendrite grows, and the dendrite functionally begins to take on many of the molecular functions of an axon (Stone et al., 2010). While this only seems to occur in axons and not dendrites (Stone et al., 2010), I decided to test to see if reduction of shaggy, anon-71Aa, and dlimk microtubule proteins would affect dendrite regeneration. Shaggy (sgg) is a protein that phosphorylates futsch, and futsch plays a role in dendritic and axonal development (Gögel et al., 2006; Hummel et al., 2000). Anon-71Aa (also known as DCX-EMAP), is a doublecortin containing microtubule-associated protein that plays a role in mechanosensory transduction in *Drosophila*. Dlimk is a kinase has been shown to regulate actin dynamics in both mammals and *Drosophila* (Ohashi et al., 2000). RNAi knockdown of these proteins did not lead to a significant reduction in dendrite regeneration (Figure 18).

Neurons with RNAi Knockdown of Various Proteins Involved in Development, Axonal Clearance and Vesicular Trafficking Show Dendrite Regeneration

I decided to test several different proteins involved in various processes: CK1-α, which is involved in the canonical wnt signaling pathway that in part controls neural patterning during embryogenesis (Komiya and Habas, 2008); sktl, which was identified in a screen for proteins involved in peripheral nervous system development (Prokopenko et al., 2000); Stat92E, a transcription factor that promotes expression of proteins to clear axonal debris after damage (Doherty et al., 2014); BcDNA, an ADP-Ribosylation factor GTPase that is involved in

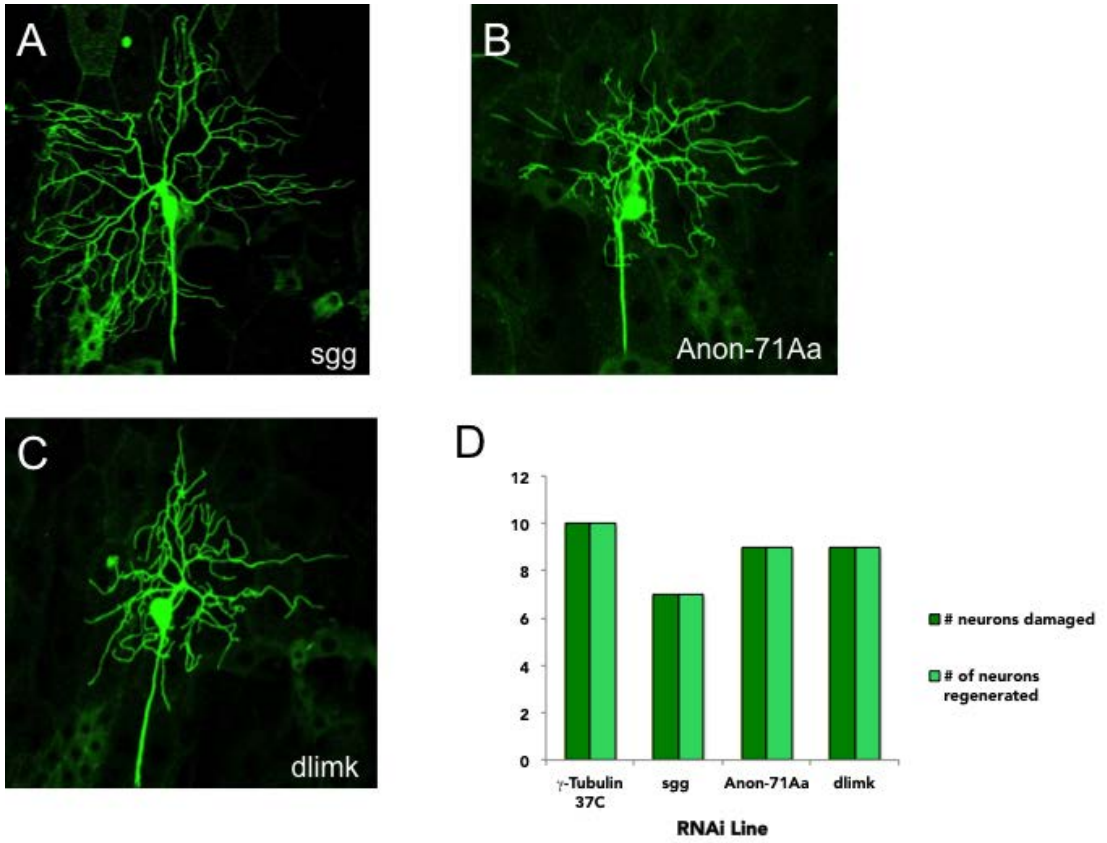

Figure 18: Neurons with RNAi knockdown of microtubule proteins showed dendrite regeneration after damage. Sgg (A), Anon-71Aa (B), and dlimk (C) were tested for their roles in dendrite regeneration using RNAi knockdown, and dendrites were able to regenerate in all tested neurons (D).

regulation of vesicular trafficking (Wennerberg et al., 2005); varicose, a protein involved in cell-adhesion (Wu et al., 2007); and src64B, a kinase found to be involved in actin organization in ovarian ring canals (Kelso et al., 2002). I hypothesized that knockdown of these proteins would result in a reduced level of dendrite regeneration or complete lack of regeneration. Upon testing these proteins, I found that none appear to regulate dendrite regeneration after injury (Figure 19 and 20).

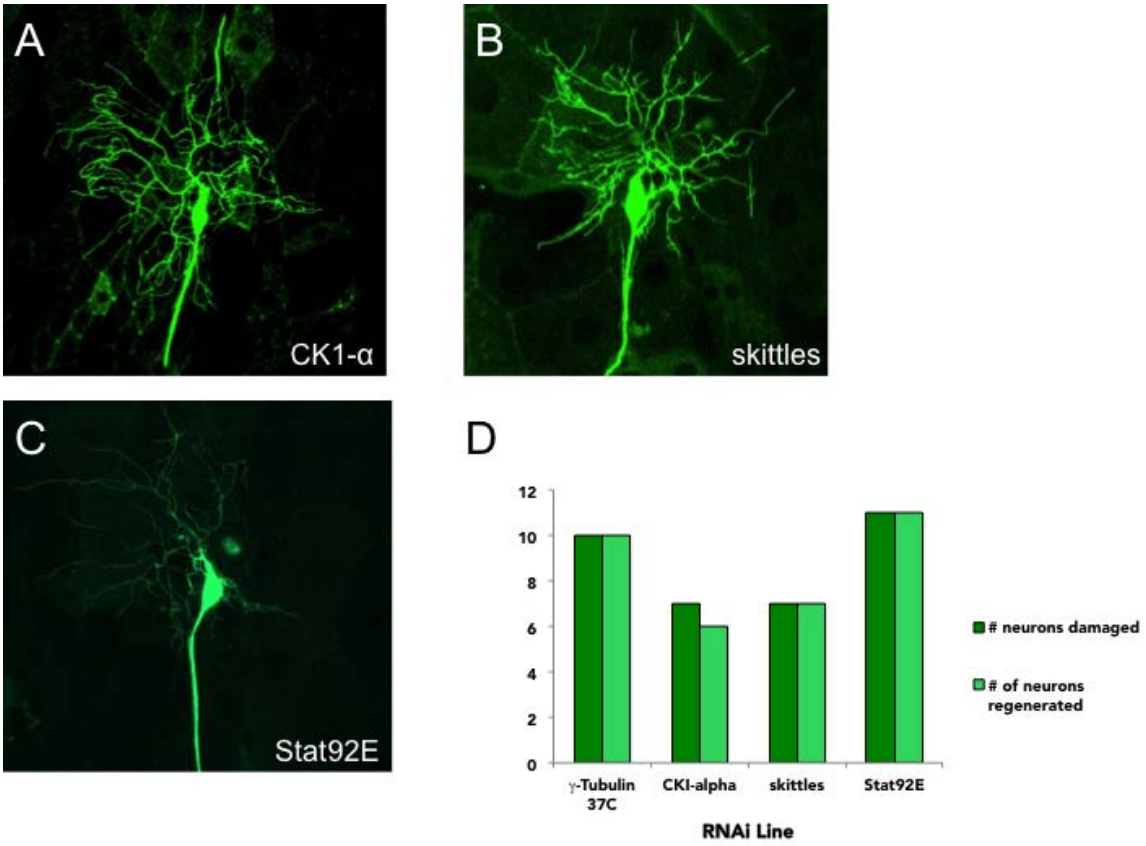

Figure 19: Neurons with RNAi knockdown of various proteins involved in development and axonal clearance showed dendrite regeneration. CK1-α (A), skittles (B), and Stat92E were tested for their roles in dendrite regeneration using RNAi knockdown, and dendrite regeneration was able to occur in almost all tested neurons (D).

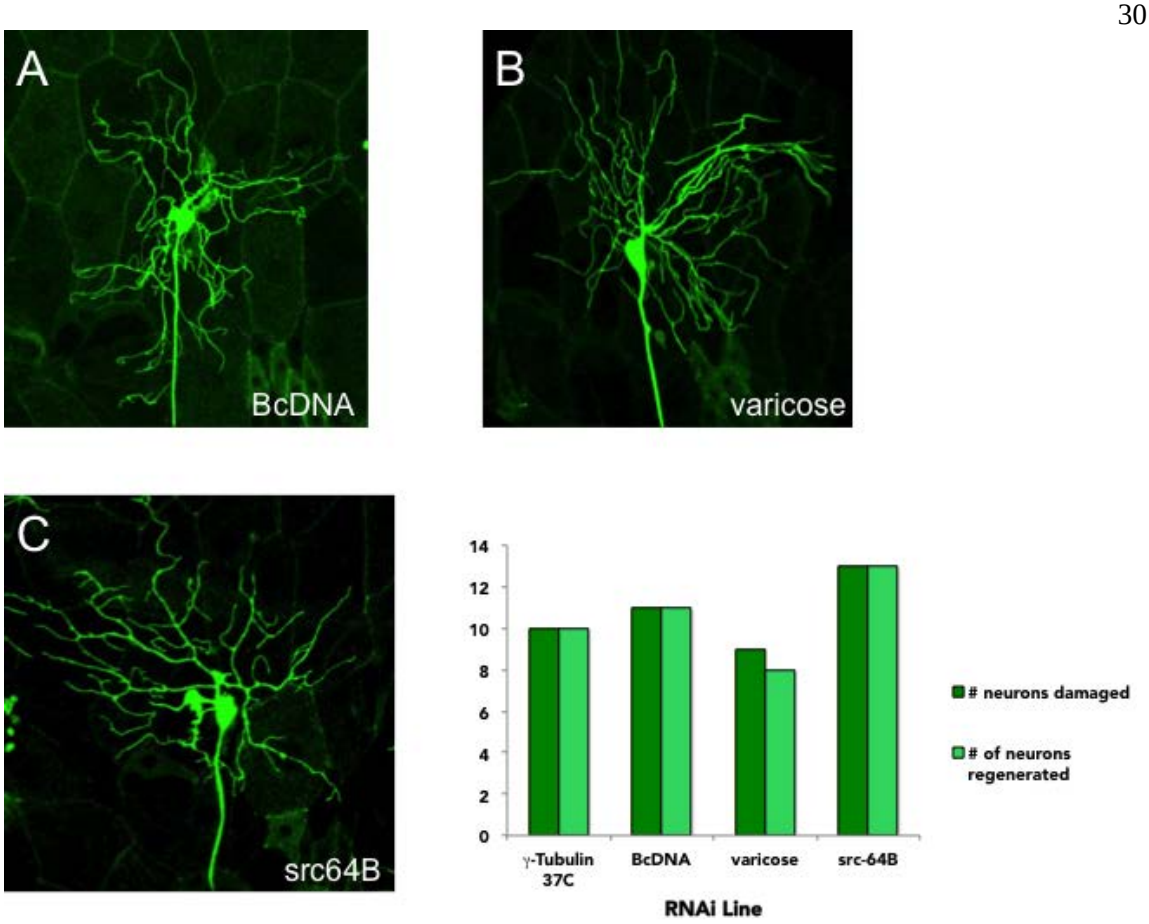

Figure 20: Neurons with RNAi knockdown of kinases involved in vesicular trafficking, cell adhesion, and actin regulation showed dendrite regeneration. BcDNA (A), varicose (B), and src64B (C) were tested for their roles in dendrite regeneration using RNAi knockdown, and dendrite regeneration was able to occur in almost all tested neurons (D).

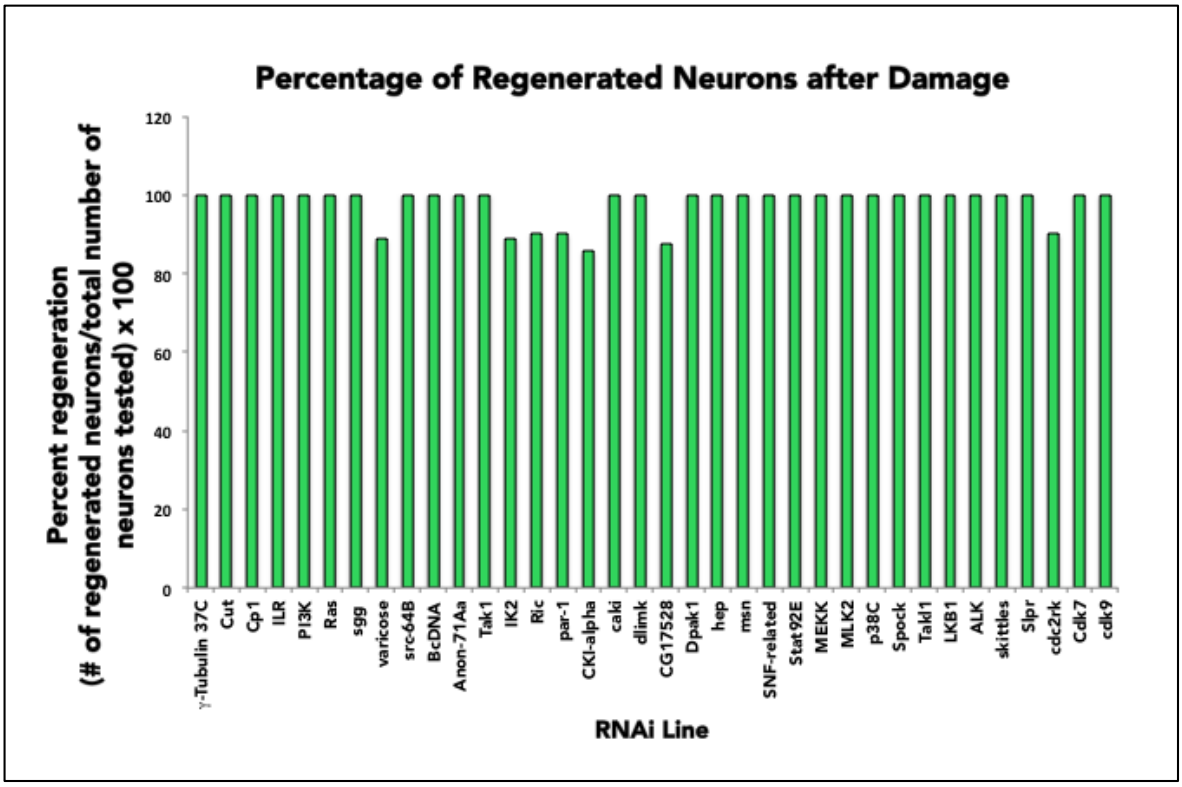

Figure 21: Dendrite regeneration occurs more than 80% of the time in each RNAi candidate. Percent regeneration was calculated as a ratio of regenerated dendrites of the total number of larvae tested. Results show that dendrite regeneration occurs after damage, with $p >> 0.05$ in each case. Statistical analysis was performed using Fisher's Exact Test.

Modeling Dendrite Injury Response in Presence of Dead Glial Cells

In order to test if dendrite regeneration can occur without glial cells, we needed to be able to visualize three different features: the glial cell membrane (to be able to see the entire shape of the glial cells), the glial cell nuclei (to cause glial cell ablation), and the class IV sensory neuron membrane (to be able to test for dendrite regeneration). One of the initial challenges to overcome was being able to visualize all of these features, as using the Gal4/UAS system would require two separate Gal4 drivers and three different upstream-activating sequences. While the Gal4 may be specific to either glial cells or sensory neurons, the UAS would not be. If both membranes are

visualized with the UAS-mcD8-GFP, then it may be difficult to differentiate between sensory neuron membranes and glial cell membranes. Additionally, the large number of transgenes would likely be detrimental to the overall health of the flies, and would interfere with many of the features that make *Drosophila* a convenient model system to work with. By using PPK-tdtomato, which has RFP directly fused to the pickpocket protein expressed on the class IV neuron membrane, I eliminated the need for a separate UAS-mcD8-RFP to label the sensory neuron. I used repo-Gal4 to drive expression of Gal4 in glial cells, UAS-redstinger to label the glial cell nuclei, and UAS-mcD8-GFP to label the glial cell membranes.

This fly line was generated in order to determine the role of glial cells in dendrite injury, but it was uncertain whether it would be feasible to perform a damage assay on both glial cells and the sensory neurons. Also, whether the glial cells would be close enough to the epithelial layer to induce damage was another practical concern. Figure 22 illustrates that I was able to develop an assay to test if glial cells are required for the dendrite regeneration response to injury. While dendrite regeneration is present 48 hours after glial cell ablation and 24 hours after dendrite injury (Figure 22), the current sample size is too low to make definitive conclusions on whether dendrite regeneration after damage will occur in absence of functioning glial cells. In addition, some of the glial cells are farther away from the epithelial layer of the larvae and thus not as easily accessible to the UV pulse laser, making it difficult to damage these glial cells. However, the assay for killing glial cells and testing for dendrite regeneration is feasible and should become streamlined with time.

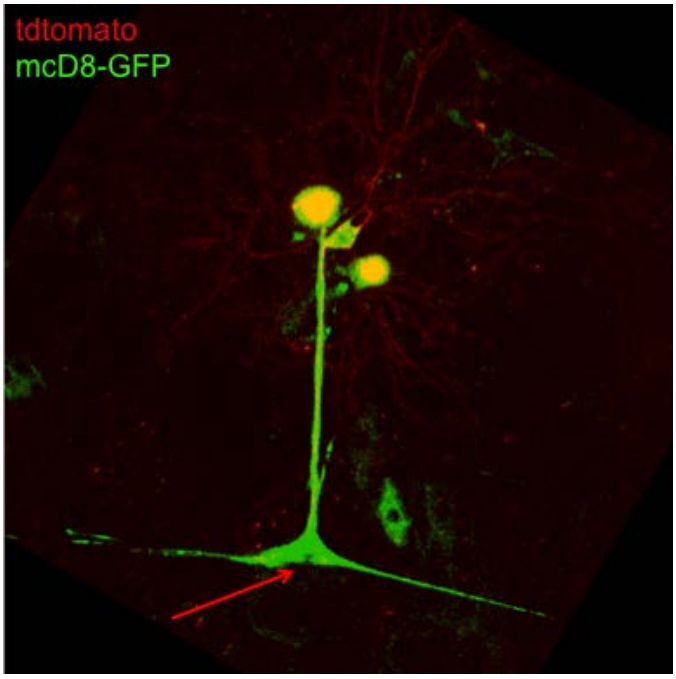

Figure 22. Modeling dendrite injury responses and glial cell ablation. Image was taken 48 hours after glial cell ablation and 24 hours after dendrite injury. Glial cells were killed using a UV pulse laser. Glial cell membranes are marked with mCD8-GFP whereas the dendrites are marked with tdtomato. The location of the targeted nucleus is indicated with the red arrow. Redstinger is not visible as this image was taken after ablation; lack of fluorescence in this region illustrates absence of nuclei and a functionally dead glial cell. After 24 hours, the dendrites were cut from the neuron, and regeneration of dendrites was observed 24 hours later.

Generation of Transgenic Flies Containing *osm-9* and *ocr-4*

Though there are orthologs to *C. elegans osm-9* and *ocr-4* in *Drosophila – inactive* and *nanchung*, respectively – cells that express *inactive* and *nanchung* do not experience cell death when exposed to NAM (Upadhyay et al., 2016). By cloning the two *C. elegans* genes *osm-9* and *ocr-4* into a plasmid vector and subsequently making transgenic *Drosophila*, I aim to develop an alternative assay to dendrite regeneration. The two genes were linked with a viral 2A sequence to allow bicistronic expression of the two proteins. Expression of two proteins with a viral 2A sequence is advantageous compared to using an internal ribosomal entry site (IRES), as using an IRES often leads to reduced expression of the second gene in the sequence (Kaufman et al.,

1991). In addition, a viral 2A sequence often is better than co-transfecting *in vivo* cells with two separate plasmids, as this application is not as practical *in vivo* as it is *in vitro* (Daniels et al., 2014)

In-Fusion cloning was used to generate a pUAST plasmid construct with *osm-9* and *ocr-4*. The pUAST *Drosophila* vector has a multiple cloning site (MCS) to allow for insertion of genes of interest. Expression of the proteins was placed under UAS-control. This is important as it will allow for us to have tissue-specific expression of *osm-9* and *ocr-4* under Gal4 in *Drosophila*. *E. coli* transformants were screened using colony PCR, and plasmid was extracted from positive transformants using a plasmid miniprep. The plasmids were digested using two different restriction enzymes, and each produced bands at the expected sizes, confirming that the plasmid contains the *osm-9*, *ocr-4*, and viral 2A genes (Figure 23 and 24). The plasmid construct was then further amplified using a midiprep and injected into *Drosophila* embryos to generate transgenic flies expressing *osm-9* and *ocr-4*. The pUAST vector has the *mini-white* selectable marker to allow for selection of flies that have taken up the plasmid. Transgenic *Drosophila* will be used to develop an assay that will potentially induce damage to the neurons by feeding nicotinamide to larvae expressing *osm-9* and *ocr-4* (Figure 24).

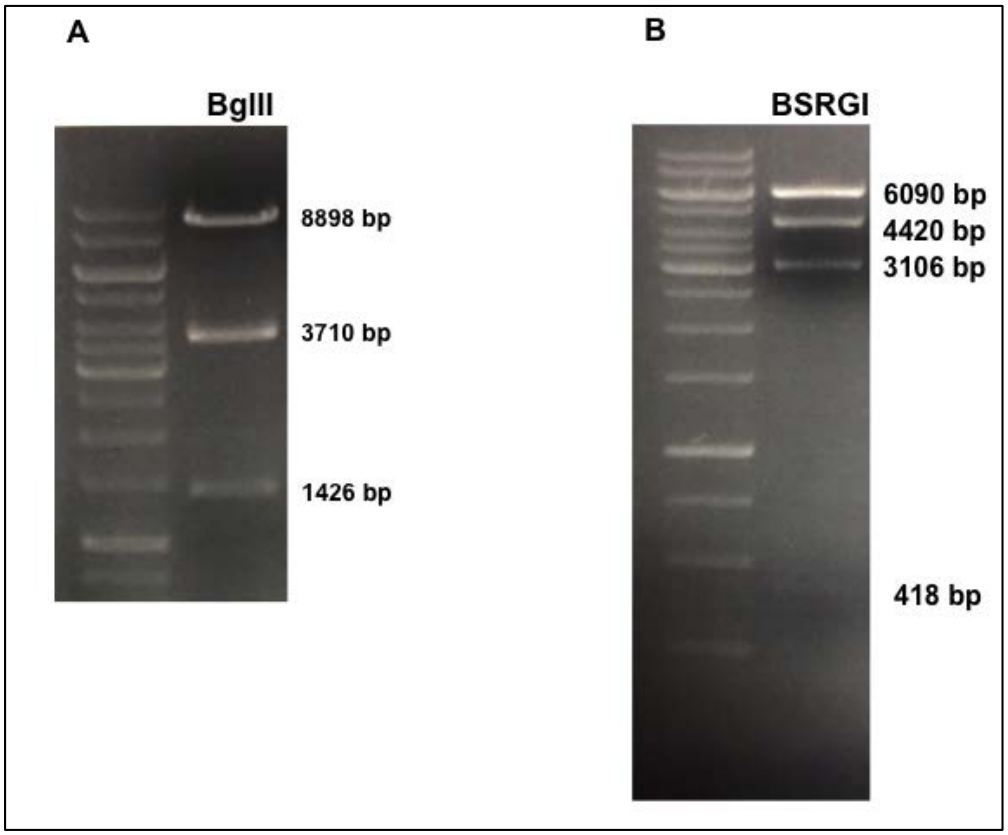

Figure 23: Restriction enzyme digestion confirms plasmid product. Plasmid construct was digested with BglII (A) and BSRGI (B). Digests produced bands at the expected sizes.

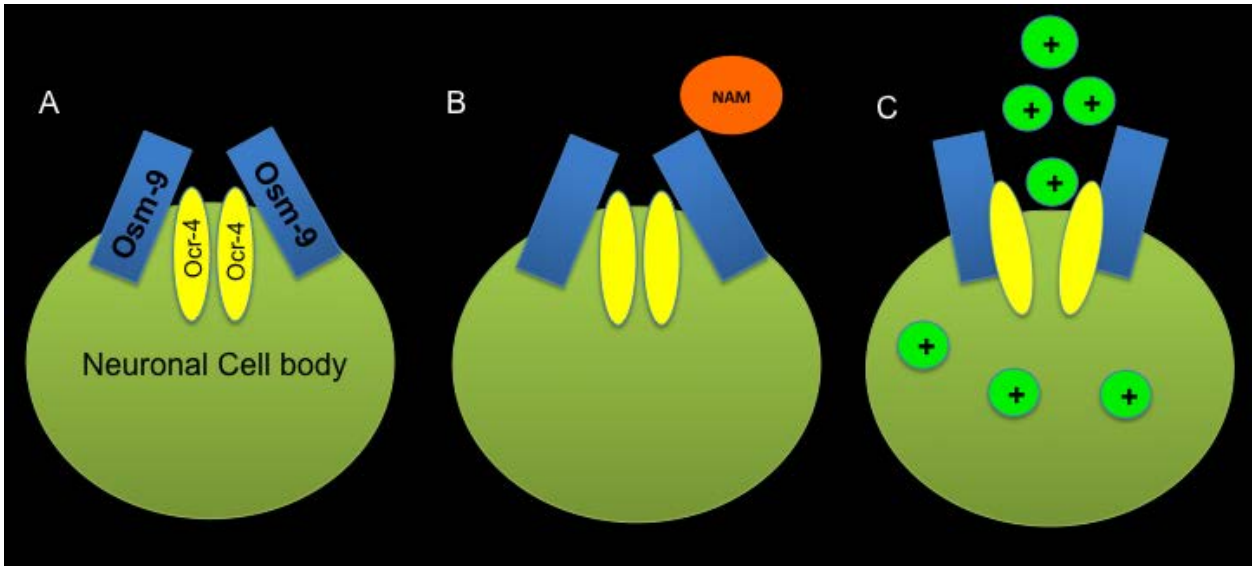

Figure 24: OSM-9, OCR-4 and NAM. Osm-9 and ocr-4 form a heterotetramer in *C. elegans* neurons (A)(Upadhyay et al., 2016). Upon binding of NAM (14B), there is a subsequent influx of calcium (14C). Calcium-dependent proteases destroy the components of the neuron required for survival (Bano and Nicotera, 2007).

Discussion and Future Directions

Tested Proteins do not Appear to Play a Role in Dendrite Regeneration

When thirty-five different proteins were knocked down using RNAi, dendrites were able to regenerate after damage in each case with no statistically significant reduction in dendrite regeneration (Figure 21). The fact that dendrites were able to regenerate suggests that these proteins do not regulate dendrite regeneration after damage. Though this was the case, we cannot definitively conclude that these proteins do not play a role in dendrite regeneration after damage. There is a small possibility that RNAi did not produce effective knockdown of one of the proteins, and that particular protein may have been an enzyme involved in the dendrite regeneration pathway. This is one of the downfalls of performing a broad genetic screen using RNAi. However, due the large number of protein candidates that may be involved in dendrite regeneration, the ability to quickly see which proteins play a role in dendrite regeneration far outweigh the risks of using RNAi.

There is also the possibility that dendrite regeneration is a process that utilizes several parallel pathways. In a parallel pathway, knockdown of one protein would not completely silence the final response of dendrite regeneration, as there would be proteins in the parallel pathways to compensate for the protein that was knocked-down. This would make it particularly difficult to identify a protein using the RNAi screen described herein. If the dendrite regeneration pathway does require a series of parallel pathways, we may need to look at alternative methods to begin to understand proteins involved in dendrite regeneration. Though the screen has not revealed any proteins involved in the dendrite regeneration pathway, the results speak as a testament to the

remarkable capability of a neuron to regenerate in response to injury. The fact that dendrite regeneration utilizes a pathway that is independent of the axonal regeneration pathway, and that none of the protein candidates produced a lack of dendrite regeneration after damage means that dendrite regeneration likely utilizes a pathway different from what we had initially expected.

Glial Cell Injury Assay and Dendrite Regeneration

We developed a fly line that has a red fluorescent protein directly fused to PPK, which has allowed us to visualize glial cells and class IV neurons using separate fluorophores. Prior to these experiments, it was unknown whether we would be able to utilize a UV pulse laser to kill glial cells and subsequently damage dendrites to test for regeneration after damage. One of the major concerns was that performing a large amount of local damage with the laser would kill the sensory neuron. Figure 22 shows that it is possible to monitor dendrite regeneration after killing the glial cells. The sample size is too small at this point (n=1) to definitively conclude whether or not glial cells play a role in dendrite regeneration after damage. However, this assay will provide the gateway to being able to understand if glial cells are necessary for the dendrite regeneration response.

There are still quirks to damaging the glial cells with the UV pulse laser. Each sensory neuron has approximately 3-4 glial cells that surround it towards the synaptic end of the axon. In some cases, these glial cells are superficial enough to the epithelial layer to induce damage with the UV pulse laser, while in other cases they are not. Typically, the larvae is mounted dorsal-side up with the two trachea facing upwards. In instances where the glial cells are not accessible for damage with the laser, I will accommodate this by mounting the larvae on a slight angle, to allow

for easier access to glial cells that are deeper in comparison to the epithelial layer. Once this assay becomes more streamlined, I plan to test to see if glial cells are required for dendrite regeneration damage using this assay we have developed.

Future Directions

From the RNAi screen, I observed that damage to a class IV sensory neuron with msn (misshapen) RNAi knockdown exhibited more complex dendritic arbors after regeneration than seen in the γ-tubulin 37C RNAi control (Figure 13D). Msn is thought to have MAPKKKK activity from sequence similarity with other MAPKKKKs (Su et al., 1998). I would like to further explore msn to see if it has complex dendritic arbors prior to dendrite injury, or if this only occurs in the regenerative response. Complexity phenotypes are interesting to explore because of their functional outcomes; a neuron with a high surface density of dendrites at the epithelial layer would have a smaller receptive field. It would be interesting to test if these class IV neurons do in fact have smaller receptive fields by performing two-point discrimination tests. It would also be interesting to see if these neurons have an increased nociceptive response from the increase in number of input processes that seem to be apparent in larvae with msn RNAi knockdown.

In addition to performing the RNAi screen, I am working with M.D./Ph.D. student Richard Albertson and utilizing a transcriptomics approach to determine global gene expression changes following dendrite injury. This is accomplished by freezing larvae at specific time points following injury. These animals can then be cryosectioned and prepared for laser capture microdissection. Neuronal somas of interest are pooled into a single sample, and a cDNA library

is generated according to protocols developed by Kevin Janes (Wang and Janes, 2013). These cDNA libraries can be extensively profiled by qPCR prior to RNA sequencing to ensure sample quality. RNA sequencing will allow determination of dendrite regeneration associated genes, and allow more focused candidate analysis using RNAi and mutant fly lines. These experiments will provide much needed insight into dendrite regeneration.

The screening process will also presumably become faster once I am able to take advantage of transgenic *Drosophila* expressing the *C. elegans osm-9* and *ocr-4* genes. By taking these transgenic flies and mating them with the tester line, I hope to be able to take advantage of larvae that have the same features as those described in this experiment (i.e. *Dicer2, mcD8-GFP* in sensory neurons) but with sensory neurons that are damaged in response to nicotinamide instead of the UV pulse laser. Through the expression of *osm-9* and *ocr-4* channels in *Drosophila* and feeding the larvae with nicotinamide, I aim to be able to induce damage to the dendrites of sensory neurons.

Naturally, developing this assay will come with certain challenges; neurons may simply die upon addition of nicotinamide. It will be necessary to determine an appropriate concentration of nicotinamide to use to induce damage without simply killing the neuron. While the aim of this experiment is to focus on damaging just the dendrites, it is also possible that both dendrites and axons will be damaged after adding nicotinamide. This could provide interesting insight into how *Drosophila* respond to loss of all processes *in vivo*, as there is little insight as to whether neurons can regenerate in response to both damaged dendrites and axons. Once I am able to make this assay become functional, we will be able to induce damage to the neurons without using the UV pulse laser, and will greatly simplify damage assays for the use of this project as well as for future projects to come.

It will also be interesting to further examine the role of glial cells in dendrite regeneration using the assay described. If dendrites do regenerate after glial cell ablation, then glial cells most likely do not play a role in the dendrite regeneration response to injury. If dendrites do not regenerate when glial cells are killed, then perhaps glial cells send a signal that is required to trigger the dendrite regeneration response. It would be interesting if this were the case, as glial cells are most often described in the context of axons and synaptic terminals. To identify proteins involved in this process, I may choose to screen for proteins involved in the glial cell response by performing RNAi knockdown in glial cells instead of the sensory neurons as in this assay.

Final Thoughts

Once we have identified a protein involved in the dendrite regeneration pathway, predictions will be made on other proteins involved in the pathway by using the known list of candidates from the cDNA analysis, and comparing this list to the known interactions of the identified protein with other proteins. By doing so, we hope to be able to discover the dendrite regeneration pathway. Understanding neural response to injury is important because dendrites are damaged during traumatic brain injury, stroke, and seizures (Gao and Chen, 2011; Murphy et al., 2008; Zeng et al., 2007). Observing the rapid and robust nature of dendrite regeneration in *Drosophila* has also raised many questions about the functionality of regenerated neurons. Are the class IV nociceptive neurons able to respond to touch with damaged and subsequently regenerated dendrites? Do regenerated axons ultimately meet with their synaptic counterparts? There are still many questions that remain about neural response to injury, specifically in regards

to dendrites. Looking forward, the experiments described herein will help to provide the tools necessary to answer the remaining questions about dendrite regeneration after injury.

References

Adelman, K., Lis, J.T., 2012. Promoter-proximal pausing of RNA polymerase II: emerging roles in metazoans. Nat. Rev. Genet. 13, 720–31.

Appenzeller, O., Palmer, G., 1972. The cyclic AMP (adenosine 3′,5′-phosphate) content of sciatic nerve: changes after nerve crush. Brain Res. 42, 521–524. doi:10.1016/0006-8993(72)90553-7

Ayaz, D., Leyssen, M., Koch, M., Yan, J., Srahna, M., Sheeba, V., Fogle, K.J., Holmes, T.C., Hassan, B.A., 2008. Axonal injury and regeneration in the adult brain of Drosophila. J Neurosci 28, 6010–6021. doi:28/23/6010 [pii]\r10.1523/JNEUROSCI.0101-08.2008

Bano, D., Nicotera, P., 2007. Ca2+ signals and neuronal death in brain ischemia. Stroke. 38, 674–6. doi:10.1161/01.STR.0000256294.46009.29

Bodmer, R., Jan, Y.N., 1987. Morphological differentiation of the embryonic peripheral neurons in Drosophila. Rouxs Arch. Dev. Biol. 196, 69–77. doi:10.1007/BF00402027

Brand, A.H., Perrimon, N., 1993. Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Development 118, 401–15. doi:10.1101/lm.1331809

Carlsen, R.C., 1982. Axonal transport of adenylate cyclase activity in normal and axotomized frog sciatic nerve. Brain Res 232, 413–424.

Chen, L., Chisholm, A.D., 2011. Axon regeneration mechanisms: Insights from C. elegans. Trends Cell Biol. doi:10.1016/j.tcb.2011.08.003

Daniels, R.W., Rossano, A.J., Macleod, G.T., Ganetzky, B., 2014. Expression of multiple transgenes from a single construct using viral 2A peptides in Drosophila. PLoS One 9. doi:10.1371/journal.pone.0100637

Doherty, J., Sheehan, A.E., Bradshaw, R., Fox, a. N., Lu, T.-Y., Freeman, M.R., 2014. PI3K Signaling and Stat92E Converge to Modulate Glial Responsiveness to Axonal Injury. PLoS Biol. 12, e1001985. doi:10.1371/journal.pbio.1001985

Gallo, K. a, Johnson, G.L., 2002. Mixed-lineage kinase control of JNK and p38 MAPK pathways. Nat. Rev. Mol. Cell Biol. 3, 663–72. doi:10.1038/nrm906

Gao, X., Chen, J., 2011. Mild traumatic brain injury results in extensive neuronal degeneration in the cerebral cortex. J. Neuropathol. Exp. Neurol. 70, 183–91. doi:10.1097/NEN.0b013e31820c6878

Ghosh-Roy, A., Wu, Z., Goncharov, A., Jin, Y., Chisholm, A.D., 2010. Calcium and Cyclic AMP Promote Axonal Regeneration in Caenorhabditis elegans and Require DLK-1 Kinase. J. Neurosci. 30, 3175–3183. doi:10.1523/JNEUROSCI.5464-09.2010

Gogel, S., Wakefield, S., Tear, G., Klambt, C., Gordon-Weeks, P.R., 2006. The Drosophila microtubule associated protein Futsch is phosphorylated by Shaggy/Zeste-white 3 at an homologous GSK3?? phosphorylation site in MAP1B. Mol. Cell. Neurosci. 33, 188–199. doi:10.1016/j.mcn.2006.07.004

Goldin, A.L., 2002. Evolution of voltage-gated Na(+) channels. J. Exp. Biol. 205, 575–584.

Grueber, W.B., Ye, B., Yang, C.-H., Younger, S., Borden, K., Jan, L.Y., Jan, Y.-N., 2007. Projections of Drosophila multidendritic neurons in the central nervous system: links with peripheral dendrite morphology. Development 134, 55–64. doi:10.1242/dev.02666

Guo, W., Wang, H., Watanabe, M., Shimizu, K., Zou, S., Lagraize, S.C., Wei, F., Dubner, R., Ren, K., 2007. Glial – Cytokine – Neuronal Interactions Underlying the Mechanisms of Persistent Pain. Area 27, 6006 – 6018. doi:10.1523/JNEUROSCI.0176-07.2007

Han, C., Song, Y., Xiao, H., Wang, D., Franc, N.C., Jan, L.Y., Jan, Y.-N., 2014. Epidermal cells are the primary phagocytes in the fragmentation and clearance of degenerating dendrites in Drosophila. Neuron 81, 544–60. doi:10.1016/j.neuron.2013.11.021

Henry, G.L., Davis, F.P., Picard, S., Eddy, S.R., 2012. Cell type-specific genomics of Drosophila neurons. Nucleic Acids Res. 40, 9691–704. doi:10.1093/nar/gks671

Hughes, C.L., Thomas, J.B., 2007. A sensory feedback circuit coordinates muscle activity in Drosophila. Mol. Cell. Neurosci. 35, 383–396. doi:10.1016/j.mcn.2007.04.001

Hummel, T., Krukkert, K., Roos, J., Davis, G., Klämbt, C., 2000. Drosophila Futsch/22C10 is a MAP1B-like protein required for dendritic and axonal development. Neuron 26, 357–370. doi:10.1016/S0896-6273(00)81169-1

Hwang, R.Y., Zhong, L., Xu, Y., Johnson, T., Zhang, F., Deisseroth, K., Tracey, W.D., 2007. Nociceptive Neurons Protect Drosophila Larvae from Parasitoid Wasps. Curr. Biol. 17, 2105–2116. doi:10.1016/j.cub.2007.11.029

Jessen, K.R., 2004. Glial cells. Int. J. Biochem. Cell Biol. 36, 1861–7. doi:10.1016/j.biocel.2004.02.023

Kaufman, R.J., Davies, M. V, Wasley, L.C., Michnick, D., 1991. Improved vectors for stable expression of foreign genes in mammalian cells by use of the untranslated leader sequence from EMC virus. Nucleic Acids Res. 19, 4485–4490. doi:10.1093/nar/19.16.4485

Kelso, R.J., Hudson, A.M., Cooley, L., 2002. Drosophila Kelch regulates actin organization via Src64-dependent tyrosine phosphorylation. J. Cell Biol. 156, 703–713. doi:10.1083/jcb.200110063

Kelso, T.W.R., Baumgart, K., Eickhoff, J., Albert, T., Antrecht, C., Lemcke, S., Klebl, B., Meisterernst, M., 2014. CDK7 controls mRNA synthesis by affecting stability of preinitiation complexes, leading to altered gene expression, cell cycle progression and

survival of tumor cells. Mol. Cell. Biol. 34, MCB.00595–14. doi:10.1128/MCB.00595-14

Kim, M.E., Shrestha, B.R., Blazeski, R., Mason, C.A., Grueber, W.B., 2012. Integrins Establish Dendrite-Substrate Relationships that Promote Dendritic Self-Avoidance and Patterning in Drosophila Sensory Neurons. Neuron 73, 79–91. doi:10.1016/j.neuron.2011.10.033

Kirilly, D., Wong, J.J.L., Lim, E.K.H., Wang, Y., Zhang, H., Wang, C., Liao, Q., Wang, H., Liou, Y.-C., Wang, H., Yu, F., 2011. Intrinsic epigenetic factors cooperate with the steroid hormone ecdysone to govern dendrite pruning in Drosophila. Neuron 72, 86–100. doi:10.1016/j.neuron.2011.08.003

Komiya, Y., Habas, R., 2008. Wnt signal transduction pathways. Organogenesis 4, 68–75. doi:10.4161/org.4.2.5851

Lee, H.-H., Jan, L.Y., Jan, Y.-N., 2009. Drosophila IKK-related kinase Ik2 and Katanin p60-like 1 regulate dendrite pruning of sensory neuron during metamorphosis. Proc. Natl. Acad. Sci. 106, 6363–6368. doi:10.1073/pnas.0902051106

Lyons, G.R., Andersen, R.O., Abdi, K., Song, W.-S., Kuo, C.T., 2014. Cysteine proteinase-1 and cut protein isoform control dendritic innervation of two distinct sensory fields by a single neuron. Cell Rep. 6, 783–91. doi:10.1016/j.celrep.2014.02.003

Murphy, T.H., Li, P., Betts, K., Liu, R., 2008. Two-photon imaging of stroke onset in vivo reveals that NMDA-receptor independent ischemic depolarization is the major cause of rapid reversible damage to dendrites and spines. J. Neurosci. 28, 1756–72. doi:10.1523/JNEUROSCI.5128-07.2008

Ohashi, K., Hosoya, T., Takahashi, K., Hing, H., Mizuno, K., 2000. A Drosophila homolog of LIM-kinase phosphorylates cofilin and induces actin cytoskeletal reorganization. Biochem. Biophys. Res. Commun. 276, 1178–85. doi:10.1006/bbrc.2000.3599

Prokopenko, S.N., He, Y., Lu, Y., Bellen, H.J., 2000. Mutations Affecting the Development of the Peripheral Nervous System in Drosophila: A Molecular Screen for Novel Proteins. Genetics 156, 1691–1715.

Stone, M.C., Albertson, R.M., Chen, L., Rolls, M.M., 2014. Dendrite injury triggers DLK-independent regeneration. Cell Rep. 6, 247–53. doi:10.1016/j.celrep.2013.12.022

Stone, M.C., Nguyen, M.M., Tao, J., Allender, D.L., Rolls, M.M., 2010. Global up-regulation of microtubule dynamics and polarity reversal during regeneration of an axon from a dendrite. Mol. Biol. Cell 21, 767–77. doi:10.1091/mbc.E09-11-0967

Stone, M.C., Roegiers, F., Rolls, M.M., 2008. Microtubules Have Opposite Orientation in Axons and Dendrites of Drosophila Neurons. Mol. Biol. Cell 19, 4122–4129. doi:10.1091/mbc.E07-10-1079

Su, Y.C., Treisman, J.E., Skolnik, E.Y., 1998. The Drosophila Ste20-related kinase misshapen is

required for embryonic dorsal closure and acts through a JNK MAPK module on an evolutionarily conserved signaling pathway. Genes Dev. 12, 2371–2380. doi:10.1101/gad.12.15.2371

Tavosanis, G., 1997. Essential role for gamma -tubulin in the acentriolar female meiotic spindle of Drosophila. EMBO J. 16, 1809–1819. doi:10.1093/emboj/16.8.1809

Tavosanis, G., Llamazares, S., Goulielmos, G., Gonzalez, C., 1997. Essential role for gamma-tubulin in the acentriolar female meiotic spindle of Drosophila. EMBO J. 16, 1809–1819. doi:10.1093/emboj/16.8.1809

Upadhyay, A., Pisupati, A., Jegla, T., Crook, M., Mickolajczyk, K.J., Shorey, M., Rohan, L.E., Billings, K.A., Rolls, M.M., Hancock, W.O., Hanna-Rose, W., 2016. Vitamin B3 / Nicotinamide is an endogenous agonist for a C. elegans TRPV OSM-9/OCR-4 channel. Revised manuscript under review at Nature Communications.

Van Baelen, K., Dode, L., Vanoevelen, J., Callewaert, G., De Smedt, H., Missiaen, L., Parys, J.B., Raeymaekers, L., Wuytack, F., 2004. The Ca2+/Mn2+ pumps in the Golgi apparatus. Biochim. Biophys. Acta 1742, 103–112. doi:10.1016/j.bbamcr.2004.08.018

Wang, L., Janes, K.A., 2013. Stochastic profiling of transcriptional regulatory heterogeneities in tissues, tumors and cultured cells. Nat. Protoc. 8, 282–301. doi:10.1038/nprot.2012.158

Wang, Z., Jin, Y., 2011. Genetic dissection of axon regeneration. Curr. Opin. Neurobiol. doi:10.1016/j.conb.2010.08.010

Wennerberg, K., Rossman, K.L., Der, C.J., 2005. The Ras superfamily at a glance. J. Cell Sci. 118, 843–846.

Wong, J.J.L., Li, S., Lim, E.K.H., Wang, Y., Wang, C., Zhang, H., Kirilly, D., Wu, C., Liou, Y.C., Wang, H., Yu, F., 2013. A Cullin1-Based SCF E3 Ubiquitin Ligase Targets the InR/PI3K/TOR Pathway to Regulate Neuronal Pruning. PLoS Biol. 11. doi:10.1371/journal.pbio.1001657

Wu, V.M., Yu, M.H., Paik, R., Banerjee, S., Liang, Z., Paul, S.M., Bhat, M.A., Beitel, G.J., 2007. Drosophila Varicose, a member of a new subgroup of basolateral MAGUKs, is required for septate junctions and tracheal morphogenesis. Development 134, 999–1009. doi:10.1242/dev.02785

Xiong, X., Wang, X., Ewanek, R., Bhat, P., Diantonio, A., Collins, C.A., 2010. Protein turnover of the Wallenda/DLK kinase regulates a retrograde response to axonal injury. J. Cell Biol. 191, 211–23. doi:10.1083/jcb.201006039

Zeng, L.-H., Xu, L., Rensing, N.R., Sinatra, P.M., Rothman, S.M., Wong, M., 2007. Kainate seizures cause acute dendritic injury and actin depolymerization in vivo. J. Neurosci. 27, 11604–13. doi:10.1523/JNEUROSCI.0983-07.2007

Ziegenfuss, J.S., Biswas, R., Avery, M.A., Hong, K., Sheehan, A.E., Yeung, Y.-G., Stanley, E.R., Freeman, M.R., 2008. Draper-dependent glial phagocytic activity is mediated by Src and Syk family kinase signalling. Nature 453, 935–9. doi:10.1038/nature06901

Academic Vita

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Education

Bachelor of Science in Biochemistry and Molecular Biology *May 2016*
Eberly College of Science/Schreyer Honors College
The Pennsylvania State University, State College, PA

Schreyer Honors Thesis: Dendrite Regeneration Mechanisms after Damage
Thesis Supervisor: Melissa Rolls

Research Experience

Undergraduate Researcher *January 2015-May 2016*

Melissa Rolls, Associate Professor of Biochemistry and Molecular Biology
The Pennsylvania State University

Conducting an RNAi screen to determine proteins involved in dendrite regeneration of damaged neurons in *Drosophila*. Tested more than 30 different proteins to examine their role in dendrite regeneration after damage. Developing a new technique to induce damage to neurons by cloning two *C. elegans* genes into *Drosophila* that cause cell damage when exposed to NAM. Testing to see if glial cells play a role in the dendrite regeneration mechanism. Utilizing a transcriptome-wide approach to observe increases in particular transcripts in damaged neurons.

NSF REU Student *June 2014-August 2014*

Paul Masters, Research Scientist, Division of Infectious Diseases
Wadsworth Center, New York State Department of Health

Researched molecular interactions of coronavirus replication through classical temperature-sensitive replicase mutants of Mouse Hepatitis Virus (MHV). Found the mutation responsible for temperature-sensitivity in a MHV replicase mutant through revertant analysis. Communicated research findings in an oral presentation at the end of the REU in front of mentors and undergraduate students.

Work Experience

Pharmacy Technician, Weis Pharmacy, Hamburg, PA *August 2011-April 2014*

Identified brand-name and generic medications and their purposes to answer customer questions; filled new prescriptions and prescription refills. Stocked and organized pharmaceuticals and dispensing vials; inventoried controlled substances and narcotics.

Physiology Supplemental Instructor *August 2013-April 2014*

Penn State Berks, Reading, PA

Led peer-assisted study sessions for students enrolled in upper-level and introductory physiology courses. Reviewed exams, took attendance, checked homework, assisted students in class and prepared laboratories.

Outreach, Leadership and Teaching Experience

Writing and Communicating Health in South Africa *Spring 2014*
Course offered at Penn State Berks (CAS 297H)
Johannesburg, South Africa

Examined through experience the ways that health issues influence the people and culture of South Africa. Volunteered at Nkosi's Haven, an NGO that provides care and support for mothers infected with HIV/AIDS, her children, or AIDS orphans.

Peer Mentor *Fall 2013*
Penn State Berks, Reading, PA

Assisted an instructor with two first-year seminars linked to a calculus course. Led class discussions, assisted students with calculus concepts. Assimilated new students to college life. Took attendance and managed grades.

PSU Berks of the American Chemical Society *Fall 2012-Spring 2014*
Penn State Berks, Reading, PA

Performed interactive chemistry demonstrations in the surrounding Reading community to get children and teenagers interested in chemistry. Assisted with coordinating the first annual *You be the Chemist* challenge, to encourage middle-school age children in surrounding areas to take an interest in chemistry.

Poster Presentations

Penn State Undergraduate Research Exhibition *April 2015*
State College, PA
Terry Hafer, Alex Grigas, Kristin Kern, Bhavik Modasia, and Dr. Melissa Rolls. *Dendrite Regeneration Mechanisms in Drosophila*,

Council on Undergraduate Research (CUR) REU Symposium *October 2014*
Arlington, Virginia
Terry Hafer and Dr. Paul Masters. *Mapping of a Classical Temperature-Sensitive Replicase Mutant of Mouse Hepatitis Virus*.

Oral Presentation

Wadsworth Center REU Student Symposium *August 2014*
Albany, New York

Terry Hafer and Dr. Paul Masters. *Mapping of a Classical Temperature-Sensitive Replicase Mutant of Mouse Hepatitis Virus.*

Scholarships, Awards, and Grants

Charles R. Gerth Scholarship in Biochemistry	*Fall 2015*
Lewis and Opal D. Gugliemelli Scholarship in Science	*Fall 2015*
Bayard D. Kunkle Scholarship	*Fall 2015*
Erickson Discovery Grant	*Summer 2015*
Gateway Scholars Program Scholarship, Schreyer Honors College	*Fall 2014*
The President Sparks Award	*Spring 2014*
President's Freshman Award	*Spring 2013*