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DEPARTMENT OF BIOCHEMISTRY AND MOLECULAR BIOLOGY

CHARACTERIZATION OF THE CELLULAR RESPONSE TO SIMULTANEOUS AXON AND DENDRITE INJURY IN DROSOPHILA SENSORY NEURONS

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ABSTRACT

Neurons are post-mitotic nerve cells that comprise both the central and peripheral nervous systems. They receive information via dendritic processes, and send information to neighboring cells by propagating signals down the axon. Without axons and dendrites, neurons are unable to function properly, though the maintenance of neuronal health and functionality is positively correlated with the health of the host. Neurons are incapable of being replaced in the event of cell death, so their ability to respond to injury is critical. Damage to both neuronal processes is likely to occur in response to stroke and traumatic brain injury. Although neurons have been shown to survive injury by regenerating damaged processes, simultaneous injury of both the axon and dendrite has rarely been performed or studied experimentally. Since neuronal survival is critical for humans, and simultaneous axon and dendrite damage is likely to occur in response to certain neurological diseases, it is crucial to understand the cellular mechanisms responsible for facilitating either the death or survival of these nerve cells. We aim to characterize the neuronal response to simultaneous axon and dendrite injury, and to determine whether neurons are capable of regeneration following such severe damage. It has previously been shown that axons regenerate via activation of a conserved kinase cascade known as the Dual Leucine Zipper Kinase (DLK) pathway. Though dendrite regeneration has been shown to be rapid and robust, the mechanism by which dendrites regenerate is not yet known. We also aim to understand whether these pathways for regeneration are mutually exclusive when the need for both arises simultaneously. Our data indicate that neurons are capable of surviving complete ablation of their axons and dendrites and are, remarkably, able to regenerate both axons and dendrites in response to this severe injury. Investigating the mechanistic machinery that facilitates regenerative responses to various types of injury will be fundamental in aiding the reparation of damaged mammalian neurons. The ability of neurons to regenerate after simultaneous axon and dendrite injury has not been demonstrated previously, so this finding has incredible potential to eventually help patients of stroke and traumatic brain injury.

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Introduction

Neuron Structure and Function

Neurons are the basic structural and functional units that provide the blueprint for the nervous system (Shepherd, 2009). They are postmitotic cells that do not proliferate once they have been differentiated from the cells that preceded them. Also known as nerve cells, neurons comprise a large circuitry, containing information that either the vertebrate or invertebrate host requires to function normally. Although there are several classifications of neurons, all nerve cells are characterized by distinct structural components: the soma (cell body), axon, dendrites, and synaptic terminals (Figure 1). Each neuronal component is assigned a unique role that ultimately allows neurons to transmit and receive information to and from neighboring cells, respectively. A neuron's ability to transport information via the conduction of electrical impulses is the characteristic of neurons that allows them to be classified as specialized cells.

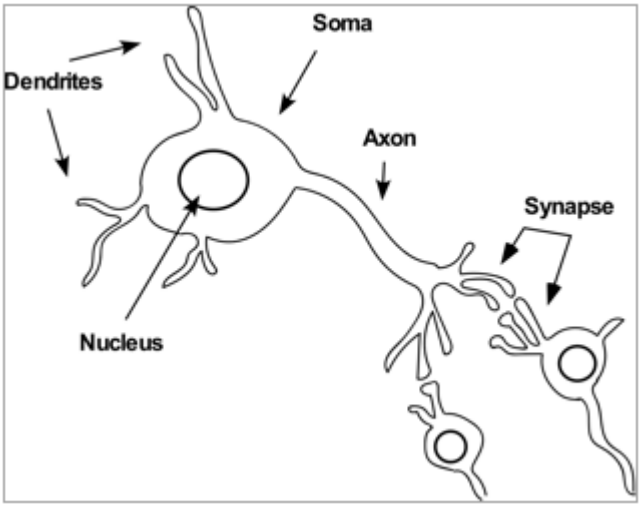

Figure 1. Neuron structure
The anatomy of a neuron, and the means by which it synapses with other neurons is depicted here. Cellular components such as the axon, dendrites, and synapse are unique to neurons.

The central element of each neuron is the cell body; it is spherical in shape and contains the nucleus—arguably the most important organelle in the cell. DNA is housed inside the nucleus and is responsible for regulating protein production and cargo transport throughout the neuron. Also attached to the soma are other critical processes that facilitate the cell's communicative abilities—the dendrites and the axon (Lodish et al., 2000). Dendrites are highly branched processes that are often easily identified by

their tree-like arborization patterns; they are responsible for receiving input from neighboring cells (W.B. Grueber, 2013). Axons, alternatively, are located between the soma and the synaptic terminals. They are long extensions of the nerve cell whose primary function is to transmit information by conducting an electrical impulse known as an action potential (Lodish et al., 2000). Action potentials propagate signals by altering the membrane potential of the cell. The resting membrane potential of a neuron is around -70mV, meaning that the inside of cell is more negatively charged than the outside. Action potentials are triggered by depolarization events, when the voltage of the cell membrane becomes less negative due to an influx of sodium (Na^+) ions ("Neurons & Synapses - Memory & the Brain - The Human Memory," n.d.). The conduction of action potentials is a function that can only be performed by axons, and it is a critical process for both the transmission of information as well as the initiation of other cellular responses.

Action potentials trigger the onset of a chemical process called neurotransmission, which occurs at the synapse, a small space between the presynaptic terminals at the end of the axon and the postsynaptic terminals at the tips of the receiving dendrites (Figure 1) (Kandel, Schwartz, & Jessell, 2000). Arrival of the action potential at the presynaptic terminals activates the release of chemicals known as neurotransmitters, which diffuse across the synaptic cleft and are received by specific receptors on the postsynaptic terminals. Synaptic transmission facilitates the transfer of information from cell to cell, and is thus critical for the unique ability of neurons to communicate with one another (Kandel, Schwartz, & Jessell, 2000).

Microtubule Polarity

Cell morphology often provides sufficient information to discern the identity of neuronal processes; dendrites are short and tend to be highly branched, while axons are thicker in diameter and extend further away from the cell body. The most effective means for distinguishing the type of neuronal process, however, is by assessing the microtubule polarity.

Microtubules are dynamic structural components of the cell that are constantly polymerizing and depolymerizing; they provide support for distinct cellular functions such as neural morphogenesis and organelle transport (Avila, 1992). The polarity of these cellular constituents has been characterized with the plus end oriented away from the cell body in the axon (plus-end-out) (Figure 2),and mixed orientation in dendritic processes. The polarity of the microtubules is relatively well conserved in *C.elegans*, *Drosophila*, and mice. However, in *Drosophila*, dendrites are classified by the prominence of minus-end-out microtubules (Figure 2), as opposed to the mixed polarity observed in other organisms (Stone et al., 2010). Microtubules typically grow from their plus ends via the addition of tubulin (Cooper, 2000). Additional explanations for the distinct polarity phenotypes are based on the functions they serve in either neurite, namely the presence of Golgi and ribosomal components are found in dendrites but not axons (Baas et al., 1988). Dendrites and axons have specified roles, and maintaining the correct microtubule polarity is necessary for performing their respective functions.

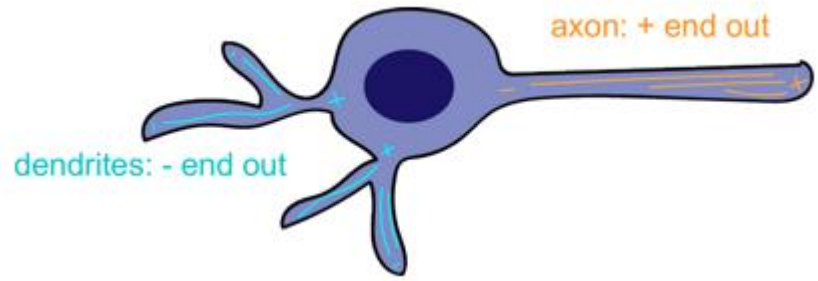

Figure 2. Microtubule polarity
Neurons have plus-end-out microtubule polarity in axons, and minus-end-out microtubule polarity in dendrites. Dendrites tend to display mixed polarity in most organisms, but are predominantly minus-end-out in Drosophila.

Drosophila dendritic arborization (da) neurons

Drosophila serve as useful model organisms for the study of neuroscience because neurons in their abdominal peripheral nervous system (PNS) line the epidermis of the animal and can be visualized during the larval stage using microscopy. Neurons in the periphery of *Drosophila* are classified as dendritic arborization (da) neurons, and are grouped into subtypes based on the complexity of their arborization. Complexity of each class, I through IV, increases in correspondence with the ascending number. We primarily study Class I and Class IV neurons. Class I neurons (ddaE and ddaD) have the

most simple arborization and are easily characterized by their 'comb-like' dendrites that project at 90° angles from the primary dendrite, resembling a hair comb. Class IV neurons (ddaC) are morphologically distinct from the Class I neurons; they typically have dendrites protruding in all directions from the cell body, and tile the entire body wall with extensive branching phenotypes (Wesley B Grueber, Jan, & Jan, 2002).

Although *Drosophila* are invertebrate organisms, much of the molecular pathways that comprise the neural networks are highly conserved in vertebrates as well (Jeibmann & Paulus, 2009). This substantiates the significance of using *Drosophila* as a model organism for understanding brain injury and disease as it applies to humans. Classification of neuronal injury response in *Drosophila* may offer modalities for molecular therapies in the future.

Neuronal response to injury

Maintenance of healthy neuronal processes is critical for the survival and functionality of the cell. As postmitotic cells that do not continuously divide, like epidermal cells for example, the ability of a neuron to repair itself in the event of damage is crucial. Fortunately, neurons have developed relatively robust regeneration mechanisms to mitigate the consequences imposed by injury.

Axon regeneration is rather well characterized. Following injury, axons are capable of regenerating via activation of a conserved kinase cascade known as the DLK pathway (Hammarlund et al., 2009). In *Drosophila*, the way in which axons regrow is dependent on where the injury occurs with respect to the soma. Axons that are injured distally (greater than 50 microns from the cell body), will regenerate by growing through the existing stump that remains. If the cell sustains complete ablation of the axon, or if the axon is injured proximal to the soma, the neuron will repurpose an existing dendrite to become an axon (Figure 3). During the respecification process, the polarity of the dendrite that will be converted to an axon is switched from minus-end-out to plus-end-out (Stone et al., 2010). Alternatively, the mechanism by which dendrites undergo regeneration is not known. In comparison to axon

regeneration, dendrite regeneration is significantly less studied, and consequently is not as well-characterized. It has, however, been shown that dendrites are capable of regeneration (Stone et al., 2014). Class I dendrites will regrow to recover branchpoint number within a 96 hr period following injury, though their morphology will not match the original uninjured cell morphology (Figure 3).

Class IV dendrites also regenerate successfully following injury, though their regeneration pattern is distinct from Class I neurons; Class IV dendrites will regrow to cover their initial domain over a 96 hr period, as opposed to branchpoint recovery. Although the pathway for dendrite regeneration is not yet defined, it is known to require a separate pathway than is necessary for axon regeneration (DLK pathway) (Stone et al., 2014).

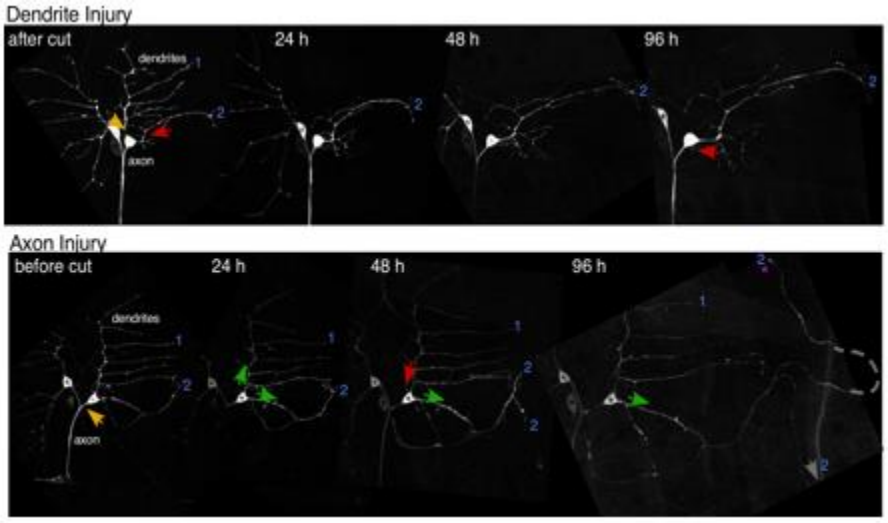

Figure 3. Characteristic dendrite and axon regeneration in sensory neurons
Dendrite injury is shown in sensory neurons, and the pattern for regeneration is demonstrated where branchpoint number is recovered over a 96 hour period. Axon injury and regeneration is also shown, where proximal axon injury causes an existing dendrite to be respecified as the new axon.

Axon and dendrite injury may result from traumatic brain injury (TBI), seizure, stroke, or excitoxicity (Zeng et al., 2007); (Gao & Chen, 2011). Understanding the molecular pathways that govern the cellular responses to these injuries is essential for characterization of the damage that occurs, but that may be undetectable with current diagnostic imaging. Defining molecular mechanisms *in vivo* that allow cells to successfully regenerate in their native environment may have future implications for molecular-based drug therapies targeting neurological disease. The neuronal response to simultaneous axon and dendrite injury has not been characterized. Studying concurrent injury may elucidate unknown characteristics of dendrite regeneration, and provide insight concerning the interaction between the axon and dendrite regeneration pathways.

Microtubule upregulation after axon injury

Since the polarity of microtubules in neuronal processes is used to definitively distinguish axons from dendrites, understanding the orientation of microtubules following injury provides information that details the cellular response. We know from prior studies that both axons and dendrites are capable of regeneration after injury, though the mechanisms that govern the regenerative potential are distinct. Quantifications of microtubule dynamics (presence and activity level), have demonstrated a 10-fold increase following axon injury, but not dendrite injury. It has been shown that axon injury activates DLK, which is upstream of c-Jun N-terminal kinase (JNK). JNK activity has been deemed the source of microtubule upregulation following axon injury, and is a necessary step in successful axon regeneration (Figure 4). Microtubule upregulation can be seen as quickly as five minutes after injury in axons, and is often seen to create a 'whirlpool effect' by 24 hours after injury, where microtubules move in a unidirectional circle around the nucleus, resembling a whirlpool. This upregulated activity is not resolved until 48 hours following injury, during which time a 'neuroprotective effect' prevents dendrite degeneration (Chen et al., 2012). The microtubule activity is definitively downregulated by 72 hours after injury, at which point the neuron has selected an existing process, and begun to alter its polarity, for generation of a replacement axon (Stone et al., 2010). The increased microtubule activity that succeeds axon injury is not observed following dendrite injury, and has thus been defined as a characteristic of the regenerative response to axon damage. The polarity of microtubules in a neuron provides substantial information about how the cell is responding to inflicted injury, what type of neuronal processes are being generated, and how existing microtubule orientation is shuffled to complete the injury-imposed tasks.

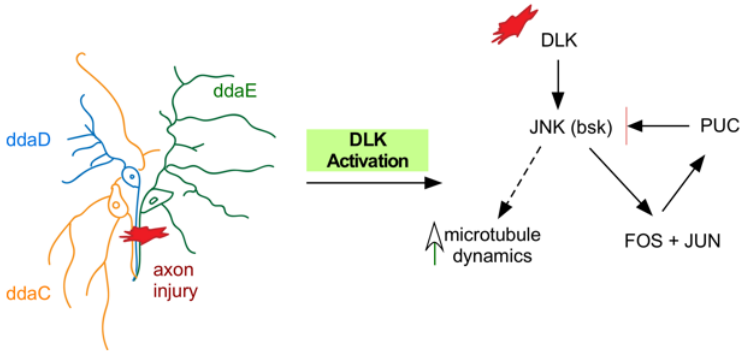

Figure 4. DLK pathway
Following axon injury, the DLK pathway is initiated to begin axon regeneration. JNK is necessary for the successful progression of axon regeneration, and is responsible for the upregulated microtubule activity observed after axon injury.

The present study: simultaneous axon and dendrite injury

Although axon and dendrite injury have historically been studied independently of one another, the response to concurrent injury of these processes is not well studied or understood. We aim to characterize the neuronal response to simultaneous axon and dendrite injury, and primarily address whether the cell is capable of surviving following concurrent axon and dendrite injury. If the cell is able to survive concurrent injury, we hypothesize that regeneration will occur in response to injury of both the axon and dendrites. If regeneration is observed, we will address the question of whether delayed activation of either regeneration pathway (for axons or dendrites) is inhibitory to the other once regeneration has already begun. Since it has been shown that the pathways for axon and dendrite regeneration are distinct, we expect to observe that they are capable of operating in parallel. We hope that elucidating the neuronal response to dual axon and dendrite injury will allow us to assess how the mechanisms for regeneration interact with one another, and potentially offer more information about the distinct regeneration processes.

Methodology

RNA interference (RNAi)

RNA interference (RNAi) is a commonly used tool in fly genetics to regulate, or suppress, gene expression. RNAi exploits an endogenous cellular pathway, where an enzyme called Dicer cleaves double stranded RNA (dsRNA) into short double-stranded segments ranging from 21-25 nucleotides in length. The central dogma of biology details the transcription of genetic material (DNA) into RNA, which is then transcribed into protein. Thus, interference with RNA can effect protein creation and gene expression. RNAi exists to protect cells from translating RNA into proteins that may be harmful to the host. Synthetic induction of double stranded RNA can cause the cell to incite the RNAi pathway, and degrade the genetic material to significantly downregulate expression of a targeted gene or protein. Our lab commonly uses knockdown of reticulon 2 (rtln2) as a control, because we have found no significant phenotypes when rtln2 is suppressed.

Binary expression: Gal4/UAS system

Drosophila melanogaster is an extremely useful model organism primarily for the ease at which the genetics may be manipulated. A gene-targeting system for use in *Drosophila* was developed in 1993 by Brand and Perrimon, and has since been a geneticist's most practical tool. As a binary system that takes advantage of a yeast transcription factor, GAL4, and a specific site to which GAL4 binds directly--Upstream Activating Sequence (UAS)—it has been termed the GAL4-UAS system. This system is particularly beneficial for studying the role of particular genes in development. There are two components that comprise the system, which are contributed from distinct fly lines and allow for the controlled expression or suppression of targeted genes in specific cell types or tissues (Brand & Perrimon, 1993). In the fly lines crossed to make the progeny of interest, one parent deemed the 'driver' must contain a specific promoter to drive the expression of GAL4 in either a subset of cells or tissue type. The other

parent is referred to as the 'responder' and contains the Upstream Activating Sequence along with the gene of interest or hairpin RNA to facilitate suppression of a targeted gene via RNA interference (RNAi) (Figure 5). UAS conveniently received its name by virtue of its location that precedes the targeted gene. When the driver and responder are crossed, GAL4 is directed to drive the expression of the specific gene by binding to UAS; the gene will only be expressed where GAL4 is promoted, which facilitates the regulated gene expression (Brand & Perrimon, 1993).

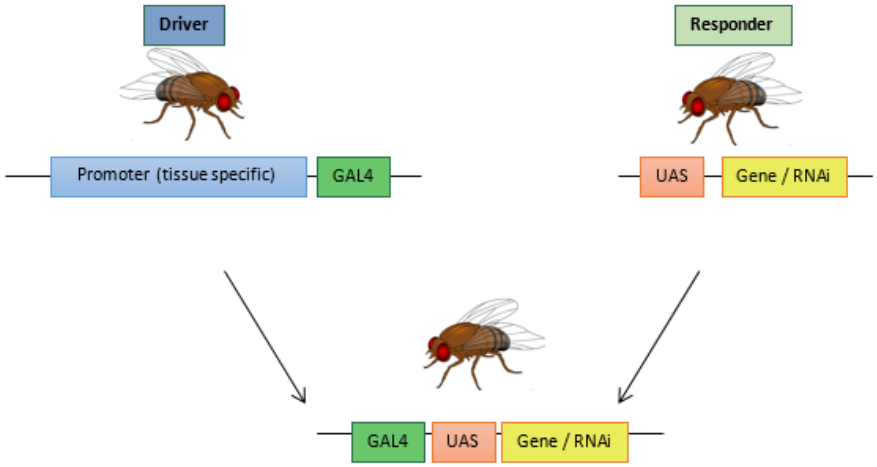

Figure 5. Gal4/UAS system
We are able to drive the expression or knockdown genes of interest by mating two flies, one which is denoted as the driver, and the other, the responder. The progeny will allow us to drive or suppress gene expression in a tissue-specific, or cell-dependent manner. Fly animation adapted from ("Why Mind the Graph? – Mind the Graph," n.d.).

Drosophila life cycle and storage

One of the many benefits of working with *Drosophila* is the ability to yield results quickly. This is a function of the rapid life cycle that is characteristic of *Drosophila Melanogaster.* When kept at a constant temperature of 25°C, *Drosophila* larvae undergo a four stage process to become an adult fly: a process that cumulatively takes five to six days. The first stage in the life cycle requires fertilization of the embryo, which yields an egg within 24 hours. Following this 24 hour period, the larval stages begin. The larvae will go through three molting stages, as it grows over a four-day period, classified as first-instar, second-instar, and third-instar respectively. When the larval stages have been completed, the larvae will

develop a chitin-based case, and is entitled pupae during this phase. Finally, on day five or six at 25°C, they become adults (Jennings, 2011).

All flies are stored in a 25°C constant temperature room. They are kept in plastic bottles with solidified fly food at the base, and enclosed with a cotton plug. The fly food contains water, yeast, soy flour, yellow cornmeal, Agar, light corn syrup, and propionic acid.

Live imaging and laser dissection confocal microscopy

The advantage of working with *Drosophila* as a model organism, in addition to their rapid life cycle and genetic manipulability, is the transparency of their cuticle during the larval stage. The translucent epidermal layer allows us to visualize neurons *in vivo*. During the third-instar larval stage, we mount *Drosophila* on a microscope slide. The larvae is gently selected with forceps from the food cap in which it has developed. It is then rinsed in either phosphate buffered saline (PBS) or water to release any food particles from the larvae. The animal is then gently mounted and compressed between a dried 3% agarose pad (on the slide) and a cover slip that is stabilized by tape. With the dorsal side facing upward, the peripheral neurons are directly accessible using a confocal microscope (Figure 6). The Zeiss LSM 800 with Airyscan and Zeiss LSM Inverted 700 microscopes equipped with Zen Software were used for cellular visualization. A Zeiss widefield microscope was used for video acquisition. Using fluorescent proteins to highlight specific subcellular components or cell types promotes the visibility of the regions of interest. Green Fluorescent protein (GFP) and Red Fluorescent protein (RFP) were used in the experiments detailed below. Fluorescence is regulated by the promoter in each fly line, and is driven to express in the areas we wish to visualize.

Neuronal damage is inflicted to specific processes using a pulsed UV laser. Once the neuron has been successfully injured, the larvae is recovered from the slide, and placed in a hydrated food bit, or a small portion of fly food hydrated with PBS. Each individual animal is recovered and stored in its own

food bit, to be saved for later imaging; larvae can be imaged up to 96 hours following the induction of initial injury, deemed the 0hr time point.

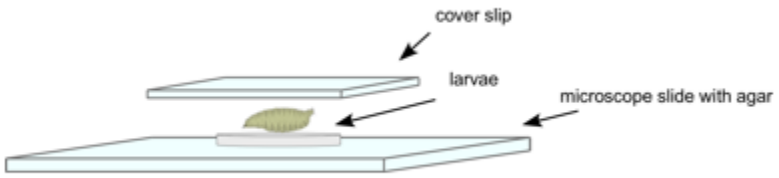

Figure 6. Live image preparation
To visualize neurons in vivo, a 3-day old larvae compressed between a microscope slide and cover slip, with the dorsal side of the animal compressed against the coverslip

Metrics for evaluating regeneration

The data collected, and inferences made regarding regeneration therein, are largely contingent upon image acquisition and analysis. Fiji imaging software was used to process videos and pictures acquired on the confocal and widefield microscopes. Using the segmented line tool in Fiji to trace the length of processes at 0 hr in the uninjured cell, and at distinct time points following the injury (i.e. 24hr, 48hr, 72hr), allowed us to assess whether growth of neuronal processes had occurred after injury. Subtracting the initial length (Lf-Li) from the final length, we calculated the change in length of extension in micrometers (μm) over a given period of time. We additionally used branchpoint number as an indication of dendrite regeneration, specifically. It has previously been shown that dendrites will either regrow to cover their entire initial domain (ddaC), or will restore the number of branchpoints they had prior to injury (Stone et al., 2014). This makes branchpoint number a strong metric for identifying the presence or absence of dendrite regeneration. Both branchpoint number and length of extension were derived from still images of the neuron.

Videos allowed for the tracking of microtubules, and thus microtubule polarity. Using EB1-GFP, that has been shown to bind to the growing microtubule plus ends. We could assess the polarity of neurites after injury, by generating a kymograph, where time is plotted against distance from the cell

body. This metric has dual functionality: 1) Determination of the neuronal process 2) Quantification of microtubule activity.

Statistical Analyses

A t-test was used to test for significant variation between the means of two data sets. This statistical test was appropriate for the majority of the experiments detailed below. Statistical significance is indicated by * $p<0.05$, ** $p<0.001$, and *** $p<0.0001$. A two-way analysis of variance (ANOVA) test was also used to test for significance when comparing the microtubule activity following full cut injuries to previously recorded microtubule activity in uninjured cells. The ANOVA allows for an assessment of statistical significance when comparing means from distinct experimental groups. All statistical analyses were run in GraphPad Prism 6 Software.

Fly lines

Table 1: Fly lines used for the listed experiments

Cross	Respective Figure
Dicer2; 221:Gal4, UAS:EB1-GFP x UAS: RTNL2 RNAi	8, 14, 15, 16
221: gal4, UAS:EB1RFPT, UAS:Dicer2 x UAS:RTNL1-GFP	11
UAS:2ace-mNeonGreen x UAS:RTLN2 RNAi	15
221:Gal4 UAS:mcd8-GFP males x UAS:bsk.DN	14, 15
477:Gal4, UAS:EB1-GFP/cyo ; Ecd/Tm6 x UAS.Red Stinger/cyo	7
UAS: Dicer2; PPK: Gal4, UAS: mCD8-GFP x UAS: JIP RNAi (or dRho1 RNAi/ dRas RNAi)	9
UAS:mCD8-mRFP1, UAS:Dicer2; 221:Gal4, Puc::GFP (trap)	10
Dicer2; 221: Gal 4, UAS: EB1-GFP/Tm6 x TD tomato	12, 13, 15

Results

Neurons survive simultaneous axon and dendrite injury

Preliminary work in our lab has demonstrated that dendrite and axon regeneration are both robust in terms of their ability to regenerate following injury, if they are damaged independently (Stone et al., 2014). Though there is significantly less known concerning the process by which dendrites regenerate, unpublished observations suggest that dendrite regeneration is extremely durable. Within our laboratory, it has become an ongoing challenge to find a means for inhibiting dendrite regeneration that may further undermine a mechanistic pathway for regeneration. The impetus of my project-to uncover the potential of neuronal survival following simultaneous axon and dendrite injury-arose from observations which demonstrate the inability of dendrites to regenerate when axon injury is induced at the same time.

To address whether neurons are capable of surviving simultaneous ablation of all dendritic processes as well as the axon**,** we created an injury assay deemed a 'full cut.' The full cut refers to concurrent severance of the axon and all dendrites in a given neuron, respectively responsible for sending and receiving information. Dendrites are severed at the primary branchpoint, while the axon is injured a distance from the soma that is approximately equivalent to the diameter of the cell body itself. We mount, image, and induce injury in larvae that are in their third-instar stage. This leaves a maximum of 96 hr (when flies are maintained at 25°C) following the induction of injury to observe the subsequent cellular response. Previous studies demonstrate that dendrites and axons can regenerate over a 96 hr period following injury if they are damaged independently. Prior observations of 'full cuts' in our laboratory displayed a lack of regeneration within a 72-hour window following injury. With the advent of injury that is inclusive of both axon and dendrite injury, we hypothesize that if the neuron is capable of regenerating after such severe injury, it may require longer than 72 hours to occur, at which point the larvae will normally pupate.

To mitigate the time limitations imposed by the rapid nature of the *Drosophila* life cycle, we employ the use of an Ecdysone mutant. Ecdysone (ECD) is a steroid hormone, which predominantly

controls the larval progression through its life cycle. Using the mutant, and storing larvae at 29°C, we are ideally able to keep the animal in its larval stage for up to one month (Yamanaka, Rewitz, & O'Connor, 2013). This enables prolonged visualization of the neuron following the onset of injury, and should allow us to define the cellular response to full cuts. All full cut assays in the ECD background were performed in class IV (ddaC) neurons, since our lab had initially begun studying dendrite regeneration in this class of neuron.

Although the Ecdysone mutant seemed to be a good solution to circumventing the constraints of larval morphogenesis, and thus visualization of the neuron past the typical allotted time, it proved to be difficult to image the cells after injury. This came as a result of the reduction in cellular fluorescence, along with the likelihood of larval death (Figure 7). Over 100 larvae were screened for this assay, and less than ten could be successfully imaged. Although the image quality was less than ideal at 24 hours following injury, it was apparent that the cell itself could survive based on upregulated microtubule activity observed in the soma that remained. However, it is unclear whether regeneration can occur, since this was seldom seen.

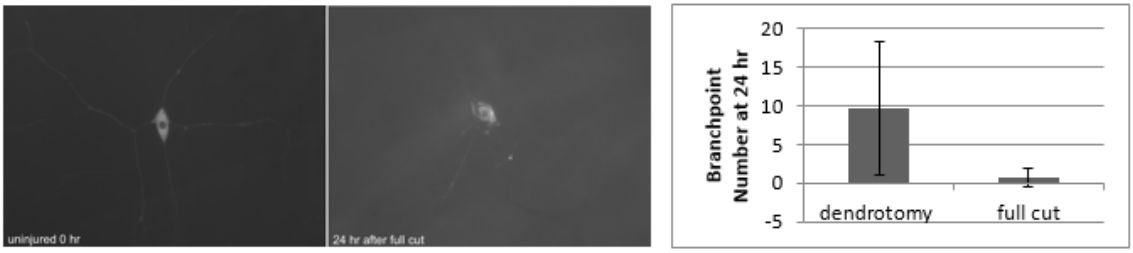

Figure 7. Full cut in ecdysone mutant
The images portray a representation of the cell visibility at 24 hr following full cuts in the ecd mutant. The image displayed to depict the cell at 24 hr following injury, demonstrates the ability to see the soma, but no regeneration otherwise. Within the data set, there were significantly more branchpoints at 24 hrs when only dendrites were injured (dendrotomy) versus when all neuronal processed were severed (full cut).

Branchpoint number has historically been used as a metric to assess dendrite regeneration, so we used this quantification to understand whether regeneration was induced following simultaneous axon and dendrite injury. Comparing branchpoint number 24 hr after injury in neurons that have sustained full cuts to those that have had all dendrites severed (dendrotomy), the results demonstrate a significant reduction in branchpoint number for cells following full cuts (Figure 7).

Knockdown of JIP, dRas, and dRho1 indicate potential for regeneration after full cuts

The ecdysone mutant, though difficult to work with, provided the understanding that the cell survives injury to all neuronal processes. Since the mutant was unhealthy, and the neurons themselves were difficult to image, we an alternate means to study the regenerative potential of a neuron that has sustained a full cut. Without the ecdysone mutant, we no longer have the ability to prolong the larval stage of the animal, and are once again constrained to imaging a larvae within a maximum of 96 hours after the induction of injury. Using the integrative multi-species prediction tool provided by Princeton, we identified JIP, Ras, and Rho as a protein and genes (respectively) that facilitate the development and morphogenesis of neurons. To determine if JIP, dRas, and dRho, which have been implicated in neuron morphogenesis, play a role in (what seems to be) the inhibition of regeneration after full cuts, we performed full cut injury assays using RNA interference to knockdown their expression.

All images were taken 24 hours after the onset of injury. In each experimental knockdown, regeneration was observed. The morphology of the newly regenerated processes at 24 hours after injury was not observed in the control RNAi (RTNL2) (Figure 8).

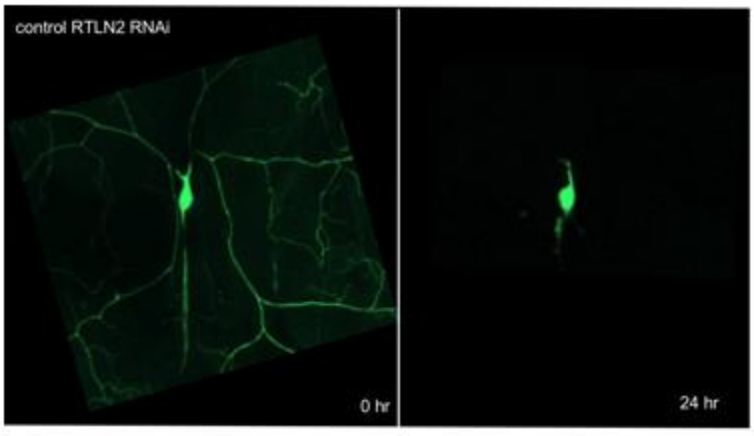

Figure 8. Control full cuts show no regeneration
This image is representative of the initial observations made 24 hours after full cuts using the control RTLN2 RNAi. No regeneration is observed here.

In our effort to comprehend the results, it became apparent that the promoter we used, pickpocket (PPK), to drive expression of the RNAi in question is inhibited by activation of the DLK pathway. This suggests that performing full cuts in these lines would shut off the promoter, and reduce the efficacy of

the RNAi. However, the images acquired at 24 hours, regardless of RNAi efficacy, demonstrate regeneration after full cuts (Figure 9). The quest to understand the cellular response to full cuts, and *now* to address whether regeneration can truly occur, continues.

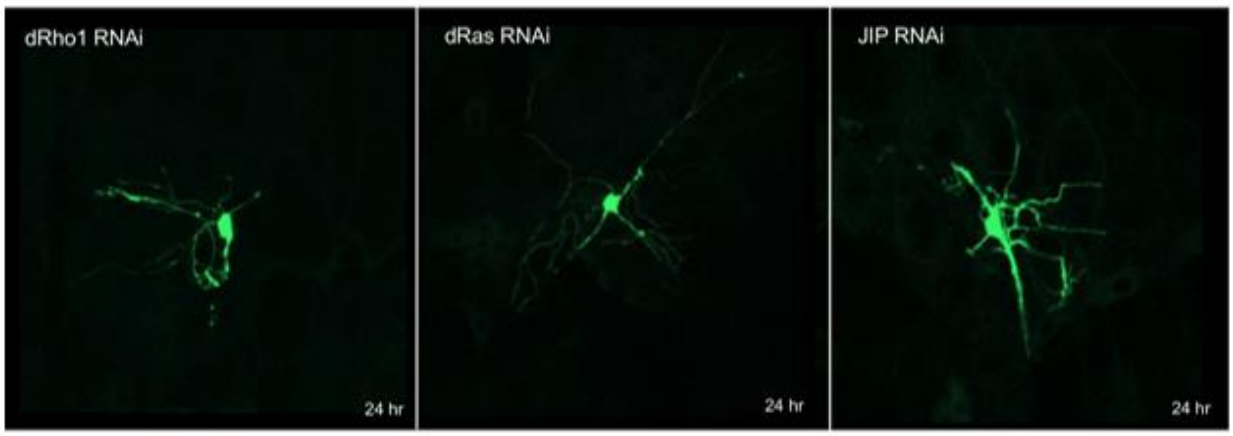

Figure 9. dRho1, dRas, and JIP RNAi demonstrate regeneration potential
dRho1, dRas, and JIP are implicated in neuron morphogenesis and development. In an attempt to suppress their expression, we observe regeneration regardless of the RNAi efficacy.

Axon regeneration (DLK) pathway is activated in response to simultaneous axon and dendrite injury

From the previous two data sets, we maintain that neurons are capable of surviving simultaneous axon and dendrite injury, but that regeneration (though possible in undefined conditions) may be halted as a result. With the understanding that dendrite regeneration is often unable to be repressed, we sought to uncover whether the defined axon regeneration pathway was being activated. If so, we propose that DLK activity may be the source of halted regeneration. To measure the activity of DLK, we use a Puckered (puc) protein trap. Puckered is a MAP (mitogen activated protein) kinase phosphatase that regulates JNK signaling in the DLK pathway. It has previously been shown that using a puc-GFP protein trap indicates the strength of the DLK pathway, and is thus a successful marker for axon regeneration. Puc-GFP has been shown to increase four-fold in nuclear fluorescence following axon injury (Stone, Albertson, Chen, & Rolls, 2014).

We measure the intensity of nuclear fluorescence 24 hours after dendrotomy, axotomy, leave one dendrite, and full cut injury types (Figure 10); the intensity of brightness is proportional to the activity of the DLK pathway (Stone et al., 2014). The dendrotomy serves as a control, since it has been noted that there are low levels of nuclear fluorescence using the puckered reporter after dendrite injury. Alternatively, we can expect to see four times the intensity of brightness after axon injury when compared to the dendrotomy. The results demonstrate that nuclear fluorescence is significantly increased after full cuts and leaving one dendrite as well as axotomy. The intensity of fluorescence observed following both full cuts and leave one dendrite assays are comparable to the brightness observed after axon injury alone. There is minimal nuclear fluorescence 24 hours after dendrite injury.

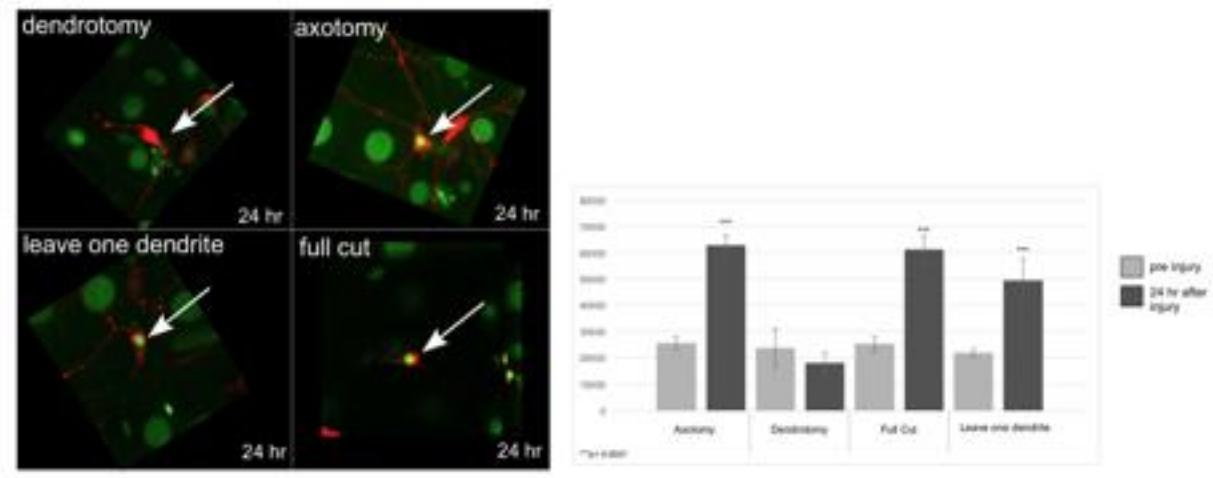

Figure 10. DLK pathway is still active after simultaneous injury
Arrows indicate nuclear fluorescence 24 hours after the specified injury Green nuclei suggest DLK pathway activity, and the brightness is proportional to the activity of the DLK pathway. Brightness is depicted in the graph, which shows a significant increase in axotomy, full cut, and leave one dendrite injuries when compared to the control dendrotomy.

Axon and dendrite regeneration are not mutually inhibitory

Although we have repetitively observed that the neuron is capable of surviving full cuts, it remains to be seen whether regeneration can follow the induction of such high throughput damage. With the observations made thus far, regeneration seems unlikely following simultaneous injury of all neuronal processes in a given cell. This leads us to hypothesize that the DLK pathway and the pathway that

facilitates dendrite regeneration after injury are mutually inhibitory, or are unable to be initiated simultaneously.

The ability of axons and dendrites to successfully regenerate on their own is well defined, but the onset of paired damage seems to halt regeneration given the observations thus far. To test this hypothesis we pose three experiments: 1) Injure the axon and dendrite simultaneously, leaving one dendrite uninjured ('leave one dendrite'); 2) Perform an axotomy at 0 hr, then sever the non-converted dendrite at 48 hr, and image the cell at 72 hr; 3) Perform a dendrotomy at 0 hr, sever the axon at 24 hr, and image the neuron at 48 hr following the initial injury. Since the injury of solely one dendrite is enough to induce the dendrite regeneration process (Stone et al., 2014), and we have previously determined that axon regeneration is initiated with dual axon and dendrite injury, experiment 1 is useful for addressing the proposed hypothesis that the mechanisms for regeneration are mutually inhibitory. Cumulatively, the results of these experiments may demonstrate the ability of regeneration to occur even when both regeneration processes are initiated, and whether the onset of one pathway while the alternate pathway has already begun, will inhibit the progression of regrowth.

Leave one dendrite

In experiment 1, the 'leave one dendrite' condition, regeneration is definitively observed 72 hours after injury. Using axon injury (axotomy) as a control, and measuring the change in the length of the furthest projection from the time of injury (0 hr) to 72 hr, we find that there is a significant increase in outgrowth observed when one dendrite is left uninjured, but both axon and dendrite cellular responses for regeneration have likely been activated. The figure (11) portrays a representative image of the cells observed 72 hours after the induction of the leave one dendrite assay. There is often a lengthy extension, with few branchpoints in comparison to the original, uninjured cell. However, there are also regions of sprouting that appear to have dendritic morphology.

The cross used to perform this experiment is 221:Gal4, UAS:RTNL1-GFP/TM6 x UAS: EB1-RFPT/cyo. Reticulon1 (RTLN1) in *Drosophila* has been shown to bind to an endoplasmic reticulum (ER) protein that facilitates endosomal trafficking, and has been shown to accumulate at the tip of a growing axon (Deshpande & Rodal, 2016; Rao et al., 2016). Increased green fluorescence is indicative of a process that may be characterized as an axon, and is observed at the tip of the lengthy extension 72 hours after injuring both the axon and one dendrite, but leaving one dendrite in tact.

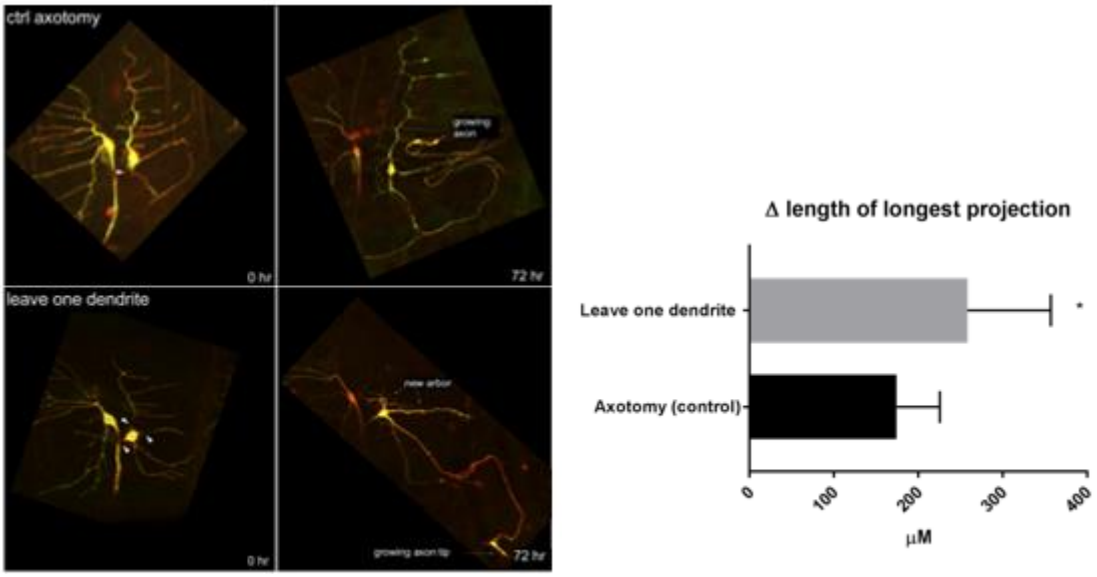

Figure 11. Increased outgrowth in leave one dendrite injury
When the axon and one dendrite were simultaneously injured, but one dendrite was left uninjured, we observed a significant increase in the length of extension at 72 hours. We compared the change in length from 0 to 72 hours. In the control, we subtracted the length of the dendrite at 0 hr that converted to the new axon, from the length of the axon at 72 hr. We similarly calculated the change in length of extension for the leave one dendrite condition.

Staggered Injury

Thus far we have determined that axon regeneration is initiated following simultaneous axon and dendrite injury, ***and*** that regeneration can occur when both mechanistic pathways of regeneration are activated. The question that follows is whether the initiation of one pathway for regeneration is inhibitory to the progression of regeneration that is already occurring. We test whether DLK activation once dendrite regeneration has begun will halt the continued process of regeneration (Figure 12). We also test the converse, beginning with axon injury, and at 48 hr severing the dendrite that has been respecified as

the new axon (Figure 13). Neuroprotection arises in response to axon injury, and the process of dendrite degeneration. Thus, neuroprotection also affects the timing of their regeneration (Chen et al., 2012). To avoid regeneration interference by neuroprotection, we waited 48 hours after axon injury to sever the newly specified axon.

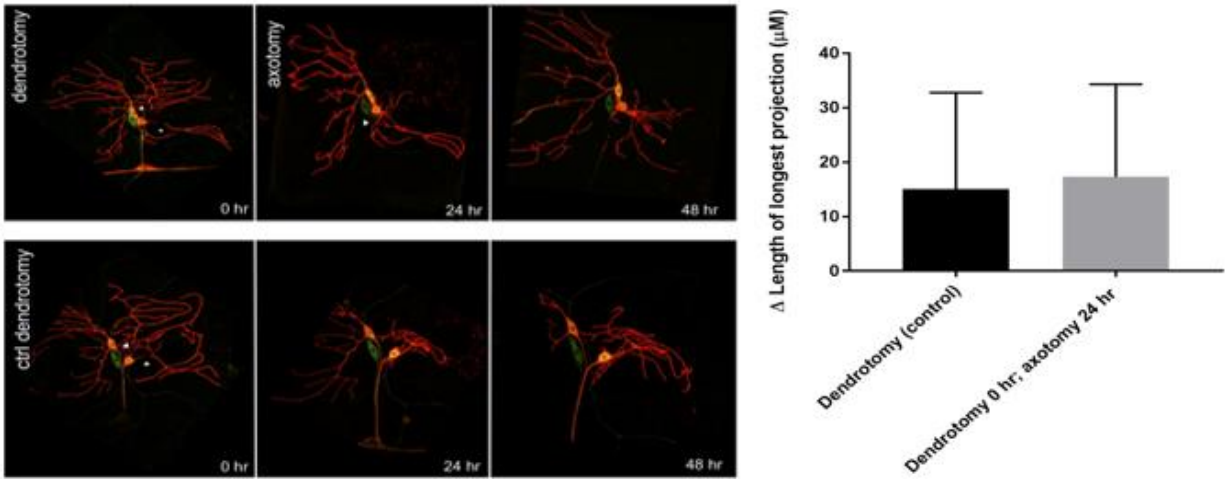

Figure 12. Axon injury does not inhibit dendrite regeneration
This image demonstrates the ability of dendrites to continue regeneration, even when axon injury is induced once dendrite regeneration has already begun. Quantitation of the change in length of projection show no difference between the control and experimental conditions. The white arrows indicate injury, and the cell is shown 24 and 48 hours after injury.

In both sets of staggered injury, regeneration was not inhibited with the activation of the opposing regeneration pathway. The results show no significant difference in the change in projection length when compared to the control. There is, however, a significant increase in branchpoint number from 24 to 48 hours when dendrotomy is performed at 0 hr, and axotomy at 24 hr. The dramatic increase in branchpoint number is not similarly observed in a dendrotomy alone, which serves as the control for this experiment.

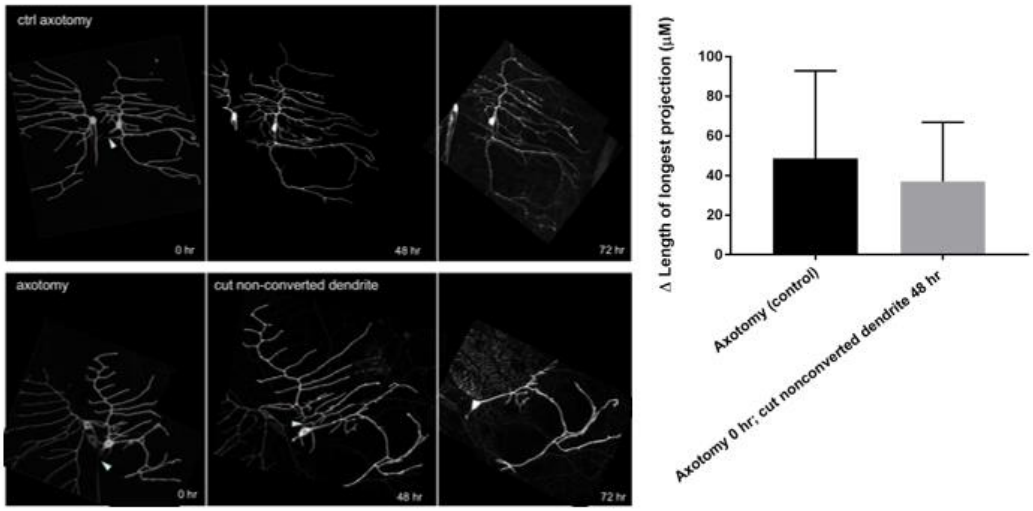

Figure 13. Dendrite injury does not inhibit the progression of axon regeneration
When the non-converted dendrite that remains after axotomy is injured 48 hours after axon injury (once the neuroprotective effect has resolved), there is no significant difference in the progression of regeneration comparing the control axotomy to the staggered injury experiment.

Neurons are capable of regenerating axons and dendrites after full cuts

Once we determined that the pathways for axon and dendrite regeneration are not mutually inhibitory, we proceeded to address why we have yet to truly observe regeneration after full cuts. We hypothesized that the cell may detach from the epithelial layer, losing connection to surrounding cells, and consequently resulting in the ability to regenerate. To test this hypothesis, we employ the use of a JNK dominant negative (JNK.DN) mutant, to mimic inhibition of JNK. Use of the JNK.DN disables the cell from carrying out axon regeneration, but allows us to determine whether detachment contributes to the lack of regeneration we observe. Images acquired 72 hours following full cuts in the JNK.DN display regeneration, and suggest that disconnection from neighboring cells does not inhibit the neuron from regenerating.

As a control for this experiment, we again suppressed the expression of RTLN2 using RNAi. This is commonly used as a control line in our laboratory, because it does not show phenotypes in response to various assays. Interestingly, we now observe regeneration in the majority of control animals that have sustained full cut damage. There were a portion of cells that did not demonstrate regeneration, but of the 13 larvae that were injured, only 2 did not regenerate (Figure 14). This suggests that regeneration occurs in almost 85% of the injured larvae. The morphology of the processes that regenerate is distinct in the JNK.DN compared to the control. In the control, when regeneration does occur, there is a long process that extends far from the cell body, and often there are small areas of dendrite-like arbor. Alternatively, since axon regeneration cannot initiate in the JNK.DN, we observe highly branched morphology after full cut injury. Quantifying the change in branchpoint number from time 0 hr to time 72 hr (after injury), we find a statistically significant different between the control and JNK.DN. In the control, we find a

significant increase in the length of extension for the longest projection from the injured neuron (Figure14).

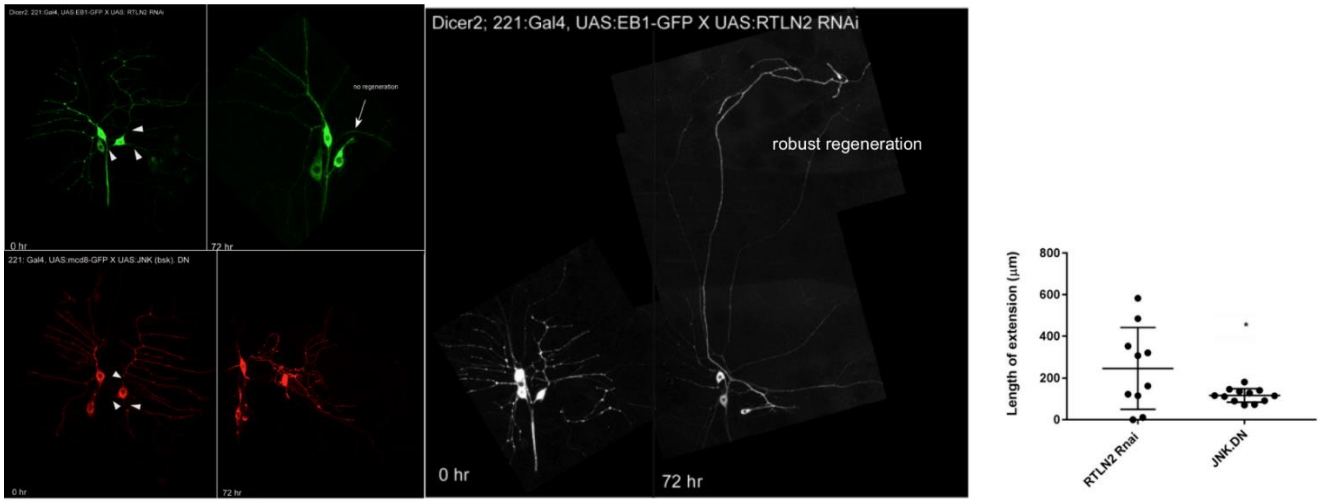

Figure 14. Regeneration occurs after full cuts
RTLN2 RNAi was used to serve as a control for this experiment. Evidently, there is variability in the phenotypes observed 72 hours after injury. We either observe robust regeneration, or none at all. In the JNK.DN, there is evident dendrite regeneration. Sites of injury are indicated by white arrows.

Given the unforeseen results in the control, we performed the same experiment in two other crosses, using other fluorophores that are often used in the lab: 2ace:mNeonGreen and TD tomato. We also observe regeneration 72 hours after injury in these crosses, validating the neurons ability to both survive and regenerate after injury to all its processes (Figure 15).

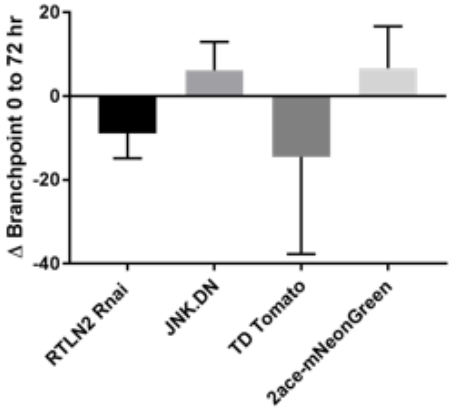

Figure 15. Change in branchpoint number for full cuts
The change in branchpoint number from 0 hours, prior to injury, to 72 hours after injury is demonstrate here. Negative values indicate that the initial branchpoint number was not restored, while positive values indicate an increase in branchpoint number 72 hours after injury. This graph showcases the variability in branchpoint number restoration following full cuts.

To determine whether the cell was capable of regenerating both axons and dendrites, we take videos 72 hours following full cuts, and track the direction of microtubule growth using EB1-GFP. We find that the long, extended process is 96% plus end out on average, which is characteristic of an axon (Baas, Deitch, Black, & Banker, 1988). We also find that the processes which appear to have dendritic morphology, maintain 72.3% plus-end-in polarity on average. Mixed polarity is characteristic of dendrites, although in *Drosophila* nearly 90% of microtubules are observed to be plus-end-in within dendrites (Stone et al., 2010). This data validates the ability of a neuron to regenerate both axons and dendrites in response to simultaneous axon and dendrite severance. Interestingly, we observe upregulated microtubule activity in both the axon and dendrites 72 hours after injury. It has previously been shown that microtubule dynamics are upregulated only in response to axon injury, but that the increased activity subsides by 72 hours (Stone et al., 2010). Here, we observe upregulated activity for an extended period of time, and in response to dendrite injury, which has not previously been seen (Figure 16).

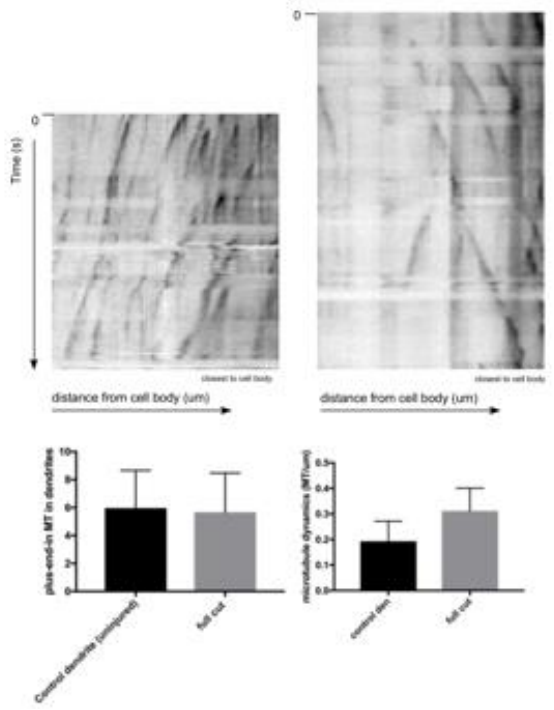

Figure 16. Microtubule polarity in regenerated processes after full cuts
The kymographs demonstrate microtubule movement in the regenerate processes, where time is plotted against microtubule distance from the cell body. These kymographs demonstrate the presence of both axons and dendrites 72 hours after injury. The bar graphs compare collected data to the control microtubule data in uninjured cells (previously collected by Alex Weiner).

Discussion

Neurons are the building blocks that maintain the structure and function of the nervous system. They are distinct from other cells with their ability to communicate by sending and receiving both chemical and electrical signals. New neurons cannot be created to replace those that have died. Thus, the maintenance of healthy neurons is critical to their survival, to the overall functionality of the nervous system, and to the information contained therein.

Although axon and dendrite regeneration have both been characterized in previous studies, simultaneous axon and dendrite injury has not been demonstrated prior to these experiments. We pilot this injury mechanism to understand a, perhaps more realistic, cellular response to injury in both types of neuronal processes. Axon and dendrite injury are likely to occur as a result of traumatic brain injury, stroke, and seizure. Defining the regenerative capabilities in response to such severe injury may ultimately offer molecular-based therapies for individuals suffering from these neurological diseases.

We demonstrate that Class I (ddaE) neurons in *Drosophila* are able to regenerate both axons and dendrites in response to the concurrent ablation of all processes protruding from the soma. Our results lead us to propose three potential explanations for the regenerative phenotype observed 72 hours after the onset of injury. We propose (a) that paired axon and dendrite injury may actually yield a pro-regenerative response, which we maintain from the significantly long axon that regenerates and extends to the regions of neighboring hemi-segments. We also consider the notion that axon regeneration may be prioritized over dendrite regeneration (b). Prioritized axon regeneration can be inferred from the data demonstrating that DLK activity, upregulated microtobule dynamics, and accumulation of RTLN1 occur after full cuts, all of which are characteristic responses to axon injury. Finally, we consider the notion that axon regeneration may appear to take precedence over dendrite regeneration because axons are regenerated first (c) (Figure 17). Perhaps, dendrite regeneration becomes more robust after the 72 hour time point observed, and there is no preference for either regeneration mechanisms.

We have answered our primary question concerning the ability of neurons to survive simultaneous axon and dendrite injury, and have demonstrated that axon and dendrite regeneration can

run in parallel. Most importantly, we have observed and described the cellular response to simultaneous axon and dendrite injury, which has not been previously shown. We demonstrate that neurons are capable of surviving concurrent damage to all neuronal processes and are, more impressively, able to regenerate both axons and dendrites in response. Since neurons cannot be generated anew, it is critical that they maintain robust repair mechanisms to mitigate the risk and occurrence of injury.

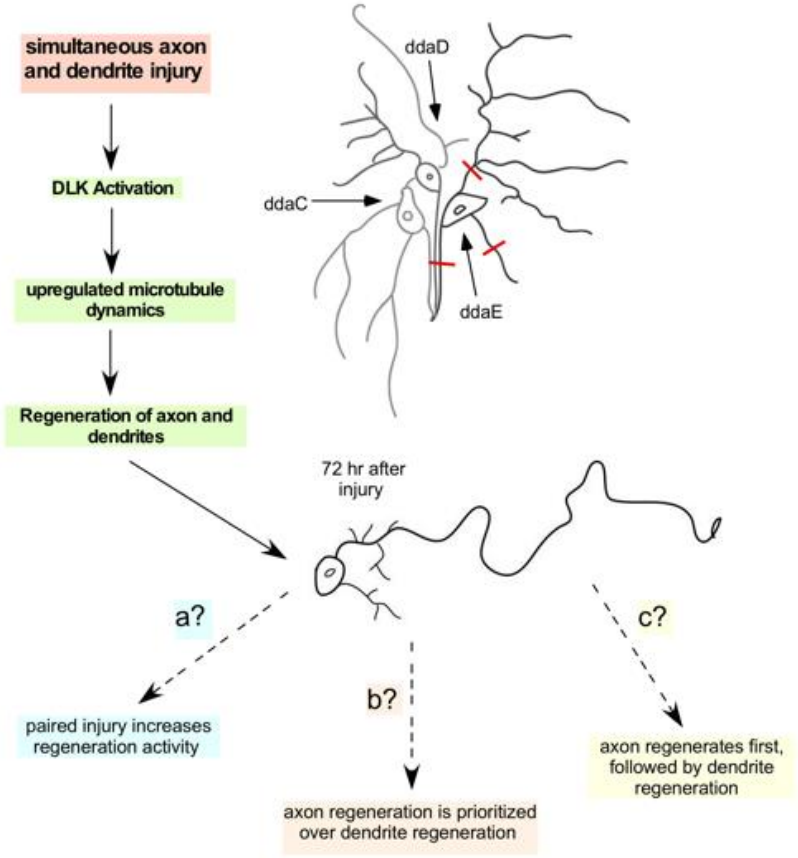

Figure 17. Schematic diagram for regenerative responses to full cuts
This diagram represents potential explanations we consider for the regenerative phenotype we observe after simultaneous axon and dendrite injury at 72 hours. Green boxes indicate processes we have shown to definitively occur after simultaneous injury.

There are over 192,000 deaths annually as a combined result of traumatic brain injury and stroke in the United States alone. Every 40 seconds an individual has a stroke, contributing to the unfathomable statistic that a stroke-related death occurs every four minutes. Traumatic brain injury is the source of 30% of injury-related deaths annually in the United States, and is a frequent occurrence even in young athletes ("Stroke Facts | cdc.gov," n.d.) (CDC, n.d.). The prevalence of these neurological morbidities is high, and

proves to have detrimental effects on the well-being of those who suffer from them. Understanding the neuronal responses that follow TBI and stroke is critical for developing molecular-based therapies in the future. Simultaneous axon and dendrite injury is a realistic simulation of these diseases; characterizing the neuronal response to such severe injury lays a foundation for future studies that may directly lessen the symptoms and death toll of patients subject to TBI and stroke.

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ACADEMIC VITA

JENNA L. MANDEL

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Education

The Pennsylvania State University, University Park, PA *Graduating May 2018*

Schreyer Honors College
Major: Biology - Vertebrate Physiology
Minor: Health Policy and Administration

Healthcare Related Experience

Organized Public Health Brigade to Nicaragua **Spring 2018**

- Recruited volunteers, handled logistics, and will be leading a trip to Nicaragua to install a clean water system for the delivery of potable water to an impoverished, disease-laden community

Medical/Public Health Brigade to Panama *Spring 2017*

- Established medical and dental clinics to increase access to health services in under-resourced communities
- Installed showers, lavatories, and latrines
- Provided hygiene education to facilitate preventive health measures

Pre-Med Program at Abington Hospital-Jefferson Health *Summer 2016*

- Provided with extensive exposure to several clinical departments within the hospital
- Attended weekly seminars covering a broad range of topics pertinent to medicine/healthcare
- Shadowed physicians in the emergency department, OB/GYN, trauma department, and observed surgeries

M.D./Ph.D. Exposure Program at Hershey Medical Center *Summer 2015*

- Selected to participate in an intensive ten-week program
- Gained exposure to life as a physician-scientist
- Studied aspects of infectious disease both in the laboratory and clinic

Medical/Public Health Brigade to Nicaragua *Spring 2015*

- Established medical and dental clinics to increase access to health services in under-resourced communities
- Installed showers, lavatories, latrines, and septic tanks
- Installed concrete floors to replace dirt floors in homes to reduce disease susceptibility

Research and Presentations

Researcher in The Rolls Lab *Fall 2015-Spring 2018*

- The lab utilizes genetics and live imaging in *Drosophila* to define fundamental cell biology associated with neuronal response to stress
- Current project requires the use of a pulsed UV laser to induce concurrent injury to axons and dendrites in an attempt to understand how neurons may regenerate. This injury type is implicated in traumatic brain injury, stroke, and seizure.

Neurons survive and regenerate after injury to both axons and dendrites *Fall 2017*

- Presented at the American Society for Cellular Biology/European Molecular Biology Organization Conference

Examining the Response to Distinct Injury in Class IV Drosophila Neurons *Spring 2016*

- Academic poster presentation at undergraduate scientific session

Investigating Host Nuclear Factors Interacting with Rous Sarcoma Virus Gag Protein *July 2015*

- Academic poster presentation at Hershey Medical Center research symposium

Leadership Experience

Global Brigades *Fall 2015-Spring 2018*

Campus chairperson (fall 2017-present), Large Event Planning (fall 2016-spring 2017), secretary (fall 2015-spring 2016)

- Global Brigades is the largest student-led sustainable development and holistic health organization in the world
- The Penn State chapter is the largest in the country and encompasses disciplines in medical/dental, public health, environmental, water/engineering, business/microfinance, and human rights
- The role of chairperson is to lead/unite the six chapters of the organization on Penn State's Campus under one common executive board
- Attended national campus chairperson conference in Wisconsin
- Attend monthly webinars hosted by national and international Global Brigades Staff
- Recruit volunteers on campus to travel abroad and empower under-resourced communities in Nicaragua, Honduras, Ghana, and Panama to rise out of poverty via the implementation of distinct projects
- Convene and lead three meetings per week

Penn State Lion Scouts Organization *Fall 2014 -Present*

Campus Tour guide

- Lion scouts is an admissions-run organization that represents Penn State to prospective students and their families
- Responsibilities include weekly meetings, a minimum of five tours per semester, and additional external relation hours
- Attend annual Lion Leadership Conference, which fosters development of leadership and networking skills via workshops led by notable individuals in the community

Awards and Honors

Erickson Discovery Grant *Summer 2017*

Phi Eta Sigma National Honors Society *Spring 2016-Current*

National Society of Leadership and Success *Fall 2016-Current*

Dean's List-every semester *Fall 2014-Current*

Extracurricular Activities

THON volunteer

- THON is the largest student-led philanthropy in the world, raising millions annually for pediatric cancer

Volunteer at Centre County PAWS Animal Shelter

MMA, kickboxing, Weight Training