THE PENNSYLVANIA STATE UNIVERSITY
SCHREYER HONORS COLLEGE

DEPARTMENT OF BIOCHEMISTRY AND MOLECULAR BIOLOGY

CALCIUM AND MICROTUBULE ACTIVITY AFTER EXPLOSIVE INJURY IN *DROSOPHILA*

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ABSTRACT

Neurons must last an entire lifetime, despite facing frequent risk of injury and stress from sources such as traumatic brain injury. Laser injury in *Drosophila* has long been used as a model to understand the axon injury response, typically by using a laser to sever neurites[1]. However, I recently observed an alternate type of axon injury, termed explosive injury, which produces an immediate, obviously distinct phenotype. In this thesis, I characterize the cellular response to explosive neuron injury in order to establish a basis for further research concerning downstream effects and regeneration. I compared microtubule activity after explosive and controlled injury in control and bsk dominant negative background and found that explosive injury causes immediate, global changes in microtubule dynamics independent of the JNK signaling pathway. GCaMP fluorescent calcium imaging was used to identify the source, scope, and duration of calcium elevation after injury. I found that explosive injury causes an immediate 4-fold spike in cytosolic calcium levels that lasts up to eight hours. A genetically encoded voltage indicator was used to verify that the calcium spike is connected to neuron depolarization and voltage change after explosive injury. Overexpressing the potassium channel Kir2.1 to suppress depolarization was found to prevent both calcium spike and microtubule activity upregulation. This leads me to propose a possible mediating role for calcium in controlling microtubule response after explosive injury. Overall, this thesis sets a foundation for future work using explosive injury as an alternative model of traumatic nervous system injuries.

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ACKNOWLEDGEMENTS

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Chapter 1

Introduction

Sub-chapter 1: Neurons respond to injury.

Neurons are the fundamental unit of the nervous system. They function to receive, process, and transmit information in response to stimuli. Neurons are highly specialized and compartmentalized, consisting of the soma, several complex branched dendrites, and a single long axon (Figure 1). Dendrites and axons are distinct in both structure and function, with signals received at the dendrites, transmitted to the soma for processing, and propagated by the axon towards other neurons or into output cells.

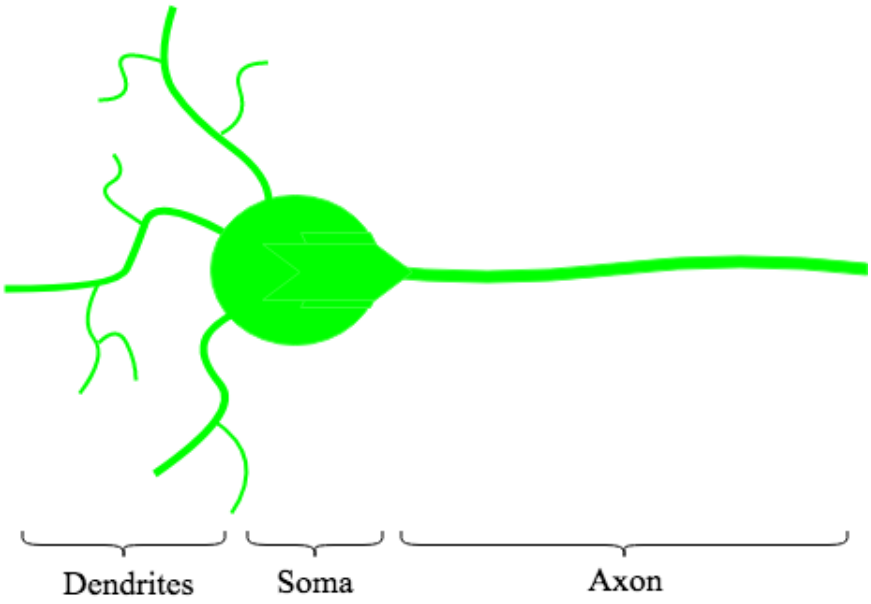

Figure 1: The neuron is a polarized cell.

Dendrites receive information and the axon propagates it.

Unlike many other cell types that are regularly replaced, neurons must last an entire lifetime despite being vulnerable to injury and stress. In particular, axon injury response is the focus of intense research, with almost 12,000 articles on PubMed. In humans, axon injury is a relatively common occurrence, with causes ranging from sports concussions to car accidents to traumatic brain injury[2].

Controlled laser injury of single neurons in *Drosophila* larvae (and other model organisms like *Caenorhabditis elegans*) has been used to investigate the axonal injury response[1,3,4]. *Drosophila* is a useful model organism for studying how neurons respond to injury because its neurons are highly polarized, similar to vertebrate neurons[1]. In addition, because the cuticle of the larva is clear, changes in fluorescently-labeled cell structures can be directly visualized in live larvae. The Rolls lab has previously used this single-cell injury method to investigate how microtubules respond to axon injury through a general upregulation in dynamics[1,5,6].

Because neurons cannot function with a damaged axon, traumatic axon injury tends to promote a vigorous response[1]. In *Drosophila* dendritic arborization neurons, axon removal results in the conversion of a dendrite to an axon through the reversal of microtubule polarity in the dendrite[1]. In other systems, a new growing axon may regenerate from the severed axon stump[7–9]. Axon injury also triggers a global upregulation in the number of dynamic microtubules as part of the rearrangement of the microtubule cytoskeleton[1]. This response requires activation of the JNK/DLK injury response pathway (Fig. 2).

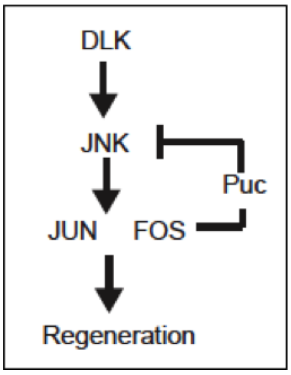

Figure 2: The DLK/JNK pathway is activated by controlled axon (but not dendrite) injury.

DLK acts as the upstream kinase, activating DLK and thus promoting regeneration.

DLK is a MAP3K dual leucine zipper kinase that acts to sense cytoskeleton disruption, such as neuronal injury or mutation in cytoskeleton proteins, and acts as the upstream activator of the Jun N-terminal kinase, JNK, which is called bsk in flies[10,11]. JNK signaling is required for up-regulation of the number of growing microtubules after controlled axon injury[1,5]. In *Drosophila* larva, controlled axon removal produces a ten-fold, JNK signaling-dependent upregulation of microtubule dynamics in the soma at eight hours post-injury[1,5]. Loss of the DLK/JNK pathway prevents normal neuronal response to axon injury, including degeneration and regeneration[1,8].

Dendrite removal is less well-characterized than axon injury, even though dendrite trauma is implicated in stroke, traumatic brain injury, and seizures[11,12]. While neurons have been found to mount a robust response to dendrite damage and some types are able to initiate regeneration, the JNK/DLK pathway is not activated in this response[11]. In fact, the microtubule activity upregulation that is characteristic of the JNK/DLK pathway is only produced by controlled axon injury, not dendrite injury[1].

Sub-chapter 2: Microtubule polarity and dynamics.

Polarity is critical to neuronal function. The cytoskeleton, including microtubules, plays a fundamental role in determining and maintaining the structure and the function of neurons[13]. Microtubules are dynamic components of the cytoskeleton, polymerizing and depolymerizing in response to cellular requirements for protein transport or reorganization. They are also important for the polarity of the neuron, as they provide tracks for the organized trafficking of organelles and proteins by motor proteins specifically to the dendrite or axon.

Microtubules are polymers of tubulin, which itself is an alpha-beta heterodimer. Tubulin dimers are arranged head to tail, thus establishing intrinsic polarity with a fast-growing plus end and a slow-growing minus end[3]. Most growth occurs at the fast-growing plus end, while the minus end is stabilized by various binding proteins[14].

Axons and dendrites have distinct microtubule orientation. Microtubules in both vertebrate and invertebrate axons are uniformly organized with the plus ends distal to the cell body (plus-end-out)[15]. In vertebrate dendrites, microtubule orientation is mixed, with both plus-end-out and minus-end-out microtubules present[16]. In contrast, proximal invertebrate dendrites have microtubules oriented with mostly minus ends distal to the soma (minus-end-out)[16]. Axons and dendrites take advantage of the intrinsic polarity of microtubules to direct compartmentalization, as various microtubule-binding proteins may have affinity specifically for the plus or minus end[17].

Microtubules play a guiding role in axonal development, and defects in polymerization have been shown to mediate axonal-based sensory neuropathy in a mouse model of ALS[18,19]. Overall, microtubules are linked at multiple levels to stress and injury response as well as pathology in neurons.

Sub-chapter 3: Calcium is an important cellular signaling molecule.

Free cytosolic calcium is normally regulated to very low levels in neurons, with a concentration difference across the plasma membrane of 10^{-7} M inside the cell and 10^{-3} M outside the cell[20]. Depolarization of the cell opens voltage-dependent calcium channels, allowing calcium to be used as an important intracellular signaling molecule or "second messenger" that mediates fundamental changes in cell activity[21]. In addition, substantial quantities of intracellular calcium are stored in organelles, mainly the endoplasmic reticulum.

Upon axon damage, this extreme level of control is perturbed to alert the neuron to the damage; a wave of calcium returns to the soma over the course of several minutes, where it promotes axon regeneration through tubulin deacetylation and DLK-dependent axon fragment fusing[11,22,23].

However, sustained elevation of cytosolic calcium is also associated with axon pathology and general cell toxicity[24–26]. In vitro, calcium has been observed to directly destabilize growing microtubules, while calcium influx has been linked in zebra fish to late axon degeneration[25,27]. Persistent elevation in calcium levels after injury has also previously been noted as a little-understood factor in neuronal pathology[26,24]. Given calcium's complex and varied interactions with injury response, being able to investigate a calcium influx phenotype in *Drosophila* would be a useful way to understand more complex consequences of neuronal injury in mammalian systems.

Sub-chapter 3: Key questions and hypotheses.

In this study, I explore a novel injury phenotype that differs from the previously described controlled injury. Controlled injury is performed by using a pulsed UV laser to sever or "melt" the neurite. The Rolls Lab recently observed an alternate type of injury, termed explosive injury, that occurs when the pulsed UV laser is applied at higher power, causing a small localized explosion on the neurite. This explosive injury was noticed to produce an immediate, obviously distinct phenotype in both calcium and microtubule imaging while potentially circumventing JNK signaling. I suspect that explosion-type injury in *Drosophila* may represent a more realistic model of traumatic nervous system injuries that occurs in real life. My goal was to characterize the cellular response to explosive neuron injury in order to establish a basis for further research concerning downstream effects and regeneration (Figure 3).

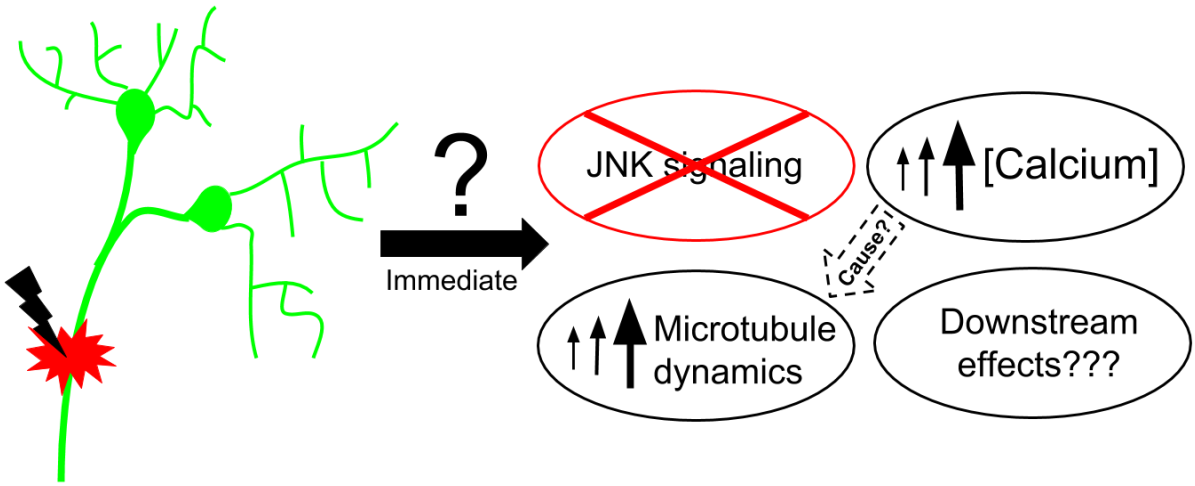

Figure 3: In this thesis, I investigate the microtubule-calcium relationship to characterize a novel explosive injury phenotype in *Drosophila*.

Three main areas of investigation include cytosolic calcium levels, microtubule dynamics, and whether the JNK signaling pathway is dispensable for the explosive injury response. I hypothesize that there may be downstream effects of explosive injury given its striking, immediate phenotype.

I investigated the scale, duration, and JNK-dependence of microtubule upregulation after explosive injury. Then, given the link between microtubule depolymerization and calcium signaling, I was called to further characterize their relationship in the explosive injury signaling response[27,28]. **I** hypothesized that the calcium spike seen after explosive injury is caused by membrane depolarization and influx of extracellular calcium**.** I used fluorescent calcium imaging as well as direct visualization of neuron voltage changes to investigate the scale and duration of calcium influx. Finally, I hypothesized that calcium plays a role in controlling the microtubule activity after explosive injury.

Chapter 2

Results

Sub-chapter 1: Explosive injury causes immediate, global changes in microtubule dynamics distinct from controlled injury.

Controlled axon removal results in a ten-fold, JNK signaling-dependent upregulation of microtubule dynamics after approximately eights hours post-injury[1,5]. This upregulation is not seen after controlled dendrite injury[11]. However, preliminary observations from Matthew Shorey hinted that explosive injury might initiate more immediate up-regulation in dynamics. I hypothesized that explosive injury is distinct from controlled injury, causing immediate global changes in microtubule dynamics independent of whether the axon or dendrite is cut.

To better characterize the microtubule response to explosive injury and contrast it to controlled injury, I took five minute videos using GFP-tagged plus-end-binding protein EB1 to visualize the microtubule activity in the comb dendrite of Class I neurons. Data was collected at baseline, after controlled non-comb dendrite injury, after explosive non-comb dendrite injury, after controlled axon injury, and after explosive axon injury. Post-injury videos were taken 15 seconds after injury.

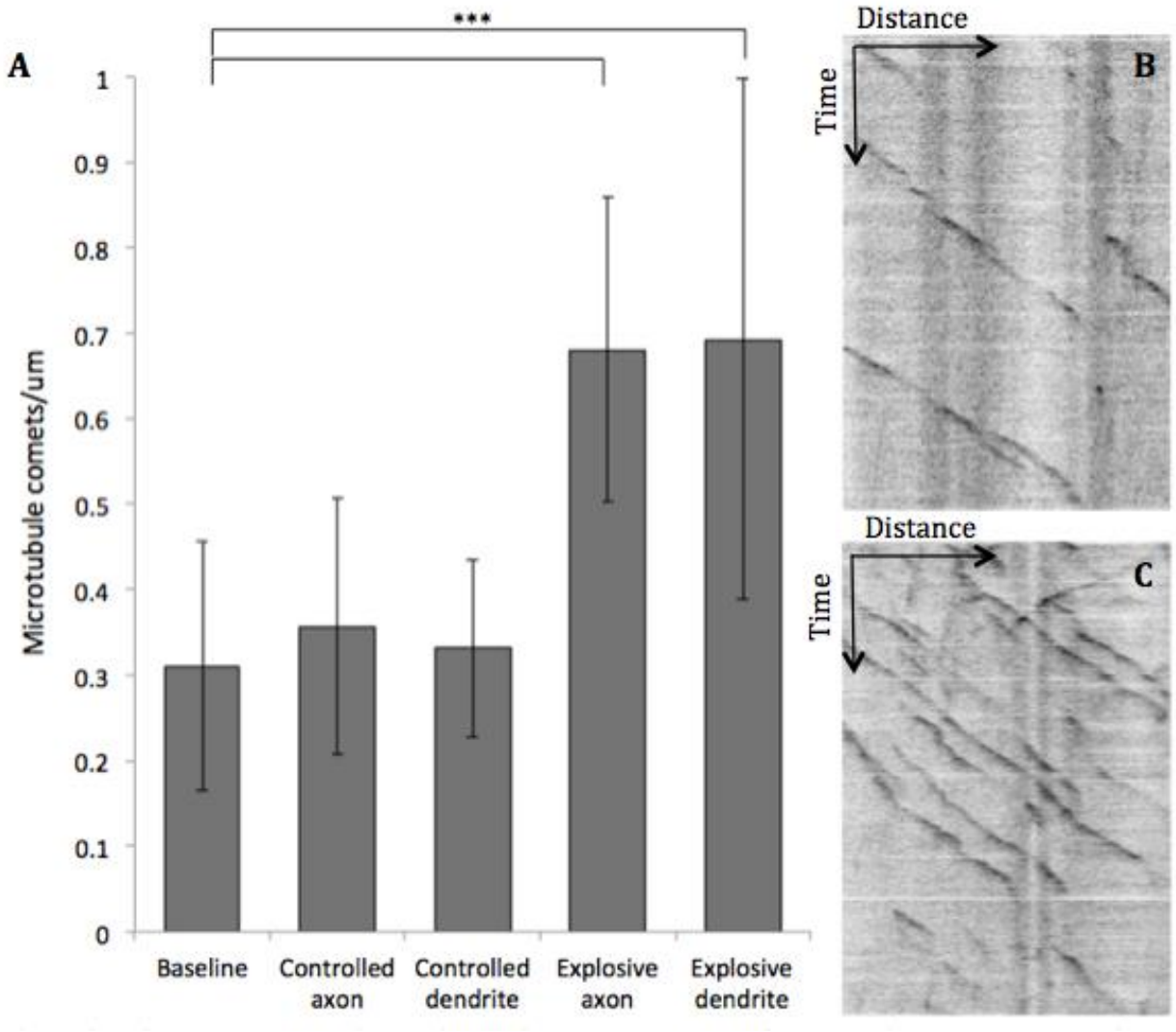

Figure 4: Microtubule dynamics are immediately upregulated after explosive axon and dendrite injury but not after controlled injury.

EB1-GFP was used to visualize the growing end of microtubules in the comb dendrite at baseline and immediately post-injury. Average microtubules/um measurements for 10 videos per injury state are shown with standard deviations. (**A**) Explosive injury to either the non-comb dendrite or the axon resulted in a statistically significant upregulation compared to baseline ($p < 0.0005$, unpaired t-test), while controlled injury did not. Sample kymographs visualize time versus distance from the soma for controlled injury (**B**) and explosive injury (**C**). Microtubule comets appear as dark lines.

Comparison of each test condition to the baseline showed two main results: both axon explosion and dendrite explosion produce a statistically significant, roughly 2-fold increase in microtubule dynamics compared to baseline at T = 15 seconds (Figure 4). In contrast, controlled

injury of both axon and dendrite produces no statistically significant difference in microtubule activity at T = 15 seconds (Figure 4).

JNK signaling is required for the up-regulation in the number of growing microtubules after axon injury. However, the JNK signaling pathway is transcription-dependent and not seen until eight hours post-injury. Because explosive injury still produced an immediate microtubule upregulation, the timing suggests that the JNK pathway is not required for the immediate explosive injury phenotype. In addition, the microtubule upregulation seen after controlled cuts is only produced by controlled axon cuts, while both axon and dendrites produce the massive upregulation in dynamics after explosive injury. This suggests that explosive injury relies on an independent signaling pathway to cause the microtubule dynamics upregulation.

Sub-chapter 2: The increase in microtubule dynamics after explosive injury is sustained independently of the JNK pathway.

The striking, immediate upregulation of microtubules after explosive injury raised the question of how long this effect is sustained in the neuron. By eight hours after a controlled axon injury, the JNK signaling pathway initiates an upregulation in microtubule dynamics. Although the initial response to explosive injury was found to be independent of the JNK pathway, the question was raised of whether the JNK pathway would eventually be activated and contribute to sustained upregulation even after explosive injury.

Three parallel time courses were performed following explosive axon injury in the rtnl2 control line, explosive dendrite injury in the rtnl2 line, and explosive axon injury in the bsk dominant negative line. The JNK pathway is axon-specific, so any microtubule upregulation seen at eight hours post-dendrite injury must be attributed to another injury response[11]. Similarly,

bskDN is a genetic line that blocks the JNK signaling pathway from initiating microtubule upregulation at eight hours. In this way, I sought to isolate the effects of the JNK pathway on the time course of microtubule activity after injury.

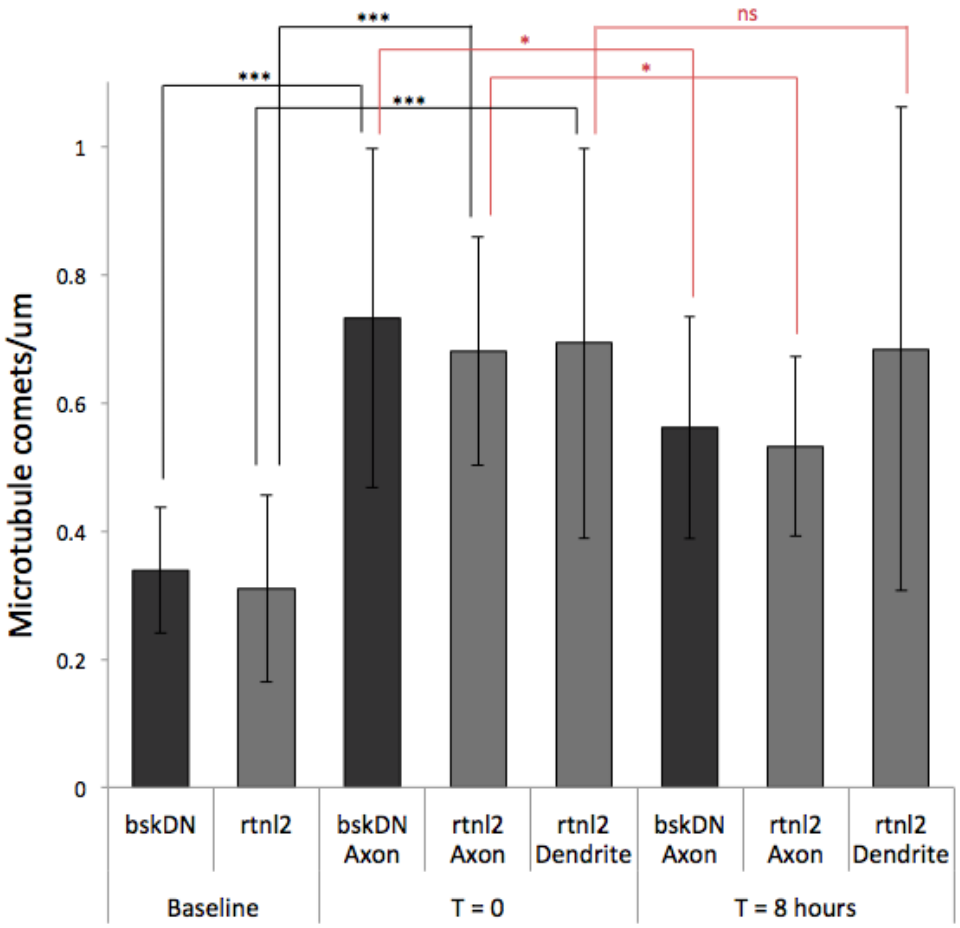

Figure 5: Microtubule time course shows extended, JNK-independent upregulation after explosive injury.

Microtubule comets per micrometer was measured immediately post-injury and at eight hours. Average microtubule comets/um measurements for 10 five-minute videos per injury state are shown with standard deviations. A significant difference was found between baseline and T=0 for all test conditions ($p < 0.001$, unpaired t-test). A significant difference was found for bsk DN and rtnl2 axon injury neurons at T = 8 hours ($p < 0.05$), but no significant difference was found eight hours after dendrite injury in rtnl2 ($p > 0.05$).

At baseline levels, bsk DN and rtnl2 neurons showed statistically similar levels of microtubule activity in microtubule comets per micrometer (Figure 5). Immediately after

explosive axon injury in rtnl2 and bsk DN, as well as after explosive dendrite injury in rtnl2, microtubule activity is elevated to twice baseline-levels. This further confirms that the JNK pathway is not necessary for the immediate response to explosive axon injury. At eight hours, the microtubule activity for all injury types is still upregulated compared to baseline levels. However, microtubule activity after bsk DN axon injury and rtnl2 axon injury has declined compared to the T = 0 time point. After dendrite injury, microtubule activity in rtnl2 neurons is not significantly different at eight hours post-injury than immediately post-injury: it remains elevated.

Sub-chapter 3: Explosive injury produces an immediate spike in cytosolic calcium levels.

Controlled injury has been observed to cause a small, temporary increase in cytosolic calcium levels in the neuron[8]. The question was raised, then, if explosive injury triggers a similar change in calcium that might correlate with the microtubule response. Because calcium is an important and tightly controlled signaling molecule, I sought to quantify its level of elevation after explosive injury, compare the effects of axon injury versus dendrite injury, and characterize the duration of its elevation.

The GCaMP assay used here is a simple and highly quantifiable method to visualize calcium levels inside the cell. GCaMP is a fluorescent calcium sensor protein that's fluorescence level is proportional to the free intracellular calcium concentration. Each cell was imaged immediately before and some time after injury and fluorescence in the cell body was quantified. The opposite neuron in the hemi-segment served as an internal control for bleaching and non-cell-specific changes in calcium levels.

In Class I neurons, explosive axon injury produces a roughly 3-fold increase in fluorescence immediately post-injury compared to uninjured baseline levels of calcium (Figure 6). In contrast, controlled axon injury produces only a 1.5-fold increase in fluorescence. This result supports that explosive axon injury produces a significantly larger calcium spike.

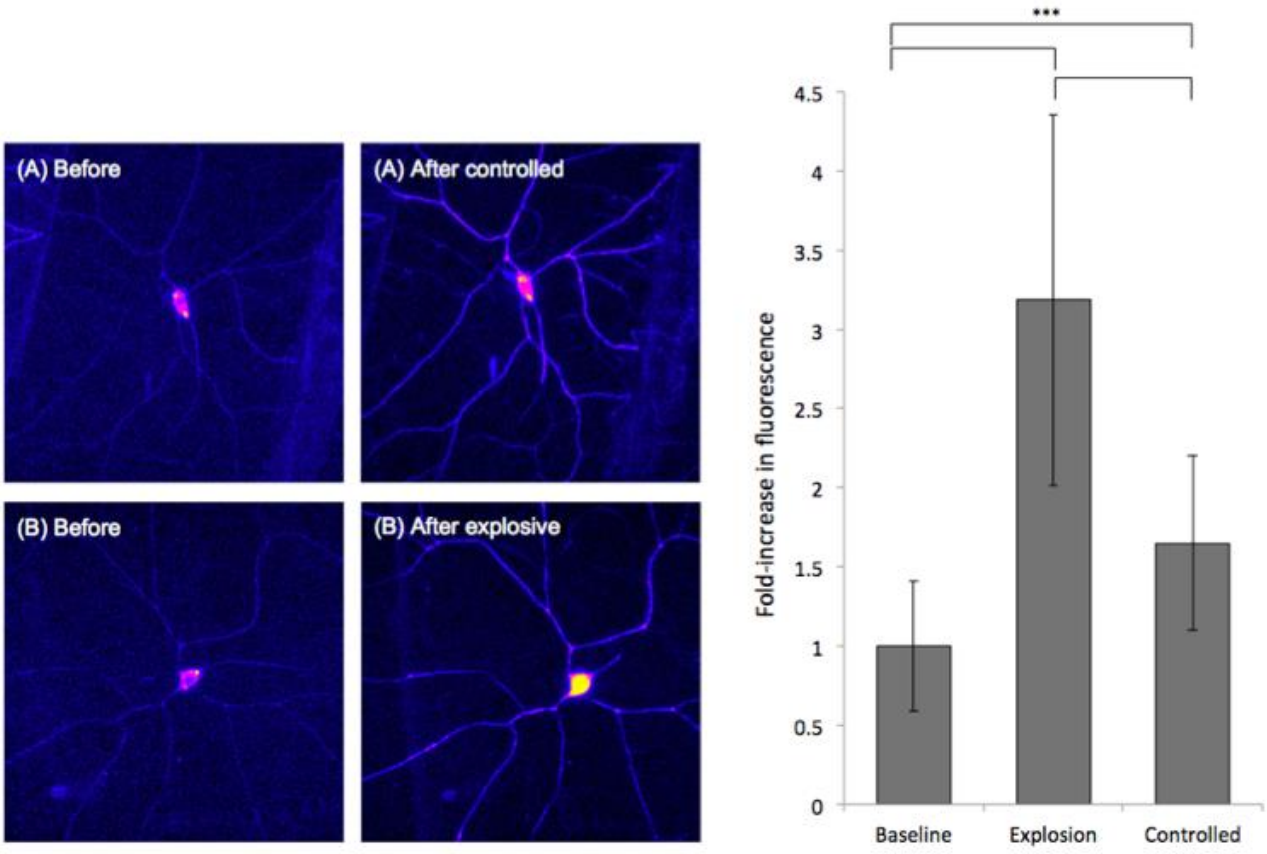

Figure 6: Explosive axon injury produces a significantly greater increase in GCaMP fluorescence than controlled axon injury in Class I neurons.

Average fold-increase measurements for 10 neurons per injury state are shown with standard deviations. **(A**) GCaMP fluorescence (represented as a heat map) provides a visualization of calcium levels before and after controlled axon injury. **(B)** Fluorescence is visibly brighter after explosive axon injury. **(C)** Explosive axon injury produces a 3.18-fold increase in fluorescence, which is significantly more than either baseline or controlled injury fluorescence ($p < 0.001$). Controlled axon injury produces a 1.65-fold increase in fluorescence over baseline.

Explosive axon injury was compared to explosive dendrite injury in class IV neurons (Figure 7). Both injury types resulted in a similar 4-fold spike in free intracellular calcium levels, which is significantly more than baseline or controlled axon injury. Confirming that axon and dendrite injury result in similar changes in calcium levels allowed us to proceed with only axon injury for further calcium-based experiments.

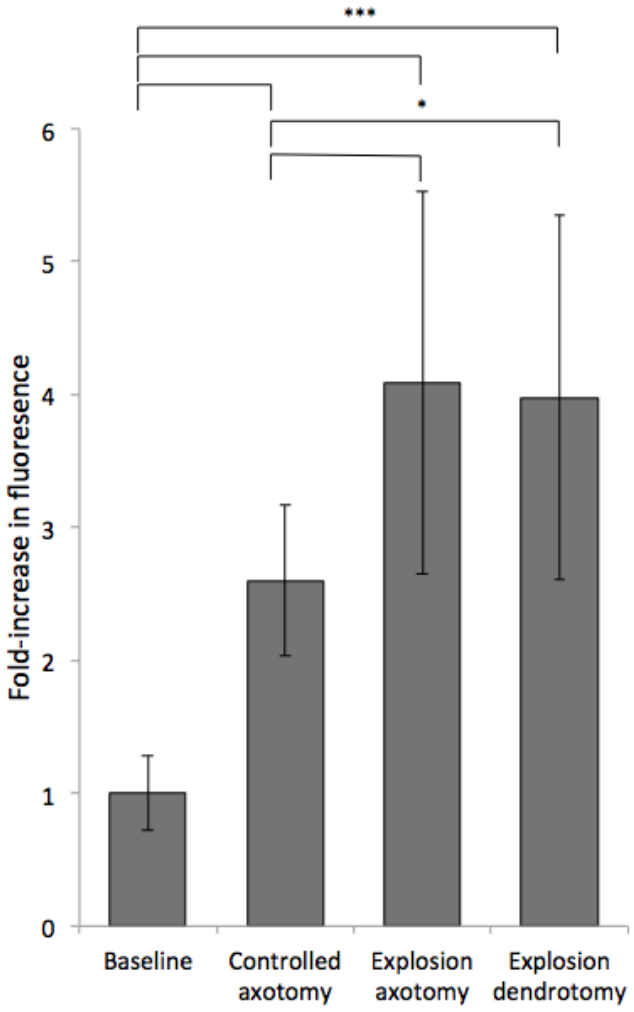

Figure 7: Explosive axon and dendrite injury produce statistically similar increases in GCaMP fluorescence in Class IV neurons.

Average fluorescence fold-increase measurements for 10 neurons per injury state are shown with standard deviations. GCaMP fluorescence is affected similarly by dendrite and axon explosive injury 15 seconds post-injury ($p < 0.001$).

Having previously established that microtubules dynamics continue to be upregulated at eight hours after explosive injury, I investigated the extent of the parallel calcium spike by performing a time course at fifteen seconds, five minutes, one hour, two hours, and eight hours. Immediately after explosive axon injury, neuron fluorescence is approximately four-fold elevated compared to baseline fluorescence in uninjured neurons (Figure 8). Calcium levels remain elevated at five minutes and are still roughly two-fold elevated between one and two hours post-injury. By eight hours post-injury, calcium levels have returned roughly to baseline.

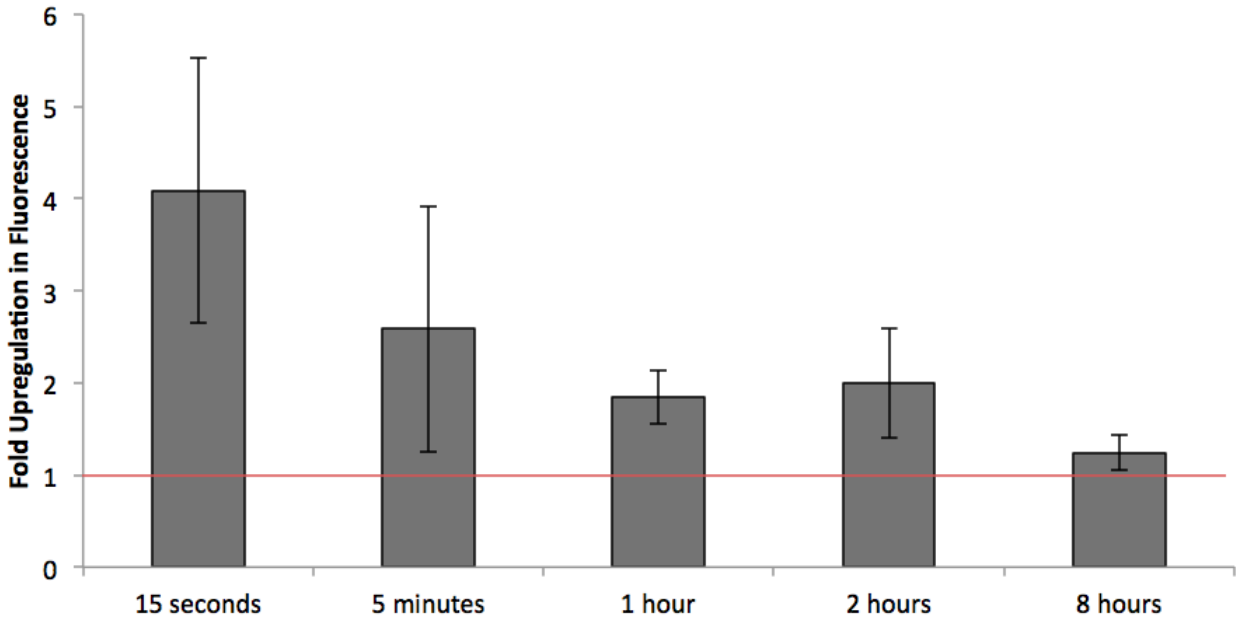

Figure 8: GCaMP fluorescence remains elevated for several hours after explosive injury.

Average fluorescence fold-increase measurements for 10 neurons per injury time are shown with standard deviations. Immediately after injury, GCaMP fluorescence increases four-fold. At five minutes, fluorescence is still nearly 3-fold increased, indicating that free cytosolic calcium is still substantially higher than baseline.

Sub-chapter 4: Voltage changes correspond to the initiation increase in microtubule dynamics.

Calcium imaging demonstrated an immediate, massive spike in cytosolic calcium levels after explosive injury. There are two main potential sources of this spike: rearrangement of intracellular calcium from intracellular stores or influx of extracellular calcium due to membrane depolarization. Calcium imaging alone cannot begin to differentiate calcium influx from internal calcium release, calling for an alternative approach.

Arclight is a genetically encoded voltage sensing fluorescent molecule that allows direct visualization of voltage changes in the neuron after injury[30]. Because intracellular calcium rearrangement upon injury would not cause a voltage change and therefore would not affect Arclight fluorescence, Arclight allowed me to test the hypothesis that the calcium spike immediately after injury is partially mediated by an influx of calcium. Controlled and explosive axon injuries were performed on Class IV neurons. Images of both test neurons and internal controls were taken immediately prior to injury, 15 seconds after injury, and 5 minutes after injury. Arclight fluorescence dims in response to neuron depolarization[30].

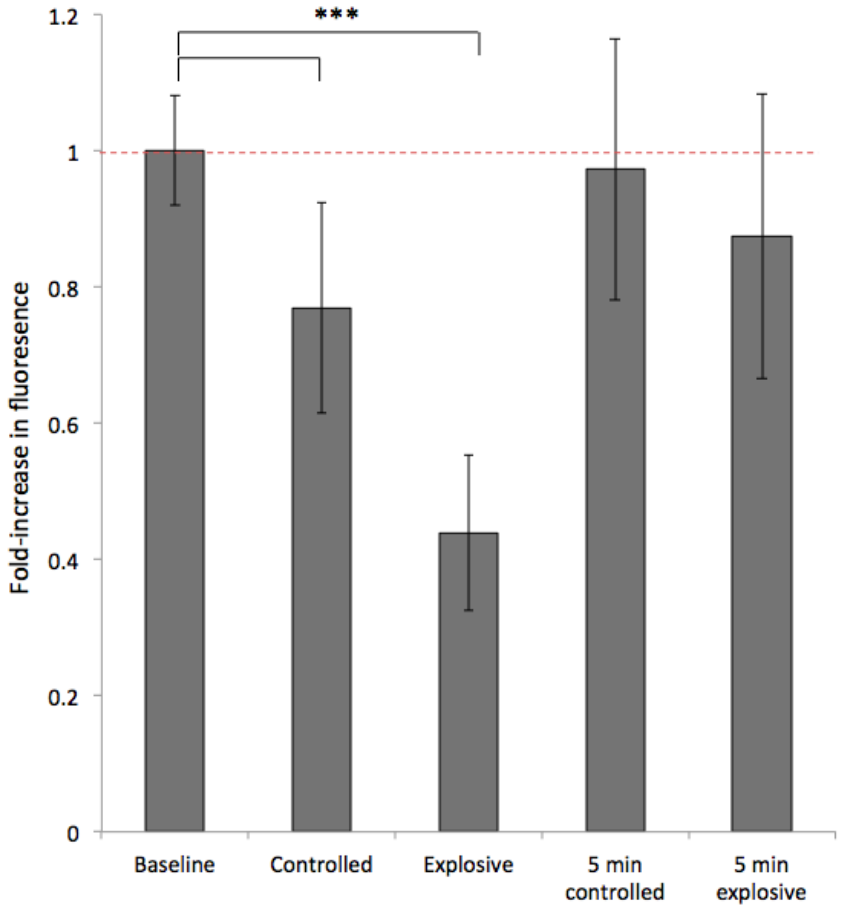

Figure 9: Fluorescent voltage sensor Arclight demonstrates significant, temporary neuron polarization after explosive injury.

Average fluorescence fold-increase measurements for 10 neurons per injury state are shown with standard deviations. Fold-increase in fluorescence was calculated for each neuron as the ratio of its fluorescence before and after injury, then divided by the before/after fluorescence ratio of each internal control neuron. Explosive injury causes a significantly greater decrease in Arclight fluorescence fifteen seconds after injury than controlled injury ($p < 0.001$). At five minutes, voltage has returned to baseline.

Controlled injury produces a small but significant decrease in Arclight fluorescence, while explosive injury produces a significantly more dramatic decrease in Arclight fluorescence (Figure 9). At five minutes, neuron voltage is not significantly different than at baseline for either injury method. This experiment provides preliminary evidence that the calcium spike—but not the sustained calcium elevation—is associated with membrane depolarization after injury.

Sub-chapter 5: Calcium spike during post-injury depolarization may mediate the up-regulation of microtubule dynamics.

I hypothesized that a calcium spike during post-injury depolarization mediates the up-regulation of microtubule dynamics after injury. To test this hypothesis, I overexpressed the membrane channel Kir2.1, a potassium channel whose overexpression causes neurons to become hyperpolarized. This prevents depolarization, thus reducing calcium influx. In the first experiment, direct visualization of calcium levels by GCaMP before and after explosive injury was used to observe if preventing calcium influx with Kir2.1 reduces the calcium spike.

Kir2.1 significantly reduced the calcium spike as measured fifteen seconds after explosive axon injury in Class IV neurons (Figure 10). GCaMP fluorescence in the control had a roughly four-fold increase after explosive injury in the control, compared to a 2.5-fold increase in the neurons expressing Kir2.1. This experiment suggests that preventing depolarization reduces the spike in cytosolic calcium after explosive injury. This result further supports that at least some portion of the calcium spike can be attributed to an influx of extracellular calcium rather than release of intracellular calcium stores.

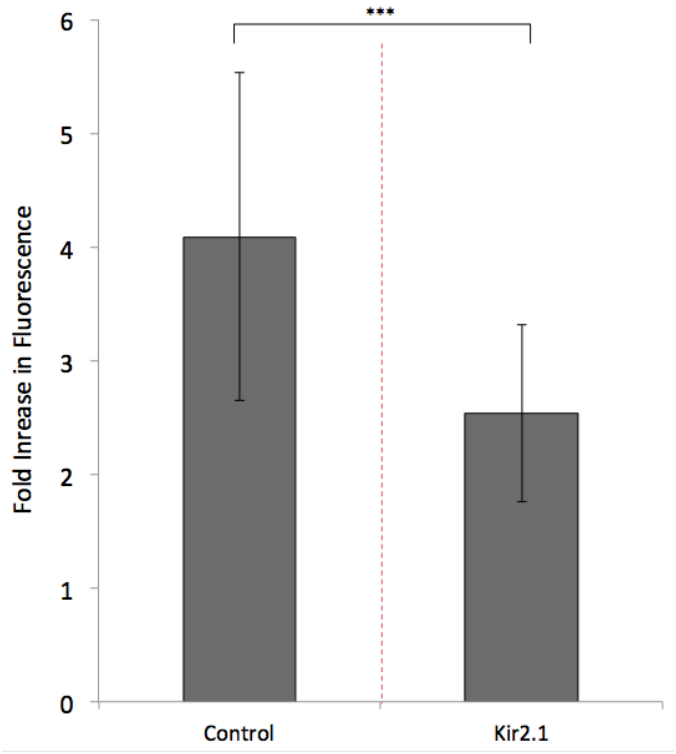

Figure 10: Overexpression of potassium channel Kir2.1 significantly reduces calcium spike after explosive injury compared to controls in Class IV neurons.

Average fluorescence fold-increase measurements for 10 neurons per injury state are shown with standard deviations. Explosive axon injury in the control causes a 4-fold increase in GCaMP fluorescence, compared to a 2.5-fold increase in neurons expressing Kir2.1. Red line indicates that the control and Kir2.1 experiments were performed separately.

Having verified that preventing neuron depolarization prevents some of the calcium spike after explosive injury, I turned to the microtubule dynamics assay to tease out the relationship between the calcium spike and the increase in microtubule dynamics. I once again overexpressed Kir2.1 to prevent neuron depolarization—thus preventing calcium influx—and observed if this prevents the increase in microtubule dynamics.

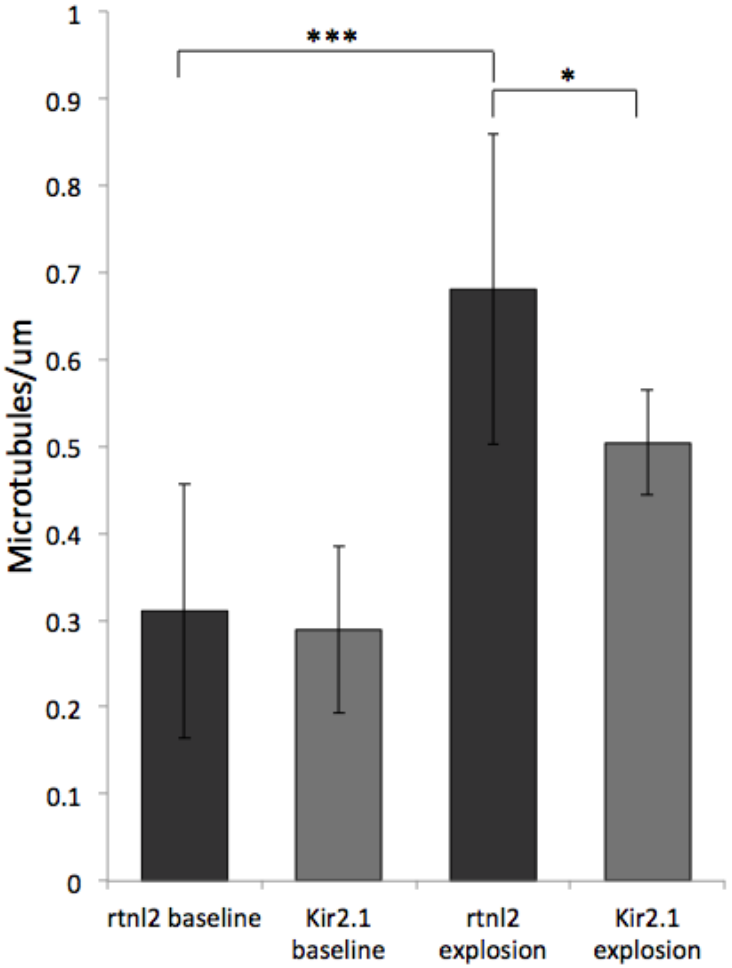

Figure 11: Preventing neuron depolarization and calcium influx reduces microtubule upregulation after explosive injury.

Average microtubule comet/um measurements for 10 neurons per injury state are shown with standard deviations. Kir2.1 prevents neuron depolarization but has no apparent baseline effect on microtubule activity compared to rtnl2 control. Kir2.1 reduces the increase in microtubule activity after explosive injury compared to rtnl2.

While results are preliminary, overexpressing Kir2.1 appears to reduce the upregulation in microtubule activity after explosive injury (Figure 11). Kir2.1 and rtnl2 have similar baseline levels of activity. Immediately after explosive injury, rtnl2 microtubule activity increases from 0.3 comets/um to 0.7 comets/um. In contrast, Kir2.1 microtubule activity increases from 0.3 to 0.5 comets/um.

Chapter 3

Discussion

In this study, I observed a new neuronal injury phenotype that does not rely on the same JNK signaling pathway as the well-characterized controlled axon injury. The experiments I performed aimed to characterize the phenotype and elucidate its general mechanism. In particular, I focused on comparing and contrasting the neuronal response to controlled versus explosive injury. My data suggests that explosive neuron injury is distinct from controlled injury in microtubule activity, cytosolic calcium level, and membrane polarization, both immediately and over the course of several hours.

To initially characterize if explosive injury was distinct from controlled injury, I investigated if explosive injury relies on the JNK signaling pathway. Controlled axon injury, which is well-characterized and considered a standard model of neuronal injury, signals through the JNK pathway to cause a transcription-dependent, moderate increase in microtubule dynamics by eight hours. I verified that controlled injury to the axon or dendrite produces no immediate upregulation of microtubule dynamics (Figure 4). In contrast, I found that explosive injury initiates an immediate (<15 seconds post-injury), significant upregulation of microtubule activity regardless of whether the axon or dendrite is injured (Figure 4). Because this upregulation was immediate and global, it could not be a result of the JNK pathway; explosive injury must signal at least initially through an alternative, most likely transcription-independent, mechanism.

However, even though the initial response to explosive injury was shown to be JNK-independent, it was not ruled out that the JNK signaling pathway might still be responsible for

the neuronal response later after injury. A time course using the bsk dominant negative line, which blocks the JNK signaling pathway and prevents microtubule upregulation eight hours after controlled axon injury, showed massive microtubule upregulation eight hours after explosive axon injury (Figure 5). Similarly, explosive dendrite injury, which should not activate the axon-specific JNK pathway, also showed massive up microtubule upregulation eight hours later (Figure 5). These results demonstrate that the injury response is not only immediate but also extended independent of the JNK pathway. Interestingly, while all types of explosive injury tested showed similar levels of upregulation immediately after injury, explosive dendrite injury behaved differently at eight hours (Figure 5). Explosive axon injury in both rtnl2 and bsk DN lines remained upregulated compared to baseline but declined significantly from T = 0 (Figure 5). In contrast, explosive dendrite injury caused microtubule activity to remain equally elevated at eight hours (Figure 5).

I found that explosive injury, to either the axon or the dendrite, immediately produces a much larger increase in cytosolic calcium levels compared to the small, transient increase after controlled injury (Figures 6 and 7). Given what is known of calcium as a critical and tightly controlled molecule in neurons, it was surprising to see how long the calcium levels remain elevated; a time course revealed that elevation was still significant at two hours and only returned to baseline around eight hours post-injury (Figure 8).

Given the magnitude and length of microtubule activity upregulation and calcium elevation, it is highly plausible that explosive injury has different effects on downstream cellular activities compared to controlled injury. With such a massive rearrangement of microtubules, we might expect to see changes in regeneration, trafficking of endosomal vesicles, actin remodeling, and others. Indeed, our lab has already seen suggestions that explosive injury produces a

different regeneration phenotype. Further investigation into downstream effects of explosive injury should prove interesting and fruitful.

Is it possible that the initial calcium spike is due to influx of extracellular calcium or is the release of intracellular stores (from the endoplasmic reticulum, etc.) the primary mediator? One portion of the answer to this question comes from the fluorescent voltage indicator protein, Arclight. Arclight showed that explosive injury causes significant, temporary depolarization compared to controlled injury (Figure 9). This suggests that an influx of intracellular calcium is involved in the spike, at least initially, because rearrangement of intracellular stores would not cause such a change in the voltage. However, I cannot currently rule out the possibility that intracellular calcium stores also contribute to or are responsible for perpetuating the elevation in calcium levels. It is likely that both intra and extracellular calcium sources play a role in explosive injury response.

Having ruled out the JNK pathway, I sought an alternative way to explain the response to explosive injury. Given that the calcium spike is so striking and corresponds in time with the microtubule upregulation, I hypothesized that the calcium spike might mediate the upregulation in microtubule dynamics. Kir2.1 overexpression was used to verify the connection between neuron depolarization (as seen using Arclight) and calcium spike (as seen with GCaMP). Kir2.1 is a potassium channel that prevents neuron depolarization when overexpressed, and the GCaMP assays performed here indicate that it also reduces calcium influx (Figure 10). Kir2.1 overexpression was also found to reduce the upregulation of microtubule activity normally after explosive axon injury (Figure 11). Since reducing calcium influx reduces microtubule dynamics, this supports that there may be a cause and effect relationship between post-injury microtubule dynamics and calcium signaling. However, these results are preliminary and there remains a

substantial amount of work to characterize this relationship. In addition, the mechanism by which calcium may mediate the microtubule response is still unknown and will be a subject of investigation for future experiments.

Given the drastically different scale and immediate result of explosive injury, it is likely to cause different downstream effects than controlled injury. For example**,** axons are known to have some capacity to regenerate after injury in *Drosophila, C. elegans*, zebra fish, and even humans[1,13,31,32]: is that rate of regeneration the same after an explosive axon injury? Does explosive axon injury trigger the same neuroprotective pathway as controlled axon injury, despite triggering a different initial signaling pathway[5]? Understanding how neurons mediate their response to explosive injury could have implications to important molecular mechanisms throughout the cell, especially because both calcium and microtubules are involved in a wide array of cellular processes.

These experiments laid a solid groundwork to establish explosive injury as a unique, additional injury model in *Drosophila*. Existing models of laser-induced neuron injury in *Drosophila* have been effective, but may be so gentle as to fail to accurately represent some kinds of damage that occurs in highly traumatic injury in humans (for example, in diffuse axonal injury). In contrast, explosive injury rips off small pieces of the neuron, which may allow us to elucidate a wider range of neuronal injury responses. In light of the severe and often lasting consequences of traumatic brain injuries in humans, successful investigation of a new way to model real-world injuries in *Drosophila* would be a useful research tool with potentially long-term implications for our understanding of nervous system injuries.

Appendix

Methodology

Sub-chapter 1: Fly stocks and injury assays.

Fly stocks were obtained thanks to the Vienna Drosophila Resource Center and the Bloomington Drosophila Stock Center at Indiana University. All *Drosophila* stocks were maintained at 25°C in standard media. Class I and class IV dendritic arborization neurons were examined in third-instar larva.

Controlled neuron injury was performed using a pulsed UV laser on medium power to precisely sever the dendrite or axon at a distance far from the soma. Explosive injury was performed using a pulsed UV laser on medium-high power to create a localized explosion on the dendrite or axon, avoiding injury to the soma.

Sub-chapter 2: Microtubule dynamics, calcium, and voltage assays.

Microtubule dynamics assays were carried out using the genotype UAS-dicer2; 221-Gal4, UAS-EB1-GFP/TM6 crossed with UAS-RNAi lines as necessary. EB1-GFP is a fluorescently tagged plus-end-binding protein allows visualization of actively polymerized microtubules. Rtnl2 RNAi was used as a control line as it has previously been found to have no significant phenotype in microtubule activity[29]. 221-Gal4, UAS-EB1-GFP/TM6 (no dicer) was used in bskDN experiments.

Microtubule activity was quantified by counting the number of growing microtubules (“comets”) between the second and fourth branch points in the comb dendrite during the five

minute video. The total comets were divided by the distance between the second and fourth branch-points. Only comets present in three or more successive frames were counted.

Calcium imaging was performed using the genotype 221 or ppk-Gal4, UAS:TdTomato X UAS:GCaMP. GCaMP is a green fluorescent calcium sensing protein. Images were quantified by measuring the green fluorescence in the soma before and after injury to calculate a fold-change in GCaMP fluorescence. The opposite neuron in the hemi-segment was imaged as an internal control for bleaching and non-cell-specific changes in calcium levels.

The genotypes 221 or ppk:Gal4, UAS:TdTomato X UAS:Arclight were used to visualize voltage changes in the neuron. Arclight is a genetically encoded voltage sensing fluorescent protein. The opposite neuron in the hemi-segment was imaged as an internal control for bleaching and non-cell-specific changes in Arclight levels.

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