Regulation of Calcium Influx in *Drosophila* Motorneuron Axon Degeneration

Undergraduate Honors Thesis
Neuroscience
Catherine Collins Lab
University of Michigan

Ross Allen Carson

Mentor: Dr. Catherine Collins
Readers: Dr. Richard Hume and Dr. Orie Shafer

Table of Contents

ABSTRACT

Wallerian degeneration is a conserved process by which axons are fragmented after injury and has implications for the treatment of both traumatic and acute neurodegenerative conditions. It has been observed that an increase in intracellular calcium is necessary for Wallerian degeneration after injury has occurred (George et al., 1995; Knöferle et al., 2010), and that injured axons can be protected from degeneration by chelating external calcium (Schlaepfer and Bunge, 1973; George et al., 1995). However, the mechanism by which extracellular calcium gains entry into the axon and its role in initiating degeneration is not well understood. This project employs novel assays which allow for the manipulation of the extracellular fluid bathing axons and calcium sensitive imaging of *Drosophila melanogaster* larvae to demonstrate that calcium plays an essential role throughout the process of axon degeneration after nerve injury. A channel mediated influx of calcium occurs within 15 minutes after injury and is maintained within the nerve for approximately three hours. At around six hours after injury a second influx of calcium takes place, and both calcium influx events appear necessary for Wallerian degeneration to occur normally. The first influx of calcium appears to be dependent upon changes in sodium and potassium currents, which affect membrane potential, causing the opening of voltage gated calcium channels. Additionally, this calcium influx is diminished in larvae expressing *Wld*^{S}, which encodes a chimeric protein that protects axons from degeneration after injury and a range of neurodegenerative conditions. Calcium entry likely takes place in part through voltage gated type 2 calcium channels present at the synapse, which open in response to a change in membrane potential after injury.

INTRODUCTION

Wallerian Degeneration and the Axon

Neurons are essential for a variety of processes such as collecting, sending and processing information which is carried in the form of an electrical impulse in a long process called an axon. At the distal end of the axon lies the axon terminal, where neurons synapse onto target cells, such as other neurons or muscle tissue, to chemically transfer information in the form of neurotransmitter release. In the human sciatic nerve, axons can reach over a meter in length. Because of their unique morphology, axons are vulnerable to a wide range of insults that could potentially separate the axon terminal from the cell body, including mechanical and chemical damage.

If an axon is damaged, the portion of the axon distal to the injury site enters a characteristic pattern of self-destruction called Wallerian degeneration. In this process, the distal portion of the axon fragments and is later cleared by surrounding glial cells. Wallerian degeneration can be initiated by a number of insults including heritable diseases, chronic metabolic disorders, exposure to toxins, and long term stress. In addition, acute injuries such as traumatic brain injury and spinal cord injury can initiate this process. The mechanisms by which Wallerian degeneration occurs are shared with a wide range of neuropathies and neurodegenerative diseases (Coleman, 2005). It is important to understand the timing and molecular basis of Wallerian degeneration in order to monitor and control the rate degeneration in pathological conditions. It is vital to note that not all axon degeneration is harmful. In many cases of peripheral nerve injury, damaged axons must first be fragmented and

cleared in order for neurons to regrow and reconnect to their targets. In this case speeding up the rate of axon degeneration would beneficial. On the other hand, slowing the rate of degeneration in conditions such as multiple sclerosis or glaucoma could significantly prevent or delay the onset of deleterious symptoms and greatly improve patient outcomes.

The Phases of Wallerian Degeneration

Wallerian degeneration after acute axotomy has been characterized as having three distinct phases: acute axon degeneration, the latency period, and granular fragmentation. Acute axonal degeneration (AAD) results in the widening of the injury site by several hundred micrometers in both directions and is completed within an hour after mechanical transection in vertebrates (Kerschensteineret al., 2005). This process is thought to be mediated by calpain which cleaves neurofilaments and microtubules, resulting in the shortening of both the proximal and distal segments of the axon. The process of AAD can largely be attenuated by the expression of the *Wld*^{S} transgene (Kerschensteineret al., 2005), suggesting a common protective mechanism for both AAD and Wallerian degeneration.

The latency period is characterized by stasis in the segment of the axon distal to the site of injury. During the first few hours of this phase, the inured axon remains electrically excitable and transport activities continue to take place (Mishra et al., 2013; Wang, 2012). The latent phase begins after acute axon degeneration and lasts for roughly 6 hours in *Drosophila melanogaster* motoneuron axons, although axon function may be lost before this point (Mishra et al., 2013). While it appears that not much is happening during this time, it is probable that metabolic changes occur which help to trigger or promote the process of granular fragmentation. One hypothesis is that protective factors may be degraded during the latency

period, resulting in the onset of Wallerian degeneration. One candidate is Nmnat, which has been implicated in many models of axon degeneration as a protective factor due to its energy conserving properties (Seng et al., 2013). Nmnat is gradually transported in an anterograde fashion in axons and down-regulated at the synapse by an enzyme called Hiw (Xiong et al., 2012). This down-regulation could result in the decrease of Nmnat activity which could act as an upstream signal of degeneration. The specific role of Nmnat in degeneration will be discussed later in the introduction. No consensus currently exists on what takes place during this stage of Wallerian degeneration.

During granular fragmentation, the distal portion of the transected axon becomes fragmented in a fashion dependent upon the size of the axon (Wang, 2012). In *Drosophila melanogaster*, initial signs of degeneration in motorneurons can clearly be seen around 8 hours after axotomy and axons are almost completely fragmented by 20 hours after injury in wild type larvae. The mechanisms by which axons become fragmented are not currently understood. After the process of fragmentation has occurred, the axolemma is cleared away by surrounding glia and macrophages (Wang, 2012). In some cases of spinal cord injury, the inflammation and release of cytokines by macrophages causes toxicity induced secondary injury to surrounding neurons and glia. This inflammation response may continue for years after the initial injury (Oudega, 2013). The stages of Wallerian degeneration represent interrelated components of a process which seeks to remove neuronal debris, and a greater understanding of each of these stages is needed to better understand the process as a whole.

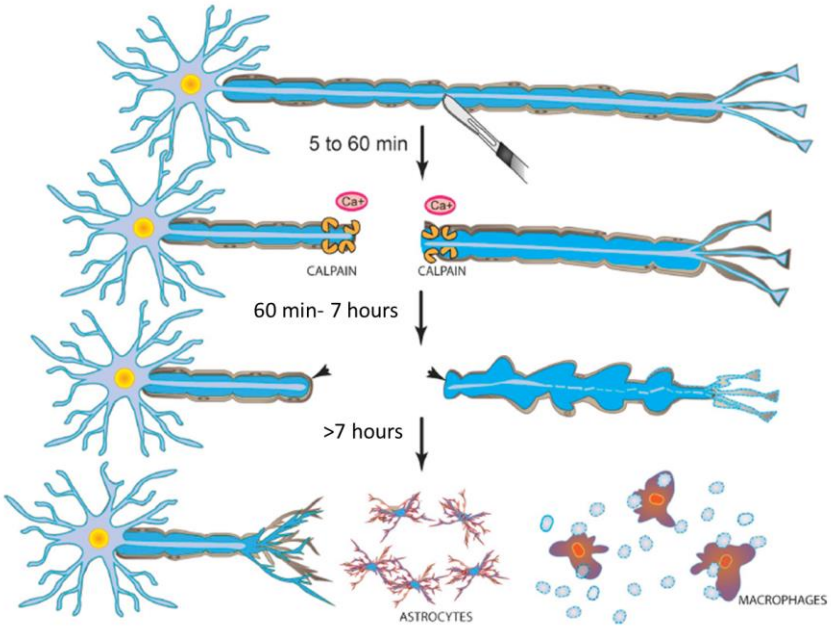


Figure 1. The phases of Wallerian degeneration in the fruit fly. From top to bottom: intact axon before injury, acute axonal degeneration, latency period, and granular degeneration. Adapted from Wang et al., 2012.

Wld^S, Powerful Tool to Study Axon Degeneration

A major breakthrough in the field of neurodegeneration was the discovery of the *Wld*^{S} mutation which drastically slows the rate of degeneration after nerve injury (Lunn et al., 1989). It was a spontaneous mutation discovered by chance and encodes a chimeric protein which includes Nmnat1 and N16. Numerous studies have demonstrated that the protective effect of Wld^S is dependent upon its ability to increase Nmnat enzymatic activity, although it there is no consensus on why Nmnat activity is important (Coleman, 2010). Normally, Nmnat is responsible for the synthesis of NAD+, which may have implications for maintaining energy balance after injury (Seng et al., 2013). Because of the protective effects of Wld^S, Nmnat and its regulatory proteins have been implicated into many models of Wallerian degeneration.

The protective effects of Wld ^{S} have been shown to be reliant upon its N terminal N16 domain, which is responsible for axonal localization (Coleman, 2010). This indicates that the protective Nmnat activity conferred by this chimeric protein takes place in the axon. Before the discovery of *Wld*^{S}, it was thought that axons relied upon connection to the cell body for stability, suggesting that Wallerian degeneration is a passive process. The discovery and study of the *Wld*^{S} mutation clearly demonstrates that axons are able to sustain themselves long after removal from the cell body in the presence of protective factors (Coleman, 2010).

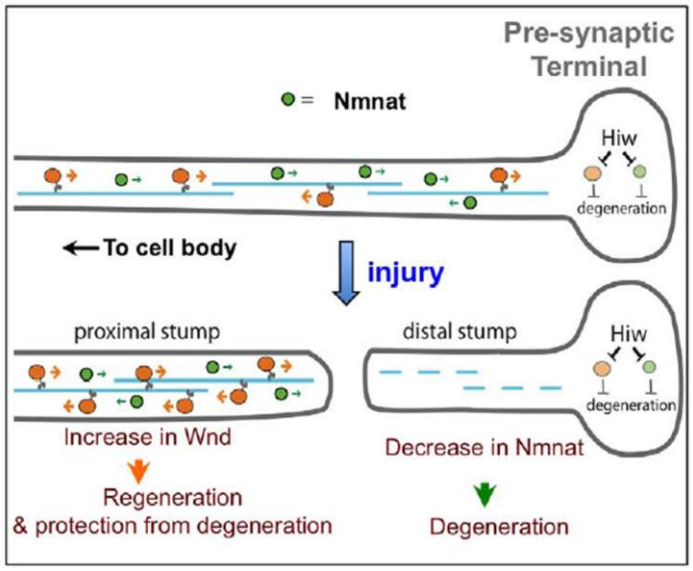


Figure 2. An active model for Wallerian degeneration. NMNAT is transported to the synapse where it is down regulated by Hiw. The absence of NMNAT is a potential trigger for the initiation for Wallerian degeneration. Adapted from Xiong et al., 2012.

In *Drosophila melanogaster*, Nmnat has been identified as a factor which may stabilize the axon due to research done on Wld^{S} (Coleman, 2010). The process of Wallerian degeneration is thought to be mediated by the down-regulation of Nmnat at the synapse by the enzyme Hiw. Since Nmnat is synthesized and transported to the axon from the cell body, damage to axons may result in an inability to transport new Nmnat to axon and any Nmnat present in the axon at the time of injury would be down-regulated by Hiw (Xiong et al., 2012). Thus, functional Nmnat would be effectively removed from the distal axon which may trigger the process of Wallerian

degeneration (Xiong et al., 2012). These findings support a model in which a neuro-protective factor stabilizes mature axons, and the absence of the factor leads to the activation of a signaling pathway resulting in Wallerian degeneration. Now it is understood that there is an axonal factor which stabilizes axons and that its absence may play a role in initiating Wallerian degeneration.

The Role of Calcium in Injury Induced Wallerian Degeneration

Intra-axonal calcium levels transiently spike within the first 90 seconds of injury induced Wallerian degeneration (Knöferle et al., 2010; Freeman et al., 2012). The immediate influx of calcium triggers acute axonal degeneration by leading to the activation of calpain which cleaves neurofilament and microtubule associated proteins (Billger et al., 1988; Johnson et al., 1991). The widening of the injury site which results from AAD can be attenuated by removing extracellular calcium or by adding calcium channels blockers. Conversely, the progression of AAD can also be hastened by adding calcium ionophores (Knöferle et al., 2010). These findings demonstrate that extracellular calcium enters the axon after injury and that calcium influx acts as a trigger for acute axonal degeneration.

In addition to triggering AAD, calcium also appears to play a role in determining the length of the latency period. It has been observed that the removal of extracellular calcium or the addition of non-specific calcium channel blockers to cell culture media delays axon fragmentation after axotomy *in vitro* (George et al., 1995). These experiments also demonstrate that calcium channels facilitate influx after injury, offering insight into how calcium enters the axon after injury (George et al., 1995). Because removal of extracellular calcium from cultured neurons attenuates acute axonal degeneration and delays granular fragmentation, it is possible

that they share a common mechanism which is triggered by calcium. Alternatively, AAD and granular fragmentation may be triggered by separate mechanisms, which are independently activated by separate calcium influx events. This question remains unresolved, but provides two testable hypotheses. The first is that a single calcium influx event is sufficient to trigger AAD and granular fragmentation simultaneously through a common mechanism after nerve injury. Another hypothesis is that calcium may enter the damaged axon multiple times to independently activate the different phases of Wallerian degeneration.

It appears that calcium may have a role in the initiation of granular fragmentation. One study which has reported that internal calcium concentrations increase several hours after injury (LoPachin et al. 1990), but it is unclear how this influx of calcium proceeds or if this is actually necessary for Wallerian degeneration. It is likely that this calcium influx is also channel mediated, but an alternative explanation is that it could simply be the result of increased membrane permeability due to the destabilization and fragmentation of the axon during granular fragmentation. Although extracellular calcium has been shown to be essential for initiating the process of Wallerian degeneration, very little is known about how this process takes place or how it is regulated. These studies provided motivation to study a potential mechanism by which calcium could enter the distal axon segment after injury and the role it might play in the cascade which triggers Wallerian degeneration.

Drosophila as a Model to Study Wallerian Degeneration

Because rodent models of neurodegeneration are highly sophisticated and have limitations for studying Wallerian degeneration on a molecular basis, simpler models were sought in order to study this complex process. To help elucidate questions about the molecular

biology of Wallerian degeneration, researchers turned to the model organism *Drosophila melanogaster* which features a simple nervous system containing many of the same cell types found in vertebrates (Fang and Bonini, 2012). More importantly, the genome of *Drosophila melanogaster* is well studied and allows a high degree of manipulation.

Drosophila is a powerful model organism used to study physiology, genetics, life history, and evolution. It has become widely used because of its short intergenerational time, high fecundity, and suite of genetic traits which allow for a high degree of manipulation. The Gal4-UAS system present in *Drosophila melanogaster* is particularly useful for easily inducing the expression of transgenes in desired tissues. This system has made use of a serendipitous discovery about the reactivity of transposons in *Drosophila melanogaster* to insert genes containing the yeast Gal4 gene downstream of chosen a chosen promoter, restricting Gal4 expression to desired organs, tissues or even cells (Brand and Perrimon, 1993). Many different lines of flies expressing Gal4 with different promoters have now been created using enhancer trap insertions of Gal4 (Duffy, 2002). This is an extremely simple and elegant system which allows for a high degree of manipulation in the *Drosophila* nervous system which can be used to study molecular phenomena much more easily than in rodent models. This approach has been used throughout this thesis to label and express transgenes in specific neurons in order to study the process of degeneration. It has also been applied to express genetically encoded calcium sensors to monitor changes in intracellular calcium after injury.

While studying events relevant to traumatic brain injury, spinal cord injury, and complex neurodegenerative disorders in the fly may seem counterintuitive considering the fly lacks the complex structures involved, many of the molecular pathways leading to degeneration are

highly conserved, including protection by Wld^S (Fang and Bonini, 2012). Additionally, the fly has led to the discovery of new molecules involved in the process of degeneration such as Nmnat and Sarm (Xiong et al., 2012; Osterloh et al., 2013). Recently, assays to study events such as acute trauma have also been developed, such as the larval nerve crush developed in the Collins Lab. This assay involves a simple acute injury inflicted by pinching nerves through the cuticle with a pair of fine tipped forceps (Xiong et al., 2012). It has a high degree of precision and is extremely replicable. In addition to highly conserved molecular mechanisms, both chronic and acute models of axon degeneration in *Drosophila* mimic the timing and patterns fragmentation observed in mammalian systems (Fang and Bonini, 2012). All things in consideration, the model organism *Drosophila melanogaster* is an extremely powerful tool for studying the process of Wallerian degeneration.

While nerve crush assay offers a simple and replicable method for damaging axons, it does not allow for the addition of pharmacological agents or manipulation of ions since the cuticle remains intact. In order to address this problem I created the *ex vivo* assay which allows for such manipulations. In this preparation, larvae are injured according to the nerve crush protocol then immediately dissected in a media resembling the larval hemolymph. Because the formula for the media in which the larvae are incubated can be altered, this assay has allowed me to manipulate extracellular ions and to add pharmacological reagents in order to probe conditions that may interfere with the process of Wallerian degeneration. The *ex vivo* assay also allows for viewing live axons. This technique has been used in combination with the genetically encoded calcium sensor GCaMP to understand the timing and mechanisms by which calcium enters the axon after injury.

Ion Channels and Excitability

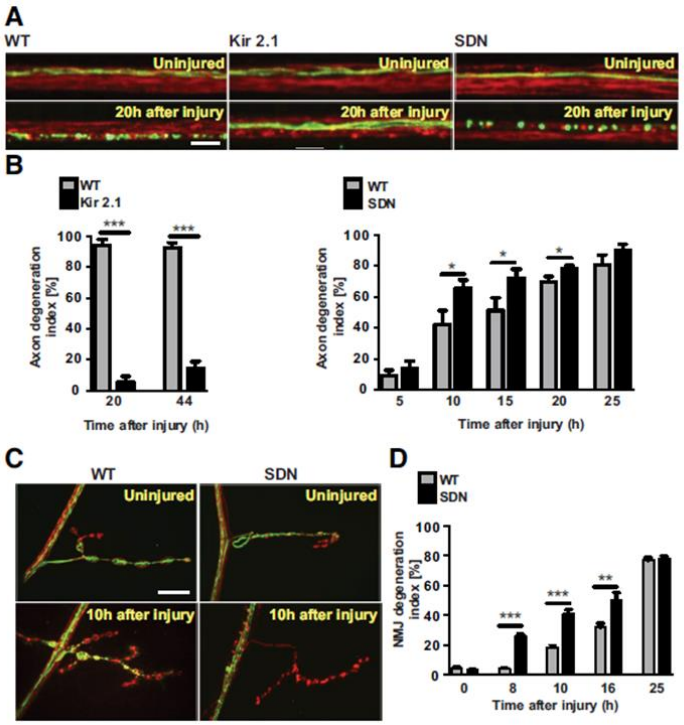
After axotomy, changes in electrical activity and excitability have been observed in the proximal as well as the distal axon segments (Titmus and Faber, 1990; Mishra et al., 2013). In a recent paper by Bibhu Mishra, a postdoc in the Collins lab, it has been observed that mutations in a number of ion channels present in the axolemma of drosophila melanogaster can change the rate at which axons degenerate after injury (Mishra et al., 2013). It has long been known that voltage gated ion channels are major players in regulating membrane excitability because they allow ions to flow across the plasma membrane (Armstrong et al., 1998). Mishra's observations suggest that Wallerian degeneration may be regulated in part by changes in sodium and potassium currents after injury, but it was not known why. Because increases in intracellular calcium concentration after injury have been shown to be channel mediated, I hypothesized that changes in membrane potential due to altered sodium and potassium currents lead to calcium influx through voltage gated ion channels after injury. I will explore this hypothesis throughout this thesis. In order to understand the manipulations performed, it is first important to understand the basics of voltage gated ion channels and the work of postdoc Bibhu Mishra. Both are summarized in this section.

Voltage gated ion channels are transmembrane channel proteins which respond to changes in membrane potential by changing conformation. Changes in conformation lead either the opening or closing of their channels and movement of ions across the plasma membrane. Change in membrane potential is detected by voltage sensors in the fourth transmembrane domain. Voltage sensors are made up of of groups of charged amino acids, which have the potential to change the shape of the ion channel. Most voltage gated ion

channels are sensitive and selective, meaning that they can respond to very small changes in membrane potential and are ion specific (Armstong et al., 1998; Jan et al., 1989). Mutations in voltage gated ion channels can change their permeability and responsiveness to changes in membrane potential, resulting in greater or lesser difficulty for ion entry into the axon. These changes alter the excitability of axons in which they are expressed and can be used to influence membrane potential (Mishra et al., 2013). Bibhu Mishra has used a number of these channels to manipulate membrane excitability in the study of Wallerian degeneration, including the Shaker Dominant Negative potassium channel, the Kir 2.1 rectifying potassium channel, and the para sodium channel.

The first channel mutation examined by Mishra et al. was the Shaker Dominant Negative (*SDN*) mutation. It decreases the function of Shaker potassium channels, which play a large role in regulating membrane excitability. This mutation causes an increase in membrane potential (Mosca et al., 2005) which Mishra has shown results in an increased rate of axonal degeneration and earlier onset of granular fragmentation (Mishra et al., 2013). Conversely, the mutant Kir 2.1 rectifying potassium channel demonstrates a decrease in membrane potential and is often used to silence neurons (Baines et al., 2001). Reduction in electrical activity delayed the onset of granular fragmentation and protected against degeneration (Mishra et al., 2013). Additionally, the para mutant has been observed to acutely inhibit voltage gated sodium channel function when shifted to a non-permissive temperature, which effectively decreases membrane potential (Siddiqi and Benzer, 1976; Wu and Ganetzky, 1980). When animals with the para mutation were shifted to the non-permissive temperature after axonal injury, the rate of axonal degeneration decreased dramatically (Mishra et al., 2013).

It has been observed that the initial, transient influx of calcium is channel mediated (Billger et al., 1988; Johnson et al., 1991), and this influx could occur through one or more voltage gated calcium channels. It is plausible that calcium influx could be regulated by changes in sodium and potassium currents after injury since the movement of ions across the membrane results in changes in membrane potential. I therefore hypothesized that changes in membrane potential due to the voltage gated channel mutations could change the rate of degeneration by influencing extracellular calcium influx after injury, which (as discussed above) is known to be necessary for the initiation of Wallerian degeneration. The channel mutations studied by Bibhu Mishra are used throughout this paper in order to better understand the relationship of sodium and potassium currents to membrane potential and calcium influx through voltage gated channels after injury.



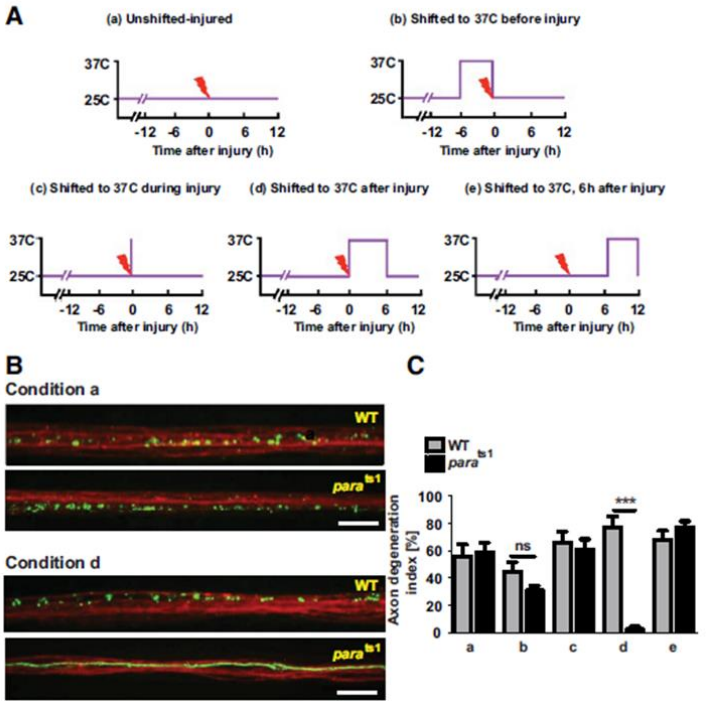


Figure 3. Sodium and potassium currents influence Wallerian degeneration. In the first panel, the Kir2.1 potassium channel causes a delay in axon degeneration, while the SDN potassium channel causes an increase in the rate of degeneration after injury. In the second panel, the para sodium channel delays degeneration. Adapted from Mishra et al., 2013. *P<.05, **P<.01, ***P<0.001. Error bars indicate standard error of the mean.

Overview

The experimental goal of this project is to understand the role of calcium in Wallerian degeneration using peripheral motor neurons in the model organism *Drosophila melanogaster*. Although it is known that calcium is important in the early stages of Wallerian degeneration, it is not known if it also plays a role during the latency period or in the initiation and propagation of granular fragmentation. To examine this, I established the *ex vivo* assay and used a number of transgenes affecting ion channels function which allowed me to use calcium chelating agents and channel blockers in combination with powerful genetic tools. My experiments confirm that calcium plays a central role in degeneration and is necessary multiple times throughout degeneration. My data suggest that sodium and potassium currents change the course of degeneration by regulating extracellular calcium influx after injury, likely through changes in membrane potential. I will then provide evidence which demonstrates that calcium influx is facilitated, at least in part, by voltage gated calcium channels which are regulated by sodium and potassium currents and that Wld^S can attenuate the influx of extracellular calcium after injury. In summary, this thesis will provide insights into the role of calcium in Wallerian degeneration and the regulation of calcium influx after injury.

METHODS

Nerve Crush Assay

The nerve crush assay was carried out according to the protocol established by Xiong et al. (2010). Third instar larvae were anesthetized using CO_2 and the injury was carried out using Dumostar® number 5 forceps by pinching the peripheral nerves through the cuticle. The site of injury was clearly visible through the cuticle and was done immediately distal to the nerve cord. After injury, larvae were kept on grape plates in a 25°C incubator until dissection and fixation.

***Ex vivo* Preparation**

Third instar larvae were selected for preparation and rinsed in distilled water. They were then anesthetized with carbon dioxide and axonal injury was induced using the nerve crush assay established by Xiong et al. (2010). The larvae were then immediately dissected and pinned down to an agar dissection plate in a solution of HL3 with 1mM of calcium, unless otherwise specified. During the dissection, only the cuticle, nervous system and muscle tissue of the body wall were left intact; all other organs were removed from the animal and discarded. The dissected larvae were left pinned on the dissection plate and incubated at 25°C in HL3 with 1mM calcium until the desired time point was reached at which time the larvae were fixed.

Immunochemistry

Dissected third instar larvae were initially rinsed in phosphate buffer solution (PBS) then fixed in either 4% paraformaldehyde in PBS for 25 minutes to view axon degeneration or in Bouin's fixative for 15 minutes to view degeneration at the neuromuscular junction. The fixative was then quickly rinsed with PBS and washed twice for 10 minutes using PBS containing 0.3%

Triton-X. The preparation were then stained using the following antibodies and dilutions: ms anti-Futch (1:100), Cy3 anti-HRP (1:500), A488 anti-ms, and A488 anti-GFP. Samples were mounted in Vectashield for viewing.

Genetics

Strains used include: *Canton- S* (WT), *m12*-Gal4 (Ritzenthaler et al., 2000), *Ok6*-Gal4 (Aberle et al., 2002), *SDN* (Mosca et al., 2005), $para^{ts1}$ (Siddiqi and Benzer, 1976; Wu and Ganetzky, 1980), $para^{ST76}$ (Siddiqi and Benzer, 1976), *UAS-Kir 2.1*(Baines et al., 2001), UAS-Wld^{s} (Hoopfer et al. 2006), and UAS-*GCaMP 3.0* (Nakai et al., 2001).

Imaging

Confocal imaging was done on an Improvision spinning disc confocal microscope at room temperature. This spinning disc system consists of a Yokagawa Nipkow CSU10 scanner and a Hamamatsu C9100-50 EMCCD camera mounted on a Zeiss Axio Observer. Either a 63x or 40x (1.3 NA) oil objective was used to take images used for quantification. Identical settings were used to compare animals with an experiment and similar settings were used across experiments of a similar nature. Velocity software (PerkinElmer) was used for acquisition.

Quantification

Axon degeneration was scored according to the fragmentation of *m12Gal4UASmcd8GFP* labeled axons within the peripheral nerves according to (Xiong and Collins, 2012). Axons were given a ranking of 0%, 25%, 50%, 75%, or 100% based on the extent of degeneration observed, with 100% being completely degenerated. All measurements constitute averages taken from a large number of axons from multiple animals while blinded to the genotype/manipulation (Figure 4A).

To quantify the extent of degeneration at the neuromuscular junction, a Futch:HRP coverage ratio was used. The Futch antibody binds to microtubules which extend throughout the terminals of the neuromuscular junction and the HRP antibody binds to membrane proteins in the axolemma. Since microtubules are cleared quickly after axonal injury while the axolemma stays intact for much longer time, the length of anti-Futch coverage divided by the length of anti-HRP coverage of the NMJ can be used to establish a ratio that indicates the extent of degeneration. Lengths were quantified using simple neurite tracer plugin of ImageJ software.

To quantify the relative amounts of intracellular calcium concentrations present, the average intensity of GCaMP 3.0 over the area of the nerve captured in the image was quantified using Volocity software (PerkinElmer). Average background intensity was subtracted from the average intensity of the nerve to account for any day to day variations in the imaging hardware (Figure 4B). All imaging was done at identical settings to ensure that the recorded data is comparable.

Drugs and Chemicals Used in *ex vivo* Preparation

Chemicals include PLTX-II (40nM) (Gu et al., 2009), EGTA (1mM) (Bett and Rasmusson, 2002), and TTX (1uM) (Lee and Ruben, 2008).

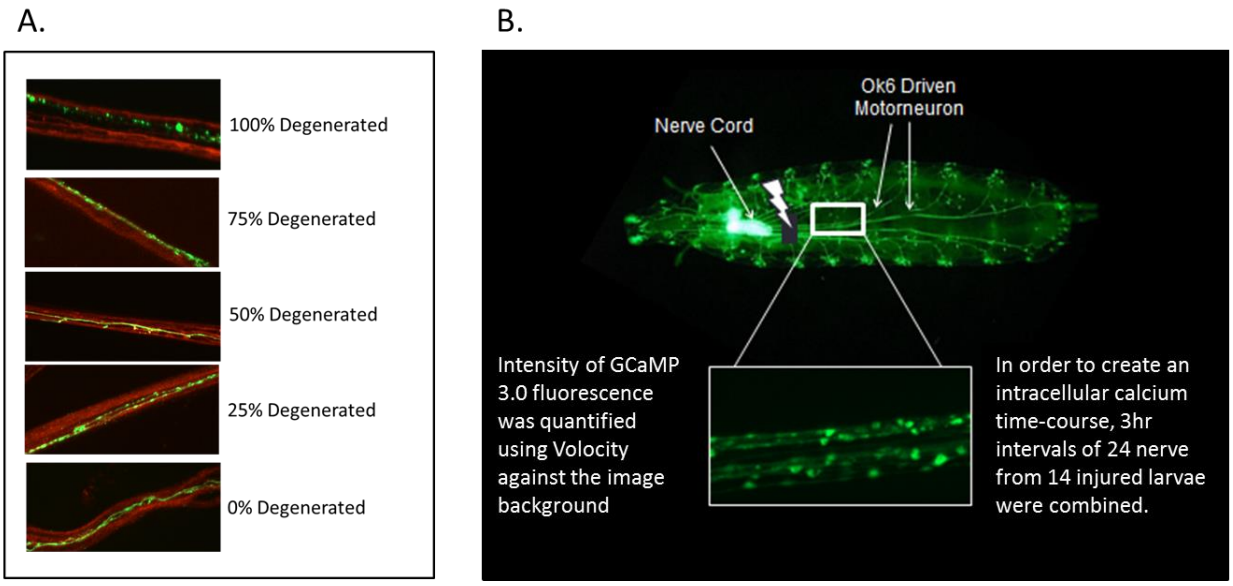


Figure 4. A) Axon degeneration index with representative images for each score. All axons scored are driven by *m12*-Gal4. While axon degeneration demonstrates continuous variability, the method used to quantify axon degeneration is discrete. B) Representative image and description of technique used to assemble the intra-cellular calcium time-course. All images were taken of *Ok6*-Gal4 driven nerves.

RESULTS

Development of the *Ex vivo* Assay

In order to study the effects of channel blocking drugs and ion chelating agents on motor neurons in *Drosophila melanogaster*, I developed a new assay in which desired ions and pharmacological agents could be added directly to intact nerves. Normally larvae were injured using the nerve crush assay then incubated at 25°C on a grape plate; the larvae are left intact but paralyzed from the injury. Later, the larvae were dissected on an agar dish and the tissue was fixed and treated for visualization. This worked well when studying the effects of genetic mutations on the process of degeneration, but in order to study the effects of chemical agents on degeneration it was necessary to establish a new assay. In order to expose the nerves to chemical agents, I created an assay in which the larvae were dissected prior to incubation in media which reflects the composition of larval hemolymph. This was essential in understanding the mechanistic question of how channel mutations change the rate of degeneration, an outstanding question posed by the post-doctoral fellow, Bibhu Mishra.

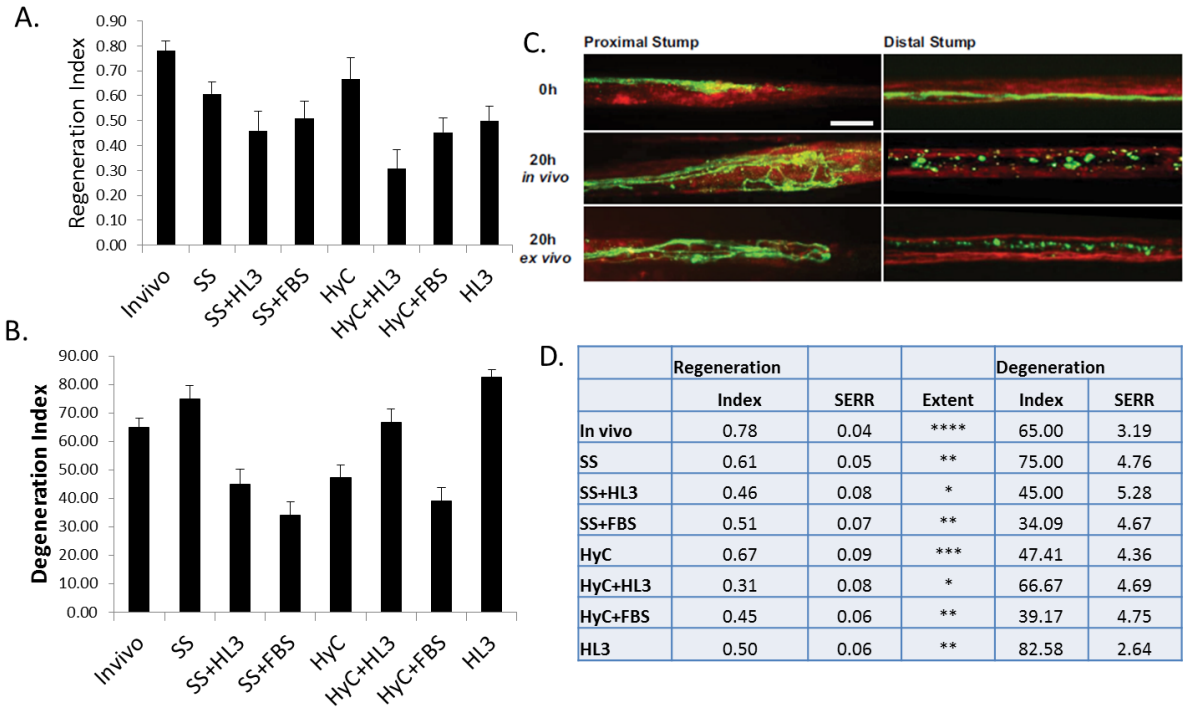
In order to develop such an assay, I needed to create a suitable environment in which exposed muscle and nervous system tissue could survive. A number of different conditions were examined including different media for incubation as well as temperature and mechanical conditions. I selected three solutions to screen for incubation including Shields and Sang Insect Media (Sigma), HyClone Insect Media (Thermo Scientific), and 1 mM calciumHL3. The first two solutions are commonly used for culturing insect neurons. I also tested the effect of adding fetal bovine serum (FBS), which is often used to encourage cellular growth and survival, to the tested

media. The third media I tested was HL3 which is used for electrophysiological recordings of neurons from drosophila larvae. A number of these solutions were also tested in combination with one another. The results are summarized in Figure 5. To test which solution worked the best I incubated larvae for 20 hours after injury at 25°C.

The results showed that HL3 had the highest index of degeneration while Shields and Sang and HyClone Insect Media resulted in the greatest index of regeneration. Interestingly, the extent of regeneration (the length to which axon sprouts had regrown) was not correlated with the index of regenerating axons (axons were defined as regenerating if they exhibited two or more axonal sprouts). The two insect media showed the highest extent of regeneration, but had the lowest index of regeneration. Unfortunately, the insect media solutions with the addition of FBS had a high occurrence of bacterial infestation which destroys results. HL3 did not have such problems.

Because my initial experiments revealed that incubating wild type larvae in a solution of 1mM Ca^{2+} HL3 at 25°C yields a similar morphological pattern of axonal degeneration to that observed in-vivo (intact larvae kept on a grape plate at 25°C after injury), a lack of bacterial infestation problems and a high regeneration index, I selected HL3 as the best medium for the *ex vivo* assay. Choosing a solution which yielded a high rate of regeneration was important because this indicates that the neurons are alive and carrying out the cellular processes involved in the injury response. While the extent of proximal segment regeneration in HL3 is less than that observed in in-vivo preparations, it was clearly visible when they were incubated in Shields and Sang or HyClone insect media indicating that regeneration was occurring. The lack of regeneration in HL3 is likely due to lower micronutrient nutrient levels or the absence of

growth factors since its composition is much simpler than either of the insect media, but since regeneration is not the focus of this study this was not necessarily a concern.



D.

	Regeneration			Degeneration	
	Index	SERR	Extent	Index	SERR
In vivo	0.78	0.04	****	65.00	3.19
SS	0.61	0.05	**	75.00	4.76
SS+HL3	0.46	0.08	*	45.00	5.28
SS+FBS	0.51	0.07	**	34.09	4.67
HyC	0.67	0.09	***	47.41	4.36
HyC+HL3	0.31	0.08	*	66.67	4.69
HyC+FBS	0.45	0.06	**	39.17	4.75
HL3	0.50	0.06	**	82.58	2.64

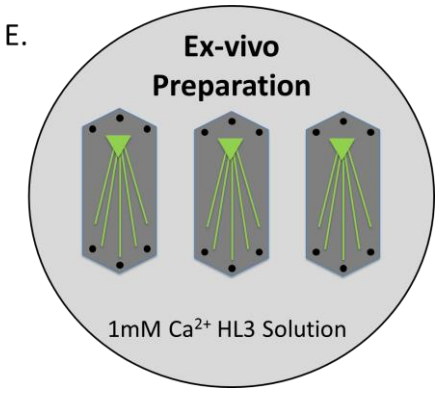


Figure 5. Summary of media testing for ex vivo assay. Abbreviations: SS (Shields and Sang), HyC (HyClone), FBS (Fetal Bovine Serum), & HL3 with 1mM Calcium HL3 A) Regeneration rate for different conditions is illustrated with standard error bars. This was calculated by the number of axons which had two or more axon sprouts at the distal end of the proximal stumps of injured axons. B) Degeneration index is illustrated with standard error bars. C) Images taken from representative animals demonstrating the presence of regeneration and degeneration in the ex vivo assay compared to in-vivo controls. D) Table demonstrating plotted data in addition to scores for the extent of regeneration. Extent was a qualitative description intended to summarize the length and number of axon sprouts extending from the proximal stump of injured axons; it does not take into consideration the regeneration index. More stars indicate more regrowth in the segment of the axon still connected to the cell body. E) Cartoon of *ex vivo* preparation

Wallerian Degeneration Proceeds Normally in the *Ex vivo* Assay

Even though the rate and morphology of degeneration in the *ex vivo* preparation was similar to that of an in-vivo preparation, one concern was that there might be a disruption of the signaling pathway involved in Wallerian degeneration in the distal stump. I next asked whether mutations that are known to affect Wallerian degeneration *in vivo* would similarly affect degeneration in the *ex vivo* assay. Similarity would suggest a shared molecular basis for Wallerian degeneration in the *in vivo* and *ex vivo* assays and validate the *ex vivo* assay's use for studying *Drosophila* mutants. I crossed four mutant backgrounds to *m12Gal4,UASmcd8GFP/TMβ6,Sb* larvae in order to compare the extent of axonal degeneration of *in vivo* to *ex vivo* preparations after axotomy: the Wallerian degeneration slow mutant (Wld^S), shaker dominant negative (*SDN*), silenced Kir 2.1 potassium channels, and $para^{ts1}$. The results are presented in Figure 6.

The Wld^S mutation encodes for a unique, chimeric protein that leads to strong protection in the distal segments of axons after axotomy (Wang et al., 2001). When observed 20 hours after axotomy, Wld^S mutants in the *ex vivo* preparation showed a decreased rate of axonal degeneration by nearly fifty percent when compared to wild type controls. Interestingly the Wld^S protection observed in the *ex vivo* assay is not as robust as protection seen *in vivo* (Figure 6A). This suggests that Wallerian degeneration in the *in vivo* and *ex vivo* assays have a shared molecular basis which is undisturbed by the steps involved in the *ex vivo* preparation.

The *SDN* mutation is a dominant negative mutation in the Shaker K^+ channel which has been shown to increase membrane excitability and lead to an increased rate of axonal degeneration after injury (Mishra et al., 2013). When observed 16 hours after injury, the

phenotype observed in the *ex vivo* preparation of the *SDN* mutant closely matched the phenotype observed by Mishra (2013) and showed a significantly higher rate of degeneration than the wild type control, nearly forty percent (Figure 6B).

Expression of Kir 2.1, a rectifying K^+ channel, effectively lowers the resting membrane potential of the neuron. Mishra had observed that expressing the Kir 2.1 channel in motor neurons slows axonal degeneration after injury, by roughly ninety percent when observed 20 hours after injury. This phenotype was replicated in the *ex vivo* preparation when observed 20 hours after injury and showed a significant decrease in the rate of degeneration when compared to the wild type control (Figure 6A).

Finally I chose to examine the $para^{ts1}$ mutant in the *ex vivo* preparation. When shifted to the non-permissive temperature for six hours after injury, the temperature sensitive $para^{ts1}$ mutation causes a decrease in the rate of axon degeneration by roughly half when observed 20 hours after injury. In the *ex vivo* preparation, this mutant demonstrates a nearly identical rate of degeneration observed *in vivo* (Figure 6C).

Phenotypes of wild type and mutant strains of *Drosophila melanogaster* in the *ex vivo* assay were observed to be nearly identical to those seen *in vivo*, providing evidence that Wallerian degeneration proceeds by the same molecular mechanisms in both instances. This assay is useful because it can be used to assess the effects of chemical agents applied to the surface of the nerves or changes in ion concentration. Unfortunately, the *ex vivo* assay cannot easily be used to visualize muscle tissue due to tissue degeneration. The mechanism by which the tissue degenerates in this assay is not well understood and makes studying degeneration at the neuromuscular junction difficult since the majority of muscles are not left intact beyond 16

hours of incubation in 1mM calcium HL3. While limits exist in the application of the assay, the strength of the *ex vivo* preparation lies in its simplicity and versatility.

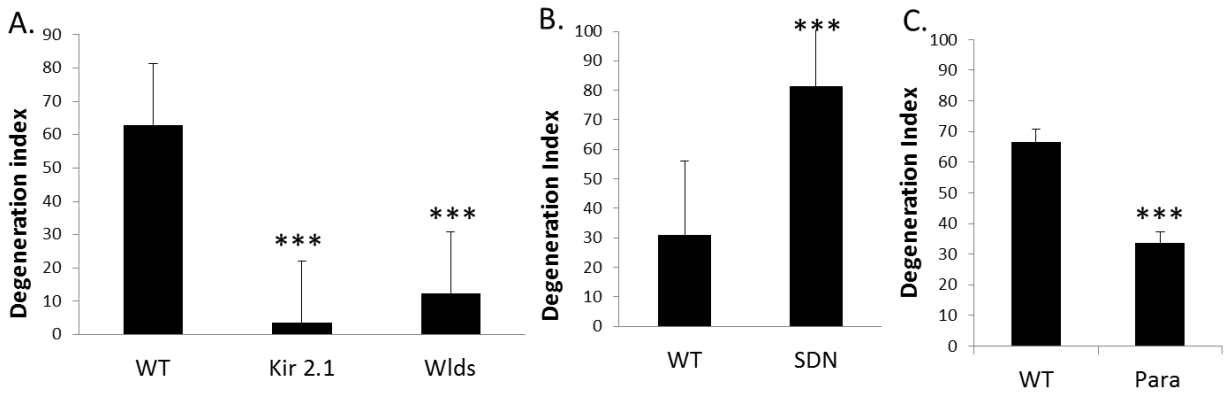


Figure 6. Mutant Wallerian degeneration phenotypes in ex vivo. A) Degeneration index for Kir 2.1 and Wlds mutants compared to wild type larvae in ex vivo assay 20 hours after injury. B) Degeneration index for SDN compared to wild type larvae in ex vivo assay 16 hours after injury. C) Degeneration index for Para^{ts1} larvae 20 hours after injury. Both the wild type and para larvae were temperature shifted from 0-6 hours after injury. *P<.05, **P<.01, ***P<0.001. Error bars indicate standard error of the mean.

Calcium is Necessary for Wallerian Degeneration in *Drosophila*

After establishing that the *ex vivo* assay was a reliable way to study axon degeneration, I sought to test the hypothesis that extracellular calcium is necessary for Wallerian degeneration. Using the *ex vivo* assay, I tested the hypothesis that extracellular calcium is necessary for axon degeneration in later stages of Wallerian degeneration including the latency period and granular fragmentation.

In order to test this hypothesis, two groups of wild type larvae were injured then dissected and incubated in HL3 solutions of either 1mM or 0mM calcium (Figure 7A). Since the larvae are injured before they are dissected, the injured nerves were briefly exposed to the

extracellular calcium present naturally in the larval hemolymph for roughly 90 seconds before the nerves were exposed to HL3. These first ninety seconds correspond with a transient spike in intracellular calcium levels. From about 90 seconds after injury to twenty hours after injury, larvae were incubated in either 1mM or 0mM calcium HL3. To ensure that there was truly no free calcium in the 0mM calcium HL3 solution, 1mM EGTA was added to the solution. EGTA selectively chelates extracellular calcium since it is not membrane permeable (Howard, 1984).

When extracellular calcium was removed after injury, the axons were robustly protected from axonal degeneration, which indicates a strong role for extracellular calcium in Wallerian degeneration even after the initial, transient influx (Figure 7A). The suggests that calcium is necessary later in degeneration and that signaling involved in acute axon degeneration is not sufficient to activate Wallerian degeneration in the absence of extracellular calcium.

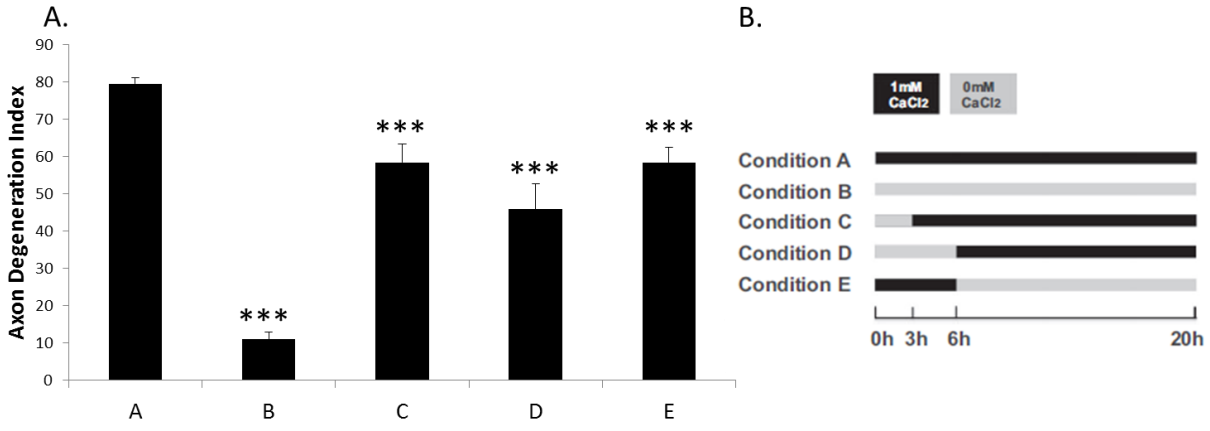


Figure 7. Effect of Extracellular Calcium Removal on Wallerian degeneration after nerve injury.. A) Graph showing extent of Wallerian degeneration after calcium removal during different phases of Wallerian degeneration including 0-20hours, 0-3hours, 0-6hours, and 6-20hours after nerve injury. Media used for incubation alternated between 1mM Ca2+ HL3 and 0mM Ca2+ HL3 with 1mM EGTA, which chelates external calcium. B) Figure legend for part A including description of calcium treatment. Calcium concentrations in all groups were 1mM and EGTA concentration in all groups was 1mM. *P<.05, **P<.01, ***P<0.001. Error bars indicate standard error of the mean.

Calcium is Important during the Latent and Granular Phases of Wallerian Degeneration

Next, I wanted to examine when calcium was most important during the process of degeneration and determine if the second calcium influx was necessary for degeneration or simply an artifact of the degenerative process. To explore this idea, I removed the calcium from solution during three intervals of an *ex vivo* experiment by altering the calcium concentrations in solutions of HL3. All animals were incubated at 25°C for 20 hours after injury, as was done in the previous experiment. The calcium removal intervals included 0-3 hours after injury, 0-6 hours after injury, and 6-20 hours after injury. The first two time intervals represent phases that correspond to the latent period of Wallerian degeneration since we have observed that in our hands the first signs of axon fragmentation do not begin until roughly six hours after injury, while the last interval corresponds to a time window that we associate with granular fragmentation.

Removal of calcium from 0-3 hours after injury resulted in modest, yet significant protection from axon degeneration (Figure 7A). Removal of calcium from 0-6 hours after injury resulted in a higher level of protection than what was seen in the 0-3 hour removal (Figure 4A). This suggests that the presence of extracellular calcium is necessary during the latent period of Wallerian degeneration in order for axon degeneration to occur.

When calcium was removed from 6-20 hours was more modest than calcium removal from 0-20 or 0-6 hours after injury, yet significant protection from degeneration was observed (Figure 7A). This finding suggests that the presence of extracellular calcium is necessary during the period of granular fragmentation as well as the latent period of Wallerian degeneration in order for axon degeneration to occur.

The protection that resulted from calcium removal from 0-20 hours was far more robust than removal during any of the other intervals, suggesting an important role for extracellular calcium throughout Wallerian degeneration. In fact, no signs of degeneration were observed when calcium was removed during the entire 0-20 hour period. It appears that calcium is necessary not only for acute axonal degeneration to occur but also the later phases of Wallerian degeneration; furthermore, calcium is necessary throughout the entire process, and removal of calcium at any of the time intervals examined significantly delayed degeneration. Alternatively, the presence extracellular calcium may promote general tissue degradation, which may explain why muscle tissue in the *ex vivo* assay appears less intact than in the *in vivo* assay.

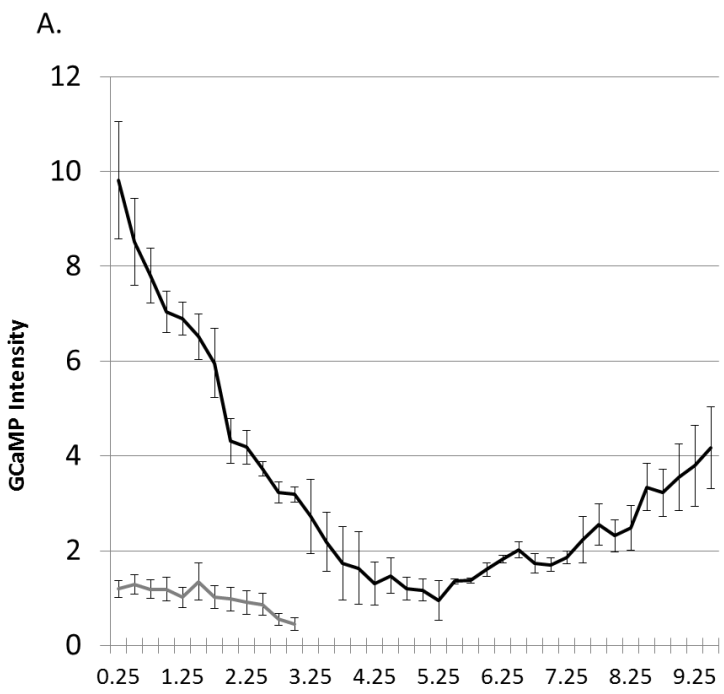
Intracellular Calcium Levels Change after Nerve Injury

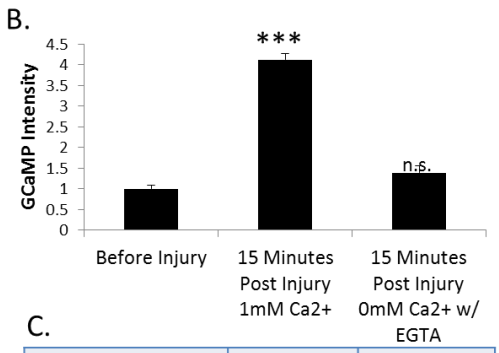
After the observation that extracellular calcium is essential during different time periods of Wallerian degeneration, I next hypothesized that changes in intracellular calcium should correspond to different events in Wallerian degeneration. Since calcium was needed in two temporally distinct time periods, it should follow that there are at least two distinct changes in intracellular calcium concentration. In order to assess this, I established a time course of what happens to intracellular calcium levels after injury in wild type larvae. Relative levels of calcium were compared using GCaMP 3.0 expressed in *OK6* driven motorneurons. But before constructing a time course, I compared the intensity of GCaMP3.0 fluorescence across different animals of the same genotype and determined they were in fact not significantly different ($p < 0.10$) (Figure 8C). This was essential since multiple animals were used to generate the graphic discussed in the following paragraph.

To construct an idea of how calcium levels fluctuate within nerves after injury, *ex vivo*

preparation larvae in 1mM calcium HL3 were imaged every 15 minutes over a three hour period of time, since after three hours the levels of fluorescence began to diminish in control animals (Figure 8A). Three hour intervals from 14 larvae were stitched together to create an image of intracellular calcium from fifteen minutes to 9.5 hours after injury. This reveals elevated levels of calcium that decrease gradually over the first three hours after injury before returning to near baseline levels. As predicted, it also shows that a second influx of calcium occurs beginning around seven hours after injury. These findings are consistent with the previous data which demonstrate an important role for calcium throughout the process of degeneration. Additionally, the second increase in calcium corresponds with the time when granular fragmentation is thought to begin.

To ensure that the fluctuations observed in the relative fluorescence of GCaMP 3.0 in the injured axons was due to extracellular calcium, larvae imaged in 0mM calcium HL3 were compared to those imaged in 1mM calcium HL3 15 minutes after injury. If the changes in fluorescence were due to influx of extracellular calcium, then no change should occur when extracellular calcium is removed. Larvae imaged in the 0mM calcium HL3 had significantly lowered levels of GCaMP 3.0 fluorescence than those imaged in 1mM calcium HL3 and were statistically equivalent to baseline measurements of GCaMP 3.0 fluorescence taken before injury (Figure 8B). These results indicate that the GCaMP 3.0 fluorescence observed in the injured larvae was dependent upon extracellular calcium.





C.

Ok6 GCaMP 3.0 Uninjured		
Animal	NC Mean	Axon Mean
1	633.73	
2	537.38	675.6
3	509.85	622.82
4	636.67	718.36
5	520.19	628.78
6	589.99	730.56
7	597.59	771.15
Mean	575.05	691.21
Standard Deviation	52.68	59.15
% Deviation	0.0916	0.0855

Figure 8. Changes in intracellular calcium level after injury. A) Graph showing average changes in intracellular calcium level after axotomy. The black line shows changes in intracellular calcium from 15 minutes after injury to 9.5 hours after injury in a wild type animal. The grey line shows the average calcium levels of uninjured larvae under the same conditions from 15 minutes to three hours after injury. B) Intracellular calcium levels before injury compared to 15 minutes after injury with 1mM calcium HL3 and 0mM calcium HL3. C) Table showing average variation in GCaMP 3.0 fluorescence in nerve cords and axons of uninjured larvae. *P<.05, **P<.01, ***P<0.001, n.s. not significant. Error bars indicate standard error of the mean.

Sodium and Potassium Currents Impact Wallerian Degeneration

To test the hypothesis that the ion channel mutations explored by Mishra alter the progression of degeneration by changing the influx of extracellular calcium after injury, I tested whether a relationship exists between the *para* mutation and calcium or if they have an additive effect. I assessed the effect of calcium removal from 0-20 hours after injury in the *para* mutant background compared to controls. The *para* mutation, which effectively reduces sodium influx,

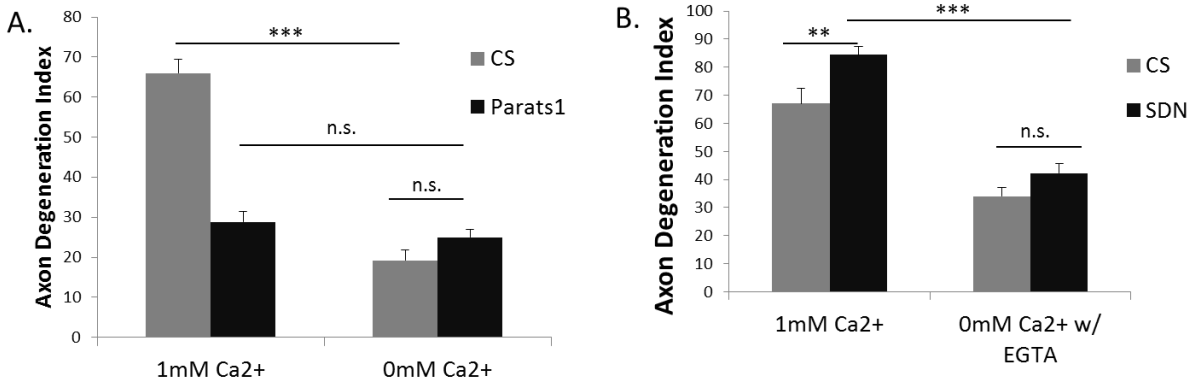
slows the rate of degeneration after axon injury (Mishra et al., 2013). Because calcium removal also slows the rate of degeneration after axon injury, removal of calcium from HL3 in the *ex vivo* assay should not enhance the protective phenotype of the *para* mutation if an epistatic relationship exists.

The results of the experiment demonstrate that calcium removal has no significant effect on the protective phenotype observed in larvae with the *para* mutation; thus, indicating the existence of an epistatic relationship (Figure 9A). This supports the hypothesis that the *para* mutation acts to slow degeneration by influencing the influx of extracellular calcium after injury.

In order to further test the hypothesis that ion channel mutations change the rate of axon degeneration by changing calcium influx after injury, I tested to see if a relationship exists between the *SDN* mutation and calcium as well. I assessed the effect of calcium removal from 0-16 hours after injury in the *SDN* mutant background compared to controls. The *SDN* mutation increases the rate of degeneration after axon injury (Mishra et al., 2013). Because the removal of calcium slows degeneration after injury, removal of calcium from solution in the *ex vivo* assay should slow the rate of degeneration compared to wild type levels if an epistatic relationship exists.

The results of the experiment indicate that *SDN* mutants incubated in 0mM calcium HL3 degenerate significantly more slowly compared to *SDN* mutants in 1mM calcium HL3 (Figure 9B). They also show that there is no significant difference in the rate of degeneration between wild type and *SDN* mutants incubated in 0mM calcium HL3, demonstrating that removal of calcium returned the rate of degeneration to levels seen in matching wild type controls (Figure 9B). This supports the hypothesis that the *SDN* mutation acts to change the rate of

degeneration by altering the influx of extracellular calcium.



C.

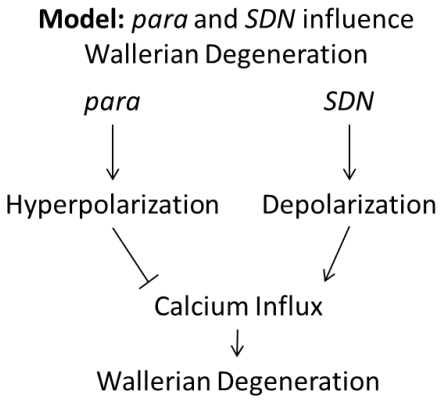


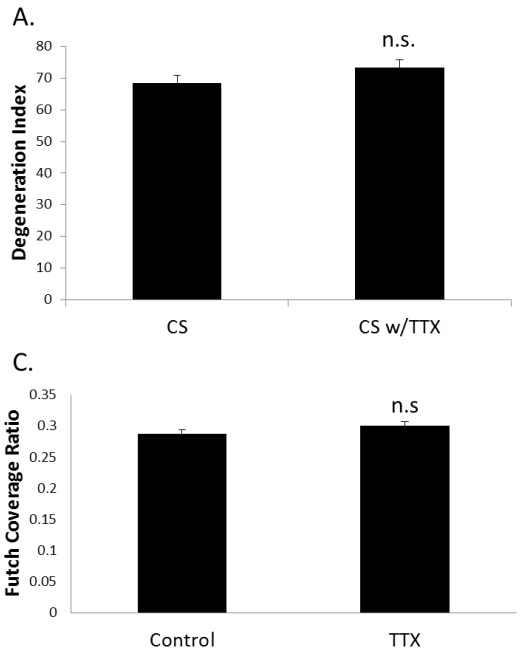
Figure 9. Channel mutations have an epistatic relationship with calcium removal for axon degeneration phenotypes. A) Degeneration index of wild type and para mutants with and without calcium 20 hours after axotomy. The temperature was shifted to 37°C from 0-6hours after injury in both the para and control animals. Controls are shown in grey and para mutants in black. B) Degeneration index of wild type and SDN mutant with and without calcium 16 hours after axotomy. Controls are shown in grey and SDN mutants are shown in black. *P<.05, **P<.01, ***P<0.001, n.s. not significant. Error bars indicate standard error of the mean. C) Model hypothesizing an epistatic relationship between the *para* and *SDN* mutations and the calcium influx event which triggers Wallerian degeneration.

Action Potentials Are Not a Signal of Axonal Degeneration

One of the phenotypes exhibited by *para* mutants is a difficulty in generating action potentials compared to wild type animals due to a sodium channel defect (Siddiqi and Benzer, 1976). Because sodium channels play a major role in the conduction of action potentials down the length of the axon and it is known that changes in membrane potential occur after injury, I wanted to test the hypothesis that action potentials could be involved in signaling the occurrence of axonal injury. In order to test this hypothesis, I used tetrodotoxin (TTX), which

blocks the voltage gated sodium channels involved in generating and conducting action potentials. After preparing larvae in the *ex vivo* assay, I incubated them in 1uM TTX in a solution of 1mM calcium HL3, a concentration known to effectively silence action potentials, for 20 hours. After preparing the tissue for imaging, I examined the degeneration index for both axons and neuromuscular junctions.

The results indicate that TTX has no effect on the rate of axon degeneration after nerve injury. Both axons and neuromuscular junctions degenerated at a similar rate to controls (Figure 10). This was not due to an inability of TTX to reach and block sodium channels since TTX was effective in preventing action potentials during electrophysiological recording (Mishra et al., 2013). These results suggest that action potentials are not necessary to initiate the process of Wallerian degeneration. It is interesting to note that blocking sodium channels with TTX does not result in the same phenotype as the *para* mutant since both treatments decrease the activity of sodium channels.



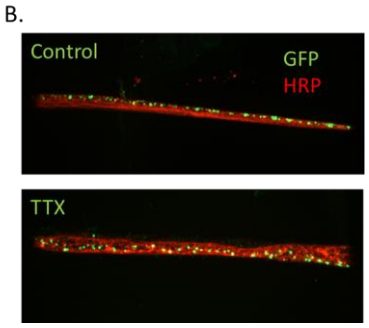


Figure 10. Action Potentials Are Not Necessary for Wallerian Degeneration A) Degeneration index comparing wild type larvae with and without 1uM TTX in the ex vivo assay from 0-20 hours after injury. B) Representative images for control animals in 1mM Ca2+ HL3 and experimental animals in 1uM TTX in 1mM Ca2+ HL3 in the ex vivo assay 20 hours after injury. C) Degeneration quantified by Futch coverage ratio at the neuromuscular junction for wild type larvae with and without 1uM TTX in 1mM Ca2+ HL3 in the ex vivo assay 20 hours after injury. *P<.05, **P<.01, ***P<0.001, n.s. not significant. Error bars indicate standard error of the mean.

Channel Mutations Alter Intracellular Calcium Levels after Nerve Injury

After collecting information on how intracellular calcium levels fluctuate in wild type larvae after injury and observing an epistatic relationship between calcium removal and channel mutations, I set out to test the hypothesis that the *para* and *SDN* mutations change the rate of degeneration by influencing the influx of extracellular calcium after injury. Because these mutations were shown by Mishra to have their greatest influence on degeneration in the first three hours after injury and the first three hours after injury showed considerable variation in GCaMP3.0 fluorescence in wild type animals after injury, I imaged the larvae before injury, 15 minutes after injury and then again three hours after injury. The amount of calcium in the nerves was visualized in nerves using GCaMP3.0 expressed in *Ok6* driven motorneurons of flies with mutant backgrounds.

I hypothesized that the *para* mutation would decrease the amount of intracellular calcium after injury, since both the *para* mutation and the removal of calcium slow the rate of degeneration after injury. The results demonstrate that after injury there is no significant increase in intracellular calcium concentration after nerve injury in the *para* mutant and that they may even decrease below pre-injury concentrations by three hours after injury (Figure 11A). Interestingly, the baseline levels of GCaMP 3.0 fluorescence taken before injury were elevated above the levels observed in the wild type, although it is not clear why such results were obtained. This data supports the hypothesis that the *para* mutation decreases the rate of degeneration after injury by preventing calcium levels from rising above their baseline concentrations.

I next hypothesized that the *SDN* mutation would increase the concentration of

intracellular calcium after injury, since it increases the rate of Wallerian degeneration after injury. The results demonstrate that calcium concentrations after injury are not significantly higher than those of wild type animals, but do reveal that calcium levels stay elevated significantly longer than would be expected in a wild type animal (Figure 11B). This data supports the hypothesis that the *SDN* mutation increases the rate of degeneration by maintaining relatively elevated intracellular calcium levels compared to wild type animals after injury.

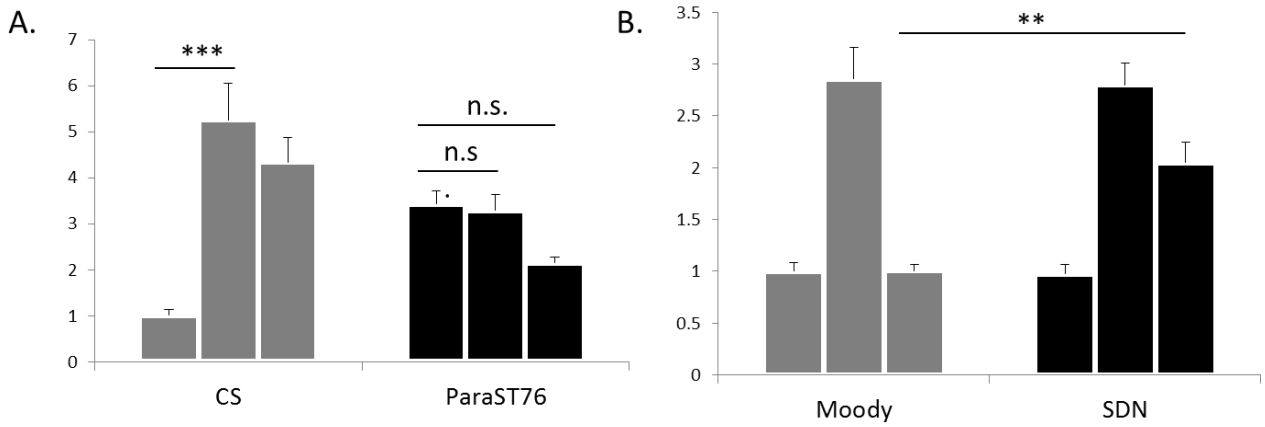


Figure 11. Channel Mutations and the Wlds protein affect intra-axonal calcium concentration after axotomy. GCaMP levels in each experiment were normalized to the intensity of fluorescence of the wild type control before injury and identical imaging settings were used for all imaging within each experiment. From left to right the three vertical bars indicate fluorescence before injury, 15minutes after injury, and three hours after injury. A) Comparison of GCaMP fluorescence control and para mutant larvae. B) Comparison of GCaMP fluorescence control and SDN mutant larvae. Moody represents the control group.*P<.05, **P<.01, ***P<0.001, n.s. not significant. Error bars indicate standard error of the mean.

Calcium Channel Blocking Drug PLTXII Delays Degeneration

Since I observed that removing calcium from solution could slow the rate of degeneration after axon injury and that the amplitude of the calcium influx of depends on the permeability of other ions, I hypothesized that a voltage calcium channel is responsible for the

influx of calcium after axon injury. Changing the permeability of sodium and potassium in the axon alters the membrane potential which influences the tendency for other charged molecules, such as calcium, to enter the axon. Because voltage gated calcium channels open and remain open in response to increased membrane potential, altered electrical activity due to the *para* and *SDN* mutations could be responsible for the changes in the rate of degeneration.

To examine this hypothesis I applied a calcium channel blocking toxin, called Plectreurys toxin II (PLTXII) which blocks type 2 calcium channels (Gu et al., 2009), to exposed nerves using the *ex vivo* preparation. 40 nM PLTXII in 1mM calcium HL3 was applied directly to nerves from 15 minutes after injury until 20 hours after injury, 15 minutes after injury to three hours after injury, or 3 hours after injury to 20 hours after injury. All larvae were fixed at 20 hours after injury and were kept in 1mM calcium HL3 solution when not exposed to PLTXII in a solution of 1mM calcium HL3. Unfortunately, PLTXII could not be applied to the nerves sooner because of the dangers of dissecting larvae in a potent neurotoxin. Degeneration of nerves was assessed by using GFP labeled axons driven by *m12*-Gal4 which is expressed in two motorneurons per nerve. In addition to axon degeneration, the effect of 40nM PLTXII on the neuromuscular junction was also accessed when applied from 15 minutes to 20 hours after injury.

The results show a weak delay in degeneration of injured NMJ's (Figure 12A) and axons (Figure 12C) when PLTXII was applied from 15 minutes after injury until fixation of the tissue, 20 hours later. Additionally axons show significant protection when PLTXII is applied only in the first three of 20 hours of incubation after injury when compared to controls, but there is only a modest delay in degeneration for axons incubated in PLTXII from three to 20 hours after injury (Figure 12C). This suggests that while the initial influx extracellular calcium may be meditated by

voltage sensitive ion channels blocked by PLTXII, the second influx may not depend on entry through these channels.

The effects of PLTXII confer less robust protection compared to complete removal of calcium may be due to the fact that the type 2 calcium channels which PLTXII is known to block are likely localized near the synapse, due its role in the stimulus secretion coupling mechanism. This would restrict the ability of calcium to quickly disperse throughout the length of the axon, suggesting that other calcium channels may be involved. Additionally, the late application of the drug to the preparation may help to explain the weak phenotype. I hypothesize that there are other mechanisms for the entry of calcium into the axon besides voltage gated calcium channels which also contribute to the elevated levels of calcium after injury such as a reversal of the sodium-calcium exchanger which has been shown to play a role in an astrocyte injury-like response (Gu et al., 2009) or a release of intracellular calcium stores from axoplasmic reticulum.

PLTXII Blocks Channel Regulated by Sodium and Potassium Currents

After observing that PLTXII had an effect on the rate of degeneration after injury, I wanted to determine if the channels blocked by this drug were regulated by sodium and potassium currents. In order to this, I incubated *para* and *SDN* mutant larvae in the *ex vivo* preparation in 40nM PLTX II in 1mM Ca^{2+} HL3 and compared their rate of degeneration relative to controls. If the voltage gated Calcium channels blocked by PLTXII is regulated by sodium and potassium currents the application of PLTXII should demonstrate an epistatic relationship to the *Para* and *SDN* mutations such that no additional protective effect of PLTXII be observed in *para* mutants compared to *para* mutants incubated 1mM Ca^{2+} HL3. Additionally, if this held true I would expect that the enhanced degeneration effect be suppressed in the *SDN* mutants in

PLTXII compared to *SDN* mutants in 1mM Ca^{2+} HL3. Degeneration index results were compared using a relative scale since incubation of *para* larvae in either 1mM Ca^{2+} HL3 or 40nM PLTXII in 1mM Ca^{2+} took place for 20 hours while incubation of *SDN* larvae took place for only 16 hours.

The results reveal that there is no significant protective effect observed in the *para* mutants incubated in PLTXII compared to *para* mutants incubated in a solution of HL3 (Figure 12E). This suggests that the voltage gated calcium channels blocked by PLTXII are regulated after injury by changes in sodium currents since an epistatic relationship exists between the two. Additionally, the results demonstrate that PLTXII suppresses the effects of the *SDN* mutation on axon degeneration. It reduced the level of degeneration seen in the *SDN* mutant to that observed in the relevant wild type controls incubated in PLTXII (Figure 12E). This result suggests that voltage gated calcium channels blocked PLTXII are also regulated by potassium currents after injury due to an epistatic relationship. Taken together these results offer additional support for the hypothesis that changes in membrane potential after axotomy facilitate the entry of calcium into the portion of the axon separated from the cell body.

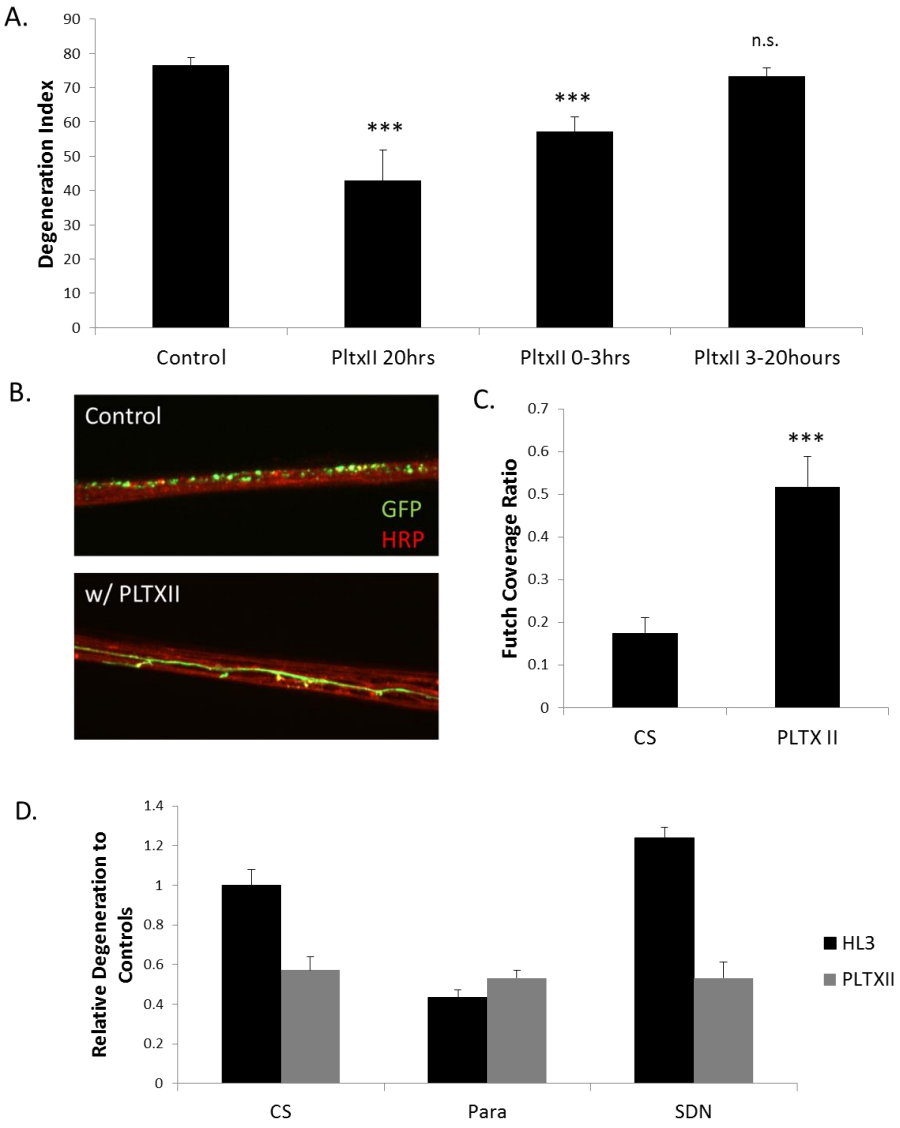


Figure 12. PLTXII blocks calcium channels and prevents axon degeneration at the axon and NMJ A) Axon degeneration index for ex vivo preparations treated with 40nM PLTXII in 1mM Ca2+ HL3 immediately after injury and fixed 20 hours after injury. Treatments include PLTXII from 0-20, 0-3, and 3-20 hours after injury. B. Representative images for axons incubated in 1mM Ca2+ HL3 with or without 40 nM PLTXII for 20 hours after injury. C. Futch coverage ratio representing the extent of NMJ degeneration after treatment of 40nM PLTXII in 1mM Ca2+ HL3 for 20 hours after injury. D. Axon degeneration scores for ex vivo preparations treated with 40nM PLTXII in 1mM Ca2+ HL3 in mutant backgrounds. Black bars indicate control treatment and grey bars indicate PLTXII treatment for different groups. para mutants were shifted from 0-6hours after injury. *P<.05, **P<.01, ***P<0.001, n.s. not significant. Error bars indicate standard error of the mean.

Wld^S Changes Intracellular Calcium Influx after Injury

The mechanism by which Wld^S acts is not well understood. I decided to test if the Wld^S mutation affects intracellular calcium concentration after nerve injury to better understand how the Wld^S protein affects the progression of axon degeneration. In order to study the effect of this enzyme on axon degeneration, I observed GCaMP 3.0 fluorescence in nerves containing

Ok6 driven motorneurons before injury, 15minutes after injury and three hours after injury in both mutant and wild type backgrounds.

The results indicate that Wld^S, which delays axon degeneration after injury, diminishes the relative increase in intracellular calcium concentration after injury (Figure 13C). This suggests that the Wld^S likely protects axons from degeneration by attenuating the increase in intracellular calcium after injury. Interestingly, the baseline levels of GCaMP 3.0 fluorescence were elevated above that of the wild type controls, similarly to the *para* mutant. Examination and comparison of Figure 11A and Figure 13A reveal that axon protective mutations diminish changes in intracellular calcium concentrations after injury, yet have elevated baseline GCaMP 3.0 fluorescence compared to controls. Unfortunately the second observation is not well understood, but could be due to compensatory mechanisms which help to counter the effects of the chronic mutations or simply be due to higher levels of GCaMP3.0 expression in these animals.

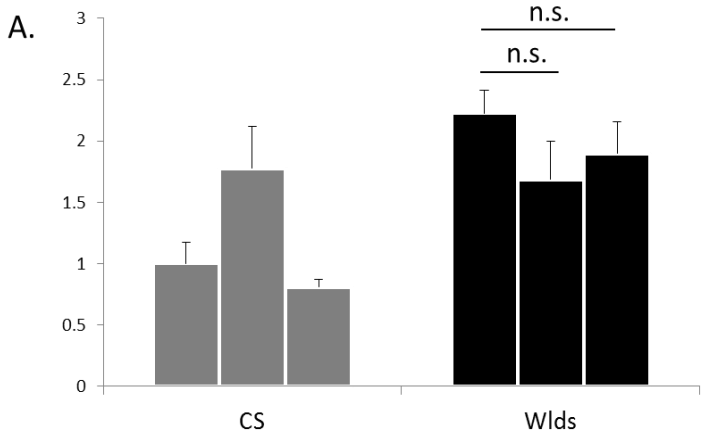


Figure 13. No change in GCaMP fluorescence in Wlds mutant larvae A. Relative GCaMP levels before, 15 minutes, and 3 hours after injury for wild type and Wlds animals. Dissected animals were kept in a solution of 1mM Ca2+ HL3 during the duration of the experiment. *P<.05, **P<.01, ***P<0.001, n.s. not significant. Error bars indicate standard error of the mean.

Discussion

Conclusion

This thesis has demonstrated a new way to study Wallerian degeneration in *Drosophila melanogaster* larvae using the *ex vivo* assay in addition to a simple preparation for observing changes in levels of intracellular calcium in peripheral motorneurons. I have used these techniques to show that extracellular calcium is necessary throughout the process of Wallerian degeneration and that the influx of calcium is regulated by sodium and potassium currents. I have shown that the influx of calcium takes place, at least partially, through voltage gated calcium channels present in the axon and that the Wld^S mutation can prevent calcium influx after injury.

Future Applications of the *Ex vivo* Assay

The *ex vivo* assay has proven incredibly useful for studying the role of calcium after nerve injury and has led to new insights in how calcium influx is regulated in Wallerian degeneration after nerve injury. Currently another undergraduate student in the lab, Grace Kim, is using the assay to study the role of reactive oxygen species in Wallerian degeneration. This assay has allowed her to apply a number of pharmacological agents, including electron transport chain inhibitors, directly to larval neurons. Her work is featured in another thesis being presented this year. The value of this assay lies in its simplicity and ease of use. One simply needs to add the desired pharmacological agents or ions to HL3 solution and observe the effects on peripheral and central neurons to study cellular processes. I have demonstrated that changes in ion balance and addition of channel blocking toxins can successfully be applied to

study neurons, but there are many more potential applications for this assay. I will provide some ideas here.

The *ex vivo* assay provides access to study neurons, both central and peripheral, in the larval *Drosophila melanogaster* nervous system. In addition to studying Wallerian degeneration in axons and at the neuromuscular junction, the process of regeneration could also easily be studied using this assay. It has been shown that is easy to visualize motorneurons which express GFP driven by both *Ok6*-Gal4 and *m12*-Gal which drive expression in the entire nerve or two motorneurons per nerve, respectively. Therefore, it should be easy to study changes in the segment of neuron still attached to the cell body after injury. While the extent of regeneration was less robust in the *ex vivo* preparation compared to controls, the addition of growth factors commonly used in cell cultures restores regeneration to near control levels. By slightly modifying the protocols outlined here, changes in ion concentration and addition of pharmacological agents could be applied to study regeneration in the larval nervous system.

In addition to leaving the nervous system intact, the *ex vivo* assay also leaves the muscles of the body walls intact. Many models of muscle degeneration have been developed in *Drosophila melanogaster* including spinal muscular atrophy, myotonic dystrophy, and a number of other neuromuscular degenerative disorders (Lloyd and Taylor, 2010). Additionally, axons could be left uninjured in the *ex vivo* preparation in order to study chronic forms of axon degeneration, like Parkinson's disease. The *ex vivo* assay could easily be applied in the study of such conditions and would allow for the combination of powerful genetic and pharmacological methods.

Calcium Influx after Nerve Injury

Using two techniques this thesis has demonstrated that extracellular calcium influx after injury is essential during both the latent period and granular fragmentation phase of Wallerian degeneration. It is clear that extracellular calcium in necessary for Wallerian degeneration to occur normally, but it is not clear whether or not this makes up the majority the of the increase in calcium observed within the axon after injury. It is possible that the initial influx of calcium through voltage gated ion channels causes the release of internal stores of calcium commonly sequestered in the axoplasmic reticulum and axonal mitochondria. In the heart, there are calcium channels which open in response to calcium binding, and it is thought that this process is involved in stimulus excitation coupling (Fabiato, 1983). If such a system existed within the axon, it would help explain the rapid increase in intracellular calcium levels after injury by providing a mechanism by which calcium influx through voltage gated ion channels at the synapse could quickly disperse throughout the entire length of the axon. Alternatively it is possible that increases in internal calcium may be mediated by calcium channels distributed along the length of the axon, which will be discussed later in the discussion.

A gradual decrease in the concentration of calcium in the axon after nerve injury has been observed from 15 minutes to around 3 hours after injury, but the functional significance of this is not yet understood. This event corresponds to the latency period of Wallerian degeneration. It seems likely that calcium is necessary during this phase since removal delays the process of degeneration.

The second increase observed in intracellular calcium around seven hours after injury is much less well documented in the literature, but hypotheses predicting a second influx have been suggested. It is possible that this second increase in intracellular calcium is also dependent

upon extracellular calcium since removal of calcium from solutions used in the *ex vivo* assay six hours after injury resulted in a delay in degeneration. It is not known whether or not voltage gated calcium channels play a large role in this process, since PLTXII application from 3-20 hours after injury was only slightly protective of axons. It could be the case that calcium influx could correspond to onset of axon degeneration in single axons. This could be tested if calcium imaging could be performed in single axons in order to more closely correlate the two events. Alternate explanations could be that calcium influx could occur through a weakened or disturbed axonal membrane to help trigger granular fragmentation. While much has been elucidated about the process of calcium influx after injury, there is still much to be learned.

Relationship between Sodium and Potassium Currents to Calcium Influx

This thesis has demonstrated a relationship between sodium and potassium currents through mutated voltage gated ion channels implicated in axon potential conduction. These channels operate through local changes in membrane potential and lead to further changes in the membrane potential. While this thesis does not directly demonstrate that wild type versions of the sodium and potassium channels studied are implicated in regulating calcium influx during Wallerian degeneration, it does support the conclusion that changes in membrane potential are likely involved in the process of calcium regulation after nerve injury. Bibhu Mishra has demonstrated that the mutations studied do result in changes in membrane potential through electrophysiological recordings (Mishra et al., 2013). Since this paper has shown that calcium influx after injury takes place, at least in part, through voltage gated calcium channels, regulation likely takes place through changes in membrane potential.

In addition to voltage gated calcium channels at the synapse there are number of other channels that may also depend on membrane potential and ion balance, which may facilitate calcium influx after injury. A potential channel which could be involved is the sodium-calcium exchanger. This is a secondary active ion channel which moves sodium ions into the cell and calcium ion out of the cell in order to keep intracellular calcium concentrations low (Gu et al., 2009). This channel operates based on the gradient established by the sodium potassium exchanger, which uses ATP driven transport to facilitate a number of cellular functions. Since the sodium and potassium channel mutations studied in this thesis were observed to change the membrane potential (Mishra et al., 2013), it is likely that the ion balance after injury is disturbed. This could form to a gradient which promotes the influx of calcium through the sodium-calcium exchanger into the cell after injury. A change in membrane potential would likely disturb a number of voltage gated channels after injury, resulting in functional changes promoting Wallerian degeneration.

Integrated mechanism for Calcium Influx and Wld^S Protection

Because Wld^S protects from many different forms of neuron degeneration, a model which provides a universally protective mechanism is highly sought after. One model which has recently been studied by Shen and colleagues is that Wld^S prevents degeneration by maintaining energy balance. They have shown that induced energy deprivation promotes an increase in membrane potential and the influx of calcium after injury and that these phenotypes can be partially attenuated by the *Wld^S* mutation (Shen et al., 2013). Their manipulation of energy balance involves removing all pathways leading to the production of ATP. This suggests the segment of the axon separated from the cell body undergoes energy

deprivation and subsequently a change in membrane potential and influx of extracellular calcium. Shen attributes the protective effect of Wld^S to its Nmnat activity, which could create an additional pathway for the emergency synthesis of ATP during a crisis, such as Wallerian or even chronic degeneration.

One potential mechanism by which a reduction in levels of ATP could lead to the influx of extracellular calcium is by decreasing the activity level of the sodium-potassium exchanger. Since this ion pump moves ions up their respective concentration gradients, it requires ATP to provide the energy for the reaction (Skou, 1989). In the absence sodium-potassium pump activity, the membrane potential and ion balance of injured neurons could become disturbed and lead to a depolarizing effect which could impact voltage gated ion channels such as the voltage gated calcium channel studied in this experiment as well as change the direction of movement of ions through secondary active transporters, such as the sodium-calcium exchanger. This model of the protective effect of Wld^S protection has been examined in acute forms of neurodegeneration such as Wallerian degeneration, but it has not yet been studied in longer models of chronic degeneration. If this model is shown to be correct, it would be in agreement with the result presented in this thesis that the Wld^S mutation prevents calcium influx after axotomy in *Drosophila melanogaster* larvae.

Acknowledgments

I would like to thank Catherine Collins for being a fantastic mentor over the last two years and for providing me with both the direction and freedom needed to complete this thesis. My experience in her lab has been educational, thought provoking and extremely rewarding.

I would also like to thank Bibhu Mishra for teaching me the skills needed to carry out my experiments and for being a spring of information about my project and the Collins lab in general. Over the last two years he has been both a mentor and friend to me.

I would like to acknowledge the entire Collins lab, past and present, that I have had the opportunity to work alongside. They have provided me with a productive space in which to perform research and think critically about my work.

I would also like to thank my readers Dr. Hume and Dr. Shafer for agreeing to help me in the completion of my thesis. You time is greatly appreciated.

References

Aberle H, Haghighi AP, Fetter RD, McCabe BD, Maga˜lhaes TR, Goodman CS (2002) Wishful thinking encodes a BMP type II receptor that regulates synaptic growth in *Drosophila*. Neuron 33:545–558.

Armstrong CM and Hille B (1998) Voltage-Gated Ion Channels and Electrical Excitability. Neuron 20:371-380.

Avery MA, Rooney TM, Pandya JD, Wishart TM, Gillingwater TH, Geddes JW, Sullivan PG, Freeman MR (2012) WldS prevents axon degeneration through increased mitochondrial flux and enhanced mitochondrial Ca2+ buffering. Curr Biol 22:596–600.

Baines RA, Uhler JP, Thompson A, Sweeney ST, Bate M (2001) Altered electrical properties in *Drosophila* neurons developing without synaptic transmission. J Neurosci 21:1523–1531.

Billger M, Wallin M, and JO Karlsson (1988) Proteolysis of tubulin and microtubule-associated proteins 1 and 2 by calpain I and II. Difference in sensitivity of assembled and disassembled microtubules. Cell Calcium. 9:33–44.

Brand AH and Perrimon N (1993) Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Development. 118:401-415.

Christie JM, Delia NC, and Craig E Jahr (2011) Ca2+-dependent enhancement of release by subthreshold somatic depolarization. Nature 14(1):62-69.

Coleman M (2005) Axon Degeneration Mechanisms: Commonality Amid Diversity. Nature Reviews 6:889-898.

Duffy JB (2002) GAL4 System in *Drosophila*: A Fly Geneticist's Swiss Army Knife. Genetics 34:1-15.

Fabiato A (1983) Calcium-induced release of calcium from the cardiac sarcoplasmic reticulum. Am J Physiol 245(1):1-14

Fang Y and Nancy M. Bonini (2012) Axon Degeneration and Regeneration: Insights from *Drosophila* Models of Nerve Injury. Annu. Rev. Cell Dev. Bio 28:575-597.

George EB, Glass JD, and John W. Griffin (1995) Axotomy-Induced Axonal Injury Is Mediated by Calcium Through Ion Specific Channels. Journal of Neuroscience 15(10):6445-6452.

Gu H, Jiang SA, Campusano JM, Iniguez J, Su H, Hoang AA, Lavian M, Sun X, and O'Dowd DK (2009) Cav2-Type Calcium Channels Encoded by *cac* Regulate AP-Independent Neurotransmitter Release at Cholinergic Synapses in Adult *Drosophila* Brain. J Neurophysiol 101:42-53.

Hoopfer ED, McLaughlin T, Watts RJ, Schuldiner O, O'Leary DD, et al. (2006) Wlds protection distinguishes axon degeneration following injury from naturally occurring developmental pruning. Neuron 50: 883–895.

Johnson GV, Litersky JM, and RS Jope (1991) Degradation of microtubuleassociated protein 2 and brain spectrin by calpain: a comparative study. J. Neurochem. 56:1630–1638.

Kerschensteiner, M, Schwab ME, Lichtman JW, and Misgeld T (2005) *In vivo* imaging of axonal degeneration and regeneration in the injured spinal cord. Nat. Med. 11:572–577.

Knöferele J, Koch JC, Ostendorf T, Michel U, Planchamp P, Vutova P, Tönges L, Stadelmann C, Brück W, Bähr M, and Paul Lingor (2010) Mechanisms of acute axonal degeneration in the optic nerve *in vivo*. PNAS 107(13):6064-6069.

Lee CH and Ruben PC (2008) Interaction between voltage-gated sodium channels and the neurotoxin, tetrodotoxin. Landes Bioscience 2(6): 407-412.

Lloyd TE and Taylor JP (2010) Flightless Flies: *Drosophila* models of neuromuscular disease. Ann N Y Acad Sci 1184:1-20.

LoPachin RM Jr, LoPachin VR, Saubermann AJ (1990) Effects of axotomy on distribution and concentration of elements in rat sciatic nerve. J Neurochem 54:320 –332.

Lunn ER, Perry VH, Brown MC, Rosen H, Gordon S (1989) Absence of Wallerian degeneration does not hinder regeneration in peripheral nerve. Eur. J. Neurosci. 1:27–33

Mishra B, Carson R, Hume RI, Collins CA (2013) Sodium and Potassium Currents Influence Wallerian Degeneration of Injured *Drosophila* Axons. The Journal of Neuroscience 33(48):18728-18739.

Mosca TJ, Carrillo RA, White BH, Keshishian H (2005) Dissection of synaptic excitability phenotypes by using a dominant-negative shaker K+ channel subunit. Proc Natl Acad Sci U S A 102:3477–3482.

Osterloh JM, Yan J, Rooney TM, Fox AN, Adalbert R, Powell EH, Sheehan AE, Avery MA, Hackett R, Logan MA, MacDonal JM, Ziegenfuss JS, Milde S, Hou Y, Nathan C, Ding A, Brown RH, Conforti L, Coleman M, Tessier-Lavigne M, Züchner S, and MR Freeman (2012) dSarm/Sarm1 Is Required for Activation of an Injury-Induced Axon Death Pathway. Science 337:481-484,

Oudega, M (2013) Inflammatory response after spinal cord injury. Experimental Neurobiology 250:151-155.

Ritzenthaler S, Suzuki E, Chiba A (2000) Postsynaptic filopodia in muscle cells interact with innervating motoneuron axons. Nat Neurosci 3:1012– 1017.

Siddiqi O, Benzer S (1976) Neurophysiological defects in temperaturesensitive paralytic mutants of *Drosophila* melanogaster. Proc Natl Acad Sci U S A 73:3253–3257.

Shen H, Hyrc KR, and Mark P. Goldberg (2013) Maintaining energy homeostasis is an essential component of Wld^S-mediated axon protection. Neurobiology of Disease 59:69-79.

Skou JC (1989) The identification of the sodium-pump as the membrane-bound Na+/K+-ATPase: a commentary on 'The Influence of Some Cations on Adenosine Triphosphate form Peripheral Nerves'. Biochim Biophys Acta 1000:435-438.

Tian L, Hires SA, Mao T, Huber D, Chiappe ME, Chalasani SH, Petreanu L, Akerboom J, McKinney SA, Schreiter ER, Bargmann CI, Jayaraman V, Svoboda K, Looger LL (2009) Imaging neural activity in worms, flies and mice with improved GCaMP calcium indicators. Nat Methods 6:875–881.

Titmus MJ, Faber DS (1990) Axotomy-induced alterations in the electrophysiological characteristics of neurons. Prog Neurobiol 35:1–51.

Wang JT, Medress ZA, and Ben A. Barres (2012) Axon degeneration: Molecular mechanisms of a self-destruction pathway. Journal of Cell Biology Review 196(1):7-18.

WuCF, Ganetzky B (1980) Genetic alteration of nerve membrane excitability in temperature-sensitive paralytic mutants of *Drosophila* melanogaster. Nature 286:814–816.

Xiong X, Hao Y, Sun K, Li J, Li X, Mishra B, Soppina P, Wu C, Hume HI, and Catherine A Collins (2012) The Highwire Ubiquitin Ligase Promotes Axonal Degeneration by Tuning Levels of Nmnat Protein. PLOS Biology 10(12):1-18.