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## Genes Required for Wallerian Degeneration Also Govern Dendrite Degeneration: A Dissertation

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"GENES REQUIRED FOR WALLERIAN DEGENERATION ALSO GOVERN  
DENDRITE DEGENERATION"

A Dissertation Presented  
By

Timothy Michael Rooney

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April 3, 2015

MD/PhD Program in Biomedical Sciences/Neurobiology

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April 3, 2015

## **Dedication**

This thesis is dedicated to my parents, Bob and Elaine Rooney.

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I would first like to thank my advisor and mentor, Marc Freeman, for taking me in and providing an excellent environment for learning to be a good scientist.

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## Abstract

Neurons comprise the main information processing cells of the nervous system. To integrate and transmit information, neurons elaborate dendritic structures to receive input and axons to relay that information to other cells. Due to their intricate structures, dendrites and axons are susceptible to damage whether by physical means or via disease mechanisms. Studying responses to axon injury, called Wallerian degeneration, in the neuronal processes of *Drosophila melanogaster* has allowed the identification of genes that are required for injury responses. Screens in *Drosophila* have identified *dsarm* and *highwire* as two genes required for axon degeneration; when these genes are mutated axons fail to degenerate after injury, even when completely cut off from the neuronal cell body. We found that these genes are also required for dendrite degeneration after injury *in vivo*. Further, we reveal differences between axon and dendrite injury responses using *in vivo* timelapse recordings and GCaMP indicators of intracellular and mitochondrial calcium transients. These data provide insights into the neuronal responses to injury, and better define novel targets for the treatment of neurodegenerative diseases.

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## Chapter I: Introduction

### The Nervous System

The nervous system is our means of interacting with our environment. It provides information about our surroundings and ourselves, from the feeling of thirst to the heat of a candle flame, and allows us to integrate that information and act appropriately and even automatically, from pouring a glass of water to jerking a finger away from the candle. Sensory input travels through the peripheral nervous system (PNS) in nerves to the central nervous system (CNS), where it is processed and integrated into behavior that is enacted by motor output from the CNS to the muscles of the body. All of this information transfer and computation occurs in the huge number of neurons and glia that make up the nervous system. The diversity and complexity of tasks performed by these cells is made possible by virtue of their numbers as well as the great variety of different kinds of neurons and glia.

Neurons are the cellular functional units that underlie our every sensation, thought, and action. To perform such tasks, they form networks of connections with many other neurons and other cells to relay information and compute outcomes. Importantly, they form these connections with two main structures: axons and dendrites. Axons are long, slender structures that transmit signals to other cells while dendrites are branched structures (“dendron” is the Greek word

for tree) that receive and integrate those signals. Both structures can exhibit extremely varied and often elaborate forms, some reaching as far as a meter from the cell body. There is a relationship between axonal and especially dendritic structure and the function they impart to neurons. For example, a neuron with a large, complex dendritic arbor with many branches can make connections with many other neurons and integrate their input signals to a single output through the axon. Some sensory neurons, such as those that sense chemicals in the environment, have a rather simple, diminutive dendrite as their function is simply to respond to the presence of the chemical: no integration required.

### **Dendrites vs. Axons**

Dendrites spatially and temporally integrate various sensory and synaptic signals by virtue of their structure, which varies between classes of neurons. In fact, neurons are often classed based on the branching pattern of their dendrites<sup>1</sup>. Similar to the look of tree branches, dendritic arbors consist of processes that ramify starting proximally near the cell body. The most proximal segment of the dendrite is the largest in caliber, and with successive branching and distance from the soma the dendrites taper in size<sup>2</sup>. Axons are largely uniform in caliber and only branch at the termini in synaptic areas. Organelle and molecular components also distinguish axons and dendrites. Nearly all cellular

organelles can be found in dendrites, including mitochondria, rough endoplasmic reticulum, Golgi bodies, and polyribosomes. This is in contrast with axons, which typically only contain mitochondria and not rough endoplasmic reticulum, Golgi bodies, or polyribosomes<sup>3,4,5</sup>. Cytoskeletal polarity is another distinguishing feature between these two compartments. Microtubules are polymeric cylinders that provide rigid structural support and have identifiable ends due to the arrangement of their  $\alpha$  and  $\beta$  subunits; these are denoted the plus and minus ends<sup>6</sup>. Axonal microtubules are aligned plus end towards the synapse and minus end towards the cell body. In dendrites, the microtubules are arranged in both directions<sup>7,8</sup>. These arrangements have implications for the transport of proteins and other cellular components as the motor proteins responsible for transport either move towards microtubule plus ends or the minus ends<sup>9</sup>. Additionally, some cytoskeletal binding proteins are only found in axons (unphosphorylated tau) or dendrites (MAP2)<sup>10,11</sup>.

Another difference between the two structures is the manner in which axons and dendrites relay information. This is due to the differential expression of ion channels in dendrites and axons. Synaptic or sensory input to dendrites results in the opening of calcium channels, causing a net influx of calcium ions into the arbor<sup>12,13</sup>. When sufficiently large, this calcium influx activates voltage-gated channels and creates a sodium and calcium-based action potential that is then transmitted towards the axon often through the cell body. At the most proximal part of the axon, this action potential activates voltage-gated sodium

channels and converts the signal into the predominantly sodium-based axonal action potential<sup>14</sup>. This difference in signal propagation also requires different mechanisms to regulate ion levels during and after activation. For example, calcium does not play a major role in the axonal action potential, and intra-axonal levels do not normally vary to the degree seen in dendrites<sup>15</sup>. In axons, calcium levels are maintained by the plasma membrane sodium/calcium exchanger and via the uptake by mitochondria<sup>16</sup>. Dendrites utilize a plasma membrane ATPase fueled by glycolysis to rapidly and actively return dendroplasmic calcium to resting levels<sup>17,18</sup>. Thus, many factors differ between axons and dendrites relating to the specialization and function of these structures.

### **The *Drosophila* Nervous System**

The fruit fly *Drosophila melanogaster* is the first established model organism, and has been used in biological research for over 100 years. The ease of maintenance, genetic tractability, and plethora of genetic tools has led to many seminal discoveries in various fields. The *Drosophila* nervous system shows remarkable conservation with the mammalian nervous system. Flies sense, move within, and interact with their environment, integrating sensory information and displaying complex behaviors such as learning – all aspects of complex nervous system function. Indeed, the basic principles of nervous system function are identical between flies and you. Accordingly, the fly nervous

system contains similar cell types as mammals, including subtypes of neurons and glia that physically resemble and function as do their mammalian counterparts<sup>19</sup>. Thus, *Drosophila* provides an excellent model to study nervous system biology *in vivo*.

### **Nervous System Dysfunction and Injury**

The nervous system is a vital and complex system that regulates most aspects of animal life. It is also susceptible to injury and disease, which causes significant pathology and suffering. Diseases may affect specific components, such as motor neurons in amyotrophic lateral sclerosis (ALS), or dopaminergic neurons in Parkinson's disease<sup>20,21</sup>. Less discriminant injuries occur due to trauma, diabetic neuropathy, and from increased intracranial pressure as found in metastatic cancer and intracranial bleeding. Axons and dendrites can also be differentially affected. For example, motor neuron disease such as gracile axonal dystrophy often leads to the dying back of axonal processes<sup>22</sup>. Similarly, dendrites can specifically degenerate due to A $\beta$  plaques in Alzheimer's disease or excitotoxicity following hypoxia/ischemia in stroke<sup>23,24</sup>.

Axons and dendrites are equally vulnerable to physical injury, such as from trauma. When injured, they stereotypically fall apart and the remains are then cleared by surrounding cells within a couple of days. This process was first described over 165 years ago in frog hypoglossal axons by Augustus Waller<sup>25</sup>.

The eponymous process of Wallerian degeneration (WD) appeared to be due to the axon being separated from its lifeline the cell body. Wallerian degeneration is classically studied in response to physical transection of axonal processes. Whether in culture or *in vivo*, the distal part of the axon is immediately cut off from the cell body. This distal stump remains unchanged for several hours to a day or so (35-44hrs in mouse sciatic nerve), depending on the neuron type and model used. The entire distal stump then undergoes a rapid, catastrophic granular disintegration, leaving only fragmented axonal debris<sup>25</sup>. Surrounding glial cells or other phagocytes then clear the debris. If not cleared appropriately, this debris can hinder the axon regeneration that often occurs in the peripheral nervous system<sup>26</sup>.

For 140 years, WD was assumed to be a passive degenerative response of the distal axonal stump to being cut off from the cell body. This assumption predominated until the fortuitous discovery of a mouse whose axons failed to degenerate after transection, and instead remained intact for weeks rather than a day<sup>27</sup>. Later called the Wld<sup>s</sup> mouse (for slow Wallerian degeneration), its discovery transformed the way we think about WD: from a passive wasting away to an active process that could be prevented.

## Slow Wallerian Degeneration

WLD<sup>s</sup> arose from a spontaneous tandem triplication event that resulted in the fusion of the full coding sequence of *Nmnat1*, a rate-limiting enzyme in an NAD<sup>+</sup> salvage pathway, and the first 70 amino acids of *Ube4b*, an E4 ubiquitin ligase, leaving the endogenous loci of *Nmnat1* and *Ube4b* intact<sup>28–30</sup>. While the short sequence derived from *Ube4b* is unlikely to have ligase activity, NMNAT1 enzymatic activity is preserved; indeed it is required for the protection of axons<sup>31</sup>. How does this mutant protein protect injured axons? Two hypotheses dominate the field. First, WLD<sup>s</sup> may substitute for an axonal survival factor that is degraded after injury and the loss of which causes degeneration. Second, WLD<sup>s</sup> may alter axon biology in such a way as to prevent a signal caused by axotomy from enacting the degeneration program. Note that these hypotheses are not necessarily mutually exclusive.

In the first case, there is evidence that WLD<sup>s</sup> takes the place of endogenous NMNAT proteins, which act as a critical signal for axon integrity. This idea stems from the trophic factor theory: the soma continuously supplies a vital factor that becomes depleted in the distal stump after axotomy due to it being cut off from the cell body<sup>32</sup>. NMNAT2 is a good candidate for this factor as it is an extremely labile protein that is transported in axons, levels decrease sharply upon axotomy, and when depleted in neurons causes degeneration that is suppressed by WLD<sup>s</sup><sup>33,34</sup>. Additionally, WLD<sup>s</sup> can rescue the postnatal lethality

and axon outgrowth defects of *Nmnat2*<sup>-/-</sup> mice<sup>33</sup>. NMNAT2, then, may indeed act as an axonal trophic factor required to stave off WD.

The second hypothesis invokes a gain-of-function role for the protein. WLD<sup>s</sup> requires enzymatic activity to protect axons<sup>31,35-38</sup>, however, no consistent link between NAD<sup>+</sup> and WD has been shown<sup>39</sup>. There is no increase in basal NAD<sup>+</sup> with WLD<sup>s30,36</sup>, and addition of NAD<sup>+</sup> to axons has shown slight protection in some studies but not others<sup>36,37,40-42</sup>. Other studies have highlighted the role of mitochondria in axon degeneration<sup>43</sup>. Additionally, a mitochondrial localization for WLD<sup>s</sup> has been observed and also correlated with increased ATP production and calcium buffering capacity<sup>35,44</sup>. Indeed, a drop in ATP precedes degeneration after axotomy<sup>45</sup>. WLD<sup>s</sup> could, therefore, enhance mitochondrial function to maintain axonal homeostasis after injury thereby preventing WD.

The influx of extracellular calcium is both necessary and sufficient for WD *in vitro* and *in vivo*<sup>46</sup>. Several studies have shown that blocking L-type calcium channels or chelating extracellular calcium prevents the intra-axonal accumulation of calcium and the fragmentation of the injured axons<sup>46,47</sup>. Calcium is thought to activate the calcium-dependent protease calpain, leading to the breakdown of cytoskeletal and axonal proteins. Inhibition by endogenous murine calpastatin *in vitro* and *in vivo* can modulate WD<sup>48</sup>. Interestingly, WLD<sup>s</sup> may prevent WD by enhanced calcium buffering by mitochondria, and was shown to attenuate a calcium spike upon laser ablation of *Drosophila* larval motor neurons<sup>44</sup>. Together with a mitochondrial localization for WLD<sup>s</sup>, as well as

increased mitochondrial ATP production<sup>35,44</sup>, these results suggest that WLD<sup>s</sup> could operate in or on mitochondria to prevent WD.

The fragment of Ube4b found in WLD<sup>s</sup> is known to interact with the valosin-containing protein (VCP)<sup>31</sup>, an ATPase involved in the ubiquitin-proteasome system, which is also found in mitochondria<sup>49</sup>. VCP is thought to redistribute WLD<sup>s</sup> from the normally nuclear localization of NMNAT1, including potentially to mitochondria<sup>50</sup>. Interestingly, both *Nmnat1* and VCP are hexamers, and the interaction of WLD<sup>s</sup> with VCP may preserve the enzymatically competent structure of NMNAT1<sup>51,52</sup>. Critically, WLD<sup>s</sup> requires its enzymatic activity as well as VCP binding to prevent WD, suggesting that this may be the case<sup>31</sup>.

Though apoptosis and WD are both active cell death processes, they are distinct. Caspase activation is not observed in WD, but is required for apoptosis<sup>53</sup>. Likewise, neither *Bax* deletion nor BCL-2 overexpression alters WD while both manipulations inhibit apoptosis<sup>54,55</sup>. Additionally, WLD<sup>s</sup> expression does not affect the apoptosis in motor neuron cell bodies after axotomy<sup>56</sup>. Developmental neurite degeneration, or pruning, is also distinct from WD. In *Drosophila* sensory neuron pruning, knock down of several genes gives pruning defects in dendrites but does not affect degeneration of these same dendrites after laser ablation. The genes include: *dronc*, *Mical*, *Kat-60L*, and *IK2*<sup>57</sup>. Additionally, WLD<sup>s</sup> expression did not alter the developmental pruning of mouse cortical and retinal axons nor fly mushroom body axons, yet protected these axons from injury<sup>58</sup>. Together, these results provide strong support that

developmental and injury-induced degeneration operate through distinct mechanisms.

Given the robust protection of severed axons by WLD<sup>s</sup>, there has been much interest in testing whether there is also neuronal protection in models of neurodegeneration. Some such studies showed little or no protection by expression of WLD<sup>s</sup>. These mostly include very strong models of motor neuron disease, such as several mutant SOD1 models of ALS where mutant SOD1 proteins are overexpressed over long periods of time (months)<sup>59,60</sup>. Several acute injury models, on the other hand, showed relatively strong protection by WLD<sup>s</sup>. Toxic neuropathy induced by taxol treatment delayed axonal degeneration and improved mouse rotarod performance, a measure of motor coordination<sup>61</sup>. In acute models of Parkinson's disease, degeneration was delayed after administration of 6-hydroxydopamine as well as MPTP, which both lead to dopaminergic cell death<sup>62-64</sup>. In a mouse model of hypoxia/ischemia, delayed degeneration was shown up to 1 week after carotid artery ligation<sup>65</sup>. As a last example, NMDA injection into the retina results in excitotoxic injury, and WLD<sup>s</sup> delayed degeneration of retinal ganglion cells for greater than 2 weeks<sup>66</sup>. These studies show the promise of inhibiting WD in the treatment of neurodegenerative diseases, particularly in acute settings.

## ***Drosophila* Models of Wallerian Degeneration**

The first *Drosophila* system used to study WD was the olfactory receptor neuron model (ORN)<sup>67</sup>. ORNs are chemosensory neurons that reside in the antennae of flies and project into the brain to synapse on spatially distinct glomeruli. MacDonald, et al showed that removal of the antennae severs the olfactory nerve, leading to WD in the ORN axons, and that this degeneration is prevented by the expression of WLD<sup>s</sup>. Distinct subsets of ORNs can be labelled using promoter-Gal4 constructs, such as the OR22a-Gal4 that drives expression in ~24 neurons in each antenna<sup>67</sup>. ORN clones can be induced by using *eyeless-flp* (*ey-flp*), which generates flippase activity early in eye and antennal development. The mosaic analysis with a repressible cell marker technique together with *eyeless-flp* can be used to create and specifically label homozygous mutant ORN clones in an otherwise heterozygous animal. The ORN model has been extensively used to characterize WD and identify genetic components of the responses involved<sup>68</sup>. For example, the initial structure-function studies of WLD<sup>s</sup> were performed in ORNs<sup>31</sup>. These studies showed that VCP binding and enzymatic activity are crucial to the protection shown by WLD<sup>s</sup>. Several studies investigating the glial response to axon injury identified the cell corpse receptor Draper and downstream signaling pathways as being critical for the recognition and engulfment of axonal debris<sup>19,69–71</sup>.

Another model of WD in *Drosophila* that is especially useful for investigating dendritic biology centers on larval multidendritic sensory neurons.

These dendritic arborization (da) neurons tile the larval body wall providing sensory input for each segment of the larvae<sup>72</sup>. They are classed I-IV according to the size of their receptive fields as well as the complexity of their dendritic arbors, class IV being the largest and most complex<sup>1</sup>. Class III da neurons project several long primary dendrites that contain microtubules<sup>73</sup>. Found along the length of these primary dendrites are projections called sensory filopodia/spikes, which are much shorter, highly dynamic, and rich in F-actin. The actin-based protrusion and retraction of the spikes, along with a complement of ion channels, is required in these cells for behavioral responses to light touch<sup>73</sup>.

Class IV da neurons have the largest and most highly branched dendritic structures of the multidendritic sensory neurons<sup>1</sup>. Their arbors tile the larval body wall, covering each larval body segment with little overlap between neighboring cells. These cells have been shown to respond to many noxious stimuli such as touch, heat, and light (especially UV light)<sup>74-76</sup>. Recently, the class IV da neurons were found to degenerate in a manner similar to axons after injury<sup>57</sup>. Further, this degeneration was prevented by WLD<sup>s</sup>, suggesting that axons and dendrites share the mechanism and/or signaling that leads to degeneration. After injury, the fragmented dendrites are engulfed by the overlying epithelium, which, similar to glia, utilize the engulfment receptor Draper<sup>77</sup>. The class IV da neurons are also able to regenerate both axons and dendrites after injury, sprouting new branches from the cell bodies or the injured proximal stump<sup>78</sup>. In contrast, class

III neurons do not regenerate axons or dendrites from injured branches<sup>78</sup>, but are able to sprout new branches from the cell body after ablation of all dendritic branches early in larval life<sup>79</sup>. Thus, there appears to be a differential response to injury between class III and class IV da neurons.

### **dSarm/SARM1**

An unbiased forward-genetic screen in the ORNs identified the first endogenous regulator of WD with protection that rivaled WLD<sup>s</sup>, *dsarm*<sup>68</sup>. *dsarm* and the mouse ortholog *Sarm1* are members of the Myd88 family of Toll/interleukin-1 receptor (TIR) domain containing immune signaling proteins<sup>80</sup>. TIR domains relay signals from Toll receptors to downstream signaling pathways such as the NFκB pathway<sup>80</sup>. Sarm proteins also contain Armadillo (ARM) repeats and a number of sterile alpha motif (SAM) domains, both of which mediate protein-protein interactions<sup>81</sup>. Sarm proteins are very conserved across phyla; *C. elegans tir-1* and *Drosophila dsarm* share 40% identity with human *Sarm1*<sup>81</sup>.

In *C. elegans*, TIR-1 acts within a pathway downstream of calcium and the calcium/calmodulin-dependent kinase CaMK and upstream of the ASK-1 MAP kinase kinase kinase in the differentiation of a pair of ORNs, as well as in the behavioral adaptation to odors<sup>82–84</sup>. Interestingly, expression of TIR-1 lacking the ARM domain showed a gain-of-function phenotype in the ORNs, suggesting that

the ARM domain acts to inhibit TIR-1 signaling<sup>82</sup>. Mouse studies of SARM1 have largely been conducted in immune assays with overexpression of the protein, which indicate a negative role for SARM1 in innate immune signaling. This regulation stems from a transcriptional regulation of another TIR domain containing adapter protein, TIR-domain-containing adapter-inducing interferon- $\beta$  (TRIF)<sup>85,86</sup>. Whether Sarm proteins also regulate WD via transcriptional changes remains to be determined.

A structure-function study of SARM1 has been conducted in mouse dorsal root ganglia culture (DRG)<sup>87</sup>. This study concluded that all three domains of SARM1 are required for proper axon degeneration. It also showed that SARM1 proteins dimerize via the SAM domains, and that the TIR domain is required for downstream signaling as expression of SARM1 lacking the TIR domain acted as a dominant negative, blocking axon degeneration. Similar to the worm data, elimination of the ARM domain in SARM1 created a dominant active version that induced the destruction of axons and cell bodies. Additionally, GFP-tagged SARM1 was found have a mitochondrial localization, suggesting that SARM1 could, like WLD<sup>s</sup>, work in or on mitochondria.

A recent study placed SARM1 upstream of a MAP kinase cascade that ultimately led to c-Jun N-terminal kinase (JNK) activation and ATP depletion in severed axons<sup>45</sup>. The pathway consists of SARM1 activating MEKK4/MLK2/DLK that then activate MKK4/MKK7 that then activate JNK1/2/3. A *Jnk1/Jnk2* double knock-out with shRNA knock-down of JNK1 afforded protection to 60% of axons

at day 6 *in vivo*; the same level of protection has been shown at 2 weeks in *Sarm1*<sup>-/-</sup> axons.

## Hiw/PHR1

Recently, a second endogenous regulator of WD was discovered. Mutations in the gene encoding the *Drosophila* E3 ubiquitin ligase Highwire (Hiw) were shown to protect ORN axons up to 2 weeks after ablation and larval motor neurons for at least 2 days after crush, during which they remained electrically competent<sup>88</sup>. It was later shown that the mouse ortholog, *Phr1*, also robustly regulates axon degeneration<sup>89</sup>. The protection seen in PHR1-deficient axons, while strong (75% at day 5, 25% at day 10), is less than seen with WLD<sup>s</sup> or *Sarm1* mutants (60% ablated axons preserved in mouse sciatic nerve at day 6)<sup>68</sup>. Hiw is a rather large protein (~5233 amino acids), and is well conserved across phyla; the *C. elegans* ortholog RPM-1 has 32% identity and 55% similarity to the human ortholog<sup>90</sup>. It contains several domains implicated in signaling, protein interactions/scaffolding, and ubiquitin ligase activity; these include a RCC1-like domain, PHR repeats, a Myc-binding domain, and a RING-H2 zinc finger motif<sup>90</sup>. PHR family proteins are involved in many neuronal processes such as axon outgrowth, synaptogenesis, and regeneration of injured axons<sup>91</sup>.

In *C. elegans*, the E3 ubiquitin ligase activity of RPM-1 regulates several neuronal cell processes including restraining synapse formation during

development<sup>92</sup>. It also has roles in negatively regulating axon regeneration, as *rpm-1* mutants have enhanced regeneration via disinhibition of the MAPKKK DLK-1<sup>93</sup>. Additionally, RPM-1 is thought to regulate receptor trafficking via endocytosis. Increased MAPK signaling in *rpm-1* mutants results in the accumulation of AMPA receptors in the dendrites of interneurons<sup>94</sup>.

In addition to participating in axon degeneration in *Drosophila*, *hiw* mutants were originally found in a screen for perturbations at the larval neuromuscular junction (NMJ). *hiw* mutants cause a pronounced overgrowth of NMJs, which has been linked with increased levels of BMP and DLK/MAPK signaling<sup>90,95</sup>.

In mice, axon guidance and synaptic development have also been linked to PHR1 E3 ligase activity in the *Magellan* mutant mouse<sup>96</sup>. These phenotypes, similar to their invertebrate counterparts, are thought to be due to increased levels/activity of DLK as well as increases in signaling by the tuberous sclerosis complex via up regulation of TSC2<sup>97</sup>. Aberrant microtubule dynamics have also been observed in *Magellan* mice; microtubules inappropriately invade axon growth cones, thereby linking overgrowth phenotypes with cytoskeletal dysregulation<sup>96</sup>.

In the context of Wallerian degeneration, it is thought that Hiw/PHR1 E3 ubiquitin ligase activity regulates the levels of NMNAT proteins, and causes their rapid loss after axotomy. Accordingly, in *hiw* mutants, dNmnat levels become

detectable via immunostaining of larval peripheral nerves, and there are higher levels of exogenously expressed NMNAT2<sup>88</sup>. Increased levels of a transduced tagged NMNAT2 are also seen in *Phr1* mutant nerves, while shRNA knock down of NMNAT2 suppresses the axon protection phenotype of *Phr1* mutant animals<sup>89</sup>. While these results are consistent with the regulation of NMNAT proteins after axotomy, the regulation of endogenous NMNAT levels by Hiw family members has not been shown in this context.

## Chapter II: Screens to Identify Novel Regulators of Wallerian Degeneration

### Introduction

Without the discovery of the *Wld<sup>s</sup>* mouse, we may not have considered the notion that axons actively degenerate following injury. The subsequent sea change in thinking about and interest in Wallerian degeneration (WD) led to many new and interesting findings. For example, we have learned that axon degeneration in response to injury is molecularly distinct from other cell death pathways, such as developmental neurite pruning and apoptosis. This fact along with the gain-of-function nature of *Wld<sup>s</sup>* necessitates the identification of endogenous regulators of axon degeneration if we are to fully understand this process. Many genes have been implicated in regulating WD, however the reported preservation is much weaker than seen with *Wld<sup>s39</sup>*. Therefore, new efforts to find WD genes are needed.

Wallerian degeneration in mammals encompasses the processes of axon degeneration as well as the clearance of axonal debris by phagocytic cells, such as macrophages in the PNS and surrounding glia in the PNS and CNS.

*Drosophila* olfactory receptor neurons (ORNs) have also been shown to degenerate after axotomy, which is prevented by the heterologous expression of

Wld<sup>s67</sup>. Additionally, glia were shown to be activated by axotomy and to engulf the degenerated axon debris. Thus, the processes of WD are well conserved between mammals and flies.

*Drosophila* as a model organism has a long history of identifying roles for genes through genetic screens. The ease of establishing and maintaining mutant stocks along with the ability to readily screen for phenotypes allows for rapid, large-scale screening *in vivo*. We therefore sought to combine these attributes with the conservation of WD to identify endogenous genes required for WD. These efforts ultimately lead to the identification of *dsarm* mutants as the first to prevent axon degeneration to a similar extent as Wld<sup>s68</sup>.

The screen for novel regulators of WD is based on the axotomy and imaging of a subset of ORNs, which reside in the 3<sup>rd</sup> segment of the fly antennae and project their axons into the brain to synapse on the olfactory glomeruli. Axotomy is performed by removing the antennal 3<sup>rd</sup> segment, completely removing the cell bodies. The brains and labelled ORN axons can then be antibody stained and imaged to assay WD. Additionally, mutations that prevent viability in adult flies can be assayed using Mosaic Analysis with a Repressible Cell Marker (MARCM)<sup>98</sup>. This technique produces and specifically labels cells homozygous for a particular chromosomal arm in an otherwise heterozygous animal. This technique was used in the screen that identified *dsarm*, which screened approximately 1911 lines on both arms of chromosome 3, and identified 4 independent alleles of *dsarm*. I therefore decided to develop the fly

stocks to screen both arms of the 2<sup>nd</sup> chromosome as well as take a candidate-based approach to finding genes that regulate WD.

In this study, I identified candidate genes from the literature and tested available mutants in the WD assay. I also identified and tested mutants in WD assays based on mouse orthologs of genes identified by our collaborators. Finally, I developed fly stocks to perform a forward-genetic screen for WD mutants, tested several deletion stocks using these lines, constructed 1100 individual mutants fly stocks, and together with Nicki Fox, PhD and later Rachel Hackett, screened the vast majority of these lines in the search for genes that regulate WD. Herein I describe 199 mutant genotypes, 22 mapped deletions, and over 2000 mutant chromosome arms that are not required for axon degeneration *in vivo*.

## Candidates Identified in the Literature

### Materials and Methods

The following fly strains were used to balance the lines tested as well as to cross in the ORN Gal4 driver along with the membrane marker mCD8::GFP: *SP/CyO* ; *OR22a-Gal4, UAS-mCD8::GFP*, *OR22a-Gal4, UAS-mCD8::GFP* ; *Dr/TM3*, and *OR22a-Gal4, UAS-mCD8::GFP* (II). Stocks with aberrations on the X chromosome were crossed to *Or22a-Gal4, UAS-mCD8::GFP* flies and males were used for the degeneration screen. Stocks for mutations on the second

chromosome were crossed to *Sp/Cyo; OR22a-Gal4, UAS-mCD8::GFP*, and the progeny were self-crossed to obtain homozygous mutant flies on the second and *OR22a-Gal4, UAS-mCD8::GFP* on the third chromosome, or crossed to the progeny of a similar cross with another allele of the same gene to obtain trans-heterozygotes. Similarly, stocks for mutations on the third chromosome were crossed to *OR22a-Gal4, UAS-mCD8::GFP; Dr/TM3* and the progeny self-crossed to obtain homozygous mutant flies or crossed to different mutant stocks to obtain trans-heterozygotes on the third chromosome and *OR22a-Gal4, UAS-mCD8::GFP* on the second chromosome. Flies were raised and maintained on standard *Drosophila* media at 25°C. All lines were obtained from the Bloomington Stock Center except the following: *Toll 6, Toll 7, and Toll 8* mutants (gifts from Yoshimasa Yagi, Nagoya University, Nagoya, Japan), *ninjurin* mutants (gifts of Andrea Page-McCaw), *IkappaB kinase-like 2* and *IMD* mutants (gifts from Neal Silverman), all *Calx* lines (gifts from Craig Montell), all *Lkb1* lines (gifts from Daniel St. Johnston), *ca-alpha-1D<sup>x10</sup>, FRT40a, cac<sup>HC129</sup>, FRT19A, cac<sup>HC129</sup>, FRT19A; ca-alpha-1D<sup>x10</sup>, FRT40A, FRTG13, Df(2R)RyR<sup>1</sup>, FRT82B, IP3R<sup>sv35</sup>, FRTG13, dOral<sup>A355</sup>, calp-B<sup>1</sup>, calpA<sup>72</sup>, calpA<sup>75</sup>, ca-beta<sup>10</sup>, and ca-beta<sup>48</sup>* (gifts from Kazuo Emoto), and mouse knock-outs of *Unc93B, Tlr3, and Tril* (gifts from Kate Fitzgerald).

For mouse lines, rearing/handling and surgeries were performed by technicians in the lab under established protocols and IACUC approval. Plastic embedded sciatic nerves that had been transected 7 days prior to harvest were

obtained from the electron microscopy core at UMass Medical School. Semi-thin 1 $\mu$ m sections were made using a glass knife, heat fixed, and stained with toluidine blue.

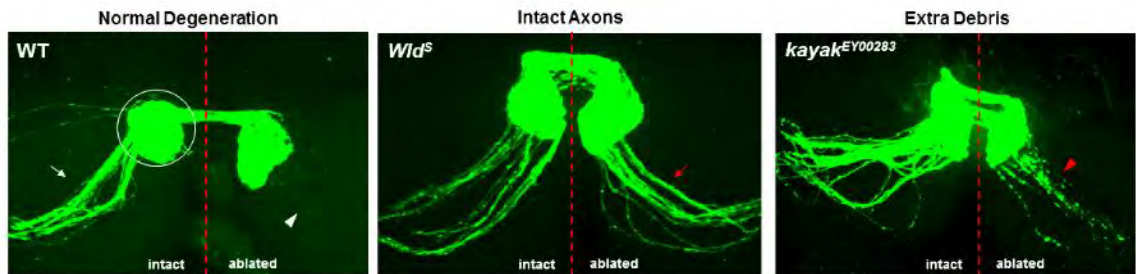
The Wallerian degeneration assay was performed as previously described<sup>67</sup>. Flies were aged for 7 days after eclosion to permit strong expression of the mCD8::GFP marker. The left third antennal segments were then removed with forceps, and the flies aged for 7 days at 25°C. Fly heads were then removed and fixed in 4% formaldehyde/PBS/0.1% Tween and the brains dissected. Fly brains were then mounted in Vectashield antifade (Vector Laboratories) and imaged on a Zeiss spinning disc confocal microscope (Intelligent Imaging Innovations, Denver, CO).

## Results

Three main phenotypes were expected from this screen: wild type (WT), where axons degenerate and are cleared fully by 7 days after ablation; intact, where the axons failed to degenerate normally, as in *Wld<sup>S</sup>*-expressing neurons; and extra debris, where axons degenerated but were not cleared. Figure II-1 gives an example of each of these phenotypes; in each case, intact axons appear on the left and injured axons on the right. In WT, injured axons have degenerated and been cleared by surrounding glia; those uninjured axons that remain synapse on their olfactory glomeruli, cross through the midline

commissure and synapse on the contralateral glomerulus. Intact axon phenotypes, such as when *Wld<sup>s</sup>* is expressed, resemble WT uninjured ORNs despite being cut off from the discarded cell bodies for 7 days. In the extra debris phenotype, axons degenerate but are not cleared. This is seen as a debris trail where axons normally reside, as on the right in the *kayak* mutant in Figure II-1.

Table II-1 lists the genes tested and the lines used to do so, and the outcome of the assay. In all, 217 lines were obtained and tested for defects in WD. With several of the lines tested the mutants used were not compatible with life in the adult fly, and these were marked as non-viable (NV) combinations. NV crosses were observed for 22 lines or combination of lines. Normal axon degeneration and clearance was observed in the majority of cases: 199/265 combinations tested. 31 genotypes tested showed debris left at day 7 after ablation. This phenotype could be due to a defect in glial activation or engulfment of the axonal debris, or it could be a failure within the axons to produce a signal to activate the glia. The strongest engulfment phenotype was seen with an allele of the *kayak* gene, which encodes the *Drosophila* c-fos, a transcription factor downstream of JNK (Figure II-1). Notably, this phenotype was subsequently confirmed in an axonal engulfment assay with RNAi knock down of glial *kayak*<sup>71</sup>.



**Figure II-1. Wallerian degeneration of *Drosophila* olfactory receptor neurons: wild-type and mutant phenotypes.**

Olfactory receptor neurons project axons (arrow) through the fly brain to synapse in the ipsilateral glomerulus (circled), cross the midline (red line), and synapse on the contralateral glomerulus. When ablated, the axons undergo Wallerian degeneration, and are cleared by 7 days post ablation. The arrowhead denotes the region where the axons were prior to degeneration. When WLD<sup>S</sup> is expressed in these neurons, ablated axons remain intact (red arrow). Some mutations, such as in *kayak*, prevent the clearance of degenerated axons, extra debris remains (red arrow head). Neurons were labelled by the olfactory receptor neuron marker *OR22a-Gal4* driving expression of a membrane-localized green fluorescent protein.

Table II-1. Literature candidate lines tested for defects in Wallerian degeneration.

Gene	Stock Number	Phenotype
		<b>NV = not viable</b>
Toll	2507	none (3238/25052), (2507/3238) some debris (25052/2507)
Toll	3238	
Toll	25052	
18Wheeler	4372	some had quite a lot of debris
18Wheeler	543	
MstProx	2057	none (2057 alone and with 7625)
MstProx	7625	
Tehao	9594	none
Toll-6	25412	NV
Toll-6	Yagi	none
Toll-7		none
Toll-7	30588	none
Tollo	Yagi	none
Toll-9	29264	none, 29264 alone-none
Toll-9	26519	
Calx	Montell	none
Calx	Montell	none
Calx	Montell	none
Calx	Montell	none
Calx	Montell	none
IK2	Silverman	none
IMD	Silverman	none
TRXT1/2	4557	none
dunce	6020	none
licorne	11880	extra debris
Clic	12267	none
CG3719	21105	none
CaMK1	16799	none
SERCA	25434	debris; weak (d5)
SERCA	25443	
SERCA	26700	debris (d5)-25434 none-25443

kayak	15018	lots of debris - strong
TRAF-like	26869	none
TRAF-4	17285	none (none 17285 alone too)
TRAF-4	9694	
TRAF-6	29989	debris (weak)
rdg-C	30627	none + 30627 alone
rdg-C	3601	
lic and hep	9058	NV
hep	6761	
hep	10082	
Mkk4	17956	NV
Mkk4	7628	
EGFR-CA	9534	degeneration with Pi3K <sup>caax</sup>
LON	27141	none
LON	7945	
gdh	7675	none(7992/20165 and 7675/20165)
gdh	7992	
gdh	20165	
diff. gdh	27841	none
diff. gdh	7990	
twinkle	26949	none
twinkle	7508	
aconitase	26445	none
aconitase	20708	
aconitase	24609	
aconitase	14849	
cacophony	8579	none
slo	29918	none
slo	4587	none
SPG7	18149	none
SPG8	18967	none
CG6724	7820	none: 7820/21128, 21128/18019
CG6724	18019	
CG6724	21128	
Tehao	27383	extra debris (d5) with 9594
Mpk2	8822	none
p38b	22319	none (14364 alone: none)
p38b	14364	
p38c	14126	none

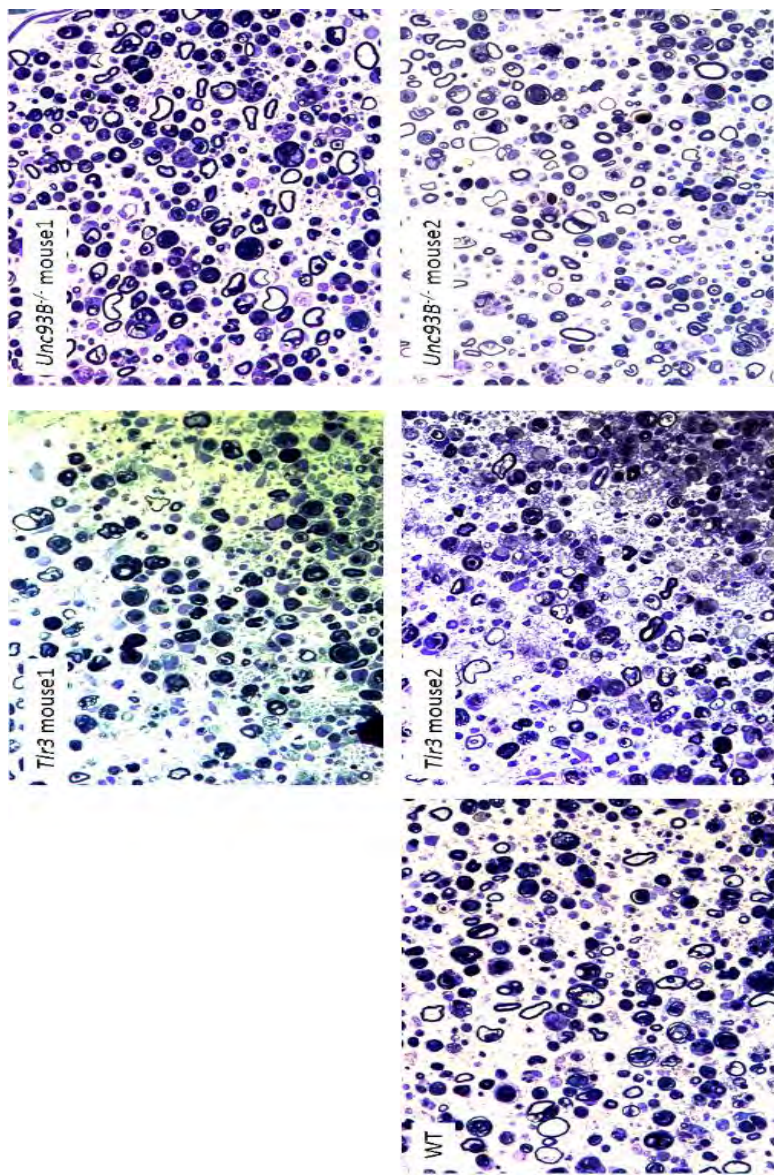
CG4709	27442	none
starvin	11501	
starvin	7598	none
chip	16378	none
CG11486	17263	
CG11486	20352	none (20352 alone - none)
CG8232	18295	lots of debris at d5
calcineurin A1	29211	
calcineurin A1	24142	none: 24142/26847, 29211/24142, 26847/29211, 29211
calcineurin A1	26847	
CG3714	17075	
CG3714	9600	none
CG10496	22415	
CG10496	11657	none: 11716/22415, 11657, 22415/11657, 11716
CG10496	11716	
CG9062	24352	
CG9062	24382	NV
GS1	6248	
GS1	27940	slight debris; wiring defect
Csp	32035	
Csp	20497	debris
Drep-1	12531	
Drep-1	26551	none
vermillion	856	
vermillion	142	
vermillion	3109	none, all combinations
det	17963	
det	25388	NV
Cyp1	10136	
Cyp1	27415	none alone, trans: ectopic connections + weak debris
Frq1	34397	34397/8939 none
Frq2	1739	unhappy axons, slight debris
Frq2	18939	none
pelle	3111	
pelle	3112	
pelle	7686	none
tube	3116	
tube	9538	none

dMyd88/Kra	14091	23665/14091 and 14091 alone: engulf/delay
dMyd88/Kra	23665	
tep3	19199	9619/19199 weak delay; 19199 none
tep3	29239	
tep3	9619	
tep4	15936	none
Pkc delta	25698	extra debris
gd	15472	none
snake	3114	NV
snake	7973	
CG9095	19310	none
CG9095	22433	
CG9095	26595	
CG6459	7890	7890/18627 none; 18627 slight debris
CG6459	18627	
iPLA2-VIA	15947	15947 wiring, sometimes debris; 24417/15947 & 24417/15947 wiring defect? Occasional axon on injured side; 24418/24417 NV
iPLA2-VIA	24418	
iPLA2-VIA	24417	
iPLA2-VIA	24417	
CG8232	9063	none
CG9906	23296	none
CG9906	26858	none
Cnx99A	17136	none
Cnx99A	8925	
crc	9227	9227/25056 NV; 13125 13125/9227 25056/13125 none
crc	25056	
crc	13125	
vha55	1617	NV
vha55	9445	NV
vha55	9446	
Vha100-2	19975	none
Vha100-2	21709	21709 some with debris; 21709/7657 some with debris; 19975/7657 none; 11656/7657 NV
Vha100-2	11656	
Vha100-2	7657	
ltd	338	23664/338 and 338 alone: some debris
ltd	24448	
ltd	23664	

stim	3658	
stim	26566	NV
olf186-f	11042	
olf186-f	<input checked="" type="checkbox"/> 20119	24362/20119 some debris; 20119 some debris;
olf186-f	24362	11042/20119; 24362/11042
deadhead	17494	extra debris
aralar	15960	
aralar	18846	15960/25007 some debris; 18846/15960 none;
aralar	25007	25007/18846 none; 18846/25008 none;
aralar	25008	25008/15960 some debris; 25007/25008 NV
CRMP	19693	none
ort (3R)	1133	
ort	29637	1133 alone and in trans: none
jafrac2	18489	debris
Ca- $\alpha$ 1D	4275	
Ca- $\alpha$ 1D	25141	
Ca- $\alpha$ 1D	25527	4275/25141 2527/4275, 25141/25527 none; 25141/25527 & 25527/4275 NV
AIF	Freeman stocks	none
NOS	18555	
NOS	24283	none Df(2L)8026 wing clones; none with 8026 & alone
14-3-3zeta	15902	some debris
CG42813	30197	
CG42813	7686	30197 none; 7686/30197 & 30197/25001 quite a bit of debris; 25001/7686 NV
CG42813	25001	
Tpi	16563	
Tpi	20699	16563/25006 none; 20699/25006 none; 20699 none;
Tpi	25006	16563 none
bsf	22624	none
rolled (erk)	386	none
Trpl	31433	NV
Trpl	29134	
Trpl	34306	34306/29134 NV
AIP1	1132	
AIP2	2371	NV
AIP3	15808	none

AIP4	17664	none
Duox	16468	NV
poe	11758	11758/11761 none
poe	11761	
CG42271	18046	none
angel	27351	NV
angel	9424	
CG9391	16929	16929 axons and glomeruli look sick; 24923/16929 none
CG9391	24923	
iav	6029	none
nan	24902	none
nan	24903	
egr	Silverman	none
egr	Silverman	none
PI3K	8294	none
sina	30724	no clones
eff	11779	none
ipk2	Freeman stocks	none
strica	McCall	none
dredd	McCall	none
dronc	McCall	none
ninjurin A	Page-McCaw	none
ninjurin B	Page-McCaw	none
ninjurin A/B	Page-McCaw	mild/moderate debris
DCP1	McCall	some minor debris
DCP1	McCall	some minor debris
LKB1	St.Johnston	none
LKB1	St.Johnston	none
LKB1	St.Johnston	none
ca- $\alpha$ 1D	Emoto	debris d8; fragmented 24hr
cacophony	Emoto	debris d8; fragmented 24hr
cac;ca- $\alpha$ 1D	Emoto	debris d8; fragmented 24hr
RyR	Emoto	none
IP3R	Emoto	none
orai	Emoto	none
calpain B1	Emoto	none
calpain A	Emoto	none
ca-beta	Emoto	debris d8; fragmented 24hr

<b>mouse genes</b>		
TLR3	Kate Fitzgerald	KO
UNC93B	Kate Fitzgerald	KO
TRIL	Kate Fitzgerald	KO



**Figure II-2. Sciatic nerves degenerate after injury in *Tlr3* and *Unc93B* mutant mice.**

Toluidine blue staining of sciatic nerves 7 days after surgical cut. In wild-type as well as *Tlr3* and *Unc93B* mutant mice, Wallerian degeneration occurs normally.

Images are of sciatic nerve distal to the cut.

## Candidates Identified by Mouse Proteomics

The following is adapted from: Wishart, et al, PLoS Genet. 012;8(8):e1002936, of which I was second author, and I performed all the *Drosophila* work below<sup>99</sup>.

## Materials and Methods

### *Drosophila* degeneration screen

*Drosophila* orthologs of the designated mouse proteins were identified, when available, using a reciprocal BLASTing approach and the Ensembl website (ensembl.org). Available mutations were identified in Flybase; the following *Drosophila* mutant stocks were obtained from the Bloomington Stock Center (Bloomington ID #): 12900, 26277, 18382, 7762, 20497, 12301, 30075, 24665, 18046, 5595, 23173, 6671, 13491, 20672, 25213, 18502, 13446, 9109, 11876, 7084, 25107, 7938, 5708, 11734, 29228, 2247, 13491, 15889, 8479, 18884, 23097, 15642, 382, 32035, and 7617. Mutant stocks on the X chromosome were crossed to *OR22a-Gal4, UAS-mCD8::GFP* flies and males were used for the degeneration screen. Stocks for mutants on the second chromosome were crossed to *Sp/Cyo; OR22a-Gal4, UAS-mCD8::GFP*, and the progeny were self-crossed to obtain homozygous mutant flies on the second and *OR22a-Gal4, UAS-mCD8::GFP* on the third chromosome. Similarly, stocks for mutants on the third chromosome were crossed to *OR22a-Gal4, UAS-mCD8::GFP; Dr/TM3* and the progeny self-crossed to obtain homozygous mutant

flies on the third chromosome and *OR22a-Gal4, UAS-mCD8::GFP* on the second chromosome.

The Wallerian degeneration assay was performed as described<sup>67</sup>ma. Briefly, flies were aged for 7 days after eclosion to allow strong labeling of ORN axons with GFP. The left 3<sup>rd</sup> antennal segment was then removed, and the flies aged for another 7 days. The right antennal ORNs served as unlesioned controls. The fly heads were then fixed in 4% formaldehyde/PBS/0.1% Tween, and the brains dissected. Fly brains were mounted in Vectashield and examined by confocal microscopy where they were phenotypically scored by an investigator who was unaware of the genotype. A minimum of 10 animals were examined and assessed per mutant strain.

Spontaneous degeneration in uninjured axons and synapses was scored by eye using the following criteria: 0 = no evidence for fragmentation of axons or loss of GFP fluorescence in the glomerulus; 1 = <10% of axons showing fragmentation and/or mild loss of GFP fluorescence in the glomerulus; 2 = <25% of axons showing fragmentation and/or mild to moderate loss of GFP fluorescence in the glomerulus; 3 = >50% of axons showing fragmentation and/or moderate to severe loss of GFP fluorescence in the glomerulus; 4 = only fragmented axons remaining and/or severe loss of GFP fluorescence in the glomerulus; 5 = no GFP signal remaining in axons or the glomerulus. Delayed degeneration in injured axons and synapses was scored by eye using the following criteria: 0 = no intact axons and no fragments remaining; 1 = no intact

axons but fragmented debris remaining; 2 = ~25% of axons intact but with extensive fragmentation of surrounding axons; 3 = ~50% of axons intact with evidence for fragmentation in surrounding axons; 4 = >75% of axons intact with only modest amounts of fragmentation in surrounding axons; 5 = intact axons and synapses with no evidence for fragmentation.

## Results

### **Identification of individual proteins capable of independently regulating synapse and distal axon degeneration *in vivo***

Although we had obtained a clear understanding of conserved molecular alterations occurring in synapse-enriched fractions undergoing neurodegeneration, it remained unclear whether or not any of the proteins and pathways identified were capable of actively modulating synaptic and axonal stability and degeneration *in vivo*. We therefore used a molecular genetic approach in *Drosophila* to screen individual proteins for a direct role in neurodegeneration.

We examined the role of individual proteins in regulating synaptic stability and degeneration using the *Drosophila* olfactory system to screen a collection of existing mutants, or lines with transposon insertions in a subset of these genes. Briefly, mutants and insertion lines were crossed in to a background that allowed visualization of a subset of olfactory receptor neurons (ORNs; *OR22a-Gal4/UAS-*

*mCD8::GFP*). Distal axons and their synaptic fields in the antennal lobe were examined in uninjured controls as well as 7 days after surgical ablation of antennae. Examining uninjured controls allowed us to screen individual mutant lines and test whether they modified basal synaptic and axonal stability (e.g. do synapses and axons degenerate spontaneously in the mutant line?).

Spontaneous degeneration was identified by the presence of fragmented axons and absence/decrease of GFP signal in the glomeruli housing synaptic terminals of ORNs and scored using a spontaneous degeneration index, where a score of 0 represented no disruption of axons or synapses in the glomerulus and 5 indicated complete spontaneous breakdown (see methods). Surgical ablation of antennae triggered rapid axonal and synaptic degeneration, which is complete within one day in wild-type controls, and axonal debris is cleared within one week after injury. Screening individual mutant lines 7 days after surgical ablation therefore allowed us to examine whether any of the mutations resulted in a delay in the rate of injury-induced degeneration, scored using a delayed degeneration index where a score of 0 indicated no delay in degeneration and 5 indicated a complete block.

From our original list of 47 synaptic proteins we obtained *Drosophila* lines for 21 different genes that harbored either defined mutations known to affect that gene, or P element insertions within the locus identified by the *Drosophila* Genome Project (see methods). Of the 34 mutant lines obtained, 14 produced viable flies suitable for analyses of axonal and synaptic stability and degeneration

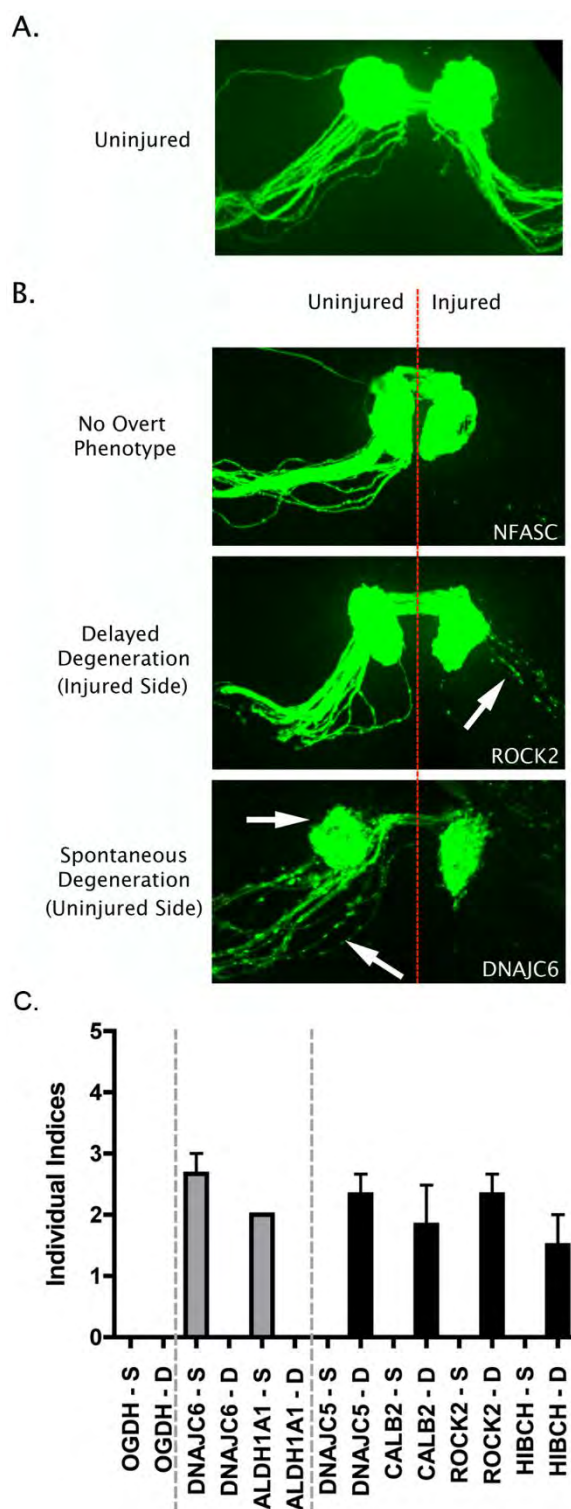
(covering a total of 13 individual proteins; Table II-2). Eight of the lines examined showed no overt phenotype in either stability or degeneration assays (Table II-2). However, 6 mutant lines were found to independently modulate stability or degeneration of distal axons and synapses in ORNs. Mutants of both ALDHA1 and DNAJC6/Auxillin caused spontaneous degeneration of distal axons and synaptic terminals in uninjured ORNs (Figure II-3). In contrast, mutations affecting CALB2/Calretinin, DNAJC5/CSP, HIBCH and ROCK2 caused a partial delay of injury-induced WD of axons and synapses (Figure II-3). In each of these lines, intact distal axons or axonal fragments were observed 7 days after experimental nerve lesion, a time-point at which axonal remnants were never observed in wild-type flies (data not shown).

<b>Protein</b>	<b>Bloomington ID</b>	<b>Observation</b>
ALDHA1	12900	Spontaneous Degeneration
Auxillin/DNAJC6	26277	Spontaneous Degeneration
CALB2/calretinin	18382	Delayed Degeneration
CFL1	7762	No Overt Phenotype
CSP/DNAJC5	20497	Delayed Degeneration
DLG1	12301	No Overt Phenotype
HIBCH	30075	Delayed Degeneration
HTT	24665	No Overt Phenotype
INPP4A	18046	No Overt Phenotype
NFASC	5595	No Overt Phenotype
OGDH	23173	No Overt Phenotype
ROCK2	6671	Delayed Degeneration
VPS29	13491, 20672	No Overt Phenotype

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TABLE II-2: List of viable *Drosophila* lines tested in the current study.

(Adapted from Wishart, et al, PLoS Genet. 2012;8(8):e1002936)



Wishart, et al, PLoS Genet. 2012;8(8):e1002936

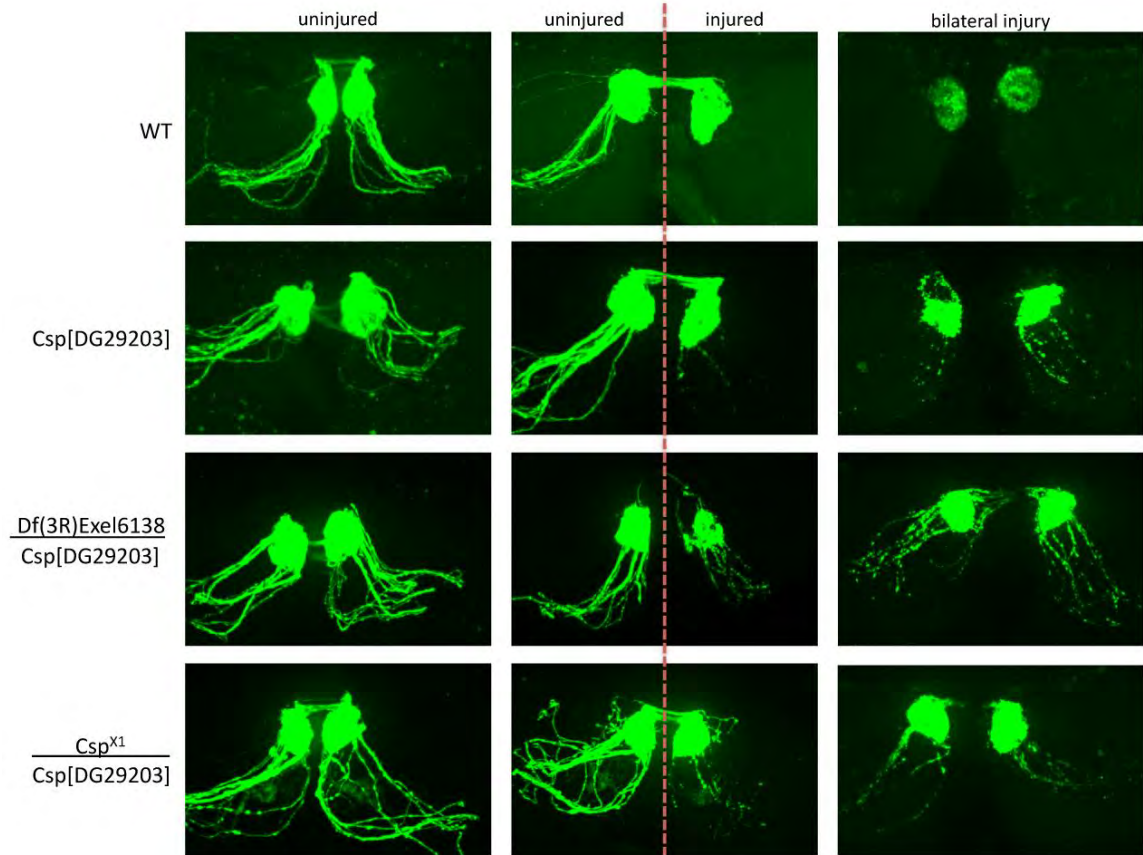
**FIGURE II-3. Overview of putative axo-synaptic degeneration phenotypes observed in *Drosophila* neurodegeneration screens.**

A. Representative confocal micrograph showing the morphology of the intact *Drosophila* olfactory receptor neuron (ORN) system, with axons and synaptic fields labeled with GFP in the *UAS-mCD8::GFP, OR22a-Gal4/+* background. Axons enter the antennal lobe laterally and project medially across the lobe to reach their target glomerulus, where synapses are located (see Figure II-1). B. Representative confocal micrographs showing three distinct phenotypic profiles observed in injured and un-injured ORN axons and synapses 7 days after unilateral (right hand side of image) antennal ablation. The top panel shows intact healthy axons and synapses on the uninjured side and complete axonal degeneration (indicated by absence of GFP labeled profiles) on the injured side (example from an NFASC mutant). The middle panel shows delayed axo-synaptic degeneration on the injured side, as indicated by the retention of GFP-labelled axon profiles 7 days after injury (white arrow; example from a ROCK2 mutant). The bottom panel shows spontaneous (i.e. not injury-induced) axo-synaptic degeneration in the uninjured axons and synapses, indicated by reduction and fragmentation of GFP labeled axons and synapses (white arrows; example from a DNAJC6 mutant). C. Bar chart (mean $\pm$ SEM) showing index scores (see methods) for spontaneous degeneration (S; grey bars) and delayed degeneration (D; black bars) in 7 mutant *Drosophila* lines. OGDH is shown as an

example of a mutant line with no overt phenotype. DNAJC6 and ALDH1A1 mutants revealed evidence for spontaneous degeneration in the absence of any injury stimulus. DNAJC5, CALB2, ROCK2 and HIBCH mutants revealed evidence for delayed degeneration following antennal ablation.

(Adapted from Wishart, et al, 2012)

To provide more robust genetic evidence for a role for one of these proteins (DNAJC5/CSP) in axonal and synaptic degeneration, we obtained two additional alleles: *csp*<sup>X1</sup>, a loss of function allele which deletes the first exon of *csp*; and *Df(3R)Exel6138*, a deletion which completely removes the *csp* locus. Both *csp*<sup>X1</sup> and *Df(3R)Exel6138* failed to complement the delay in axonal degeneration observed with our original allele (*csp*<sup>DG29203</sup>), thereby mapping this phenotype to the *csp* locus (Figure II-4). We further note that the severity of the delay in axonal degeneration appeared to be enhanced when *csp*<sup>DG29203</sup> was placed over either of these null alleles of *csp*, which argues that *csp*<sup>DG29203</sup> is a weak loss of function allele.



Wishart, et al, PLoS Genet. 2012;8(8):e1002936

**FIGURE II-4. Detailed genetic analysis confirms DNAJC5/CSP as a robust regulator of axo-synaptic degeneration *in vivo*.**

Representative confocal micrographs showing axon degeneration profiles in wild-type (WT) flies and additional DNAJC5/CSP lines: *csp*<sup>X1</sup>, a loss of function allele which deletes the first exon of *csp*; and *Df(3R)Exel6138*, a deletion which completely removes the *csp* locus. Examples are shown of uninjured axons (left panels), unilaterally injured axons (middle panels) and bilaterally injured axons (right panels). Both *csp*<sup>X1</sup> and *Df(3R)Exel6138* failed to complement the delay in axonal degeneration observed with our original allele (*csp*<sup>DG29203</sup>), thereby mapping this phenotype to the *csp* locus. Note how the severity of the delay in axonal degeneration was enhanced when *csp*<sup>DG29203</sup> was placed over either of these null alleles of *csp*, suggesting that *csp*<sup>DG29203</sup> is a weak loss of function allele. Neurons were labelled by the olfactory receptor neuron marker *OR22a-Gal4* driving expression of a membrane-localized green fluorescent protein.

(Adapted from Wishart, et al, 2012)

Thus, several individual proteins initially identified as a result of having modified expression levels in synapse-enriched fractions undergoing neurodegeneration appear capable of directly influencing synaptic and axonal stability and degeneration in *Drosophila*. In addition, our work rigorously defines the role of DNAJC5/CSP as an in vivo regulator of synaptic and axonal degeneration.

### **Unbiased Screening of the *Drosophila* 2<sup>nd</sup> Chromosome**

The MARCM system allows for the analysis of cells homozygous for a particular chromosomal arm in an otherwise heterozygous animal<sup>98</sup>. This is done using flippase-based mitotic recombination of entire chromosomal arms that have Flippase Recognition Target (FRT) sites inserted near the centrosome. During mitosis, heterozygous cells give rise to daughter cells homozygous for one parental chromosomal arm via flippase-mediated recombination – one cell inherits two copies of the same parental arm and the other cell inherits the two copies of the other parental arm. In the MARCM animal, one parental arm typically carries a lethal mutation while the homologous arm contains a tubulin-driven Gal80 that acts as an inhibitor of Gal4 drivers. In the heterozygous animal, Gal4 is silenced by Gal80 in all cells. Upon mitotic recombination by flippase, cells that inherit two copies of Gal80 remain unmarked. The cells that

inherit two copies of the other (typically mutant) arm do not inherit Gal80, and are thus marked by the Gal4 driver being used. Thus, the MARCM clones observed occur in the intersection between the pattern of expression of the flippase and that of the Gal4 driver.

Ethyl methanesulfonate (EMS) is used as a mutagen in flies to create randomly mutagenized stocks that can be screened for various phenotypes<sup>100</sup>. EMS induces mutations by reacting with guanine nucleotides in DNA. The subsequent adducts are either repaired incorrectly or cause incorrect base pairing during DNA replication, causing random DNA mutations.

Chromosomal deletions, also called deficiencies (Df), are useful tools in the mapping and characterization of novel mutations<sup>100</sup>. The Exelixis company has created whole collections of deletions with well-mapped endpoints that tile over 50% of the entire fly genome, providing precise information about which genes are uncovered by these deletions<sup>101</sup>. I used part of this collection to test whether any of the deletions would show a defect in Wallerian degeneration. If so, then I could test all of the small number of genes known to be uncovered by that deletion with other available reagents, or create new reagents targeting those genes.

## MARCM Analysis of WD Using Chromosome 2L Deficiency Lines

### Materials and Methods

MARCM-ready *Drosophila* stocks with deletions on chromosome 2L were previously established in the laboratory of Marc Freeman, PhD by recombining the deletions onto a WT FRT chromosome. An isogenized stock of *FRT40A* on the left arm of chromosome 2 was recombined with the individual stocks from the Exelixis collection of deletions on chromosomal arm 2L that is available from the Bloomington Stock Center. The generic genotype for these lines is *Df(2L)Exel####,FRT40A*. These lines were crossed to the following MARCM tester stock: *ey-FLP, UAS-mCD8::GFP ; tub-Gal80, FRT40A ; OR22a-Gal4, UAS-mCD8::GFP*. *ey-FLP* induces flippase activity in the developing eye and antennal discs, resulting in MARCM clones in the adult structures. A subset of ORNs were labelled with the OR22a promotor driving expression of a membrane-bound GFP. The Wallerian degeneration assay was performed as previously described<sup>67</sup>. Briefly, flies were aged for 7 days after eclosion to permit strong expression of the mCD8::GFP marker. The left third antennal segments were then removed with forceps, and the flies aged for 7 days at 25C. Fly heads were then removed and fixed in 4% formaldehyde/PBS/0.1% Tween and the brains dissected. Fly brains were then mounted in Vectashield antifade (Vector

Laboratories) and imaged on a Zeiss spinning disc confocal microscope (Intelligent Imaging Innovations, Denver, CO).

## Results

When crossed to the tester stock: *ey-FLP, UAS-mCD8::GFP ; tub-Gal80, FRT40A ; OR22a-Gal4, UAS-mCD8::GFP*, clones homozygous for the deficiency were generated in the developing eyes and antennae, a subset of which were marked with the olfactory receptor neuron driver *OR22a-Gal4*. These animals were tested in the ORN Wallerian degeneration assay, as described above. Four main phenotypes were expected from this screen: 1 – no phenotype, where axons degenerate and are cleared normally; 2 - debris, where axons degenerated but were not cleared. Due to the cell autonomy afforded to the neurons via MARCM, this could be due to a defect in “eat-me” cues where degenerating axons fail to signal to surrounding glia; 3 - intact, where the axons failed to degenerate normally; and 4 – no clones/cell lethal when no clones were observed in the adult brain. This could be due to either a failure in MARCM clone production, or to cell lethality of the homozygous deletion. Table II-3 lists the lines tested and results. Most of the lines did not produce testable clones in the ORN system – 35 out of 57 tested, even when repeated. In the other 22 lines, no phenotype was observed, meaning axon degeneration and glial

clearance of the debris proceeded normally. In these 22 lines, the genes uncovered likely do not participate in WD.

No Phenotype		No Clones/Cell Lethal	
Df(2L)Exel6011	Df(2L)Exel7042	Df(2L)Exel6001	Df(2L)Exel6277
Df(2L)Exel6014	Df(2L)Exel7046	Df(2L)Exel6002	Df(2L)Exel7002
Df(2L)Exel6016	Df(2L)Exel7048	Df(2L)Exel6003	Df(2L)Exel7006
Df(2L)Exel6029	Df(2L)Exel8008	Df(2L)Exel6004	Df(2L)Exel7007
Df(2L)Exel6030	Df(2L)Exel8010	Df(2L)Exel6005	Df(2L)Exel7008
Df(2L)Exel6038	Df(2L)Exel8016	Df(2L)Exel6006	Df(2L)Exel7014
Df(2L)Exel6256	Df(2L)Exel8019	Df(2L)Exel6007	Df(2L)Exel7015
Df(2L)Exel7010	Df(2L)Exel9031	Df(2L)Exel6008	Df(2L)Exel7016
Df(2L)Exel7022	Df(2L)Exel9032	Df(2L)Exel6009	Df(2L)Exel7021
Df(2L)Exel7029	Df(2L)Exel9038	Df(2L)Exel6012	Df(2L)Exel7023
Df(2L)Exel7040	Df(2L)Exel9064	Df(2L)Exel6015	Df(2L)Exel7027
		Df(2L)Exel6021	Df(2L)Exel7038
		Df(2L)Exel6022	Df(2L)Exel7039
		Df(2L)Exel6027	Df(2L)Exel7043
		Df(2L)Exel6028	Df(2L)Exel7059
		Df(2L)Exel6034	Df(2L)Exel8012
		Df(2L)Exel6036	Df(2L)Exel8021
			Df(2L)Exel8034

Table II-3. Deficiency lines tested for defects in Wallerian degeneration.

## A Forward-Genetic Screen for Wallerian Degeneration Mutants

### Materials and Methods

#### *Drosophila* Stocks and Injury Protocol

The following *Drosophila* strains from the Bloomington stock center were used in this study: *OR22a-Gal4,pUAST-mCD8::GFP; ey-flp; tub-Gal80,FRT2A; FRT82B,tub-Gal80; FRT2A,FRT82B; tub-Gal80,FRT40A; FRTG13,tub-Gal80; FRT40A,FRTG13*. The Wallerian degeneration assay was performed as described above.

#### Generation of 2<sup>nd</sup> Chromosome EMS-induced Mutant Stocks

To make the lines needed to test for WD mutants on the 2<sup>nd</sup> chromosome of *Drosophila*, I first isogenized the double-FRT chromosome (*FRT40A,FRTG13*) so that all subsequent lines arose from one single wild type chromosome to limit the effects of random drift in the original stock. The double-FRT chromosome contains FRT sites on both arms of chromosome 2, allowing for the independent MARCM analysis of both arms of a single chromosome (of a single mutant stock). The isogenization step is important because the deep sequencing results of any mutants found are compared to those of this original stock as a reference genome to identify polymorphisms (hopefully in the causative gene). A single

male of the stock *FRT40A,FRTG13/CyO ; OR22aGal4,UAS-mCD8::GFP/TM3*  
 was crossed to virgins of the stock *Sp/CyO ; OR22aGal4,UAS-mCD8::GFP/TM3*.  
 4 Individual male progeny were selected and crossed as follows:

*FRT40A,FRTG13/CyO ; OR22aGal4,UAS-mCD8::GFP/TM3*

X

*Sp/CyO ; OR22aGal4,UAS-mCD8::GFP/TM3*.

The progeny of these individual crosses were self-crossed to make several stocks of: *FRT40A,FRTG13<sup>iso</sup>/CyO ; OR22aGal4,UAS-mCD8::GFP/TM3*.

To make MARCM-competent tester stocks for screening, the following lines were created:

*eyFLP, CD8::GFP ; tub-Gal80, FRT40A/CyO*

*eyFLP, CD8::GFP ; FRTG13, tub-Gal80/CyO*

*eyFLP* induces flippase activity in developing eye and antennal structures<sup>102</sup>,  
*mCD8::GFP* is a Gal4-driven membrane-bound GFP, and *tub-Gal80* is a  
 ubiquitously expressed Gal80 that suppresses the Gal4 activation of UAS.

These lines were then used to test the different isogenized lines to ensure that normal WD occurred and to test for efficient clone induction. 10 adults from the following crosses were assayed in the standard WD assay as described above:

For 2L:

*FRT40A, FRTG13<sup>iso</sup>/CyO ; OR22aGal4, UAS-mCD8::GFP/TM3*

X

*eyFLP, CD8::GFP ; tub-Gal80, FRT40A/Cyo*

And for 2R:

*FRT40A, FRTG13<sup>iso</sup>/CyO ; OR22aGal4, UAS-mCD8::GFP/TM3*

X

*eyFLP, CD8::GFP ; FRTG13, tub-Gal80/Cyo*

The percentage of adults with ORN clone induction for each line tested was determined for each chromosome arm:

Line 1: FRT40A (2L): 88%

FRTG13 (2R): 33%

Line 2: FRT40A (2L): 60%

FRTG13 (2R): 43%

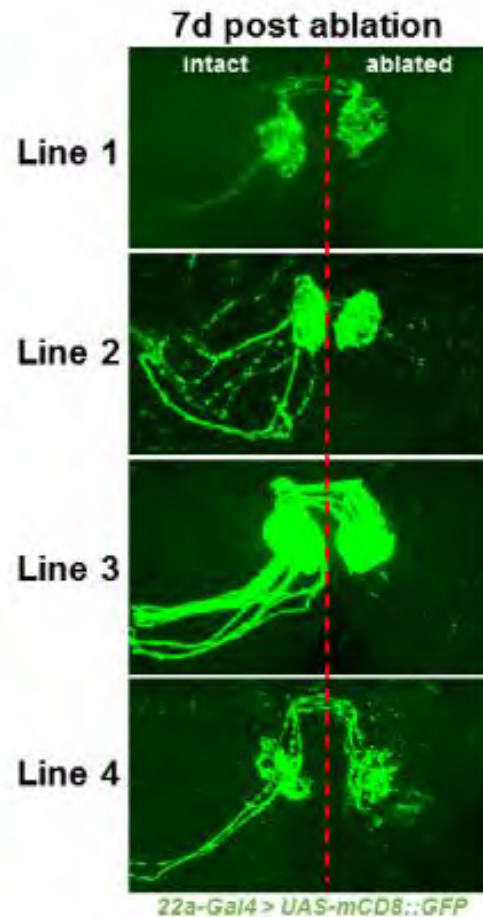
Line 3: FRT40A (2L): 100%

FRTG13 (2R): 100%

Line 4: FRT40A (2L): 100%

FRTG13 (2R): 50%

As line 3 gave had the best clone induction for both arms of chromosome 2, that line was used further and the others were discarded. Figure II-5 shows the representative results of the WD assay, as well as clone induction in the intact side. There are approximately 24 *OR22a-Gal4*-labelled ORNs per antennae, and due to axon bundling, typically 11-12 are discernable in the brain preparations shown<sup>102</sup>. Line 3 had the best clone induction overall, and in all lines WD proceeded normally as there is no debris left on the ablated side 7 days after injury.



**Figure II-5. MARCM clone induction and Wallerian degeneration in olfactory receptor neurons.**

MARCM clones were labelled by the olfactory receptor neuron marker *OR22a-Gal4* driving expression of a membrane-localized green fluorescent protein.

Representative images from each line show the variation in clone induction (see intact sides on the left). All lines showed normal Wallerian degeneration 7 days after ablation (right side of the images).

To make EMS-induced mutant stocks, I first made the following stock:

*Sp,hs-hid/CyO ; OR22aGal4,UAS-mCD8::GFP/TM3*

This line eliminates the need to collect virgins from each individual mutant cross to make stocks. Rather, the crosses are heat-shocked which induces the expression of the pro-apoptotic gene *hid*. Any progeny in the crosses that inherits the *Sp,hs-hid* chromosome will therefore die and the remaining progeny will be appropriately balanced.

I then performed the following protocol twice. First, dozens of males from the genotype *FRT40A,FRTG13<sup>iso</sup>/CyO ; OR22aGal4,UAS-mCD8::GFP/TM3* were starved by placing them in empty bottles with a damp Kimwipe in the morning. In the evening, I added 12.5µL EMS to 5mL 1% sucrose and mixed very well (EMS is oily) for each bottle of males (50-100 males per bottle). This dilution was determined by the characterization of induced mutagenesis prior to the construction of mutant stocks for the 3<sup>rd</sup> chromosome screen. The EMS/sucrose solution was then added to a Kimwipe at the bottom of an empty bottle and the flies were incubated in the bottle overnight. The next morning, the flies were transferred to fresh bottles of food and allowed to clean themselves for 3-4 hours. The males were subsequently crossed with hundreds of virgins of *Sp/CyO ; OR22aGal4,UAS-mCD8::GFP/TM3*. After 3 days, males were removed and females were flipped into new bottles. The females were flipped into new bottles after 3 days twice more.

From these crosses 1994 males were collected and individually crossed with 4-5 virgins of the genotype: *Sp,hs-hid/CyO ; OR22aGal4,UAS-mCD8::GFP/TM3*. 3 days later, the flies were discarded and the vials heat-shocked in a 37°C water bath for 1hr, and again the next day for 1hr. Only 3 stocks made in this fashion had the *Sp* marker, indicating excellent negative selection of *Sp,hs-hid*. The surviving 1100 stocks with EMS-induced mutations had the following genotype:

*\*,FRT40A,FRTG13<sup>iso</sup>,\* / CyO ; OR22aGal4,UAS-mCD8::GFP / TM3*

I, Nicki Fox, PhD, and later Rachel Hackett then tested these stocks in the standard WD assay as described above. Stocks with homozygous viable 2<sup>nd</sup> chromosomes, as determined by loss of the *CyO* balancer, were tested as 7 day old adults. The 3<sup>rd</sup> chromosomal ORN driver and GFP reporter allowed us to test both chromosomal arms simultaneously in the 199 (18%) 2<sup>nd</sup> chromosome homozygous viable stocks recovered. The remaining 901 (82%) stocks had homozygous lethal 2<sup>nd</sup> chromosomes, or synthetic lethal interactions that required MARCM analysis to determine if they were competent in WD. Each stock was crossed to a tester stock for each chromosomal arm.

For 2L:

*\*,FRT40A,FRTG13<sup>iso</sup>,\* / CyO ; OR22aGal4,UAS-mCD8::GFP / TM3*

X

*eyFLP, CD8::GFP ; tub-Gal80, FRT40A/Cyo*

And for 2R:

*\*,FRT40A,FRTG13<sup>iso</sup>,\* / CyO ; OR22aGal4,UAS-mCD8::*GFP* / TM3*

X

*eyFLP, CD8::*GFP* ; FRTG13, tub-Gal80/Cyo*

Progeny of these crosses were collected and the genotypes below were assayed in our WD protocol (as above).

For 2L:

$$\frac{+}{eyFLP, CD8::*GFP*}; \frac{*,FRT40A,FRTG13^{iso},*}{tub-Gal80, FRT40A}; \frac{22aGal4,UAS-mCD8::*GFP*}{+}$$

And for 2R:

$$\frac{+}{eyFLP, CD8::*GFP*}; \frac{*,FRT40A,FRTG13^{iso},*}{FRTG13, tub-Gal80}; \frac{22aGal4,UAS-mCD8::*GFP*}{+}$$

We felt it was important to screen enough animals per cross to make a solid call whether or not WD was affected in that particular line, especially considering that clone induction was quite variable fly-to-fly with the EMS lines. We therefore dissected and screened at least 7-10 brains per cross. In lines that required repeating due to a lack of observed clones, anti-GFP antibody staining

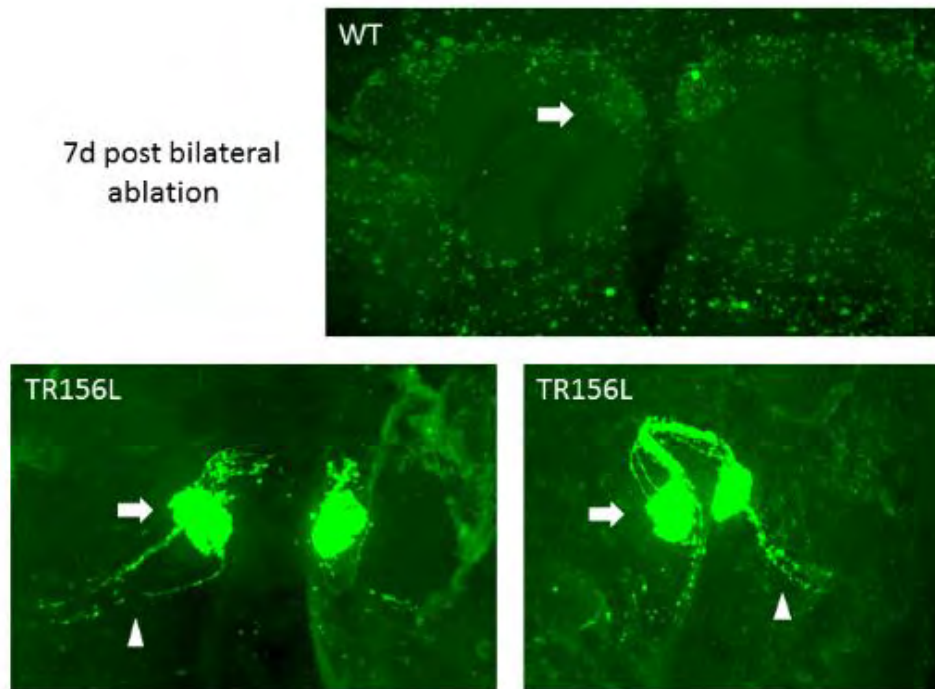
was performed to boost any weak signal; these added steps revealed weakly labelled clones in some crosses.

## Results

Altogether, we were able to screen through 1593 chromosomal arms. In 400 of these arms, no clones were visible, even after a repeat cross with anti-GFP staining. 330 arms were tested by another graduate student, Tom Burdett, who tested them in the wing assay developed later<sup>103</sup>, for a grand total of 1923 2<sup>nd</sup> chromosomal arms tested. Much to our great dismay, none of these arms harbored a mutation that affected axon degeneration.

While most of the tested chromosomal arms had no phenotype, around 20 were classified as having a confirmed clearance defect upon repeating. For the viable arms tested, this could be due to loss of a neuronal eat-me cue, resulting in axon degeneration with a failure in the clearance by glia or it could be due to a defect in the glial response to axon injury. The lethal arms tested, by virtue of the MARCM system, are homozygous only in the labelled neurons and any cells that may have expressed *ey-flp* when dividing in the developing eye and antennal discs. As glia proliferate in the larval brain and migrate into newly developed brain regions like olfactory lobes during metamorphosis, and do not express *ey-flp*, the cell-autonomy of the clearance defect of any lethal arms lies with the neurons<sup>104</sup>. One example of a mutant of this class is TR156L. By 24hrs after

ablation, the homozygous mutant TR156L axons are fragmented (data not shown). However, at 7 days after ablation, debris remains; Figure II-6 shows the results of ablating both antennae compared to the same ablation in WT. Arrows point to the synaptic zones and arrowheads point to debris. While not all brains had large amounts of debris at day 7, all had more than is seen in WT (essentially none).



**Figure II-6. Line TR156L exhibits a failure in debris clearance after axotomy.**

Representative MARCM clones showing wild-type (WT) and mutant (TR156L) phenotypes. In WT, axonal debris is cleared by 7 days after axotomy, with minor synaptic debris visible on the glomeruli (arrows). TR156L shows extra synaptic (arrows) and axonal (arrow heads) debris 7 days after axotomy. MARCM clones were labelled by the olfactory receptor neuron marker *OR22a-Gal4* driving expression of a membrane-localized green fluorescent protein.

## Discussion

I have not failed 10,000 times. I have not failed once. I have succeeded in proving that those 10,000 ways will not work. When I have eliminated the ways that will not work, I will find the way that will work.

-Thomas Edison, attributed

## Candidates from the Literature

The objective of this work was to identify genes that when mutated gave an axon degeneration phenotype. The genes tested were gleaned from the literature as candidates based on their involvement in other cell death or signaling pathways, on being implicated to interact with SARM proteins, or on their involvement in other pathways that could play a role in axon degeneration based on assumptions about the process of degeneration and how Wld<sup>s</sup> might prevent it. As none of the 213 lines tested resulted in intact axons after axotomy, most were not investigated further. Genotypes that resulted in extra debris remaining fall into 2 broad categories: immune/kinase signaling, and calcium regulation.

Genes in the immune signaling category include: *Toll*, *18wheeler (Toll2)*, *Tehao (Toll5)*, and *Traf6*. Toll receptors are a large family of receptors that

regulate many aspects of immunity<sup>80</sup>. The mammalian ortholog of *Traf6* is an adapter of the TNF receptor, and has been linked with JNK signaling in necrotic cell death<sup>105</sup>, and also PKC $\delta$  /JNK/c-Jun in cell adhesion<sup>106</sup>. Interestingly, both *Pkc $\delta$*  and *kayak* mutants had extra debris in the screen. *Kayak* is the *Drosophila* ortholog of Fos that partners with Jun to form the AP-1 transcription factor that regulates the transcription of many genes in various cell processes<sup>67</sup>. Alterations in these pathways could indeed delay axon degeneration or interfere with the production of an eat-me cue. However, since whole-animal mutants were tested, the cell-autonomy of the defect cannot be determined. It is more likely, given the immune function of most of the genes in the category, that glial clearance of debris is compromised. In support of this hypothesis, the *kayak* allele tested, which had the strongest phenotype in the screen, was later shown to be required in glia for the appropriate clearance of degenerated axons<sup>71</sup>. Therefore, it is likely that these other genes also work in glia to regulate the clearance of degenerated axons.

The other category centers on the regulation of calcium ions in the cell. These were tested because the influx of extracellular calcium is necessary and sufficient for axon degeneration. The genes include: *SERCA*, *ca- $\alpha$ 1D*, *cacophony*, and *ca- $\beta$* . *SERCA* is a calcium ATPase that regulates endoplasmic reticulum calcium levels, and is required for calcium signaling in *Drosophila* neurons<sup>107</sup>. Though influx of extracellular, and not intracellular store-based release are required for axon degeneration, endoplasmic reticulum is intimately

linked with calcium dysregulation in cell death pathways and is also found in axons<sup>107</sup>.

*ca-α1D*, *cacophony*, and *ca-β* all encode voltage-gated calcium channel subunits, and were tested as they could be part of the channels that allow calcium influx after axotomy. *Ca-α1D* is the *Drosophila* ortholog of mammalian α1 subunit of the Cav1 L-type channels, *Cacophony* is the orthologous α1 subunit of the Cav2 P/Q, N, and R-type channels, and *Ca-β* is the β subunit of Cavβ channels<sup>108</sup>. The *ca-β* mutants are viable, and both resulted in debris at day 8 indicating a defect in clearance. However, axons were degenerated by 24hrs after injury, indicating that there was not a delay in axon degeneration. That debris remains at day 8 could mean that the glia are not activated appropriately or that they are incompetent in clearing the axonal debris. *ca-α1D* and *cacophony* mutants are both lethal, and so were tested with MARCM individually as well as in a double MARCM system to generate neurons that are homozygous mutant for both genes. In all cases, similar to the *ca-β* mutants, axons were fragmented by 24hrs with debris remaining by 8 days after axotomy. It is thus possible that voltage-gated calcium channels participate in the production of injury-induced eat-me cues that signal to surrounding glia to clear degenerated axonal debris. The notion that L-type calcium channels are required for axon degeneration has only been tested pharmacologically; therefore these assays were an attempt to test them genetically. The results suggest that these channels do not participate in the calcium influx required for

axon degeneration. In epithelial injury in *C. elegans*, TRPM gates the calcium influx<sup>109</sup>. It is thus possible that TRPM also operates in axon degeneration to increase intra-axonal calcium levels leading to degeneration. We are currently testing mutants of *Trpm* in the axotomy assay.

### **Candidates Identified by Mouse Proteomics**

The objective of this work was to test genes identified by our collaborators in the axotomy assay to see if they could delay WD, which encompasses axon degeneration as well as the clearance of axonal debris. Our collaborators identified mouse proteins that were differentially regulated during WD when comparing WT and *Wlds* mouse synaptosomal preparations<sup>99</sup>. I identified and tested mutant lines in the orthologous *Drosophila* genes in the ORN axotomy assay. While none of the genotypes had intact axons at 7 days after axotomy, 4 showed axonal debris and 2 showed spontaneous degeneration of intact axons at this time point. All but HIBCH, which causes a progressive neuropathy when mutated, have been implicated in synaptic function<sup>110</sup>. DNAJC5/CSP has been shown to participate in the assembly of SNARE complexes<sup>111</sup>, DNAJC6 likely participates in vesicle recycling<sup>112,113</sup>, and ROCK2, CALB2/Calretinin, and ALDHA1 participate in synaptic plasticity<sup>114–116</sup>. The identification of genes involved in synaptic plasticity and function is consistent with the fact that these

genes were identified via proteomic analysis of mouse synaptosomal preparations. The way these mutants influence WD is unclear.

We chose to further examine *csp* mutants as the level of debris was considerable at day 7. An additional loss of function allele and a deletion line gave stronger phenotypes, and together with phenotypic complementation assays directly indicate DNAJC5/CSP as a regulator of WD. The caveat of cell autonomy still applies, as these were whole-animal mutants that were tested. However, the results imply a conservation in genes required during WD for the appropriate degeneration and clearance of axonal and synaptic debris.

### **MARCM Screening of EMS-generated Mutants on the *Drosophila* 2<sup>nd</sup> Chromosome**

The objective of this work was to screen for mutations that prevent the appropriate degeneration of axons after axotomy. This screening strategy has so far identified one novel *bona fide* regulator of axon degeneration: *dsarm*. Many mutations were also found in the *hiw* gene, however *hiw* and the mouse homolog *Phr1* were already known to regulate axon degeneration.

Some mutant chromosomal arms tested exhibited the interesting phenotype of neuronal cell-autonomous defects in glial clearance of axonal debris, the strongest being TR156L. As MARCM was used to analyze the line, the phenotype appears to be cell-autonomous to the neurons. Dominant effects

of a mutation on the right arm (or other chromosomes) of the mutant stock could possibly affect glial clearance of debris remaining. Examining WD in the mutant stock as-is or crossed to WT would rule out this possibility. This mutant and the reference stock were deep sequenced and the SNPs identified on each left 2<sup>nd</sup> chromosome arm compared. Unfortunately, no divergent coding changes were identified. Given the lack of other stocks/complementation groups with which to compare, the investigation into this line was not further pursued.

Screening through the mutant lines was hindered by the number of arms that did not give MARCM clones, precluding any phenotypic analysis. Approximately 240 arms (11%) were not able to be screened for this reason. These arms are presumed to have cell- or ORN-lethal mutations that prevent homozygous mutant clones. They could also have EMS-destroyed FRT sites, which would prevent the flip event that creates a MARCM clone.

The vast majority of the stocks harbored homozygous lethal mutations (approximately 849 / 1049 total, 80%), indicating that the EMS-induced mutagenesis was adequate. We used a titer of EMS that would produce many mutations per chromosome in order to test multiple genes per chromosomal arm. However, this “multitasking” should be balanced with the ability to produce assayable clones. There are approximately 24 OR22a-labelled ORNs per antenna, however there are ~600 ORNs total in each 3<sup>rd</sup> antennal segment. It seems possible that increasing the number of labelled cells in the system could result in a greater chance of creating induced clones. Using a pan-ORN driver

such as *OR83b-Gal4* could therefore increase the number of testable lines showing MARCM clones.

Though this screening effort failed to produce novel regulators of axon degeneration, I still believe that in the study of Wallerian degeneration the most important work remains in delineating the pathway that controls this process.

Thomas Edison's words seem well suited to inspire persistence, though possibly they pertain better to iterative rather than repetitive processes. Writing this chapter, I cannot help but be reminded of my screening anthem that seemed to be played more than randomly as I sat day after day in the fly room in my (screening) chair:

Sitting on an angry chair  
Angry walls that steal the air  
Stomach hurts and I don't care

What do I see across the way, hey  
See myself molded in clay, oh  
Stares at me, yeah I'm afraid, hey  
Changing the shape of his face, aw yeah...

...Loneliness is not a phase  
Field of pain is where I graze  
Serenity is far away

Excerpted from: Alice in Chains. "Angry Chair." *Dirt*.

## Chapter III: Genes Required for Wallerian Degeneration also Govern Dendrite Degeneration after Injury

### Introduction

Neuronal connectivity, and therefore function, relies on the proper maintenance and operation of neurite processes. Dendrites and axons can exhibit extremely lengthy, varied, and elaborate forms. As such, they are vulnerable to various insults resulting from trauma, hypoxia/ischemia, and neurodegenerative diseases. When injured, dendrites and axons follow a stereotyped process of degeneration and clearance by neighboring cells, called Wallerian degeneration (WD)<sup>57</sup>. This process in axons is regulated by calcium, as the influx of extracellular calcium is both necessary and sufficient for WD<sup>46</sup>.

WD was long thought to be the result of the neurite being cut off from its lifeline the cell body. 25 years ago the fortuitous discovery of the *Wld<sup>s</sup>* mouse (for slow Wallerian degeneration) questioned the assumption that WD was a passive process of deterioration<sup>27</sup>. Eventually we came to understand that the *Wld<sup>s</sup>* mutation created a new gene, that when expressed in neurons could preserve the structure and function of a severed axon for weeks instead of 1-2 days<sup>28</sup>. This preservation of neurites by WLD<sup>s</sup> is now known to extend from fly axons and dendrites to mouse axons and even human cells<sup>117</sup>. It is not known

exactly how WLD<sup>s</sup> protects neurites, but recent studies have linked increased mitochondrial motility, ATP production, and axonal calcium buffering to axon preservation<sup>118</sup>.

The protection afforded dendrites and axons by WLD<sup>s</sup> is remarkable. However, the exogenous gain of function nature of WLD<sup>s</sup> makes it inherently difficult to study the process of WD. Therefore, identifying endogenous regulators of WD is important to parse out this process as well as provide therapeutic targets for neurite insults resulting from trauma or disease. Recently, we and others identified *dsarm/Sarm1* as an endogenous, robust regulator of axon degeneration<sup>68,87</sup>. Mutations in this gene allow severed axons to remain intact several weeks in flies and mice, a level similar to that seen with WLD<sup>s</sup>. The highly conserved Sarm proteins contain ARM and SAM protein-protein interaction domains as well as a TIR domain, and require all of these for proper regulation of WD. In *C. elegans*, the ortholog TIR-1 acts downstream of an influx of extracellular calcium to activate a MAP kinase pathway<sup>82</sup>. Similarly, in mice, SARM1 acts upstream of a MAP kinase cascade activated by axon injury<sup>45</sup>.

Recently, another potent regulator of WD was discovered. Mutants of *Drosophila hiw*, and later its mouse ortholog *Phr1* were shown to protect severed axons to an extent similar to *Wld<sup>s</sup>* and *dsarm/Sarm1*<sup>88,89</sup>. *Hiw* and *PHR1* are part of a highly conserved family of E3 ubiquitin ligases with prominent roles in axon guidance, synapse formation, and axon regeneration<sup>91</sup>. In addition to protein degradation, *Hiw* family members govern these processes through signaling

events and cytoskeletal rearrangement<sup>96</sup>. Interestingly, Hiw family proteins have also been shown to negatively regulate a MAP kinase cascade in various contexts. In WD, it is proposed that Hiw and PHR1 regulate levels of NMNAT enzymes, which are thought to act as survival factors for axons<sup>88,89</sup>.

*dsarm* and *hiw* both endogenously regulate the degeneration of severed axons. Do these genes also regulate dendrite degeneration? *Drosophila* dendritic arborization neurons (da) extend large, complex dendritic branches and are an excellent system to use to address this question. The da neurons are classed I-IV based on the complexity of their branches<sup>1</sup>. Class III and IV neurons have the most complex branch patterns, and reside in the larval body wall allowing direct visualization of their dendrites in live, intact animals. Class III da neurons respond to gentle touch, and do not regenerate ablated dendrites<sup>73,78</sup>. Class IV da neurons respond to noxious stimuli including heat and UV light, and regenerate both dendrites and axons if they are ablated<sup>76,78</sup>. Using these CIII and CIV da neurons, we provide evidence that both *dsarm* and *hiw* are required for dendrite degeneration after injury. We also use time-lapse recording of dendrites expressing GCaMP to investigate the role of these genes in dendritic and mitochondrial calcium handling after dendritic injury. Finally, we show that in *hiw* mutants, protection is branch-specific, distinguishing it from the protection afforded by *Wlds* and *dsarm* mutants, and the protection of axons by all three.

## Results

### Genes required for Wallerian degeneration also govern dendrite degeneration after injury

Upon injury, axons undergo a stereotyped sequence of degeneration and clearance called Wallerian degeneration (WD)<sup>25</sup>. The mutant protein slow Wallerian degeneration (WLD<sup>s</sup>) strongly inhibits this process in both axons and dendrites<sup>57</sup>. Recently, two endogenous regulators of WD were described that robustly prevent injury-induced degeneration in *Drosophila* axons: *dsarm* and *hiw*. We therefore wanted to determine if these genes also govern dendritic responses to injury. To assay dendritic injury, *ppk-Gal4* was used to label class IV multidendritic neurons in the *Drosophila* larval body wall. The dendrites of these sensory neurons were injured with a pulsed-UV laser and imaged immediately after injury and 24hrs later. The ImageJ plugin NeuronJ was used to trace and analyze the images; tracings appear below their respective images (Figure III-1 A-D). As previously shown by Tao and Rolls, injured WT dendritic branches degenerate and are cleared by 24hrs after dendriotomy (Figure III-1A)<sup>57</sup>. WLD<sup>s</sup> strongly protects severed dendritic branches, with greater than 70% of the total branch length remaining at 24hrs (Figure III-1B). The injured dendrites remaining at 24hrs are somewhat simplified, having lost a portion of their terminal branches. Mutations in an endogenous regulator of axon degeneration, *dsarm*, also protected dendrites after injury to a degree equivalent

with WLD<sup>s</sup> (Figure III-1C), suggesting that endogenous regulators of WD cell-autonomously govern neurite degeneration.

Surprisingly, mutations in *hiw*, which confer protection to axons similar to WLD<sup>s</sup> and *dsarm*, afforded only 40% protection to the severed dendrites (Figure III-1D & E). Interestingly, the partial protection of *hiw* mutants seems to be cell specific, as only 60% of the ablated cells had protected dendrites as compared with 100% of WLD<sup>s</sup> and *dsarm* mutant cells (Figure III-1F). If only those *hiw* mutant cells showing protection are analyzed, the dendrite protection seen is on par with WLD<sup>s</sup> and *dsarm* mutants (Figure III-1G). This result suggest that the partial protection of *hiw* mutant cells occurs on a cell-by-cell basis and results in an all-or-none phenotype. Taken together, these data argue that endogenous pathways that govern axon degeneration also govern dendrite degeneration after injury.

Class IV da neuron dendrites are capable of regenerating after sustaining an injury, unlike other larval sensory neuron dendrites, such as those of CIII da neurons<sup>78</sup>. To determine if genes required for WD govern dendrite degeneration in a different neuronal subtype that has different regeneration responses to injury, we examined whether WLD<sup>s</sup>, *dsarm*, and *hiw* mutants also protect injured dendrites in class III multidendritic sensory neurons. Class III da neurons also reside in the larval body wall and are marked by the *19-12-Gal4* driver. Their dendrites consist of long, microtubule-enriched primary branches (green in the tracings) and short, actin-rich sensory filopodia or spikes that originate from and

line the primary branches (magenta in the tracings). To assay WD genes in the degeneration of CIII dendrites, primary branches were ablated close to the cell bodies. Images and tracings of the branches were recorded as above. In WT, branches degenerated and were cleared by 24hrs after ablation, as occurs with CIV cells (Figure III-2A). Also similar to CIV cells, *Wld<sup>s</sup>* and *dsarm* mutant branches failed to degenerate (Figure III-2B and III-2C). *hiw* mutant branches were also preserved, but to a lesser extent than *WLD<sup>s</sup>* or *dsarm*, or even *hiw* CIV cells as only ~30% of CIII cells showed preservation of dendritic branches (Figure III-2D and III-2E). Interestingly, the persistent CIII branches in all genotypes show a different pattern of preservation than the CIV branches. The primary branches were largely intact, and did not show a simplification as do the CIV branches (Figure III-2F). The sensory filopodia/spikes, however, were greatly reduced in number, though not average length, 24hrs after injury (Figure III-2G, III-2H). Surprisingly, *hiw* mutants, when the branches failed to degenerate, had significantly more spikes preserved than *WLD<sup>s</sup>* or *dsarm*. Overall, these results argue that WD genes operate in different neuronal cell types to govern dendrite degeneration.

## Injury induces intra-dendritic calcium transients

The influx of extracellular calcium has been shown to be both necessary and sufficient for normal axon degeneration *in vitro* and *in vivo*<sup>46</sup>. Upon transection of axons, intra-axonal calcium rapidly increases and then returns toward baseline<sup>118</sup>. This initial burst in axonal calcium level may initiate WD, as pharmacologically blocking L-type calcium channels or chelating extracellular calcium prevents degeneration. Recently, WLD<sup>s</sup> was shown to attenuate the rise in axonal calcium after a laser ablation injury, likely by increased mitochondrial calcium buffering<sup>118</sup>. Calcium handling in dendrites is different than axons; calcium transients represent the main signal found in post-synaptic or sensory activation of dendrites whereas they contribute little to the action potentials of normal extra-synaptic axonal function<sup>12,13</sup>. We therefore wanted to determine whether dendrite injury also results in calcium transients upon injury as in axons. To do this, we expressed GCaMP6S in CIV da neurons with the *ppk-Gal4* promoter. Co-expression of myr-td-Tomato allowed visual confirmation of dendriotomy. Data were collected by time-lapse confocal microscopy and mean  $\Delta F/F_0$  values per time point were plotted. In resting WT cells, intra-dendritic calcium, as imaged with GCaMP6S, is maintained at a steady and low level (Figure III-3A). Laser ablation of a dendritic branch near the cell body resulted in a robust GCaMP signal in the branch both proximal and distal to the cut (Figure III-3B). This spike in intra-dendritic GCaMP signal was transient, and levels returned toward baseline levels as before the injury. In Figure III-3C, GCaMP

tracings of the branch distal to the cut (grey) and the mean (red) are plotted versus time.

### **Dendritic calcium transients upon injury in Wallerian degeneration mutants**

To determine whether dendritic GCaMP signals are attenuated by WLD<sup>s</sup> as in axons, we expressed WLD<sup>s</sup> or a catalytically inactive form of WLD<sup>s</sup> (WLD<sup>s-dead</sup>) that was shown to be inactive in both axon protection and the attenuation of a GCaMP spike after axon injury<sup>118</sup>. Surprisingly, unlike axonal GCaMP spikes, dendrites expressing WLD<sup>s</sup> showed no difference in peak signal or time to return to baseline than WLD<sup>s-dead</sup> in the dendrite distal to the cut. These results could indicate differences between axons and dendrites in the way calcium is handled, or reveal differences in degeneration between these two cellular compartments. We next wanted to determine if endogenous WD genes affect calcium transients in dendrites after injury. Dendrite branches of WT and *dsarm* MARCM clones expressing myr-td-Tomato and GCaMP6S were laser ablated and imaged. The resulting  $\Delta F/F_0$  GCaMP tracings showed no difference between WT and *dsarm* in peak signal or return to baseline (Figure III-3E), suggesting that the dendrite protection seen in *dsarm* mutants does not depend on the attenuation of a calcium spike upon injury. Similarly, *hiw* mutant dendrites had comparable peak and return to baseline  $\Delta F/F_0$  values as *hiw* / + controls (Figure III-3F). These

results together suggest that both *dsarm* and *hiw* act downstream or independently of a calcium spike after dendrite severing.

### **Mitochondrial calcium transients upon dendrite injury in Wallerian degeneration mutants**

Mitochondria are important regulators of intracellular and intra-axonal calcium levels, and have been implicated to be the subcellular compartment where WLD<sup>s</sup> operates<sup>36,118</sup>. Since extracellular calcium influx is required for WD, and since mitochondria buffer and store calcium, we reasoned that mitochondrial calcium levels could change after dendrite injury and that mutants that prevent WD could alter that process. To investigate calcium dynamics in mitochondria *in vivo*, we created a GCaMP5a that is targeted to the mitochondrial matrix via the mitochondrial import sequence from human cytochrome C oxidase subunit VIII. This mitoGCaMP was expressed in CIV da neurons together with myr-td-Tomato to visualize dendriotomy and analyzed as above. In WT cells, mitoGCaMP labelled mitochondria in the dendrites, cell bodies, and axons of CIV da neurons (Figure III-4A). Upon laser injury to a dendrite close to the cell body, mitoGCaMP fluorescence rapidly increased in mitochondria throughout the cell and returned toward baseline in a manner similar to soluble GCaMP6S (Figure III-4B). Additionally, the dendritic mitochondria appeared to shrink after injury; a morphological change that has been reported in axons<sup>117</sup>.

To determine if mutants affecting WD also alter mitochondrial calcium dynamics after injury, we expressed either WLD<sup>s</sup> or WLD<sup>s-dead</sup> as well as mitoGCaMP and myr-td-Tomato in CIV da neurons. Upon laser injury, there was a spike in mitoGCaMP fluorescence and a subsequent return to baseline (Figure III-4C). No difference was observed between the WLD<sup>s</sup> and WLD<sup>s-dead</sup> groups in  $\Delta F/F_0$  peak and return to baseline values (Figure III-4C). Similarly, there was no difference between *hiw* mutants and the heterozygous control animals (Figure III-4D). These results suggest that WD mutants may not alter mitochondrial matrix calcium handling after injury to dendrites.

### ***hiw* mutants reveal cell-autonomous branch-specific degeneration of dendrites**

The partial protection afforded to dendrites by a *hiw* mutant is at odds with the data for axon degeneration in flies and mice<sup>88,89</sup>. Robust protection of axons after injury in *hiw* mutants has been reported for nearly the entire lifespan of the fruit fly (~50 days) and strong (but incomplete) protection for at least 10 days in transected mouse sciatic nerve. Additionally, cell-to-cell variability at a time point as early as 24hrs in any system has not been reported for *hiw* mutants. We therefore sought to investigate these discrepancies further. We took advantage of the fact that the *hiw*<sup>AN</sup> allele is homozygous viable<sup>119</sup>, and injured dendritic branches on adjacent CIV da neurons. All of the branches, as well as the axons,

were severed in four neighboring cells (Figure III-5A, top). Interestingly, some dendritic branches, but not others failed to degenerate in each cell, suggesting that degeneration is branch-specific rather than cell specific (Figure III-5A, bottom). The branches were severed in a clockwise fashion starting at the 12 o'clock position in each cell to ascertain if a recent prior injury to the cell could influence the degeneration of other branches. As no clear pattern emerged in the order of branch preservation, the degeneration appears to not be influenced by a previous injury. All the severed axons remained, similar to what has been reported previously in other neurons<sup>103</sup>. It is possible that length of a dendrite branch determines whether it will degenerate normally in this mutant background; therefore branch length was quantified and related to degeneration status. There was no significant difference in the average branch length between degenerated and preserved branches, indicating that this possibility is not a likely explanation for the partial protection seen in *hiw* mutants (Figure III-5B).

The *hiw* allele thus far used to examine dendrite degeneration after injury, *hiw*<sup>ΔN</sup>, is a viable truncation that removes the N terminus of the gene. While production of a truncated, and possibly partially active, protein has so far not been formally excluded, phenotypes such as neuromuscular junction overgrowth and a robust defect in axon degeneration after injury have been reported with this allele<sup>120</sup>. However, we wanted to test other alleles to exclude such possibilities. Recently, many alleles of *hiw* were recovered in a forward-genetic screen for axon degeneration mutants<sup>103</sup>. We therefore tried an additional allele, *hiw*<sup>587</sup>,

which encodes an early stop in the gene. This allele is homozygous lethal, so we used MARCM to generate clones labelled by *ppk-Gal4*. MARCM also allowed us to test the cell autonomy of *hiw*<sup>587</sup> to suppress degeneration in CIV da neuron dendrites. Clones were identified and all dendrite branches, as well as axons, were severed near the cell body and imaged as above. In the MARCM clones, some dendrites failed to degenerate, and branches showing protection were preserved to a level equivalent with *hiw*<sup>ΔN</sup> (Figure III-5C and D). In all cases, the severed axons were preserved by 24hrs (Figure III-5C and D). Similar results were obtained with a third allele, *hiw*<sup>275</sup>, which also encodes an early stop in the gene (data not shown). Altogether, these results indicate a cell-autonomous role for *hiw* in the branch-specific degeneration of dendrites after injury.

## Discussion

In this study, we found that endogenous regulators of axon degeneration also regulate the degeneration of injured dendrites. Surprisingly, the preservation of the complex and dynamic dendritic structures of CIV and CIII da neurons revealed differences between *WLD*<sup>s</sup>/*dsarm* and *hiw*. Several mutations in *hiw* provided cell-autonomous but seemingly branch-specific degeneration in CIV dendrites, suggesting that this process could be stochastic or delayed in these backgrounds. We also found that dendrites, like axons, experience a large calcium transient in response to injury. In contrast to that seen in axons, *WLD*<sup>s</sup>

does not lessen the transient; nor do *dsarm* or *hiw* mutants. A similar pattern was observed with a mitochondrially-targeted GCaMP5a, suggesting that regulating mitochondrial matrix calcium handling as can be assayed by GCaMP after injury is not a mechanism of dendrite protection for either *Wld<sup>s</sup>* or *hiw*.

Protection of axons from Wallerian degeneration has been observed for some 25 years, however only recently endogenous regulators of this process have been identified. Mutations in *dsarm/Sarm1* and *hiw/Phr1* robustly protect axons after axotomy. We show that this protection extends to dendrites in *Drosophila* sensory neurons. Two different classes of dendritic arborization neurons with different injury-induced regeneration responses were tested. Class IV da neurons robustly regenerate their dendrites (and axons) after laser ablation while class III da neurons do not regenerate an injured dendrite. However, our analyses show that degeneration responses to injury are equivalent between these two classes of neurons, suggesting that the degeneration and regeneration responses to injury operate through distinct mechanisms.

In both classes of neurons, we observed a simplification of preserved dendrites after injury. In mutant CIV da branches, terminal branches were lost while the majority of the dendritic structure was maintained. In mutant CIII da branches, actin-rich spikes were diminished in number while the microtubule-rich primary dendrites were completely preserved. Comparing these two patterns of preservation could indicate that labile, actin-based branches are less preserved and may be the first structures to degenerate after injury. The loss of these

branches could also be explained by a change in branch dynamics, resulting in an imbalance between extension and retraction. CIII spikes are extremely dynamic in most larval stages, and in *singed* mutants a perturbation in dynamics leads to decreased spike numbers<sup>121</sup>. Interestingly, in some cases with all three mutants, dynamic spikes could be observed 24hrs after injury, suggesting that dynamics are preserved after injury and that an imbalance in extension/retraction could participate in spike loss (scan for QR code at end of paragraph for an example). Many termini of CIV da neurons are also actin-rich, dynamic, and lack microtubules<sup>122</sup>. Microtubule invasion into these labile structures stabilizes the termini, whereas retraction typically occurs if this invasion/stabilization fails to occur. Altogether, these results suggest that microtubule-based structures are protected in WD mutant dendrites while actin structures are largely lost, whether through *bone fide* degeneration or through dynamic imbalance.



Preserved dynamics in *dsarm*<sup>896</sup>-protected class III dendrite  
24hrs after ablation.

Extracellular influx of calcium into an injured axon has been shown to be necessary and sufficient for axon degeneration<sup>46</sup>. We and others have previously shown that there is a large spike in axonal calcium upon injury<sup>118,123</sup>. WLD<sup>s</sup> reduces this spike, likely through enhanced mitochondrial buffering of calcium that leads to an attenuation of the degeneration signal. Here, we show

that dendrites also have a large injury-induced calcium spike. We also show that dendrite injury results in a calcium spike within mitochondria. Puzzlingly, though WLD<sup>s</sup> protects both dendrites and axons, it did not diminish the dendritic calcium transients. This discrepancy could be due to differences between calcium handling in axons versus dendrites. In axons, extra calcium is largely cleared by mitochondria and the passive sodium/calcium exchanger<sup>124</sup>. Accordingly, mitochondria accumulate in axonal regions of high metabolic activity, calcium influx, and sodium influx (which causes the Na/Ca exchanger to import calcium), such as at nodes of Ranvier<sup>125</sup>. Dendrites, however, rely almost exclusively on glycolysis and the plasma membrane calcium ATPase to clear the dendroplasm of excess calcium<sup>18</sup>. Therefore, enhancing mitochondrial calcium buffering may not alter dendritic responses to large injury-induced calcium spikes as it does in axons.

It will be important to test the mitochondrial calcium responses to axon injury with these mutants. Interestingly, higher levels of intra-mitochondrial calcium have been correlated with decreased mitochondrial motility<sup>125</sup>. Mitochondrial motility is also suppressed upon axon injury, but in WLD<sup>s</sup>-expressing axons mitochondrial motility is maintained<sup>118</sup>. The interesting link between mitochondrial motility, calcium buffering, and axon protection could, therefore, distinguish axon and dendrite degeneration.

*dsarm* and *hiw* mutants also do not change dendritic calcium responses to injury. An interpretation of these results is that these genes act downstream of a

degeneration-inducing calcium signal. In *C. elegans*, the dSarm ortholog Tir-1 acts downstream of a calcium signal in a pathway that determines left/right asymmetry in a pair of neurons<sup>82</sup>. Additionally, SARM1 in mouse neurons was proposed to function downstream of calcium-induced increase in mitochondrial reactive oxygen species production<sup>126</sup>. Hiw, on the other hand, has been implicated in the regulation of several calcium-signaling proteins including AMPA receptors, adenylate cyclases, and TRPV1<sup>91</sup>. However, since calcium transients are not changed in *hiw* mutants, it is unlikely that Hiw acts upstream of these calcium regulating proteins to prevent degeneration. Therefore, dSarm and Hiw likely act downstream or independently of calcium spikes in neurite degeneration.

Hiw is a highly conserved E3 ubiquitin ligase with roles in axon and synaptic development. Recently, mouse *Phr1* and fly *hiw* were shown to be required for the degeneration of axons after injury<sup>88,89</sup>. We show that *hiw* is also required for the proper degeneration of injured dendrites. However, the protection afforded by *hiw* mutants is variable: some dendrite branches are preserved while others completely degenerate. This is in contrast to axon protection within the same cells and with other axons tested in flies and mice. There are no paralogs of Hiw, but it is possible that a functional paralog compensates for the loss of Hiw; for example there are at least 50 identified E3 ubiquitin ligases in the *Drosophila* genome<sup>127</sup>. Ubiquitin ligase activity is the best studied function for the Hiw family, and the down regulation of dNmnat and NMNAT2 has been invoked to explain Hiw/PHR1 function in axon

degeneration<sup>39</sup>. However, due to the lack of reagents to adequately detect endogenous dNmnat or NMNAT2, these studies rely on differences in overexpressed protein levels between WT and *hiw/Phr1* mutant neurons after injury. It remains to be seen whether endogenous dNmnat levels are increased in *hiw* mutant axons and/or dendrites upon injury.

It is possible that the down regulation of dNmnat is not the mechanism *Hiw* uses to promote neurite degeneration. *Hiw/PHR1/RPM-1*, similar to *SARM1/TIR-1*, have been placed upstream of a MAP kinase cascade by negatively regulating the MAPKKK *Wnd/DLK/DLK-1* that then positively regulate their respective MAP kinase cascades<sup>91</sup>. The resulting increase in MAPK signaling has been implicated in receptor trafficking, axon regeneration, and synapse development. Interestingly, mutations in *wnd* were recently shown to modestly delay axon degeneration as compared with *dsarm* and *hiw* mutants<sup>88</sup>. It will be important to test whether mutations in *wnd* and downstream components also confer protection to injured dendrites.

The maintenance of dendrites is critical to sustain neural connectivity and nervous system function. Explicit dendritic pathology and loss is observed in many disease states, such as Alzheimer's, Parkinson's, ALS, and during excitotoxicity following trauma or stroke<sup>10,24,128–130</sup>. Therefore, the ability to prevent pathological dendrite degeneration would be a powerful tool to combat functional loss in neurological disease. Here, we reveal two endogenous regulators of dendrite injury *in vivo*. The partial protection of injured dendrites

seen in *hiw* mutants precludes its usefulness as a drug target; yet the robust protection of dendrites as well as axons by *dsarm* mutants highlight SARM1 as an attractive target for drug development. These studies, therefore, have revealed new roles for WD genes and reinforced the validity of SARM1 as a therapeutic target in the search for new treatments for neurological disease.

## Materials and Methods

### Fly stocks

All fly stocks were maintained at 25°C on standard cornmeal-molasses agar medium. The following strains from the Bloomington *Drosophila* stock center were used in these studies: *ppk-Gal4*, *UAS-mCD8::GFP*, *UAS-mCD4::tdTomato*, *UAS-GCaMP6S*. *UAS-mitoGCaMP5a* was generated by PCR amplifying the full 1354bp of GCaMP5a from pGP-RSET-EcoRV-GCamp3-T302L R303P 7.36 (a gift from Doug Kim, Janelia Farm) and ligating it into the pJFRC7-20xUAS-mito plasmid with NotI / Xba. Additional lines used were: *UAS-Wld<sup>s</sup>* and *UAS-Wld<sup>s-dead</sup>*<sup>31</sup>, *dsarm*<sup>896</sup>, *dsarm*<sup>4621 68</sup>, *hiw*<sup>587</sup>, *hiw*<sup>275 103</sup>, *hiw*<sup>ΔN</sup> (a gift from Cathy Collins, University of Michigan), and *19-12-Gal4* ; *repo-Gal80*.

### Laser Ablation Assay and Analysis

Early 3<sup>rd</sup> instar larvae (~96hr AEL) were collected and anesthetized with isofluorane. They were then mounted in halocarbon oil-27 (Sigma) and placed under a coverslip with a 0.25mm silicone slotted gasket (Warner Instruments, Hamden, CT). Dendritic arborization neurons were then imaged on a Zeiss M1 upright spinning disc confocal (Intelligent Imaging Innovations, Denver, CO) and neurites were severed using a Micropoint laser ablation unit at 63x magnification (Andor Technology, South Windsor, CT). Larvae were subsequently recovered on culture media, stored at 25°C for 24hrs and imaged.

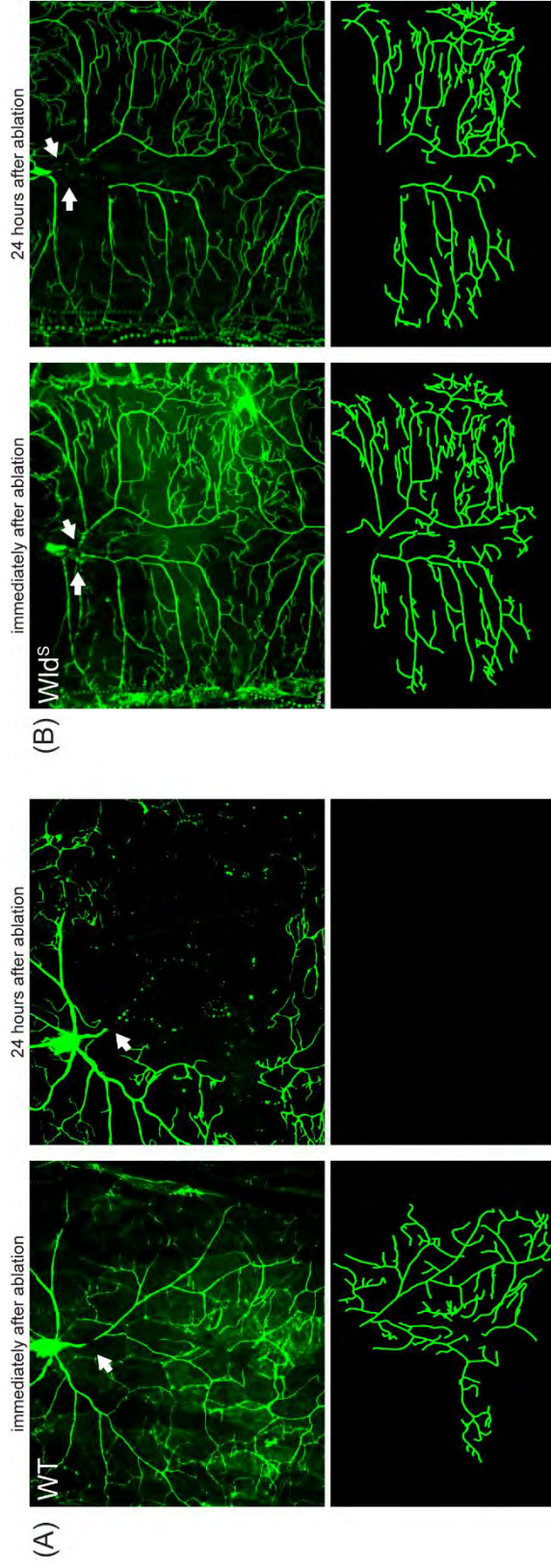
Compressed Z stack images from each time point were exported from Slidebook 6 (Intelligent Imaging Innovations, Denver, CO) into ImageJ (National Institutes of Health). The ImageJ plugin NeuronJ (REF) was used to trace dendrite lengths. Graphing and one-way ANOVA/Tukey's multiple comparison test analyses were performed using GraphPad Prism 6.02. Image composites were made in Adobe Photoshop CS6.

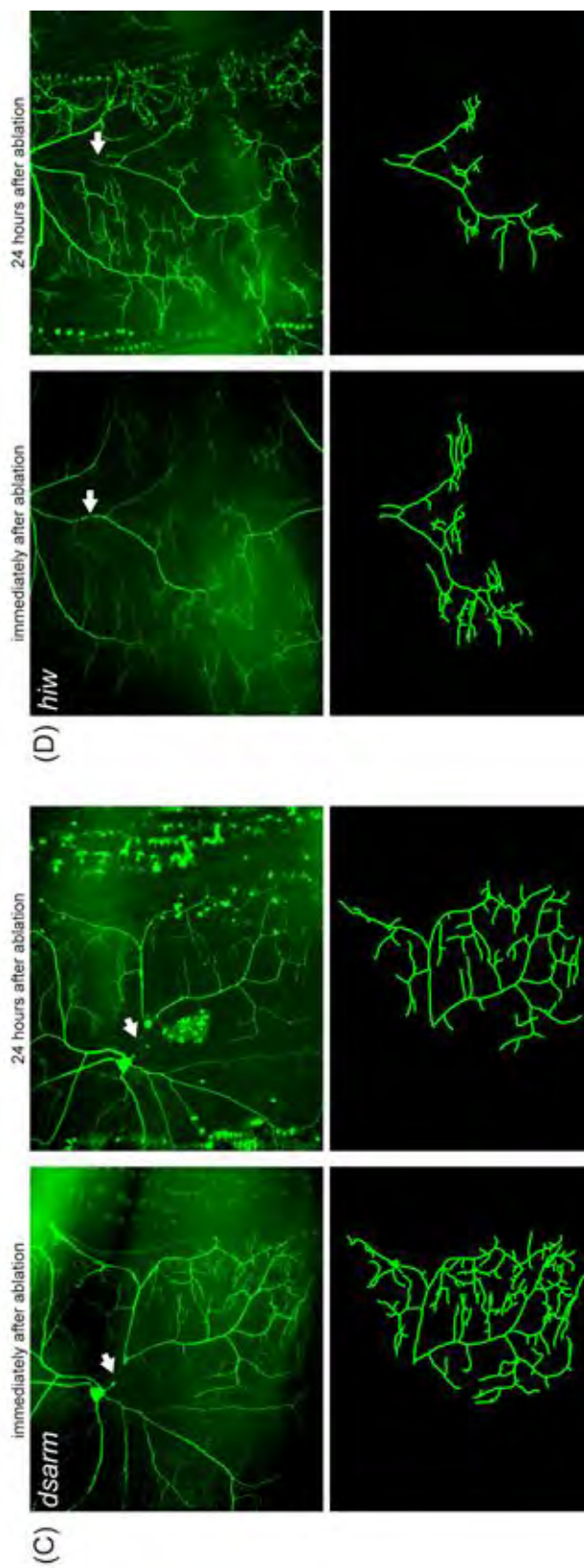
### Timelapse Calcium Imaging Assay and Analysis

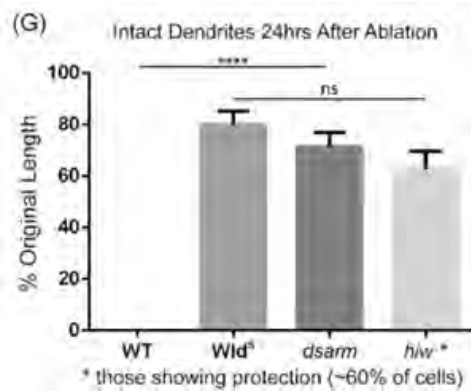
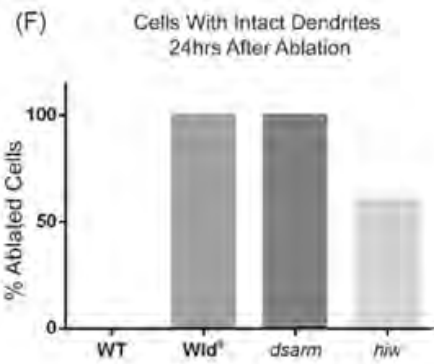
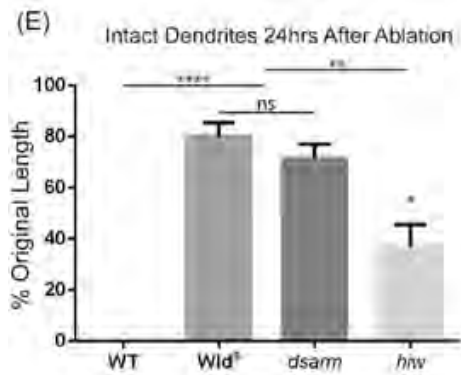
Wandering 3<sup>rd</sup> instar larvae were collected, anesthetized, imaged, and injured as described above. For the timelapse studies, 11µm Z stacks with a step size of 1 µm were defined to encompass the dendrite of interest. Frames were collected every 50ms (essentially without pause), with laser exposure times

of 250ms for 488nm and 50ms for 561nm. The timelapse was allowed to proceed for 30s and then the dendrite branch was ablated with the Micropoint system followed by additional imaging.

Compressed Z stacks were then analyzed using Slidebook6. A region that did not contain dendrites in any time point was used to subtract the background. Another region was drawn around the distal stump and the red channel was used to gate the dendrite. Mean intensity of GCaMP signal within the gated dendrite was then analyzed. For mitoGCaMP data, the mean intensities of the mitochondria, as defined by the area within the mito-Tomato signal, were quantified. Data were then statistically analyzed in GraphPad Prism using unpaired t test.



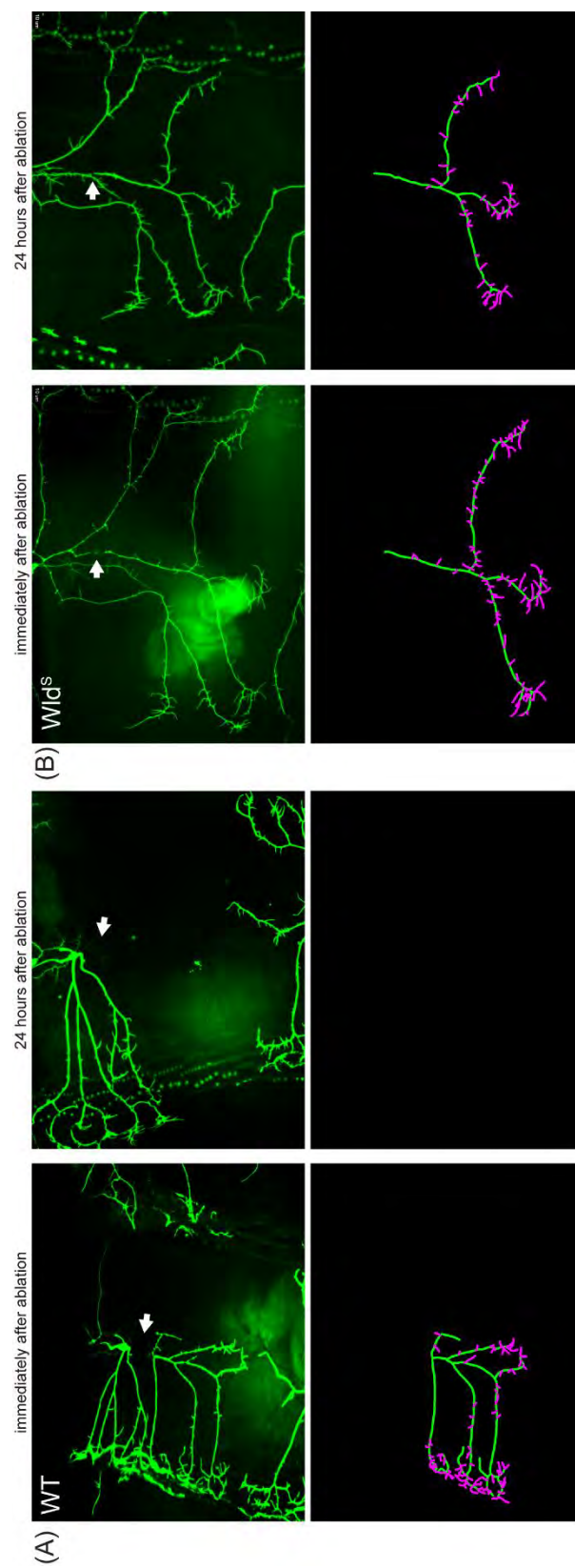


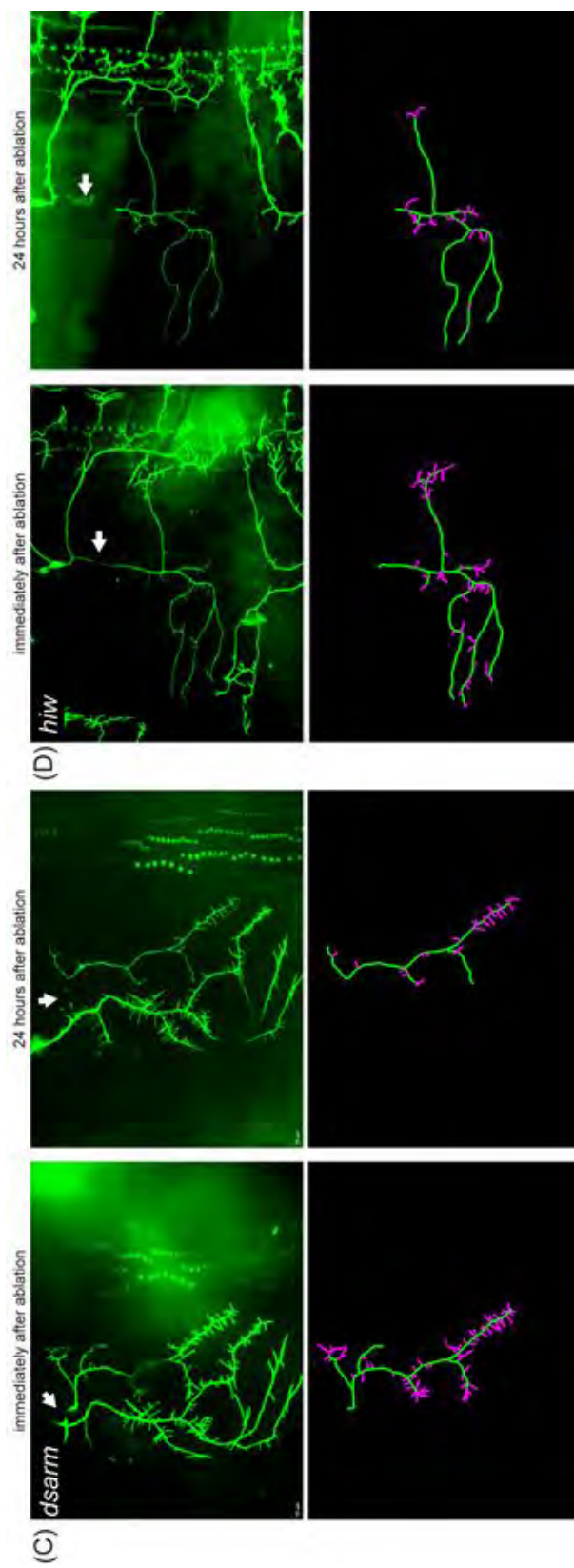


**Figure III-1. Degeneration of *Drosophila* class IV da neuron dendrites requires *dsarm* and *hiw*.**

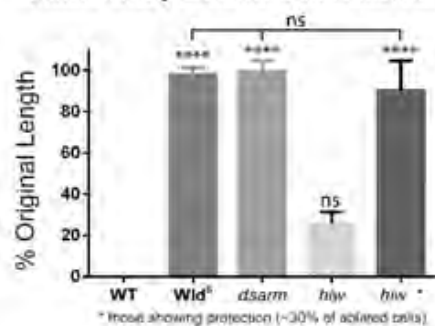
Class IV dendritic arborization neurons are labelled with *ppk-Gal4* driving mCD8::GFP. When ablated, WT dendrites undergo degeneration, and are cleared by 24 hours post ablation (A). The arrow denotes the region where the dendrites were ablated. When WLD<sup>s</sup> is expressed in these neurons, ablated dendrites remain intact (B). In *dsarm* (C) or *hiw* (D) mutants, ablated dendrites also fail to degenerate. The level of protection in *hiw* mutants is significantly less than with WLD<sup>s</sup> or *dsarm* mutants (E). All cells tested with WLDs and *dsarm* mutants showed protected dendrites, while only ~60% of *hiw* mutant cells showed protection (F). There are equivalent levels of protection between WLD<sup>s</sup>, *dsarm* mutants, and those *hiw* mutants showing protection (G). Error bars = SEM

\*\*\*\* p<0.0001 \*\* p<0.01 \* p<0.05

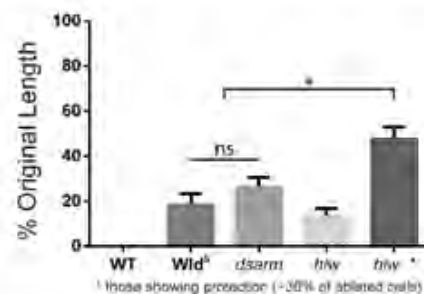




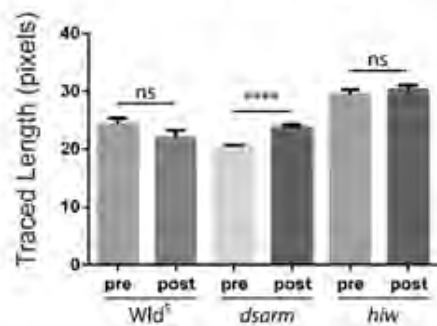
(E) Intact Primary Dendrites 24hrs After Ablation



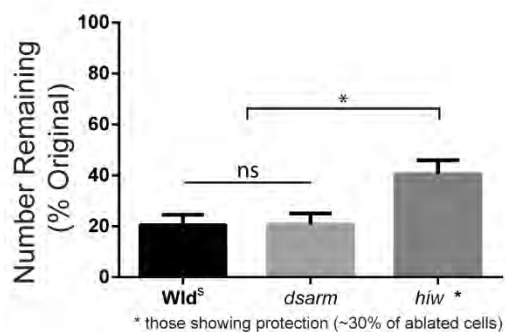
(F) Intact Dendritic Spikes 24hrs After Ablation



(G) Dendritic Spike Length

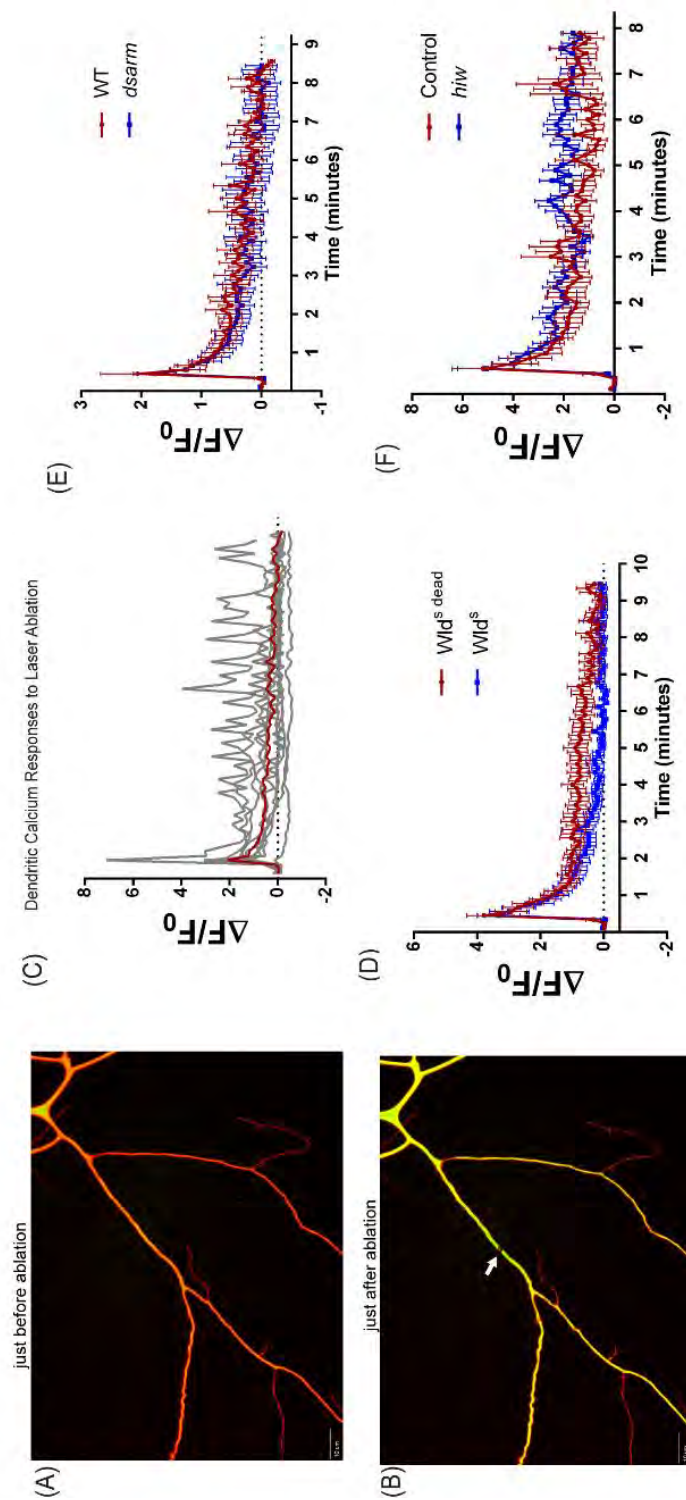


(H) Intact Spikes 24hrs After Ablation



**Figure III-2. Degeneration of *Drosophila* class III da neuron dendrites requires *dsarm* and *hiw*.**

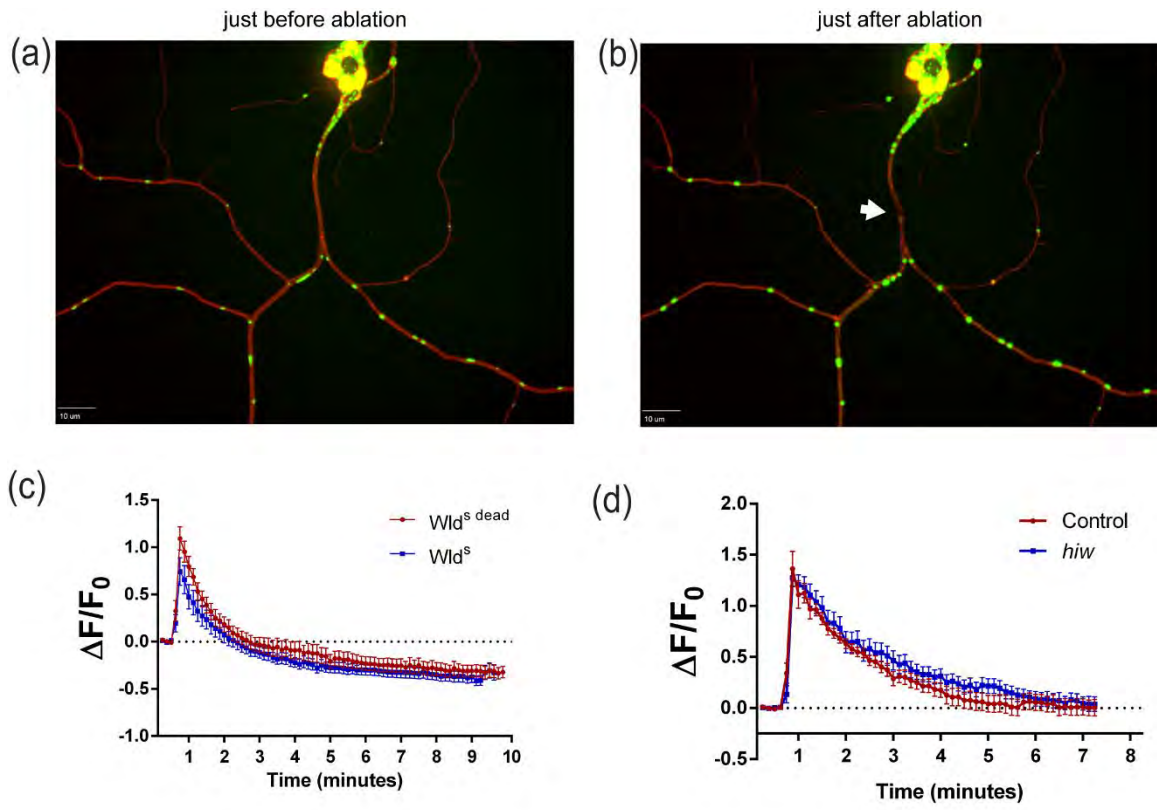
Class III dendritic arborization neurons are labelled with *19-12-Gal4* driving mCD8::GFP. When ablated, WT dendrites undergo degeneration, and are cleared by 24 hours post ablation (A). The arrow denotes the region where the dendrites were ablated. When WLD<sup>s</sup> is expressed in these neurons, ablated dendrites remain intact (B). In *dsarm* (C) or *hiw* (D) mutants, ablated dendrites also fail to degenerate. The level of primary dendrite protection in *hiw* mutants is significantly less than with WLD<sup>s</sup> or *dsarm* mutants; however if only cells showing protection are quantified, the levels are equivalent between WLD<sup>s</sup> and mutants of *dsarm* and *hiw* (E). Total mean dendritic spike lengths are greatly reduced after injury in all genotypes (F). The reduction of dendritic spike length is not due to decreased length of individual spikes (G), but rather due to a reduced number of spikes (H). Error bars = SEM \*\*\*\* p<0.0001 \*\* p<0.01 \* p<0.05



**Figure III-3. Injury-induced calcium transients in *Drosophila* class IV da neuron dendrites are not changed in WD mutant dendrites *in vivo*.**

Class IV dendritic arborization neurons are labelled with *ppk-Gal4* driving myr-tdTomato and GCaMP6S (A). Ablation causes calcium transients in WT dendrites (B). The arrow denotes the region where the dendrites were ablated. C, Plot of GCaMP  $\Delta F/F_0$  values from timelapse recordings with ablation of individual WT dendrites (grey) and the mean (red). WLD<sup>s</sup> expression does not change the mean  $\Delta F/F_0$  values of injury-induced calcium transients (D). In *dsarm* (E) or *hiw* (F) mutants, these transients are also not different than the control values. Scan QR code for a representative video.

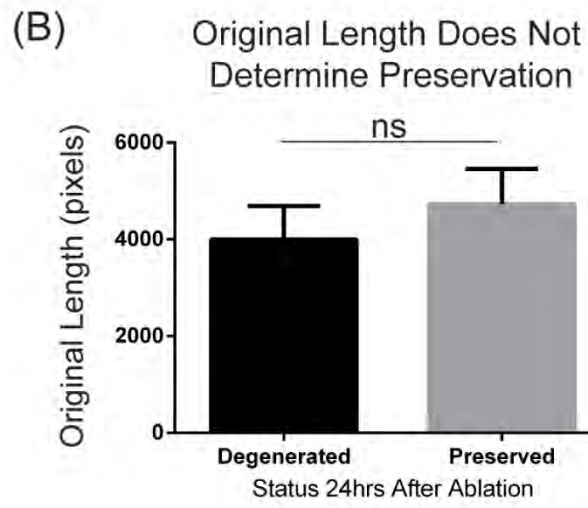




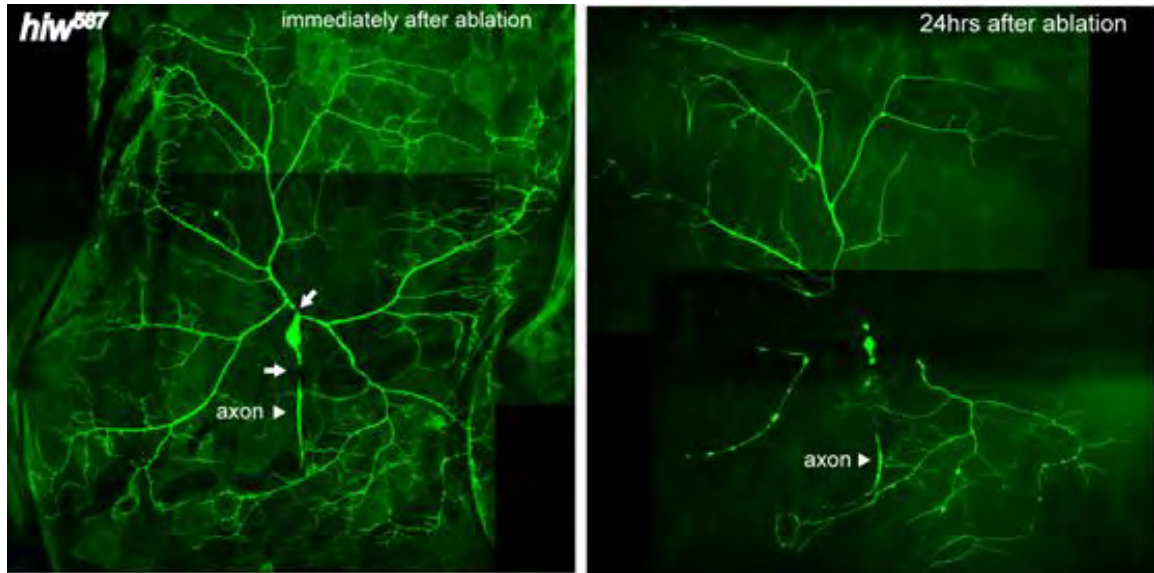
**Figure III-4. Injury-induced mitochondrial calcium transients in *Drosophila* class IV da neuron dendrites are not changed in WD mutant dendrites *in vivo*.**

Class IV dendritic arborization neurons are labelled with *ppk-Gal4* driving myr-tdTomato and mito-GCaMP5a (A). Ablation causes calcium transients in WT dendritic mitochondria (B). The arrow denotes the region where the dendrites were ablated. C, WLD<sup>s</sup> expression does not change the mean  $\Delta F/F_0$  values of injury-induced calcium transients (D). In *hiw* (D) mutants, these transients are also not different than the control values.

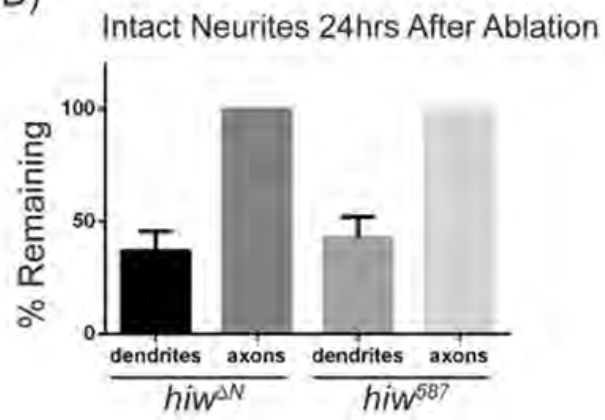




(C)



(D)



**Figure III-5. *hiw* mutants exhibit cell-autonomous branch-specific degeneration after injury.**

A, Class IV dendritic arborization neurons are labelled with *ppk-Gal4* driving *myr-tdTomato*. Clockwise ablation of all dendrite branches and the axon (arrowhead) of four adjacent class IV da neurons. 24 hours after ablation (below), axons are preserved (arrowheads) but ~50% of dendritic branches degenerated and were cleared. B, no difference is seen between average traced lengths just after injury of degenerated or preserved dendrites. C, MARCM clone of *hiw*<sup>587</sup> labelled with *ppk-Gal4* driving *mCD8::GFP*. All dendrite branches and the axon were ablated (arrows, ablation; arrowhead, axon). Right, 24hrs after ablation showing preservation of some branches but not others, as well as an intact axon (arrowhead). D, average percent of remaining dendrites (length) and axons (number) in *hiw*<sup>ΔN</sup> and *hiw*<sup>587</sup> mutants. Error bars = SEM

## Chapter IV: Discussion

### Endogenous regulators of Wallerian degeneration

That the degeneration of a transected cellular process can be inhibited is remarkable. This realization led to the concept of Wallerian degeneration (WD) as an active process, initiated by injury to axons and dendrites. Though most neural injury is not as acute or severe as a clear transection, pathology yet ensues. Many disease processes, such as in neurodegenerative diseases, result in the dying back of axonal processes. This degeneration morphologically resembles WD and is therefore called Wallerian-like degeneration. Examples include mouse models of motor neuron disease and multiple sclerosis, as well as diabetic neuropathy in patients<sup>131–133</sup>. Such disease processes could activate WD at a low, chronic level that leads to gradual neural destruction over long periods of time. The axon and dendrite protective WLD<sup>s</sup> protein has been shown to ameliorate pathology in some neurodegenerative disease models, including those mentioned above, suggesting that inhibiting WD could indeed help to maintain neural function and lessen disease burden.

Expression of WLD<sup>s</sup> shows even greater protection of axons in models of acute injury<sup>39</sup>. Some of these insults, such as acute multiple sclerosis attacks,

are transient, and inhibiting WD could give the injured cells time to recover without initiating degeneration. Examples of acute axon injury include hypoxia/ischemia, excitotoxicity, and exposure to toxins such as taxol and vincristine, all of which are attenuated by WLD<sup>s</sup> <sup>61,65,134,135</sup>.

However, the molecular nature of how WLD<sup>s</sup> protects neurites is not known. Since WLD<sup>s</sup> is not an endogenous protein, it cannot be targeted by pharmaceuticals. Therefore, the identification of endogenous regulators of WD is of utmost importance to develop therapeutics to prevent or treat neuronal injury.

To search for regulators of WD, I first scoured the literature for candidates based on their involvement in cell death pathways or their potential relation to dSarm/SARM1 or WLD<sup>s</sup>. Testing of strong mutants, such as confirmed genetic nulls, allows genes to be excluded from having a role in WD. In my candidate list, several categories with strong mutants showed no role for the caspases, immune signaling proteins, or calcium regulating proteins tested. The three initiator caspases in *Drosophila* (Dronc, Strica, and Dredd, alone and in double-mutant combinations) as well as an effector caspase (Dcp-1) were tested, as there are non-apoptotic roles for certain caspases in axon degeneration during developmental processes<sup>136</sup>. These mutants did not alter WD, supporting independent pathways for these degenerative processes as previously suggested by *Bax* deletion and BCL-2 overexpression<sup>54,55</sup>.

SARM1 likely relays a degenerative signal via its TIR domain, a domain well conserved for signaling in Toll-like receptor (Tlr) pathways<sup>80,87</sup>. Therefore, I tested *Drosophila* Tlr mutants in degeneration. No strong phenotype was observed using the lines in Table II-1, however, some debris was seen with *Toll2* (*18Wheeler*) and *Toll5* (*MystProx*) mutants, suggesting that they may play a role in glia in the clearance of degenerated axons. Similar results and later independent verification of glial defects were obtained with a *kayak* mutant<sup>71</sup>. These results are consistent with reported roles for TLR4 in the clearance of degenerated mouse neurons *in vitro* and *in vivo*<sup>137,138</sup>.

As the influx of extracellular calcium is necessary and sufficient for WD, several genes that regulate intracellular calcium were also tested. Normal WD was seen in null mutants of the sole *RyR*, *Orai*, and *IP3R* genes in the fly, arguing against a role for store-operated calcium release. This is in contrast with a report that axoplasmic reticulum calcium release triggers WD<sup>139</sup>. However, this effect was observed with chemical inhibitors and siRNA in culture 12hrs after injury, as compared with 7 days *in vivo* with reported nulls of *RyR*, *Orai*, and *IP3R*. Inhibition of these receptors could modulate WD to an extent, but are not likely to be part of the main pathway that includes *dsarm* and *hiw*.

The conclusion that influx of extracellular calcium is involved in WD is largely centered on the delay in degeneration by pharmacological inhibition of L-type calcium channels<sup>46,47</sup>. Several *Drosophila* L-type calcium channel subunit mutants were tested in the WD assay and found to not affect the degeneration of

axons, but rather delayed the clearance of the axonal debris. Interestingly, *cacophony* and *ca- $\alpha$ 1D* mutants were tested neuronal cell-autonomously, which could indicate a role for the production of an axonal eat-me cue after degeneration.

These results are in contrast with the conclusions of some studies using pharmacological inhibition of mammalian L-type calcium channels *in vitro* and *in vivo*<sup>46,47,140</sup>. However, the calcium channel blockers used, such as nifedipine, could alter calcium regulation or have other roles in degeneration independent of L-type calcium channel inhibition. Such an effect was observed with nifedipine in calcium regulation not involving L-type calcium channels in vascular smooth muscle<sup>141</sup>. Additionally, there are differences between *Drosophila* and mammalian L-type calcium channels<sup>142</sup>, and these drugs have not been evaluated in *Drosophila* axon degeneration. Nevertheless, nulls of these channels failed to prevent axon degeneration, suggesting that they do not play a major role in this process in *Drosophila*.

Many of the other lines tested above are potentially not complete loss of function alleles, which precludes the ability to make solid conclusions of their role in axon degeneration. They remain worthwhile candidates, and better screening techniques could investigate their role in WD further. Novel gene targeting techniques such as CRISPR are vastly reducing the time and effort of gene deletion<sup>100</sup>. It would not be surprising to see a genome-wide gene deletion effort

produce genetic nulls of most *Drosophila* genes, which could then be tested for the prevention of WD.

I also performed a forward-genetic screen of the *Drosophila* 2<sup>nd</sup> chromosome to identify novel regulators of Wallerian degeneration. Unfortunately, no lines screened showed defects in axon degeneration. This could be due to a number of potential issues with the screen. The lines constructed could be incorrect; however the characterization of these lines as shown above (Figures II-5 and II-6, WT panel) makes this possibility unlikely. Additionally, since 82% of the lines had a homozygous lethal 2<sup>nd</sup> chromosome, there was likely adequate EMS-induced mutagenesis.

Redundancy in signaling components can greatly decrease the likelihood of recovering strong phenotypes in a screen. For example, if two redundant genes reside on different chromosomes, or even different chromosomal arms then mutants in these genes will not be recovered using MARCM as this system can only test one arm at a time. It is possible to make MARCM clones that are homozygous for two different chromosomal arms, but as this requires additional transgenes and results in less clone induction, it would be prohibitively complicated in a screen setting. An example of redundancy in Wallerian degeneration signaling was recently shown in the identification of a MAPK cascade downstream of SARM1 in axon degeneration<sup>45</sup>. In each level of the pathway, from MAP kinase kinase kinase to the 3 downstream JNK paralogs, elimination of more than one kinase added to the axon protection phenotype.

The strongest phenotype, where 60% of axons were preserved at 6 days after axotomy, was seen with MKK7 shRNA knock-down in a *MKK4*-mutant background. Since our screen looked at 7 days post ablation, we may have missed weak phenotypes in redundant genes. We also lack the ability, in a screen setting, to test for redundancy other than that produced by mutants on the same chromosomal arm.

One of the largest hurdles to identifying mutant phenotypes by screening is the throughput of the system. The throughput and therefore likelihood of identifying mutant phenotypes relies heavily on screening through large numbers of animals. For example, 2194 chromosome 3L arms were screened to reveal 4 separate alleles of *dsarm*. This corresponds to approximately one hit per 550 arms tested. In the ORN screens that identified *dsarm* and were performed in the 2<sup>nd</sup> chromosome in this work, a sustainable and sanity-preserving rate is ~10 lines screened per day. The screens performed were fairly laborious owing to the maintenance of stocks and crosses as well as the collection, ablation, fixing, dissecting, antibody staining, and imaging of fly brains. Newly developed systems such as in the wing increase the throughput 10-20 times by eliminating the need to fix, dissect, and stain brains. Additionally, so called F1 screens test the progeny of the EMS-mutagenized males (F1 animals) and only create an F2 stock from those flies exhibiting the phenotype of interest. Recovery of the mutant in the F2 stock is, however, difficult as less than 15% of mutants have

been recovered in the Freeman lab (personal communication from Tom Burdett and Jon Farley on 3-17-15).

EMS surely affects separate genomic regions in distinct ways, causing higher mutation rates in some areas compared to others. For example, 8 alleles of *hiw* were identified in ~1500 lines corresponding to a hit rate of approximately 1 in 188. Even accounting for size – *Hiw* is huge (~5233 amino acids) compared to *dSarm* (1637 amino acids) - this hit rate resulting in coding changes is still almost four times that of *dsarm*. It is therefore possible that WD genes on the 2<sup>nd</sup> chromosome could be less affected by EMS mutagenesis and therefore fall below our numbers of screened chromosomes. However, the Freeman lab has continued to screen the 2<sup>nd</sup> chromosome without any recovered hits, and numbers now total 7449 2L arms and 4013 2R arms, suggesting that there are few, if any, mutable non-redundant genes involved in WD on the 2<sup>nd</sup> chromosome.

The identification of new roles for genes in regulating WD requires screening, be it by candidate or unbiased approaches. New technology, such as CRISPR gene targeting could greatly accelerate this screening. For example, the *Drosophila* 4<sup>th</sup> chromosome is typically not screened by unbiased forward-genetic methods. This is partly due to the lack of appreciable recombination due to the small size of the 4<sup>th</sup> chromosome. The lack of recombination has precluded MARCM studies, as recombination is routinely used to obtain lethal mutants and the repressive Gal80 on a chromosome with an FRT site. Using

CRISPR, it would be possible to direct the insertion of an FRT site near the centrosome of the 4<sup>th</sup> chromosome. The tub-Gal80 construct could then be distally engineered/inserted on that FRT chromosome. Mutants in candidate genes could also be engineered on the FRT chromosome to reconstitute the basic lines needed for MARCM. Several interesting candidates reside on the 4<sup>th</sup> chromosome. For example, both *Drosophila* calcium/calmodulin-dependent protein kinases are located on chromosome 4, and no mutants are available for either. In *C. elegans* the CaMKII ortholog UNC-43 signals through TIR-1 (the dSarm ortholog) to activate a MAP kinase cascade, making CaMKII an attractive candidate to test in WD assays<sup>82,143</sup>. Through advanced targeted genome editing techniques, it would be possible to test mutants in either or both CaMK genes in a MARCM setting. Such advances, as well as continued screening efforts, are sure to reveal other genes that are required for axon degeneration.

## **Dendrites**

Dendrites are the primary structures used by neurons to receive and process inputs in the many varied information processing networks in the nervous system. This input could be from other connecting neurons or from sensory input that is directly sensed by the dendrites. Sensory neurons that line the *Drosophila* larval body wall provide information about the environment, such as light, heat, and pressure or touch. These neurons extend large dendrites

to completely cover the larva in sensory endings. The dendritic branches reside in the body wall between the basal aspect of the epidermal cells and the extracellular matrix. The epidermal cells also engulf degenerating dendrites as occurs in developmental pruning or after physical injury to the branches. With their superficial localization and extensive dendritic arbors, the multidendritic da neurons provide an ideal model to study responses to dendrite injury.

Dendrites rival axons for their size and diversity of structure; some can account for greater than 80% of the cell surface such as in dentate granule neurons<sup>4</sup>. Often containing complex branching structure, several features distinguish them from axons. Dendrites have a mixed polarity of microtubules, whereas axonal microtubules are plus-end out. Within dendrites, all cellular organelles such as mitochondrial, rough endoplasmic reticulum, polyribosomes and Golgi outposts can be found, while axons typically only contain mitochondria. Intrinsic calcium homeostasis mechanisms are also different between these two cellular compartments. Axons rely on mitochondria for calcium buffering, while dendrites utilize membrane-bound ATPases that are fueled primarily by glycolysis rather than mitochondria.

The finding that WLD<sup>s</sup> does not attenuate a calcium spike after sensory dendriotomy also differs from that seen in motor axons<sup>118</sup>. This difference could in fact be due to the differences in calcium handling between these two compartments. For example, if WLD<sup>s</sup> primarily works primarily by enhancing mitochondrial calcium buffering, then it may not be expected to affect the calcium

buffering in dendrites which does not rely on mitochondria as in axons. It could also be due to differences between motor and sensory cell types. Though a calcium transient was observed in the axons after dendriectomy, it remains to be seen whether calcium transients are altered by WLD<sup>s</sup> or *dsarm* and *hiw* mutants in sensory axons or motor neuron dendrites after injury *in vivo*. It also remains to be seen whether WLD<sup>s</sup>, or mutations in *dsarm* or *hiw* alter axoplasmic and mitochondrial calcium transients after axonal injury. If WLD<sup>s</sup> allows mitochondria to more efficiently buffer calcium, then either increases or decreases might be observed in mitochondrial matrix-targeted GCaMP signal depending on the mechanism used. For example, if WLD<sup>s</sup> increases the ability to store calcium as an insoluble conjugate with inorganic phosphate<sup>144,145</sup>, a reduction in GCaMP signal might be observed. Conversely, if WLD<sup>s</sup> simply allows the level of matrix calcium to increase without forming the mitochondrial permeability transition pore<sup>146</sup>, then increased GCaMP signal would be observed. Matrix calcium levels are, however, remarkably stable<sup>147</sup>, and no change in calcium transients might reflect efficient clearance from this compartment as well.

Dendrites and axons are both extreme extensions of the neuronal cell body that are designed to transmit signals. They also degenerate after injury, and it seems that the degeneration of both cellular compartments is due to a shared signaling pathway. This pathway is inhibited by exogenous expression of the WLD<sup>s</sup> protein, as well as by mutations in *dsarm* and *hiw*. Yet several mutations in *hiw* that robustly protect axons confer only partial protection of

dendrites that is branch-specific. The fact that some branches are preserved while others completely degenerate indicates that there are intrinsic differences between individual branches of the same neuron. As the degeneration of certain branch is not influenced by a prior injury (Figure III-5A), these differences are likely present even before the injury. Branch-specific changes in dendrites have recently been described, and are associated with differential input affecting calcium transients and plasticity in learning paradigms<sup>148,149</sup>. These changes, then, are in response to exogenous input, and are not engendered by intrinsic differences between dendritic branches. The branch-specific degeneration revealed by *hiw* mutants seems unique to dendriotomy.

How could dendritic branches differ in *hiw* mutants? Hiw family proteins regulate many processes and pathways in neurons. For example, Hiw/Phr1 has been shown to interact with and regulate the cytoskeleton as well as regulate different signaling pathways, potentially accounting for branch-specific differences<sup>91</sup>. Markers showing microtubule polarity such as the plus-end binding protein EB1 as well as markers for organelles such as mitochondria and Golgi outposts should be examined in the *hiw* mutant backgrounds. Variances from the wild type patterns might indicate how different branches in these mutant backgrounds could differ from one another, leading to mechanistic understandings of dendrite degeneration. Perturbations in signaling pathways regulated by PHR1, such as the tuberous sclerosis complex and BMP/Smad<sup>91</sup>, can also be evaluated in the *hiw* mutants. There are no known paralogs of either

*hiw* or *Phr1*, but certainly functional paralogs could operate in dendrites or axons after injury. Perhaps screening efforts will uncover such a gene.

Hiw family proteins down regulate many other proteins through their function as E3 ubiquitin ligases. It is possible that in the context of injury, Hiw also down regulates degeneration-related proteins such as dNMNAT or other novel targets. The concept of a role for the ubiquitin-proteasome system (UPS) in axon degeneration was first brought about when VCP was found to interact with WLD<sup>s</sup>. However, manipulations of the UPS such as with chemical inhibitors has little effect on axon degeneration. And while the UPS is required for aspects of developmental dendrite pruning in class IV da neurons, *hiw* mutants do not have pruning defects, suggesting that these processes are distinct<sup>136,150</sup>.

Interestingly, mutants in *hiw* were recently recovered that are thought to eliminate or mutate the RING domain and therefore E3 ligase activity<sup>103</sup>. These mutants dominantly inhibit axon degeneration, suggesting that Hiw's E3 ligase function is required for degeneration. If these mutants bind to substrates, but do not mark them for degradation, then they could potentially be used to biochemically identify the substrates that are down regulated during axon and dendrite degeneration. It will be interesting to see whether these mutants also dominantly inhibit dendrite degeneration after injury.

Is the difference in degeneration between dendritic branches in *hiw* mutant cells due to some simply degenerating slower than others? In mouse, *Phr1*<sup>-/-</sup>

axons are robustly protected but to a lesser extent than with WLD<sup>s</sup> or *Sarm1*<sup>-/-</sup> at the same time points *in vitro* and *in vivo*<sup>89</sup>. As sensory dendrites in *Drosophila* degenerate by 12hrs after ablation, looking at earlier time points could mirror the mouse data. However, the protected branches that remain show equivalent protection to both WLD<sup>s</sup> and *dsarm* mutants. If degeneration were simply delayed, then all branches might be expected to show similar levels of degeneration instead of the all-or-nothing pattern observed. It would be informative to take these studies to longer endpoints in all mutant backgrounds. It would be possible to extend the time course by ablating the dendrites earlier, or possibly by ablating adult sensory dendrites that are found on the abdomen of the fly. By looking at later time points, we might be able to discover differences in the levels of protection in severed dendrites between these mutants.

### **Towards an inhibitor of SARM1 proteins**

The ultimate goal of these investigations is the identification of targets that can be pharmaceutically modulated to lessen or prevent disease. So far, WLD<sup>s</sup> has shown promising levels of axon and dendrite protection in several disease and toxicity models, including in human cell culture. As the mechanism that WLD<sup>s</sup> employs remains elusive, it is difficult to design therapies that mimic WLD<sup>s</sup>.

Hiw/PHR1 is a poor target due to its partial protection of dendrites (this study), and its involvement in other important cellular functions such as the

regulation of ion channel levels<sup>91</sup>. Conversely, dSarm/SARM1 has several qualities that make it an attractive target. *Sarm1*<sup>-/-</sup> mice are viable and healthy, with little or no developmental or degenerative phenotypes known. The only strong phenotype observed in *Sarm1*<sup>-/-</sup> mice is the failure of axon degeneration after injury<sup>68</sup>. The Sarm proteins are therefore potential candidates for inhibition by therapeutics. In advance of the development of such drugs, our lab and others are currently investigating Sarm1 null mice in various neurodegenerative disease models.

Toll/interleukin-1 receptor (TIR) domains are conserved signaling motifs in TLRs that link receptor dimerization with downstream signaling<sup>80</sup>. SARM1 contains a TIR domain, which has been shown to be required for axotomy-induced degeneration<sup>87</sup>. In the same study, SARM1 proteins were shown to dimerize, which could lead to activation of the TIR domains. Inhibition of TIR domain signaling has been demonstrated using peptide mimics of the TLR4 TIR domain BB loop that are thought to interfere with TIR dimerization and downstream signaling<sup>151,152</sup>. To see if this strategy could be used to inhibit SARM1, I designed peptides analogous to those used in the inhibition of TLR4 signaling. I first modeled the tertiary structure of the mouse SARM1 TIR domain using two different programs: Phyre2 and Jpred3. The results appear in Appendix I. Using these models, I identified the conserved BB loop, which differs in only 2 amino acids between mouse SARM1 and fly dSarm (2 lysines in mice vs. 2 arginines in flies; both are positively charged amino acids). The sequence

encompassing this loop from SARM1 was used to identify the peptide sequence: DVEKLEAGKF.

Small peptides can be extremely labile in neurons due to the preponderance of neuronal endopeptidases. The retro-inverso approach has been used to stabilize small peptides within neurons, greatly extending their half-lives<sup>153</sup>. Retro-inverso peptidomimetics utilize D-amino acids in reverse order in the peptide instead of the L enantiomers used in nature. Using the reverse order of the amino acids mitigates the effect of the inverted chirality, maintaining the spatial organization of amino acid side groups while inverting the order of the peptide bonds. This arrangement allows for approximate preservation of peptide structure while conferring resistance to endopeptidase degradation. I therefore chose to employ the retro-inverso strategy in the design of the SARM1 peptidomimetic.

A good inhibitor should be specific for and have adequate access to the target protein. It is not known if SARM1 works within axons to promote degeneration, but it has been observed to be elevated in injured axons<sup>154</sup>. Also, WLD<sup>s</sup> protein is able to prevent axonal degeneration if transduced directly into the axon up to several hours after axotomy<sup>155</sup>. SARM1 is thought to be an intracellular signaling protein, and thus drugs with access to the neuronal cytoplasm are likely able to interact with and inhibit SARM1. Cell-penetrating peptides (CPPs) are short amino acid sequences with the ability to traverse membranes and even carry proteins and other conjugated structures with them

through the membrane<sup>156</sup>. CPPs, such as those derived from HIV TAT and the *Drosophila* Antennapedia proteins, have been used to deliver peptide inhibitors of JNK into neurons<sup>157</sup>. I thus designed the SARM1 inhibitor to contain the TAT sequence that permits cellular uptake (underlined); the final sequence of the retro-inverso CPP became: FKGAE**LKEVDPPRRRQRRK**RG.

I tested the SARM1 CPP in dorsal root ganglia (DRG) cultures. While neither of the 3 concentrations tested prevented axotomy-induced degeneration by 24hrs after axotomy, as seen in *Sarm*<sup>-/-</sup> DRG, there was a dose-dependent increase in the amounts of debris remaining (Appendix I). This could indicate a small delay in degeneration with the CPP, although testing a scrambled version of the CPP would be required to conclude that result. Further studies including testing the L-amino acid version of the peptide may provide a useful starting point in the development of a SARM1-based therapeutic.

### **Remaining Questions**

What constitutes the signaling pathway(s) that enact Wallerian degeneration? *Sarm1* was recently shown to act upstream of a MAP kinase cascade that involves several MAP kinases leading to activation of JNK1/2/3 and resulting in energy deficit after axotomy<sup>45</sup>. It seems plausible that energy deficits could lead to unregulated and fulminant calcium influx resulting in the catastrophic granular fragmentation of axons long observed in the WD field.

Does this progression also occur in dendrites? Certainly, calcium dysregulation has been linked to dendrite pathology, especially in the context of excitotoxicity<sup>24</sup>. In light of the differences in calcium handling between axons and dendrites discussed above, the final outcome of degeneration may proceed via independent mechanisms in these two cellular compartments.

Independent mechanisms are also suggested by the differential protection afforded by *hiw* mutants in dendrites compared to axons. Where does *hiw* fit into the pathway? *Hiw/PHR1* is thought to regulate *dNMNAT/NMNAT2*, a proposed axonal survival factor, though this has not been shown directly in axotomy or at all in dendrites. Also, the down regulation of over-expressed *NMNAT2* occurs very soon after axotomy, yet *WLD<sup>s</sup>* can prevent WD even when transduced 4hrs after axotomy<sup>33,155</sup>. It remains to be seen whether *Hiw/PHR1* can regulate endogenous *NMNAT* proteins and whether this is indeed the trigger for degeneration in axons and dendrites.

If the trigger for WD is a decrease in *NMNAT* levels and *Hiw/PHR1* regulate this directly, then they should be upstream or in parallel to *Sarm* proteins. When the N-terminus of *SARM1* is deleted but the *SAM* and *TIR* domains remain, this protein acts as a dominant active and induces the degeneration of axons and cell bodies in the presence or absence of endogenous *SARM1*<sup>87</sup>. Testing whether *Phr1* mutant or *WLD<sup>s</sup>* expressing neurons are rescued from this gain of function protein could help delineate whether *PHR1/NMNAT2* lie upstream of *SARM1*. In fact, in ORNs, *WLD<sup>s</sup>* can

suppress the axon degeneration caused by this activated dSarm (my unpublished results). This suggests that WLD<sup>s</sup> acts downstream of SARM1 activation to prevent WD. Similarly, it is possible that, assuming WLD<sup>s</sup> substitutes for endogenous NMNAT activity, that SARM1 lies upstream of the NMNAT activity that preserves axonal integrity. Further characterization of the suppression of activated SARM by WLD<sup>s</sup> could lead to insights into the mechanism and pathway(s) operating in WD.

It would also be possible to design suppressor screens of this gain of function SARM1 to search for genes downstream of SARM1 in this form of degeneration. For example, expressing the activated dSarm (dSARM SAM-TIR) in ORN MARCM clones causes axon degeneration and kills the neurons. By simply modifying the ORN degeneration screen described in Chapter 2 above so that the clones also express dSarm SAM-TIR, one could screen for suppressors by simply looking for intact axons – no ablation required. The *Drosophila* X chromosome could be interrogated in an even simpler scheme by using attached X. The attached X chromosome allows male flies to inherit their X chromosomes from their fathers. In this way, males could pass the mutagenized X chromosomes to their male offspring expressing dSarm SAM-TIR in all neurons, which normally kills the animal. Loss-of-function suppressors of dSarm SAM-TIR could then be identified by the presence of male flies in the progeny that can survive despite expressing activated dSarm.

Finally, the activated SARM1 could be used to screen for chemicals that inhibit its function. For example, cells lines integrated with an inducible SARM1 SAM-TIR could be exposed to FDA-approved drugs (or any drugs) in culture. Induction of SARM1 SAM-TIR would then kill any susceptible cells, while those that remain would indicate candidate inhibitors of SARM1 signaling. In this way, drugs may ultimately be identified that can prevent SARM1 signaling, which would make them interesting candidates to test in the context of neurodegenerative diseases.

Is this degeneration pathway specific to neurons? All cells can be injured. Is the reason that we are studying WD in neurons simply because of their hyper-extended processes? Intriguingly, WLD<sup>s</sup> expression was shown to improve glucose tolerance in type I and II diabetes models in mice<sup>158</sup>. Additionally, most of the signaling functions attributed to *hiw* family members were originally found in human cancer cell lines, and expression of the human ortholog MYCBP2 was detected in all tissues<sup>159</sup>. In *C. elegans*, the *dsarm* ortholog *tir-1* is proposed to signal downstream of epidermal injury or fungal infection to activate a MAP kinase cascade, similar to that recently described for axon degeneration<sup>160</sup>. Interestingly, I observed that knockdown of dSarm in glia resulted in a strong engulfment phenotype with debris remaining 2 weeks after axotomy (Appendix II, unpublished results). These results suggest that dSarm works in glia to drive the engulfment of axonal debris after axotomy. Altogether, these data suggest that dSarm could act in other non-neuronal cell types after injury.

To begin to test this idea further, I made MARCM clones in *Drosophila* tracheal cells and laser ablated their cellular processes. (Appendix III). Trachea are comprised of epithelial cells that constitute the *Drosophila* respiratory organ<sup>161</sup>. These cells extend cellular processes reminiscent of neurites, and terminal branches can be completely severed as with axons or dendrites. Both wild-type and *dsarm*<sup>896</sup> clones have inconsistent degeneration of the tracheal branches at 24hrs, precluding a comparison at this time point. Whether or not these cellular processes have a degeneration program akin to neurons may yet be discovered by further study of tracheal cells in these mutant backgrounds. It will be interesting to learn if Wallerian degeneration is indeed a conserved and universal cellular process or whether it exists solely in the domain of neurons.

### **Concluding Remarks**

In this study, I have sought to understand the process of Wallerian degeneration. I describe screening strategies to find endogenous regulators of WD and highlight several genes required for the efficient processing of axonal debris and its clearance, as well as candidates that are not involved in axon degeneration. I then show that endogenous regulators of axon degeneration are also required for the injury-induced degeneration of dendrites in *Drosophila*. In doing so I found unexpected differences between injury responses in axons and dendrites, which may reveal interesting differences between these neuronal

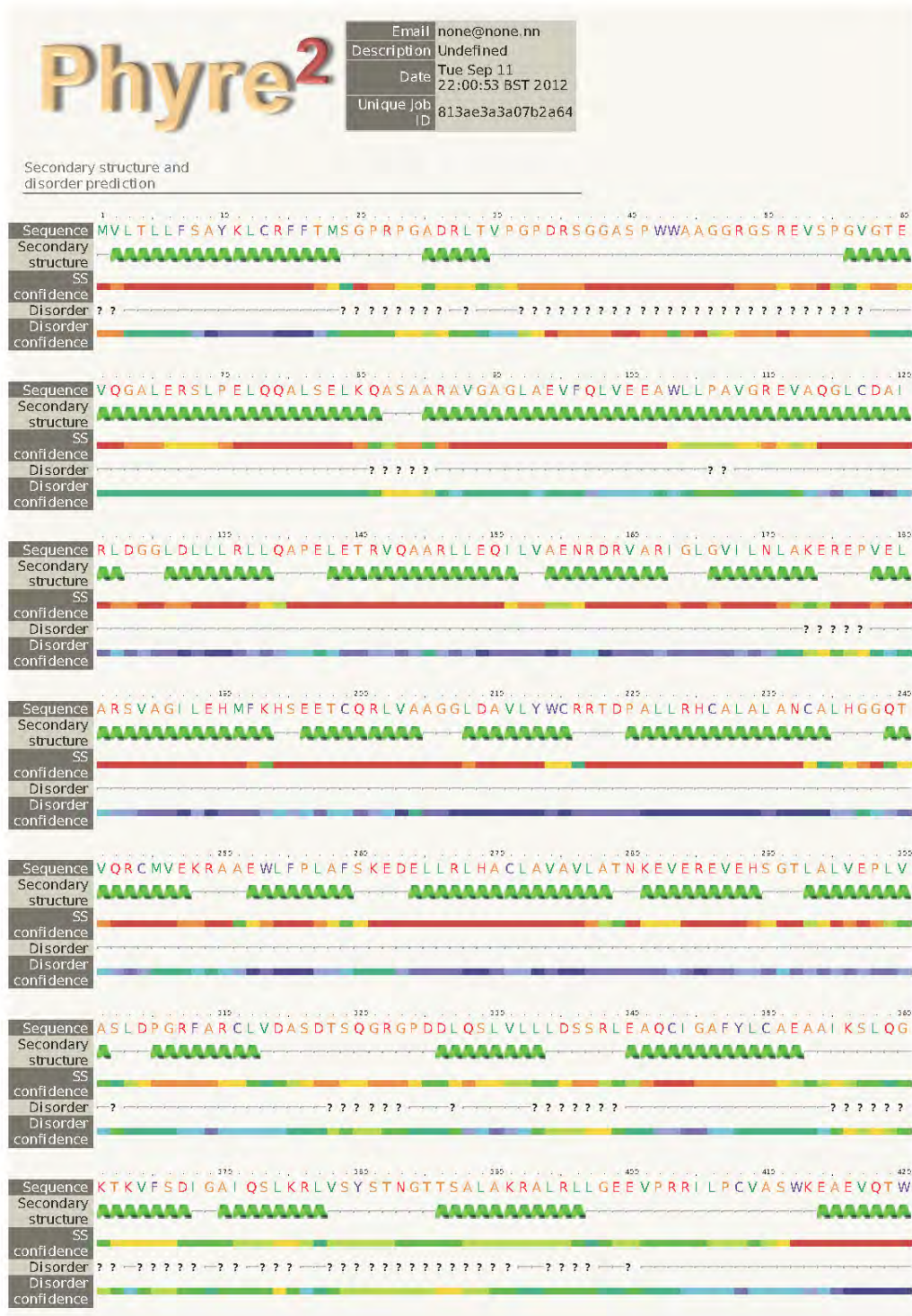
processes. I also found unexpected differences in dendrite degeneration between mutants of *Wld<sup>s</sup>/dsarm* and *hiw*. My preliminary investigations into developing a SARM1 inhibitor as well as examining WD in non-neuronal cells have provided a framework to further these avenues of research.

The study of WD is of great importance in the fight against neurodegenerative disease, a concept not lost on the founding father of the field, Augustus Waller. I believe that the delineation of pathways and mechanisms operating after injury and in disease will ultimately lead to our ability to decrease the suffering inflicted by such illnesses.

It is impossible not to anticipate important results from the application of this inquiry to the different nerves of the animal system. But it is particularly with reference to nervous diseases that it will be most desirable to extend these researches. If one conviction impresses itself more firmly on the mind than another, it is that what we term functional diseases of the nerves are in reality owing to certain organic and physical changes in the tubular fibre, which it will be the province of the microscope to ascertain.

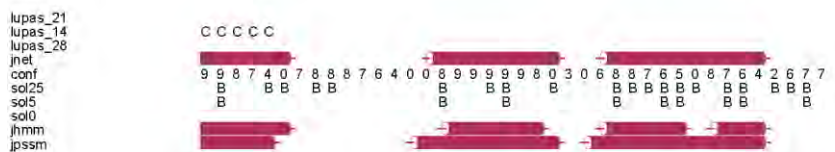
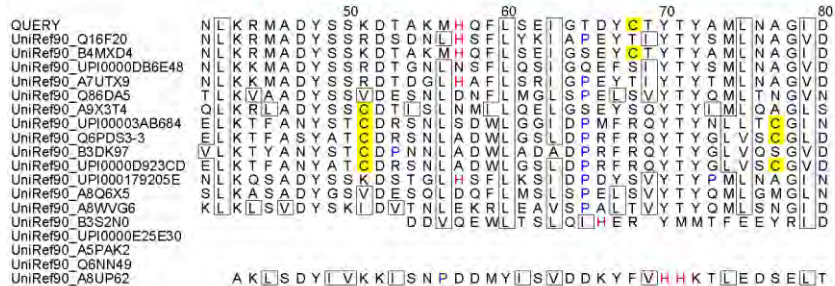
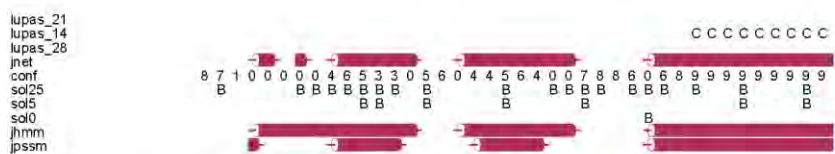
-Augustus Waller, 1850 (pp. 428-9)

## Appendix I: Towards an Inhibitor of SARM1



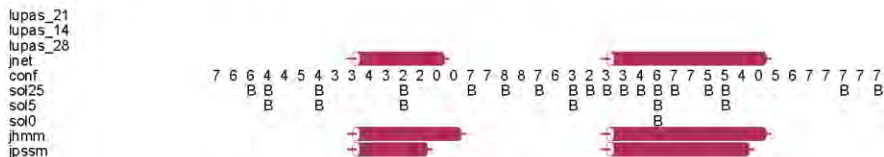


## Jpred3 alignment and tertiary structure prediction of the SARM1 TIR domain



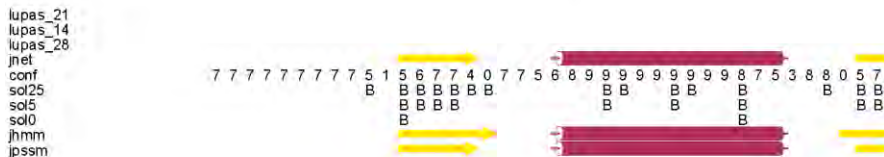
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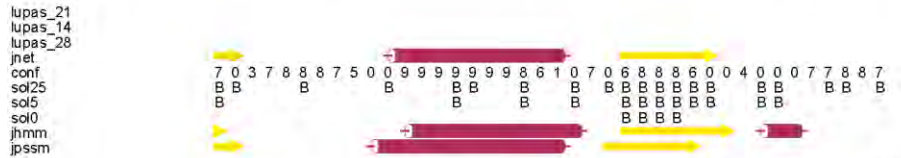
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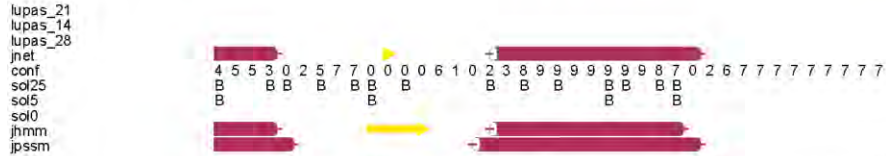
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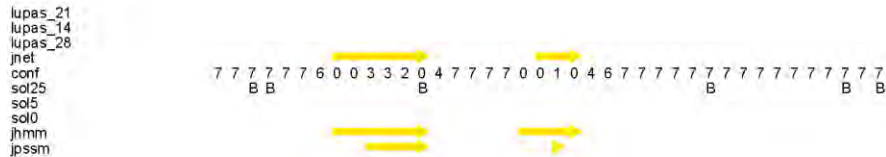
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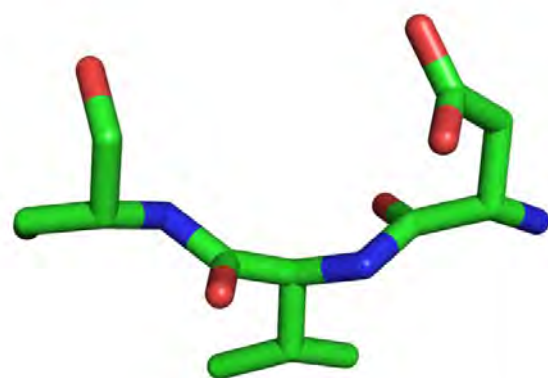


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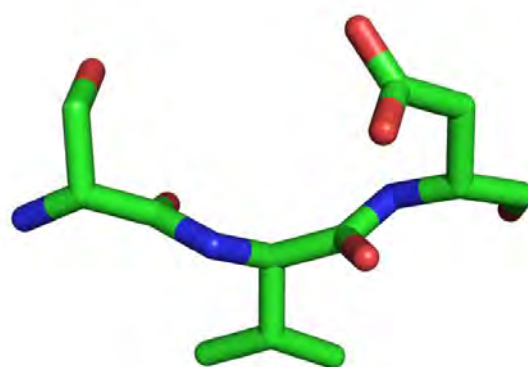


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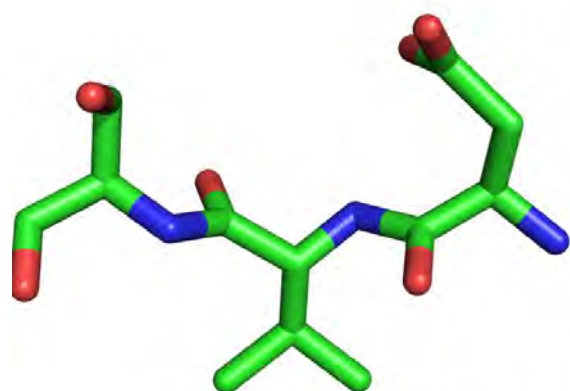




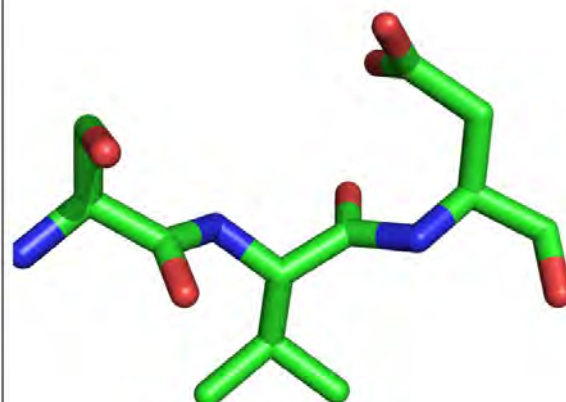
(1) L(Asp-Val-Ser)



(2) D(Ser-Val-Asp)

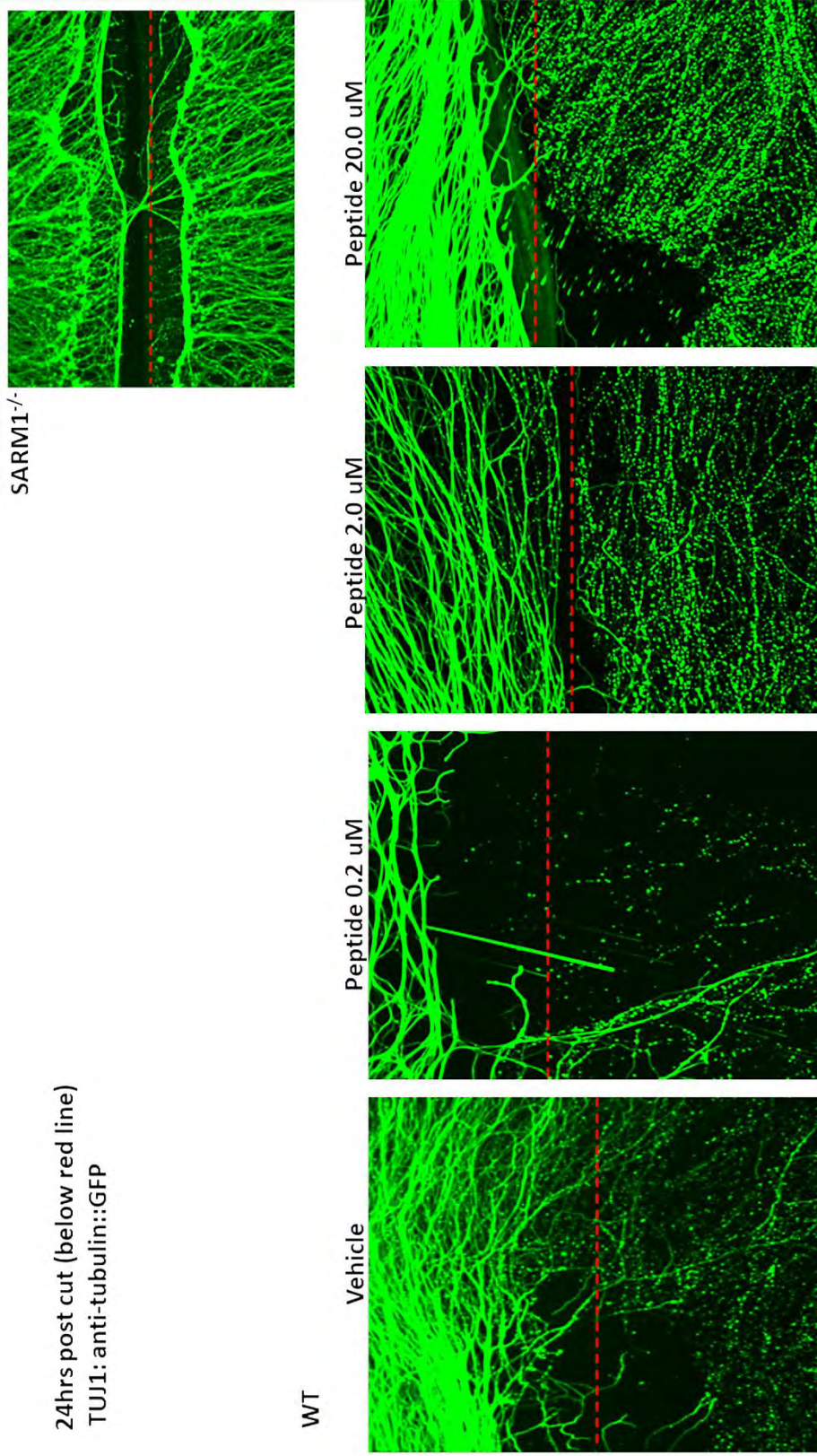


(3) D(Asp-Val-Ser)

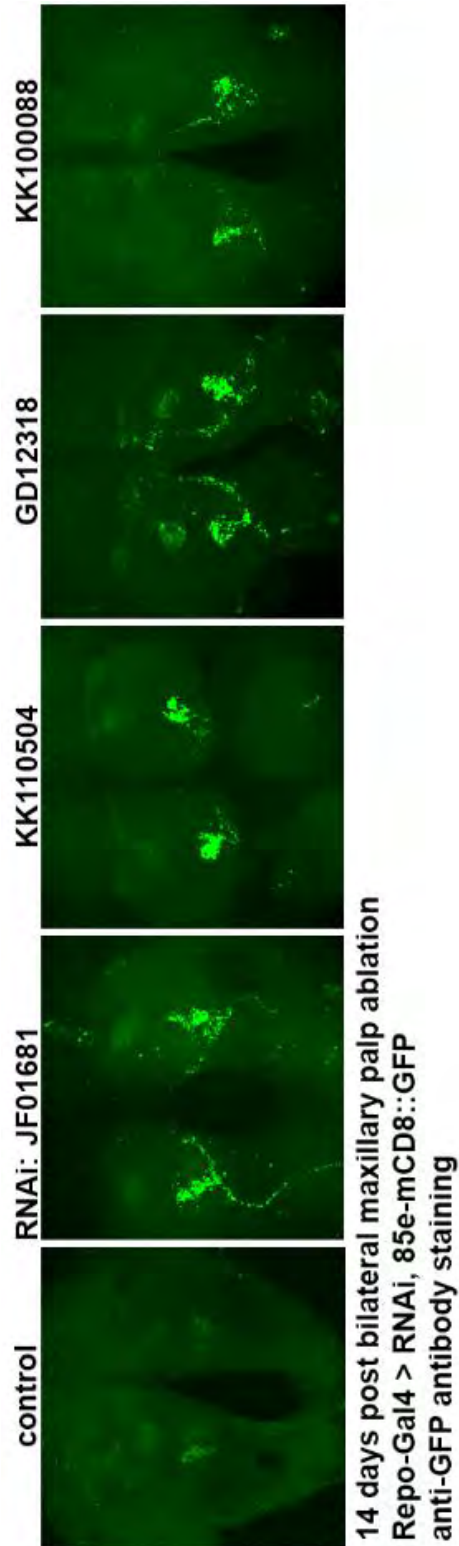


(4) L(Ser-Val-Asp)

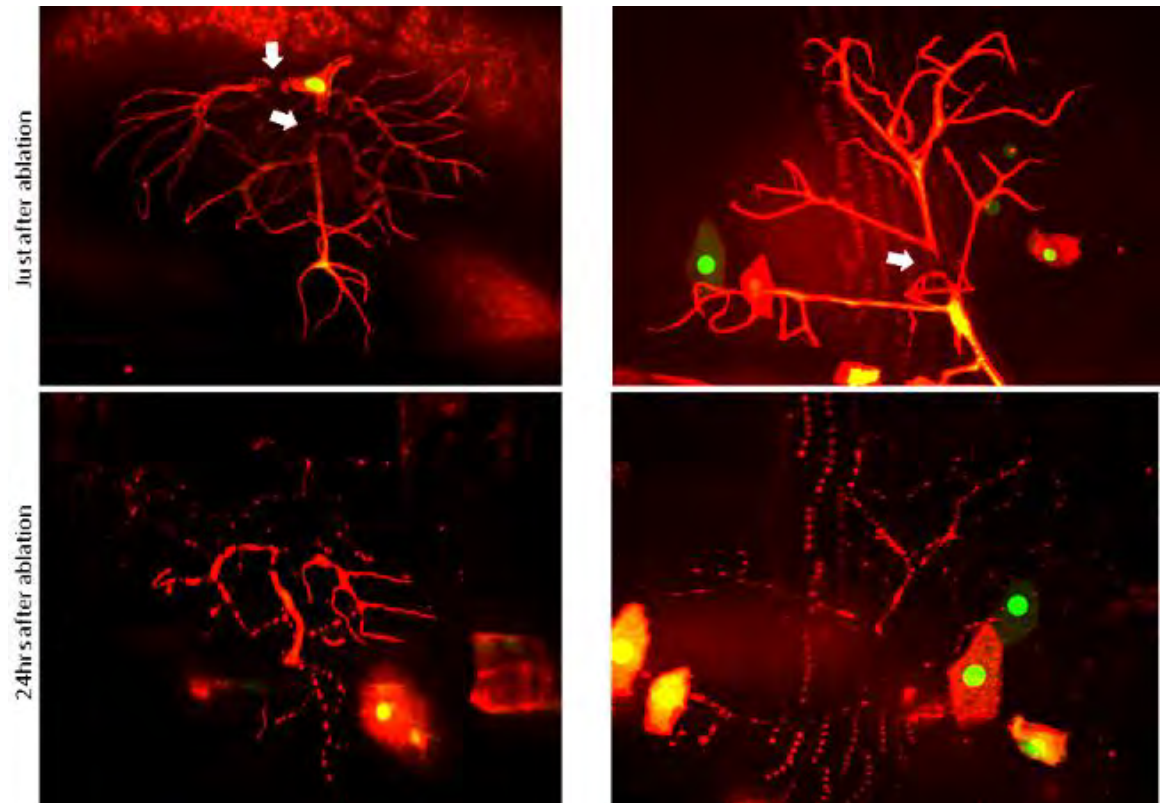
Example of retro-inverso with a 3 amino acid peptide.  
Credit: <http://en.wikipedia.org/wiki/Peptidomimetic>



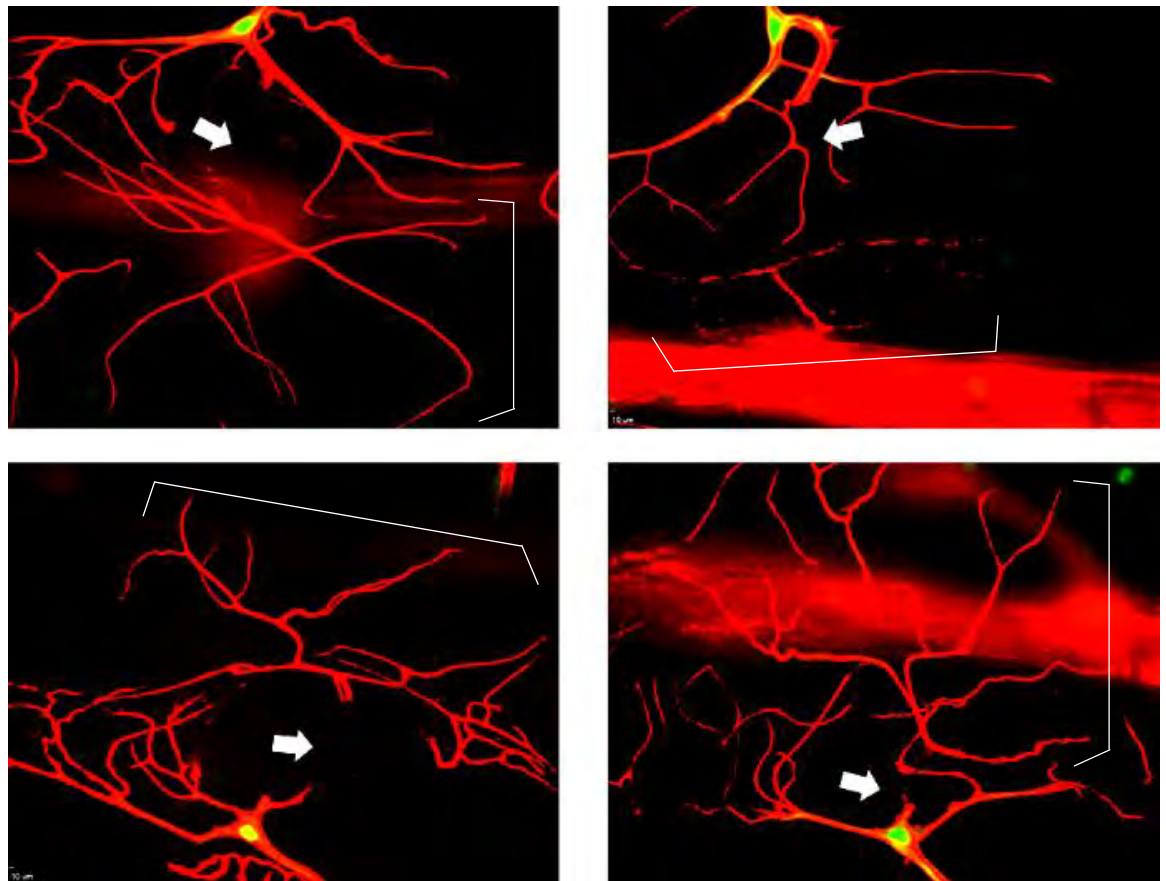
## Appendix II: dSARM RNAi in Glia



### Appendix III: Ablation of Tracheal Clones



Wild-type tracheal clone ablation (arrows, top), and the same cells 24 hours after ablation (bottom). Cells are labelled with *Btl-Gal4* driving myr-tdTomato and GFP-nls.



Wild-type tracheal clones 24 hours post-ablation (arrows). Brackets highlight severed branches. Cells are labelled with *Btl-Gal4* driving myrtdTomato and GFP-nls. Branch degeneration is only seen in 1 out of the 4 clones at this time point.

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