ROLES FOR THE HIGHWIRE/WALLENDA PATHWAY IN REGENERATIVE AND DEGENERATIVE RESPONSES TO AXOANL INJURY

by

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To my husband and son

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PREFACE

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ABSTRACT

Axons allow neurons to communicate over long distances, and their long length makes them vulnerable to injury. Neurons make several important responses to axonal injury. One is that the axons that are still connected to the cell body can initiate new axonal growth. This requires transcriptional reprogramming, and an important question is how this nuclear event can be induced by an injury in a distal site of the axon. Another response is that the distal stump, which is disconnected from the cell body, degenerates through a process called Wallerian degeneration. This self-destruction process is poorly understood, but is misregulated in some neurodegenerative diseases.

To study these responses in a model organism, I developed an axonal injury assay in Drosophila. I have characterized the function an axonal signaling pathway, which is regulated by the ubiquitin ligase Hiw, and its conserved axonal target, Wnd. I found that Hiw and Wnd, through a downstream signaling cascade, regulate several important responses to axonal injury. First, the Hiw/Wnd signaling pathway is activated by axonal injury and mediates the transcriptional changes that facilitate new axonal growth from the proximal stump. Second, this signaling pathway also inhibits Wallerian degeneration, allowing for neurons that have been injured once to have increased resilience to a second injury. This demonstrates that Hiw and Wnd control remarkable plasticity in the axonal degeneration process. Third, Hiw performs an additional role in

degeneration by regulating the levels and localization of NMNAT. This NAD+ synthesizing enzyme plays an essential role in dramatic inhibition to degeneration when *hiw* is mutant. Since the Hiw/DLK pathway is highly conserved, these findings may ultimately be of clinical interest for understanding and treating nerve damage in humans.

CHAPTER 1

GENERAL INTRODUCTION

The capability to respond to the surrounding environment is a basic feature of living beings. In this study, I am particularly interested in how our nervous system copes with the common type of environmental insult, traumatic injury. Neuronal injuries have severe consequences, including paralysis and even death, so there is great interest in understanding the mechanisms of these injury responses and in developing strategies to repair neuronal circuits after damage. This chapter will give a general introduction to how neurons, as the basic units of our nervous system, make responses to injury, in particular, injury to axons. The first part of this chapter will focus upon mechanisms that promote axonal regrowth after damage, with particular emphasis on the required transcriptional changes and how these are induced upon axonal injury. The second part of this chapter will focus on the self-destruction process happens in the axon that is separated from the cell body. The clearance of this distal axon is required for the proximal axon to regenerate. Also, this axonal degeneration process also takes place in many neurodegenerative diseases. A summary of the injury responses in different parts of an injured neuron is shown in Figure 1.1. The third part of this chapter will introduce a conserved signaling pathway, which has become the focus of my thesis work. The body

of the thesis will describe roles that I have discovered for this signaling pathway in both regenerative and degenerative responses to axonal injury.

1.1 Injury signaling and regeneration

Neurons are highly polarized cells with distinct neurite structures. The task of relaying information over long distance by neurons is achieved by their axon structures, which can be over 1000 times the length of a cell body. The long length of axons makes them particularly vulnerable to injury, and axonal damage leads to the disruption of neural circuits, which may have severe consequences for the organism. In some circumstances, the axons that remain connected to their cell body can regrow to repair the damaged circuit. This is referred to as ‘axonal regeneration’. However axon regeneration only happens in some, not all, circumstances. Therefore, understanding the molecular and cellular mechanisms that regulate axon regeneration is of great clinical significance.

1.1.1 Intrinsic and extrinsic factors for regeneration

Whether a neuron is able to regenerate its axon after damage depends on the balance between its intrinsic growth capability and its inhibitory extracellular environment. It has been known for a long time that axons in the peripheral nervous system (PNS) can regenerate after injury, while axons in the central nervous system (CNS) fail to regenerate (Ramon y Cajal, 1928). This is why spinal cord injury usually causes paralysis without any improvement even after many years. The different

regeneration capabilities of CNS neurons and PNS neurons provide a great platform for identifying the intrinsic and extrinsic regeneration factors that differ between these neuron types.

An extrinsic difference is that CNS axons are myelinated by oligodendrocytes while axons in PNS are wrapped by Schwann cells. Several myelin-associated inhibitors in the CNS have been identified more than a decade ago. These include Nogo (Chen et al., 2000; GrandPre et al., 2000; Prinjha et al., 2000), MAG(myelin-associated glycoprotein) (McKerracher et al., 1994) and OMgp (Oligodendrocyte myelin glycoprotein) (Wang et al., 2002). These molecules can inhibit axon regeneration by signaling through Nogo Receptors (NgRs) (Schwab, 2004). In addition to myelin inhibitors, another source of inhibition is the glial scar that forms after CNS injury. Many astrocytes at the injury site can release inhibitory extracellular matrix molecules. A known class of these molecules is the Chondroitin sulphate proteoglycans (CSPGs) (McKeon et al., 1991; Morgenstern et al., 2002). A recent study found that CSPGs can also bind to Nogo receptors, suggesting that myelin-associated inhibitors and CSPGs might inhibit axon regeneration via some shared mechanisms (Dickendesher et al., 2012).

However, mutations that reduce the function of these inhibitors and their known receptors only allow for modest regeneration in the CNS (Yiu and He, 2006). This suggests that the intrinsic growth capability is also different between PNS and CNS neurons. Understanding the intrinsic mechanisms that promote axon regeneration in PNS neurons will hopefully help to improve the regeneration ability of CNS neurons.

1.1.1 Intrinsic regeneration promoting factors: injury signaling pathways in neurons

Many efforts have been devoted in various model systems to study the intrinsic mechanisms that regulate axon regeneration. An important aspect of regeneration is the formation of new axonal structures to repair what has been damaged. Therefore, regeneration requires new protein synthesis, which largely depends on the transcriptional changes in the nucleus. This subsection will mainly focus on discussing the mechanisms by which these transcriptional changes are regulated in response to axonal injury.

Previous studies with mouse DRG neurons suggest that the ability of the cell body to make transcription-dependent changes in gene expression correlates well with the neuron's ability to regenerate. DRG neuron axons have two branches, a peripheral branch that regenerates after cutting, and a central branch that innervates spinal cord and fails to regenerate after cutting (Ramon y Cajal, 1928). Interestingly, the cell body responds differently to injuries at these two branches. Injury to the peripheral branch triggers transcriptional changes that lead to expression of growth associated proteins (Hoffman and Cleveland, 1988), while cutting central branch does not (Chong et al., 1994; Richardson and Issa, 1984; Smith and Skene, 1997). Strikingly, a prior conditioning lesion to the peripheral branch enables the central branch to regenerate (Richardson and Issa, 1984). Figure 1.2. shows a schematic illustration of this finding. This remarkable observation suggests that the transcriptional changes induced by peripheral branch injury may increase the intrinsic growth capacity of the neuron to enable regeneration at the central branch (Hoffman, 2010; Richardson and Issa, 1984).

One fundamental question is how does the cell body sense axonal damage in order to initiate transcriptional changes when the damage takes place at a far distance from the cell body. Or in other words, how does the injury information spread from the injury site to the cell body? In order for the cell body to receive accurate and comprehensive information about the site and extent of axonal injury, the injured neuron might apply multiple injury signaling pathways to convey the injury information. Some progress has been made towards characterizing the signaling pathways that are induced by axonal injury.

One important signaling pathway involves cAMP, whose levels in the cell body elevate after PNS axon injury, and this increase is required for the initiation of axon regeneration (Hannila and Filbin, 2008) Artificially increasing cAMP levels is sufficient to improve axon regeneration in CNS neurons (Spencer and Filbin, 2004). The function of cAMP in regeneration involves the transcription factor CREB (cAMP response element binding protein) (Sands and Palmer, 2008). However, the upstream factors that lead to cAMP increase in the cell body are less understood. Because adenylate cyclases can be regulated by calcium, an attractive model is that an intracellular ca2+ wave, which spreads from injury site to the cell body, may lead to an induction of cAMP levels (Ghosh-Roy et al., 2010).

Another well-characterized factor induced by axon injury is the transcription factor c-Jun. c-Jun can be activated via phosphorylation by the MAP (Mitogen-activated protein) kinase JNK (c-Jun NH2-terminal kinase) and its function is required for axon regeneration (Lindwall and Kanje, 2005; Raivich et al., 2004). Studies with mouse sciatic nerve have demonstrated that nerve injury induces local activation of JNK via

phosphorylation, and that phosphorylated JNK can travel retrogradely from the injury site to cell body (Cavalli et al., 2005; Lindwall and Kanje, 2005). How does the phosphorylated JNK travel in the axon? Previous studies have identified an important role for the scaffolding protein JIP3/Sunday Driver, which can link JNK to dynein components of fast axon transport machinery (Cavalli et al., 2005). Fast axonal transport refers to the microtubule based cargo trafficking in axons, which could be bi-directional including anterograde transport towards axon terminus by kinesin motor proteins and retrograde transport towards cell body by dynein motor proteins.

Additional components which may be transported retrogradely in axons include transcription factors themselves. Surprisingly, multiple transcription factors have been detected in transected sciatic nerves, including ATF2, ATF3 and STAT3, and they appear to be transported in the axons (Ben-Yaakov et al., 2012; Hanz and Fainzilber, 2006; Lindwall and Kanje, 2005). A few studies have suggested that transcription factors might be transported in axons in a NLS and importin dependent manner (Perry and Fainzilber, 2009). Importin proteins are the core components of classical nuclear import machinery that have high affinity to NLS-containing proteins. The importin α/β complex is induced by injury and can associate with dynein complex (Hanz and Fainzilber, 2006; Hanz et al., 2003). By binding to importins, transcription factors would gain access to retrograde transport machinery and thus be able to reach the nucleus.

In summary, injury induces a number of molecular events within axons, some of which ultimately mediate retrograde signaling to the nucleus. However, it is still unclear how axonal damage leads to activation of these pathways. Chapter 2 of this thesis will

describe a conserved MAPKKK that functions to sense axonal damage and regulate a retrograde nuclear response pathway that promotes axon regeneration.

1.2 Degeneration

When axons are injured, the portion of the axon that is separated from the cell body, or distal stump axon, undergoes degeneration. This process is termed as wallerian degeneration, following Augustus Waller's original description of nerve transection experiments(Waller, 1850). After a period of lag time (typically 1.5 days in veterbates), injured axons rapidly degenerate, marked by the beading and swelling of axonal membrane, and the breakdown of cytoskeleton structure, followed by clearance of axon debris by glial cells and immune cells (Griffin et al., 1995; Vargas and Barres, 2007; Waller, 1850).

1.2.1 Wallerian degeneration is a conserved active destruction process

It has long been thought that axons disconnected from the cell body degenerate as a result of lack of new material supply. However, the discovery of a spontaneous mutation in mouse, slow wallerian degeneration (WldS), suggests that the degeneration may be an active process (Lunn et al., 1989). In these mice, the distal axons stay morphologically intact and can conduct action potential for several weeks after axon transection. Therefore, the wallerian degeneration process likely involves a regulatory cellular pathway, about which little is known.

Since the WldS mutation strongly delays the onset of wallerian degeneration, studying how it functions will contribute to our understanding of the regulatory mechanism of wallerian degeneration. Intriguingly, expression of the mouse WldS mutant protein in flies is sufficient to delay wallerian degeneration for several weeks in fly olfactory sensory neurons (Hoopfer et al., 2006; MacDonald et al., 2006). The strong protection of axon degeneration in flies by a mouse mutant protein suggests that the process of wallerian degeneration is conserved from invertebrates to vertebrates. Therefore, by studying wallerian degeneration in flies we should gain mechanistic insights to the mechanism of this process in vertebrates.

Axon degeneration can be caused by various insults including metabolic disorders, toxins, loss of myelination, or inherited neurodegenerative diseases (Coleman, 2005; Coleman and Freeman, 2010). Importantly, the WldS mutation can also suppress axon degeneration in mouse models of glaucoma, multiple sclerosis, diabetic neuropathy, parkinson's disease, as well as some models of Alzheimer's disease (Coleman, 2005; Coleman and Freeman, 2010; Wang et al., 2012). This suggests that a universal mechanism appears to be shared between wallerian degeneration induced by injury and axon degeneration in many diseases. Therefore, the information that we learn from wallerian degeneration will likely be more broadly important for neuropathies and disease.

1.2.2 Different forms of axonal degeneration

Many models are established for studying the mechanism of wallerian degeneration in mammals as well as in invertebrate model organisms. Wallerian

degeneration was initially observed after nerve transection in mice. The study of wallerian degeneration with mouse neurons has extended to in vitro assays using cultured primary neurons. Besides physical axon damage, including axon transaction and axon crush, other chemical interferences can also induce wallerian-like degeneration. These include applying neurotoxin vincrinstine, taxol and cochicine to the neuronal cultures, as well as depriving the neurotrophic factors that are required for integrity maintenance in neurons. NGF (nerve growth factor) is a target derived neurotrophic factor that controls the survival of sympathetic ganglia neurons during development (Levi-Montalcini and Cohen, 1956). In *in vitro* experiments, culturing neurons in a compartmentalized chamber allows environmental isolation of axons with their cell body (Campenot, 1982). When nerve growth factor (NGF) is deprived from only the axon compartment of the cultured sympathetic neurons, the axons undergo wallerian-like degeneration (Campenot, 1982). Because the axon and cell body could be assayed separately, the compartmentalized chamber model is especially informative for distinguishing the factors that act locally in axons with those that function via transcriptional regulation.

Another category of axon degeneration happens during development, termed axon pruning, in which specific axonal branches are destroyed to refine their wiring pattern and build precise connectivity with their targets. This process is observed in multiple organisms ranging from worms to mammals (Luo and O'Leary, 2005). Axon degeneration during pruning shares some common mechanism with wallerian degeneration. For example, inhibiting the UPS, which delays wallerian degeneration, can also inhibit axon degeneration during pruning (Hoopfer et al., 2006). However, the

Wlds mutant, which potently inhibits wallerian degeneration, does not protect axons during pruning (Hoopfer et al., 2006). This difference indicates that the regulatory mechanism for developmental degeneration is divergent from that for wallerian degeneration, although they share some pathways and are morphologically similar processes. In this study, I will mainly focus on the injury-induced wallerian degeneration, and will compare its mechanism with that of other degeneration models.

1.2.3 The mechanism of the WldS mutation and the protective action of Nmnat

Extensive studies have been carried out to understand how the WldS mutation can protect axons from degeneration. The WldS mutation is caused by spontaneous chromosome rearrangement that leads to the creation of a fusion protein that contains a complete sequence of Nmnat1 (Nicotinamide mononucleotide adenylyltransferase 1) and the N-terminal 70 amino acid of the E4 ubiquitin ligase Ube4b, linked by a unique 18 amino acid sequence generated by the read-through of Nmnat 5' UTR (Conforti et al., 2000; Mack et al., 2001).

Consensus has now been reached that Nmnat1 is the core component of the WldS mutation (Coleman and Freeman, 2010; Wang et al., 2012). Nmnat1 is a key enzyme of the NAD+ salvage pathway. Mutation of either the ATP binding site of Nmnat1 or the NMN+ binding site, both of which block the enzymatic function of Nmnat1, potently diminish the ability of Wlds to protect injured axons (Conforti et al., 2009; Yahata et al., 2009). However, despite the agreement that Nmnat activity is important, it is less clear whether NAD+ is the protective enzyme product. The total NAD+ levels in brain extracts from the Wlds mouse are not significantly altered (Mack et

al., 2001), and genetically manipulating the NAD+ synthesis pathway to increase NAD+ levels has no protective effect (Sasaki et al., 2009b). To further complicate matters, a study in flies has found that the enzymatic activity of Nmnat is dispensable for its neuroprotective function, and has proposed instead that Nmnat may function as a chaperone to protect against neurodegeneration (Zhai et al., 2008).

The cellular location of WldS's protective action has been the subject of much debate. Wlds protein predominantly resides in the nucleus since Nmnat1 contains a nuclear localization sequence and is normally localized exclusively in the nucleus (Magni et al., 2004). Recent studies have reported that trace amount of Wlds protein have been detected in the axoplasm and mitochondria (Beirowski et al., 2009; Yahata et al., 2009). The extranulcear localization of Wlds is likely due to interaction between the Ube4b sequence and cytoplasmic VCP protein (Laser et al., 2006). Emerging findings suggest that this pool of extra-nuclear Wlds protein is the critical component for neuroprotection. Overexpressing Nmnat1 is not sufficient to protect injured axons. However, relocating Nmnat1 outside the nucleus by deleting its NLS or fusing it to an axon targeting sequence endows axons with a resiliency to degeneration after injury (Babetto et al., 2010; Beirowski et al., 2009; Sasaki et al., 2009a). Moreover, direct delivery of Nmnat1 protein to severed axons can protect axons from degeneration (Sasaki and Milbrandt, 2010). A recent study has provided direct evidence that the axonal pool of Wlds is necessary for axon protection with a chemical genetic method, which enables selective destabilization of axonal pool of Wlds in compartmented neuronal culture (Cohen et al., 2012).

Despite the plethora of studies on WldS, which is a gain-of-function mutation, less is know about the endogenous function of Nmnat proteins in axon degeneration. There are three Nmnat genes in mouse, Nmnat 1, 2, 3. Studies in both mouse and flies have demonstrated their different protective abilities when over-expressed. As mentioned above, Nmnat1 is the core component of Wlds that normally resides in the nucleus that can protect when artificially re-localized to extra-nucleus (Babetto et al., 2010; Beirowski et al., 2009; Sasaki et al., 2009a). Nmnat3 localizes to mitochondria (Berger et al., 2005). When overexpressed, Nmnat3 can potently protect axons in the extent comparable to Wlds in both vertebrates and drosophila models (Reviewed in (Wang et al., 2012)). However, it is not clear whether Nmnat3 is endogenously present in neurons. Nmnat2 is the most labile Nmnat isoform that is required for maintenance of axon integrity (Gilley and Coleman, 2010). Endogenous Nmnat2 is present in neurons and undergoes rapid turnover in transected mouse SCG neurites in a proteasome dependent manner. It has been proposed that reducing Nmnat2 below a certain threshold in injured distal stump is necessary for axon degeneration, suggesting its potential endogenous roles in regulating wallerian degeneration (Gilley and Coleman, 2010). However, the molecular mechanism that regulates endogenous Nmnat2 level in neurites is not known.

There is only one Nmnat gene in fly, which produces two splice forms. Chapter 4 of this thesis will describe a similar turnover of fly Nmnat protein in degenerating axons. I have found that a conserved E3 ubiquitin ligase regulates the endogenous Nmnat level in axons, and its function is necessary for the reduction of Nmnat in injured distal stumps, as well as for axons to degenerate. This E3 ubiquitin ligase can also down-

regulate exogenously expressed mouse Nmnat2 protein, implying a conserved mechanism for this regulation.

1.2.4 Mediators and regulators of wallerian degeneartion

Since wallerian degeneration is a self-destruction process, researchers have wondered whether it involves some known cellular destruction programs. Defined pathways for cellular self-destruction includes apoptosis (programmed cell death,) and autophagy, which performs bulk protein degradation and destruction of organelles. There are also some fine cellular destruction pathways that degrade particular proteins, including ubiquitin proteasome system (UPS) and proteases. In the following, I will discuss the relationship between these destruction programs and wallerian degeneration.

Although both wallerian degeneration and apoptosis are active self-destruction processes and share morphological similarities, they are molecularly distinct, since potent cell death inhibitors fail to block axon degeneration, and severed Wlds axons can survive for weeks even after the cell body death (Deckwerth and Johnson, 1994; Raff et al., 2002). Caspases are the main regulators and executioners for apoptosis. They are involved in axon degeneration after NGF deprivation in neuronal cultures, as well as during developmental pruning of mouse DRG sensory neurons (Nikolaev et al., 2009; Schoenmann et al., 2010). Caspases have also been implicated in synaptic retraction, triggered by cytoskeletal mutations, at the Drosophila neuromuscular junction. However, as discussed previously, Wallerian degeneration and developmental degeneration may involve different mechanisms. In further support of this difference, several studies

suggest that caspases are not required for Wallerian degeneration (Finn et al., 2000; Osterloh et al., 2012; Zhai et al., 2003).

Autophagy is another self-destruction pathway that can lead to bulk degradation of proteins and cellular structures. Links have been made between defects in autophagy and many neurodegenerative diseases. However, there is no evidence that autophagy is involved in wallerian degeneration. Mutants in autophagy essential genes fail to protect severed axons from degeneration in fly olfactory neurons (Osterloh et al., 2012).

As a main pathway mediating protein degradation, the UPS has been identified to play a critical role in regulating wallerian degeneration. Adding proteasome inhibitors to cultured rat sympathetic superior ganglia (SCG) neurons in vitro or applying to severed optic nerves in vivo delays the onset of wallerian degeneration for up to 3 days (Zhai et al., 2003). In addition, expressing yeast deubiquitinase UBP2 in Drosophila olfactory sensory neurons can potently delay wallerian degeneration for more than 10 days after injury (Hoopfer et al., 2006). Since inhibiting UPS arrests degeneration at a relative early stage, it is likely that the UPS plays a regulatory role in the initiation of wallerian degeneration. One explanation for its role is that the UPS mediates bulk protein degradation via the combined action of many ubiquitin ligases. However, a more likely model is that one or several specific E3 ligases target the destruction of key inhibitors of the degeneration process. The chapter 4 will describe a specific E3 ligase that plays essential role in regulating the initiation of wallerian degeneration.

Protease mediated degradation is another pathway for bulk protein destruction. It has been well characterized that the Ca2+ dependent protease, Calpain, can breakdown cytoskeleton components during wallerian degeneration (Billger et al.,

1988). After injury, intracellular ca2+ level increases both in proximal and distal stump axons. It is reported that this increase of ca2+ level is both necessary and sufficient for the initiation of wallerian degeneration. Reducing extracellular ca2+ concentration of cultured neurons delays axon degeneration for several days after axotomy, while adding ca2+ ionophores is sufficient to induce degeneration in uninjured neurites (Billger et al., 1988; George et al., 1995; Schlaepfer and Bunge, 1973). Increased ca2+ in the axon can activate the serine-threonine protease Calpain, which cleaves cytoskeleton components after being activated (Billger et al., 1988). However, chemically inhibiting Calpain activity leads to much milder delay of axon degeneration than reducing external Ca2+ (George et al., 1995; Glass et al., 1994; Schlaepfer and Bunge, 1973), suggesting that additional Ca2+ dependent pathways may also be involved in wallerian degeneration.

Despite that other cellular destruction processes are involved in wallerian degeneration, the molecular mechanisms that initiate and regulate this process are poorly understood. The majority of the previous studies have centered on the WldS gain-of-function mutation while only few endogenous regulators have been identified. Studies in mice have identified JNK as a positive regulator of wallerian degeneration. Adding JNK inhibitors to cultured DRG neurons delays wallerian degeneration of transected axons by 48 hours (Miller et al., 2009). JNK is locally activated in axons after injury, and the regulation of wallerian degeneration by JNK is likely to be a local action because adding JNK inhibitors within 3 hours after axon transaction is sufficient to delay degeneration (Miller et al., 2009). In jnk1 and jnk3 mutant mice, degeneration of damaged cortical spinal tract (CST) axons after thoracic spinal cord injury is also

prevented (Yoshimura et al., 2011). Despite the compelling role for JNK in degeneration, the substrates of JNK which mediate degeneration are still unknown.

DLK is a MAPKKK that can act upstream of JNK. Loss of function mutations in *dlk* modestly delay wallerian degeneration in both mouse DRG neurons and fly olfactory neurons (Miller et al., 2009). In my study, however, as described in chapter 3, the fly homolog of DLK, Wallenda, plays opposite roles and instead inhibits axonal and synaptic degeneration in fly larval motoneurons. This will be discussed further in chapter 3 and chapter 5. A recently reported new and essential regulator of wallerian degeneration is the conserved Toll receptor adaptor Sarm (sterile α/Armadillo/Toll-Interleukin receptor homology domain protein) (Osterloh et al., 2012). Mutations in Sarm potently suppresses Wallerian degeneration in both fly and mouse neurons to an extent that is comparable to Wlds (Osterloh et al., 2012). Chapter 4 of this thesis will describe another essential regulator of wallerian degeneration, Highwire, since mutations in hiw also lead to a strong protection phenotype that is comparable to Wlds and Sarm. In future work, it will be interesting to study the relationship of Sarm and Highwire, as well as their relationship with Wlds.

Besides intrinsic pathways, the extracellular environment of injured neurons, majorly comprised of glial cells, also plays important roles in wallerian degeneration, predominantly to clear fragmented axonal debris. In the CNS, astrocytes extend processes towards injured axons several hours after axotomy (Bechmann and Nitsch, 1997), and microglia undergo rapid proliferation and migrate towards injured axons (Petersen and Dailey, 2004). Likewise, in the PNS, macrophages are rapidly recruited to the injured nerves to clear degenerating axons and myelin (Hall, 2005). Drosophila

glial cells undergo similar morphological changes in response to axon transection. After olfactory sensory neuron axotomy, glial cells change their morphology and extend their membrane processes towards injured axons and subsequently engulf degenerating debris. The glial engulfment requires the glia receptor Draper, and could be blocked when inhibiting the initiation of axon degeneration by WldS (MacDonald et al., 2006).

While glial cells can function as dustmen to clear debris at the late stage of degeneration, they are poised to potentially function as more active regulators in the initiation of degeneration. There is currently very little data that directly addresses this hypothesis for Wallerian degeneration, however a recent study of synaptic retraction at the fly NMJ has implicated a glial derived TNF-α homologue and its TNF- α receptor (TNFR), expressed on neuronal membranes, in wallerian-like degeneration triggered by a cytoskeletal mutation (Keller et al., 2011). This study suggests that non-cellautonomous pro-degenerative signals may also regulate wallerian degeneration.

1.3 The Highwire/Wallenda pathway

During the course of my thesis work, I have found that a conserved pathway plays essential roles in both regeneration and degeneration after axon injury. This pathway has been previously identified to be involved in regulating axonal and synaptic morphology in multiple organisms. In this sub-section I will introduce this pathway and its previously identified roles in synaptic development.

The development and plasticity of neuronal circuits depends upon the assembly of synapses, including the pre-synaptic structures and their apposed post-synaptic

structures. The structure of the pre-synaptic nerve terminus is important for determining the site and amount of neurotransmitter release.

Previous studies in multiple model organisms have implicated a role for a highly conserved E3 ubiquitin ligase in regulating the morphology of the pre-synaptic axon terminus. Mutations in this gene, known as *highwire (hiw)* in *Drosophil*a, lead to dramatic axonal sprouting phenotype at the larval neuromuscular junction (NMJ) (Wan et al., 2000). Mutations in homologues *in C. elegans (rpm-1)*, zebrafish (*esrom*) and mice (*phr1*) also lead to defects in axon guidance and synaptogenesis (Bloom et al., 2007; D'Souza et al., 2005; Lewcock et al., 2007; Schaefer et al., 2000; Zhen et al., 2000), hence this gene appears to play a conserved role in regulating the structure and behavior of axons. Hiw is a large protein, which shares a RING finger domain with E3 ubquitin ligases. Biochemical and functional studies in both *Drosophila* and *C. elegans* suggest that Hiw functions within a complex of ubiquitin ligases (Liao et al., 2004; Wu et al., 2007; Wu et al., 2005).

Previous genetic screens have identified Wallenda (Wnd) kinase as a critical target of regulation by Hiw (Collins et al., 2006). Wnd is a member of the mixed lineage family of kinases, which act as Mitogen-activated protein (MAP) Kinase Kinase Kinases (MAPKKKs). Wnd has conserved homologues in *C.elegans* and vetebrates named dileucine zipper kinase (DLK). Hiw down-regulates the levels of Wnd protein in axons, and over-expression of *wnd* phenocopies the axonal sprouting defects of *hiw* mutants (Collins et al., 2006). This regulatory mechanism is conserved in *C. elegans* (Nakata et al., 2005). However the relationship between vertebrate homologues Phr1 and DLK may be more complex (Bloom et al., 2007; Lewcock et al., 2007).

MAP kinases are an important class of serine/threonine-specific protein kinases that are involved in various essential biological processes, including but not limited to cell proliferation, differentiation, mitosis, cell survival, apoptosis as well as in response to a large array of environmental stimuli. MAP kinases and their upstream kinase usually form a kinase cascade, in which a MAPKKK activates a MAPKK via phosphorylation, and the activated MPAKK could then in turn activate downstream MAPK (reviewed in (Chang and Karin, 2001)). This hierarchical kinase activation mechanism enables complex signaling transduction as well as signal magnification. In *Drosophila*, Wnd promotes synaptic growth through the MAPKK Hep and the MAPK JNK, by regulating the downstream transcription factor Fos (Collins et al., 2006). The involvement of the Fos transcription factor implies that Hiw and Wnd regulate axonal sprouting by regulating a gene expression program. The schematic illustrations of the Wnd pathway, as well as the synaptic morphology phenotype caused by mis-regulation of this pathway are shown in Figure 1.3.

Although its down-regulation by Hiw is critical for synaptic development, no role had been identified for Wnd itself in synaptic development. My initial project was to detect the function of Wnd during neural development. However, I was not able to identify obvious defect with axon guidance or synaptic formation in several classes of neurons. Nor could I detect an obvious change in the activity of Wnd signaling pathway through developmental stages. Thereafter, I became more and more attracted to the hypothesis that Wnd doesn't function in the normal development, but instead may function in stress responses, given the involvement of its downstream JNK in various stress conditions. A common type of stress to axons and synapses is injury. After

establishing a nerve crush assay to observe responses to axonal injury in Drosophla larve, I have found that Wnd pathway indeed plays important regulatory roles in both axon regeneration and axon degeneration. This will be overviewed in the next subsection.

1.4 Overview

In this thesis, I will describe my finding that the Highwire/Wallenda pathway plays a central role in regulating multiple injury responses.

Chapter 2 will mainly focus on the proximal part of an injured neuron including the cell body and the axon stump that still connects to it. This part of thesis will discuss about how injury information is carried to the cell body after axonal damage to activate transcriptional changes, which are important for axon regeneration. In this part, I will demonstrate the essential roles of Wnd pathway in mediating the retrograde injury responses and promoting regeneration.

Chapter 3 will mainly focus on degeneration of the distal axons and synapses. In this part, I will describe a Wnd pathway-mediated protective mechanism that allows neurons to be more resilient when encountering recurrent injuries.

Chapter 4 will focus on the molecular mechanisms that regulate the initiation of wallerian degeneration in the distal axons and synapses. In this part, I will demonstrate the essential role of Hiw in regulating wallerian degeneration. To explain the molecular mechanisms for Hiw's role in regulating degeneration, I will introduce the newly identified target for Hiw, the Nmnat, whose mouse homologue is the core component of

Wlds. Reduction of Nmnat protein level mediated by Hiw might be an important initiation event for wallerian degeneration.

Chapter 5 will summarize the findings of this study and discuss about other relevant studies, as well as potential future directions.

1.5 Figures

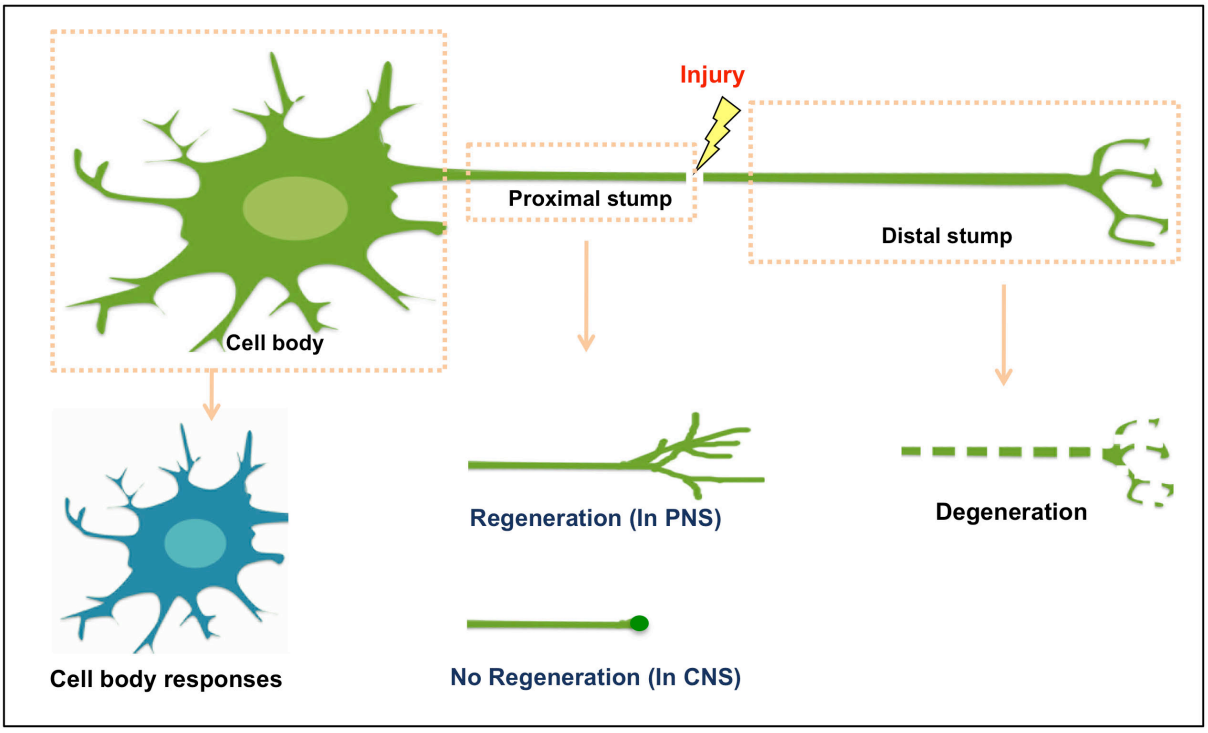


Figure 1.1. Axonal injury responses

A series of changes will take place in the neuron when axonal injury occurs. These responses include changes in the cell body, especially changes in gene expression and protein synthesis. The proximal stump usually can regenerate when the injured axon are in the peripheral nervous system (PNS), but does not regenerate in the central nervous system (CNS). Distal stump axons that are separated from the cell body usually undergo a self-destruction process, termed wallerian degeneration.

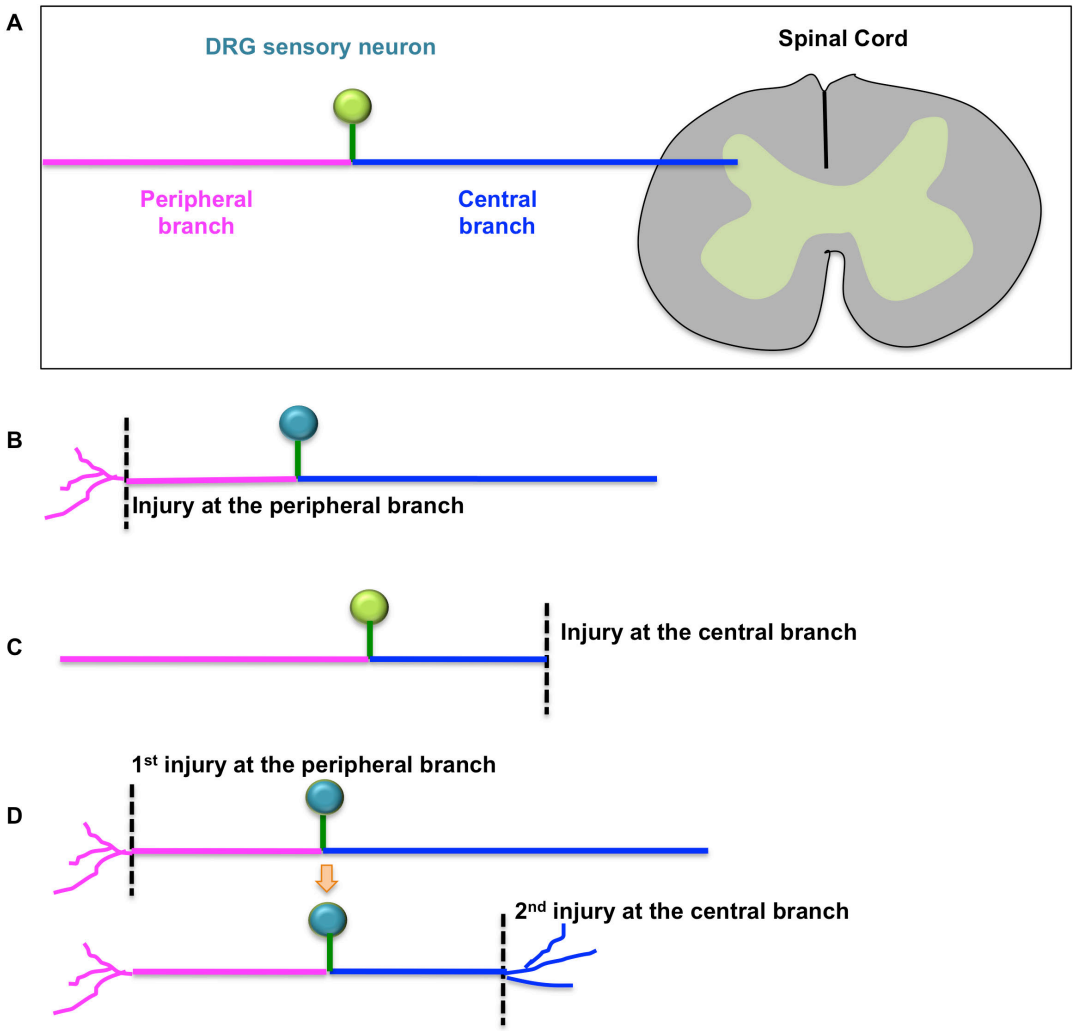


Figure 1.2 The conditioning lesion experiment (Richardson and Issa,1984)

(A)Sensory neurons in the dorsal root ganglion (DRG) have two axonal branches. A peripheral branch, which are located entirely in the peripheral nervous system (PNS), and a central branch, which extends from the DRG to spinal cord.
(B-C) The two axonal branches respond differently to injury. Peripheral branches are capable of effective regeneration. Central branches in the spinal cord does not regenerate after axotomy.
(D) Regeneration of the central branches can be enhanced re-injured 1-2 weeks after the intrinsic growth state has been increased by a prior conditioning lesion (CL) at the peripheral branch.

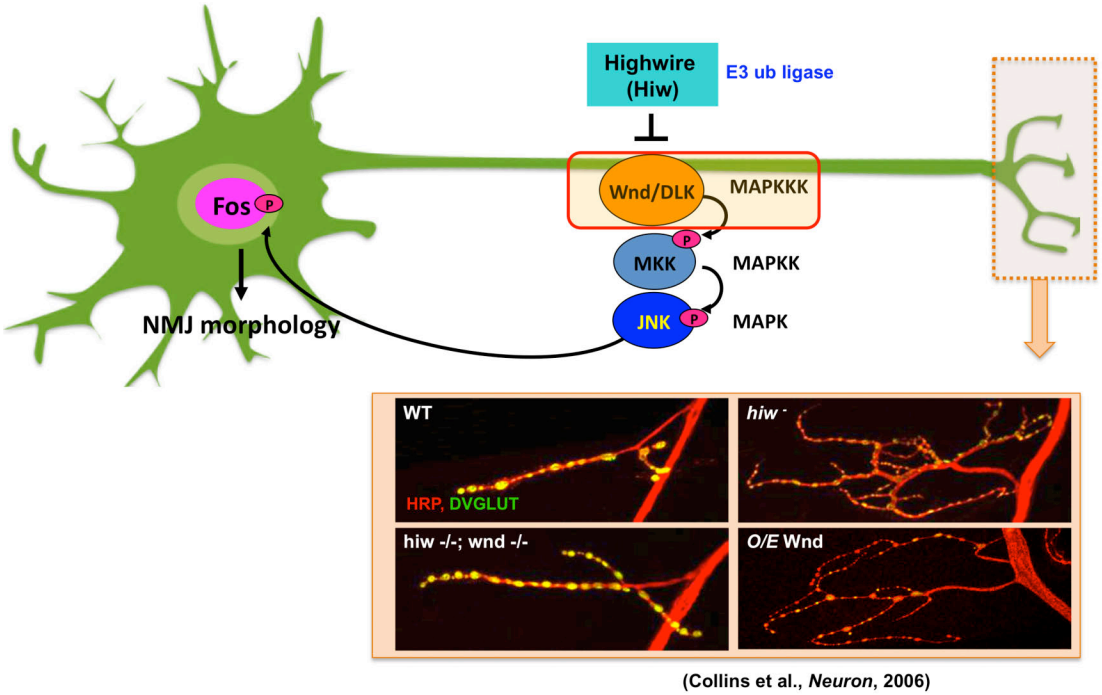


Figure 1.3 The Highwire/Wallenda pathway regulates synaptic morphology (Collins et al., 2006)

Wallenda (Wnd) is an axonal MAPKKK that regulates synaptic morphology via its downstream kinase cascade and the transcription factor Fos. Wnd is down-regulated by the E3 ubiquitin ligase Highwire (Hiw) at the protein level. Loss of function in *hiw* causes massive overgrowth of the neuromuscular junction (NMJ). Wnd is both necessary for this overgrowth phenotype in *hiw* and sufficient to promote synaptic overgrowth.

CHAPTER 2

PROTEIN TURNOVER OF THE WALLENDA/DLK KINASE REGULATES A RETROGRADE RESPONSE TO AXONAL INJURY

2.1 Abstract

Regenerative responses to axonal injury involve changes in gene expression, however little is known about how such changes can be induced from a distant site of injury. Here we describe a nerve crush assay in *Drosophila* to study injury signaling and regeneration mechanisms. We find that Wallenda (Wnd), a conserved MAPKKK homologous to DLK, functions as an upstream mediator of a cell autonomous injury signaling cascade that involves the JNK MAP Kinase and Fos transcription factor. Wnd is physically transported in axons, and axonal transport is required for the injury signaling mechanism. Wnd is regulated by a conserved E3 ubiquitin ligase, named Highwire (Hiw) in *Drosophila*. Injury induces a rapid increase in Wnd protein, concomitantly with a decrease in Hiw protein. In *hiw* mutants, injury signaling is constitutively active and neurons require less time to initiate a regenerative response. We conclude that regulation of Wnd protein turnover by Hiw is a damage surveillance mechanism for detecting and responding to axonal injury.

2.2 Introduction

Regenerative responses to axonal injury require a transcriptional reprogramming of the injured neuron, and there is great interest in understanding how this reprogramming is activated and controlled (Richardson et al., 2009; Sun and He, 2010). Because axons are long (as long as 1 meter for human motoneurons), a key element of an injury response mechanism is the ability to signal to the nucleus that the axon has been damaged. A number of studies suggest that signaling molecules are physically transported in axons via microtubule based motors, such as the minus-end directed motor dynein (reviewed in (Abe and Cavalli, 2008; Hanz and Fainzilber, 2006)). The transported molecules include transcription factors, intermediate filaments, and activated MAP Kinases ERK and JNK. How such molecules are orchestrated to detect and respond to axonal damage is poorly understood.

Recent findings have brought attention to a conserved MAPKKK, DLK, as a candidate regulator of axonal damage signaling. DLK is an upstream regulator of MAP Kinase signaling which can localize to axons (Eto et al., 2009; Hirai et al., 2005) and is functionally required for regeneration after axotomy in *C. elegans* and mice (Hammarlund et al., 2009; Itoh et al., 2009; Yan et al., 2009). Interestingly, DLK is also required for Wallerian degeneration of the distal stump after injury (Miller et al., 2009). The dual function in degeneration as well as regeneration is best suggests that DLK may be acutely activated by axonal injury to mediate these various downstream injury responses.

In *Drosophila* we have previously found that the homologue of DLK, Wallenda (Wnd), regulates synaptic bouton growth and morphology at the larval NMJ, via a

nuclear signaling pathway that requires JNK and the transcription factor Fos (Collins et al., 2006). Wallenda and its homologue in *C. elegans* are regulated by a conserved E3 ubiquitin ligase, named Highwire (Hiw) in *Drosophila* (Collins et al., 2006; Nakata et al., 2005) Mutations in *hiw* lead to increased levels of Wnd protein in axons (Collins et al., 2006), and this mis-regulation of Wnd leads to increased branching and bouton growth at the motoneuron nerve terminus (Wan et al., 2000).

Here we test the hypothesis that Hiw and Wnd function to regulate an injury response pathway. For this, we developed an axon injury and regeneration assay in *Drosophila* motoneurons. Importantly, a downstream molecular reporter, whose induction coincides with the initiation of regeneration, allows us to dissect the steps required for neurons to mount a transcriptional response to injury. Our findings indicate that Wnd acts as a key upstream mediator of a nuclear signaling pathway, which is activated by axonal damage and promotes axonal sprouting. Furthermore, transport and destruction of Wnd in axons are important elements of a damage surveillance mechanism. By regulating the levels of Wnd in axons, the Hiw ubiquitin ligase plays a key role in regulating a retrograde injury signaling pathway.

2.3 Methods

Genetics

The following strains were used in this study: Canton-S (wild type), *puc*-lacZ^{E69}(Martín-Blanco et al., 1998), BG380-Gal4 (Budnik et al., 1996), OK6-Gal4 (Aberle et al., 2002), m12-Gal4 (P(GAL4)^{5053A}) (Ritzenthaler et al., 2000), RRa(eve)-

Gal4 (Fujioka et al., 2003), *hiw*^{DN} (Wu et al., 2005), *hiw*^{ND8} (Wan et al., 2000), *wnd*^{1}, *wnd*^{3}, and UAS-*wnd* from (Collins et al., 2006), UAS-Fos^{DN} (Fbz) and UAS-Jun^{DN} (Jbz) from (Eresh et al., 1997), slpr^{BS06} and UAS-*slpr(WT)* from (Polaski et al., 2006), UAS-Bsk(JNK)^{DN} (Weber et al., 2000), UAS-p150(*Glued*)^{DN}, 96B (Allen et al., 1999), UAS-GFP-Hiw (Wu et al., 2005), *sw*^{1} (Boylan and Hays, 2002), *khc*^{8} (Hurd and Saxton, 1996), *khc*^{27} (Brendza et al., 1999), *with a*^{3} (Aberle et al., 2002), and *with a*^{4} (Aberle et al., 2002), which was recombined with *puc*-lacZ^{E69}. UAS-GFP- *wnd*^{KD}, was generated directly from UAS-*wnd*^{KD} (Collins et al., 2006), which contains the K188A mutation in kinase domain. UAS-*wnd-RNAi* and UAS-*bsk-RNAi* were acquired from the Vienna RNAi center (Dietzl et al., 2007)

Table 2.1 Guide to genotypes used

	With *puc*-lacZ^{E69}	Without *puc*-lacZ^{E69}
WT	BG380-Gal4; *puc*-lacZ^{E69}/+	Canton S
wnd	------	*wnd1/wnd3*
wnd RNAi	BG380-Gal4, UAS-Dcr2; UAS-Wnd RNAi/ *puc*-lacZ^{E69}	BG380-Gal4, UAS-Dcr2; UAS-Wnd RNAi/+
Fos^{DN}	BG380-Gal4; UAS-Fos^{DN}/ *puc*-lacZ^{E69}	BG380-Gal4; UAS-Fos^{DN}/+
JNK^{DN}	BG380-Gal4; UAS-JNK^{DN}/*puc*-lacZ^{E69}	
Jun^{DN}	BG380-Gal4; UAS-Jun^{DN}/*puc*-lacZ^{E69}	
O/E wnd	BG380-Gal4; UAS-*wnd*/*puc*-lacZ^{E69}	BG380-Gal4; UAS-*wnd*
slpr	*slpr*^{BS06}*; puc*-lacZ^{E69}/+	
wit	*wit*^{HA3}*, puc*-lacZ^{E69}*/witA*^{HA4}	
O/E slpr	BG380-Gal4; UAS-*slpr/*+; *puc*-lacZ^{E69}/+	
Glued^{DN}	BG380-Gal4; UAS-Glued^{DN}/+; *puc*-lacZ^{E69}/+	BG380-Gal4; UAS-Glued^{DN}/+
OE wnd; Glued^{DN}	BG380-Gal4; UAS-Glued^{DN}/+;UAS-*wnd*/*puc*-lacZ^{E69}	BG380-Gal4; UAS-Glued^{DN}/+; UAS-*wnd*/+
hiw	*hiw*^{ND8}, BG380-Gal4; *puc*-lacZ^{E69}/+	*hiw*^{ND8}
hiw; wnd	*hiw*^{ND8}*; wnd-1/ puc*-lacZ^{E69}	*hiw*^{ND8}*; wnd*^{1}*/wnd*^{3}
hiw; Fos^{DN}	*hiw*^{ND8},BG380-Gal4; UAS-Fos^{DN}/ *puc*-lacZ^{E69}	*hiw*^{ND8},BG380-Gal4; UAS-

		Fos^{DN}/+
hiw; Glued^{DN}	*hiw^{ND8}*, BG380-Gal4; UAS-Glued^{DN}/+; *puc*-lacZ^{E69}/+	*hiw^{ND8}*, BG380-Gal4; UAS-Glued^{DN}/+

with m12-Gal4 driver	
WT	UAS-mCD8GFP/+; m12-Gal4/+
wnd RNAi	UAS-Dcr2; UAS-mCD8GFP/+; UAS-*wnd* RNAi/m12-Gal4
Fos^{DN}	UAS-mCD8GFP/+; UAS-Fos^{DN}/m12-Gal4
JNK^{DN}	UAS-mCD8GFP/+; UAS-JNK^{DN}/m12-Gal4
Glued^{DN}	UAS-Glued^{DN}/UAS-mCD8GFP; m12-Gal4/+
hiw	*hiw^{DN}*; UAS-mCD8GFP/+; m12-Gal4/+

Immunocytochemistry

Wandering 3rd instar larvae were dissected in PBS and fixed in either 4% paraformaldehyde in PBS, or Bouin's fixative, for 15-30 minutes, depending upon the antibodies used. Antibodies were used at the following dilutions in PBS with 5% normal goat serum: Rb anti-Wnd A1 (Collins et al., 2006) was used at 1:100, Rb anti-DVLGUT (Daniels et al., 2004) was used at 1:5,000, Rat anti-elaV (7E8A10, Developmental Studies Hybridoma Bank) at 1:50, ms anti-lacZ (40-1a, also from Developmental Studies) at 1:100 , Cy3-Gt anti-HRP (from Jackson labs) at 1:1000, A488-Rb anti-GFP (from Molecular Probes), ms anti-phospho-JNK (from Cell Signaling) at 1:1000. For secondary antibodies Cy3 and A488 conjugated Goat anti-Rb and anti-mouse (from Invitrogen) were used at 1:1000.

Imaging and Quantification

Confocal images were collected on an Improvision spinning disk confocal system, consisting of a Yokagawa Nipkow CSU10 scanner, and a Hamamatsu C9100-50 EMCCD camera. Similar settings were used to collect all compared genotypes. All quantitation was conducted with Volocity software.

To quantify the average intensity of *puc*-lacZ expression, the neuronal nuclei that lie along the dorsal midline of the nerve cord, in segments A3-A7, were selected based on staining for elaV. Due to an NLS sequence fused to lacZ, the reporter protein also localized to nuclei. The average lacZ intensity per nucleus was measured for at least 8 animals for each genotype, and normalized against analogous measurements in the control (wild type) genetic background. A similar strategy was used to quantify the intensity of staining for phosphorylated JNK in motoneuron nuclei for Figure 2.6.

The total intensity GFP-Wnd^{KD} (for Figure 2.12.D) was measured in 100 mm of each segmental nerve adjacent to the site of exit from the ventral nerve cord. To measure the density of GFP-Wnd^{KD} (Figure 2.12.E) and GFP-Hiw (Figure 2.13.B) particles in this area, we used Volocity software to select and count the number of particles above a threshold pixel intensity and size (Intensity > 2000, Size > 1mm^2 for GFP-Wnd^{KD}; Intensity > 2000, Size > 2.5mm^2 for GFP-Hiw).

Live imaging

Adapted from (Barkus et al., 2008), third instar larvae were dissected quickly in PBS, and then laid on microscope slides with the cuticle side against the glass

containing coverslip fragments as spacers. A 22 X 40 mm coverslip was then placed over the specimen and anchored at the corners with nail polish. The remaining space around the specimen was then filled with PBS. Dissection and imaging was performed at room temperature. Imaging was initiated within 10 minutes after dissection.

GFP-Wnd^{KD} particles in segmental nerves were imaged continuously with a frame collected every 500 milliseconds. To generate kymographs, the collection of single frames spanning one minute of imaging time was processed using the 'Multiple Kymograph' plug-in for Image J, submitted by J. Rietdorf and A. Steitz, EMBL, Heidelberg. A total of 342 anterograde and 270 retrograde segments were measured from 16 one-minute long kymographs.

Nerve crush and regeneration assay

The segmental nerves of early third instar larvae were visualized through the cuticle under a standard dissection stereomicroscope. While larvae were anesthetized with CO_2 gas, the segmental nerves were pinched tightly through the cuticle for 5 seconds with Drumostar number 5 forceps. The segment injured was varied as described in Figure 2.2.A, with injury site 1 at segment A2, injury site 2 at segment A4, and site 3 at A6. After successful injury, the posterior half of the larva was paralyzed. Larvae were then transferred to a grape plate and kept alive for varying periods of time at 25^{o}C.

To observe the regenerative response, we used the Gal4/UAS system to express mCD8-GFP in subsets of motoneurons, using either the m12-Gal4

(P(GAL4)^{5053A}) (Ritzenthaler et al., 2000), or RRa(eve)Gal4 (Fujioka et al., 2003) driver lines, each of which drive expression in only two fasciculated axons per segmental nerve. The segmental nerve contains a bundle of many motoneuron and sensory neuron axons, which cannot otherwise be distinguished from one another. To measure the regeneration ratio (For Figure 2.7. 2.10. and 2.15.), we defined a regenerating axon as an injured fiber that has more than 5 branches of at least 10 mm in length. The fraction of injured axons that were regenerating was counted for at least 10 animals per genotype, while blinded to the genotype.

Biochemistry

Adult flies of *hiw*^{DN} genotype were frozen in liquid nitrogen and heads were separated from the body through a brass sieve as described in (van de Goor et al., 1995). Extracts were generated by dounce homogenization in acetate buffer (10 mM Hepes pH 7.4, 5 mM EGTA, 100 mm potassium acetate, 3 mM magnesium acetate, 1 mM DTT) with protease inhibitor cocktail (Roche) and proteosome inhibitor (0.05M MG115, Sigma). Debris was then removed by centrifugation two times at 3,000g for 10 minutes at 4^{o}C to generate the PNS (post-nuclear supernatant).

The differential centrifugation steps are cartooned in Figure 2.9.A. The PNS was centrifuged at 10,000g, 4^{o}C for 10 minutes to generate S2 (supernatant 2) and P2 (pellet 2, which is predicted to contain 'heavy membranes' (Littleton et al., 1999)). The S2 fraction was then subjected to high-speed centrifugation, 115,000g for 1 hour in a TLA100 rotor to generate fractions S3 (supernatant 3) and P3 (pellet 3, which contains 'light membranes', small vesicles such as synaptic vesicles (Littleton et al., 1999)).

Equal amounts of each fraction were compared by Western blot.

For the sucrose flotation assay, S2 (supernatant from 10,000g spin), prepared as described above from approximately 4.5 mg of fly heads, was brought to a total volume of 500 ml in 1.375 M (40%) sucrose in acetate buffer. This comprised the bottom of sucrose step gradient, which was layered with 500 ml of 1.77M (35%), then 300 ml of 0.241 M (8%) sucrose, and centrifuged in a TLS55 rotor for 1.5 hours at 50,000 rpm at 4 ^{o}C. Equal amounts of each fraction are compared by Western blotting in Figure 2.9.C. This protocol is similar to that used in (Haghnia et al., 2007).

For detection of Wnd protein level in injured verses uninjured nerve cords (Figure 2.12.A, B), the whole brain and connected axons was carefully dissected from 3rd instar larvae. The two brain lobes were then removed, and the remaining ventral nerve cord (VNC) and connected axons were processed for Western Blotting (20 VNCs per lane).

For Western Blotting, Rb anti-Wnd A3-1, 2 (Collins et al., 2006) was used at 1:700, Rb anti APPL #952 (Luo et al., 1990)(from Kalpana White) was used at 1:500, ms anti a-tubulin (Developmental Studies) at 1:10,000, and ms anti-Fas II (Developmental Studies) at 1:500.

2.4 Results

A nerve crush injury assay in *Drosophila*

To study axon injury response in the *Drosophila* system, we established a nerve crush assay for larval segmental nerves (Figure 2.1.A, B and Materials and Methods), which contain motoneuron and sensory neuron axons. Because they run close to the

ventral midline, the segmental nerves can be visualized through the cuticle in early 3rd instar larvae under a standard dissection stereomicroscope. After anesthetization with CO_2, the nerves and surrounding cuticle are pinched tightly with number 5 forceps. The injury leads to paralysis in the posterior segments, however the animal is still able to feed and, remarkably, survives pupation and ecloses as a fully motile adult. Figure 2.1.D shows an example segmental nerve 24 hours after nerve crush. The nerve becomes stretched and thin, and vesicular cargoes accumulate at the crush site but do not pass through. Staining for MAP1B (Futsch) indicates a loss of microtubule structure at the crush site, and single neuron labeling indicates that individual axons are transected by the injury (Figure 2.1.C). Distal to the crush site, nerves and synaptic neuromuscular junctions degenerate within 48 hours after the injury (Figure 2.1.E).

Nuclear and cell body responses to the nerve crush injury

To study the mechanism of nuclear injury response we first needed to identify a reporter for cellular changes induced by injury. The JNK phosphatase *puckered*, whose expression can be reported via a lacZ enhancer trap (Martín-Blanco et al., 1998), is an attractive candidate, since it has been previously observed to be induced around sites of traumatic brain injury in *Drosophila* (Leyssen et al., 2005). *Puc*-lacZ is expressed at very low levels in uninjured neurons (Figure 2.2.B), however nerve crush induces a dramatic increase in *puc*-lacZ expression in injured motoneurons (Figure 2.2.C). The induction of *puc*-lacZ by injury is cell autonomous. This is shown by varying the location of injury (cartooned in Figure 2.2.A) to different segments in the animal, which induces *puc*-lacZ only in the motoneurons whose axons have been injured (Figure 2.2.C1-C3).

To quantify the change in *puc* promoter activity, the average intensity of lacZ staining was measured in the neurons that lie along the dorsal midline, most of which are motoneurons (Sanyal, 2009). When segmental nerves are crushed at injury site 1, *puc*-lacZ expression begins to increase within 8 hours after injury (at 25°C), and continues to increase linearly until pupation (Figure 2.2.D).

A well-documented response to injury in vertebrate neurons is a reduction in synaptic transmission (Navarro et al., 2007). In *Drosophila* motoneurons the vesicular glutamate transporter (DVGLUT) plays a critical role in neurotransmission (Daniels et al., 2006), and is robustly detected by antibody staining in motoneuron cell bodies ((Daniels et al., 2008). We found that injury induces a dramatic reduction in the cell body levels of DVGLUT (Figure 2.2.E).

Wallenda and downstream signaling via JNK and Fos mediates the nuclear response to axonal injury

With *puc*-lacZ and DVGLUT as downstream molecular responses to axonal injury, we could then use these markers as reporters to dissect the mechanism of injury signaling. We first tested the role of the *Drosophila* DLK homologue Wallenda (Wnd) in injury response, since DLK has recently been implicated in both regenerative and degenerative injury responses in other organisms (Hammarlund et al., 2009; Itoh et al., 2009; Miller et al., 2009; Yan et al., 2009).

Due to the chromosomal location of *wnd* (near the centromere), we were unable to recombine *wnd* mutations with the *puc*-lacZ reporter. We therefore used a transgenic

RNAi knockdown strategy to reduce *wnd* transcript (Dietzl et al., 2007). A UAS-*wnd*-RNAi transgene was co-expressed with *Dcr2* using the neuronal BG380-Gal4 driver line, which drives strong expression in motoneurons (Sanyal, 2009). This reduced the total levels of Wnd protein in whole brain extracts (Figure 2.5.A), and was sufficient to recapitulate other *loss-of-function* phenotypes for *wnd* in motoneurons, such as disruption of axonal transport (Horiuchi et al., 2007)(Figure 2.5.B) and suppression of synaptic overgrowth caused by mutation in *hiw* (Collins et al., 2006), (Figure 2.5.C). Expression of *wnd*-RNAi completely inhibited the induction of *puc*-lacZ by injury (Figure 2.3.B and Figure 2.4.A). We note that *wnd-RNAi* also resulted in a slight reduction in basal *puc*-lacZ expression in uninjured animals, however the reduction in injury-induced *puc*-lacZ expression was much more dramatic (Figure 2.3.B). In addition, wnd^{1}/+ heterozygotes partially inhibited the effect of injury upon *puc*-lacZ (Figure 2.5.D). In wnd^{1}/wnd^{3} loss-of-function mutants, the effect of injury upon DVGLUT was abolished (Figure 2.3.B and Figure 2.4.C). Conversely, over-expression of *wnd* in uninjured neurons causes a dramatic increase in *puc*-lacZ and decrease in DVGLUT (Figure 2.3.D and Figure 2.4.B), mimicking the effect of the nerve crush. We conclude that Wnd is a critical upstream regulator of the cellular responses to injury.

Because Wnd was previously found to promote synaptic overgrowth via JNK and Fos (Collins et al., 2006), we then tested the requirement of JNK and Fos in the injury signaling mechanism. Expression, using BG380-Gal4, of dominant negative transgenes JNK^{DN} and Fos^{DN} inhibited the injury-induced effects upon both *puc*-lacZ and DVGLUT (Figure 2.3.C and Figure 2.4.A). In contrast, Jun^{DN} had no effect (Figure 2.4.A), which parallels previous observations that Fos^{DN} but not Jun^{DN} inhibits synaptic overgrowth

(Collins et al., 2006).

Two controls further address the specificity of Wnd's role and effect. First the other mixed lineage kinase family member in *Drosophila*, Slpr, has no effect upon *puc*-lacZ in neurons either when it is mutant or over-expressed (Figure 2.4.B), despite the fact that Slpr regulates *puckered* in epithelial cells during dorsal closure (Stronach and Perrimon, 2002). Second, mutations in BMP signaling, which also affect NMJ morphology and development via a nuclear signaling pathway (Keshishian and Kim, 2004; Marques and Zhang, 2006), do not affect the *puc*-lacZ induction by injury (Figure 2.4.B). Furthermore, injury has no effect upon levels of nuclear phospho-MAD (data not shown).

The *puc*-lacZ reporter is regulated by JNK signaling in other developmental contexts (Bosch et al., 2005; Dobens et al., 2001; Galko and Krasnow, 2004; McEwen and Peifer, 2005; Stronach and Perrimon, 2002). To address directly whether JNK is activated by axon injury and whether this activation requires Wnd, we probed larvae with an antibody against phosphorylated JNK (p-JNK). In uninjured animals, p-JNK is prominent in the neuropil of the larval ventral nerve cord (Figure 2.6.A). We verified that this staining is specific for JNK, since transgenic RNAi-knockdown of the *Drosophila* JNK homologue, *bsk,* in neurons abolishes the p-JNK staining (Figure 2.6.B). Dramatically, nerve crush induces the appearance of p-JNK in injured motoneuron cell bodies (Figure 2.6.A). Similarly to *puc*-lacZ, p-JNK appears only in the cell bodies of injured neurons. However its accumulation is more transient, beginning around 6 hours (for injury site 1), peaking around 12 hours, and decreasing thereafter, concomitant with the induction of the *puckered* phosphatase (Figure 2.2.D). Importantly, p-JNK did not

appear in the cell body in *wnd* mutants after injury (Figure 2.6.C, D), which indicates that Wnd is required for JNK activation (or localization) in injured neurons. Because *wnd* mutants did not have reduced p-JNK staining in the neuropil (Figure 2.6.C) or segmental nerves (not shown), we infer that Wnd is only one of the regulators of JNK in axons. However Wnd is a critical upstream regulator of JNK in response to axonal injury.

The Wallenda pathway is required for axon regeneration after injury

What is the physiological role of the nuclear and cell body response to injury? An attractive model is that this injury-signaling pathway induces a regenerative response in the injured neuron. To test whether larval motoneuron axons regenerate after injury, we used the Gal4/UAS system to label single motoneuron axons within each segmental nerve by expression of UAS-mCD8-GFP. Two different driver lines were used for this purpose. 'm12'-Gal4 (Ritzenthaler et al., 2000) drives strong expression in only two axons per segmental nerve, which are tightly fasciculated with one another. RRa(eve)-Gal4 (Fujioka et al., 2003) drives specific expression, albeit more weakly, in RP2 and aCC motoneurons in 3rd instar larvae.

With either subset of labeled motoneurons, extensive branching is observed from the proximal stump within 14 hours of injury (Figure 2.7.A). Within 24 hours, branches can be longer than 100 mm (Figure 2.7.D). Because the lesion is a great distance (>1 mm) from the target muscles, and the time before pupation is limited (< 3 days), we do not observe functional reconnection of the injured axons their targets. Likewise, reconnection to the distal stump also does not occur since the distal stump degenerates. Nonetheless, the axonal branching represents new axonal growth in response to injury,

which can be considered as an attempted regenerative response. To quantify this response, we counted the number of injured nerves that showed more than 5 branches of at least 10 mm in length at the injury site (arrows in Figure 2.7.A), while blinded to genotype. Because only two axons per nerve are labeled by each Gal4 driver, the new branches must have arisen from new remodeling/growth of these injured axons. By this criteria, 70-80% of the injured axons (in a wild type genetic background) show signs of regenerative growth within 14 hours after injury (Figure 2.7.C, E).

To test whether the Wnd/JNK/Fos signaling pathway is required for this regenerative response, we used the strong m12-Gal4 driver to expresss *wnd*-RNAi, JNK^{DN}, or Fos^{DN} in the labeled motoneurons (Figure 2.7.B). We find that disruption of each component of the pathway significantly inhibits the formation of new axonal branches at the injury site (Figure 2.7.B, C).

Because we were unable to isolate recombinants between m12-Gal4 and *wnd*, we used the RRa-Gal4 driver to label single axons in segmental nerves in a wnd^{1}/wnd^{2} mutant background. We find that regeneration is dramatically inhibited when *wnd* is mutant (Figure 2.7.D, E). These observations indicate that the Wnd pathway plays an important role in the regenerative response to injury. We note that the formation of branches at the injury site bears similarities to the induction of *puc*-lacZ in the requirement for JNK and Fos. The timing of the branch formation, which begins to appear about 8 hours after injury, is also similar to the timing of *puc*-lacZ induction (Figure 2.2.D). We conclude that the nuclear response to injury, which is mediated by Wnd signaling, induces new axonal growth/branching of the injured neuron.

Wallenda protein is transported in Axons

Since Wnd is an upstream mediator of an injury response pathway, an attractive hypothesis is that Wnd is locally activated in axons. Consistent with this hypothesis, the vertebrate homologue of Wnd, DLK, can be detected in axons, growth cones, and synapses (Eto et al., 2009; Hirai et al., 2005; Mata et al., 1996). We therefore tested whether Wnd localizes in axons in *Drosophila*.

Endogenous Wnd is barely detectable by immunocytochemistry in a wild type background (Figure 2.8.A). However, when axonal transport was inhibited by over-expressing a truncated form of the dynactin subunit p150/Glued (Glued^{DN}) in motoneurons (Allen et al., 1999), cargo for axonal transport accumulate in axonal swellings (Martin et al., 1999), and Wnd protein can be detected in these swellings (Figure 2.8.A). No such axonal accumulations are observed when transport is disrupted in a *wnd*^{3} mutant (Figure 2.8.A), when the Wnd antibody epitope is absent (Collins et al., 2006). Also consistent with transport in axons, Wnd protein accumulates at both sides of the site of nerve crush within two hours of axonal injury (Figure 2.8.B), similarly to other cargo for axonal transport (Barkus et al., 2008; Horiuchi et al., 2005). To assay directly the transport of Wnd protein, we used the Gal4/UAS system to drive expression of a UAS-GFP-*wnd*^{KD} transgene exclusively in motoneurons using OK6-Gal4 (Sanyal, 2009). The GFP-*wnd*^{KD} transgene contains a mutation in the kinase domain ('kinase-dead'), which allows the protein to be expressed at detectable levels for live imaging without causing lethality. Importantly, the GFP-*wnd*^{KD} transgenic protein can be visualized in live dissected 3rd-instar larvae by rapid time-lapse imaging. Figure 2.8.C shows a single frame from a representative

movie (Supplemental Movie) and the kymograph generated from a single axon tract. The GFP-Wnd^{KD} protein localizes to discrete puncta, many of which move anterogradely and/or retrogradely, with average segment velocities of 0.83 +/-0.02 mm/sec (n = 342), and 0.62 +/-0.02 mm/sec (n = 271) respectively. These velocities are comparable to other cargo for axonal transport machinery in *Drosophila* axons (Barkus et al., 2008; Haghnia et al., 2007; Miller et al., 2005).

The localization of Wnd to discrete motile particles suggests that Wnd associates with vesicles. Wnd and its homologues in vertebrates have no trans-membrane domains and are predicted to be cytoplasmic proteins. However, biochemical characterization of DLK from mouse brain homogenates indicates that a significant fraction of the protein co-fractionates with membranes (Mata et al., 1996). Differential centrifugation and sucrose floatation assays from *Drosophila* head extracts indicate that Wnd behaves biochemically like a membrane-associated protein (Figure 2.9.). We conclude that Wnd is transported in axons while associated with vesicles.

Injury signaling and regenerative response via Wnd requires axonal transport machinery

If the axonal localization of Wnd is functionally relevant for the injury signaling mechanism, a prediction is that mutations that disrupt axonal transport machinery would inhibit both the nuclear and the downstream regenerative responses to injury. We disrupted axonal transport using mutations in either the minus-end directed motor dynein (Figure 2.11.D) or the dynactin complex (Figure 2.10. and Figure 2.11.A-C), which plays a critical role in dynein cargo binding (Schroer, 2004). Dynactin was

disrupted by expressing a dominant negative truncated subunit p150/Glued (Glued^{DN})(Allen et al., 1999) in only the relevant motoneurons. This resulted in a strong inhibition to the regenerative response (Figure 2.10.). However this was not particularly informative alone, because axonal transport may also be required for steps downstream of the nuclear injury signal, such as the transport of new material into the regenerating axon. The *puc*-lacZ reporter allowed us to directly test the requirement for dynactin and dynein in injury signaling independent of downstream events. Both Glued^{DN} (Figure 2.11.A, C) and *sw^{1}* (Figure 2.11.D) mutations cause a near complete block to the induction of *puc*-lacZ by injury. The cell body/nuclear accumulation of phospho-JNK is also inhibited (data not shown). We conclude that dynein and dynactin are required for the transduction of the injury signal to the nucleus.

Interestingly, the requirement for dynactin in injury signaling cannot be simply bypassed by over-expressing *wnd* in neurons. That is, while over-expression of *wnd* is sufficient to activate *puc*-lacZ and down-regulate DVGLUT (Figure 2.3.D), it is not sufficient when axonal transport is inhibited by Glued^{DN} (Figure 2.11.B, C). We interpret that localization of Wnd alone to the cell body is not enough to induce the signaling pathway. Rather, Wnd may need to be transported into axons in order to become activated or to encounter a necessary co-factor or substrate. Because mutations in components of axonal transport machinery inhibit both plus and minus end directed transport in *Drosophil*a motoneurons (Allen et al., 1999; Barkus et al., 2008; Haghnia et al., 2007)(and data not shown), it cannot be distinguished whether anterograde, retrograde, or bidirectional transport is required for the signaling. Furthermore, additional cellular processes may be disrupted by the Glued^{DN} and *sw^{1}* mutations (Levy

and Holzbaur, 2006). Nonetheless, these finding imply that axonal localization and transport is an important element of Wnd's function in regulating an axon-to-nucleus signaling cascade.

Injury regulates Wnd protein turnover

The above observations suggest that injury signaling involves local activation of Wnd in axons. Previous studies in vertebrate cells suggest that DLK protein is activated via dimerization (Nihalani et al., 2000). Since over-expression of *wnd* alone can activate downstream signaling (Figure 2.3.D), a potential mechanism of activation is to increase local levels of Wnd so that it can dimerize and self-activate. Previous studies suggest that Wnd protein levels are tightly regulated by protein turnover (Collins et al., 2006; Nakata et al., 2005; Wu et al., 2007). The Hiw E3 ubiquitin ligase plays an important role in this regulation, since mutations in *hiw* lead to increased levels of Wnd protein in axons (Collins et al., 2006). We therefore tested whether axonal injury induces changes in Wnd protein levels.

Ventral nerve cords (VNCs) and connected segmental nerves from uninjured or injured larvae were micro-dissected and processed for Western blotting at different time points after injury. Intriguingly, we observed an 80% increase in Wnd protein within 4 hours of injury (Figure 2.12.A, B). This short time frame (much shorter than the time required for *puc*-lacZ induction) suggests that increased Wnd levels may be an earlier event in the injury signaling mechanism.

To test whether the effect of injury upon Wnd protein level is post-transcriptional,

we used the Gal4/UAS system drive expression of GFP-Wnd^{KD} ectopically in motoneurons. Because expression from the OK6-Gal4 driver is not affected by injury (data not shown), any changes to GFP-Wnd^{KD} should be post-transcriptional, reflecting altered localization, translation, or protein turnover. We find that injury induces a dramatic increase in the amount of GFP-Wnd^{KD} in axons. This can be detected both in axonal segments within the ventral nerve cord (arrows in Figure 2.12.C), and in the segmental nerve (Figure 2.12.C bottom panel), where a greater than 3 fold increase in average intensity (Figure 2.12.D) and particle density (Figure 2.12.E) was observed 4 hours after injury. In contrast, we measured no significant change in the intensity of GFP-Wnd^{KD} in cell bodies after injury (Figure 2.12.F, $p > 0.45$). Because the total levels of Wnd increase after injury (Figure 2.12.A, B), and because transgenic Wnd levels also increase in axons, we hypothesize that Wnd levels increase via an alteration in stability or protein turnover.

An attractive model is that injury induces Wnd by inhibiting its regulation by Hiw. We therefore investigated whether injury altered the levels or localization of Hiw protein. UAS-GFP-*hiw* was expressed in motoneurons using the OK6-Gal4 driver. This transgenic protein, which is capable of rescuing the *hiw* mutant phenotype of synaptic overgrowth (Wu et al., 2005), localizes to both axons and cell bodies (Figure 2.13.A). While injury does not induce significant changes in the levels of GFP-Hiw in cell bodies (Figure 2.13.C, $p > 0.2$), it induces a clear reduction in axonal GFP-Hiw (Figure 2.13.A, B). While the localization of endogenous Hiw is not known, we suspect that the axonal Hiw is a functionally relevant pool, since mutations in *hiw* lead to increased levels of Wnd in axons and because the vertebrate homologue Phr1 localizes in axons of

cultured neurons (Lewcock et al., 2007). Because injury leads to a reduction in this axonal GFP-Hiw, we propose that injury induces activation of Wnd by down-regulating Hiw, which allows Wnd protein levels to increase in axons.

Highwire negatively regulates a retrograde injury response pathway

Because Hiw negatively regulates Wnd protein level (Collins et al., 2006), we hypothesized that the injury signaling pathway would be constitutively activated when *hiw* is absent. Two observations support this model.

First, when *hiw* is mutant, *puc*-lacZ is increased >5 fold above basal levels, while DVGLUT expression is dramatically decreased in motoneuron cell bodies (Figure 2.14.A, B), resembling neurons that have been injured (Figure 2.2.). These effects can be suppressed by removing a single copy of *wnd,* (Figure 2.14.A, B). The induction of *puc*-lacZ by mutation of *hiw* also requires Fos and Glued (Figure 2.14.B). We conclude that when *hiw* is mutant, the retrograde injury signaling pathway is ectopically active.

Second, when *hiw* is mutant, injured neurons require less time to initiate a regenerative response (Figure 2.15.). When wild type animals are injured by nerve crush, new axonal branches are detected around 10-12 hours after injury. This coincides with the timing of the nuclear signaling pathway measured by *puc*-lacZ induction (Figure 2.2.D). However in *hiw* mutants, neurons exhibit new branching and large lamellodia-like structures within 6 hours after injury (Figure 2.15.). This acceleration of about 5 hours resembles the time required for phospho-JNK to appear in

cell bodies after injury. This time could be bypassed in the *hiw* mutant because the injury signaling pathway is constitutively active. Of note, mutants in the homologue of *hiw* in *C. elegans, rpm-1,* also cause enhanced regeneration (Hammarlund et al., 2009). So this role in regulating an injury response pathway is likely an evolutionarily conserved function for the Hiw/Rpm-1/Phr1 E3 ubiquitin ligase.

2.5 Discussion

In this study we describe an axonal injury and regeneration assay in *Drosophila* larval motoneurons, which creates a powerful paradigm for studying the molecular events and requirements for axonal regeneration of defined neurons within an intact living animal. Importantly, a molecular reporter (the JNK phosphatase *puckered*), whose expression is induced by axonal injury in a cell autonomous fashion allows us to dissect the steps required for neurons to mount a transcriptional response to injury. We use this new assay to reach several insights into the function of a conserved MAPKKK, Wnd/DLK, and its mechanism of activation during injury signaling and regeneration.

First, we show that Wnd functions as an upstream regulator of a nuclear signaling pathway that is activated by axonal injury. Second, we show that activation of this signaling pathway promotes axonal growth and branching of the injured neuron. Third, we demonstrate that Wnd is transported in axons, peripherally associated with membrane-bound vesicles, and the injury signaling mechanism requires functional axonal transport machinery. Fourth, we find that injury signaling is regulated by the Hiw E3 ubiquitin ligase, and that protein turnover of Wnd in axons may be a surveillance mechanism for detecting axonal damage. Injury induces a rapid increase in Wnd protein,

concomitant with a decrease in Hiw protein. In *hiw* mutants, the nuclear response to injury is constitutively active, and neurons require less time to initiate a regenerative response to injury. We conclude that the regulation of Wnd by Hiw in axons comprises an important mechanism for detecting and responding to axonal injury.

The Wnd/DLK kinase regulates a regenerative response to axonal injury

Our findings indicate that an important component of Wnd/DLK's role in regeneration is to regulate a retrograde signaling pathway, linking injury, which activates the Wnd pathway in axons, to a downstream transcriptional response in the nucleus. In the vertebrate nervous system, not all neurons are capable of regenerating after axonal injury. Conditioning lesion studies in DRG neurons suggest that the capacity to regenerate in the CNS is linked to the ability to mount a cellular and transcriptional response to injury (Hannila and Filbin, 2008; Hoffman, 2010). DLK is therefore an attractive candidate regulator of injury signaling in PNS axons. It will be interesting to determine the function of DLK in different neuronal types.

The role for Wnd in activating a nuclear injury response does not rule out additional functions for Wnd in the axon. Recent studies suggest in *C. elegans* suggests that DLK regulates translation in axons (Yan et al., 2009), which is important for both injury signaling and regeneration (Gumy et al., 2010; Hanz et al., 2003; Yudin et al., 2008). Wnd/DLK may also regulate local cytoskeletal changes, since JNK signaling is known to regulate microtubules in axons (Barnat et al., 2010; Bogoyevitch and Kobe, 2006; Gelderblom et al., 2004; Stone et al., 2010). An attractive hypothesis, which is consistent with growth cone phenotypes for vertebrate homologues of *hiw* (Hendricks

and Jesuthasan, 2009; Lewcock et al., 2007), is that Wnd signaling locally alters cytoskeleton in order to form or modify growth cones after injury. In addition to these roles in regeneration, we should also consider the involvement of JNK in cell death and degeneration (Johnson and Nakamura, 2007). It is possible that that the activation of Wnd by injury could have negative consequences in some scenarios.

Mechanism of activation of axonal injury signaling

1. Wnd is transported in axons

Wnd is transported in axons, in the form of particles which move at a speed similar to other cargoes for fast axonal transport. Because endogenous Wnd protein co-fractionates with membranes, this cytoplasmic kinase may associate peripherally with vesicles. An interesting future pursuit is to determine the molecular nature of these vesicles. Studies in the vertebrate sciatic nerve demonstrate that the JNK scaffolding protein JIP3/Sunday Driver associates with a large, multivesicular organelles that travels retrogradely in response to injury (Abe et al., 2009; Cavalli et al., 2005). Since vertebrate DLK also associates with a membrane compartment (Mata et al., 1996), it is possible that DLK associates with a retrograde signaling cargo or a precursor to such cargo. Future tools are needed to follow the transport and associations of kinase-active and endogenous Wnd after injury.

2. Wnd is activated in axons

Previous studies of the role of DLK in regeneration in *C. elegans* (Hammarlund et al., 2009; Yan et al., 2009), and in degeneration in cultured DRG neurons (Miller et al.,

2009), suggest that DLK functions acutely at the time of injury. The most attractive model for both observations is that DLK/Wnd is locally activated in axons by injury to mediate different responses in different contexts.

Our findings strongly support this model. Both endogenous and GFP-tagged Wnd localize to axons, and functional axonal transport machinery is required for transduction of the injury signaling. Even if Wnd is ectopically over-expressed, when presumably some protein could localize to the cell body, the downstream response requires functional axonal transport machinery. We interpret that the axonal localization and transport of Wnd is functionally relevant for its signal transduction mechanism. It may need to be localized in axons to become activated, or to encounter a necessary co-factor or substrate.

3. Injury regulates protein turnover of Wnd

Injury leads to an increase in the total levels of endogeneous Wnd protein. Furthermore, the levels of transgenically expressed GFP-Wnd^{KD} are also increased, particularly in axons. Since the OK6-Gal4 driver is not affected by injury, the increase in GFP-Wnd^{KD} must take place post-transcriptionally, either as increased protein synthesis or decreased protein turnover. Several observations favor the model that injury activates Wnd by inhibiting its turnover in axons. First, the GFP-Wnd^{KD} transgene lacks 5' and 3' UTRs, which usually function in regulation of translation. Second, Wnd and its DLK homologue in *C. elegans* are down-regulated by the Hiw E3 ubiquitin ligase, (Collins et al., 2006; Nakata et al., 2005), which decreases in axons after injury. Third, regulation of the levels of Wnd is a viable mechanism for regulating its activation because over-expression of Wnd is sufficient to activate this signaling pathway.

Our data suggest that down-regulation of Hiw could be part of the injury signaling mechanism. However, further studies are required to understand the mechanism of Hiw regulation after axon injury. Intriguingly, a recent study suggests that Hiw is regulated by autophagy (Shen and Ganetzky, 2009), which could potentially be induced by axonal injury.

Rescue studies indicate that Hiw function is required throughout the larval stage to down-regulate Wnd (Wu et al., 2005), hence Wnd is constantly made, transported and destroyed in axons. It has previously been perplexing that despite the amount of energy required to regulate this signaling pathway, there was not an obvious function of Wnd in synaptic development or function. We propose that the regulation of Wnd by Hiw in axons could be part of a damage surveillance mechanism for the cell to detect and respond to axonal injury.

Connections between injury response, synaptic growth, and synaptic maintenance

Previous studies in *Drosophila* suggest that Hiw functions to regulate nerve terminal growth at the NMJ. The 5-fold increase in number of pre-synaptic boutons in *hiw* mutants, is one of the most dramatic phenotypes described at the larval NMJ, and is due to the mis-regulation of Wnd protein. This synaptic overgrowth may simply be the outcome of a mis-regulated regenerative response within an intact, uninjured circuit.

However, it is also interesting to consider the possibility that the Wnd signaling pathway, which is constantly regulated by Hiw in axons, normally performs other

functions. An injury, which disrupts an axon's connections with its target, may share some similarity with other insults that affect the functional connections at the synapse. Along this line, a recent study found that Hiw-regulated signaling can counteract synaptic retraction caused by loss of the spectrin cytoskeleton, and that loss of spectrin induces expression of *puckered* (Massaro et al., 2009), While the role of Wnd in this remains to be addressed, an intriguing possibility is that loss of synaptic adhesion induces the injury signaling/regeneration pathway. This could then counteract the loss of synaptic contacts by promoting more growth. It is of great interest to understand in more detail the mechanism by which this molecular pathway is regulated, and the downstream consequences of its activation. Our data, combined with others in *Drosophila* and other model organisms, point to Hiw and Wnd as important upstream regulators of a therapeutically interesting cellular response in neurons.

2.6 Figures

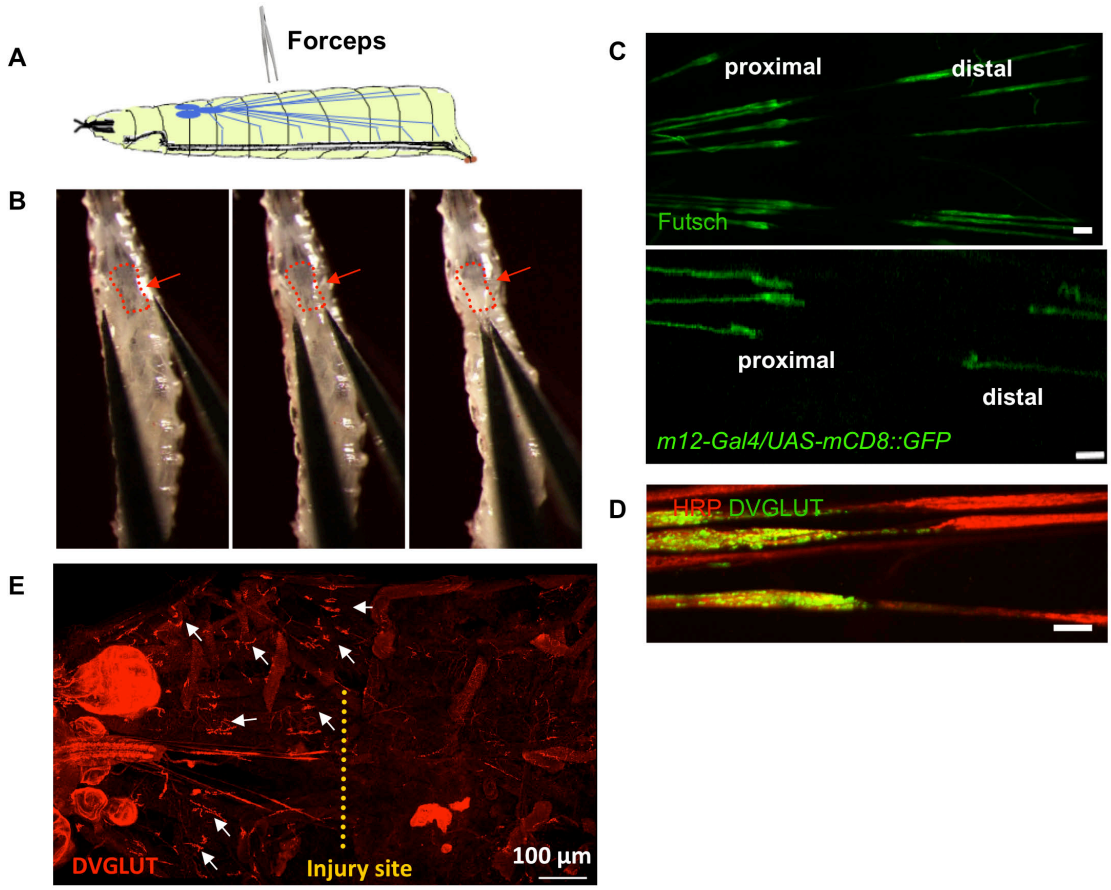
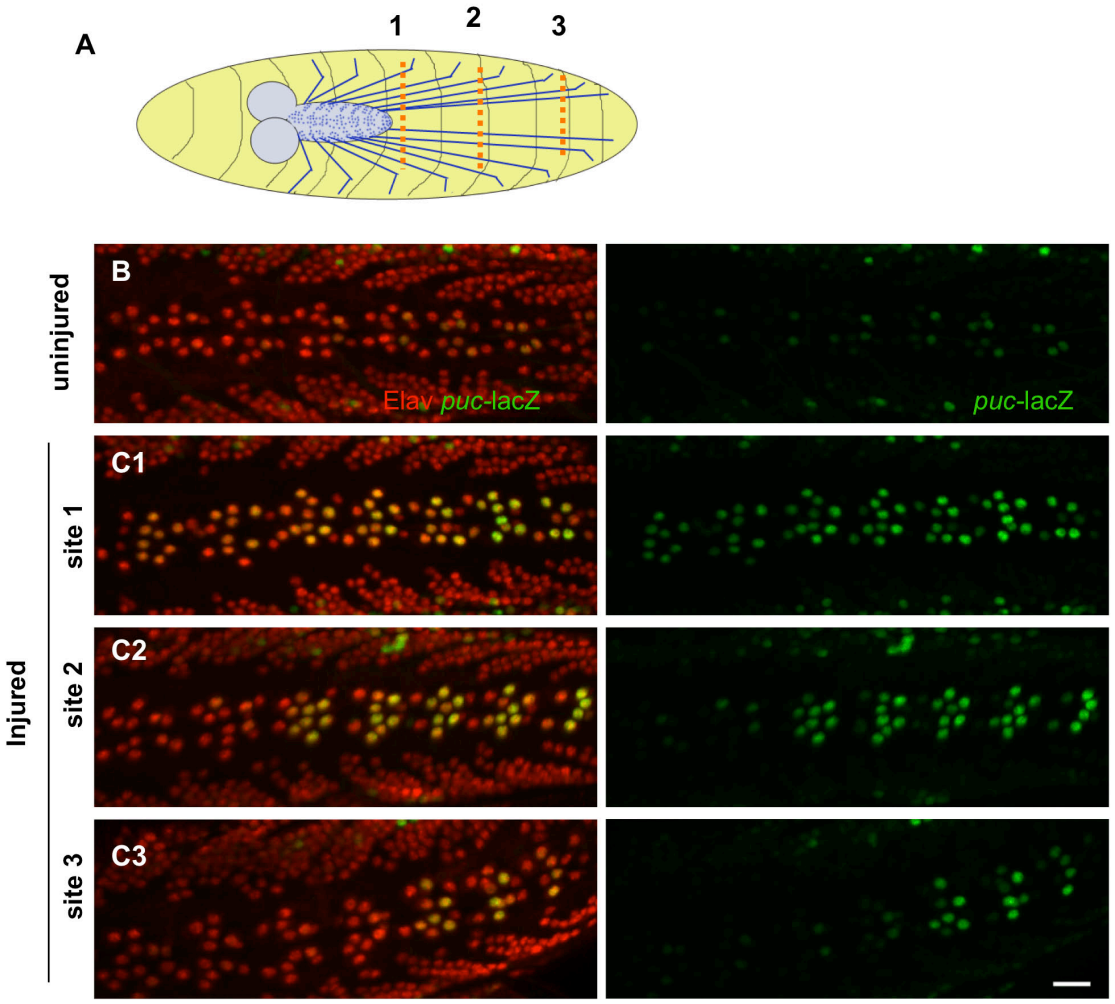
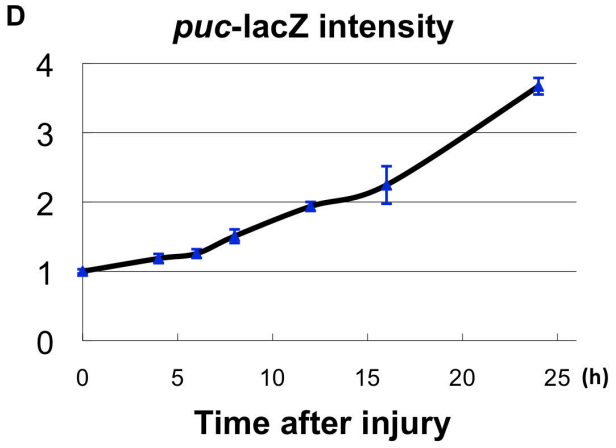


Figure 2.1 The nerve crush assay

(A) Schematic of the nerve crush assay. The segmental nerves of an early 3rd instar larva are crushed by pinching the ventral cuticle with forceps.
(B) Frames from a video of the nerve crush assay, viewed from the ventral side of the early 3rd instar larva, using injury site 1. The brain and nerve cord are highlighted by red dotted lines and arrow.
(C) The Futsch (MAP1B) labeled microtubule structure in segmental nerves is transected at the site of crush (white rectangle). Single axons labeled by m12-Gal4; UAS-mCD8-GFP become discontinuous after injury. Scale bar = 25 µm
(D) Injured segmental nerves 24 hours after nerve crush. Synaptic vesicle precursors detected by staining for DVGLUT staining (green) accumulate at the proximal side of the crush site (blue arrow).
(E) The entire larva is filleted and stained with DVGLUT antibodies 48 hours after nerve crush at injury site 2 (Figure 2.2.A). By this time, DVGLUT labeled synapses are completely absent from NMJs distal to the crush site. White arrows point to example NMJs anterior to injury site, which do not degenerate.
Scale bar = 100 µm


A
1
2
3
B
uninjured
Elav puc-lacZ
puc-lacZ
Injured
C1
site 1
C2
site 2
C3
site 3


D
puc-lacZ intensity
4
3
2
1
0
0
5
10
15
20
25 (h)
Time after injury

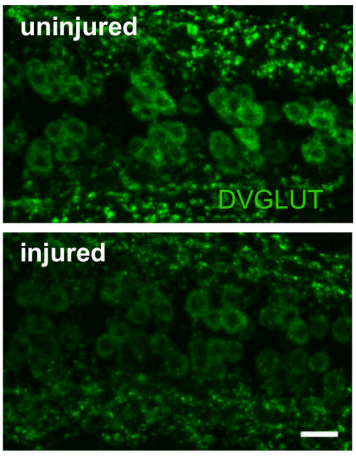

E
uninjured
DVGLUT
injured

Figure 2.2 Axon injury induces transcriptional changes in the motoneuron cell body

(A) Cartoon of neuron cell bodies (blue dots) and segmental nerves (blue lines). Different crush sites (dashed red lines) injure a predictable number of motoneurons. Crush site 1 injures more cells than crush site 2 and 3.
(B) In uninjured animals, *puc*-lacZ expression is barely detectable. A nuclear localization signal on lacZ (green) localizes the reporter to the nucleus, and neuronal nuclei are detected by staining for the ElaV (red) marker.
(C1-3) Injury induces *puc*-lacZ expression. 24 hours (at 25C) after injury at sites 1-3 induces expression of *puc*-lacZ in a defined subset of motoneurons as predicted by the anatomy cartooned in panel (A).
(D) Time course quantitation of *puc*-lacZ. The average intensity of *puc*-lacZ is measured as described in Materials and Methods for the dorsal midline neurons. 24 hours after injury, *puc*-lacZ intensity is increased 3.5 fold compared uninjured animals (n > 15).
(E) Axon injury leads to a decrease in DVGLUT protein in motoneuron cell bodies. Scale bars = 25μm

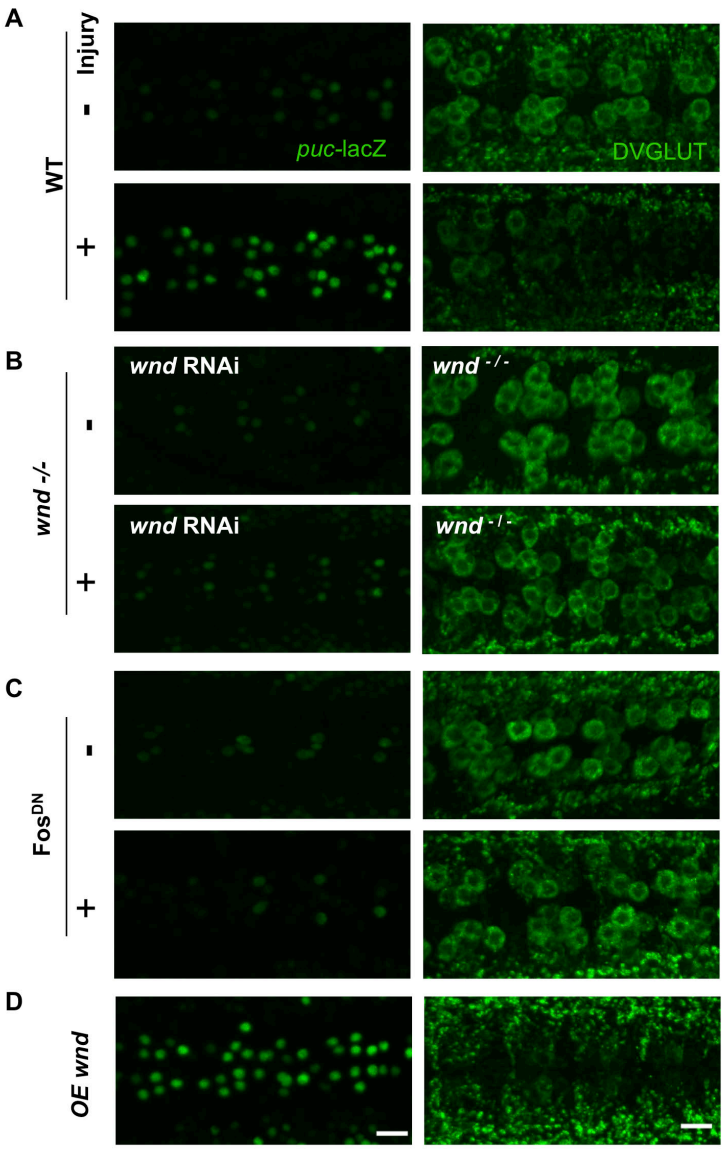


Figure 2.3 The nuclear and cell body response to axonal injury specifically requires Wallenda and downstream signaling components.

(A-D) *puc*-lacZ expression (left) and staining for DVGLUT (right) in ventral nerve cords uninjured and 24 hours after injury.
(A) The nerve crush injury induces an increase in *puc*-lacZ expression (left) and decrease in staining for DVGLUT (right).
(B) The response to injury requires Wnd function. No obvious change in *puc*-lacZ (left) and DVGLUT (right) is observed after injury when Wnd is disrupted.
(C) The response to injury is inhibited by Fos^{DN}.
(D) Overexpression of *wnd* in neurons is sufficient to activate the injury response, including induction of *puc*-lacZ and reduction in DVGLUT staining.
Scale bars = 25μm

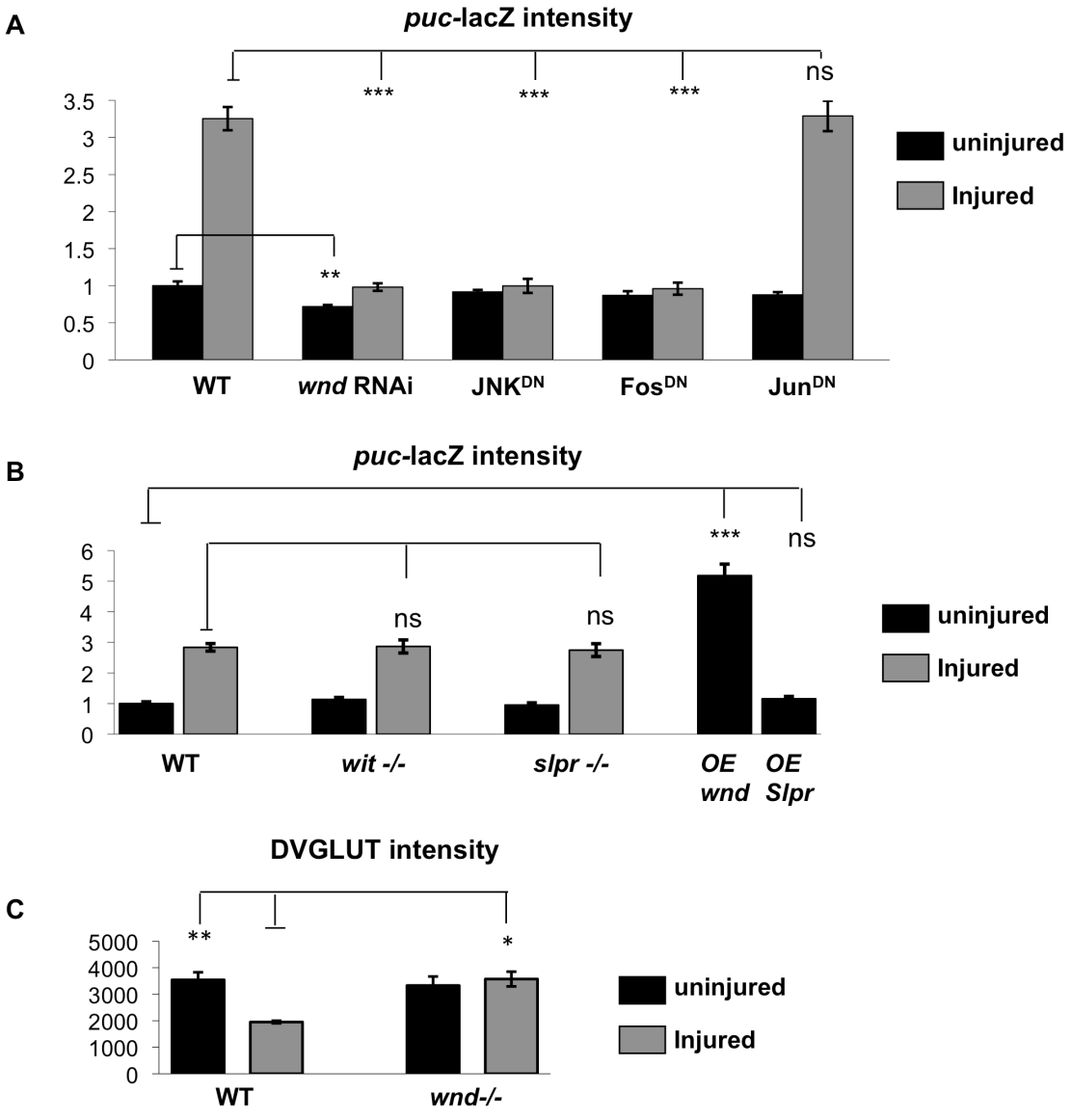


Figure 2.4 Quantifications of *puc*-lacZ and DVGLUT intensity in motoneurons

(A-B) Quantification of the *puc*-lacZ expression level, before (gray bars) or 24 hours after injury (black bars) in different genotypes.
(C) Quantation of DVGLUT intensity in the cell body of injured and uninjured wild type or wnd^{1}/wnd^{3} mutant larvae.
ns: $p > 0.05$, * $p < 0.05$, **$p<0.001$, ***$p<0.0001$

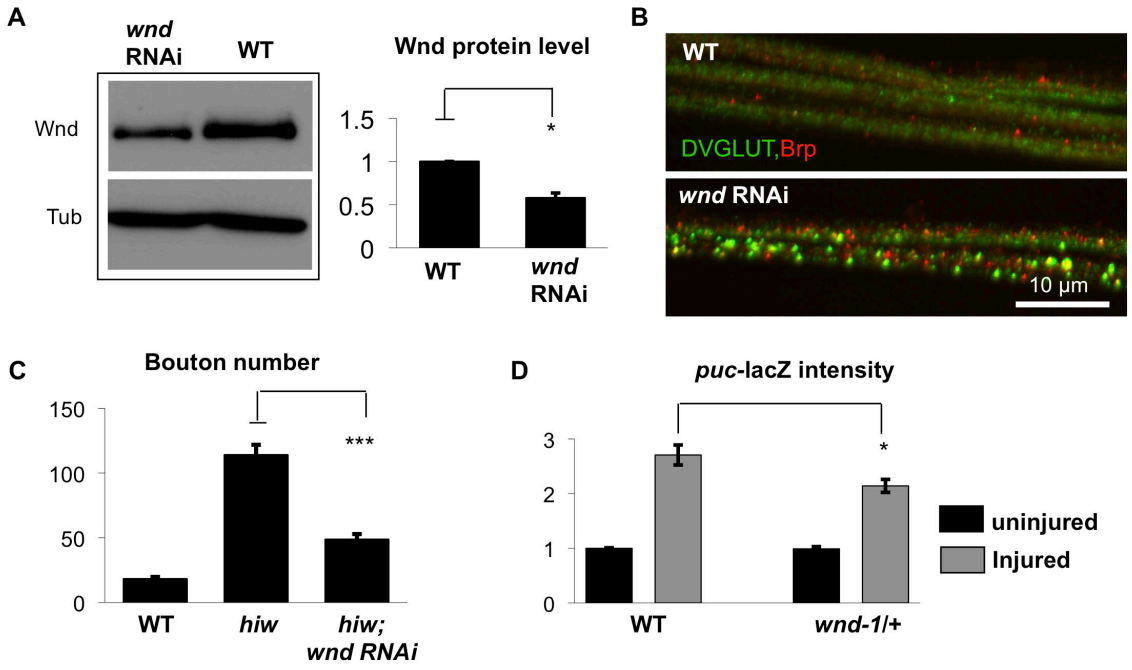


Figure 2.5 Further evidence that Wnd is required for the injury response

(A) Western blot of extracts from whole brains of WT and *wnd*-RNAi 3rd instar larvae (UAS-*wnd*-RNAi is expressed using the BG380-Gal4 driver).
(B) Expression of *wnd*-RNAi causes an axonal transport defect, similar to as described for *wnd loss-of-function* mutations in (Horiuchi et al., 2007). Synaptic markers DVGLUT (green) and Brp (red) in accumulate in axonal jams within segmental nerves.
(C) Expression of *wnd*-RNAi suppresses the synaptic overgrowth phenotype of *hiw* mutants, as in (Collins et al., 2006).
(D) wnd^{1}/+ heterozygous mutations cause a partial reduction in the *puc*-lacZ induction by injury.

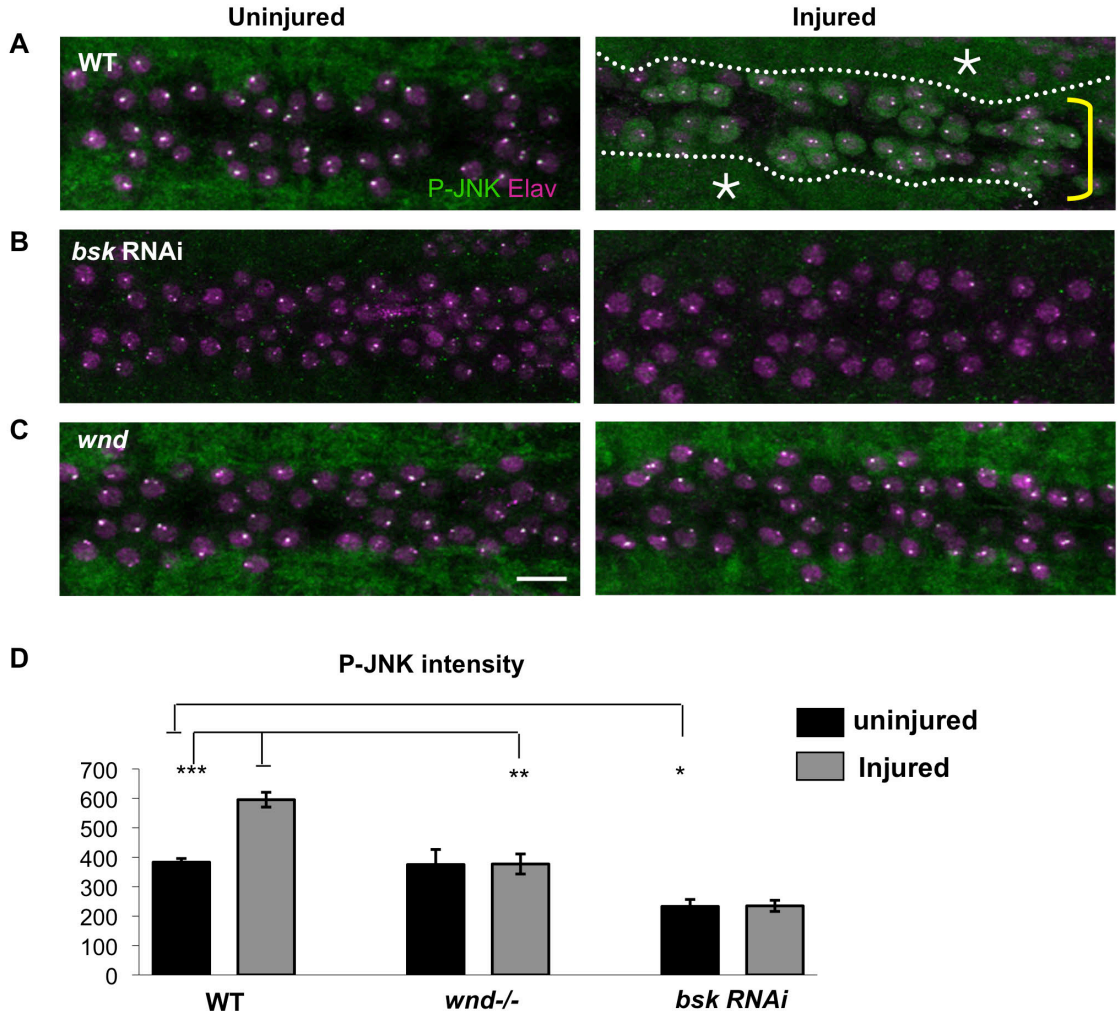


Figure 2.6 Injury induces phospho-JNK accumulation in the cell bodies and nuclei of injured neurons.

(A-C) Ventral nerve cords are co-stained for p-JNK (green) the nuclear marker Elav (magenta). In both uninjured and injured animals, p-JNK stains the neuropil, which can be seen both above and below the cell bodies (asterisks and white dotted lines in A).
(A) p-JNK appears in motoneuron cell bodies within 12 hours after injury (yellow bracket highlights cell bodies).
(B) This p-JNK staining is abolished by expression of *bsk*-RNAi (Dietzl et al., 2007) in neurons.
(C) Mutations in wnd (wnd^{1}/wnd^{3}) inhibit the cell body accumulation of p-JNK after injury.
(D) Quantification of p-JNK intensity in the cell body before (gray bars) and 12 hours after injury (black bars).
Scale bar=25μm * $p<0.05$, ** $p<0.001$, *** $p<0.0001$. The bright dots in the nuclei are fixation artifacts.

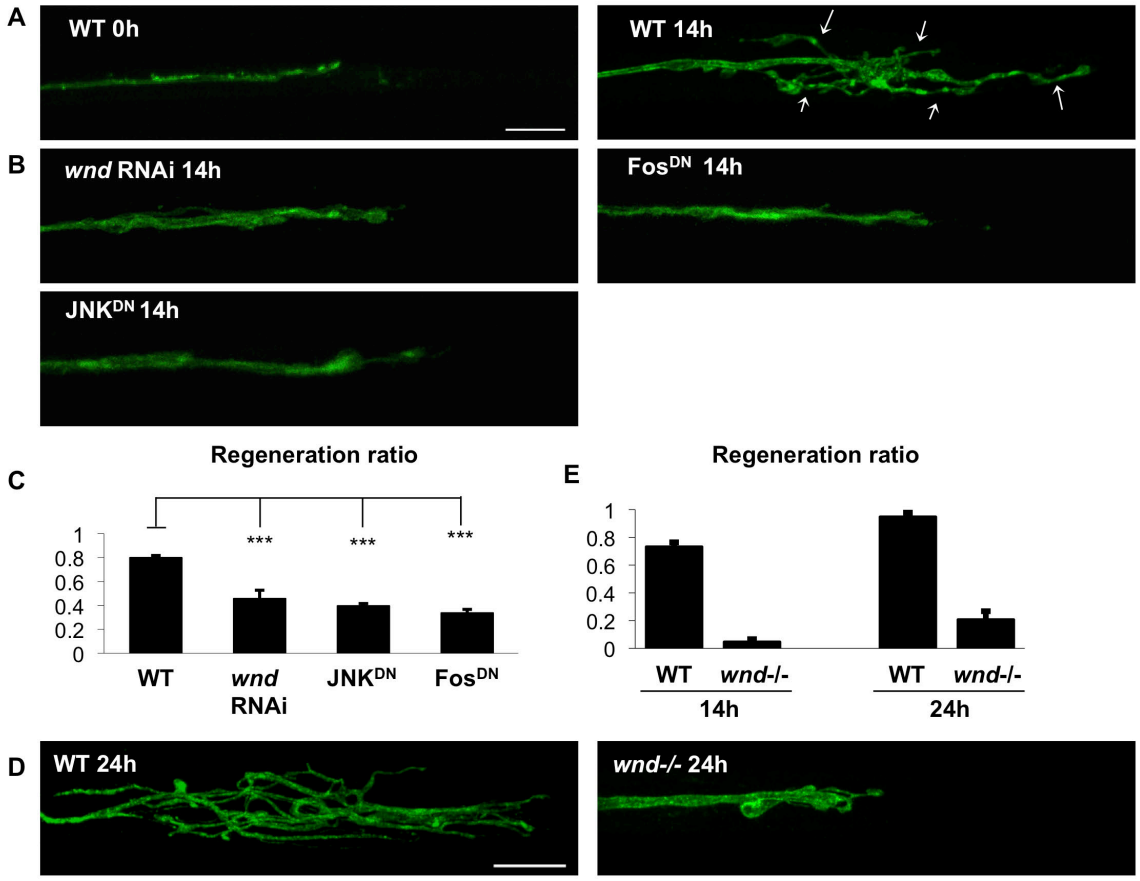


Figure 2.7 Wallenda/JNK/Fos injury signaling is required for new axonal growth after injury.

Axons are labeled by driving expression of UAS-mCD8-GFP by m12-Gal4 in (A-C), and by RRa(eve)-Gal4 in (D,E).
(A) In wild type (WT) animals, the proximal stump forms extensive new branches (white arrows) by 14 hours after injury.
(B) Sprouting after injury is inhibited by expression of *wnd*-RNAi, JNK^{DN} or Fos^{DN} in the GFP-labeled neurons.
(C) Quantification of regeneration ratio (as described in Materials and Methods) for different genotypes, 14 hours after injury.
(D) At 24 hours after injury, branches from the proximal stump are even longer in the WT background, however this sprouting remains strongly inhibited in the wnd^{1}/ wnd^{2} mutant background.
(E) Quantification of the regeneration ratio for WT and $wnd^{1/2}$ at 14 and 24 hours after injury.
Scale bar=25μm, **p<0.001, ***p<0.0001

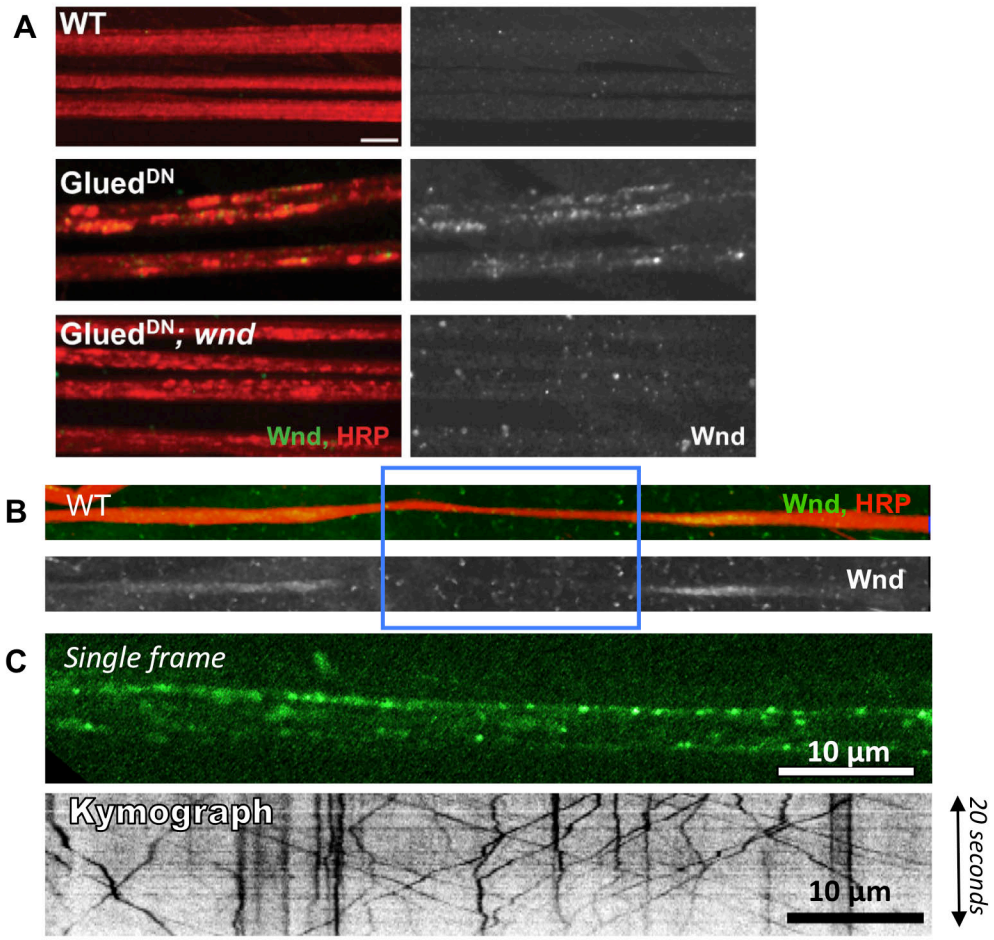


Figure 2.8 Wallenda protein is transported in axons

(A) Wnd protein accumulates in segmental nerves when axonal transport is disrupted. Segmental nerves from 3rd instar larvae are stained for axonal membrane (anti-HRP) in red, Wnd (anti-Wnd) in green. The right panels show Wnd staining alone.
(B) Wnd protein (green in upper panel and alone in lower panel) accumulates at the injury site (blue rectangle).
(C) Live imaging indicates that Wnd particles rapidly translocate both anterogradely and retrogradely in axons. (See also the Supplementary Movie). The GFP-*wnd*^{KD} transgene was expressed in motoneurons using the OK6-Gal4 driver. The top panel shows a single 0.5 second exposure. The bottom panel shows a representative kymograph from a single axon.
Scale bars = 10µm. (Figure 2.8.A and 2.8.B were prepared by Dr.Catherine Collins, Figure 2.8.C was prepared by Xin Wang).

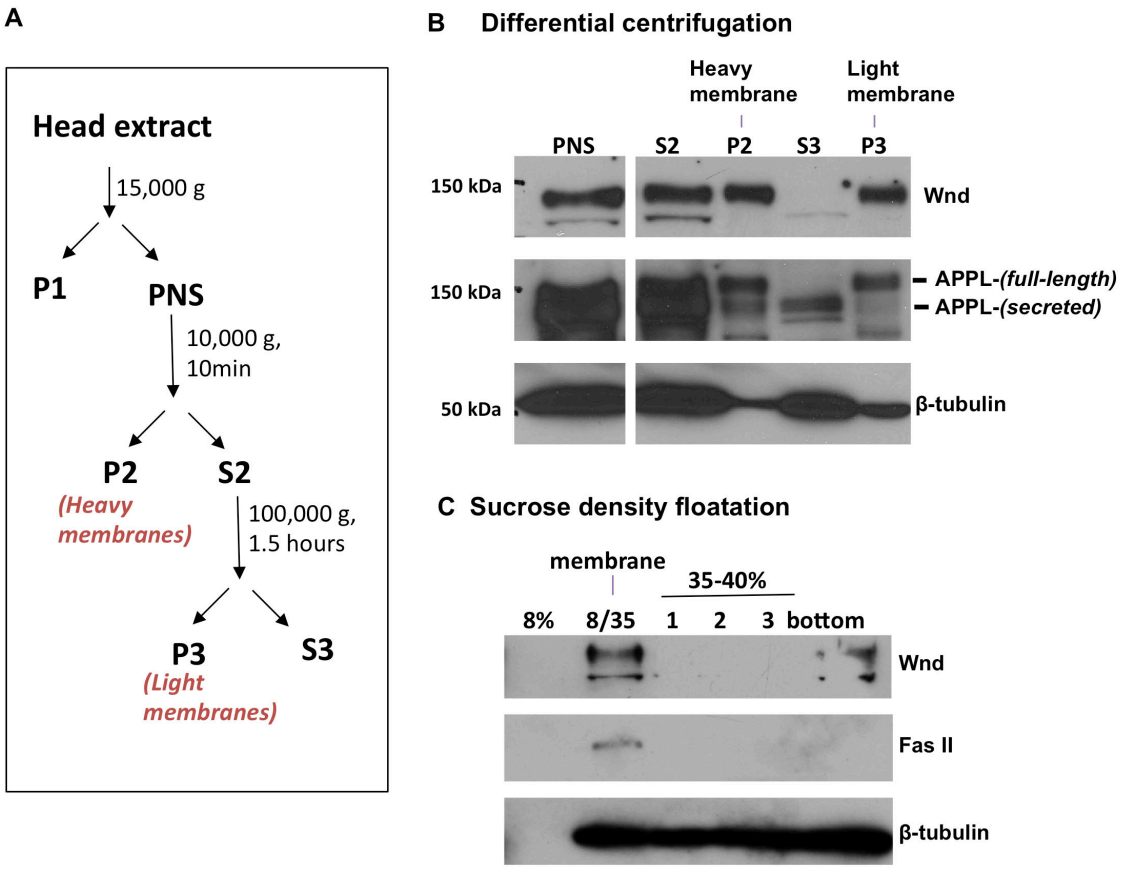


Figure 2.9 Wnd protein associates with membrane. (Figure 2.9 was prepared by Ronny Ewanek)

(A) Cartoon of the differential centrifugation protocol, which is described in detail in Materials and Methods.
(B) Differential centrifugation indicates that Wnd protein associates with heavy particles. The majority of Wnd protein partitions equally to the light (P3) and heavy (P2) membrane fractions, while controls, α-tubulin and the secreted form of APPL, partition to the soluble (S3) fraction.
(C) A sucrose floatation assay indicates that Wnd protein co-fractionates with membranes. Equal amounts of each fraction from a sucrose step gradient are compared by Western Blotting. Wnd protein partitioned to the 8/35 interface along with other membrane-associated proteins such as Fas II, and to the bottom of the gradient.

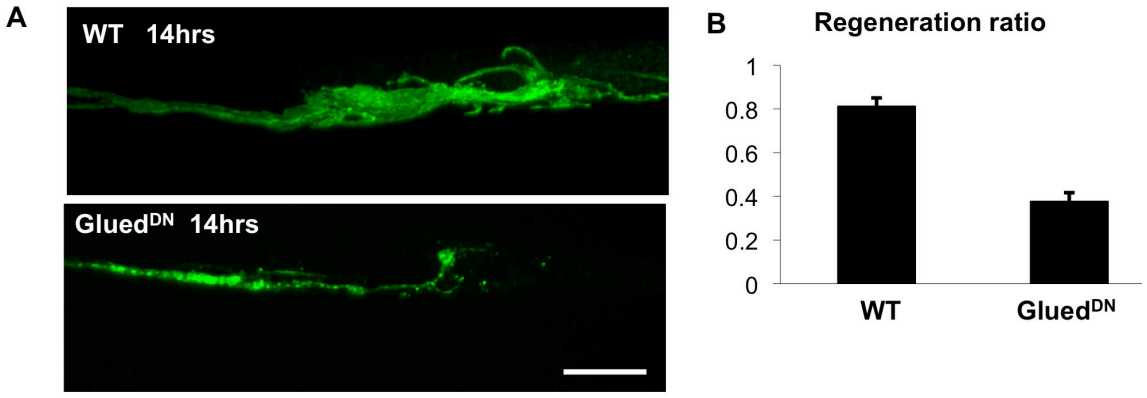


Figure 2.10 Axonal transport is required for axon regeneration.

(A) Inhibition of axonal transport inhibits regeneration after injury. Expression of the p150-Glued dynactin subunit, UAS-Glued^{DN} , inhibits the formation of new branches at the injury site.
(B) Quantiation of the regeneration ratio, similarly to in Figure 2.7.
Scale bar=25μm

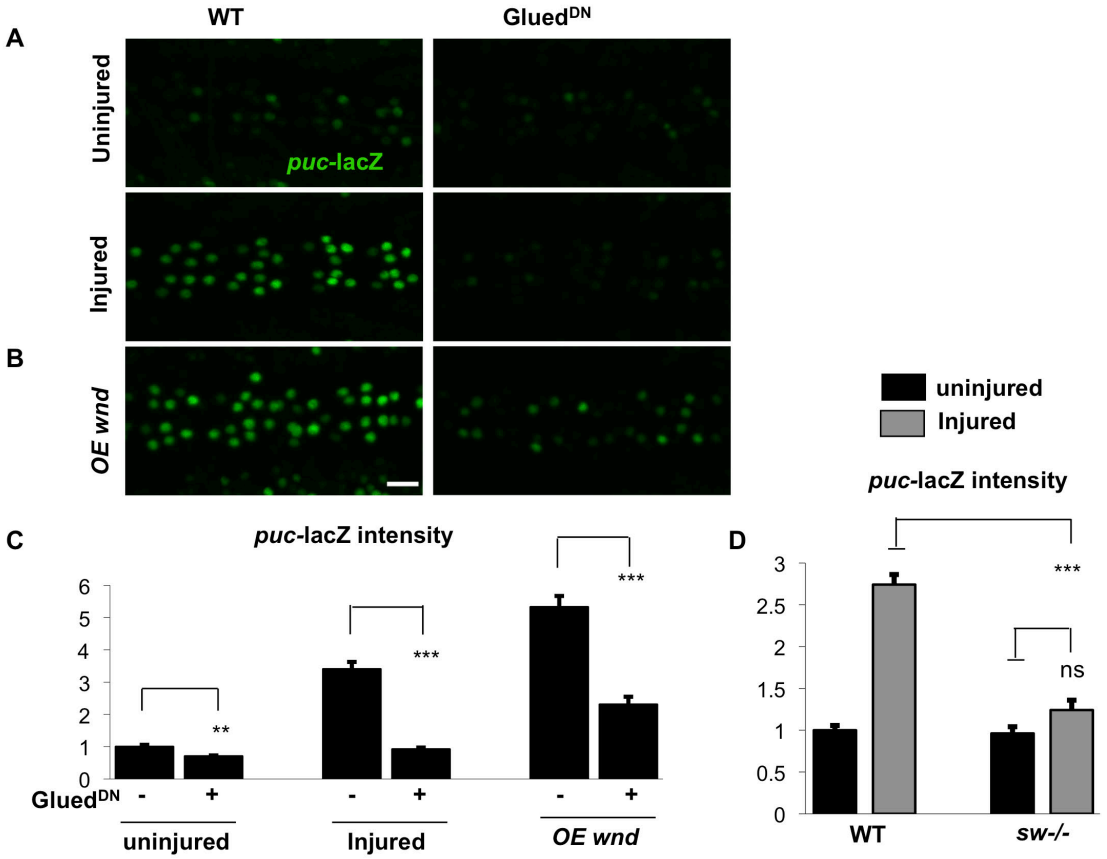


Figure 2.11 Axonal transport is required for injury and Wnd-induced nuclear signaling.

(A) Inhibition of axonal transport inhibits the effect of axon injury on *puc*-lacZ expression. (B) Ectopic Wnd signaling also requires axonal transport. The induction of *puc*-lacZ by over-expression of *wnd* is suppressed by co-expression of GluedDN.
(C) Quantification of *puc*-lacZ intensities for genotypes described in A and B.
(D) The *sw*^{1} allele of the dynein intermediate chain subunit is temperature sensitive (Boylan and Hays, 2002). Animals were raised at the non-permissive temperature (30^{o}C) for 4 days in order to detect a strong inhibition to transport by live imaging. The average intensity of *puc*-lacZ was quantified in midline neurons of injured and uninjured wild type or *sw*^{1} mutant larvae.
Scale bar=25μm, ns: p >0.05, **p<0.001, ***p<0.0001

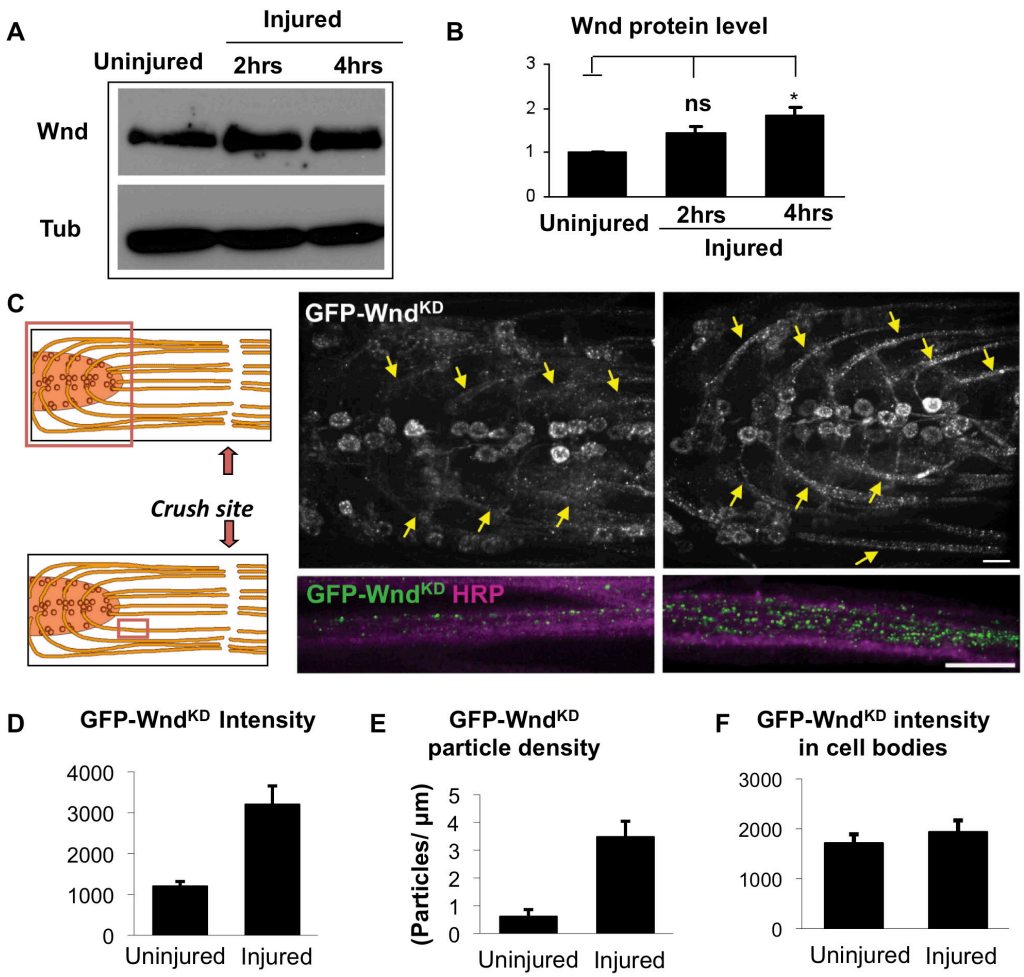


Figure 2.12 Injury induces an increase in Wnd protein in axons

(A) Western blot of extracts from uninjured and injured nerve cords to detect endogenous Wnd protein level (20 VNCs per lane).
(B) Quantitation of changes in Wnd protein level based on Wnd:Tubulin ratio from five independent experiments.
(C) GFP-Wnd ^{KD} particles in nerve cords and segmental nerves before and 4 hours after injury. UAS-*GFP-wnd* ^{KD} is expressed in motoneurons by OK6-Gal4. The cartoon shows the anatomy of nerve cord and segmental nerves with blue rectangle indicating the sites that are shown in upper and bottom panels. Upper panel: Nerve cords of uninjured and injured animals. Injury induces dramatic increase of GFP-Wnd ^{KD} intensity in the axons projecting from motoneurons (yellow arrows) Bottom panel: Segmental nerves proximal to injury site.
(D-E) After injury, the segmental nerves contain a higher average intensity (D) and density (E) of GFP-Wnd^{KD} particles (the quantification method is described in Methods).
(F) The average intensity of GFP-Wnd^{KD} in the cell bodies for segments A3-A7 in injured (4 hours) and uninjured animals is not significantly different.
Scale bar=25µm, ns: $p>0.05$, * $p<0.05$

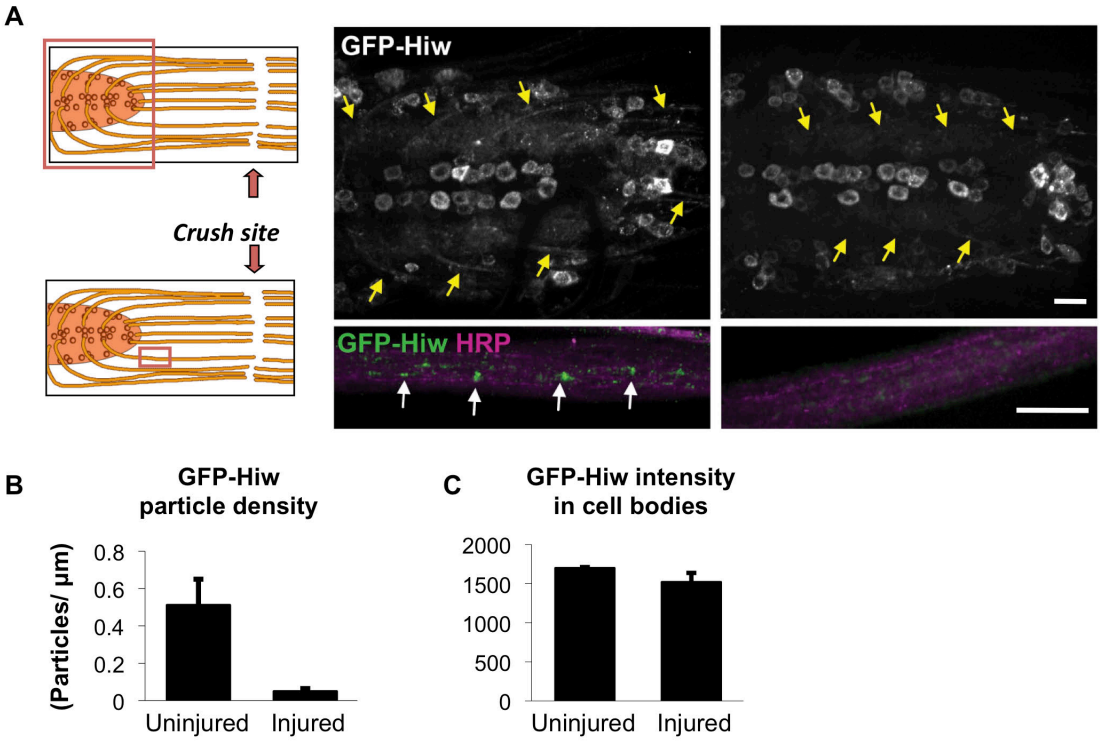


Figure 2.13 Injury induces a decrease in Hiw protein in axons

(A) GFP-Hiw (driven by OK6-Gal4) in nerve cords and segmental nerves in uninjured and injured (4 hours) animals. Upper panel: Injury induces overall decrease of GFP-Hiw in indicated axons (yellow arrows). Bottom panel: GFP-Hiw is present in puncta in uninjured axons, while most these puncta are no longer present 4 hours after injury.
(B) Measurement of GFP-Hiw particle density in segmental nerves for injured (4 hours) and uninjured animals.
(C) Measurement of average GFP-Hiw intensity in the cell bodies for segments A3-A7 in injured (4 hours) and uninjured animals.
Scale bar=25µm,the quantification method is described in Methods.

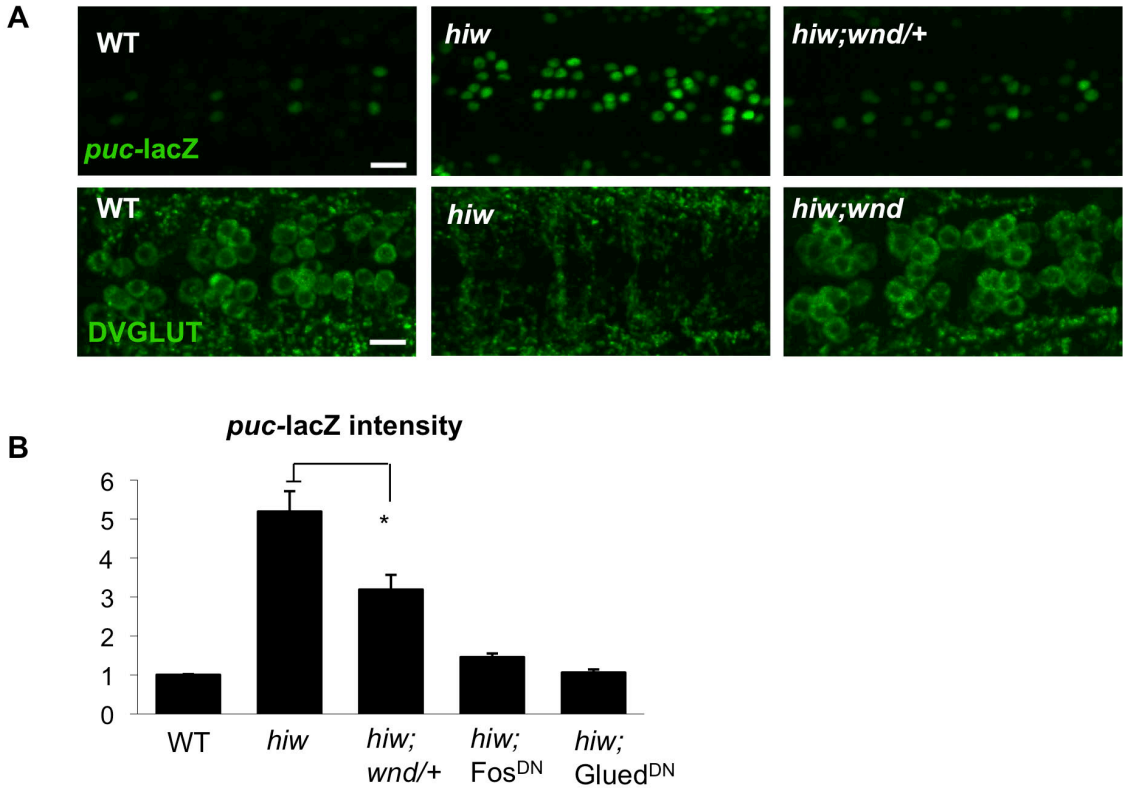


Figure 2.14 The Hiw E3 ubiquitin ligase negatively regulates injury signaling

(A) *hiw*^{DN} mutant shows a dramatic increase of *puc-lacZ* and decrease of DVGLUT intensity in motoneuron cell bodies, and these changes require *wnd* function.
(B) Quantitation of *puc*-lacZ intensity for genotypes in (A), as well as when Fos and Glued are inhibited by expressing UAS-Fos^{DN} or UAS-Glued^{DN} in the *hiw*^{DN}*; puc-lacZ* mutant background. These results suggest that hiw regulates a retrograde signaling pathway.
Scale bar=25μm

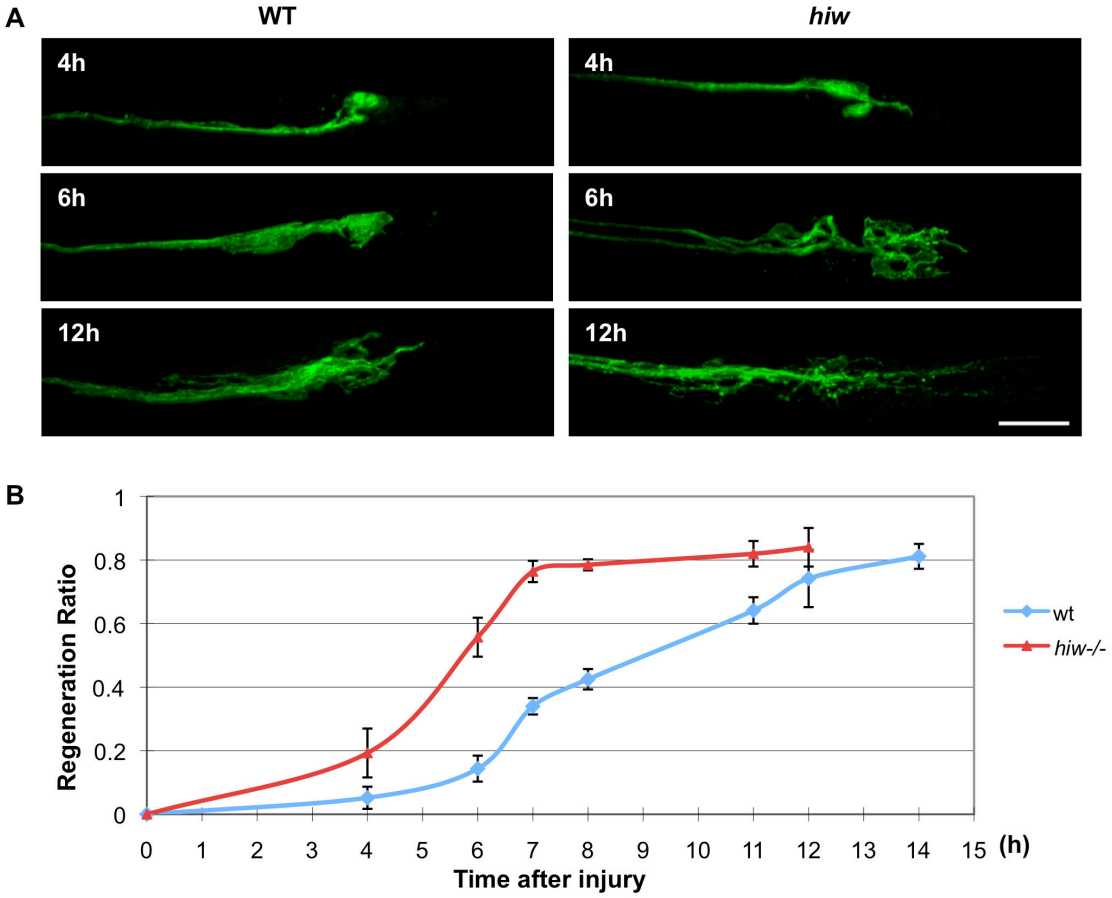


Figure 2.15 Axon regeneration is accelerated in *hiw* mutant

(A) Examples of the proximal stump morphology at different time points after injury. In hiw^{DN} mutants, most injured axons form long new branches within 6 hours injury. In contrast, sprouting is not readily apparent in wild type axons until 12 hours after injury.
(B) Time-course of regeneration ratio comparing wild type (blue) and hiw^{DN} (red) mutants.
Scale bar=25μm

CHAPTER 3

A CONDITIONING LESION PROTECTS AXONS FROM DEGENERATION VIA THE WALLENDA/DLK MAP KINASE SIGNALING CASCADE

3.1 Abstract

Axons are vulnerable components of neuronal circuitry, and neurons are equipped with mechanisms for responding to axonal injury. A highly studied example of this is the conditioning lesion, in which neurons that have been previously injured have an increased ability to initiate new axonal growth(Hoffman, 2010). Here we investigate the effect of a conditioning lesion upon axonal degeneration, which occurs in the distal stump after injury, and also occurs in neuropathies and neurodegenerative disorders (Coleman, 2005). We find that *Drosophila* motoneuron axons which have been previously injured have an increased resiliency to degeneration. This requires the function of a conserved axonal kinase, Wallenda (Wnd)/DLK, and a downstream transcription factor. Because axonal injury leads to acute activation of Wnd (Xiong et al., 2010), and over-expression studies indicate that increased Wnd function is sufficient to promote protection from degeneration, we propose that Wnd regulates an adaptive response to injury which allows neurons to cope with axonal stress.

3.2 Introduction

When the continuity of an axon is disrupted, the distal 'stump' degenerates through a regulated self-destruction process called Wallerian degeneration, whose cellular mechanism is poorly understood. Recent studies suggest that a conserved dileucine zipper kinase, DLK, plays a role in this degeneration process (Ghosh et al., 2011; Miller et al., 2009). However, this kinase has also been found to promote regenerative growth of injured axons (Ghosh-Roy et al., 2010; Hammarlund et al., 2009; Itoh et al., 2009; Xiong et al., 2010; Yan et al., 2009).

To further understand the relationship of these pro-degenerative and pro-regenerative functions, we have investigated the role of the *Drosophila* homologue of DLK, *Wallenda* (Wnd) in Wallerian degeneration of *Drosophila* motoneurons and synapses. We have previously described how Wnd, which is localized in axons, becomes activated after axonal injury and regulates a nuclear signaling cascade whose action promotes new axonal growth in the proximal stumps of injured motoneuron axons (Xiong et al., 2010). Here we describe the role of Wnd in the distal stump of these same neurons.

Contrasting to previous observations in other neurons (Ghosh et al., 2011; Miller et al., 2009), we find that Wnd does not promote degeneration of *Drosophila* motoneuron axons. Instead, Wnd acts as an inhibitor for Wallerian degeneration of these neurons. Because Wnd becomes acutely upregulated in axons after injury (Xiong et al., 2010), we hypothesized that Wnd promotes an adaptive response in these neurons that is protective against further damage. Using a conditioning lesion assay, we found that motoneuron axons which have been previously injured have an increased

resilience to degeneration, and this requires the cell-autonomous function of Wnd and the transcription factor Fos. These findings implicate conserved axonal signaling molecules in a mechanism that controls remarkable plasticity in the axonal degeneration process.

3.3 Methods

Genetics

The following strains were used in this study: Canton-S (wild type), BG380-Gal4 (Budnik et al., 1996), m12-Gal4 (P(GAL4)^{5053A}) (Ritzenthaler et al., 2000),*wnd*^{1}, *wnd*^{2}, and UAS-*wnd* from (Collins et al., 2006), UAS-*Fos*^{DN} (Fbz) from (Eresh et al., 1997), UAS-*Bsk(JNK)*^{DN} (Weber et al., 2000), UAS-*Wld*^{s} (Hoopfer et al., 2006). UAS-*Dcr2* was gift from Stephan Thor, and UAS-*wnd-RNAi* was acquired from the Vienna RNAi center (Construct ID 13786) (Dietzl et al., 2007). Male larvae were utilized for all experiments using the BG380-Gal4 driver. For other experiments larvae of both sexes were used.

Immunocytochemistry

Larvae were dissected in PBS and fixed in either 4% paraformaldehyde in PBS, or Bouin's fixative, for 15-30 minutes, depending upon the antibodies used. Antibodies were used at the following dilutions in PBS with 0.3% TritonX-100 and 5% normal goat serum: ms anti-Futsch 1:100, ms anti-Brp 1:50, Rb anti-GluRIII 1:1000, Rb anti-DVGLUT 1:5000, Cy3-Gt anti-HRP (from Jackson labs) at 1:500, A488-Rb anti-GFP

(from Molecular Probes) at 1:1000. For secondary antibodies Cy3 and A488 conjugated Goat anti-Rb and anti-mouse (from Invitrogen) were used at 1:1000.

Nerve crush assay

The segmental nerves of third instar larvae were subjected to nerve crush injury as previously described (Xiong et al., 2010). For conditioning lesion assay, larvae were injured at segment A4 for the first injury, and at segment A2 for the second injury. The larvae are kept at 25^{o}C on a grape plate after or between injuries.

Imaging and Quantification

Confocal images were collected at room temperature on an Improvision spinning disk confocal system (Perkin Elmer). Similar settings were used to collect all compared genotypes and conditions.

To quantify the extent of degeneration of the NMJ, we measured disappearance of Futsch from NMJ boutons. This MAP1B homologue binds stable microtubules, which in uninjured axons extend through all but the most terminal boutons (Roos et al., 2000). The 'NMJ degeneration index' is defined the length of NMJ without Futsch staining, divided by the total length of the nerve terminal, defined by HRP staining (which is coincident with post-synaptic markers (data not shown)). Total lengths were measured using the 'simple neurite tracer' plugin of Image-J Fiji software. All quantifications shown represent the average from > 40 NMJs from >10 animals.

To quantify axonal degeneration, we scored while blind to genotype the fragmentation of m12-Gal4, UAS-mCD8:GFP labeled axons within segmental nerves according to one of five categories (described in Figure 3.1.E). All measurements indicate the average from > 100 axons.

3.4 Results

Wallerian degeneration at the *Drosophila* NMJ and in motoneuron axons

The facile genetics and highly characterized neuroanatomy of *Drosophila* make it a powerful model organism to study degeneration mechanisms. We have recently developed a nerve crush assay in which the segmental nerves of 3rd instar larvae are pinched with forceps, resulting in acute transection of motoneuron and sensory neuron axons (Xiong et al., 2010). After nerve crush, Wallerian degeneration can be observed in both axons and nerve terminals (NMJs) distal to the site of transection (Figure 3.1.).

At the NMJ, synaptic boutons from injured neurons became disconnected from one another, and then devoid of pre-synaptic proteins, including cytoskeletal proteins, active zone components and synaptic vesicles components (Figure 3.1.A-C and data not shown). This disappearance occurred in a distal-to-proximal manner: at 12 hours after injury, the most distal boutons were fragmented and empty, and by 24 hours after injury, all presynaptic components had completely disappeared from the NMJ. We exploited this distal-to-proximal disappearance to quantify the extent of NMJ degeneration over a time course (Figure 3.1.C) by measuring the length of the NMJ nerve terminal that did not contain the MAP1B homologue Futsch (described in

materials and methods). The disappearance of Futsch is synchronous with other pre-synaptic markers (data not shown). In contrast, post-synaptic proteins did not disappear, even 24 hours after injury (Figure 3.1.B). This process is quite distinct from the way in which the NMJ becomes dismantled during metamorphosis (Liu et al., 2010).

Within segmental nerves, endogenous Futsch became fragmented into spherical structures within 12 hours after injury (Figure 3.1.D), which were less abundant by 24 hours, suggesting clearance (data not shown). To quantify axonal degeneration we assessed the continuity of single motoneuron axons, labeled by expression of mCD8::GFP using the Gal4/UAS system (Figure 3.1.D,E). For both axonal and synaptic degeneration, we observed a temperature dependent lag phase followed by a rapid degeneration phase (Figure 3.1.C) that is synchronous throughout the axon.

Both axonal and synaptic degeneration were inhibited by expression of the mouse *WldS* mutant protein (Figure 3.1.C,F), emphasizing that the degeneration program shares mechanistic similarities from vertebrates to invertebrates (Hoopfer et al., 2006; MacDonald et al., 2006). Based on these observations, motoneuron axons and synapses in *Drosophila* larvae can be used as a model system to study the cellular mechanisms of degeneration after injury.

The Wnd/DLK kinase protects axons from Wallerian degeneration

A previous study in mice and *Drosophila* olfactory neurons has implicated a role for the Wallenda/DLK kinase in promoting the process of Wallerian degeneration (Miller et al., 2009). However our assay for Wallerian degeneration in *Drosophil*a motoneurons

reveals no requirement for Wnd in either synaptic or axonal degeneration after injury (Figure 3.2.A,B,D). In contrast, we observed that over-expression of *wnd* caused a dramatic delay in both synaptic and axonal degeneration (Figure 3.2.). When *wnd* was over-expressed, synaptic boutons and axons remained significantly intact (33.2% and 85.8%, respectively) even 24 hours after injury, when in comparison wild type synapses and axons had completely degenerated. Over-expression of a kinase-dead version of Wnd had no effect upon degeneration (data not shown). These observations suggest that the action of this kinase has a protective effect in motoneuron axons and synapses.

Previous studies indicate that Wnd regulates synaptic growth and the initiation of new axonal growth after injury via a nuclear signaling cascade (Collins et al., 2006; Xiong et al., 2010; Yan et al., 2009). Similar to previously described *wnd* over-expression phenotypes (Collins et al., 2006; Xiong et al., 2010), we find that the protection from degeneration by Wnd is inhibited by over-expression of a dominant negative Fos transgene (Fos^{DN}) in motoneurons (Figure 3.2.B,C,D). This finding suggests that the protective action of Wnd involves changes in gene expression. Interestingly, over-expressing *wnd* does not have protective effect in adult olfactory neurons or larval type IV sensory neurons (Figure 3.3.).

Wnd/DLK is thought to function as an upstream regulator of the JNK MAP Kinase in vertebrates and *Drosophila* (Collins et al., 2006; Ghosh et al., 2011; Hirai et al., 2002; Nihalani et al., 2001; Xiong et al., 2010). While JNK is required for nuclear signaling by Wnd (Xiong et al., 2010), we could not address the role of JNK in protection by Wnd, because inhibition of JNK alone also led to protection from axonal degeneration (Figure 3.2.B, D, yellow line). This observation parallels observations in vertebrate axons

(Barrientos et al., 2011; Miller et al., 2009; Yoshimura et al., 2011). However, because mutation or knockdown of *wnd* did not delay degeneration, we interpret that additional and independent regulators of JNK may function in promoting degeneration of the distal stump after injury.

A previous injury protects axons from Wallerian degeneration

Our findings indicated that activation of Wnd signaling in motoneurons could increase their resiliency to axonal degeneration. While Wnd is normally kept at very low levels in larval motoneurons (Collins et al., 2006), recent studies suggest that Wnd protein becomes upregulated and activated in axons after axonal injury (Xiong et al., 2010). These observations led us to hypothesize that endogenous Wnd protein may promote a protective response in neurons which have sustained an injury. Studies in vertebrate neurons suggest that a conditioning lesion can dramatically alter the response of a neuron to subsequent injuries. (Hoffman et al.,2010). We hypothesized that Wnd function, which becomes activated by axonal injury in the PNS, allows for neurons which have been previously injured to have an increased resiliency to degeneration. To test this, we conducted a conditioning lesion assay (Figure 3.4.A), in which the proximal stump after an initial injury was injured a second time, and degeneration was measured in the 'middle stump' that is distal to the second injury site. We found that if an axon had been injured at least 6 hours previously, then degeneration after the second injury was significantly delayed (Figure 3.4.B,C). Similar to the effect of conditioning lesion upon regeneration in vertebrate neurons (Hoffman, 2010), the protective effect of the conditioning lesion required time, in this case 6 hours

to reach partial effect, and 12 hours to reach maximal effect (Figure 3.4.C). We conclude that a conditioning injury to axons induces changes in the cell that lead to protection of axons from degeneration after future injuries.

The Wallenda signaling cascade mediates an adaptive response in neurons

Importantly, the time requirement for the protective effect of the conditioning lesion matches other cellular events which have been previously characterized in this nerve crush injury assay (Xiong et al., 2010). For instance, phosophorylated JNK is induced in the cell body around 6 hours after injury, and transcriptional changes can be detected in the nucleus around 8 hours after injury. All of these events are preceded by an increase in the levels of Wnd protein within 4 hours of injury. We therefore tested whether the protective effect of the conditioning lesion requires Wnd function (Figure 3.5.). When we examined axons in the middle stump after the conditioning lesion assay, axons from wnd^{1}/wnd^{2} mutants degenerated similarly to axons which received the control (unconditioned) double lesion (Figure 3.5.A), suggesting that the conditioning lesion had no effect in *wnd* mutants. Furthermore, expression of *wnd* RNAi or Fos^{DN} specifically in the mCD8::GFP labeled motoneurons (Figure 3.5.B,C) abolished the protective effect of the conditioning lesion (Figure 3.5.C). The neighboring axons which did not express the transgenes still degenerated (Figure 3.5.B), demonstrating the cell autonomy of the pathway. We conclude that a signaling cascade downstream of the Wnd kinase, which becomes activated by axonal injury, promotes a protective response in axons that inhibits Wallerian degeneration after injury (Figure 3.5.D).

3.5 Discussion

Our findings suggest that the Wnd kinase plays a protective role in *Drosophila* motoneuron axons. Wnd protein is normally kept at a low level in these axons, but becomes rapidly induced by axonal injury (Xiong et al., 2010). This leads to activation of a nuclear signaling cascade that promotes an increased resiliency of axons to degeneration after a second injury. The requirement for Wnd in the protective effect of a conditioning lesion leads us to propose that Wnd regulates a stress response pathway that allows neurons to adapt to axonal injury.

A pathway that inhibits axonal degeneration would be very advantageous in instances where the integrity of the axon is compromised but not completely lost, such as anoxia, loss of myelination, or defects in axonal transport. Such problems are a concern not only for neuronal injuries, but also neurodegenerative disorders, whose onset may be influenced by neuronal stress (Mattson and Magnus, 2006). Along these lines, mutations that lead to activation of Wnd/DLK signaling can suppress a synaptic retraction phenotype in *Drosophila* (Massaro et al., 2009).

The conditioning lesion is classically studied for its role in facilitating axonal regeneration, although it has been noted that a conditioning lesion can also attenuate degeneration of motoneurons in a rat model of ALS (Franz et al., 2009). In vertebrates, a conditioning injury of axons in the PNS induces widespread cellular changes, including chromatolysis, changes in gene expression, translation, trafficking, cytoskeleton, and physiology (Cragg, 1970; Hoffman, 2010). Studies in multiple model organisms have already implicated Wnd/DLK in nuclear signaling (Ghosh et al., 2011; Xiong et al., 2010; Yan et al., 2009), translation (Huang et al., 2009), axonal transport

(Horiuchi et al., 2007), and cytoskeletal dynamics (Bounoutas et al., 2011; Eto et al., 2010; Hirai et al., 2011), hence this kinase may function as an upstream regulator of multiple downstream responses to axonal injury. The exact cellular events that lead to protection from degeneration, and their relationship to the regenerative response, remain to be determined.

Importantly, the action of Wnd/DLK is not always beneficial. In contrast to motoneuron axons, Wnd is unable to protect olfactory neuron axons in the adult (Figure 3.3.A). Instead, Wnd plays a modest role in promoting degeneration in these neurons (Miller et al., 2009). Also in contrast, the vertebrate homologue DLK plays a pro-degenerative role in DRG neurons, both after injury (Miller et al., 2009) and after nerve growth factor withdrawal (Ghosh et al., 2011). The distinct downstream outcomes may depend upon distinct features of downstream signaling events in different cell types, developmental contexts, and cellular location. For instance, the protective role of Wnd requires time, the neuronal cell body, and downstream gene expression, while the distal stump of injured axon would be unable to receive the benefits of a nuclear signaling cascade induced by injury. However even the nuclear signaling cascade in the proximal stump is not always beneficial: in cultured DRG neurons DLK promotes apoptosis after nerve growth factor withdrawal (Ghosh et al., 2011). This negative outcome of DLK action shares mechanistic similarities with the beneficial outcome of Wnd for regeneration in that both depend upon retrograde signaling to the nucleus after an axonal stimulus (Ghosh et al., 2011; Xiong et al., 2010).

Similar to previous studies (Barrientos et al., 2011; Miller et al., 2009; Yoshimura et al., 2011), our findings suggest that JNK plays a pro-degenerative role in *Drosophila*

motoneuron axons, however this action may occur independently of Wnd regulation. Indeed, a Wnd-independent pool of phosphorylated JNK has been described in *Drosophila* axons (Xiong et al., 2010). While JNK plays an important role in the regenerative response to injury (Xiong et al., 2010), it is not clear whether JNK functions in the injury-induced protection from degeneration. An equally likely candidate mediator, based on studies in *C. elegans* (Nakata et al., 2005; Nix et al., 2011), is the other stress activated MAPK, p38. Future characterization of the mechanism of Wnd/DLK signaling, including the cofactors and effectors for its different functions in different contexts, will be important for delineating the molecular differences between positive and negative responses to axonal damage.

3.6 Figures

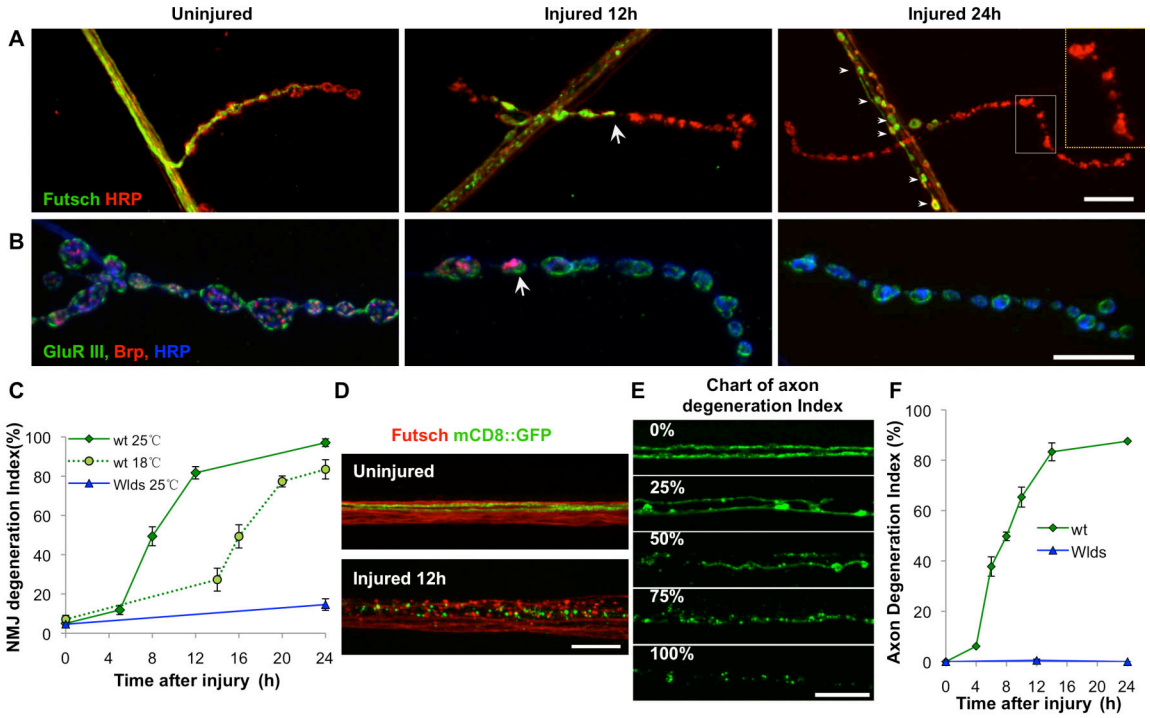


Figure 3.1 Characterization of degeneration at the *Drosophila* NMJ and in motoneuron axons after injury.

(A) The axonal membrane (red) at muscle 4 NMJ synapses is labeled by immunostaining with anti-HRP antibodies. The MAP1B homologue Futsch (green) disappears most rapidly from distal boutons and concentrates within proximal boutons (12 hours, arrow). By 24 hours, Futsch has completely disappeared from boutons and forms sphere-shaped fragments in segmental nerves (arrowheads) and HRP staining becomes discontinuous (inset).
(B) Pre-synaptic active zones, stained by anti-Brp antibodies (red) also disappear from NMJ boutons in a distal-to-proximal manner (12 hours, arrow). Postsynaptic receptors, detected by GluRIII staining (green) become diffuse but do not disappear (24 hours).
(C) The time course of NMJ degeneration was quantified by measuring the length of muscle 4 NMJ nerve terminus (defined by HRP staining) which does not contain Futsch (as described in Materials and Methods). The onset of degeneration is significantly delayed at the lower temperature (dotted line), and inhibited by expression of the *WldS* mutation (blue line).
(D) In segmental nerves distal to the injury site, Futsch (red) and mCD8::GFP-labeled single axons, using the m12-Gal4 driver (green) are extensively fragmented by 12 hours after injury. Nerves are oriented with the side proximal to the injury on the left.
(E-F) To quantify the time course of axonal degeneration, axons were scored while blinded to genotype according to the following guide, ranging from completely continuous (0%), continuous with varicosities (25%), partially discontinuous (50%), mostly fragmented with a few segments of continuity (75%) to completely fragmented (100%). Scale bars = 25μm.

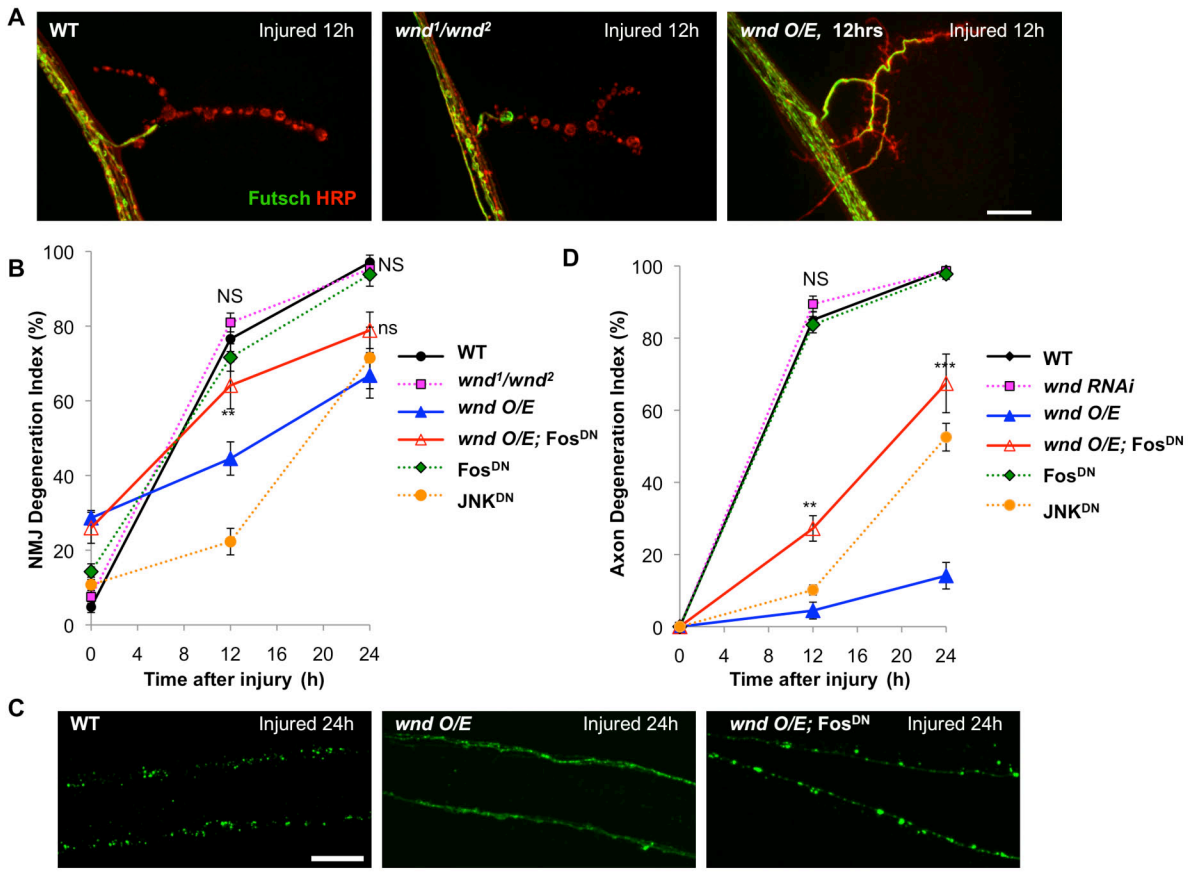


Figure 3.2 The Wallenda/DLK kinase inhibits Wallerian degeneration.

(A) Representative muscle 4 NMJs are shown for wild type (*Canton S*), wnd mutant (*wnd*^{1}/*wnd*^{2}), and over-expressed *wnd* (BG380-Gal4; UAS-*wnd*/+) 12 hours after injury.
(B) Quantification of the NMJ degeneration index (defined by absence of Futsch staining) over a time course for different genotypes. *wnd* mutant (magenta) and Fos^{DN} (green),(BG380;;UAS-Fos^{DN}/+), degenerate with a similar time course to wild type axons. NS = no significant difference from wild type. However over-expression of *wnd* (blue) dramatically delays degeneration. This inibition of degeneration is strongly reduced when Fos^{DN} is co-expressed (red), (BG380;UAS-*wnd*/+; UAS-Fos^{DN}/+). ** p < 0.01, compared to *wnd O/E*. An additional phenotype of *wnd* over-expression is a reduction of Futsch in terminal boutons, which results in a higher 'degeneration index' in uninjured animals. However this phenotype is not suppressed by co-expression of Fos^{DN}.
(C) Representative images of single axons in wild type axons (UAS-mCD8::GFP/+; m12-Gal4/+) which have degenerated within 12 hours of injury, compared to axons which remain intact 24 hours after injury when *wnd* is over-expressed (UAS-*wnd*/UAS-mCD8::GFP; m12-Gal4/+). This protection from degeneration is inhibited by co-expression of Fos^{DN} (UAS-*wnd*/UAS-mCD8::GFP; m12-Gal4/UAS-Fos^{DN}).
(D) Quantification of axon degeneration in different genotypes. ** p < 0.01, ***p < 0.001 compared to *wnd O/E.* Scale bars=25µm.

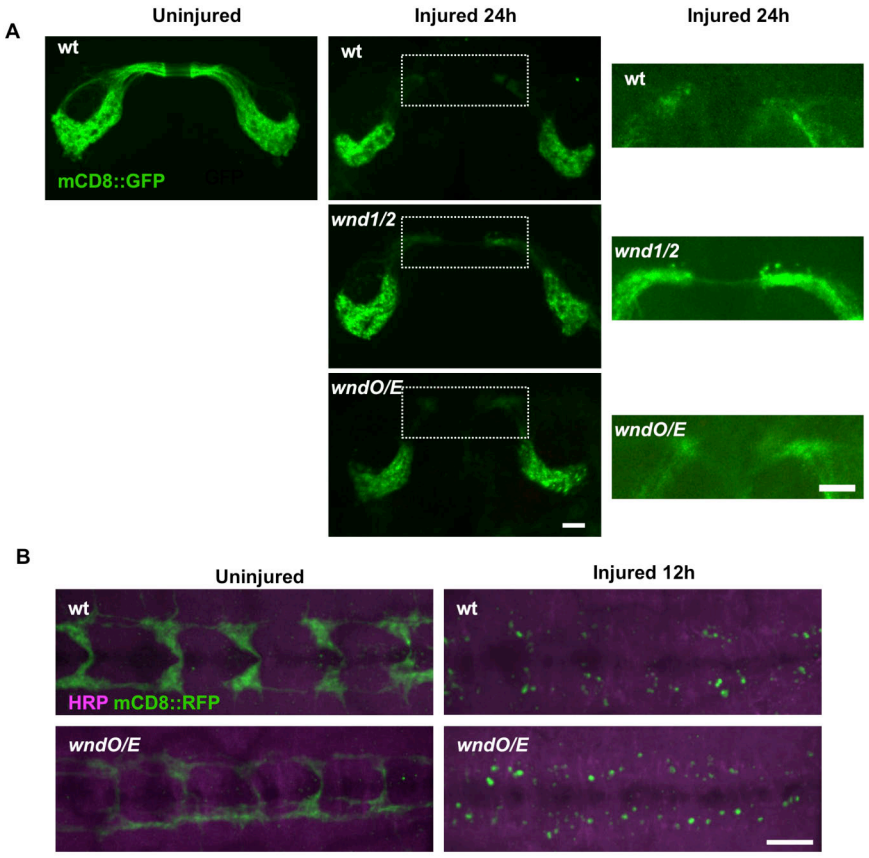


Figure 3.3 Wnd does not protect all neurons from degeneration after injury.

(A) The degeneration of olfactory neuron axons, labeled by Or47b Gal4, UAS-mCD8::GFP, after removal of cell bodies in the antennae was studied as previously described (Hoopfer et al., 2006; MacDonald et al., 2006). We confirmed the previous observation (Miller et al., 2009) that *loss-of-function* mutations in *wnd* (*wnd*^{1}/*wnd*^{2}) cause a slight delay in degeneration of these neurons. This can be detected as a small segment of axon continuity in the commissural region (rectangles, enlarged on right). Over-expression of *wnd* in these neurons does not lead to a detectable inhibition in the disappearance of these axons.
(B) The degeneration of larval class IV sensory neuron axons, labeled by ppk-Gal4, UAS-mCD8::RFP, is also not delayed by overexpression of *wnd*. These axons are injured similarly to larval motorneurons in the nerve crush assay, and the axon terminals in the nerve cord are examined.

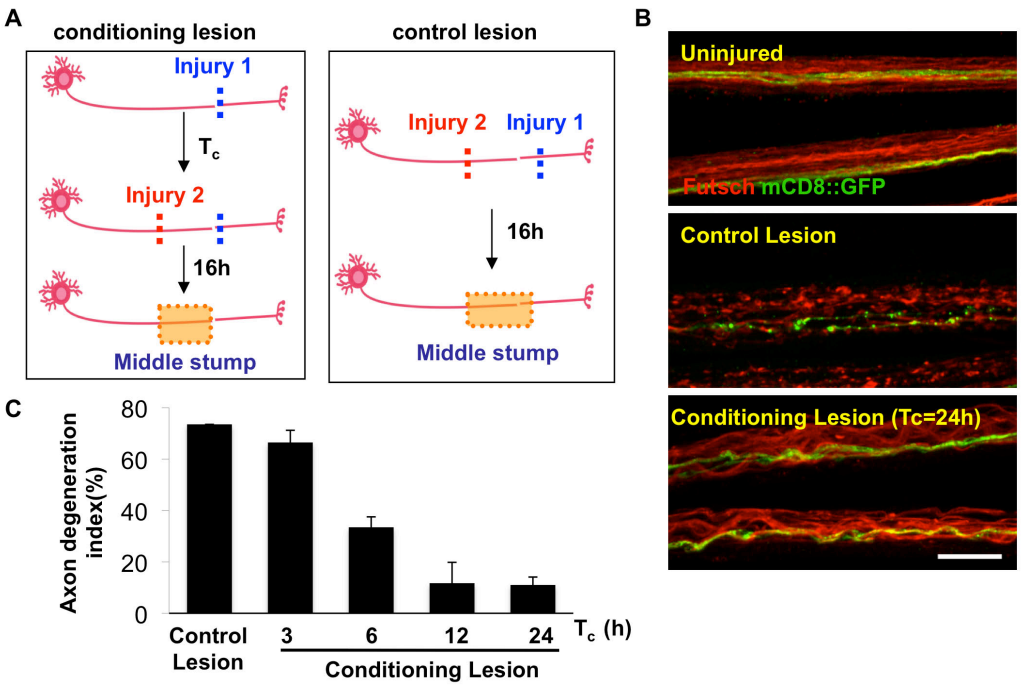


Figure 3.4 A conditioning lesion protects axons from degeneration after a second injury.

(A) Schematic of the conditioning lesion and control lesion experiments. A conditioning lesion is done at injury site 1 (see materials and methods) for a variable period of time, Tc, before the second injury at site 2, which is within the proximal stump of the previously injured axon.
(B) Uninjured axons or middle stump axons in control and conditioning lesion are stained for Futsch (Red) and GFP (green). Single axons, labeled by driving *UAS-mCD8::GFP* expression with *m12-Gal4*, and endogenous Futsch are fragmented at 16 hours after the control lesion, but intact after the conditioning lesion. Scale bar = 25μm.
(C) Quantification of the axon degeneration index, measured at 16 hours after the second injury. For the conditioning lesion, the time between the first and second injuries, Tc, was varied.

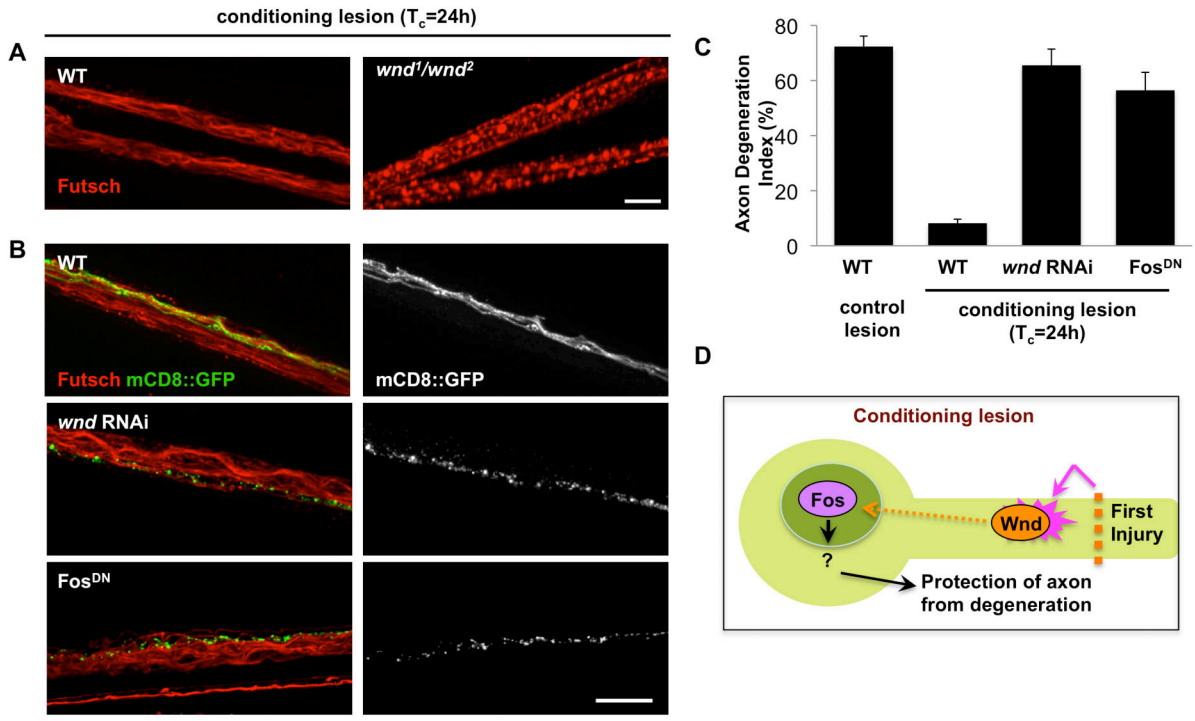


Figure 3.5 The protective effect of the conditioning lesion requires Wallenda.

(A) Staining for endogenous Futsch in the segmental nerves of axons treated with conditioning lesion, as outlined in Figure 3A. In *wnd*^{1}/*wnd*^{2} mutants, endogenous Futsch is fragmented similarly to control, singly injured axons (Figure 3.1.C).
(B) The *m12-Gal4* driver line is used to drive expression of *wnd*-RNAi (along with *UAS-Dcr2*) or Fos^{DN} in the GFP-labeled axons. The mCD8::GFP labeled axons are shown alone in grayscale in the right panels. All axons were treated with a conditioning lesion with Tc of 24 hours, and the middle stump is shown 16 after the second injury. Scale bars = 25µm.
(C) Quantification of the axon degeneration index indicates that expression of *wnd* RNAi or Fos^{DN} nearly completely abolishes the protective effect of the conditioning lesion.
(D) Model for the role of Wallenda, which becomes activated in axons by injury, and mediates a signaling cascade that requires time and the Fos transcription factor. The downstream cellular changes that alter resiliency to degeneration remain to be determined.

CHAPTER 4

THE HIGHWIRE UBIQUITIN LIGASE PROMOTES AXONAL DEGENERATION BY TUNING LEVELS OF NMNAT PROTEIN

4.1 Abstract

Axonal degeneration is a hallmark of many neuropathies, neurodegenerative diseases, and injuries. Here, using a *Drosophila* injury model, we have identified a highly conserved E3 ubiquitin ligase, Highwire (Hiw), as an important regulator of axonal and synaptic degeneration. Mutations in *hiw* strongly inhibit Wallerian degeneration in multiple neuron types and developmental stages. This new phenotype is mediated by a new downstream target of Hiw: the NAD+ biosynthetic enzyme nicotinamide mononucleotide adenyltransferase (Nmnat), which acts in parallel to a previously known target of Hiw, the Wallenda dileucine zipper kinase (Wnd/DLK) MAPKKK. Hiw promotes a rapid disappearance of Nmnat protein in the distal stump after injury. An increased level of Nmnat protein in *hiw* mutants is both required and sufficient to inhibit degeneration. Ectopically expressed mouse Nmnat2 is also subject to regulation by Hiw in distal axons and synapses. These findings implicate an important role for endogenous Nmnat and its regulation, via a conserved mechanism, in the initiation of

axonal degeneration. Through independent regulation of Wnd/DLK, whose function is required for proximal axons to regenerate, Hiw plays a central role in coordinating both regenerative and degenerative responses to axonal injury.

4.2 Introduction

Axon degeneration can be induced by a variety of insults, including injury. When an axon is transected from the cell body, the distal axon "stump" degenerates through a regulated self-destruction process called Wallerian degeneration (Waller, 1850). This process appears to be actively regulated in axons; however, the endogenous cellular machinery that regulates and executes this degeneration process is poorly understood.

Previous studies have implicated a role for the ubiquitin proteasome system (UPS) in Wallerian degeneration, since inhibition of UPS leads to a delay in the early stages of degeneration (Hoopfer et al., 2006; Zhai et al., 2003). One explanation for this result is that the UPS mediates bulk protein degradation via the combined action of many ubiquitin ligases. However an alternative model is that one or several specific E3 ligases target the destruction of key inhibitors of the degeneration process. Here, using an in vivo assay for Wallerian degeneration in *Drosophila*, we identify an essential role for a specific E3 ubiquitin ligase in promoting Wallerian degeneration.

The ligase, known as Highwire (Hiw) in *Drosophila,* Phr1 in mice, is well known from studies in multiple model organisms for its conserved functions in regulating axonal and synaptic morphology during development (Bloom et al., 2007; Burgess et al., 2004; D'Souza et al., 2005; Hendricks and Jesuthasan, 2009; Lewcock et al., 2007; Po et al.,

2010; Schaefer et al., 2000; Wan et al., 2000; Zhen et al., 2000). We found that mutations in *hiw* strongly inhibit the initiation of Wallerian degeneration in multiple neuronal types and developmental stages. Until recently (Osterloh et al., 2012; Wakatsuki et al., 2011), such a strong loss-of-function phenotype has not been reported for this process.

Mutations in *hiw* also inhibit synaptic retraction caused by disruption of the cytoskeleton structure in spectrin mutant(Massaro et al., 2009). However the finding that Hiw promotes axonal degeneration was originally perplexing, since a known target of Hiw, the Wallenda (Wnd) MAP kinase kinase kinase (also known as dileucine zipper kinase [DLK]) (Collins et al., 2006; Nakata et al., 2005), was found to promote Wallerian degeneration in mouse DRG and *Drosophila* olfactory neurons (Miller et al., 2009). In *hiw* mutants Wnd levels are increased (Collins et al., 2006; Lewcock et al., 2007; Nakata et al., 2005), however degeneration is inhibited. A partial explanation for these opposing results is that Wnd plays a protective role in some neuronal types (Fang et al., 2012; Xiong and Collins, 2012). However this alone could not account for the essential role of Hiw in Wallerian degeneration of all neuron types. These findings pointed to the existence of additional targets for Hiw.

Recent studies in vertebrate cultured neurons have suggested the NAD+ synthase enzyme nicotinamide mononucleotide adenyltransferase 2 (Nmnat2) as an attractive target of post-translational regulation in axons (Gilley and Coleman, 2010). Nmnat2 is transported in axons, where it has a short protein half-life, and neurons depleted for Nmnat2 undergo axonal degeneration (Gilley and Coleman, 2010). Moreover, many gain-of-function studies suggest that increasing the activity of an

Nmnat enzyme in axons can effectively delay Wallerian degeneration (Coleman and Freeman, 2010; Wang et al., 2012). The most classic example of this comes from studies of the Wallerian degeneration Slow (*WldS*) gain-of-function mutation in the *Nmnat1* locus, which causes a greater than 10-fold delay in the degeneration of injured axons (Mack et al., 2001). However, despite the plethora of studies examining the effect of overexpressing Nmnat enzymes (Coleman and Freeman, 2010), very little is known about the role of the endogenous Nmnat enzymes in axons and how their activity may be regulated.

In contrast to the three isoforms in vertebrates, the *Drosophila* genome contains a single *nmnat* gene, for which two splice forms are annotated. *nmnat* is an essential gene, whose depletion in neurons causes neurodegeneration (Fang et al., 2012; Wen et al., 2011; Zhai et al., 2006). Here we find that Hiw and ubiquitination negatively regulate the levels of axonal Nmnat in vivo. Moreover endogenous Nmnat is required, in parallel to Wnd, for mutations in *hiw* to inhibit degeneration. By down-regulating the levels of Nmnat protein, Hiw promotes the initiation of Wallerian degeneration in axons and synapses. Moreover, through co-regulation of the Wnd/DLK kinase, whose function is required for proximal axons to initiate new axonal growth (Hammarlund et al., 2009; Itoh et al., 2009; Shin et al., 2012; Xiong et al., 2010; Yan et al., 2009), Hiw coordinates both regenerative and degenerative responses to axonal injury.

4.3 Methods

Fly Stocks

The following strains were used in this study: *Canton-S* (wild-type), *hiw*^{ND8} (Wan et al., 2000), *hiw*$^{\Delta N}$, *UAS-hiw* and *UAS-hiw-ΔRING* from (Wu et al., 2005), *OK6-Gal4* (McCabe et al., 2004), *BG380-Gal4* (Budnik et al., 1996) *m12-Gal4* (P(GAL4)^{5053A}) (Ritzenthaler et al., 2000), *ppk-Gal4* (Kuo et al., 2005), *Or47b-Gal4* (Vosshall et al., 2000), *UAS-UBP2* (DiAntonio et al., 2001), *UAS-DTS5*, and *UAS-DTS7* from (Belote and Fortier, 2002), *wnd*^{1}, *wnd*^{3}, and *UAS-wnd* from (Collins et al., 2006). *UAS-HA::nmnat* (Zhai et al., 2006), *UAS-WldS* (Hoopfer et al., 2006), *UAS-mNmnat1::myc*, *UAS-mNmnat2::myc*, and *UAS-mNmnat3::myc* (Avery et al., 2012; Avery et al., 2009), and *UAS-Dcr2* were gifts from Grace Zhai, Liqun Luo, Marc Freeman, and Stephan Thor. *UAS-wnd-RNAi* (Construct ID 13786) and *UAS-nmnat-RNAi* (construct ID 32255) were acquired from the Vienna RNAi center (Dietzl et al., 2007).

Nerve Crush Assay

The segmental nerves of third instar larvae were visualized through the cuticle under a standard dissection stereomicroscope. While larvae were anesthetized with CO_2 gas, the segmental nerves were pinched tightly through the cuticle for 5 s with Dumostar number 5 forceps. After successful injury, the posterior half of the larva was paralyzed. Larvae were then transferred to a grape plate and kept alive for varying periods of time at 25 °C. Also see (Xiong et al., 2010).

Immunocytochemistry

Larvae were dissected in PBS and fixed in 4% paraformaldehyde or formaldehyde in PBS for 25 min for the following antibodies used: ms anti-Futsch (1:100), guinea pig (gp) anti-NMNAT (Zhai et al., 2006), (gift from Hugo Bellen and Grace Zhai, 1:1,000), rat anti-HA (Roche, 1:100), rat anti-elav (1:50), or fixed in Bouin's fixative for 15 min for the following antibodies: ms anti-Brp (1:200), Rb anti-GluRIII (1:1,000 (Marrus et al., 2004)), Rb anti-DVLGUT (1:10,000, (Daniels et al., 2004)). Rat anti-elav (7E8A10) and ms anti-Brp (NC82) were obtained from Developmental Studies Hybridoma Bank, University of Iowa. The conjugated secondary antibodies are used and diluted as follows: Cy3-Gt anti-HRP and Cy5-Gt anti-HRP (from Jackson labs) at 1:200, A488-Rb anti-GFP (from Molecular Probes) at 1:1,000. For secondary antibodies Cy3 and Alexa-488 conjugated Goat anti-rb/mouse/rat/gp (from Invitrogen) were used at 1:1,000. All antibodies were diluted in PBS-0.3%Triton with 5% normal goat serum.

Imaging

Confocal images were collected at room temperature on an Improvision spinning disk confocal system, consisting of a Yokagawa Nipkow CSU10 scanner, and a Hamamatsu C9100-50 EMCCD camera, mounted on a Zeiss Axio Observer with 25× (0.8 NA) multi and 40× (1.3NA), 63× (1.5NA), and 100× (1.46 NA) oil objectives. Similar settings were used to collect all compared genotypes and conditions. Volocity software (Perkin Elmer) was used for all measurements of average and total intensities.

For measurement of Nmnat intensity in the neuropil, the neuropil area was

selected based on co-staining for the synaptic marker Brp. Objects meeting intensity criteria of >0.8 standard deviations above the mean were selected within a 140-μm long region of the ventral nerve cord and then summed for total intensity. The average intensity of the HA-Nmnat staining in muscle 4 NMJs was measured within the synaptic area defined by HRP staining after subtraction of background intensity for each image. The average intensity of the HA-Nmnat staining in motoneuron axons and sensory nerve terminus was measured with a similar protocol. Likewise for neuronal nuclei, the average intensity for Nmnat staining was measured in neuronal nuclei defined by staining for the neuronal marker Elav. Numbers are shown normalized to the average intensity of the control for each figure.

Quantification of Degeneration

To quantify axonal degeneration, we scored (while blind to genotype) the fragmentation of *m12-Gal4*, *UAS-mCD8-GFP* labeled axons within segmental nerves according to one of five categories between 0 and 100% (with 100% meaning completely degenerated) as described in (Xiong and Collins, 2012). All measurements indicate the average from >100 axons.

To quantify the degeneration of the NMJ, NMJs were stained for the MAP1B homologue Futsch and axonal membrane marker HRP, and were scored into one of three categories: (1) complete degeneration, defined by a complete loss of Futsch staining from the NMJ and fragmentation of the axonal membrane, (2) partial degeneration, defined by a partial loss of Futsch staining from the NMJ and partial membrane fragmentation, and (3) no degeneration, in which there was no fragmentation

of the membrane or Futsch, similar to uninjured control animals. All quantifications shown represent the average scores from multiple NMJs from >six animals quantified in duplicate by two independent observers who were blind to the genotype.

Degeneration of ORN axons was quantified following the previously defined method (Hoopfer et al., 2006; MacDonald et al., 2006) by calculating the percentage of brains for each genotype in which contralateral axon projections could still be detected.

For all the statistical analysis, Student's *t* test was applied.

Electrophysiology

Intracellular recordings were made from muscle 6 in segments A3 and A4 of third-instar male larvae. The larvae were visualized with oblique illumination on an Olympus BX51W1 fixed stage upright microscope with a 10× water immersion objective. Sharp electrodes (15–25 MΩ), made of borosilicate glass (outer diameter 1.2 mm) were filled with 3 M KCl. The signal was amplified with a Geneclamp 500B (Molecular Devices), digitized with a Digidata 1322A interface (Molecular Devices), and stored on a PC with pClamp 10.2 (Molecular Devices). Recordings were performed in HL3 Stewart saline (Stewart et al., 1994) that contained (in mM) 70 NaCl, 5 KCl, 20 $MgCl_2$, 10 HCO_3, 5 trehalose, 115 sucrose, 5 HEPES, 1 $CaCl_{2,}$, the pH was adjusted to 7.2. For all genotypes the resting membrane potentials and input resistances were similar, with average resting potentials of −73 ± 4 and input resistances of 6 ± 0.2 MΩ. To elicit evoked excitatory junction potentials (EJPs), the nerve was drawn into a tight-fitting suction electrode and stimulated with brief (1 ms) depolarizing pulses controlled with

Digidata interface. The stimulus amplitude was set to 125% of the amplitude necessary to activate the higher threshold of the two excitatory axons that innervate the muscle. For injured wild-type larvae (in which nerve stimulation did not produce evoked synaptic responses) the stimulus amplitude was set to double the amplitude used for un-injured larvae. However evoked responses were not observed, even at the largest stimulus amplitude that the equipment could produce. For analysis of evoked responses, 100 events per cell recorded at 0.2 Hz were measured using the cursor feature in Clampfit 10.2 (Molecular Devices) and then averaged. For analysis of spontaneous miniature EJPs, at least 50 consecutive events were measured per cell using MiniAnal (Synaptosoft). mEJP frequency was calculated from the first 30 s of recording time.

Cell Culture

S2R+ cells were cultured in Schneider's medium (Gibco) which contains 10% (v/v) FBS (Gibco) and 1% penicillin-streptomycin (Invitrogen). For plasmid transfection, cells were transfected using FuGENE 6 (Promega) following the manufacturer's instructions. Copper sulfate solution (0.5 mM) was added 6 h after transfection to induce plasmid expression. Cell lysates were collected after 24 h. Plasmids used for transfection were pMT-Gal4 (Klueg et al., 2002), pUAST-eGFP, pUAST-GFP-Hiw (Wu et al., 2005), pUAST-HiwΔRING (Wu et al., 2005), and pUAST-HA-Nmnat (Zhai et al., 2006).

To inhibit the UPS, cells were treated with MG132 (InSolution, Calbiochem) or DMSO as control using several different conditions: 25 μM for 6 h, 5 μM for 20 h, and 5

μm for 36 h. All of these conditions led to an increase in the levels of polyubiquitinated proteins, detected by Western blots probed with FK1 antibodies.

Biochemistry

The following antibodies were used for Western blotting: rb anti-Hiw (ref, 1:2,000), rat anti-HA (Flourochem, 1:2,500), ms anti-b-tubulin (1E7) and ms anti-b-catenin (armadillo, N27A1) from Developmental Studies Hybridoma bank (University of Iowa), ms anti-polyubiquitin, (FK1, Enzo Life Sciences, 1:1,000), and ms anti-ubiquitin (P4D1, Cell Signaling, 1:1,000). Westerns were probed with IRDye 800CW and 680RD conjugated secondary antibodies (LiCor biosciences, 1:10,000) and imaged for quantitative analysis via a LiCor Odyssey imaging system.

S2R+ cells were transfected with either pUAST-HiwΔRING or pUAST-HiwΔRING and pUAST-HA-Nmnat. Cells from 6-cm dishes were harvested in 500-μl ice-cold lysis buffer (20 mM HEPES [pH 7.5]), 200 mM KCl, 0.05% Triton X-100, 2.5 mM EDTA, 5 mM DTT, 5% glycerol and Complete proteinase inhibitor [Promega]). 1.5 mg Protein G conjugated Dynabeads (Invitrogen) were used to capture 10 μl mouse monoclonal anti-HA antibody (HA-7 ascites fluid, Sigma) at room temperature for 30 min, and were then incubated with cell lysates for 30 min at room temperature. The immunoprecipitates were then washed three times with ice-cold lysis buffer and subjected to Western blotting analysis.

4.4 Results

Highwire Plays an Essential Role in Wallerian Degeneration

We used a previously described nerve crush assay (Xiong and Collins, 2012; Xiong et al., 2010) to study the degeneration of motoneuron and sensory neuron axons within segmental nerves in third instar *Drosophila* larvae. To quantify the degeneration of motoneuron axons, we used the *m12-Gal4* driver to label only a subset of motoneurons with *UAS-mCD8-GFP* (Figure 4.1.A, B, and Methods). In wild-type (WT) animals, these axons are completely fragmented within 24 h after injury (Figure 4.1.A) (Xiong and Collins, 2012).

Hiw is a large, highly conserved protein thought to function as an E3 ubiquitin ligase (Han et al., 2008; Nakata et al., 2005). Previous studies have suggested that Hiw regulates the ability of axons to regenerate after injury (Hammarlund et al., 2009; Xiong et al., 2010). Here we investigated whether Hiw plays a role in degeneration after injury.

In both *hiw* null ($hiw^{\Delta N}$) and hypomorph (hiw^{ND8}) mutant animals, axonal degeneration was strongly inhibited. Even 48 h after injury (which is the latest time that can be visualized before pupation) the distal stump of injured axons remained intact in *hiw* mutants (Figure 4.1.A,B). The protection from degeneration was also recapitulated in neurons that expressed the dominant negative mutation, *hiw-ΔRING* (Figure 4.1.B), but not in adjacent neurons that did not express Gal4. These results strongly suggest that Hiw performs a cell-autonomous function in promoting axonal degeneration after injury. Similarly, we found that overexpression of the de-ubiquitinating enzyme *UBP2* (Baker et al., 1992) delayed degeneration of *Drosophila* motoneuron (Figure 4.1.B).

The *hiw* mutation also inhibited degeneration of the NMJ (Figure 4.1.C). In wild-type animals, pre-synaptic proteins, such as the MAP1B homologue Futsch, disappeared completely from all NMJ boutons within 24 h after injury while the axonal membrane, detected with anti-HRP antibodies, fragmented into individual spheres (Figure 4.1.C). In *hiw* mutants, all markers of NMJ structure remained intact (Figures 4.1.C and 4.2.). Expression of *hiw* cDNA in motoneurons restored their ability to degenerate after injury (Figure 4.1.D).

To test whether the distal stump of *hiw* mutants remained functional, NMJ synapses at muscle 6 were subjected to a standard electrophysiology recording paradigm either before or after injury (Figure 4.1.E–H). At 24 h after injury, wild-type NMJs were completely silent: no evoked excitatory junction potentials (EJPs) were observed (Figure 4.1.H), and only one single spontaneous miniature event (mEJP) was observed in all ten recordings (Figure 4.1.F). In contrast, at 24 h after injury, recordings in *hiw* mutant NMJs showed robust spontaneous mEJPs and evoked EJPs, resembling uninjured *hiw* NMJs (Wan et al., 2000). Hence axons and synapses are functionally intact and resilient to degeneration in *hiw* mutants.

We then tested whether Hiw promotes axonal degeneration in other neuron types (Figure 4.3.). The sensory neuron axons in larval segmental nerves were also injured in the nerve crush assay, and their distal axons also degenerated in a Hiw-dependent manner (Figure 4.3.A). We then tested the role of Hiw in degeneration of adult neurons, which can be studied over a longer window of time. In wild-type animals, the distal stumps of olfactory neuron axons in the antennal lobe degenerated within 1 d after their cell bodies were removed by antennal lobe transection (Hoopfer et al., 2006;

MacDonald et al., 2006). In contrast, in *hiw* null mutants, olfactory neuron axons remained in the antennal lobe even 20 d after cell body removal (Figure 4.3.B,C), which is comparable with the extent of protection by the *WldS gain-of-function* mutation (Hoopfer et al., 2006; MacDonald et al., 2006). These dramatic phenotypes in multiple neuron types suggest that Hiw plays a fundamental role in the initiation of axonal degeneration after injury.

The Wallenda MAPKKK Is Only Partially Required for the *highwire* Degeneration Phenotype

To understand the mechanism for Hiw in Wallerian degeneration we first considered a previously characterized target of Hiw regulation, the Wnd/DLK kinase. A previous study in mouse DRG and *Drosophila* olfactory neurons found that degeneration is delayed in *wnd(dlk)* mutants (Miller et al., 2009). However, in larval motorneurons, we found the opposite result, since mutations in *hiw* lead to increased levels of Wnd kinase in axons (Collins et al., 2006), and overexpression of *wnd* in motoneuron axons can delay Wallerian degeneration (Xiong and Collins, 2012). Consistent with Wnd playing a protective role against degeneration downstream of Hiw, the protection from degeneration in *hiw* mutants was suppressed in *hiw; wnd* double mutants, although the suppression was only partial (Figure 4.4.). In contrast, the synaptic overgrowth and overbranching phenotype in *hiw* mutants was completely suppressed in the *hiw;wnd* double mutants (Collins et al., 2006). We also noticed that while *hiw* mutations inhibited degeneration in multiple neuron types, overexpression of *wnd* did not protect olfactory neuron and sensory neuron axons from degeneration

(Xiong and Collins, 2012). Hence the degeneration phenotype for *hiw* mutants could not be accounted for by Wnd alone. This suggested the existence of additional downstream effectors of Hiw during axonal degeneration.

Nmnat Is a Downstream Target of Highwire during Wallerian Degeneration

A well-known and intensively studied negative regulator of Wallerian degeneration is Nmnat (Coleman and Freeman, 2010). An increased activity of this enzyme, first discovered in the *WldS* mutation, can strongly inhibit degeneration after injury (Lunn et al., 1989). This gain-of-function phenotype for *nmnat* bears a striking resemblance to the *hiw* loss-of-function phenotype in its ability to delay the onset of Wallerian degeneration.

There is only one *nmnat* gene in *Drosophila* and it has been shown to be required for neural integrity (Fang et al., 2012; Wen et al., 2011; Zhai et al., 2006). To disrupt expression of this essential gene post-embryonically, we used the Gal4/UAS system to express double-stranded RNA (Dietzl et al., 2007) targeting *nmnat*, (*UAS-nmnat-RNAi)*, in neurons. Immunostaining with an anti-Nmnat antibody (Zhai et al., 2006) indicated that the knockdown of Nmnat was effective (Figure 4.5.A); however, it was unlikely to be complete, since neuronal clones that are homozygous mutant for *Nmnat* undergo spontaneous degeneration in uninjured animals (Wen et al., 2011; Zhai et al., 2006). In contrast, RNAi-mediated knockdown of *nmnat* in larva motoneurons did not affect the development or stability of axons and synapses (Figure 4.5.B), and only modestly affected the time course of degeneration after injury (Figure 4.5.D). However knockdown of *nmnat* strongly suppressed the *hiw* protective phenotype, both in axons

(Figure 4.5.C,D) and NMJ synapses (Figure 4.5.E,F). Similarly, reduction of Nmnat also suppressed the protection from degeneration caused by overexpression of *UBP2* (Figure 4.10.C,D). These results suggest that Nmnat function is an important component of Hiw's role in the degeneration process. Interestingly the NMJ synaptic overgrowth phenotype of the *hiw* mutants was not suppressed by RNAi knockdown of *nmnat* (Figure 4.5.E,G). This implies that Hiw regulates synaptic morphology independently of Nmnat function, or at least through a mechanism that is less sensitive to Nmnat function than degeneration. In contrast, Wnd is required for synaptic overgrowth in *hiw* mutants, and data presented below suggest that Nmnat and Wnd function independently.

Wallenda and Nmnat Function in Parallel Downstream of Highwire

To further probe the relationship between Wnd and Nmnat, we conducted genetic epistasis analysis. Overexpression (*O/E*) of either *wnd* or *nmnat* cDNA can delay Wallerian degeneration in *Drosophila* motoneurons (Figure 4.6.), so we tested whether the phenotype of *O/E nmnat* required *wnd*, and vice versa, whether the phenotype of *O/E wnd* required *nmnat*.

We found that disruption of *wnd* function had no effect upon the protection from degeneration by *O/E nmnat* (Figure 4.6.A,B). For the converse experiment, we tested whether knockdown of *nmnat* by expression of UAS-*nmnant-RNAi* affected the protection by *O/E wnd* (Figure 4.6.C,D). While this method for disrupting Nmnat suppressed the *hiw* degeneration phenotype (Figure 4.5.), it had no effect upon the *O/E*

wnd phenotype (Figure 4.6.C,D). These observations suggest that Nmnat and Wnd protect axons from degeneration through independent mechanisms.

We then tested whether knockdown of *nmnat* and *wnd* by RNA interference had additive effects in suppressing the *hiw* degeneration phenotype (Figure 4.7.). Since *nmnat-RNAi* rescues the *hiw* phenotype very strongly on its own at 24 h after injury, we assayed earlier time points, 12 and 18 h after injury, for additive effects with *wnd-RNAi*. Expression of *wnd-RNAi* alone in the *hiw* mutant background caused 42% of the NMJs to degenerate (including complete degeneration and partial degeneration) within 18 h of injury, while expression of *nmnat-RNAi* alone caused 59% of the *hiw* mutant NMJs to degenerate at this time point. Combined knockdown of both *nmnat* and *wnd* led to a nearly complete suppression of the *hiw* degeneration phenotype, with 92% of the NMJs degenerating (Figure 4.7.A,B). Together, these results suggest that Wnd and Nmnat function independently downstream of Hiw in the Wallerian degeneration process (Figure 4.7.C).

Highwire Regulates the Levels of Nmnat Protein

Hiw and its homologues are known to function within an E3 ubiquitin ligase complex (DiAntonio et al., 2001; Han et al., 2008; Liao et al., 2004; Nakata et al., 2005; Saiga et al., 2009; Wu et al., 2007). An attractive hypothesis is that Hiw promotes ubiquitination and protein turnover of endogenous Nmnat protein. Consistent with this hypothesis, we found that knockdown of *nmnat* suppressed the protection from degeneration caused by overexpression of the de-ubiquitinating enzyme *UBP2* (Figure 4.10.C,D). We therefore asked whether mutation in *hiw* leads to an increase in the

levels of Nmnat protein. Most strikingly, we noticed an appearance of Nmnat protein in the synapse and neurite-rich neuropil of *hiw* mutants, which was not detectable in a wild-type background (Figure 4.8.A,B). We also observed complex changes in the distribution of Nmnat in neuronal nuclei and glia (Figure 4.8.C).

To test whether Hiw regulates Nmnat in neurons via a post-transcriptional mechanism, we drove expression of transgenic HA-tagged *nmnat* cDNA in neurons via an ectopic Gal4/UAS promoter. In *hiw* mutants, the total level of HA-Nmnat protein, as detected on Western blots, increased in both larval brains (3.1 ± 0.6-fold) and adult heads (5.2 ± 1.1-fold) (Figure 4.9.A). By immunocytochemistry, the HA-Nmnat protein (which represents a splice form that lacks the nuclear localization sequence could readily be detected in motoneuron cell bodies (Figure 4.9.B,E) and axons within segmental nerves (Figure 4.9.C,F), but is barely detectable at NMJ synapses (Figure 4.9.D,G). In *hiw* mutants, the levels of HA-Nmnat increased in all compartments, however the 5-fold increase quantified at NMJ synapses was most striking (Figure 4.9.E-G). The increase in Nmnat protein levels remained in the *hiw;wnd* double mutant background (Figure 4.9.E-G), adding further support to the model that Hiw regulates Nmnat protein independently of Wnd.

Highwire Regulates Nmnat Via Ubiquitination

The *hiw* mutation led to an increase in the levels of transgenic Nmnat, which was expressed via the ectopic Gal4/UAS promoter. We confirmed that the *hiw* mutation did not increase expression from the different Gal4 drivers used (ppk-Gal4, OK6-Gal4, and BG380Gal4, unpublished data). Hence the regulation of Nmnat by Hiw takes place post-

transcriptionally. To test whether Nmnat is regulated by ubiquitination, we overexpressed the yeast ubiquitin protease *UBP2* in neurons, which can counteract the function of ubiquitin ligases (Baker et al., 1992; Kuo et al., 2005). We found that co-expression of *UBP2* in neurons with the *HA-nmnat* transgene caused an increase in the levels of HA-Nmnat protein (Figure 4.10.A,B), resembling the *hiw* mutant (Figure 4.9.). This suggests that the levels of *Drosophila* Nmnat are controlled by ubiquitination.

We next tested whether the action of the Hiw E3 ubiquitin ligase is sufficient to modify Nmnat protein level in axons and synapses. Co-overexpression of *hiw* cDNA (*O/E hiw*) with *HA-nmnat* caused a strong decrease in HA-Nmnat protein in motoneuron axons (Figure 4.11.A,B). Because Nmnat protein was difficult to detect at the NMJ (Figure 4.9.D), we also examined the nerve terminals of class IV sensory neurons, whose concentrated location in the ventral nerve cord was easier to visualize. *O/E hiw* caused a reduction in HA-Nmnat protein in sensory axon terminals (Figure 4.11.C,D). In contrast, co-expression of the dominant negative *hiw-ΔRING* mutation caused an increased level of HA-Nmnat in the sensory axon terminals (Figure 4.11.C,D). Further evidence that Hiw function is sufficient to down-regulate Nmnat comes from studies in S2R+ cells, which do not express Hiw endogenously. Co-expression of Hiw, but not of Hiw-ΔRING, led to down-regulation of HA-Nmnat protein (Figures 4.12.A and 4.13.A). These findings suggest that Hiw plays a direct role in regulating the levels of Nmnat protein.

Curiously, we were unable to obtain evidence that Hiw down-regulates Nmnat via the UPS. Inhibition of the proteasome by addition of MG132, using several different concentrations and periods of time that affect known targets to the UPS (Materials and

Methods), did not affect the down-regulation of Nmnat by Hiw in S2R+ cells (Figure 4.13.A). To inhibit the proteasome in vivo we co-expressed dominant-negative proteasome subunit mutations, *DTS5* and *DTS7*, which in previous studies had been shown to lead to allow targets of the UPS to accumulate (Belote and Fortier, 2002; Pandey et al., 2007; Speese et al., 2003). This led to only minor (7%) changes in the levels of HA-Nmnat in sensory neuron terminals (Figure 4.13.B). Surprisingly, inhibition of the proteasome had a much greater effect upon HA-Nmnat levels in a *hiw* null mutant than in a wild-type background (Figure 4.13.C). This observation does not favor a simple model that Hiw regulates Nmnat via the UPS. Instead, the data suggest that additional ubiquitin ligases may regulate Nmnat, and that the regulation of Nmnat may be more sensitive to the UPS when *hiw* is absent.

While the above data indicate that ubiquitination is important for the regulation of Nmnat, the detailed mechanism by which Hiw regulates Nmnat remains to be determined. The mechanism may involve a direct interaction, since co-immunoprecipitation experiments indicate that Nmnat can robustly interact with the enzyme dead Hiw-ΔRING protein in S2R+ cells (Figure 4.12.B).

Highwire Promotes Destruction of Nmnat in the Distal Stump of Injured Axons

A recent study using vertebrate cultured neurons suggested that the disappearance of Nmnat2, which has a short half-life, from the distal stump of axons may serve as a trigger for the Wallerian degeneration process (Gilley and Coleman, 2010). This leads to an attractive hypothesis that Hiw promotes the disappearance of Nmnat protein from the distal stump. Supporting this model, we observed that HA-

Nmnat levels become significantly reduced in axons (Figure 4.15.A) and synapses (Figure 4.14.A) distal to the injury site. In contrast, HA-Nmnat levels increase in the proximal stump after injury (Figure 4.15.A), consistent with the model that a cytoplasmic form of this enzyme is transported in axons from the cell body (Gilley and Coleman, 2010). Within 4 h after injury, the majority of HA-Nmnat in sensory axon terminals had disappeared (Figure 4.14.B). By comparison, a significant amount of green fluorescent protein (GFP)-Hiw remained at this time point (Figure 4.15.B).

When *hiw* was mutant, the levels of HA-Nmnat in the distal stump did not decrease significantly below its starting level, even 24 h after injury (Figure 4.14.A,B). Expression of *UBP2* had a similar effect upon HA-Nmnat in the distal stump after injury (Figure 4.14.A,B). These findings indicate that Hiw and the ubiquitination are required for the disappearance of Nmnat protein in the distal stump.

Highwire Can Specifically Down-regulate Mouse Nmnat2 Protein in *Drosophila* Neurons

Vertebrates utilize three distinct Nmnat enzymes, which localize to distinct subcellular locations. We tested whether Hiw was capable of influencing the levels of ectopically expressed mouse Nmnat1, which localizes to nuclei, mouse Nmnat2, which co-localizes with golgi and late endosome markers, or mouse Nmnat3, which localizes to mitochondria (Berger et al., 2005), by crossing UAS-mNmnat1::myc, UAS-mNmnat2::myc, and UAS-mNmnat3::myc transgenes (Avery et al., 2012; Avery et al., 2009) into the *hiw* mutant background. Intriguingly, mutations in *hiw* resulted in increased levels of mNmant2-myc protein within axons and synaptic terminals of class

IV sensory neurons (Figure 4.16.). This finding implies that mNmant2-myc protein can be transported to distal axons and synapses, and that mouse Nmnat2 shares a conserved protein feature with *Drosophila* Nmnat that allows it to be regulated by Hiw. In contrast, loss of *hiw* had no effect upon the levels of mNmnat1 or mNmnat3. We interpret that the distinct subcellular localization of mNmnat2 may make this protein more susceptible to regulation by Hiw, and that that a conserved mechanism, involving Hiw homologues, may regulate Nmnat2 in vertebrate neurons.

4.5 Discussion

Highwire Promotes Degeneration by Down-regulating Nmnat Protein

Since the discovery of the dramatic inhibition of degeneration by the *WldS* mutation, many studies have focused upon the action of the NAD+ biosynthetic enzyme isoforms, Nmnat1, Nmnat2, and Nmnat3, which in some circumstances can confer protection against axonal degeneration (reviewed in (Coleman and Freeman, 2010; Wang et al., 2012)). Most of these studies involve gain-of-function overexpression experiments; it has been difficult to address the role of endogenous Nmnat enzymes in this process. Recent observations indicate that endogenous Nmnat activity plays an essential role in neuronal survival, and its depletion leads to neurodegeneration (Fang et al., 2012; Gilley and Coleman, 2010; Wen et al., 2011; Zhai et al., 2006). In addition, recent studies in vertebrate neurons suggest that the cytoplasmic isoform, Nmnat2, has a short half-life in neurons (Gilley and Coleman, 2010). An attractive model proposes that Nmnat2 is rapidly turned over in axons, and that its loss in the distal stump of an

axon, which has become disconnected from its cell body, leads to the initiation of Wallerian degeneration (Gilley and Coleman, 2010).

Some aspects of this model are supported by our current in vivo characterization in *Drosophila*. We have identified Hiw, a highly conserved protein with features of an E3 ubiquitin ligase, as an important regulator of Wallerian degeneration. Hiw's role in this process involves the Nmnat protein, whose levels in axons and synapses are regulated post-transcriptionally by Hiw function. In *hiw* mutants, Wallerian degeneration is strongly inhibited, and the increased level of Nmnat protein in *hiw* mutants is both required and sufficient to inhibit degeneration.

The Relationship of Highwire and the UPS

Studies almost a decade ago suggested a role for the UPS in the initiation of Wallerian degeneration (Zhai et al., 2003). It is tempting to propose that this role is manifested by the regulation of Nmnat by Hiw. However our observations caution against a simple interpretation that Hiw regulates Nmnat via the UPS, since Hiw can promote disappearance of Nmnat protein in cells in a manner unaffected by proteasome inhibitors (Figure 4.13.A). Moreover, in vivo, inhibition of the proteasome had only a minor effect upon Nmnat levels in a wild-type background (Figure 4.13.B,C). However in *hiw* mutants, Nmnat levels were very sensitive to the function of the proteasome (Figure 4.13.C). We interpret that additional ubiquitin ligases and the UPS may regulate Nmnat independently of Hiw.

Regardless of the role of the proteasome, our observations suggest that ubiquitin plays an important role in Nmnat regulation. Overexpression of the yeast de-ubiquitinating protease *UBP2* leads to increased levels of Nmnat protein and inhibition of Wallerian degeneration, in a manner that requires endogenous Nmnat (Figure 4.10.). Future studies of the mechanism by which Hiw regulates Nmnat will therefore consider potential proteasome-independent roles of ubiquitination. We should also consider the possibility that Hiw regulates Nmnat indirectly: since we have thus far been unable to detect any ubiquitinated Nmnat species, it is possible that an intermediate, yet unknown, regulator of Nmnat may be the actual substrate of ubiquitination. Nevertheless, co-immunoprecipitation studies from S2R+ cells indicate that Hiw and Nmnat have the capacity to interact (Figure 4.12.B).

Highwire Can Regulate Mouse Nmnat2

The mechanism and cellular location of Nmnat's protective action is a highly debated subject. Observations in the literature point to both NAD+-dependent and NAD+-independent models for the strong protection by the *WldS* mutation (Coleman and Freeman, 2010). The location of its protective action may be the mitochondria, since mitochondrially localized Nmnat can protect axons from degeneration (Avery et al., 2012; Avery et al., 2009; Yahata et al., 2009). However golgi/endosomal localized Nmnat2 can also be protective (Fang et al., 2012; Feng et al., 2010; Gilley and Coleman, 2010; Ljungberg et al., 2012). Our findings suggest that mutation of *hiw* leads to an increase in the pool of endogenous Nmnat that functionally impacts degeneration.

While the site of endogenous Nmnat function during axonal degeneration remains to be identified, we found that the levels of ectopically expressed mouse Nmnat2 were specifically increased in the *hiw* mutant background. In contrast, the levels of nuclearly localized mNmnat1 or mitochondrially localized mNmnat3 were unaffected by Hiw. Since Nmnat2 has a short half-life in vertebrate neurons (Gilley and Coleman, 2010), it is intriguing to propose that it is regulated by Hiw orthologs via an analogous mechanism.

Since Nmnat2 does not appear to localize to mitochondria, does this favor a non-mitochondrial activity, such as function as a chaperone (Ali et al., 2012; Zhai et al., 2008), for the protective action. It remains challenging to determine the exact location of protection, since the most apparent changes in Nmnat protein may not necessarily be the functionally relevant changes.

Multiple Roles of Highwire in Responses to Injury

A previously characterized target of Hiw regulation is the Wnd MAP kinase kinase kinase (Collins et al., 2006; Nakata et al., 2005). This axonal kinase is also capable of inhibiting Wallerian degeneration in motoneurons (Xiong and Collins, 2012). The protective action of Wnd requires a downstream signaling cascade and changes in gene expression mediated by the Fos transcription factor (Xiong and Collins, 2012). Loss of *nmnat* does not affect this signaling cascade (unpublished data) nor does it affect the protective action of Wnd (Figure 4.6.C,D). Conversely, loss of *wnd* does not affect the protection caused by overexpressing *nmnat* (Figure 4.6.A,B). Importantly, the regulation of Nmnat by Hiw does not appear to require Wnd function, and Wnd and

Nmnat can protect axons independently of each other. These findings favor the model that Wnd and Nmnat are both regulated by Hiw and influence axonal degeneration through independent mechanisms.

The Wnd kinase plays additional roles in neurons, which can be genetically separated from Nmnat function. These include regulation of synaptic growth: a dramatic synaptic overgrowth phenotype in *hiw* mutants is fully suppressed by mutation of *wnd*, but is not at all affected by knockdown of *nmnat* (Figure 4.5.G). Wnd/DLK also promotes axonal sprouting in response to axonal injury (Xiong et al., 2010), which is also unaffected by *nmnat* knockdown (unpublished data). It is therefore clear that by regulating both Wnd and Nmnat, Hiw regulates multiple independent pathways in neurons.

It is intriguing that the actions of both Wnd and Nmnat promote cellular responses to axonal injury. Axonal regeneration requires an initiation of a growth program within the axon, which depends upon the function of Wnd and its homologues (Hammarlund et al., 2009; Shin et al., 2012; Xiong et al., 2010; Yan et al., 2009). Equally important is a clearance of the distal stump to make room for the regenerating axon (Bisby and Chen, 1990; Brown et al., 1992; Martin et al., 2010). The actual location in which Hiw regulates Nmnat remains to be determined. As an upstream regulator of both sprouting in the proximal stump and degeneration of the distal stump, Hiw may play a central role in regulating the ability of a neuron to regenerate its connection after injury.

Importantly, the protective action of Nmnat may not be limited to Wallerian degeneration. The *WldS* mutation can protect neurons from degeneration in a wide

variety of paradigms, from models of neurodegenerative disease, diabetic neuropathy, excitotoxity, and loss of myelination (Coleman and Freeman, 2010; Wang et al., 2012). These findings suggest that action and regulation of Nmnat function is broadly important for neuronal function and maintenance. As a critical regulator of Nmnat, the Hiw ubiquitin ligase and its vertebrate homologues deserve further scrutiny for potential roles in human health and disease.

4.6 Figures

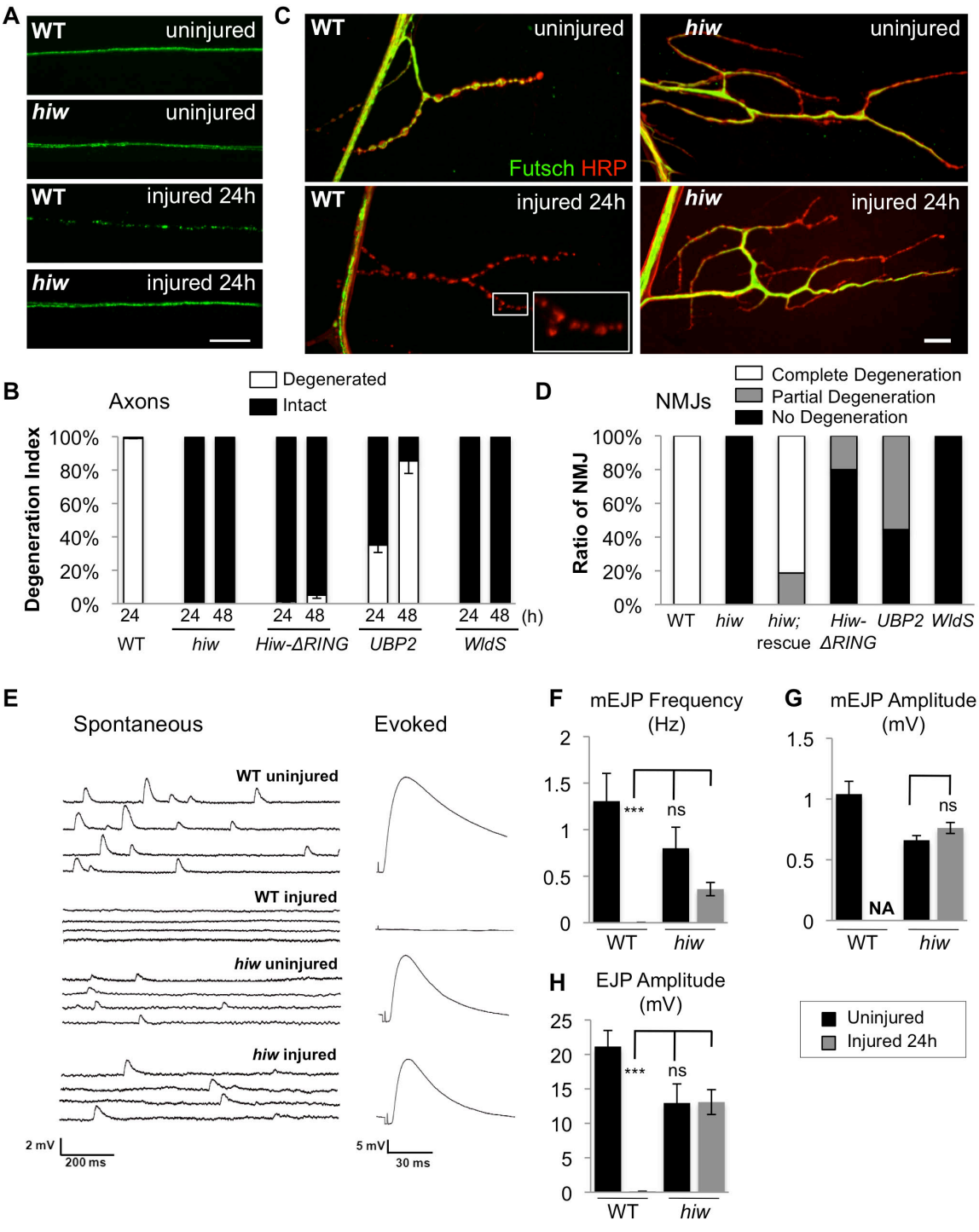

A
WT uninjured
hiw uninjured
WT injured 24h
hiw injured 24h
C
WT uninjured
hiw uninjured
Futsch HRP
WT injured 24h
hiw injured 24h
B
Axons
Degenerated
Intact
Degeneration Index
100%
80%
60%
40%
20%
0%
24
24 48
24 48
24 48
24 48 (h)
WT
hiw
Hiw-ΔRING
UBP2
WldS
D
NMJs
Complete Degeneration
Partial Degeneration
No Degeneration
Ratio of NMJ
100%
80%
60%
40%
20%
0%
WT
hiw
hiw; rescue
Hiw-ΔRING
UBP2
WldS
E
Spontaneous
Evoked
WT uninjured
WT injured
hiw uninjured
hiw injured
2 mV
200 ms
5 mV
30 ms
F
mEJP Frequency (Hz)
2
1.5
1
0.5
0
***
ns
WT
hiw
G
mEJP Amplitude (mV)
1.5
1
0.5
0
ns
NA
WT
hiw
H
EJP Amplitude (mV)
25
20
15
10
5
0
***
ns
WT
hiw
Uninjured
Injured 24h

Figure 4.1 Mutations in *hiw* strongly delay Wallerian degeneration of motoneuron axons and synapses after injury.

(A) In *Drosophila* third instar larvae, single axons are labeled by expression of *UAS-mCD8::GFP* with the *m12-Gal4* driver. In a wild-type (WT) background, axons distal to the injury site have completely degenerated within 24 h after nerve crush injury, however axons remain intact in the $hiw^{\Delta N}$ mutant background.
(B) Quantification of axon degeneration index in different genotypes. (See Materials and Methods and (Xiong and Collins, 2012) for quantification methods). The degeneration index (percent degenerated) is shown with white bars, while black bars show the complementary percentage (percent intact). The genotypes are: (*UAS-mCD8::GFP/+; m12-Gal4/+*), ($hiw^{\Delta N}$*;UAS-mCD8::GFP/+; m12-Gal4/+*), (*UAS-mCD8::GFP/+; m12-Gal4/UAS-hiw-ΔRING*), (*UAS-mCD8::GFP/UAS-UBP2; m12-Gal4/+*), (*UAS-mCD8::GFP/+; m12-Gal4/UAS-WldS*).
(C) Representative images of muscle 4 NMJs in wild-type (WT) or $hiw^{\Delta N}$ mutants 24 h after injury. In WT animals, the presynaptic marker Futsch (green) completely disappears, while the neuronal membrane, labeled with antibodies to HRP (red), remain in discontinuous fragments. In contrast, NMJs in the *hiw* mutant (which are overgrown in an uninjured animal (Wan et al., 2000)) remain continuous and intact after injury. Of note, Futsch staining does not completely cover some synaptic branches in *hiw* mutant, but quantification of the extent of Futsch coverage (as in (Xiong and Collins, 2012)) shows no significant difference between injured and uninjured *hiw* mutants (unpublished data).
(D) Quantification of NMJ degeneration. White bars represent percentage of NMJs that have completely degenerated, defined by a complete loss of Futsch staining from the NMJ. Gray bars represent the percentage of NMJs that are partially degenerated, defined by a partial fragmentation Futsch staining and neuronal membrane (see Materials and Methods). Black bars represent the percentage of NMJs that are intact. The genotypes are: *(Canton S)*, $(hiw^{\Delta N})$, $(hiw^{ND8}$*, BG380-Gal4; UAS-hiw/+)*, *(BG380-Gal4;; UAS-hiw-ΔRING/+)*, *(BG380-Gal4; UAS-UBP2/+)*, *(BG380-Gal4;; UAS-WldS/+)*.
(E) Representative traces of evoked and spontaneous neurotransmitter release recorded from wild-type *(Canton S)* and *hiw* mutant $(hiw^{\Delta N})$ larvae before or 24 h after injury. Calibration: 200 ms, 2 mV for spontaneous release; 30 ms, 5 mV for evoked release. (F–H) Histograms showing (F) average spontaneous miniature EJP frequency, (G) spontaneous miniature EJP amplitude, and (H) evoked EJP amplitude, either from uninjured (black bars) or injured (24 h after injury, gray bars), in *Canton S* (WT) or $hiw^{\Delta N}$ larvae. n = 10 recordings for each genotype. In WT injured larvae, only one single miniature event (amplitude 2 mV) was observed in all ten recordings. Of note, in uninjured larvae the amplitudes of evoked and miniature EJPs were smaller in *hiw* mutant, as previously reported (Wan et al., 2000).
Scale bars = 12.5 μm, error bars represent standard error; ***$p < 0.001$; ns, not significant, $p > 0.05$ in t-test. (Figure 4.1.E-H were prepared by Bibhudatta Mishra)

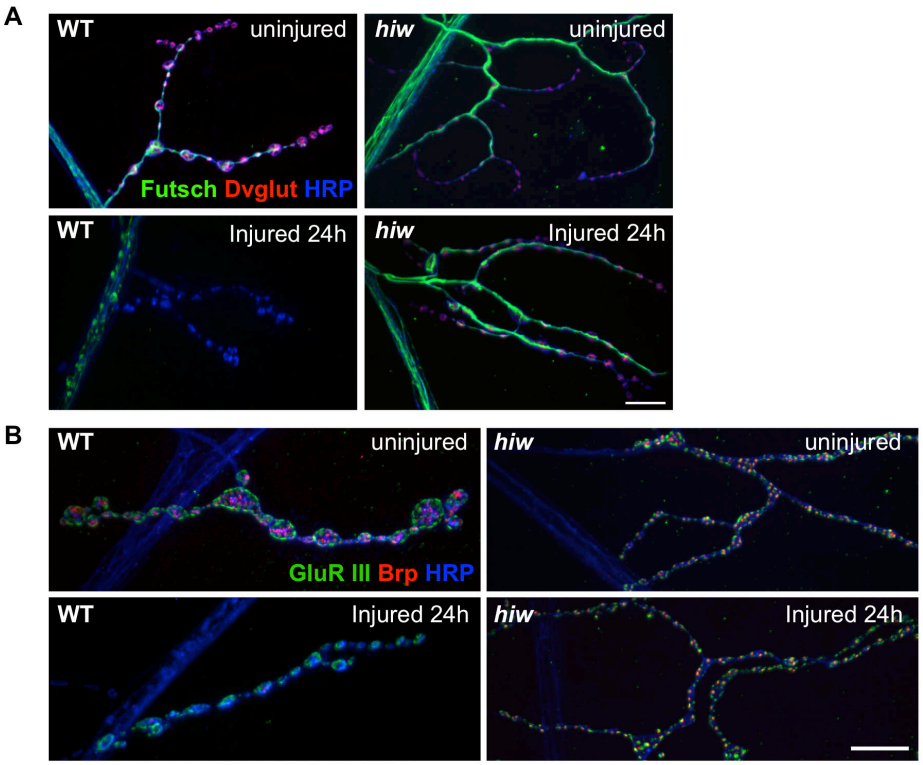


Figure 4.2 Synaptic markers remain intact in *hiw* mutants after injury.

(A) Representative muscle 4 NMJs for WT (*Canton S*) or *hiw* (*hiw*$^{\Delta N}$) mutants stained for Futsch (green), Dvglut (synaptic vesicles, red), and HRP (neuronal membrane, blue). (B) NMJs are stained for GluRIII (post-synaptic GluR receptor subunit [49], green), Brp (pre-synaptic active zones, red) and HRP (axonal membrane, blue), before or 24 h after injury. While *hiw* mutants have reduced Dvglut staining (Xiong et al., 2010) and smaller synaptic Brp and GluRIII puncta, there is no noticeable difference between the uninjured and injured NMJs.
Scale bars = 12.5 μm

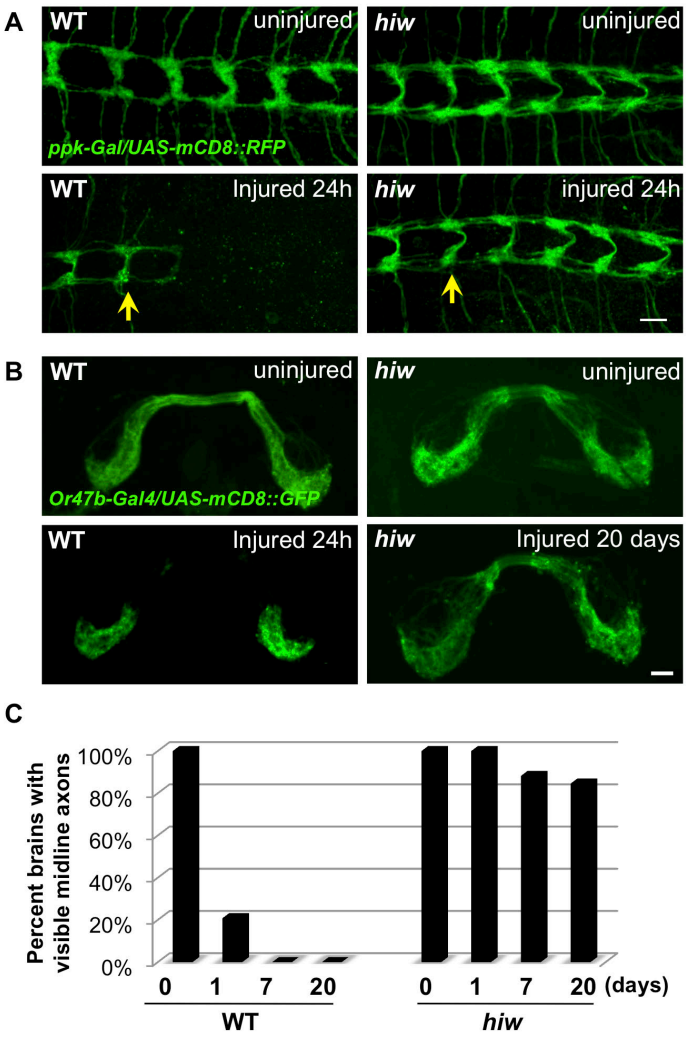


Figure 4.3 Wallerian degeneration in neurons of different neural types and developmental stages is strongly arrested in *hiw* mutants.

(A) The nerve terminals of class IV sensory neurons in the ventral nerve cord, visualized by driving *UAS-mCD8::RFP* with *ppk-Gal4*, are completely degenerated and cleared within 24 h after injury in wild-type (WT) animals, however these injured axons remain intact in the $hiw^{\Delta N}$ mutant. (Because the site of injury was in segment A2, all axons whose terminals are to the right of the yellow arrows have been injured).
(B) Olfactory neuron axons in adult flies are labeled by driving expression of *UAS-mCD8::GFP* with *OR47b-Gal4*. These axons degenerate within 1 d after antenna removal in wild-type flies, however in $hiw^{\Delta N}$ mutants these axons remain intact even 20 d after axotomy.
(C) Quantification of the percentage of animals which retain GFP-labeled commissural axons (scored as described in (Hoopfer et al., 2006; MacDonald et al., 2006)), in a time course after axotomy.
Scale bars = 12.5 μm. (Kan Sun helped with dissections for preparing Figure 4.3.B-C)

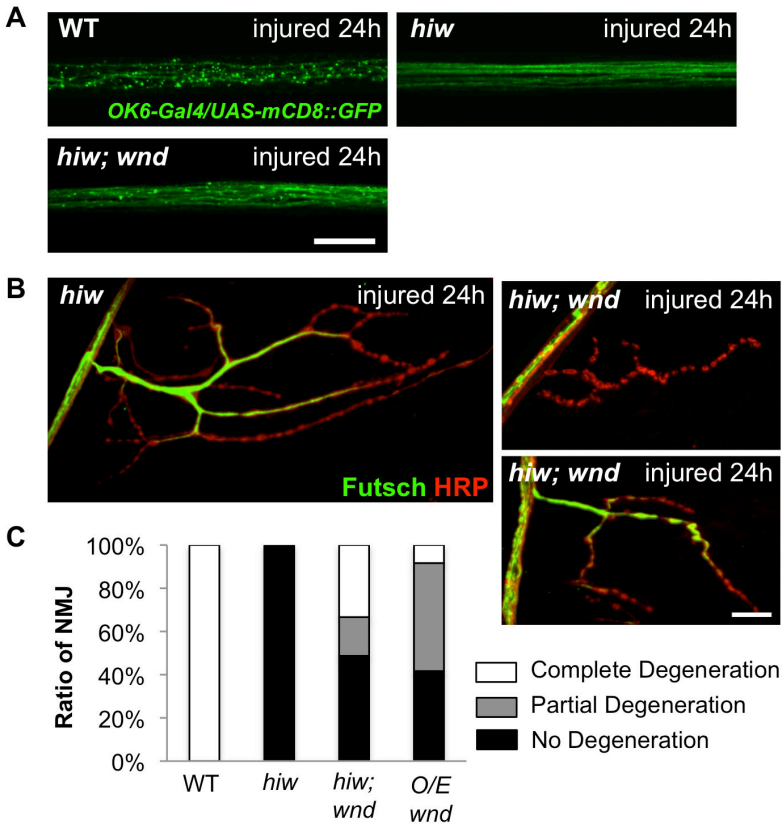


Figure 4.4 Role of the Wnd/DLK MAPKKK in Hiw-regulated degeneration.

(A) *OK6-Gal4, UAS-mCD8-GFP* labeled motoneuron axons (green) are severely fragmented in wild-type (WT) axons 24 h after injury, while they remain intact in *hiw*$^{\Delta N}$ mutants *(hiw*$^{\Delta N}$*; OK6-Gal4/UAS-mCD8::GFP)*. Axons in *hiw; wnd* double mutants (*hiw*$^{\Delta N}$*; OK6-Gal4/UAS-mCD8-GFP; wnd*^{1}*/wnd*^{2}) are only mildly fragmented 24 h after injury, implying that mutation of *wnd* only partially suppressed the *hiw* mutant degeneration phenotype.
(B) Representative muscle 4 NMJs labeled by immunostaining for Futsch (green) and HRP (neuronal membrane, red) in *hiw*$^{\Delta N}$ mutants or *hiw; wnd* double mutants *(hiw*$^{\Delta N}$*;;wnd*^{1}*/wnd*^{2}*)*. At 24 h after injury, NMJs have completely degenerated in wild-type (Figure 1) but are intact in *hiw* mutants. In *hiw;wnd* double mutants, some NMJs have completely degenerated (upper panel), while others remain intact (lower panel).
(C) Quantification of the percentage of NMJs that are completely degenerated, partially degenerated, or intact (see Materials and Methods) for the following genotypes: *(Canton S), (hiw*$^{\Delta N}$*), (hiw*$^{\Delta N}$*;;wnd*^{1}*/wnd*^{2}*), (BG380-Gal4; UAS-wnd/+)*.
Scale bars = 12.5 μm

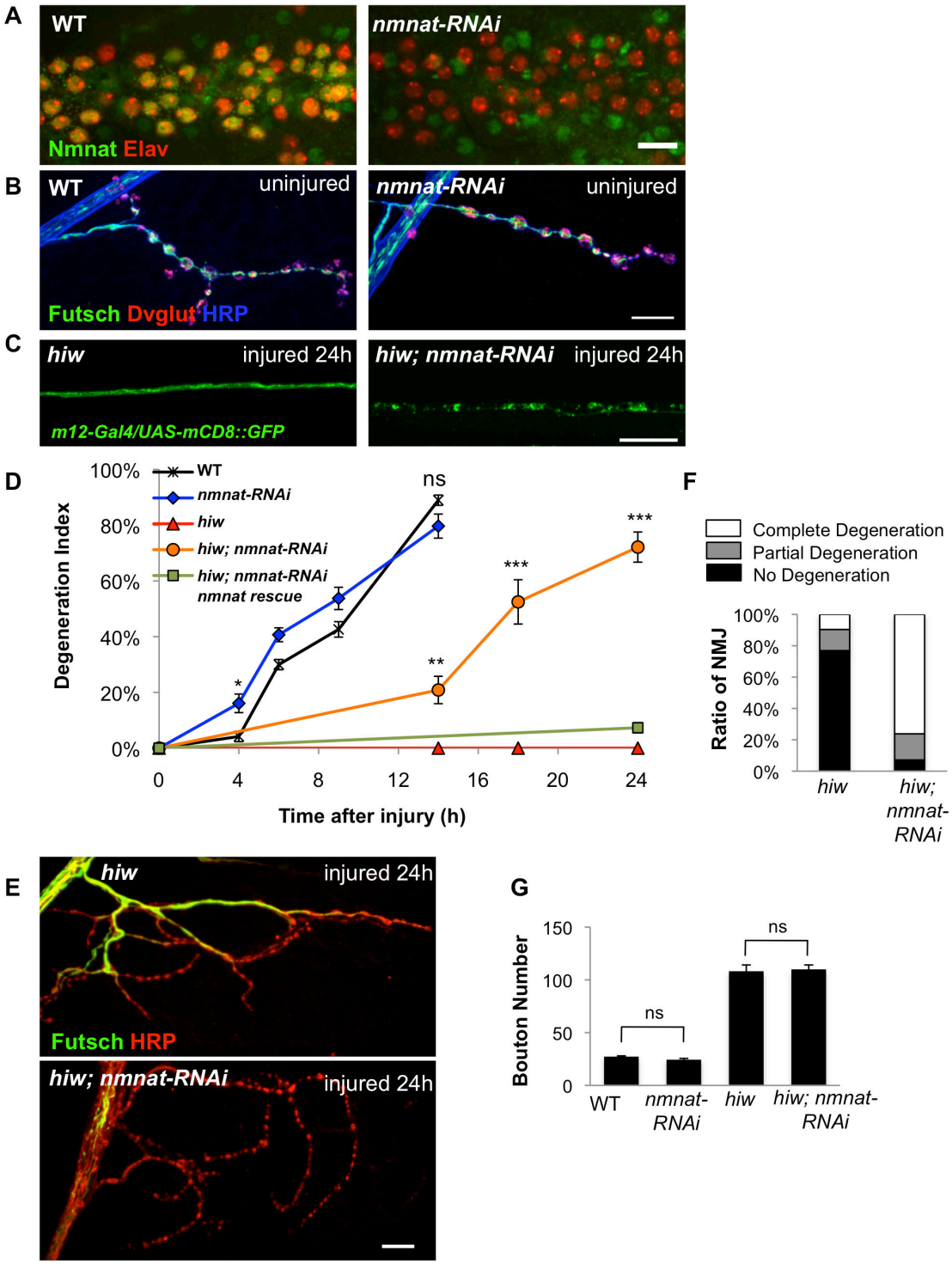

A
WT
Nmnat Elav
nmnat-RNAi
B
WT
uninjured
Futsch Dvglut HRP
nmnat-RNAi
uninjured
C
hiw
injured 24h
m12-Gal4/UAS-mCD8::GFP
hiw; nmnat-RNAi
injured 24h
D
Degeneration Index
100%
80%
60%
40%
20%
0%
WT
nmnat-RNAi
hiw
hiw; nmnat-RNAi
hiw; nmnat-RNAi
nmnat rescue
ns
*
**
***
***
0
4
8
12
16
20
24
Time after injury (h)
F
Complete Degeneration
Partial Degeneration
No Degeneration
Ratio of NMJ
100%
80%
60%
40%
20%
0%
hiw
hiw; nmnat-RNAi
E
hiw
injured 24h
Futsch HRP
hiw; nmnat-RNAi
injured 24h
G
Bouton Number
150
100
50
0
ns
ns
WT
nmnat-RNAi
hiw
hiw; nmnat-RNAi

Figure 4.5 Regulation of Wallerian degeneration by Hiw depends upon endogenous Nmnat .

(A) Depletion of Nmnat in larval motoneurons by expression of *nmnat-RNAi*. Expression of UAS-*nmnat-RNAi* with a pan-neuronal Gal4 driver (*BG380-Gal4, UAS-Dcr2; UAS-nmnat-RNAi/+)* depletes Nmnat staining (green) in neuronal nuclei (marked by co-staining with Elav, red) but not in neighboring glial cells (for which Nmnat staining increased). Quantification of the reduced staining in neuronal nuclei suggested that the Nmnat levels were reduced to 49.3% of wild-type levels in motoneurons ($p < 0.01$, $n = 6.7$).
(B) Depletion of *nmnat* by expression of *nmnat-RNAi* in neurons does not affect NMJ morphology. Representative muscle 4 NMJs stained for Futsch (green), Dvglut (synaptic vesicles, red), and HRP (neuronal membrane, blue). We did not observe spontaneous axonal or synaptic degeneration when *nmnat* was depleted by RNAi, probably because the depletion was not complete
(C) Nmnat is required for the protective phenotype of *hiw. m12-Gal4,* UAS-*mCD8::GFP* labeled axons (green) 24 h after injury in animals either mutant for *hiw* (*hiw^{ND8} ,UAS-Dcr2; UAS-mCD8::GFP/+; m12-Gal4/+*) or mutant for in *hiw* mutant and depleted for *nmnat* by RNA interference *(hiw^{ND8}, UAS-Dcr2; UAS-mCD8::GFP/UAS-nmnat-RNAi; m12-Gal4/+).*
(D) Degeneration index for the *m12-Gal4, UAS-mCD8::GFP* labeled single axons at different time points after injury in the following genotypes: *(UAS-Dcr2; UAS-mCD8::GFP/+; m12-Gal4/+), (UAS-Dcr2; UAS-mCD8-GFP/UAS-nmnat-RNAi; m12-Gal4/+), (hiw^{ND8}, UAS-Dcr2; UAS-mCD8::GFP/+; m12-Gal4/+), (hiw^{ND8}, UAS-Dcr2; UAS-mCD8::GFP/UAS-nmnat-RNAi; m12-Gal4/+), (hiw^{ND8}, BG380-Gal4; UAS-nmnat/UAS-nmnat-RNAi; m12-Gal4,UAS-mCD8::GFP/+)*. RNAi depletion of *nmnat* alone only modestly affects the rate of degeneration (compare blue to black), however it strongly inhibits the protection observed in the *hiw* mutant (compare orange to red).
(E) Representative images of NMJs at muscle 4 24 h after injury in *hiw* mutants *(hiw^{ND8}, BG380-Gal4; UAS-Dcr2/+)* or *hiw* mutants depleted for *nmnat* in neurons *(hiw^{ND8}, BG380-Gal4; UAS-Dcr2/UAS-nmnat-RNAi)*. Futsch staining in green labels cytoskeleton structure and HRP staining in red labels neuronal membrane.
(F) Quantification of the percentage of NMJs that are completely degenerated, partially degenerated or intact in the above genotypes.
(G) Quantification of average bouton numbers per NMJ at muscle 4 in the following genotypes: (*BG380-Gal4, UAS-Dcr2), (BG380-Gal4, UAS-Dcr2; UAS-nmnat-RNAi/+), (hiw^{ND8},BG380-Gal4; UAS-Dcr2/+), (hiw^{ND8}, BG380-Gal4; UAS-Dcr2/UAS-nmnat-RNAi)*. Scale bars = 12.5 μm, error bars represent standard error; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ns, not significant, $p > 0.05$ in *t*-test.

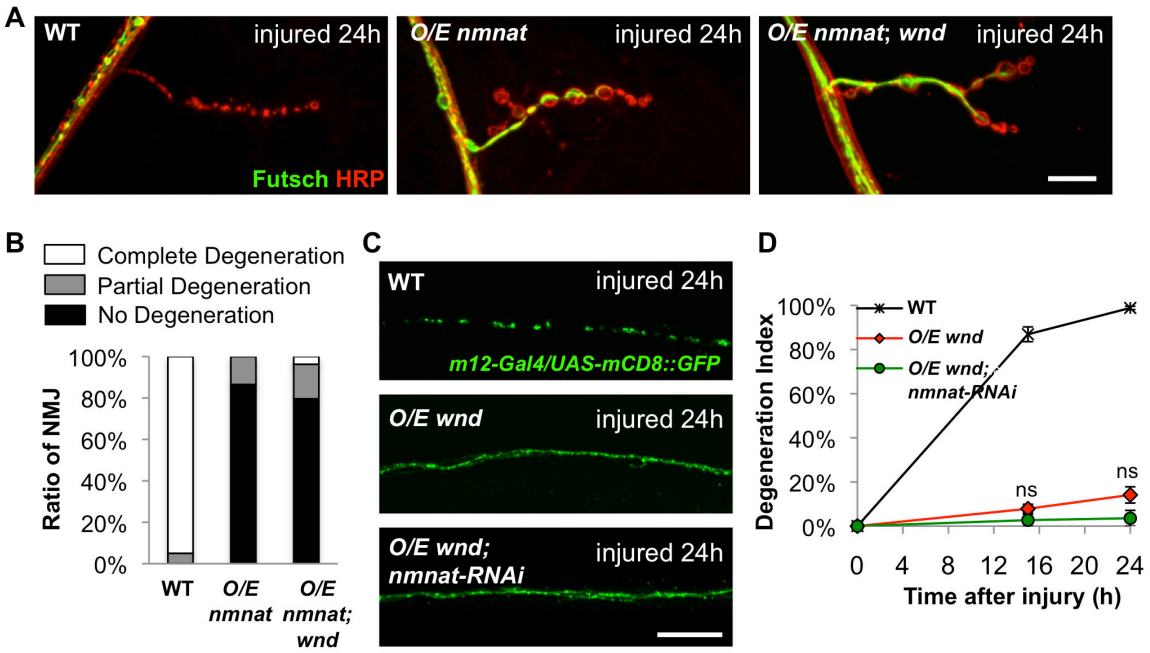


Figure 4.6 Wnd/DLK and Nmnat protect axons through independent mechanisms.

(A) Representative muscle 4 NMJs at 24 h after injury immunostained for Futsch (green) and HRP (neuronal membrane, red) for the following genotypes: WT *(Canton S),* neuronally overexpressed *nmnat (BG380-Gal4; UAS-HA-nmnat/+),* or overexpressed *nmnat* in a *wnd* mutant background *(BG380-Gal4; UAS-HA-nmnat/+; wnd*^{1}*/wnd*^{2}*)*. Overexpression of *nmnat* protected NMJs from degeneration and this protection was not compromised by mutations in *wnd*.
(B) Quantification of NMJ degeneration in the above genotypes.
(C) *UAS-mCD8::GFP/+; m12-Gal4/+* labeled singles axons (green) 24 h after injury in WT *(UAS-Dcr2; UAS-mCD8::GFP/+; m12-Gal4/+)*, when overexpressing *wnd (UAS-Dcr2; UAS-mCD8-GFP /+; m12-Gal4/UAS-wnd)*, or when overexpressing *wnd* in conjunction with *nmnat RNAi (UAS-Dcr2; UAS-mCD8-GFP /UAS-nmnat-RNAi; m12-Gal4/UAS-wnd).* Reducing Nmnat levels by this method had no effect upon the protection caused by overexpression of *wnd*.
(D) Degeneration index of the *m12-Gal4, UAS-mCD8::GFP* labeled single axons at different time points after injury in the above genotypes.
Scale bars = 12. 5μm, error bars represent standard error; ns, not significant, $p > 0.05$ in *t*-test.

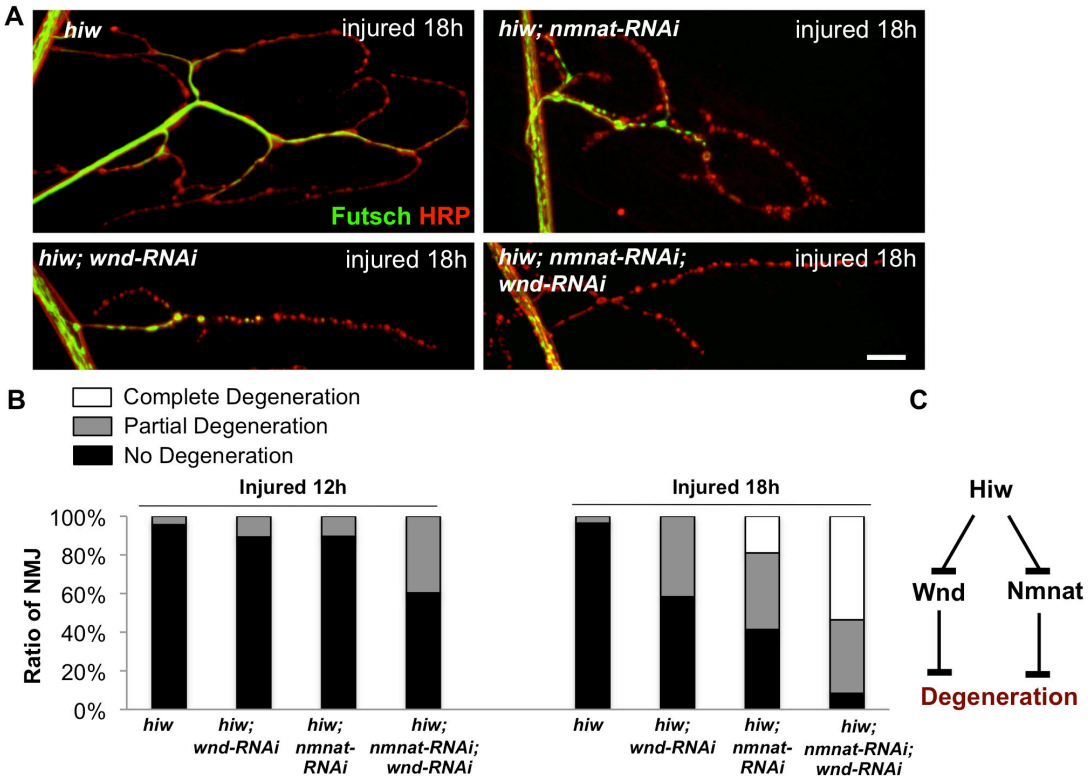


Figure 4.7 Wnd/DLK and Nmnat function in parallel downstream of Hiw.

(A) Representative images of NMJs at muscle 4 18 h after injury stained for Futsch (green) and HRP (red) in the following genotypes: *(hiw^{ND8},BG380-Gal4; UAS-Dcr2/+), (hiw^{ND8}, BG380-Gal4;UAS-Dcr2/+;UAS-wnd-RNAi/+), (hiw^{ND8}, BG380-Gal4; UAS-Dcr2/UAS-nmnat-RNAi), (hiw^{ND8}, BG380-Gal4; UAS-Dcr2/UAS-nmnat-RNAi; UAS-wnd-RNAi/+).*
(B) Quantification of the percentage of NMJs that are completely degenerated, partially degenerated, or intact at 12 h or 18 h after injury, for the genotypes described above.
(C) Model: Wnd and Nmnat inhibit axonal degeneration through independent pathways downstream of Hiw.
Scale bars = 12. 5μm

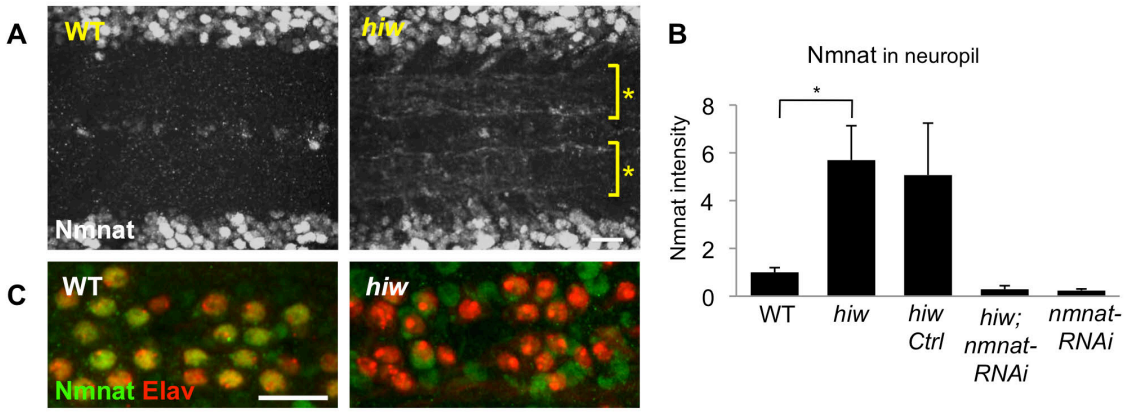


Figure 4.8 Hiw negatively regulates endogenous Nmnat protein levels in neurites.

(A) Hiw regulates endogenous Nmnat protein in neuropil. In *hiw*$^{\Delta N}$ mutants, Nmnat protein can be detected within the neuropil of the ventral nerve cord, denoted with asterisks. This area of the nerve cord is devoid of cell bodies and enriched in neurites and synapses.
(B) Quantification (relative levels) of Nmnat staining in neuropil, for WT (*w118*), *hiw* mutant (*hiw*$^{\Delta N}$), *hiw*,*Ctrl* (*hiw*$^{\Delta N}$, *BG380-Gal4*), *hiw*, *nmnat-RNAi* (*hiw*$^{\Delta N}$, *BG380-Gal4, UAS-nmnat-RNAi*), and *nmnat-RNAi* (*BG380-Gal4, UAS-nmnat-RNAi*). See Materials and Methods.
(C) In *hiw (hiw*$^{\Delta N}$*)* mutants, endogenous Nmnat (green) is reduced in neuronal nuclei. Similarly to the *nmnat RNAi* knockdown in (Figure 4.5.A), Nmnat staining increases in neighboring glial cells.
Scale bars = 12. 5μm, error bars represent standard error; *$p < 0.05$ in *t*-test.
(Figure 4.8.B was prepared by Jiaxing Li)

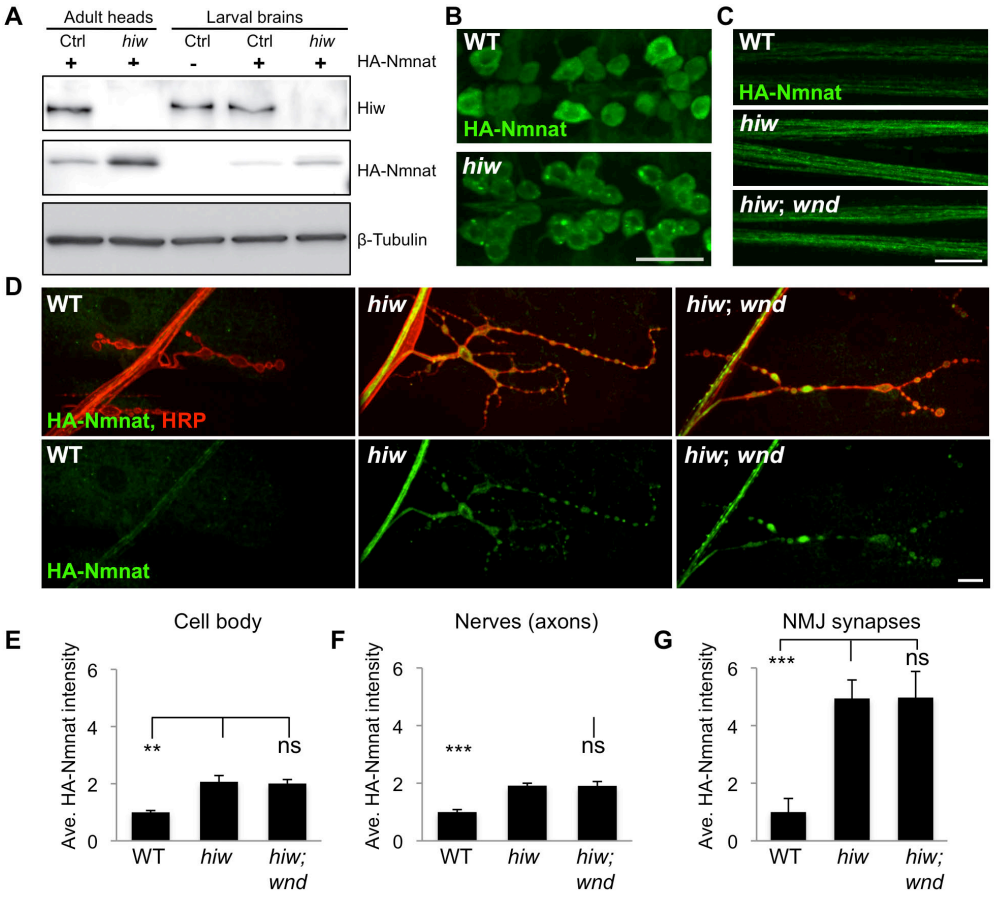


Figure 4.9 Hiw negatively regulates transgenic Nmnat protein levels in axons and synapses

(A) Western blot with adult heads or larval brains to compare total protein levels of HA-Nmnat in wild-type (ctrl) and *hiw* mutant backgrounds. The *UAS-HA::nmnat* transgene is expressed in neurons with the *BG380-Gal4* driver, and males are used for all experiments.

(B–D) The UAS-HA-Nmnat transgene was expressed in motoneurons with the OK6-Gal4 driver, in wild-type *(OK6-Gal4/UAS-HA::nmnat), hiw* mutant (*hiw*$^{\Delta N}$;*OK6-Gal4/UAS-HA::nmnat)* and *hiw; wnd* double mutant *(hiw*$^{\Delta N}$*;OK6-Gal4/UAS-HA::nmnat;wnd*^{1}*/wnd*^{2}*)* backgrounds. HA-Nmnat protein is detected by immunostaining for HA.

(B) Representative images of HA-Nmnat in motoneuron cell bodies, (C) segmental (peripheral) nerves, and (D) NMJ synapses, stained for anti-HA (green) and HRP (neuronal membrane, red).

(E–G) Quantification of the average HA-Nmnat intensity for the above genotypes in (E) cell bodies, (F) segmental nerves, and (G) NMJ synapses at muscle 4. See Materials and Methods for details about quantification methods. In *hiw* mutants, Nmnat intensity is increased, particularly at NMJ synapses. Loss of *wnd*, in *hiw;wnd* double mutants, has no effect upon this increase.

Scale bars = 12.5 μm, error bars represent standard error; **p* < 0.05; ****p* < 0.001; ns, not significant, *p* > 0.05 in *t*-test. (Figure 4.9.A was prepared by Dr. Chunlai Wu and Xia Li)

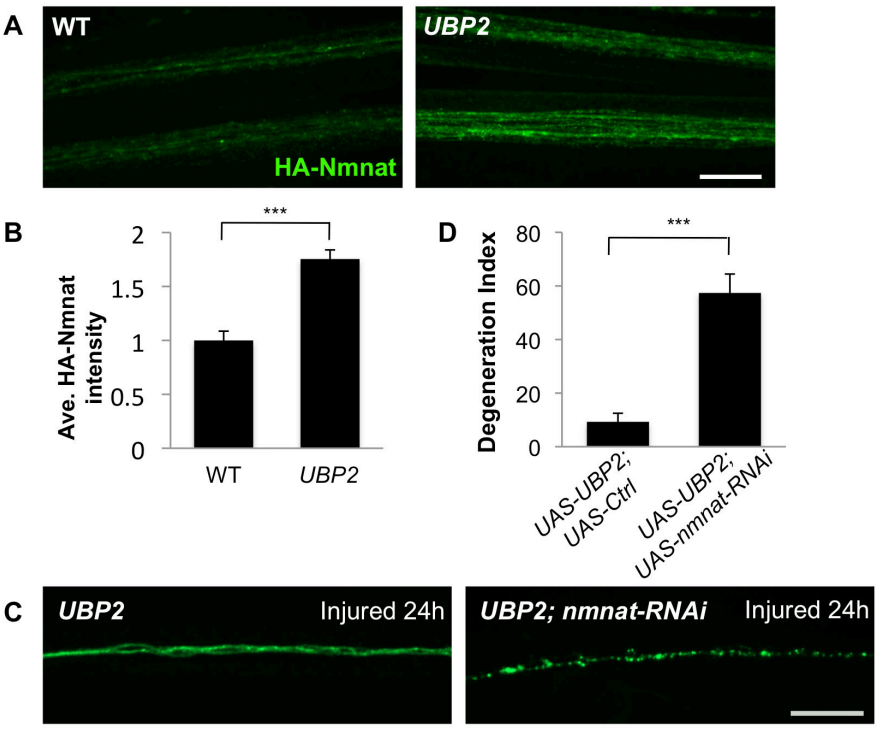


Figure 4.10 Ubiquitination down-regulates Nmnat protein.

(A) The UAS-HA-nmnat transgene is expressed in motoneurons with *OK6-Gal4* driver in a wild-type (WT) genetic background (*OK6-Gal4,UAS-HA::nmnat/+*) or when the yeast deubiquitinase *UBP2* is co-expressed (*OK6-Gal4,UAS-HA::nmnat*/UAS-*UBP2)*. Segmental nerves stained with anti-HA antibody (green).
(B) Quantification of the average HA-Nmnat intensity in segmental nerves.
(C) *m12-Gal4,* UAS-*mCD8::GFP* labeled single axons (green) 24 h after injury in animals co-expressing *UBP2* with a control UAS line *(UAS-Dcr2; UAS-UBP2/+; UAS-nls::DsRed-(Ctrl)/m12-Gal4, UAS-mCD8::GFP)* or co-expressing *UBP2* when with UAS-*nmnat RNAi* to reduce endogenous Nmnat *(UAS-Dcr2; UAS-UBP2/UAS-nmnatRNAi; m12-Gal4, UAS-mCD8::GFP/+)*.
(D) Degeneration index for the *m12-Gal4, UAS-mCD8::GFP* labeled axons for genotypes in (C).
Scale bars = 12.5 μm; error bars represent standard error; ***$p < 0.001$ in *t*-test.

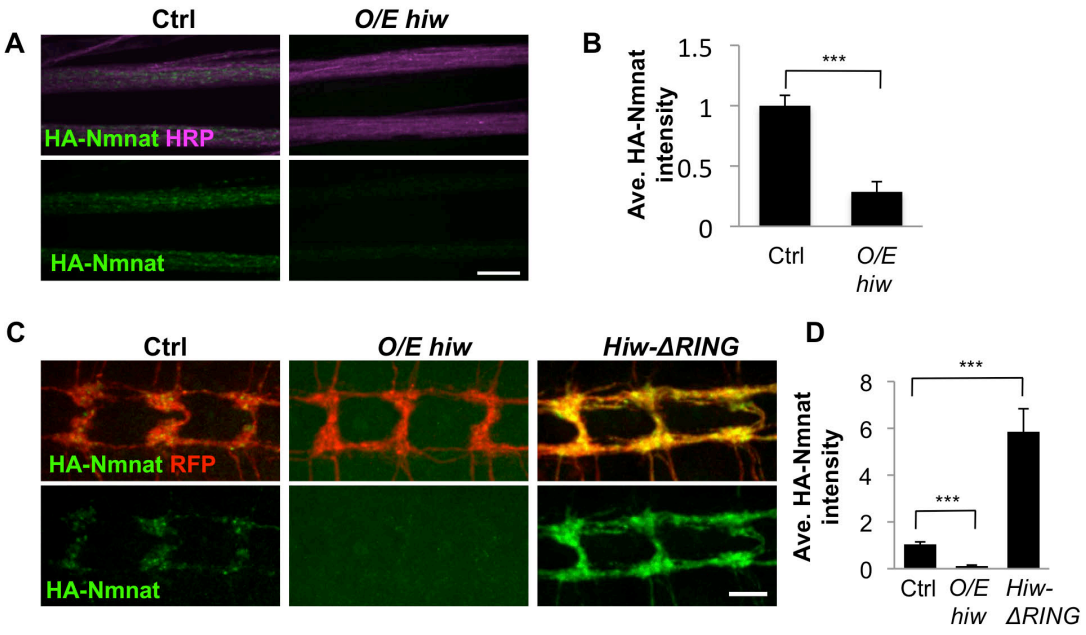


Figure 4.11 Hiw down-regulates Nmnat protein

(A) Transgenic *HA-nmnat* is co-expressed with a control *UAS-* construct *(BG380-Gal4; UAS-HA::nmnat/+; UAS-nls::GFP/+)* or with the full-length *hiw* cDNA *(BG380-Gal4; UAS-HA::nmnat/UAS-hiw)*. Segmental nerves stained for HA (green) and HRP (neuronal membrane, violet).
(B) Quantification of the average HA-Nmnat intensity in segmental nerves in (A). The average intensity is normalized to the control for each experimental group.
(C) Distal axons and axon terminals of *ppk-Gal4,UAS-mCD8::RFP* labeled sensory neurons stained for HA (green) and RFP (red). The UAS-HA-Nmnat transgene is co-expressed with a control *UAS-* construct *(UAS-HA::nmnat/+; ppk-Gal4,UAS-mCD8::RFP/UAS-nls::GFP),* or the full-length *hiw* cDNA *(UAS-HA::nmnat/UAS-hiw; ppk-Gal4,UAS-mCD8::RFP/+),* or a dominant negative *hiw* transgene mutated for conserved cysteines in the RING domain (Wu et al., 2005) *(UAS-HA::nmnat/+; ppk-Gal4,UAS-mCD8::RFP/UAS-hiw-ΔRING).*
(D) Quantification of the average HA-Nmnat intensity in the sensory neuron axon terminals for the above genotypes. See Materials and Methods for the quantification method.
Scale bars = 12.5 μm; error bars represent standard error; ***$p < 0.001$ in *t*-test.

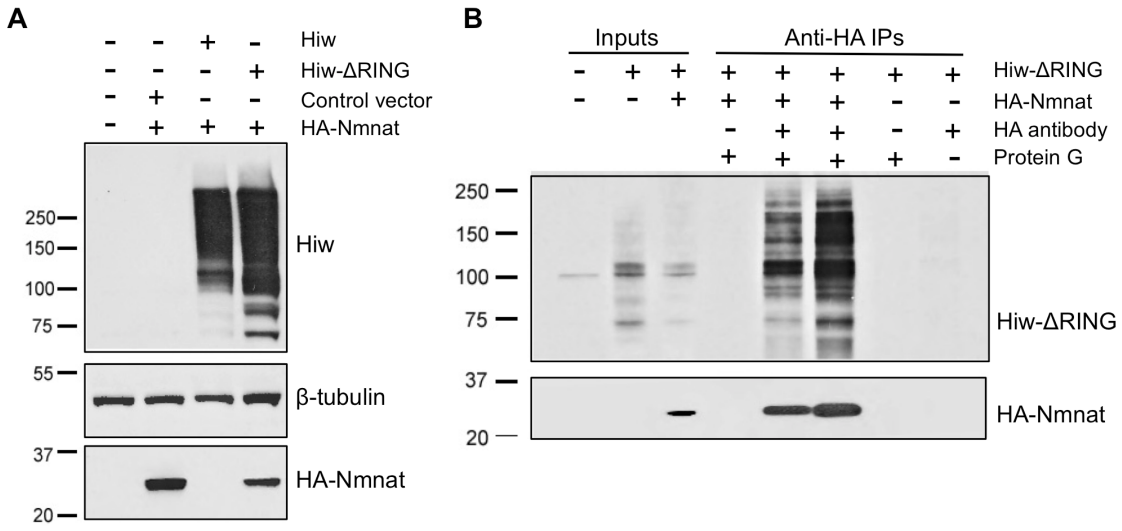


Figure 4.12 Hiw down-regulates and associates with Nmnat protein (Figure 4.12 was prepared by Yan Hao)

(A) Hiw can down-regulate Nmnat protein in S2R+ cells. S2R+ cells were co-transfected with pUAST-HA::Nmnat, and either pUAST-GFP (control vector), pUAST-Hiw, or Hiw-ΔRING. All cells were co-transfected with pMT-Gal4 and induced with 0.5 mM copper sulfate for 24 h. Hiw is not expressed endogenously in S2R+ cells, and numerous breakdown products are observed for the ectopically expressed Hiw protein. The reduction in ectopic HA-Nmnat levels in lane 3 indicates that Hiw is capable of post-transcriptionally regulating Nmnat, and that the RING domain is required for this activity (lane 4).
(B) Nmnat and Hiw-ΔRING form a physical interaction. Co-immunoprecipitation assays were performed from S2R+ cells lysate either co-transfected with pUAST-HA::Nmnat and pUAST-Hiw-ΔRING (lane 5) or mixed lysates from individual pUAST-HA::Nmnat and pUAST-Hiw-ΔRING transfections (lane 6). HA-Nmnat was immunoprecipitated by mouse anti-HA antibody against the HA tag on Nmnat. Despite the fact that Hiw-ΔRING was significantly degraded in S2R+ cell lysate (detected by Western blotting for Hiw antibody), a significant portion of Hiw-ΔRING protein co-immunoprecipitated with HA-Nmnat. The Input lanes (1–3) represent 1/25 of the total extract used for each immunoprecipitation.

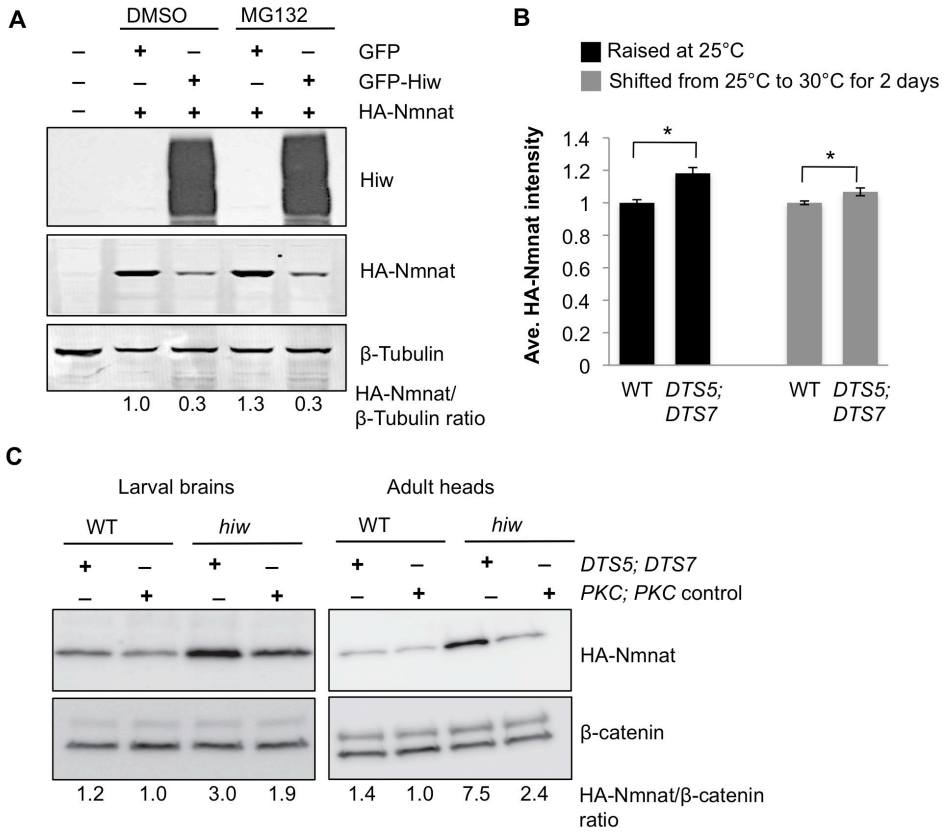


Figure 4.13 Hiw down-regulates Nmnat independently of the UPS.

(A) The decrease in Nmnat levels promoted by Hiw induction in S2R+ cells was not diminished when the proteasome was inhibited. S2R+ cells co-transfected with pUAST-HA::Nmnat and pUAST-GFP or pUAST-GFP::Hiw, then were incubated with DMSO vehicle or 5 μM MG132 for 20 h. The levels of HA-Nmnat are compared by Western blotting for the HA epitope. Relative levels compared to the β-tubulin standard were measured on the LiCor Odyssey system.

(B) Inhibition of the proteasome in sensory neurons only modestly changes HA-Nmnat levels. Average HA-Nmnat intensity in ppk sensory neuron axon terminals was compared between wild-type(*UAS-HA::nmnat/+;ppk-Gal4,UAS-mCD8::RFP /+*), and animals co-expressing *DTS5* and *DTS7* to inhibit the proteasome *(UAS-HA::nmnat/UAS-DTS5; ppk-Gal4,UAS-mCD8::RFP/UAS-DTS7)*. Data are shown for two conditions: flies raised continuously at 25 °C, and flies raised at 25 °C, then shifted to 30 °C for 2 d. Error bars represent standard error. **p* < 0.05.

(C) Hiw and the UPS may influence Nmnat levels cooperatively. Total protein from third instar larval brains or young adult heads processed for Western blot from animals co-expressing *DTS5* and *DTS7* (Belote and Fortier, 2002) to inhibit the proteasome (raised at 25 °C), and compared to animals co-expressing two control UAS-*PKC* transgenes. Relative levels of HA-Nmnat protein, compared to the β-catenin standard, were measured on the LiCor Odyssey system. Combination of the *hiw* mutation with inhibition of the proteasome leads to much higher levels of HA-Nmnat.

Error bars represent standard error; **p* < 0.05 in *t*-test. (Figure 4.13.A was prepared by Yan Hao, Figure 4.13.C was prepared by Dr. Chunlai Wu and Xia Li)

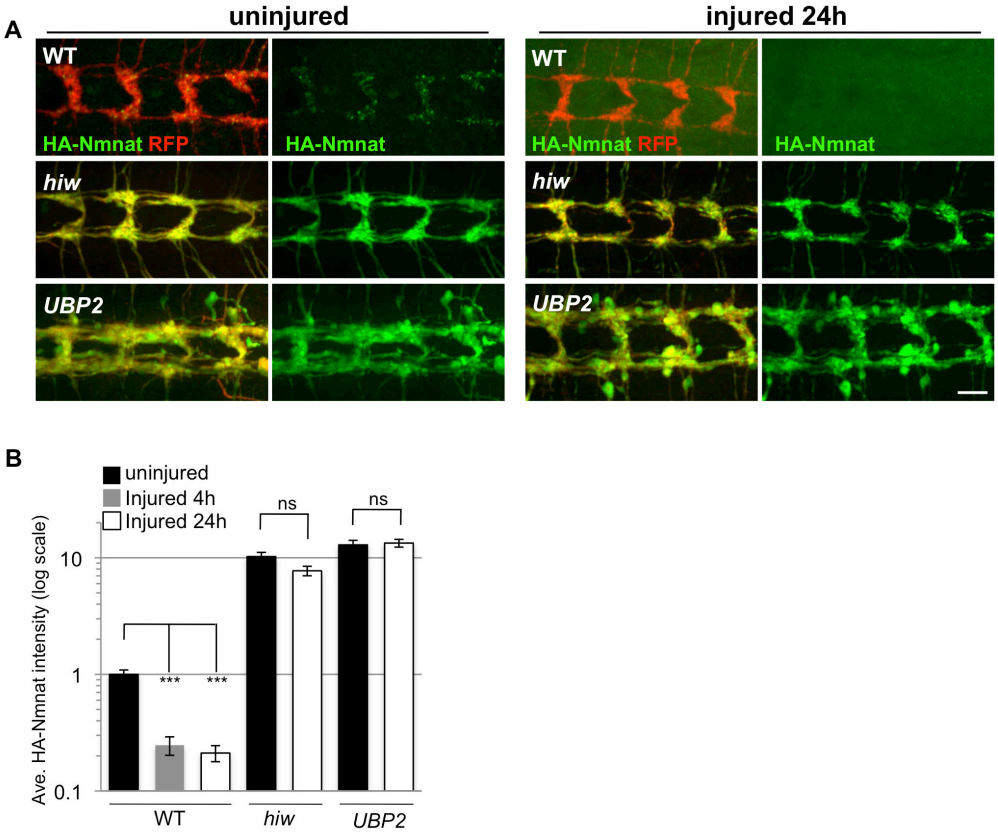


Figure 4.14 Hiw promotes Nmnat protein turnover in the injured distal axons and synapses

(A) Distal axons and synapses of *ppk-Gal4,UAS-mCD8::RFP* labeled sensory neurons, located in the ventral nerve cord, either before or 24 h after injury. Transgenic *HA::nmnat* is expressed in a wild-type (WT) genetic background *(UAS-HA::nmnat/+; ppk-Gal4,UAS-mCD8::RFP/+), hiw*$^{\Delta N}$ mutant *(hiw*$^{\Delta N}$*; UAS-HA::nmnat/+; ppk-Gal4,UAS-mCD8::RFP/+)* or co-expressed with *UBP2* (*UAS-HA::nmnat/UAS-UBP2; ppk-Gal4,UAS-mCD8::RFP/+)*. Consistent with observations in motoneurons (Figure 6), mutation of *hiw* or co-expression of *UBP2* causes a dramatic elevation in HA-Nmnat protein, and this does not disappear after injury, in dramatic contrast to the disappearance of HA-Nmnat in wild-type (WT) animals. Of note, the nerve terminals of the *ppk-Gal4* labeled axons appear to be overgrown in *hiw* and *UBP2* expressing mutants, similar to previous descriptions in other neuron types (DiAntonio et al., 2001; Wan et al., 2000). Hence the quantification shown in
(B) is normalized to the size of the nerve terminals (labeled by mCD8-RFP). Due to the expression of *HA-nmnat*, no axons are degenerating in any of the above genotypes. (B) Quantification of the average HA-Nmnat intensity in *ppk-Gal4* expressing sensory neuron axon terminals, within the most posterior four segments of the ventral nerve cord.
Scale bars = 12.5 μm, error bars represent standard error; ***p < 0.001; ns, not significant, p > 0.05 in t-test.

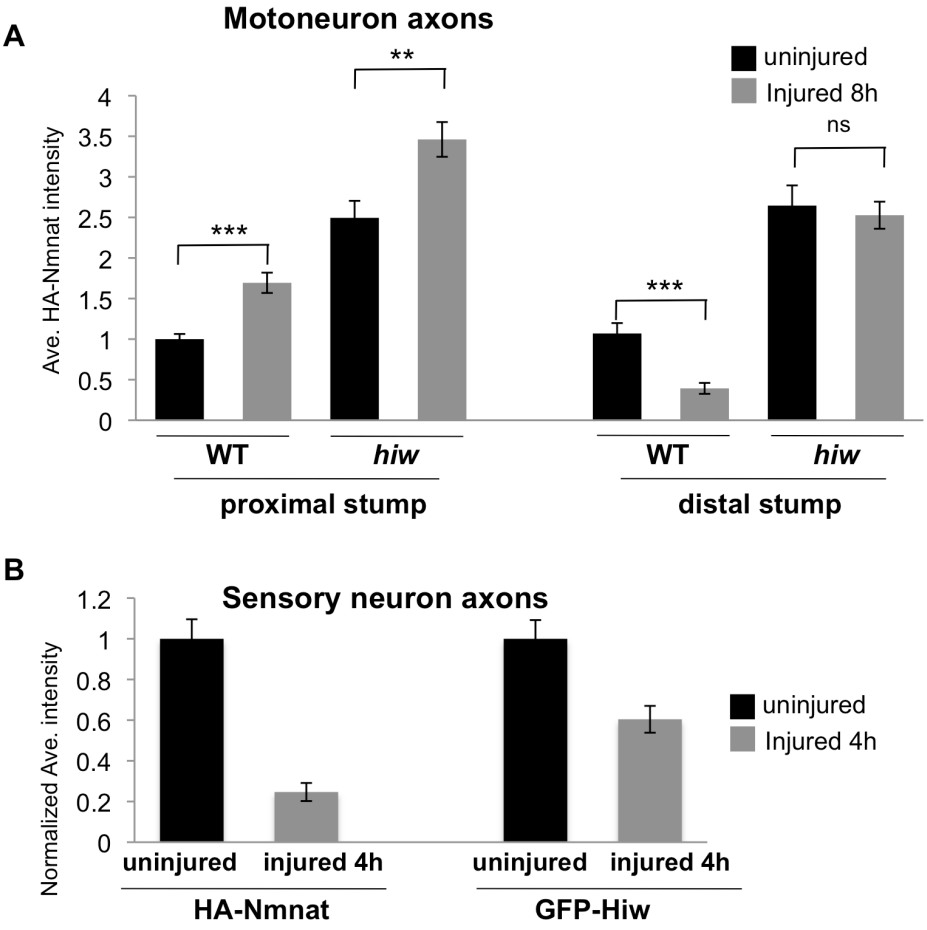


Figure 4.15 Changes in HA-Nmnat protein level in proximal and distal stumps of injured motoneurons, contrasted with the level of GFP-Hiw.

(A) Quantification of average HA-Nmnat intensity in motoneurons axons for wild-type *(OK6-Gal4/UAS-HA::nmnat) or hiw* mutants (*hiw*$^{\Delta N}$;*OK6-Gal4/UAS-HA::nmnat)* before (black) or 8 h after (gray) injury. HA-Nmnat levels in both proximal and distal axons were quantified and normalized to the average HA-Nmnat intensity in uninjured WT animals as described in Materials and Methods. Injury induces an increase of HA-Nmnat in the proximal stump in both wild-type and *hiw* mutant backgrounds. However, in the distal stump, the levels of HA-Nmnat reduced by 60.7% within 8 h in WT animals, but remained constant in *hiw* mutants.
(B) Quantification of average HA-Nmnat and GFP-Hiw intensity in axon terminals of *ppk-Gal4,UAS-mCD8::RFP* labeled sensory neurons before or 4 h after injury.
Error bars represent standard error; ** $p < 0.01$; *** $p < 0.001$; ns, not significant, $p > 0.05$ in *t*-test.

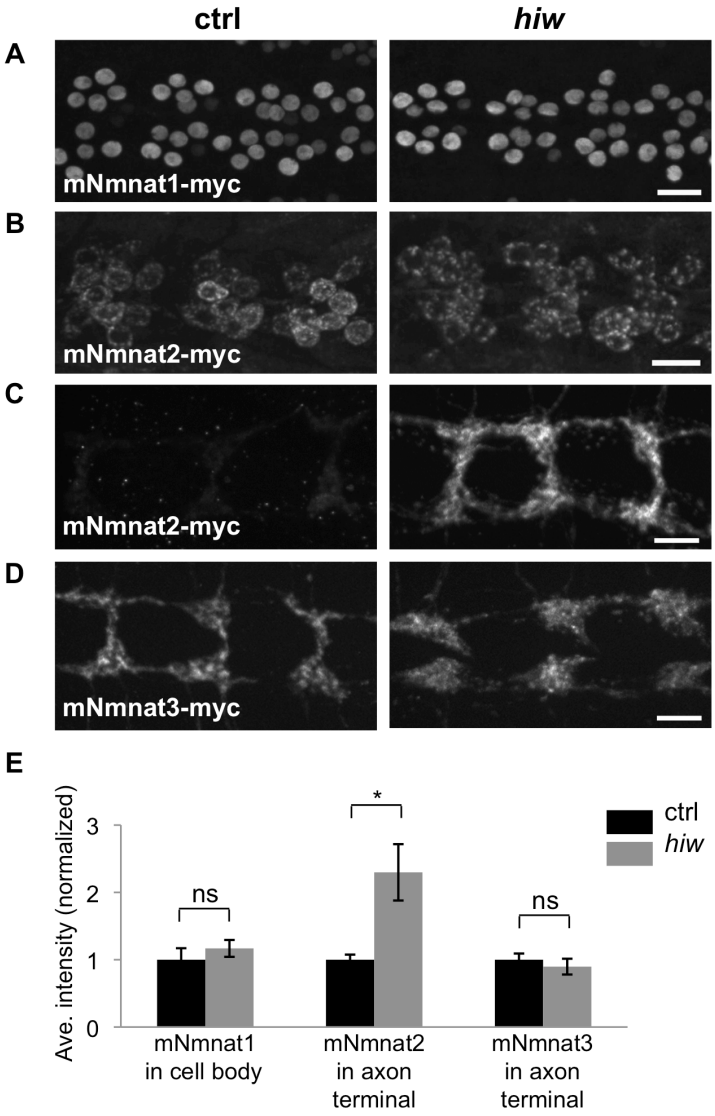


Figure 4.16 Hiw is capable of regulating mouse Nmnat2, but not mouse Nmnat1 or Nmnat3, protein. (Figure 4.16 was prepared by Jiaxing Li)

(A-B) UAS-mNmnat1::myc and UAS-mNmnat2::myc transgenes, expressed in moto-neurons with the BG380-Gal4 driver, show no difference in cell body levels between control and hiw^{ND8} mutants.
(C-D) UAS-mNmnat2::myc, and UAS-mNmnat3::myc were expressed with ppk-Gal4 in order to visualize localization at sensory neuron axon terminal in the nerve cord. The levels of mNmnat2-myc protein at axon terminals were increased in hiw^{ND8} mutants. In contrast, mNmant3-myc protein levels were similar in between control and hiw^{ND8} mutant genotypes.
(E) Quantification of average intensity of mNmnat1 in cell body, and mNmnat2 and mNmnat3 in axon terminals from (A–D).
Scale bars = 12.5 μm, error bars represent standard error; *$p < 0.05$; ns, not significant, $p > 0.05$ in *t*-test.

CHAPTER 5

CONCLUDING REMARKS

Once neural circuits are established, most neurons maintain their function for a lifetime. Hence, the ability to deal with various environmental interferences is especially important for neurons. Neurons are highly polarized cells, and their unique neurite structures enable the passage of information over great distances. Axon projections can be extremely long, which makes them vulnerable for insults such as traumatic injury. Neurons can take various strategies to cope with axonal injuries. In many cases, neurons undergo apoptosis after injury, which can lead to even more severe damage to neural circuits. In contrast, some neurons can make positive actions towards repairing the damaged neural circuit by regenerating their axons. What responses neurons make after injury may vary from neuron to neuron, and the decision also depends on the nature of injury, such as its extent and location. It is important to understand the molecular mechanisms that regulate injury responses. A series of responses are observed in neurons that regenerate. These include transcriptional responses in the nuclei, changes in protein synthesis, regenerative growth of the axon stumps that are still connected to the cell body, and degeneration at the axon stumps that are separated from the cell body to clean the path for regenerating axons. In this study, I used *Drosophila* as a model system to study the molecular and cellular mechanisms that

regulate these responses to traumatic axon injury, as well as how these responses are coordinated. Using a newly developed injury assay, I have demonstrated the central role of a highly conserved pathway in mediating retrograde cell body responses to axonal injury, promoting axon regeneration, as well as regulating degeneration in the disconnected axons and synapses.

5.1 Nerve crush assay provides a platform for studying axon injury responses

In this study, I have established a nerve crush assay in *Drosophila* larvae, which can efficiently introduce traumatic injury to nerves, and allows for in vivo studies of responses of individual neurons to axonal injury, both proximal and distal to the injury site. Prior to this study, no such traumatic nerve injury method had been described for *Drosophila* larvae. There are many advantages for this crush assay. First, the translucent larval cuticle enables live imaging of injured axons in a live animal so that the consequent injury responses could be direct observed (Ghanned-Razaie et al., 2012). Second, this crush assay injures motoneuron as well as sensory neuron axons. Therefore, injury responses in these different neuron types can be observed and compared in the same animal. Third, the assay allows studying of the degeneration process at the neuromuscular junctions, which are well-characterized synapses and have access to electrophysiology experiments for assessing synaptic function. Fourth, the nerve crush assay is easy to execute and introduces injuries to a large amount of neurons. This makes the system amenable to some biochemical studies, as well as future genetic screens.

Like all other assays, there are also some disadvantages for the nerve crush assay. First, this assay injures axons in larval stage, rather than in the adult stage. Thus there might be some developmental effects that one may need to take into consideration while interpreting the results. For example, the intrinsic growth status of developing neurons might differ from mature neurons, which may affect axon regeneration. However, the neurons have nevertheless reached their target and participate in a fully functional circuit in larvae. Second, the short time window of larval stage before metamorphosis diminishes our ability to observe events that take longer than 3 days. A recent study has reported that the duration of the third instar larval stage can be extended to 9.5 days by knocking down the prothoracicotropic hormone receptor, torso, in the ring gland of developing larvae (Miller et al., 2012). This strategy should provide further improvement to this assay and may potentially broaden its application.

5.2 Other injury models in *Drosophila*

Very recently, several other injury assays have been established in *Drosophila*. In larva, axotomy by high power laser can introduce injuries to both axons and dendrites of sensory neurons (Song et al., 2012; Stone et al., 2010), or to the axons of motoneurons (Ghanned-Razaie et al., 2012). In the laser-cutting assay, a specific fluorescent-labeled neurite could be precisely transected in a live animal that is immobilized under the microscope. This assay is especially powerful in combine with time-lapse image to precisely describe the changes to the injured neurite. In addition,

the precise control of the laser enables dendriotomy as well for studying regeneration and degeneration in dendrites, which cannot be achieved with other injury methods.

Previous studies also used cultured adult brain to study injury responses. In this injury assay, traumatic brain damage is created by poking a brain region with a needle (Ayaz et al., 2008). This approach allows studies of injury responses in the CNS environment. However, due to the large injury area, it is difficult to study cell autonomous events in the injured neurons. In the meantime, the injury responses in cultured brains may differ from what happens in vivo.

A new injury model has recently been established in adult wing (Fang et al., 2012). The axons of sensory neurons whose cell bodies sit in the adult wing are injured by cutting the wing edge with a scissor. The translucent wing structure allows time-lapse image to track changes of injured axons in a live animal. The ease of this injury procedure also enables large-scale genetic screenings. However, because many axons of different lengths and from different cells are tightly fasciculated for this group of neurons, the individual subcellular events cannot yet be resolved. Therefore, better labeling methods are need for clearer interpretations of the results in this assay.

Several other approaches are currently taken to study wallerian degeneration in *Drosophila*. The most commonly used is the antenna removal assay (Hoopfer et al., 2006; MacDonald et al., 2006), in which the cell body of olfactory sensory neurons are removed, leaving the distal transected axons to degenerate in the brain. Recently, a pharmalogical approach has been taken to induce wallerian-like degeneration in *Drosophila* larvae (Bhattacharya et al., 2012). In this study, larvae are fed with taxol, which leads to the degeneration of sensory neurons axons. This approach may serve as

a model for studying neuropathy caused by chemotherapy. My work, together with work by summer student Xiaoxue Chen, suggests that the mechanisms that regulate axon degeneration caused by taxol treatment may differ from injury induced degeneration, since in wnd mutants, the degeneration of class IV sensory neuron axons caused by taxol treatment is delayed (Bhattacharya et al., 2012), but wallerian degeneration remains unaffected (table 5.1.).

All of the above injury models are able to take advantage of the powerful genetics in *Drosophila* to study different aspects of axon injury responses in various developmental stage, neuron types and conditions.

5.3 Retrograde injury responses and axon regeneration

It has been known for almost a century that neurons in the peripheral nervous system can regenerate their axons after injury while neurons in the central nervous system cannot (Ramon y Cajal, 1928). Therefore, traumatic injuries to the brain and spinal cord can cause very severe consequences, such as permanent paralysis. To overcome this challenge in treating spinal cord injuries, we need a better understanding of the mechanisms that regulate axon regeneration. Previous studies have demonstrated the indispensable roles of transcriptional responses in the nucleus for axon regeneration. Therefore, it is meaningful to understand how the transcriptional responses in the nucleus are induced by the injury in the axon since the injury site can be distant away.

I found that a conserved kinase pathway plays a fundamental role in mediating pro-regenerative transcriptional response to axonal injury. A core component of this pathway is the MAPKKK Wallenda (Wnd) whose protein level is regulated by the E3 ubiquitin ligase Highwire (Hiw) (Collins et al., 2006). Wnd is highly conserved and has homologues in c.elegans and mouse, named DLK. Wnd protein localizes to and is physically transported in axons. Axon injury leads to dramatic increase of Wnd protein levels in axons by downregulating Wnd protein turnover. Elevation of Wnd level could induce the activation of downstream kinase cascade including MKK7 and JNK, which then regulate nuclear responses via the transcription factor Fos. I have identified two genes, *puckered* and *dvglut*, whose expression levels are changed after injury, and this change requires the Wnd kinase pathway. Moreover, inhibition of any component of the Wnd pathway greatly diminishes an axon's ability to regenerate. The Wnd protein travels in axons, and axonal transport machinery is required for the transcriptional responses mediated by Wnd pathway. Therefore, we proposed the model in which the Wnd injury response pathway can sense axonal damage and send the information back to the cell body via axonal transport to activate transcriptional events in the nucleus that promote axon regeneration. Interestingly, in *hiw* mutants, in which the Wnd pathway is constitutively active, the initiation of regeneration process is accelerated. This further demonstrated a role for the Wnd pathway in promoting axon regeneration.

Recently, a study reported that the Wnd homologue in mouse, DLK, is necessary for the activation of an intrinsic regenerative program in response to axonal injury. Upregulation of the transcription factors STAT3 and c-Jun in the cell body after sciatic nerve injury is abolished in *DLK* knock out mouse, and so is the acceleration of

regeneration induced by a preconditioning lesion (Shin et al., 2012). DLK is required for the retrograde transport of p-STAT3 (Horiuchi et al., 2007; Shin et al., 2012), supported by a previous study which found that Wnd/DLK is involved in regulating axonal transport (Horiuchi et al., 2007). *C.elegans* homologue of Wnd, DLK-1 is also essential for axon regeneration after laser transection (Hammarlund et al., 2009; Yan et al., 2009). DLK-1 regulates the local translation of the transcription factor CEBP-1 in axons by stabilizing its mRNA (Yan et al., 2009). Local translation of CEBP-1 in axons can be induced by axotomy and is required for axon regeneration. Therefore, DLK-1, by facilitating the local translation of CEBP-1, promotes axon regeneration. Another study from the same group suggests that DLK-1 can regulate microtubule growth by regulating microtubule dynamics and posttranslational modifications after injury in a CEBP-1 independent manner (Ghosh-Roy et al., 2012). All of these axonal activities of DLK combined with our observation that Wnd is transported in axons suggest that Wnd/DLK becomes acutely activated in axons by axotomy and can promote axon regeneration via its local roles in axons, possibility functions as an axonal injury detector.

The requirement of Wnd/DLK for axon regeneration in multiple model organisms suggests that this kinase plays conserved roles in promoting axon regeneration. Thus, Wnd/DLK may ultimately become a pharmaceutical target for improving axon regeneration, hence this discovery may have great clinical significance.

5.4 Wnd signaling protects axons from recurrent injuries

While the mechanism by which distal axons degenerate is poorly understood, I found that this process can be regulated by transcriptional signaling downstream of

Wnd. Injury, which activates Wnd signaling, induces a response in the cell body and remaining proximal stump, which makes the axon more resilient to a second injury. This observation suggests that neurons have plasticity in their degenerative responses to recurrent injuries. This is especially meaningful in the scenario in which the neuron has to cope with recurrent insults from the environment. This idea is supported by a recent study with *Drosophila* sensory neurons, in which dendritic arborizations are more resistant to injury-induced degeneration if the axons were severed previously or when neuron are stressed by expressing expanded polyglutamine (poly-Q) proteins (Chen et al., 2012). Interestingly, this protection requires JNK, and correlates with a JNK dependent increase in microtubule dynamics, which can be triggered by axonal injury or neuronal stress (Chen et al., 2012). These findings suggest that pre-conditioning insults might activate a common neuroprotective program, and that changes in the cytoskeleton may play an important role in the protective mechanism. It would be interesting to test whether the Wnd pathway can also protect neurons from other insults besides axonal injury.

Contrasting to Wnd being neuroprtotective, another study with *Drosophila* olfactory neurons suggests a pro-degeneration role for Wnd. This surprisingly different finding motivates me to investigate the role of Wnd in degeneration further in other neuronal types. Interesting, the Wnd pathway does not appear capable of protecting all neuron types. Rather, my findings combined with others' suggest that Wnd plays diverse roles in different cell types, developmental stages, as well as in different injury models. Table 5.1. summarizes the current findings of how degeneration in *Drosophila* neurons is affected by loss-of-function and gain-of-function in *wnd*. Wnd play pro-

degenerative roles in adult olfactory neurons after axon transection (Miller et al., 2009) as well as in larval type IV sensory neurons after taxol treatment (Bhattacharya et al., 2012). While wnd doesn't seem to be involved in wallerian degeneration of larval type IV sensory neurons, it plays protective roles in larval motoneurons (Figure 3.2.). In mice, the homologue of Wnd, DLK, promotes degeneration in transected sciatic nerves and cultured DRG neurons (Miller et al., 2009).

The variation of Wnd's function in degeneration might be because different degeneration mechanisms are used by neurons of different types and developmental stages. Another possibility is that Wnd promotes different downstream signaling events in different scenarios. My study in larval motoneurons found that the protection by Wnd requires the downstream transcription factor Fos, suggesting that the Wnd pathway may indirectly regulate degeneration via its downstream gene products that can affect degeneration in the distal axons. However, a study in vetebrate cultured DRG neurons suggests that the downstream kinase of DLK, JNK, regulates degeneration via local action in the distal axons, because inhibiting JNK after axon transection is sufficient to suppress axon degeneration (Miller et al., 2009). Despite the possibility that JNK may regulate denegation with a different mechanism than DLK, this finding proposes that this kinase pathway may regulate wallerian degeneration via transcription independent mechanisms.

5.5 Highwire promotes degeneration in distal axons and synapses by down-regulating Wallenda and Nmnat

Previously, the majority of our understanding about the mechanism of wallerian degeneration came from the studies of the gain-of-function mouse mutation Wlds mutation. Prior to my work, there were no examples of loss-of-function mutations with comparable strength to the Wlds mutation, hence the endogenous machinery that promotes Wallerian degeneration has remained unknown.

In this study, I have found that the wallerian degeneration of both axons and synapses is strongly arrested at the initiation stage in the absence of Hiw, suggesting an essential role of Hiw in promoting degeneration. However, Wnd alone cannot account for the dramatic protective phenotype in *hiw* mutant. First, loss of function in *wnd* can only partially rescue the *hiw* mutant protection phenotype in motoneurons. Second, axon protection by *wnd* over-expression is much milder compared to the protection in *hiw* mutant. Third, *wnd* over-expression can only protect axons in larval motoneurons while *hiw* mutant have strong protection in all examined neuron types. These observations suggest the existence of other downstream targets for Hiw in regulating wallerian degeneration.

The comparable extent of strong inhibition by *hiw* mutant and Wlds points towards the hypothesis that they may function through some shared mechanisms. The major component of Wlds mutation is the Nicotinamide adenylytransferase (Nmnat) 1 (Conforti et al., 2000; Mack et al., 2001), which is the core enzyme in NAD+ salvage synthesis pathway. There is a single Nmnat gene in Drosophila (Zhai et al., 2006). With genetic and biochemical approaches, I have demonstrated that Hiw promotes

degeneration by down-regulating Nmnat protein. Loss function in *hiw* causes dramatic increase in Nmnat protein level and this increase is required for protection phenotype in *hiw* mutant.

A previous study in mouse neurons has proposed a model in which Nmnat2 protein rapidly turns over, and the decrease of Nmnat2 protein in the injured distal axons is important for the initiation of wallerian degeneration (Gilley and Coleman, 2010). Consistent with model, I found that Nmnat protein levels in distal axons and synapses rapidly reduce after injury and Hiw is required for this reduction. However, my findings were not entirely consistent with this hypothesis. When Nmnat is over-expressed in neurons, axon structures are protected long after the disappearance of Nmnat protein. Reducing Nmnat protein level by overexpressing Hiw does not affect the initiation of degeneration. It is possible that trace amount of Nmnat protein is sufficient to protect from degeneration. It is also formally possible that the basal levels of Nmnat before injury, rather than the disappearance of Nmnat after injury, is an important determinant of degeneration.

Nmnat turnover in distal axons and synapses is greatly inhibited in *hiw* mutants or when ubiquitination is inhibited by expressing the yeast deubiquitinase UBP2. While ubiquitination is important for Nmnat regulation by Hiw, we were not able to conclude that UPS is involved in this regulation.

5.6 Hiw plays a central role in coordinating multiple injury responses

After injury, we observed both increased Wnd protein level in the proximal stump, and decreased Nmnat protein level in the distal stump. The elevation of Wnd level activates the retrograde injury signaling pathway that facilitates regeneration, while the decrease in Nmnat protein level may be important for the initiation of wallerian degeneration. How are the opposite regulations of these two proteins by Hiw at different parts of a severed axon simultaneously achieved? While the localization of endogenous Hiw in *Drosophila* is less clear, homologues in mice and *C. elegans* have been detected in axons and at synapses (Abrams et al., 2008; Lewcock et al., 2007). Figure 5.1. proposes a model in which Hiw functions in the axon terminus. In this model, axon injury separates the distal axon with the rest of the neuron. Therefore, lack of Hiw's action in the proximal stump leads to accumulation of Wnd there. Meanwhile, the action of Hiw in the axon terminus could promote destruction of Nmnat in the distal stump. Future follow-up studies to identify the mechanism for Nmnat down-regulation by Hiw may provide further evidences to validate this model.

Studies in vertebrates suggest that the degeneration of severed axons is important for clearing the way for regenerating axons (Martin et al., 2010). Therefore, by regulating retrograde injury signaling pathway that promotes regeneration and, at the same time, promoting degeneration of the distal stump, Hiw plays a central regulatory role in coordinating multiple injury responses to facilitate the repair of a damaged neural circuit.

Table 5.1 The role of Wnd in degeneration varies in different neurons.

Developmental Stage	Neuron type	Insult type	Degeneration phenotype	
			wnd mutant	O/E wnd
larva	motoneuron	axotomy	no phenotype	protection
larva	bodywall sensory neuron	axotomy	no phenotype	no phenotype
larva	bodywall sensory neuron	taxol feeding	protection	N/A
adult	olfactory sensory neuron	axotomy	protection	no phenotype

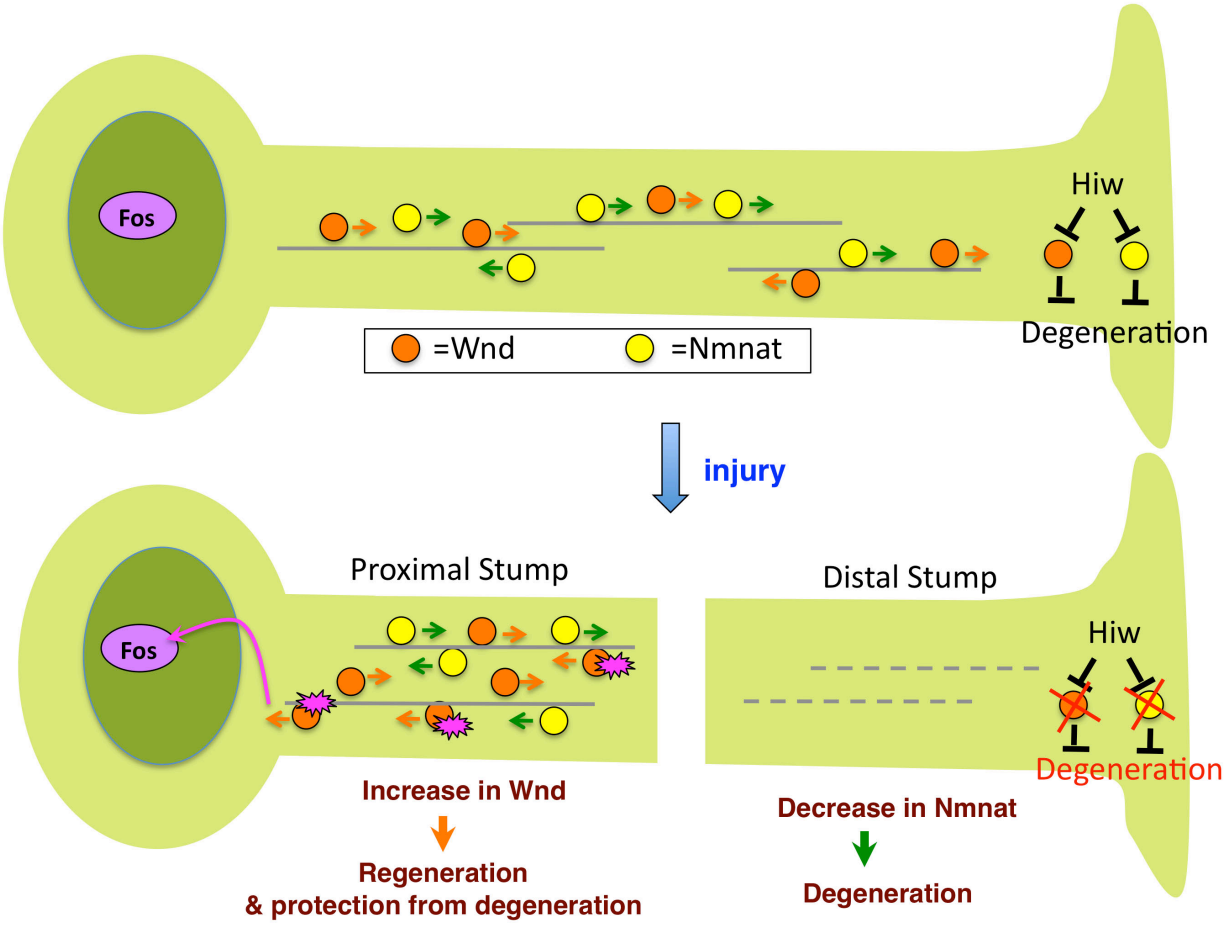


Figure 5.1 The working model that Hiw play a central role in coordinating multiple injury responses

Both Wnd protein (orange circles) and Nmnat protein (yellow circles) can be transported in axons (Gilley and Coleman, 2010; Xiong et al., 2010). In this model, Hiw functions in the distal axon terminus and destructs both Wnd and Nmnat. In the uninjured axons (upper panel), since Wnd and Nmnat could be constantly delivered from the cell body while being destructed by Hiw, they are maintained at certain levels. After axonal injury, Nmnat protein level in the distal stump decreases due to Hiw function, which might be an important event for degeneration. In the proximal stump, due to lack of Hiw function, Wnd protein accumulates and activates the downstream nuclear response pathway, which is required to promote new axonal growth. As an upstream regulator of both sprouting in the proximal stump and degeneration of the distal stump, Hiw may play a central role in regulating the ability of a neuron to regenerate its connection after injury.

APPENDIX

FUTURE DIRECTIONS

1. The mechanism of Nmnat regulation by Highwire

Nmnat has been identified as a new downstream target for the Hiw E3 ubiquitin ligase. The absence of Hiw leads to significant increase of Nmnat protein levels in neurons. Injury induced Nmnat decrease in distal axons and synapses is inhibited in *hiw* mutant. These data, together with observations that Hiw homologues localize in synapses, favor the model that Hiw regulates Nmnat locally in the axon terminus. However, the mechanism for Hiw regulating Nmnat is not known and will be an interesting direction for future studies.

There are two splice forms of Nmnat in *Drosophila*. For the cDNA clones we used to make transgenic Nmnat flies, one contains a nuclear localization sequence (NLS) (Nmnat-A) and the other does not (Nmnat-B). Of note, the annotation for the two Nmnat splice forms indicates that both of them contain a NLS (shown in Figure 6.1.A). Further studies are needed to figure out whether the natural form of Nmnat-B contains a NLS. Transgenic Nmnat-A and Nmnat-B localize differently when expressed in neurons (Figure 6.1.B). The comparisons between transgenic proteins of the two Nmnat splice forms are shown in Table 6.1. I found that in motoneurons, the splice form that doesn't contain the NLS (Nmnat-B) fills the cell body and axons, but it is excluded from the neuromuscular junction (NMJ). In *hiw* mutants, the increase of Nmnat protein level is much more dramatic for NMJs than cell body or axons (Figure 4.9.). This raises the possibility that synapses may be the site of Nmnat down-regulation action by Hiw.

Interestingly, the two different splice forms of Nmnat have dramatically different sensitivities to regulation by Hiw. When over-expressed, both of the isoforms can enter axons. In sensory neuron axon terminals, the level of the splice form that contains the NLS (Nmnat-A) does not change significantly in *hiw* mutants, while the level of the splice form that doesn't contain the NLS (Nmnat-B) is increased over 10 fold (Figure 6.1.C, D). The localization of Nmnat-A and Nmnat-B to different cellular compartments may account for this difference. It is also possible that the protein sequence that differs between these two splice forms may affect the recognition by Hiw. As shown by the sequence alignment of Nmnat-A and Nmnat-B in Figure 6.1.A, the N-terminal 31 amino acid of Nmnat-A is not present in Nmnat-B, and the c-terminal 12 amino acid is unique to Nmnat-B. Further study of why Hiw regulates the two Nmnat splice forms differently will provide clues for the mechanism of Nmnat regulation by Hiw.

Nmnat-B levels decrease dramatically in the distal axon and synapses after injury, which is inhibited in *hiw* mutant. Actually, Nmnat-A level at the sensory neuron

nerve terminals also decreases after injury. However, Hiw is dispensable for this change because Nmnat-A level decreases in the *hiw* mutant (Figure 6.1.E). Of note, Nmnat-B level also slightly decreases after injury in *hiw* mutant (not statistically significant with our quantfication method, but is visibly different Figure 4.14.), suggesting that Hiw might not be the only regulator that accounts for the decrease of Nmnat at the distal stump after injury. Instead, other regulators of Nmnat may play roles in this regulation, and may be able to regulate both Nmnat-A and Nmnat-B splice forms.

There are three Nmnat genes in vertebrates, Nmnat-1, Nmnat-2 and Nmnat-3. Their protein products localize to nucleus, Golgi compartments and mitochondria respectively (Berger et al., 2005). My colleague Jiaxing Li has found that in *hiw* mutants, among the three exogenously expressed mouse Nmnat proteins, only Nmnat-2 is increased in the axon terminus (Figure 4.16.). This observation that the fly Hiw protein can down-regulate mouse Nmnat-2 suggests a conserved mechanism may take place to regulate Nmnat2 by Hiw homologues in vertebrates. Moreover, the specificity of Nmnat2 regulation by Hiw provides possible tools for dissecting the mechanism how Hiw regulates Nmnat.

Although we have adequate evidence that Nmnat is negatively regulated by Hiw and that ubiquitination is important for this regulation, we are less certain about how ubiquitination affects Nmnat protein level. The most common manner that ubiquitination regulates protein level is via degradation by the Ubiquitin Proteasome System (UPS). However, we were not able to find any evidence that supports this model, nor could we detect poly-ubiquitinated species of Nmnat protein. Therefore, Nmnat might be regulated via other mechanisms that are mediated by ubiquitination, such as via other degradation pathway, or regulation of protein stability. It will be informative to test whether Nmnat is ubiquitinated, since it is formally possible that ubiquitination may indirectly regulate Nmnat via other protein targets.

2. The regulation of Nmnat by Wnd

In chapter 4, I found that Wnd is not involved in Nmnat regulation by Hiw, and Wnd functions separately with Nmnat in regulating wallerian degeneration. To further test whether Wnd itself can regulate Nmnat, I have checked the levels of Nmnat protein when manipulating Wnd. Interestingly, endogenous nuclear Nmnat dramatically increases in *wnd* mutants, and almost disappears when Wnd is overexpressed (Figure 5.2.). Consistent with Wnd being over active in *hiw* mutants, I have observed a decrease in nuclear Nmnat in the *hiw* mutant (Figure 4.8.). This decreased nuclear Nmnat level in *hiw* mutant contrasts with the increased axonal and synaptic Nmnat level. A similar decrease of nuclear Nmnat has been observed after axonal injury (Figure 5.2.), suggesting that nuclear Nmnat decrease might be an injury response mediated by Wnd pathway. This regulation of Nmnat by Wnd could be post-transcriptional, because the transgenic Nmnat protein could also be regulated by Wnd. Axonal and synaptic transgenic Nmnat protein also decreases when overexpressing Wnd, suggesting that the regulation by Wnd is not restrained for nuclear Nmnat (Figure 5.2.). What is the molecular mechanism that Wnd regulates Nmnat? A straightforward question is whether

this regulation requires the downstream kinases of Wnd including JNK and p38, and whether this regulation is phosphorylation dependent.

What is the physiological function of Nmnat regulation by Wnd? This regulation is less likely to be involved in regulating Wallerian degeneration since overexpressing Wnd, despite leading to decreased Nmnat level, can protect axons from degeneration. Based on data that is not shown in this thesis, knocking down Nmnat with RNAi does not affect the nuclear injury response indicated by puc-lacZ induction, nor does it affect axon regeneration at the proximal axon stump. Since the downstream MAP kinase of Wnd, JNK, is involved in responses to a variety of stress conditions besides axonal injury, Wnd's role in regulating Nmnat might be involved in other unknown functions of Wnd. Regulation of Nmnat, an enzyme for NAD+ synthesis, might be important for metabolic adjustments in responses to environmental cues, including food, oxygen and temperature changes. Consistent with this idea, a recent study suggests that Nmnat might have important roles in mediating thermotolerance and surviving hypoxia (Ali et al., 2011).

It is very interesting that Nmnat could be differently regulated by Hiw and its downstream target Wnd. Although Wnd is negatively regulated by Hiw, both of them could negatively regulate Nmnat. This complex regulation may lead to comprehensive spatial and temporal control of Nmnat levels in response to a variety of environmental stimuli. Understanding the function of Nmnat regulation by Wnd will help understanding the physiological significance for this paradox relationship between the regulations by Hiw and Wnd.

3. The mechanism that Wnd regulates degeneration

Chronic activation of the Wnd pathway by genetic manipulation, or acute activation of Wnd by a preconditioning lesion, could protect motoneuron axons from degeneration (chapter 3). What is the mechanism by which Wnd mediates axon protection?

Although the cellular mechanism of axon degeneration is still unclear, mitochondria have been hypothesized to play roles in multiple aspects of axon degeneration and serve as a central sensor for axonal degenerative stimuli (Court and Coleman, 2012). Interestingly, I found that Wnd function can affect both mitochondria morphology and motility in axons (data not shown). Therefore, I hypothesize that Wnd may regulate axon degeneration by regulating mitochondria function. However, the relationship between mitochondria morphology and function is poorly understood. Further study to test whether Wnd affects mitochondria function, such as ATP synthesis, will be informative.

Mitochondria can both have prodegenerative roles and protective roles. Protecives roles include their ability to buffer calcium and to generate ATP. Pro-degenerative roles include that mitochondria are a major source of reactive oxygen species and are gate-keepers of the apoptotic caspase cascade (Court and Coleman, 2012). I have observed that mutations in miro, which disrupt axonal transport of mitochondria into axons, can delay degeneration process, adding another evidence that malfunction of mitochondria might be a major event in degeneration process. By regulating mitochondria morphology, motility and potentially function, Wnd may affect

degeneration by increasing the capability of mitochondria to resist axonal stresses and thus delay the onset of degeneration.

What is the molecular mechanism that Wnd regulates mitochondria morphology and motility? A straightforward question to pursue is whether this regulation requires the downstream kinase of Wnd, and whether the transcription events are involved in this regulation. Previous studies suggest that Ca2+ concentration in axons is an important regulator for mitochondria motility (Wang and Schwarz, 2009), and meanwhile Ca2+ is a key factor in axon degeneration. Since Wnd regulates both mitochondria motility and axon degeneration, it is possible that Wnd regulates both by affecting Ca2+. This hypothesis could be tested by comparing the axonal and mitochondrial Ca2+ concentration in wt, *wnd* mutant and when overexpressing wnd respectively using the Ca2+ sensors. It is also possible that Wnd regulates mitochondrial proteins that mediate mitochondria fusion/fission event as well as transport. Potential Wnd targets in this regulation might be identified by proteomics approach to compare the mitochondria protein profile in different genetic background.

Wnd might play local roles in distal stump axons during degeneration via other cellular events. Current studies in *C. elegans* suggest that Wnd can regulate microtubule dynamics after axonal injury (Ghosh-Roy et al., 2012) as well as local translation (Yan et al., 2009). A recent study in *Drosophila* suggests that increased microtubule dynamics have neuroprotective effect (Chen et al., 2012). Therefore, it is possible that Wnd may delay degeneration by increasing the microtubule dynamics in axons. Local translation roles of Wnd may also be involved in regulating degeneration in the distal stump, which is separated from the cell body. Because Wnd have different effect on axon degeneration between motoneurons and sensory neurons (Table 5.1.), the cellular events downstream of Wnd might be more sensitive to Wnd's regulation in motoneurons than sensory neuron. Comparisons between the degeneration mechanisms of different neural types can serve as an approach to study the molecular mechanisms that Wnd regulates axon degeneration.

Table 6.1.

Differences	**Nmnat-A**	**Nmnat-B**
Major location	Nucleus	All through the neuron (exclude NMJ)
Co-localization with mitochondria	No	Yes
Protection from degeneration	Weak	Strong
Regulation by Hiw	No	Yes
Common feature	**Nmnat-A**	**Nmnat-B**
NMJ localization	No	No
Decrease after injury in distal stump	Yes	Yes

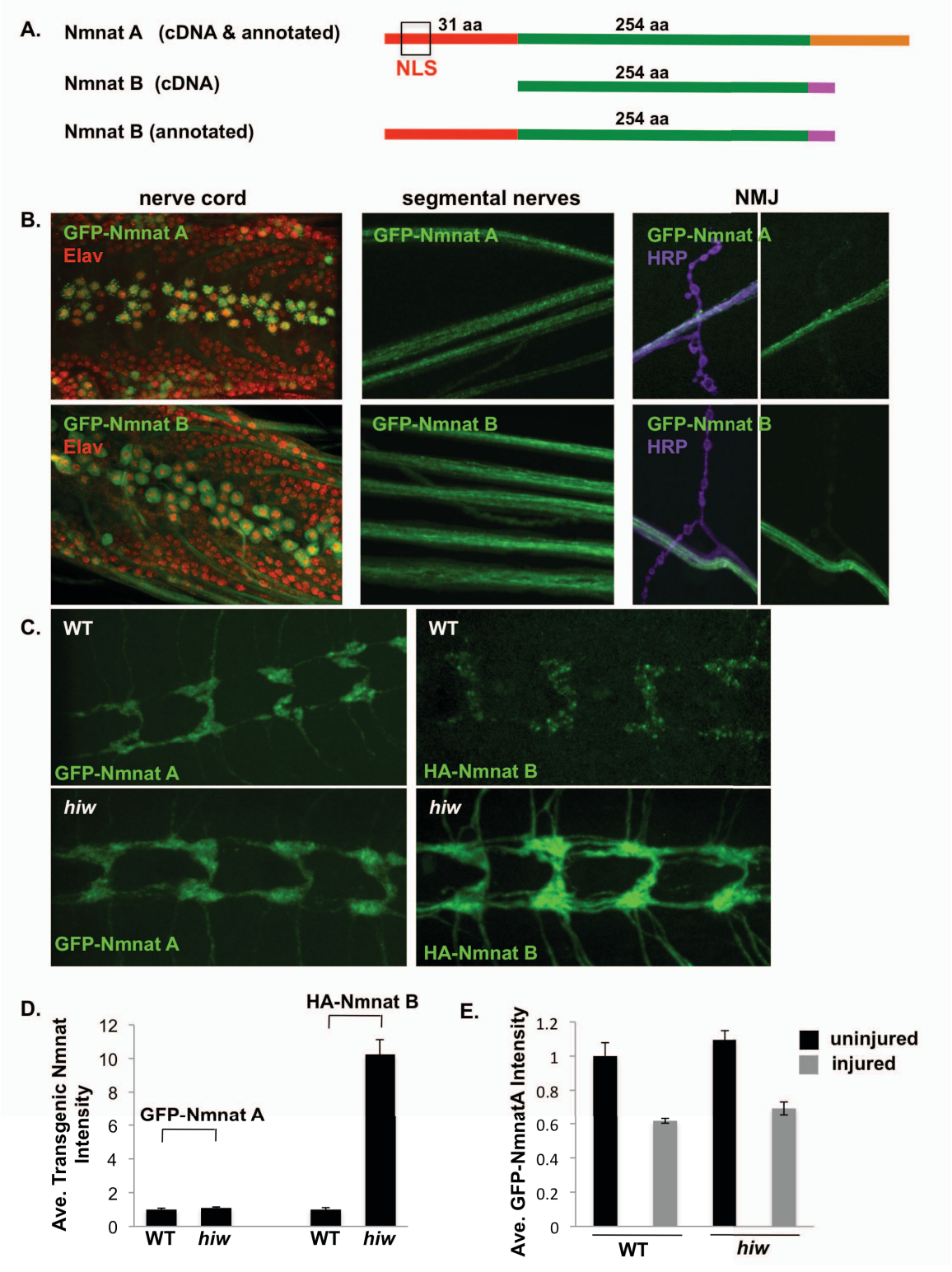

A.
Nmnat A (cDNA & annotated)
31 aa
254 aa
NLS
Nmnat B (cDNA)
254 aa
Nmnat B (annotated)
254 aa
B.
nerve cord
segmental nerves
NMJ
GFP-Nmnat A
Elav
GFP-Nmnat A
GFP-Nmnat A
HRP
GFP-Nmnat B
Elav
GFP-Nmnat B
GFP-Nmnat B
HRP
C.
WT
GFP-Nmnat A
WT
HA-Nmnat B
hiw
GFP-Nmnat A
hiw
HA-Nmnat B
D.
Ave. Transgenic Nmnat Intensity
12
10
8
6
4
2
0
GFP-Nmnat A
HA-Nmnat B
WT
hiw
WT
hiw
E.
Ave. GFP-NmnatA Intensity
1.2
1
0.8
0.6
0.4
0.2
0
uninjured
injured
WT
hiw

Figure 6.1. The features of the two splice forms of *Drosophila* Nmnat

(A) Schematic alignment of the protein sequences of Nmnat-A and Nmnat-B.
(B) Localizations of the transgenic Nmnat-A and Nmnat-B protein.
(C) Transgenic Nmnat-A and Nmnat-B protein levels in wildtype background or in *hiw* mutant.
(D) Quantification of the average transgenic Nmnat protein levels in (C). Nmnat-B increases dramatically in *hiw* mutant while Nmnat-A doesn't.
(E) GFP-NmnatA protein level before or after injury in wildtype or *hiw* mutant. For both wildtype and *hiw* mutant, GFP-NmnatA protein level decreases after injury.

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