Shared Regulators of Axon Degeneration and Synaptic Structure

by

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Ask1 - Apoptosis Signal-Regulating Kinase 1
ATPsynG - ATP Synthase Subunit G
cADPR - Cyclic ADP-Ribose
Dcr2 - Dicer 2
DLK - Dual Leucine Zipper Kinase
DPI - Days Post-Injury
ERK - Extracellular Signal-Regulated Kinase
HPI - Hours Post-Injury
JIP1 - JNK-Interacting Protein 1
JNK - Jun N-Terminus Kinase
Miro - Mitochondrial Rho GTPase
MKK4/7 - Mitogen-Activated Protein Kinase Kinase 4/7
NAD+ - Nicotinamide Adenine Dinucleotide
NAM - Nicotinamide
NMN - Nicotinamide Mononucleotide
NLS - Nucleus Localization Signal
NMNAT - Nicotinamide Mononucleotide Adenylyl Transferase
Pyk - Pyruvate Kinase
SARM1 - Sterile Alpha and TIR Motif-Containing Protein 1
Shg - Shotgun
TIR - Toll/Interleukin Receptor
Trc - Tricornered
VNC - Ventral Nerve Cord
Wld^{S} - Wallerian Degeneration Slow
Wnd - Wallenda
Wnk1 - With No Lysine (K) Kinase 1
WT - Wild Type

Abstract

Axons are a fundamental feature of the nervous system. By extending over long distances in the brain and body, axons enable neural communication fundamental for sensory perception, muscle movement, and cognition. Their long length is considered a vulnerability to injury and to cellular processes needed to maintain metabolism at great distances from the cell body. Intricate intracellular signaling is required to manage these structures, including mechanisms that regulate the structure of synaptic terminals at great distances from cell bodies. This thesis work focuses on the mechanisms that regulate the integrity and degeneration of axons, and their relationship with signaling pathways in neurons.

Chapter 1 reviews molecular machinery responsible for initiating the degeneration of axons. A key regulator of axonal degeneration is the Sterile Alpha and TIR Motif-Containing Protein 1 (Sarm1), which in its activated form functions as a NADase enzyme and drives the destruction of a key electron carrier NAD+. Before the discovery of its enzymatic activity, Sarm1 was known to function in intracellular signaling pathways that regulate diverse functions, including neuronal morphology and innate immunity. Chapter 1 considers the mechanistic relationships of Sarm1's roles in axonal degeneration and signaling, including how other signaling pathways may intersect with Sarm1 to regulate the structure and integrity or degeneration of axons.

Chapter 2 focuses on the relationship of *Drosophila* Sarm1, dSarm, with Jun N-terminal kinase (JNK) signaling. A negative regulator of JNK signaling, the transmembrane protein Raw, was discovered to regulate the ability of axons to degenerate in *Drosophila* motoneurons (Hao et al. 2019). Our genetic data indicate that Raw functions upstream of dSarm, and influences the levels of dSarm protein in axons. These data also suggest an additional role for both Raw and dSarm in the regulation of synaptic structure. However, in this role Raw and dSarm share a different functional relationship. Both appear to regulate a common signaling pathway that involves JNK and the transcription factor Fos. However, the functional role of JNK in the regulation of synaptic structure does not match its roles in nuclear signaling and its protein levels. Rather than the expected role of JNK in promoting synaptic growth, evidence points toward dSarm and Raw regulating a JNK pathway that inhibits growth at the NMJ. These findings suggest a complex regulatory system that both activates and inhibits JNK activity to regulate nuclear signaling, NMJ development, and axon degeneration.

Chapter 3 investigates the effect of energy metabolism manipulation on the degeneration of injured axons in *Drosophila*. We investigated the effect of genetic knockdown of critical enzymes in ATP synthesis pathways in *Drosophila* motoneurons: Pyruvate Kinase, a key regulator of glycolysis, and ATP-Synthase. Surprisingly, disruption of either enzyme results in an impaired ability of injured axons to undergo Wallerian degeneration. Both genetic manipulations also induce the expression of *puc*-lacZ, a reporter for activated JNK signaling in *Drosophila*. However, other manipulations that disrupt JNK signaling fail to rescue the impairment to degeneration

caused by Pyruvate Kinase and ATP-synthase knockdown. These findings imply that the mechanisms of axonal degeneration are intimately linked to mechanisms that maintain energy homeostasis in axons and identify unanticipated methods to prolong the integrity of damaged axons.

Chapter 4 covers a series of distinct experiments centered around the relationship of Raw, dSarm, and MAP kinase signaling. We show evidence that Raw regulates nuclear JNK signaling through a mechanism that can be separated from injury-induced signaling. In fact, interference with the Wallenda (Wnd) and JNK Interacting Protein 1 (JIP1) further delay axon breakdown post-injury, rather than rescuing it, suggesting that this pathway may promote degeneration in the context of *raw* loss. We expanded the search for Raw's mechanism to known partners in its dendritic role, but find no evidence of overlap in Raw regulation of dendrite patterning and axon degeneration. These results set the stage for future work on identifying Raw's methods of regulation.

The findings in this thesis advance the understanding of how axon degeneration, kinase signaling, and synapse development are regulated, with a focus on intersections between these processes. Unexpected results connecting metabolic disturbance with delayed neurodegeneration raise important questions on the balance between stress and stress response. Overall, this work contributes to and discusses the intricate links between degeneration and development, which may seem to be opposite processes, yet rely on common signaling regulation.

Chapter 1: Introduction

Abstract

Since it was first documented in the 19th century, our understanding of Wallerian degeneration has changed dramatically and informed the study of many other forms of neurodegeneration. This type of degeneration is the breakdown of axons following injury, which was originally viewed as a passive “falling apart” death process. However, the discovery of a mouse strain that showed significantly delayed Wallerian degeneration transformed our understanding of Wallerian degeneration from a passive process to an active mechanism that requires genetically-encoded regulation to proceed. Further work would identify two proteins pivotal to the fate of injured axons: the protective protein Nicotinamide Mononucleotide Adenylyl Transferase (NMNAT) and the prodegenerative Sterile Alpha Motif-Containing Protein 1 (Sarm1). This chapter will describe the role of these proteins in controlling axon degeneration, as well as their respective regulations. Particular attention will be paid to Mitogen Activated Protein Kinase (MAPK) signaling which is central to the axon injury response and has several links to NMNAT and Sarm1. The current state of the field suggests that future research will bring fascinating insights into the late stages of axon degeneration that transition a living cellular extension into fragmenting debris.

Roles for Axonal Degeneration Machinery in Mechanisms of Repair and Development

Introduction

Axons enable neural communication over great distances in the nervous system. Tuned to the vulnerability of long axons, decades of studies (including work by Ramon y Cajal and Augustus Waller in prior centuries) have showcased the plasticity of the nervous system through the varied responses made by individual cells adapting to nervous system damage. These responses include the ability to regrow damaged axons, to repair lost connections and/or sprout new axonal branches, make alternative connections, and, in some cases, to induce apoptosis of the damaged neuron. Also critical for nervous system plasticity is the degeneration and clearance of the distal axon 'stump' which has lost connectivity with the cell body.

Unless the refusion of axons can occur (which has been documented to occur robustly following damage to axons in *C. elegans* (Neumann et al. 2011)), the distal stump has no clear function in its original circuit and no obvious way to function without a cell body. However, continued existence of the severed axon may be enabled by local mechanisms of axon protein synthesis, energy supply from axonally localized mitochondria (and other organelles), and support from local glia. Indeed, countless electrophysiology preps take advantage of the fact that an axon stump disconnected from its cell body perdures for a defined amount of time before the axon degenerates into individual cellular fragments of debris that are then cleared by surrounding cells.

While it is logical that the stump should ultimately degenerate, since it would be unable to receive new organelle components or the products of new gene expression from the cell body, it is now clear that the mechanism by which most axons degenerate is not simply a passive rundown of cellular function. Rather, axons degenerate through an active and regulated 'self-destruction' pathway akin to apoptosis, but involving distinct cellular machinery. We will briefly review current understanding of this pathway following a flurry of recent insights in the field. However, our focus for this review is on how our growing understanding of axonal degeneration mechanisms intersects with other signaling pathways, with an emphasis on the enzyme Sarm1.

Roles for Sarm1 in Multiple Injury Responses

Exciting work over the past two decades has revealed a regulated cellular pathway that, when activated, can trigger the rapid degeneration of axons, as well as the death of cells (reviewed in (Sambashivan and Freeman 2021; M. D. Figley and DiAntonio 2020)). A key destroyer in this pathway is the Sterile Alpha Armadillo containing protein Sarm1. This role was first discovered through a forward genetic screen for cell autonomous regulators of axon degeneration following injury (Osterloh et al. 2012). Prior to this discovery, Sarm1 was known for its role as a regulator of MAP Kinase signaling (Chuang and Bargmann 2005; Inoue et al. 2013; Gerdts et al. 2016). In cases where it has been probed, the kinase Ask1 appears to be an important mediator (Chuang and Bargmann 2005; Julian and Byrne 2020; Ding et al. 2022; Herrmann et al. 2022; Brace et al. 2022). Sarm1-gated intracellular signaling has been linked to a variety of cellular

functions, including activation of innate immunity pathways (Couillault et al. 2004; Liberati et al. 2004; Foster et al. 2020), and, in neurons, the organization of cytoskeleton and neurite structure (Izadifar et al. 2021; Kim et al. 2007; McLaughlin et al. 2016). Sarm1's signaling roles are also closely tied with other elements of axonal injury response (**Figure 1-1**). These include regulation of the ability of injured axons to regenerate (in motor axons of *C. elegans*)(Julian and Byrne 2020), and stimulation of an immune response via the induction of cytokine expression from the axotomized neurons (noted in mammalian DRG neurons following peripheral nerve injury (Q. Wang et al. 2018)).

In addition to the above roles in injured neurons, Sarm1-regulated signaling also acts in other non-injured cells to regulate responses to injury (**Figure 1-1)**. Hsu et al documented a role for *Drosophila* Sarm1 in slowing axonal transport in non-injured 'bystander' axons (Hsu et al. 2021). Signaling downstream of Sarm1 also regulates the phagocytic capacity of glial cells during Drosophila development (McLaughlin et al. 2019), which may be relevant for their ability to clear debris following injury.

Events downstream of Sarm1 activation also trigger non-cell autonomous responses. A striking example of this was shown in a model of glaucoma, where Sarm1 participates in necroptosis signaling (Ko, Milbrandt, and DiAntonio 2020). In this case, inhibiting Sarm1 activation within stressed retinal ganglion cells is sufficient to rescue the death of oligodendrocytes in the optic nerve. Sarm1's role in promoting axonal degeneration may also play impactful non-cell autonomous roles at the injury site, since it is assumed that

clearance of degenerated debris from the injury site is prerequisite for the regrowth of axons into the site of damage. Supporting this idea, multiple studies have noted that axonal regeneration in the peripheral nervous system is impaired when Wallerian degeneration is inhibited (Lunn et al. 1989; McKerracher et al. 1994; Mukhopadhyay et al. 1994; Schäfer et al. 1996; Bisby and Chen 1990; Brown et al. 1991, 1994; Rotshenker 2011).

The Sarm1 Enzyme

While TIR domains typically mediate protein-protein interactions with other TIR domains to scaffold intramolecular complexes, Sarm1's TIR domains have the capacity to dimerize into an activated state that has potent enzymatic activity (**Figure 1-2**). In its activated form, the Sarm1 enzyme cleaves Nicotinamide Adenine Dinucleotide (NAD+) into Nicotinamide Mononucleotide (NAM) and cyclic (and non-cyclic) ADP-Ribose (cADPR) (Essuman et al. 2017). NAD+ is an allosteric inhibitor of Sarm1 (M. D. Figley et al. 2021). This may serve as a check to limit its activity in healthy cells. However, loss of NAD+, coupled with buildup of its precursor NMN, can drive Sarm1 into a feed-forward loop of chronic activation. The rundown of NAD+, a key electron carrier for cellular metabolism, is expected to lead to metabolic catastrophe (Z. Y. Zhao et al. 2019; M. D. Figley et al. 2021). Sarm1 is required and responsible for axon degeneration under a variety of stress and disease conditions (M. D. Figley and DiAntonio 2020; M. P. Coleman and Höke 2020; Sarkar, Kumari, and Mukherjee 2021), and also serves as an executioner of a form of cell death named 'sarmoptosis' (reviewed in (Fricker et al. 2018)). Sarm1-mediated neuron death can be triggered by

mitochondrial toxins (Summers, DiAntonio, and Milbrandt 2014), and Toll-Like Receptor (TLR) signaling (Mukherjee et al. 2015, 2013). It has also been documented in non-neuronal cells including as a form of programmed cell death to terminate activated T-cells (Panneerselvam et al. 2013).

In addition to NAD+ loss, the products of Sarm1's enzymatic reaction may also carry out additional functions, either to facilitate degeneration or additional roles in cellular signaling. Cyclic ADPR, a robustly measurable product of Sarm1 enzymatic activity, is a known regulator of the ER Ryanodine receptor and TRPM2 calcium channels (Galione and Chuang 2020; Galione and Ruas 2005). This function makes cADPR a prime candidate mediator to changes in intracellular signaling, as well as calcium influx. Of note, the final stages of axonal degeneration are closely accompanied by an increase in intracellular calcium (Ko et al. 2021; Mishra et al. 2013; Geffen and Hughes 1972; Schlaepfer and Bunge 1973; Schlaepfer 1974; Schlaepfer and Hasler 1979)(Ko et al. 2021). While some findings did not detect a role for cADPR in Wallerian degeneration of injured axons *in vitro* (Yo Sasaki, Engber, et al. 2020), recent work has shown a role for cADPR in triggering calcium flux and degeneration in a model of chemotherapy-induced peripheral neuropathy (Y. Li et al. 2021). Hence cADPR may also contribute to the degenerative outcome of Sarm1 activation.

Beyond the traditional breakdown of NAD+ into NAM and cADPR, recent work has shown that the Sarm1 enzyme can carry out multiple base exchange reactions (Angeletti et al. 2022). This includes base exchanges between NAD (and NADP) and

the pyrimidines 3-acetylpyridine, vacour, and nicotinic acid. In addition to being a point of vulnerability to neurotoxins (Loreto et al. 2021; Wu et al. 2021), the wide range of reactants and products may enable Sarm1 to engage multiple signaling pathways and cellular responses.

Structure-function studies in several model organisms indicate that Sarm1's TIR domains are essential for its signaling as well as degenerative functions (Gerdts et al. 2015; Chuang and Bargmann 2005). A recent study in *Drosophila* found that dSarm's enzymatic activity tracks with its signaling functions (Brace et al. 2022). However another recent study generated dSarm mutants that disrupt its ability to promote axonal degeneration but not its signaling function (Herrmann et al. 2022). These studies imply that Sarm1's NADase activity is tolerated and functionally important in healthy cells, and that much remains to be learned about Sarm1's signaling mechanism.

Regulation of Sarm1

Since unmitigated Sarm1 enzymatic activity can drive cellular degeneration, Sarm1's enzymatic activity is tightly controlled in healthy cells. The catalytic domain of the enzyme is formed by self-assembly of multiple TIR domains from multiple subunits, which structural studies indicate oligomerize into an octomeric ring (Sporny et al. 2020; Bratkowski et al. 2020; Jiang et al. 2020) (**Figure 1-2**). The TIR domains are held in an inactive state through intramolecular interactions, which are stabilized by allosteric binding of NAD+. The inhibition by NAD+ and activation by NMN provide an explanation for the widely documented sensitivity of axons to the NAD+ biosynthesis enzyme

NMNAT, Nicotinamide Mononucleotide Adenylyl Transferase. A cytoplasmically localized isoform of this enzyme (NMNAT2), is constantly transported to axons to balance its local degradation (Gilley and Coleman 2010). Reduction or loss of NMNAT2 leads to an activation of Sarm1-dependent axonal degeneration (M. P. Coleman and Höke 2020; M. D. Figley et al. 2021; Gilley et al. 2015; Yo Sasaki, Kakita, et al. 2020). Conversely, ectopic elevation of the levels of NMNAT inhibits axonal degeneration (Yo Sasaki, Araki, and Milbrandt 2006; Araki, Sasaki, and Milbrandt 2004; Yahata, Yuasa, and Araki 2009).

NMNAT2 is therefore considered to be a critical ‘survival factor’ for axons (Gilley and Coleman 2010). Multiple important mechanisms regulate the stability, turnover and/or localization of NMNAT enzymes, and thereby influence the stability and/or degeneration of axons. An evolutionarily conserved E3 Ubiquitin ligase regulates the levels of NMNAT/NMNAT2 in both Drosophila and mammalian peripheral axons (Xiong et al. 2012; Babetto et al. 2013). NMNAT levels are also controlled by JNK signaling (Walker et al. 2017), which becomes activated following axonal damage (Miller et al. 2009). Another important point of regulation is palmitoylation, which is required for NMNAT2’s protective abilities (Milde, Gilley, and Coleman 2013) and also for JNK signaling (Holland et al. 2016) .

It is expected that similarly to NMNAT, there should be additional mechanisms that directly regulate Sarm1. Sarm1 is phosphorylated, which has been shown to stimulate its NAD+ cleavage activity (Murata et al. 2018; Xue et al. 2021) Recent work suggests

that Sarm1 activation state tracks with a phase transition; in *C. elegans* intestinal epithelial cells, fluorescently labeled Sarm1 orthologue (TIR-1) could be observed to coalesce into visible puncta during conditions of stress that activate its downstream signaling (Loring et al. 2021). The phase transition and activation of downstream signaling can be triggered in genetic conditions that result in cholesterol scarcity, suggesting a cellular mechanism for how conditions of cellular stress can lead to Sarm1 activation (Peterson et al. 2022).

Sarm1 activation also occurs in additional conditions of high translational relevance, which include treatment with chemotherapy agents that induce neuropathy (Bosanac et al. 2021), and TNF-alpha triggered inflammation in an inflammatory model of glaucoma (Ko, Milbrandt, and DiAntonio 2020). Importantly, Sarm1 inhibition rescues axon function and slows both axon degeneration and cell death. In the Ko et al study, the induction of necroptosis signaling via Mixed Lineage Kinase domain-Like pseudokinase (MLKL) was shown to be responsible for Sarm1 activation, and subsequent Sarm1-mediated axonal degeneration. This Sarm1 activation can be rescued by inhibiting MAP Kinase/JNK signaling (Ko, Milbrandt, and DiAntonio 2020), which, as discussed above, can regulate the levels NMNAT2 (Walker et al. 2017). Therefore Sarm1 activation during necroptosis signaling may converge with its regulation by NAD+/NMN, discussed above.

Intersections with Axonal Injury Signaling Pathways

Axonal damage triggers the activation of multiple signaling pathways within the damaged neurons, which influence cellular responses ranging from neuronal death to the initiation of new axonal growth for facilitating repair and/or the ability to form alternative synaptic connections. Stress-associated signaling via Jun N-Terminus Kinases (JNK) is known to be activated on multiple aspects of axonal injury response (Jin and Zheng 2019; Asghari Adib, Smithson, and Collins 2018). These activated JNK kinases, along with their upstream regulators, have been observed appearing within damaged axons at short time periods after nerve injury (in mouse, phosphorylated JNK appears in injured sciatic nerves between 3-6 hours post-injury, and both activated JNK and MKK4 appear by 15 minutes post optic nerve injury) (Lindwall and Kanje 2005; Yang et al. 2015; Cavalli et al. 2005).

Multiple observations have documented an intimate web of interactions between JNK signaling and axonal degeneration machinery. First, several studies have pointed to roles for JNK signaling in the execution of distal stump degeneration. Acute application of JNK inhibitors to axomized DRG neurons in culture concomitant with injury is capable of delaying the onset of degeneration (Miller et al), suggesting a local and acute role for JNK in axotomized axons. One pro-degenerative role for JNK is promoting the turnover of NMNAT2 ((Walker et al. 2017)). In addition, all 3 JNK kinase isoforms are capable of phosphorylating Sarm1, and phosphorylation of Sarm1 promotes its enzymatic activity and subsequent neurodegeneration (Murata et al. 2018; Xue et al. 2021). While the above studies suggest upstream regulatory roles for JNK signaling in driving the

activation of Sarm1, other studies have suggested a converse relationship where JNK signaling activation in damaged axons may be triggered downstream of Sarm1 activation (Yang et al. 2015; Q. Wang et al. 2018).

It is also plausible that signaling gated by Sarm1 acts together with its enzymatic activity to drive degeneration. One study has posited a role for JNK downstream of Sarm1, since genetic disruption of all 3 JNK isoforms (or both MKK4/7) could inhibit degeneration caused by ectopic activation of Sarm1's TIR domains (Yang et al. 2015). The dual upstream and downstream relationships of JNK signaling with Sarm1 (and vice versa) suggests the possibility that they cycle together in a feed-forward relationship, acting to both promote axonal degeneration and detect the presence, likelihood, or possibility of axonal degeneration following Sarm1 activation. These relationships suggest intimate links between axonal damage signaling and Sarm1 that we have yet to understand on a molecular level. (Figley et al. 2021; Waller and Collins 2021).

One more set of twists in the intertwined relationship of JNK signaling with Sarm1 come from observations in invertebrates that JNK signaling activation following axonal damage can have protective functions in neurons. This includes the inhibition of degeneration in both axons and dendrites (Xiong and Collins 2012; Hao et al. 2019), which contrasts with the pro-degenerative role described above for JNK in mammalian axons. The mechanism for this protective role is not yet clear. However, it appears to require the transcription factor Fos and may therefore be mediated by downstream

changes in gene expression. This could possibly promote multiple changes to axonal metabolic mechanisms or cytoskeleton that influence the propensity of axons to degenerate. While it is plausible that the Fos-dependent pathway could influence the expression level or localization of NMNAT enzyme isoforms(s) (L. Chen et al. 2016), a recent study has described an NMNAT-independent form of JNK-mediated protective signaling (Hao et al. 2019).

Potential New Players in Sarm1 Signaling Mechanisms

Recent work has brought to light some additional players in NMNAT-Sarm1 signaling. One intriguing player is Axed, short for 'Axundead', which was discovered in a forward genetic screen in *Drosophila* (akin to the screen that first discovered dSarm as an essential mediator of axonal degeneration)(Neukomm et al. 2017). Similar to mutants in dSarm, neurons lacking *axed* function fail to undergo Wallerian degeneration. Remarkably, *axed* mutants inhibit axonal degeneration even in conditions that are expected to result in unmitigated NADase enzymatic activity by dSarm. This includes deletion of NMNAT, which normally leads to axonal and neuronal degeneration, but is rescued by mutation of Axed. From these striking genetic data it is proposed that Axed promotes axonal degeneration downstream of Sarm1's enzymatic activity.

How Axed may do this remains mysterious. It contains a BTB domain, which could participate in scaffolding signaling interactions, but has no known enzymatic activity of its own. Axed protein is abundantly localized near presynaptic terminals in *Drosophila*

motoneurons. The relevance of this localization to its function in axonal degeneration is both unclear and intriguing.

Another intriguing new set of players are the Lysine Deficient Protein (WNK) kinases. WNK kinases are broadly expressed, best known for roles in the regulation of ion transport and blood pressure regulation (Murthy, Kurz, and O'Shaughnessy 2017). However a recent study, also resulting from a genetic screen in *Drosophila*, identified developmental roles for dWnk in stabilizing axonal branches, in addition to regulating branching during neurite outgrowth (Izadifar et al. 2021). Knockdown of dWnk leads to both axon branching defects and early-onset degeneration. This degeneration is dependent on Axed and is rescued by dNMNAT activity (similarly to Sarm1-mediated Wallerian degeneration). In axon maintenance, dWnk promotes dNMNAT activity while negatively regulating both Axed and dSarm based on genetic epistasis data. Therefore WNK appears to be an important regulator of at least two functions for signaling surrounding Sarm1. Future study of the cellular mechanism of its interactions with Sarm1 may provide important insights into Sarm1's functions in signaling as well as degeneration.

Conclusion

The human health impacts of Sarm1 research are clear, with Sarm1 inhibition being a promising intervention for a variety of neurodegenerative diseases. However, given the vital role of Sarm1 in neurodevelopment and the immune system, care must be taken in disrupting this enzyme's activity. The potential separation of Sarm1's degenerative and

developmental signaling is an elusive research target that offers the possibility of nuanced, context-specific control.

Wallerian Degeneration: Discovery and Early Characterization

Wallerian degeneration was first documented in the frog tongue following mechanical injury, seen as fragmentation of the severed nerves (Augustus Volney Waller 1850). Originally thought to be a passive process, it is now understood that Wallerian degeneration is a carefully regulated series of events that requires active signaling in the transected nerve (Zhang, Jiang, and Fang 2021), and is distinct from other forms of degeneration like axon apoptosis and axon pruning (Geden and Deshmukh 2016). Early work revealed fragmentation of the endoplasmic reticulum, shortening of neurofilaments, and swelling of mitochondria in sectioned rat saphenous nerves (Vial 1958). One of the first molecules identified in this process was calcium, as Wallerian degeneration is inhibited when transected nerves are placed in calcium-free media and is enhanced when nerves are placed in medium with added calcium (Schlaepfer 1974). Much of this initial work pointed towards Wallerian degeneration being a passive death process, with simple physical manipulations being capable of speeding or slowing degeneration.

This passive view was radically changed by the discovery of a mouse strain showing delayed degeneration in severed sciatic nerves (Lunn et al. 1989). Initial uncertainty of whether this phenotype was mono- or polygenic was clarified by an inheritance study suggesting the cause was a single autosomal dominant mutation (Perry et al. 1990).

The locus responsible for the phenotype was mapped to chromosome 4 and named "Wld^S" (Wallerian degeneration, slow) (Lyon et al. 1993). Wld^S is a chimeric protein containing the N-terminal fragment of Ube4b (Ubiquitination Factor E4B) fused to NMNAT1 (Nicotinamide Mononucleotide Adenylyltransferase 1) (Mack et al. 2001). The Ube4b fragment and NMNAT1's enzymatic activity are both required for the delayed degeneration seen in Wld^S mutants (Avery et al. 2009; Laura Conforti et al. 2009), though overexpression of NMNAT1 alone is sufficient to delay degeneration in explant DRG (Dorsal Root Ganglion) neurons (Araki, Sasaki, and Milbrandt 2004). Wld^S was initially proposed to have an indirect effect on degeneration by acting in the nucleus (Mack et al. 2001). This hypothesis was supported by findings that Wld^S influences gene expression (Gillingwater et al. 2006), as well as the discovery that elevated NAD+ leads to delayed degeneration via SIRT1 (Sirtuin 1) (Araki, Sasaki, and Milbrandt 2004). However, unlike in cultured cells, overexpression of NMNAT1 (nucleus isoform) in mice did not recapitulate the strong delay in Wallerian degeneration seen in Wld^S mutants (L. Conforti et al. 2006). In contrast, mice overexpressing NMNAT3 (mitochondrial isoform) showed delayed Wallerian degeneration (Yahata, Yuasa, and Araki 2009), and when the NLS (Nuclear Localization Signal) of Wld^S is mutated, both axonal localization and the delay in degeneration is enhanced compared to the original Wld^S (Beirowski et al. 2009). Similarly, mutation of NMNAT1's NLS localizes the protein to the cytoplasm and axons, and shows delayed Wallerian degeneration beyond that seen with Wld^S (Y. Sasaki et al. 2009). Comparable results were found when NMNAT1 is modified with an axonal targeting peptide from APP (Amyloid Precursor Protein) to localize to the axons and synapses (Babetto et al. 2010). While overexpressed NMNAT3 and modified

NMNAT1 are both able to delay degeneration, it was ultimately NMNAT2 that was identified as the isoform which localizes to the axon and prevents spontaneous axon degeneration under uninjured, healthy conditions (Gilley and Coleman 2010),

While these data point toward NMNAT2's importance in axon integrity, the mechanism of how loss of NMNAT2 is tied to degeneration remained elusive. This changed upon the discovery of a pro-degenerative role for the enzyme SARM1 (Osterloh et al. 2012), which has since rapidly expanded our understanding of NMNAT2's axonal function, and the molecular events that drive axon degeneration.

NAD+/NMN Balancing Act by SARM1 and NMNAT2 Controls Axon Degeneration[1]

Mechanisms to destroy and clear neuronal processes are important for the ability of nervous systems to adapt to damage, stress and viral infection. Studies of the degeneration of injured axons have unraveled a cell autonomous axon death pathway whose mechanism is distinct from apoptosis (reviewed in (M. P. Coleman and Höke 2020)). 'Wallerian degeneration' named after its first description by Augustus Waller in 1851, appears to be intimately linked with the synthesis and breakdown of nicotinamide adenine dinucleotide (NAD+), a key coenzyme for metabolism and cellular respiration.

The first hint of this pathway came from the fortuitous discovery of a spontaneous mutation in the C57BL/6/Ola strain background that enabled prolonged survival (up to several months) of damaged axonal stumps severed from their cell body.

[1] This section is adapted from the following manuscript:

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Characterization of the '*Wallerian degeneration Slow*' (*WldS*) mutation revealed that the protection is conferred by the ectopic localization of the NAD+ biosynthesis enzyme NMNAT1 to axons. Later work by Michael Coleman's group found that a cytoplasmic version of the enzyme, NMNAT2, is rapidly turned over in axons. Injury and/or disruption of axonal transport interrupts the supply of NMNAT2 to distal axons (**Figure 1-3**), and its loss leads to degeneration (of axons and/or cells, depending on the extent of NMNAT2 loss). NMNAT2 is therefore considered to function as a critical 'protective' factor for axons.

But how does an NAD+ biosynthesis enzyme protect axons from degeneration? And why are axons so sensitive to its loss? Exciting work over the past 10 years has identified the sterile-α and Toll/interleukin 1 receptor (TIR) domain containing protein 1 (SARM1) as an essential executioner of axonal degeneration and death due to NMNAT loss. TIR domains are found in proteins across most kingdoms of life: in animals, plants and bacteria. They are commonly present in proteins that function in innate immunity, where the ability of TIR domains to self-associate facilitates protein interactions. Strikingly, work in 2017 by Milbrandt and DiAntonio groups discovered that the self-associated TIR domain of SARM1 (as well as TIR domains in other proteins) has enzymatic activity as a NADase (Essuman et al. 2017). By cleaving NAD+ (into Nicotinamide and ADP-Ribose), SARM1's enzymatic activity leads to rapid depletion NAD+ to the point of metabolic catastrophe. By implicating a new enzyme in the execution of axonal degeneration, these findings highlight the existence of an active

self-destruction pathway that can accelerate what might otherwise be a passive process of metabolic rundown in distal axons removed from their cell bodies.

As an enzyme that is required for axonal death but lacking other strong *loss-of-function* phenotypes on its own, SARM1 has become an attractive therapeutic target (Krauss et al. 2020). A key question is to understand how it is normally restrained in healthy axons, and the molecular conditions that lead to its activation (M. P. Coleman and Höke 2020). An N-terminal Armadillo/HEAT Motif (ARM) domain plays an important autoinhibitory role by preventing dimerization of the TIR domains, which is essential for their enzymatic activity. A flurry of recent structural studies have delineated SARM1 in its inactivated state, which assembles into an octomeric ring (Sporny et al. 2020; Jiang et al. 2020; Bratkowski et al. 2020). These studies have revealed a compelling role for NAD+ in stabilizing the inactive state of the enzyme: NAD+ binds to an allosteric site within the ARM domain, which facilitates the 'lock' interactions between the ARM and TIR domains (**Figure 1-3**).

It is attractively logical that Sarm1 should be regulated by NAD+, however the exact relationship between NAD+ levels and axon degeneration has remained a cloudy area in the field (reviewed in (M. P. Coleman and Höke 2020)). While ectopic induction of SARM1's NADase activity is sufficient for degeneration and cell death, manipulations that indirectly reduce NAD+ levels, by inhibiting the synthesis of the NAD+ precursor, NMN, lead to protection rather than degeneration of injured axons. Hence moderate NAD+ reduction can be uncoupled from degeneration, and it has been proposed that

rather than-or in addition to–loss of NAD+, build-up of the NMN precursor leads to axonal degeneration. Indeed, ectopic elevation of NMN can potently stimulate axonal degeneration, and recent studies suggest this is due to direct activation of SARM1 by NMN (Z. Y. Zhao et al. 2019). However conditions that elevate NMN do not always lead to degeneration (Yo Sasaki et al. 2016). Of note, in these conditions NAD+ levels are also high.

A recent study by Figley, Gu, Nanson, Shi and colleagues (M. Figley et al. 2021) establishes an elegant solution to this long standing puzzle, by elucidating how NMN leads to activation of SARM1's enzymatic activity. In addition to more cryo-EM structures of the full length inactive enzyme, Figley and colleagues present a 1.7 Angstrom crystal structure of the ARM domain alone (from Drosophila dSarm1) bound to NMN. Strikingly, this structure shows NMN occupying the same binding pocket as NAD+, however conferring an altered conformation of the ARM domain. This change is predicted to disrupt the ARM-TIR lock, disrupting the interactions of the outer ring (Sporny et al. 2020), freeing the TIR domain to dimerize into an enzymatically active NADase (**Figure 1-3**).

While the complete structure of the activated enzyme is not yet resolved, the new view of structural changes in the ARM domain leads to a compelling model that NMN and NAD+ compete to switch SARM1 between active and inactive states through binding to a common allosteric site. Figley tested this model with manipulations that independently alter NMN or NAD+ levels in cultured primary neurons, following the production of

SARM1's unique enzymatic product cyclic ADP ribose (cADPR) by mass spectrometry, and through flux assays with heavy-labeled nicotinamide. They found that manipulations that alter the ratio of NMN/NAD+, either by elevating NMN levels or decreasing NAD+, can equally trigger Sarm1 NADase activity. Further work with purified SARM1 *in vitro* demonstrated that the NAD+ and NMN can directly compete for regulation of SARM1's enzymatic activity (M. Figley et al. 2021).

The insight that the *ratio* of NMN/NAD+, rather than either nucleotide alone, controls SARM1 activation provides a solution to the mystery of how SARM1 is controlled by NMNAT2. The conversion of the NMN precursor to NAD+ by this enzyme tidily promotes a low NMN/NAD+ ratio, which favors Sarm1 inhibition (**Figure 1-3**). It is perhaps not an accident that ATP is also a substrate for NAD+ synthesis by NMNAT2, linking SARM1 inhibition to sufficient levels of ATP as well as NMNAT2 in axons. NMNAT2 appears to have a short half-life in axons and must be continuously supplied by transport from the cell body. Injury, or other methods that disrupt axonal transport, such as the presence of neuropathy inducing chemotherapy agents that disrupt axonal cytoskeleton, lead to a reduction of Nmnat2 in distal axons (**Figure 1-3**). The reduction in NAD+ synthesis should lead to a higher NMN/NAD+ ratio, favoring the activation of SARM1's NADase activity. If the distal axon lacks ability to resynthesize NAD+, then SARM1's NADase activity remains unchecked and drives an accelerated loss in NAD+, which should ultimately lead to metabolic catastrophe and degeneration.

In contrast to damaged neurons, Figley and colleagues present further analysis of the relationship between SARM1 activation and NMNAT2 function in healthy cultured primary neurons pulsed with nicotinamide riboside (NR) (while overexpressing the NMN-synthesizing NR kinase (NRK1)). While this popular nutritional supplement is used for an end result of increasing cellular NAD+, the pulse first leads to elevated NMN levels, which activates SARM1's NADase activity, and dampens the ability of NR to increase total NAD+ levels. Eventually (after 24 hours) NAD+ levels do increase, presumably due to NMNAT function and re-silencing of the SARM1 NADase, which can explain why neurons pretreated with NR together with NRK1 show an increased resilience to degeneration (M. Figley et al. 2021; Yo Sasaki et al. 2016). These observations emphasize that conditions that activate SARM1's NADase activity do not necessarily represent a point of no return. Instead, healthy cells (which should have NAD+ biosynthesis capacity) can tolerate some level of SARM1 NADase activity, and it is indeed detectable at modest levels in healthy neurons (Yo Sasaki et al. 2016). This basal activity may be important for signaling functions of SARM1 outside of degeneration. Multiple studies in different model organisms have detailed roles for SARM1 and its orthologues in regulating MAP Kinase signaling pathways relevant for cell fate, neuronal plasticity and innate immunity in both neurons and glial cells. Whether (and how) SARM1's NADase activity and its regulation by NMN/NAD+ ratio influences its other signaling functions is an interesting area for future investigation.

While there are likely additional layers of SARM1's regulation to uncover, the current insight further increases the attractiveness of Sarm1 as a target to inhibit axonal loss in

neuropathies associated with chemotherapy, diabetes, mitochondrial dysfunction, and potentially in neurodegenerative diseases such as ALS and FTD (Krauss et al. 2020). Recent work has shown that SARM1 inhibition at a time point after mitochondrial damage has occurred is sufficient to rescue these axons from degenerating (Hughes et al. 2021). The finding that Sarm1's enzymatic activity may be tuned by altering NMN/NAD+ ratios offers a rich new platform of potential methods to regulate SARM1, with hopes towards rescuing axons and synapses in different translational settings.

Figures

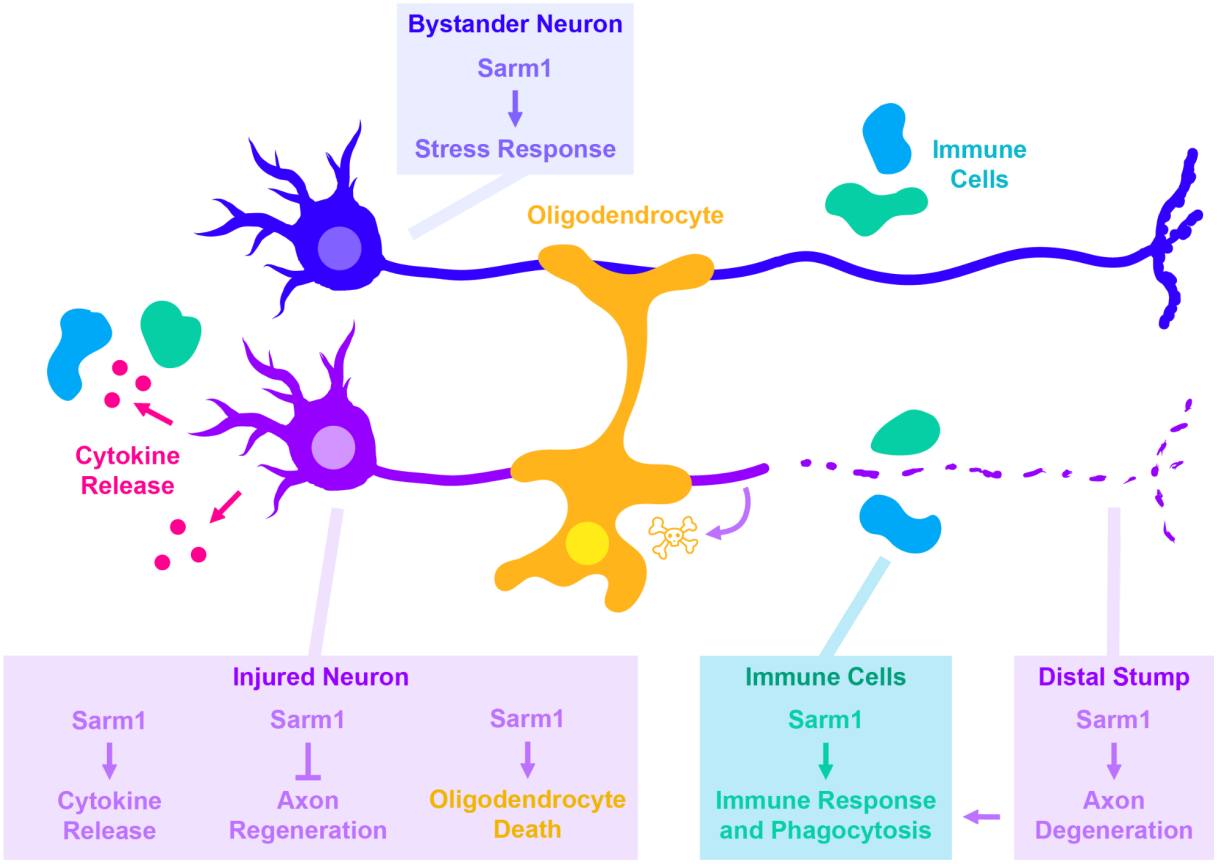


Figure 1-1: Autonomous and non-autonomous roles for Sarm1 in responses to injury

Sarm1's cell autonomous roles in injured neurons are shown in magenta. In addition to its most well known for its role in promoting degeneration of the distal stump, Sarm1 is also required for additional responses made by injured neurons. These include the release of cytokines (Q. Wang et al. 2018), the ability of neurons to regenerate in C. elegans (Julian and Byrne 2020), and in promoting death of oligodendrocytes in a glaucoma model (Ko, Milbrandt, and DiAntonio 2020). Sarm1 also functions within uninjured cells that participate in responses to injury, including uninjured 'bystander' neurons (dark blue blue) (Hsu et al. 2021), and immune cells that react to the damage (light blue) (McLaughlin et al. 2019).

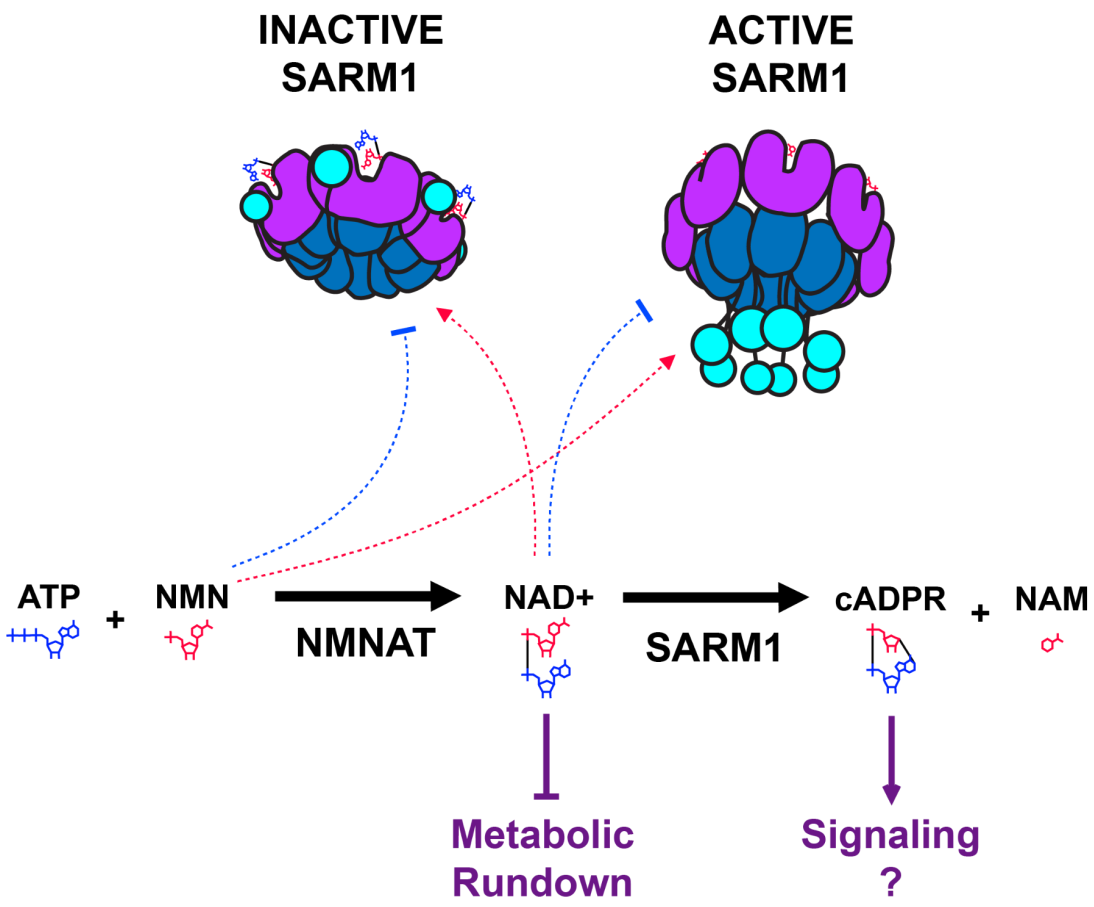


Figure 1-2: Enzymatic reactions of Sarm1 and NMNAT tune NAD+ in axons
The activated form of the Sarm1 enzyme cleaves NAD+ (Nicotinamide Adenine Dinucleotide) into ADP-Ribose (ADPR), which can be in a cyclic form (cADPR), and NAM (Nicotinamide). The loss of NAD+ caused by constitutive Sarm1 activity is expected to drive metabolic rundown, which precedes axon breakdown (J. Wang et al. 2005). cADPR buildup is a measurable proxy of Sarm1's enzymatic activity (Yo Sasaki, Engber, et al. 2020). As it is known to influence ryanodine and TRPM2 cation channels (Galione and Chuang 2020; Galione and Ruas 2005), cADPR is an attractive candidate mediator of signaling mechanisms downstream of Sarm1. However the role of cADPR in Sarm1's signaling functions (examples shown in Figure 1) requires further testing. Sarm1's enzymatic activity is carried out by its Toll/interleukin-1 receptor (TIR) domains (light blue), which must form dimers or oligomers in order to be active. Its TIR domains are normally held in an inactive state within an oligomeric holoenzyme. The inactive state is stabilized by the presence of NAD+, which binds to an allosteric regulatory site in the Armadillo/HEAT Motif (ARM) domain (violet) (M. D. Figley et al. 2021). The NAD+ precursor NMN competes with the allosteric NAD+ binding site and has the opposite effect of promoting Sarm1's active state. Sarm1's catalytic state is therefore potently

tuned by the function of the NAD+ biosynthetic enzyme NMNAT, which is a critical protective factor in axons and neurons (Gilley and Coleman 2010). This regulation also enables a feed-forward mechanism of Sarm1 activation in injured axons and cells that lack NMNAT function, since once Sarm1 is activated it can promote its own further activation by lowering NAD levels.

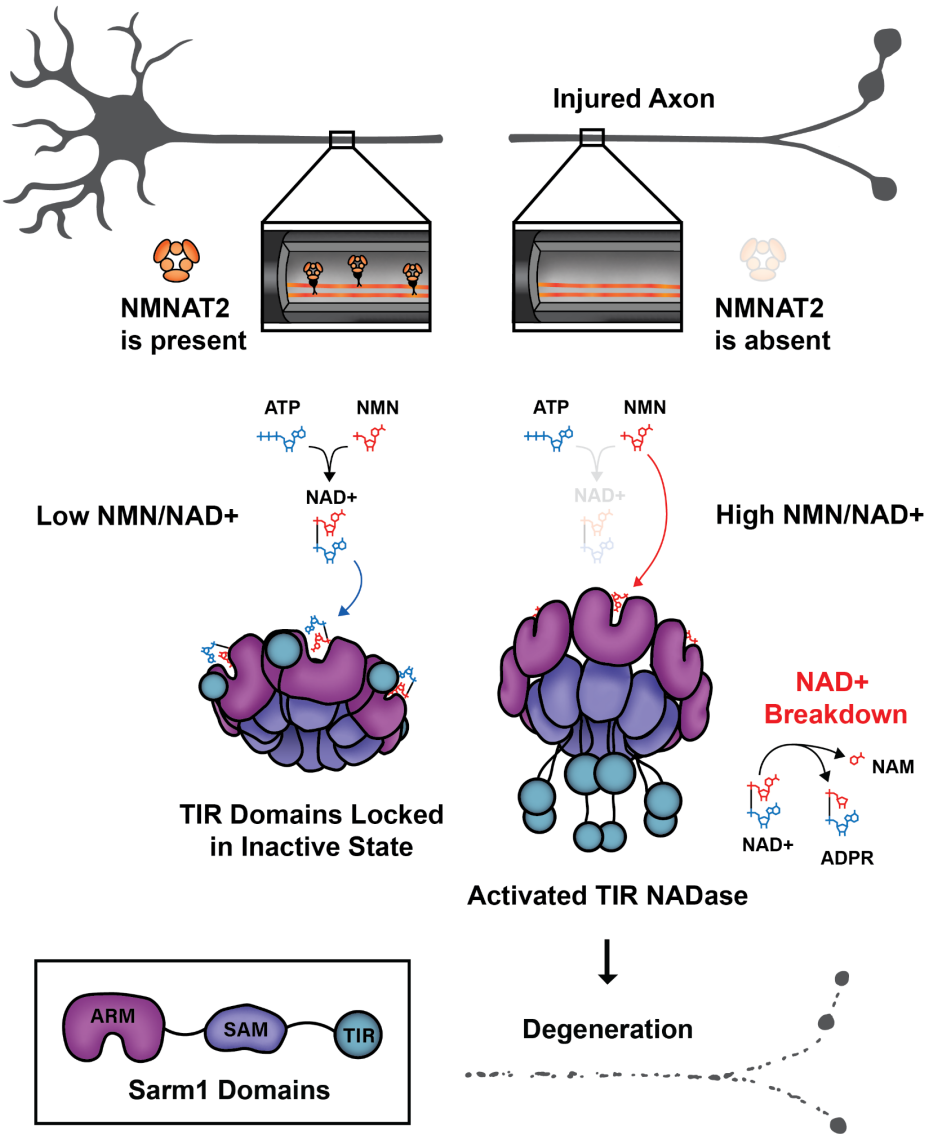


Figure 1-3: Sarm1 is regulated by rato of NMN and NAD+[2]

NAD+ binding to a regulatory site in SARM1's ARM domain stabilizes an ARM-TIR interaction that locks the TIR domains in an inactive state. The NAD+ precursor NMN binds to this same site, but switches the ARM conformation to disrupt the ARM-TIR lock, freeing the TIR domains to associate into an activated NADase enzyme. In healthy axons the presence of NMNAT2 maintains NAD+ levels while consuming NMN as a reactant, favoring the NAD+-bound locked state. In contrast, in distal axons disconnected from the cell body by injury and/or disrupted axonal transport, reduced

[2] This figure is adapted from the following manuscript:

TJ Waller and Catherine A. Collins. (2021). An NAD+/NMN balancing act by SARM1 and NMNAT2 controls axonal degeneration. Neuron. Preview. https://doi.org/10.1016/j.neuron.2021.03.021

NAD+ biosynthesis favors NMN binding to unlock the NADase enzyme. NAD+ breakdown in the absence of NMNAT to reduce the NMN/NAD+ ratio leads to unmitigated NADase activity by activated SARM1, which leads to metabolic catastrophe and axonal degeneration. The Sarm1 cartoon is adapted from (Sporny et al. 2020).

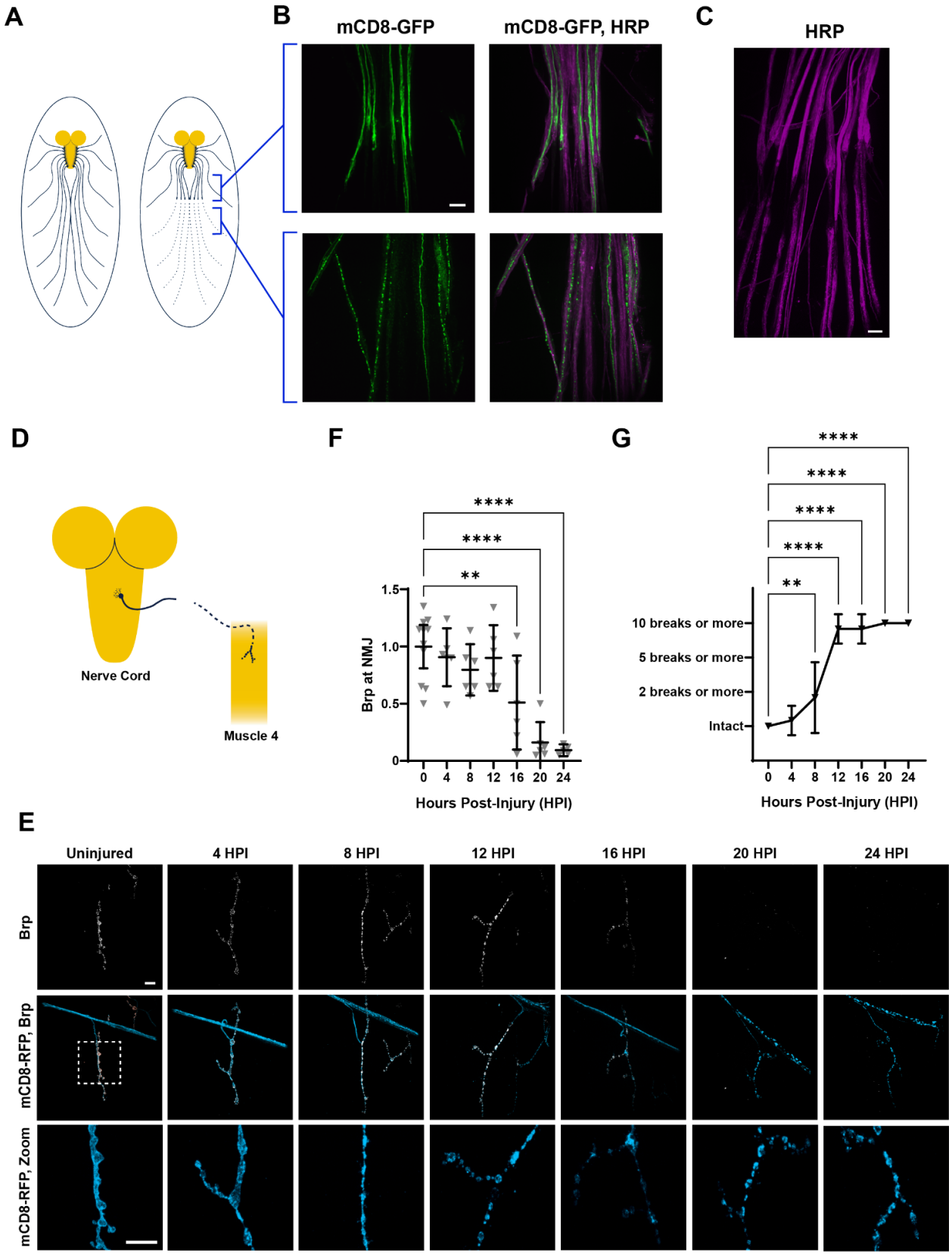

A
B
mCD8-GFP
mCD8-GFP, HRP
C
HRP
D
Nerve Cord
Muscle 4
F
Brp at NMJ
Hours Post-Injury (HPI)
G
10 breaks or more
5 breaks or more
2 breaks or more
Intact
Hours Post-Injury (HPI)
E
Uninjured
4 HPI
8 HPI
12 HPI
16 HPI
20 HPI
24 HPI
Brp
mCD8-RFP, Brp
mCD8-RFP, Zoom

Figure 1-4: Larva Injury and Degeneration Timecourse[3]

A-C) Cartoon (**A**) and images (**B**) of example motoneurons in the Drosophila larvae labeled by expression of UAS-mCD8::GFP from the m12-Gal4 (tey5053A) driver (Ritzenthaler, Suzuki, and Chiba 2000) in dark blue. Within 20 hours following the crush injury, motoneuron axons distal to the crush site undergo Wallerian degeneration, while the ending of the proximal stump shows sprouting and accumulation of mCD8::GFP. At the injury site, GFP expressed in individual axons is no longer visible (**B**), however HRP labeling shows that some connective tissue in the nerve is still present (**C**). **D**) Cartoon of the motoneuron that innervates Muscle 4; following nerve crush the degeneration of the nerve terminal at the NMJ can be visualized in (**E**). **E**) Example images of muscle 4 NMJs from D42-Gal4, UAS-mCD8::RFP animals stained for RFP (blue) to label the neuronal membrane, and the active zone protein Brp (Nc82, white). Brp is visible for up to 12 HPI, however axonal fragmentation becomes apparent around 8 HPI. Brp is cleared by 20 HPI, however fragments of axonal membrane remain much longer; full clearance of membrane is not observed before the animals undergo pupation. **F**) Quantification of the total intensity of Brp staining at muscle 4 NMJ terminals reveals the time course of its disappearance following nerve injury. **G**) Membrane fragmentation at NMJ terminals was scored using a visual scoring assay. Individual NMJs were binned according to whether they had more than two, five or 10 visible breaks in membrane continuity at the NMJ. The mean score was plotted for 1 NMJ from 5-6 injured animals per time point. All scale bars are 20 μm.

[3] This figure and legend are adapted from the following manuscript (all data shown was produced by TJ Waller):

TJ Waller*, Laura J. Smithson*, Catherine A. Collins. (*In Press*). Study of axonal injury and degeneration in Drosophila. Drosophila Neurobiology: A Laboratory Manual. *Authors contributed equally.

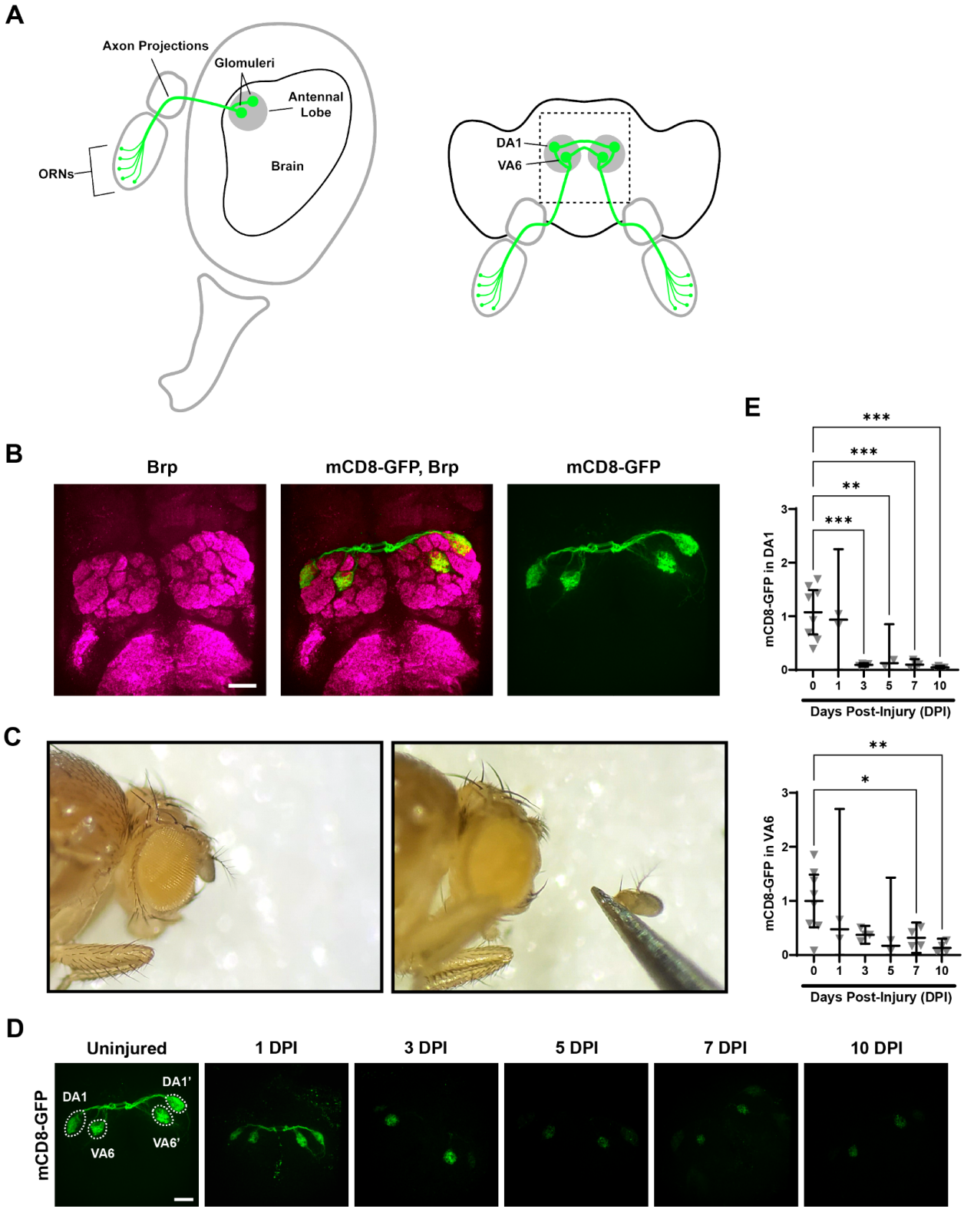

A
Axon Projections
Glomuleri
Antennal Lobe
Brain
ORNs
DA1
VA6
B
Brp
mCD8-GFP, Brp
mCD8-GFP
C
D
Uninjured
1 DPI
3 DPI
5 DPI
7 DPI
10 DPI
mCD8-GFP
DA1
DA1'
VA6
VA6'
E
mCD8-GFP in DA1
Days Post-Injury (DPI)
mCD8-GFP in VA6
Days Post-Injury (DPI)

Figure 1-5: Adult Antennal Injury and Degeneration Timecourse[4]

Schematic showing the location and anatomy of an example subset of ORNs that innervate DA1 and VA6, labeled by the Or76d-Gal4 driver. Cell bodies lie in the antennae, while axons bilaterally innervate either DA1 or VA6 glomeruli in the antennal lobe. While VA6 ORNs are not thought to express endogenous Or76d, the strong expression of Gal4 in these cells allows their terminals to be followed in parallel. The dashed square in the front view indicates the region shown in panel B. (A similar cartoon noting the ORNs was shown in (Gillingwater et al. 2006). **B**) The glomeruli in the olfactory lobes are visualized by staining with a monoclonal antibody (Nc82) against the pan-synaptic marker Brp (Wagh et al. 2006), while the terminals of ORNs innervating glomuleri DA1 and VA6 are labeled by mCD8-GFP using Or67d-Gal4. **C**) Photos of the antennae before and after removal from an anesthetized adult fly. **D**) Time course of labeled glomeruli degenerating and being cleared following double antennectomy. Dotted outlines indicate the regions of interest (ROIs) used to measure the remaining ORN membrane in DA1 and VA6 glomeruli. **E**) Quantification of clearance was carried out separately for DA1 and VA6 glomeruli pairs, from the merged projections of confocal stacks that covered the entire olfactory bulb. Using Volocity software, ROIs were drawn around the visible GFP terminals, and the same region was copied to an alternate (non-innervated) glomerulus to measure background. The total intensity of pixels derived from the mCD8-GFP channel was summed for each ROI. Total minus background (for either the DA1 or VA3 pair) was normalized to this value for uninjured animals and plotted for each fly. All scale bars are 20 μm.

[4] This figure and legend are adapted from the following manuscript (all data shown was produced by TJ waller):

TJ Waller*, Laura J. Smithson*, Catherine A. Collins. (*In Press*). Study of axonal injury and degeneration in Drosophila. Drosophila Neurobiology: A Laboratory Manual. *Authors contributed equally.

Chapter 2: Raw and dSarm Regulate Degeneration and NMJ Structure

Abstract

The degeneration of injured axons and the development of synapses are regulated across diverse species by common conserved molecules. These molecules include the sterile armadillo TIR domain containing protein Sarm1, the Jun N-terminal kinase JNK, and additional regulators of these proteins. It is not known how the actions of these shared molecular players are tuned to promote synapse development without also activating degeneration (and vice versa). In this study, we identify a role for the pro-degenerative transmembrane protein Raw as an inhibitor of synaptic growth at the *Drosophila* neuromuscular junction (NMJ). Loss of Raw leads to excessive synaptic growth coupled with reduced levels of synaptic proteins, dependent on the Fos transcription factor. The same signaling pathway is engaged by overexpression of the *Drosophila* Sarm1 homologue dSarm, which leads to similar synaptic phenotypes. While Raw and dSarm counteract each other to regulate synaptic growth, they work together to regulate axonal degeneration. Raw regulates the levels of dSarm localized to axons and promotes the activation of dSarm-mediated axonal degeneration. The combined genetic data suggest that Raw and dSarm regulate multiple intersecting signaling pathways. Our findings support a multifaceted model of JNK coordination by Raw and dSarm that regulates nuclear signaling, NMJ structure, and axon degeneration.

Introduction

Axons are a vital component of the nervous system and are required for even the basic functioning of many organisms. These cellular extensions often span incredible distances to form synapses, which can make them vulnerable to injury, toxins, and other stressors. When an axon is severed from its cell body, it undergoes a cell autonomous self-destruction process known as Wallerian degeneration (Augustus Volney Waller 1850), akin to apoptosis, but invoking distinct molecular machinery (Gerdts et al. 2016; M. P. Coleman and Höke 2020). A key enzyme that drives axonal degeneration is the Sarm1 NADase (Osterloh et al. 2012). Sarm1 contains catalytic Toll Interleukin Receptor-like (TIR) domains which break down the electron carrier Nicotinamide Adenine Dinucleotide (NAD+). Unmitigated activation of the TIR1 domain leads to NAD+ loss and metabolic catastrophe (Gerdts et al. 2015).

Coupled with great interest in progress in understanding the structure and mechanism of Sarm1, many questions remain regarding its role in axonal degeneration. One set of questions revolve around the role of Sarm1's enzymatic activity in the axonal degeneration process. The breakdown of NAD+, a key metabolite, is expected to be a catastrophic point of no return for axons and cells. However, some genetic observations have suggested the existence of additional events downstream of NAD+ loss required for axonal degeneration (Neukomm et al. 2017). One example is the requirement for the BTB-domain containing protein Axundead (Axed) in Sarm1-induced degeneration. Loss-of-function mutations in *axed* can rescue the degeneration of axons that are constitutively activated for Sarm1's NADase enzymatic activity (Neukomm et al. 2017).

In another example, complete inhibition of JNK signaling (by genetic deletion of all three isoforms of JNK) blocks the ability of ectopically-activated TIR NADase to drive the degeneration of DRG axons in culture (Yang et al. 2015). These observations suggest the existence of additional events critical for axonal degeneration.

Before the discovery that Sarm1's TIR domains have enzymatic activity as a NADase, Sarm1 was known to function as a regulator of MAP Kinase signaling. Another set of outstanding questions about Sarm1's mechanism revolve around the relationship of its roles in signaling with its roles in axonal degeneration. All studies to date suggest that Sarm1's enzymatic activity is also required for its signaling functions. Moreover, cADPR, a product of the Sarm1 NADase reaction, is a known regulator of calcium flux and degeneration (Höke 2022; Y. Li et al. 2022), hence is an attractive candidate co-factor for Sarm1's signaling functions. However, the cellular mechanisms of Sarm1's functions in signaling are not well understood, and could conceivably involve distinct mechanisms that regulate its activation, or distinct cofactors that regulate the enzyme in its signaling context.

Here we investigate the regulation of Sarm1 in degenerative signaling, and the relationship of its role in degeneration with its role in regulating synapse growth. Our previous work identified the transmembrane protein Raw as an important regulator of Wallerian degeneration in *Drosophila* motoneuron axons (Hao et al. 2019). We found that Raw-regulated signaling, presumably via JNK and the Fos transcription factor, regulates a protective pathway in *Drosophila* motoneuron axons that delays/impairs

Wallerian degeneration following injury (Hao et al. 2019). Contrasting to previously known roles for JNK signaling (Walker et al. 2017), *raw* mutants do not appear to affect or show sensitivity to the levels of NMNAT, a known upstream regulator of Sarm1 (Waller and Collins 2021).

In this study we probled the relationship of Raw with *Drosophila* Sarm1 (dSarm). Both Raw and dSarm regulate MAPK degenerative signaling (Hao et al. 2019; Yang et al. 2015). Moreover, a distant orthologue of Raw, olrn-1 in *C. elegans*, was identified as a genetic regulator of TIR signaling (Foster et al. 2020). Our genetic data from this study point to a role for Raw in regulating axonal degeneration upstream of dSarm. However, we also report an additional role for Raw-regulated signaling in the control of synaptic structure. This signaling function also dovetails with signaling regulated by dSarm. However, the relationship of dSarm and Raw in synaptic structure contrast with their roles in axonal degeneration. Our data point to multifaceted roles of the downstream signaling components JNK and Fos in the regulation of synaptic structure and axonal degeneration.

Materials and Methods

Fly Stocks. W118 [WT], UAS-Luciferase [Control Protein] (BDSC 35788), UAS-*luciferase*-RNAi [Control RNAi] (BDSC 31603), UAS-*lexA*-RNAi [Control RNAi] (BDSC 67947), QUAS-gRNA [Control gRNA] (BDSC 67539), UAS-Homo-TIR (gift from DiAntonio lab), UAS-*raw*-RNAi (VDRC 101255), $raw^{dcp\text{-}1}$ (Hao et al. 2019), $raw^{134.47}$ (Jemc et al. 2012), UAS-NMNAT (Zhai et al. 2006), UAS-dSarm (BDSC 17144),

UAS-dSarm::GFP (Osterloh et al. 2012). UAS-Fos^{DN} (II) (BDSC 7214), UAS-Fos^{DN} (III) (BDSC 7215), UAS-Bsk^{DN} (BDSC 9311), UAS-Bsk-HA (F003890), Puc-LacZ (Martín-Blanco et al. 1998), dSarmΔTIR (gift from Broihier lab), dSarm-gRNA (gift from Dickman lab), BG380-Gal4 (Budnik et al. 1996), D42-Gal4 (Sanyal 2009), M12-Gal4 (Ritzenthaler, Suzuki, and Chiba 2000), UAS-Dcr2 (BDSC 24650), UAS-Cas9 (BDSC 58985).

Larvae Rearing. All larvae were reared on yeast-agar food at 29°C.

Nerve crush injury. To study degeneration, we made use of a previously described nerve crush assay (Xiong et al. 2010). Early 3rd instar larvae were anesthetized in a PBS ice bath for 20 minutes before being placed on an inverted petri dish. Dumostar number 5 forceps were used to pinch the larval nerves through the cuticle to damage the axons. Injured larva were placed in a petri dish with yeast-agar food and incubated at 29°C for the indicated time lengths.

Injury-Induced Degeneration Scoring. Axon motoneuron pairs were labeled by M12 GAL4-expressed mCD8-GFP (or RFP) and scoring was done on fixed and stained larval filets. Conditions were scored blind using the following scale: Completely Intact (0%); Continuous with Varicosities (33%); Partially Fragmented and Partially Continuous (66%); Fully Degenerated (100%). Sample sizes reported are the total number of axon pairs scored, from at least 5 animals per condition.

Homo-TIR-Induced Degeneration and Clearance Scoring. Homo-TIR was expressed in pairs of motoneuron axons labeled with GAL4-expressed mCD8-RFP. Axon pairs from the ten nerves innervating segments A3-A7 of the larva were scored (20 axons total) on the following scale: Continuous or Mostly Continuous (0%); Fully Degenerated (50%); Cleared (100%). A single average value was calculated for each larva.

Puc-LacZ JNK Reporter. Larvae containing the Puc-LacZ reporter (Ring and Arias 1993) were dissected, fixed, and stained for β-galactosidase. Midline VNC nuclei corresponding to motoneurons that innervate segments 4-7 of the larvae were quantified, with 8-10 nuclei being summed within each section. Background readings were taken on either side of each midline motoneuron cluster, avoiding other stained nuclei (3 values per section, 12 total per animal). Values are relative to the mean of the control animals (normalized).

Neuromuscular Junction Quantifications. Between one to three Muscle 4 NMJs per larva were imaged from within segments 3-5 of the animal. Each NMJ was manually outlined and the total membrane marker intensity was quantified, along with any synaptic stains; a background reading was made in an empty area of the muscle nearby and was subtracted from the NMJ reading. All reported values are relative to the membrane marker/synaptic staining intensity of the control condition. NMJs are plotted individually, from at least 5 total animals per condition.

Tagged Protein Quantifications (Nerves). Overexpressed protein levels in the axons/nerves were quantified by imaging immediately posterior of the nerve cord in fixed and stained larva. Three nerves corresponding to segments 5-7 of the larvae were stamped with five 50 μm diameter ROI cycliners (for 15 total readings per animal, averaged into a single value). Nine background readings were taken from around the nerves and were averaged and subtracted from the mean nerve reading, then the ratio of the tagged protein to the membrane marker was calculated. All reported values are relative to the level of the normalized control condition. Each data point is the average from a single larva.

Tagged Protein Quantifications (Motoneuron Cell Bodies and Axons). Both motoneuron cell bodies and axons (immediately dorsal of the VNC) that innervate Muscles 26, 27, and 29 were imaged. Cell bodies were quantified by manually outlining ROIs around motoneuron soma pairs from sections of the VNC that innervate segments 5-7 of the larval body. Background readings were made by dragging the ROIs to empty areas around the VNC. The background sums were subtracted from the sum of the cell bodies, then the ratio of any tagged proteins to the membrane marker was calculated. Measurements of the axons corresponding to these neurons were done by stamping axon pairs with five 20 μm circular ROIs (15 readings total) with three background readings around each axon pair (nine total). The mean of the sums of the axons was calculated, with the background subtracted. The ratio of the tagged protein to the membrane marker was calculated. This was compared to the normalized ratio of the control (to give relative axon levels of the tagged protein). Additionally, the ratio of the

ratio of the tagged protein levels in the axon compared to the cell body was calculated, and were made relative to the ratio of ratios in the control (to give the relative axon localization of the tagged protein compared to levels in the cell body). Each data point represents the ratio of three motoneuron pairs in a single larva.

Microscopy. All images were taken on a spinning disk confocal (Improvision) with a C9100-50 EMCCD camera (Hamamatsu), a Nipkow CSU scanner (Yokogawa), and an Axio Observer (Zeiss). All conditions/genotypes within a repeat of an experiment were imaged at the same time with the same laser and capture settings to limit signal variation due to equipment. The Volocity software was used for all imaging and quantification.

Dissections and Staining. Larva were placed in 1xPBS on ice 20 min prior to dissection. After dissections, larval filets were fixed with 4% paraformaldehyde at RT for 20 min, then washed three times with 1xPBS. Fixed filets were then blocked with 5% normal goat serum (NGS) diluted in 1xPBS with 0.25% Triton X (1xPBST) at RT for one hour, then were stained with antibodies in 5% NGS in 1xPBST. Samples were incubated with primary antibodies overnight at 4°C, while secondary antibody staining was done at RT for 2 hours (with three 10 min 1xPBST washes between antibodies). Following three 10 min washes with 1xPBST, all samples were then mounted using Prolong Diamond mounting media and were given at least 24 hours to set before imaging. Primary antibodies used in this study: Rat anti-mCD8 (Invitrogen MCD0800), Mouse anti-β-Galactosidase (DSHB 40-1A), Mouse anti-GFP (Invitrogen 3E6), Mouse anti-CSP

(DSHB AB49), Mouse anti-Brp (DSHB NC82), Rabbit anti-HA (Cell Signaling 3724s). Secondary (including conjugated) antibodies used in this study: 488 Rabbit anti-GFP (Fisher), Cy3 anti-Rat (Fisher), 568 anti-Mouse (Fisher), 488 anti-Mouse (Fisher), 647 anti-Rabbit (Jackson).

Dominant-Negative Constructs. Fos^{DN} contains the bZIP (basic Leucine Zipper) domain of Fos (Kay) required for DNA binding and dimerization with other transcription factors (including Jun and TATA Box-Binding Protein) (Ransone et al. 1993), but missing the rest of the protein required for activating transcription (Lloyd, Yancheva, and Wasylyk 1991). Bsk^{DN} is a Bsk construct with a mutated ATP-binding site, shown to inhibit JNK signaling in *Drosophila*, (Weber, Paricio, and Mlodzik 2000).

Results

Relationship of Raw with dSarm in Regulating Axonal Degeneration

To probe the relationship between Raw and dSarm, we first considered the possibility that Raw regulates degeneration downstream of dSarm's catalytic activity. To induce dSarm's NADase activity, we used the Gal4/UAS system to ectopically express a self-dimerizing construct of the catalytic TIR domain from human Sarm1 in Drosophila motoneuron pairs (Gerdts et al. 2015), using the M12-Gal4 driver. The larval axons and NMJ nerve terminals in TIR-expressing MNs underwent spontaneous degeneration by 3rd instar (**Figure 2-1A**). Consistent with previous findings and the model that reduced NAD+ levels drive the degeneration process (Gerdts et al. 2015), we observed that co-expression of NMNAT significantly delayed the degeneration of axons expressing the

TIR domain (**Figure 2-1A**). While mutations in *raw* (*raw*$^{dcp\text{-}1}$/*raw*$^{134.47}$) cause a significant delay in axonal degeneration ((Hao et al. 2019) and **Figure 2-1B**), *raw* mutations had no impact on the degeneration of TIR-expressing MN axons (**Figure 2-1B**). These observations suggest that Raw is unlikely to influence degeneration at a step downstream of the catalytic activity of dSarm. Instead, these data favor a role for Raw either upstream or in parallel to the action of dSarm in promoting axonal degeneration.

In contrast to the TIR domain alone, ectopic overexpression full length dSarm::GFP (using the same expression system) does not lead to spontaneous degeneration (**Figure 2-1E**), However, this method of elevating dSarm levels enabled rescue of the delayed degeneration caused by loss of Raw (**Figure 2-1E**). We interpret that Raw may function upstream of dSarm to influence its levels, localization, and/or catalytic activity. Though we also note that the rescue of the *raw*-RNAi degeneration phenotype by dSarm::GFP overexpression is incomplete (**Figure 2-1E**). This leaves open the possibility that Raw influences degeneration through additional mechanisms.

We next asked whether the levels or localization of dSarm::GFP were altered by manipulations to Raw. Intriguingly, we observed that MNs co-expressing *raw*-RNAi to deplete Raw showed a reduced intensity of dSarm::GFP levels in axons, both relative to control axons, and to the level of dSarm::GFP in the cell bodies of the same genetic condition (**Figure 2-1G**). The apparent influence of Raw on the localization of dSarm to axons provides an attractive potential mechanism for Raw's role in axonal degeneration. However, a simple model that Raw promotes degeneration by promoting dSarm

localization is not consistent with other genetic data. We previously noted that the delay in degeneration in *raw*-deficient neurons was rescued by expression of a dominant negative inhibitor of Fos (**Figure 2-1G**, (Hao et al. 2019)). We found that co-expression of Fos^{DN} led to a decrease in axonal dSarm::GFP levels. This decrease was shared in *raw*-RNAi and Fos^{DN} co-expressing axons, in which the axonal degeneration defect in *raw* mutants is rescued. Therefore. the localization of dSarm::GFP in axons fails to correlate with axonal degeneration in all genetic conditions. These data suggest that the localization of the dSarm::GFP construct may not reflect all aspects of dSarm's regulation and function. In addition, Raw and signaling downstream of Raw could have multiple points of intersection with the regulation of dSarm (**Figure 2-8**).

Raw and dSarm Regulate NMJ Structure Through a Common Pathway

To further explore how Raw and Fos may functionally intersect with dSarm, we considered potential signaling functions of dSarm. Previous studies have noted roles for dSarm in regulating the structure of the neuromuscular junction terminals (McLaughlin et al. 2016). Strikingly, we noticed that RNAi-knockdown of *raw* results in altered NMJ morphology that is Fos-dependent, seen as massive overgrowth, areas of connected branches, and loss of clear bouton definition (**Figure 2-2A).** The total intensity of a UAS-mCD8-GFP is greatly increased and the NMJ is seen to cover a greater surface area of the muscle, suggesting that Raw normally acts to restrain a growth pathway or in some other way inhibits NMJ size. We also observed that levels of synaptic proteins at the NMJ terminal were strongly reduced, including both the synaptic vesicle associated protein Cysteine-String-Protein (CSP) and the active zone component

Bruchpilot (Brp) (**Figure 2-2A-D**). We have qualitative observations that Futsch does not extend out into the filopodia-like structures seen in a *raw* knockdown, while Fasciclin II is found along the entire NMJ, similar to WT (data not shown). Co-expression of Fos^{DN} to inhibit Fos restored normal NMJ growth in a *raw* knockdown (**Figure 2-2A-D**). Therefore, some of these synaptic defects appear to be mediated by signaling that requires the Fos transcription factor.

Overexpression of dSarm caused a strikingly similar NMJ phenotype to *raw* knockdown (**Figure 2-2E-H**), including increased mCD8-GFP levels and reduced levels of CSP and Brp. These defects were also rescued by co-expression of Fos^{DN} (**Figure 2-2A-H**). The shared phenotypes and genetic rescue by Fos^{DN} suggest that both Raw and dSarm manipulations influence synaptic morphology via a common signaling pathway.

Much has been reported on Raw's regulation of JNK signaling in its many roles (Bates, Higley, and Letsou 2008; Jemc et al. 2012; Luong, Perez, and Jemc 2018; Zhou, Edgar, and Boutros 2017). Sarm1/dSarm has also been connected to kinase pathways in both the axon injury response (Yang et al. 2015), as well as in innate immunity (Couillault et al. 2004; Foster et al. 2020; Liberati et al. 2004). As regulation of the NMJ involves kinase signaling (J. Li et al. 2017; Collins et al. 2006), and both Raw and dSarm act through Fos in some aspects of their NMJ regulation (**Figure 2-2**), we next tested their impact on a known transcriptional reporter for JNK. We used the *puc*-lacZ enhancer trap reporter of puckered expression, which is known to be activated downstream of JNK signaling in a Fos-dependent manner (Martín-Blanco et al. 1998). We found that both

raw knockdown and dSarm overexpression simulate the expression of puc-lacZ reporter (**Figure 2-4**). This induction was blocked by co-expression of dominant-negative Bsk (JNK) and by co-expression of Fos^{DN}. We conclude that dSarm and Raw regulate a common transcriptional pathway.

dSarm is Not Downstream of Raw in the Regulation of Synaptic Growth

The effect of Raw and dSarm manipulations on synaptic structure, compared to their roles in axonal degeneration, implied contrasting functional relationships. In degenerating axons Raw appears to promote dSarm function; however, in the regulation of synaptic structure Raw and dSarm appear to have opposing (rather than aligned) functions. To better understand the opposing relationship of Raw and dSarm in synapse regulation, we asked whether dSarm is required for the synaptic overgrowth phenotype of *raw* knockdown in motoneurons. We used two different genetic reagents to inhibit dSarm function, which we first confirmed are able to delay injury-induced degeneration in motoneurons. We used a dSarm loss-of-function mutant which is homozygous lethal but delays degeneration as a heterozygote (**Figure 2-1J**), and a dSarm guide RNA that also delays degeneration when expressed with Cas9 (**Figure 2-1K**). While both *loss-of-function* manipulations to dSarm impair its function in axonal degeneration, neither altered the NMJ defects caused by *raw* knockdown (**Figure 2-3**). Therefore, dSarm does not function downstream of Raw to regulate NMJ structure. Consistent with this finding, we observed that the elevated puc-lacZ expression in *raw*-RNAi neurons was also not affected by knockout or heterozygote mutations in dSarm (**Figure 2-5A-D**).

These observations leave the question of how both proteins act to regulate a common set of phenotypes**.**

To explore the mechanism by which Raw and dSarm influence JNK-Fos signaling, we tested whether manipulation of either would affect the localization or protein levels of Bsk, the *Drosophila* homologue of JNK. We used a UAS-HA-Bsk transgene from the FlyORF project to drive overexpression of the single Drosophila isoform of JNK (BsK). HA-Bsk showed increased levels in axons in neurons expressing *raw*-RNAi, and this phenotype was shared for neurons over-expressing dSarm (**Figure 2-5E-F**). Consistent with a correlation between axonally localized Bsk and the synaptic overgrowth phenotype, we noted that co-expression of Fos^{DN}, which inhibits the overgrowth, also led to a small decrease in HA-Bsk (**Figure 2-5G-L**).

Dual Roles of JNK Signaling in the Regulation of Synaptic Structure.

The observations thus far suggested a model that Raw and dSarm regulate synaptic structure via a JNK signaling pathway. The effects of Raw and dSarm manipulations on the levels of Bsk in axons and on a transcriptional reporter for Bsk suggested an attractive model that Raw and dSarm promote JNK signaling in axons. This model would predict that inhibition of JNK should inhibit the synaptic overgrowth phenotypes. However, while testing this hypothesis we instead observed that dominant-negative Bsk led to an increase in the membrane marker at the NMJ, and a decrease in the levels of endogenous CSP and Brp (**Figure 2-6A-D**). Similarly to phenotypes of *raw*-RNAi and dSarm overexpression, the membrane overgrowth and reduced CSP and BRP levels

caused by Bsk-DN were all rescued by co-expression of Fos^{DN} (**Figure 2-6A-D**). These results suggested a role for JNK signaling in restricting synaptic growth (**Figure 2-8**).

Consistent with a role for JNK in restricting synaptic growth, we observed that overexpression of Bsk (JNK) could rescue the synaptic overgrowth defect caused by dSarm (**Figure 2-7**). However, overexpression of Bsk on its own did not lead to reduced synaptic growth. Instead, overexpression of Bsk led to synaptic overgrowth, similarly to Bsk^{DN}. These observation suggest that JNK/Bsk engages multiple pathways, some of which are regulated by Raw and dSarm, and that synaptic structure is sensitive to a balance of these pathways (**Figure 2-8**).

Discussion

The importance of JNK signaling in promoting synaptic growth has been well documented (Wan et al. 2000; J. Li et al. 2017; Collins et al. 2006). In this study we made initial observations in support of these past models, where JNK activation (based on a genetic reporter) seemed to be promoting NMJ overgrowth in dSarm and Raw manipulations. However, our finding that Bsk (JNK) inhibition led to similar phenotypes as overexpressed Bsk caused us to reevaluate our model. Our subsequent result that overexpression of Bsk could reduce dSarm-induced NMJ overgrowth indicated a potential inhibitory role for JNK on synaptic growth. This would strongly suggest the existence of multiple JNK pathways regulating the synapse, with conflicting effects (**Figure 2-8**). Previous studies have described a requirement for JNK/Bsk in NMJ growth (J. Li et al. 2017; W. Shen and Ganetzky 2009); however, a role for JNK/Bsk in

restraining NMJ growth, which we also found in this study, has not been previously reported for the *Drosophila* NMJ. We speculate that a reason for this is the use of our more sensitive and quantitative method of measuring synaptic growth. Most studies count boutons and bouton branches. However, boutons may vary in size and structure, and are subjective to identify. This leaves limited functional meaning for bouton number. The quantitative measurement of total intensity can be made equally for all manipulations, including genotypes like *raw*-RNAi that may lack identifiable boutons.

The role for Bsk/JNK in restraining NMJ growth appears to involve an additional nuclear signaling pathway, since the Bsk^{DN} phenotypes are rescued by Fos^{DN}. It is likely that when this Bsk activity is interrupted, other signaling becomes uninhibited and acts via Fos to promote NMJ growth (**Figure 2-8**). One potential candidate is the extracellular receptor kinase (ERK) *Drosophila* homologue Rolled, which also activates signaling through Fos (Ciapponi et al. 2001)**.** A gain-of-function mutation in Rolled results in increased bouton number at the NMJ (Fischer et al. 2009), and JNK signaling is capable of inhibiting ERK activation (Y. H. Shen et al. 2003). This also ties into axon trafficking, which is strongly linked to both neurodegeneration and synaptic development, and is regulated by JNK and ERK signaling (M. Coleman 2011; Guedes-Dias and Holzbaur 2019). We also note that while the inhibition of Bsk may enhance some growth pathways, it is still expected to interfere with Wnd signaling to the NMJ (which also promotes growth, (Collins et al. 2006)). The potential activation and inhibition of multiple competing pathways may be resulting in a collection of

intermediate/mixed phenotypes at the synapse that will require more precise manipulations to piece apart.

In addition to these opposing roles for JNK at the synapse, our data also supports a multifaceted model of JNK regulation by dSarm and Raw, which impacts the NMJ, nuclear signaling, and axon degeneration. Raw is a well established as a negative regulator of JNK, and Sarm1 has been shown to activate MAPK signaling in multiple contexts and organisms (Bates, Higley, and Letsou 2008; Jemc et al. 2012; Luong, Perez, and Jemc 2018; Zhou, Edgar, and Boutros 2017; Yang et al. 2015; Inoue et al. 2013). Initially, our results followed with these earlier findings; knockdown of *raw* and overexpression of dSarm each appeared to act through JNK activation (based on a genetic reporter) to promote NMJ overgrowth. However, our findings that both overexpression of Bsk (JNK) and a dominant-negative construct of Bsk give similar NMJ phenotypes (as well as dSarm expression-induced synaptic overgrowth being rescued by co-expression of Bsk) suggests that it may instead be inhibition of Bsk in some synaptic role that is behind the Raw and dSarm phenotypes. Rather than one simple JNK pathway with multiple effects on nuclear signaling, degeneration, and NMJ regulation, we find that there may be multiple pathways with opposite regulations.

The connection between Raw and Sarm1 when it comes to degeneration appears especially intricate. Since the discovery of Sarm1's role in axon degeneration, there has been much interest in understanding its mechanism and regulation (Osterloh et al. 2012; M. P. Coleman and Höke 2020). Recent work has shed light on how Sarm1's

enzymatic activity is regulated by allosteric intermolecular interactions (Z. Y. Zhao et al. 2019; M. D. Figley et al. 2021). However, how the Sarm1 protein may be regulated via localization, transport, or turnover has received little attention. Our findings hint at conditions (and potential regulatory pathways) that influence the localization of dSarm into axons. We see that axonal levels of expressed dSarm::GFP are decreased in a Raw knock down. This is consistent with a model of Raw promoting degeneration through dSarm (in contrast to their opposite influences on JNK signaling). However, the levels of dSarm in axons do not always correlate with their ability to undergo Wallerian degeneration. The delayed degeneration of *raw* mutant axons is rescued by inhibiting the Fos transcription factor (Hao et al. 2019), but inhibiting Fos also decreases the localization of dSarm into axons. These genetic data suggest that additional mechanisms downstream of both Raw and Fos control the ability of axons to undergo Wallerian degeneration. It remains unclear how relevant decreased levels of local dSarm are for this process, though the rescue of degeneration in *raw*-RNAi by dSarm overexpression suggests its importance.

One possibility for why Raw and dSarm have oppositive influences on JNK signaling and the NMJ, but both promote degeneration, could be a divergence in the mechanisms of dSarm. Axonal degeneration is strongly linked to the enzymatic activity of Sarm1/dSarm as a NADase. Recent studies have revealed insight into how Sarm1's enzymatic activity is regulated by NAD+ and NMN, which influence the activation of Sarm1 in the form of an octameric ring (M. D. Figley et al. 2021). However, it is not known whether Sarm1 also functions in the same octomeric ring for its signaling

functions, whether other mechanisms lead to its activation in signaling contexts, and whether other mechanisms in addition to its activity as a NADase influence its signaling functions. While Raw appears to act through JNK regulation in all of the phenotypes we examined in this study, it is difficult to speculate how much Sarm1's NADase activity can be separated from its signaling (if it can at all). This seems to be central to the difference between Raw and dSarm's otherwise parallel (though opposite) effects. dSarm may activate JNK signaling, which data from the *C. elegans* homologue TIR-1 suggests can even be protective against degeneration (Ding et al. 2022), but its local role in driving axon degeneration through NAD+ destruction appears to be dominant in this context. The exact mechanism of how Raw regulates dSarm and protective JNK signaling, and how these effects influence each other, awaits future study. Our findings expand the understanding of how MAPK signaling is controlled for NMJ regulation, and hint at how this signaling may interact with Sarm1-dependent degeneration.

Figures

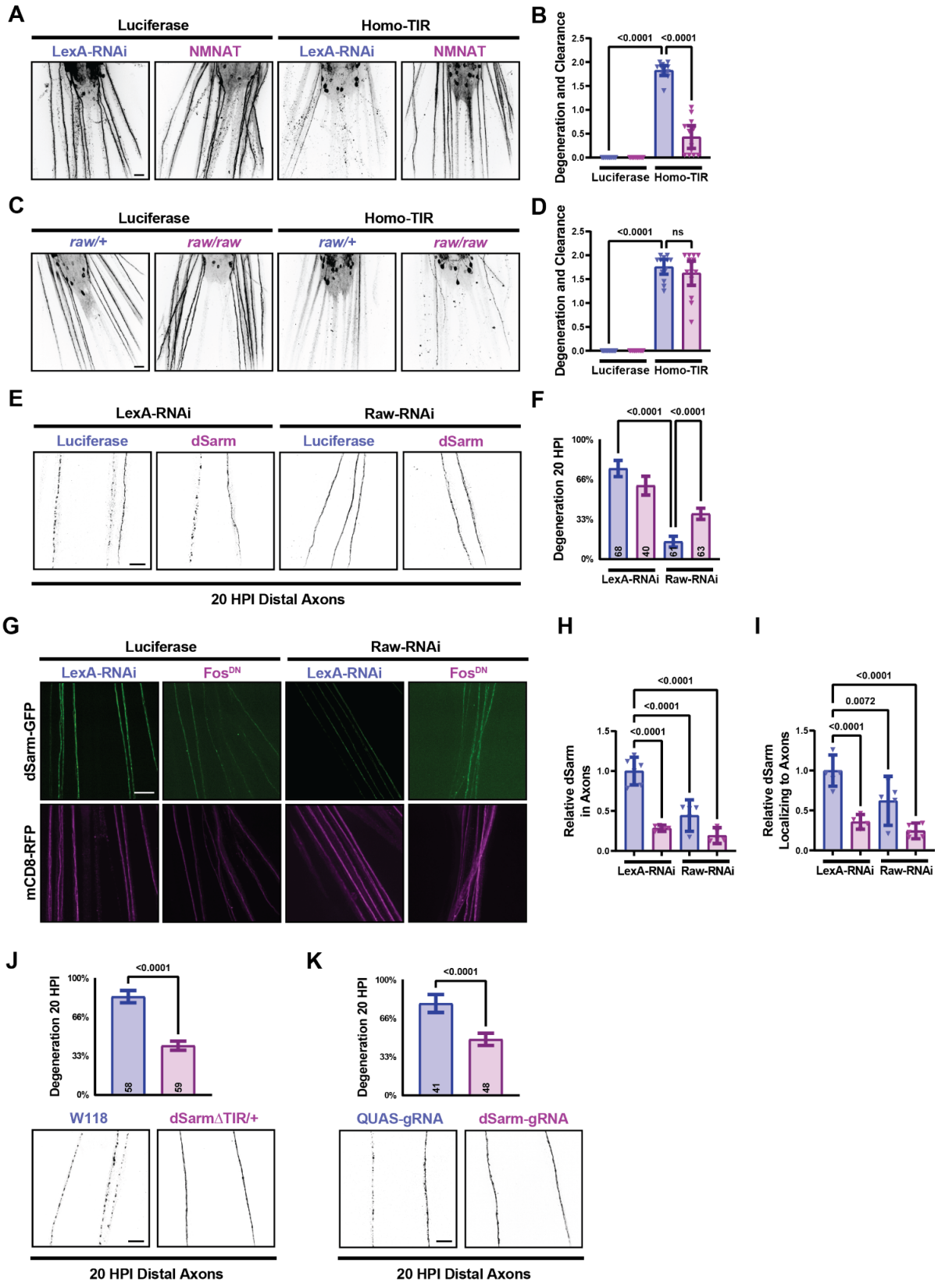

A
Luciferase
Homo-TIR
LexA-RNAi
NMNAT
LexA-RNAi
NMNAT
B
Degeneration and Clearance
<0.0001
<0.0001
Luciferase
Homo-TIR
C
Luciferase
Homo-TIR
raw/+
raw/raw
raw/+
raw/raw
D
Degeneration and Clearance
<0.0001
ns
Luciferase
Homo-TIR
E
LexA-RNAi
Raw-RNAi
Luciferase
dSarm
Luciferase
dSarm
20 HPI Distal Axons
F
Degeneration 20 HPI
<0.0001
<0.0001
68
40
61
63
LexA-RNAi
Raw-RNAi
G
Luciferase
Raw-RNAi
LexA-RNAi
Fos^DN
LexA-RNAi
Fos^DN
dSarm-GFP
mCD8-RFP
H
Relative dSarm in Axons
<0.0001
<0.0001
<0.0001
LexA-RNAi
Raw-RNAi
I
Relative dSarm Localizing to Axons
<0.0001
0.0072
<0.0001
LexA-RNAi
Raw-RNAi
J
Degeneration 20 HPI
<0.0001
58
59
W118
dSarmΔTIR/+
20 HPI Distal Axons
K
Degeneration 20 HPI
<0.0001
41
48
QUAS-gRNA
dSarm-gRNA
20 HPI Distal Axons

Figure 2-1: dSarm Acts Downstream of Raw in Axon Degeneration
A-B) Expression of Homo-TIR in motoneuron pairs results in degeneration and clearance of most axons by the 3rd instar stage, which is inhibited by co-expression of NMNAT. These data are consistent with previous finding from a similar assay (Gerdts et al. 2015). **C-D**) In contrast, expression of Homo-TIR in a $raw^{dcp\text{-}1}/raw^{134.47}$ mutant shows no significant delay in degeneration compared to Homo-TIR expressed in a $raw^{134.47}$ heterozygote. **E-F**) Overexpression of dSarm::GFP partially rescues degeneration of distal axons following injury in a *raw* knockdown, compared to the delayed degeneration seen with *raw*-RNAi and a Luciferase control. **G-I**) Knockdown of *raw* or expression of a dominant-negative Fos both result in decreased levels of expressed dSarm::GFP in the axons compared to the control (as well as decreased dSarm localizing to the axons compared to the cell bodies), and a combination of both transgenes gives a similar result to either alone. **J-K**) Confirmation that either a heterozygote dSarm loss-of-function mutant (shared by the Broihier lab) or a dSarm-gRNA (shared by the Dickman lab) expressed with Cas9 result in delayed degeneration post-injury in distal axons. The driver for all panels is M12-Gal4. Panels E-I contains Dcr2 for RNAi efficiency. Axons are visualized using the mCD8 membrane marker transgene driven by Gal4, tagged with either RFP or GFP. All scale bars are 20 μm.

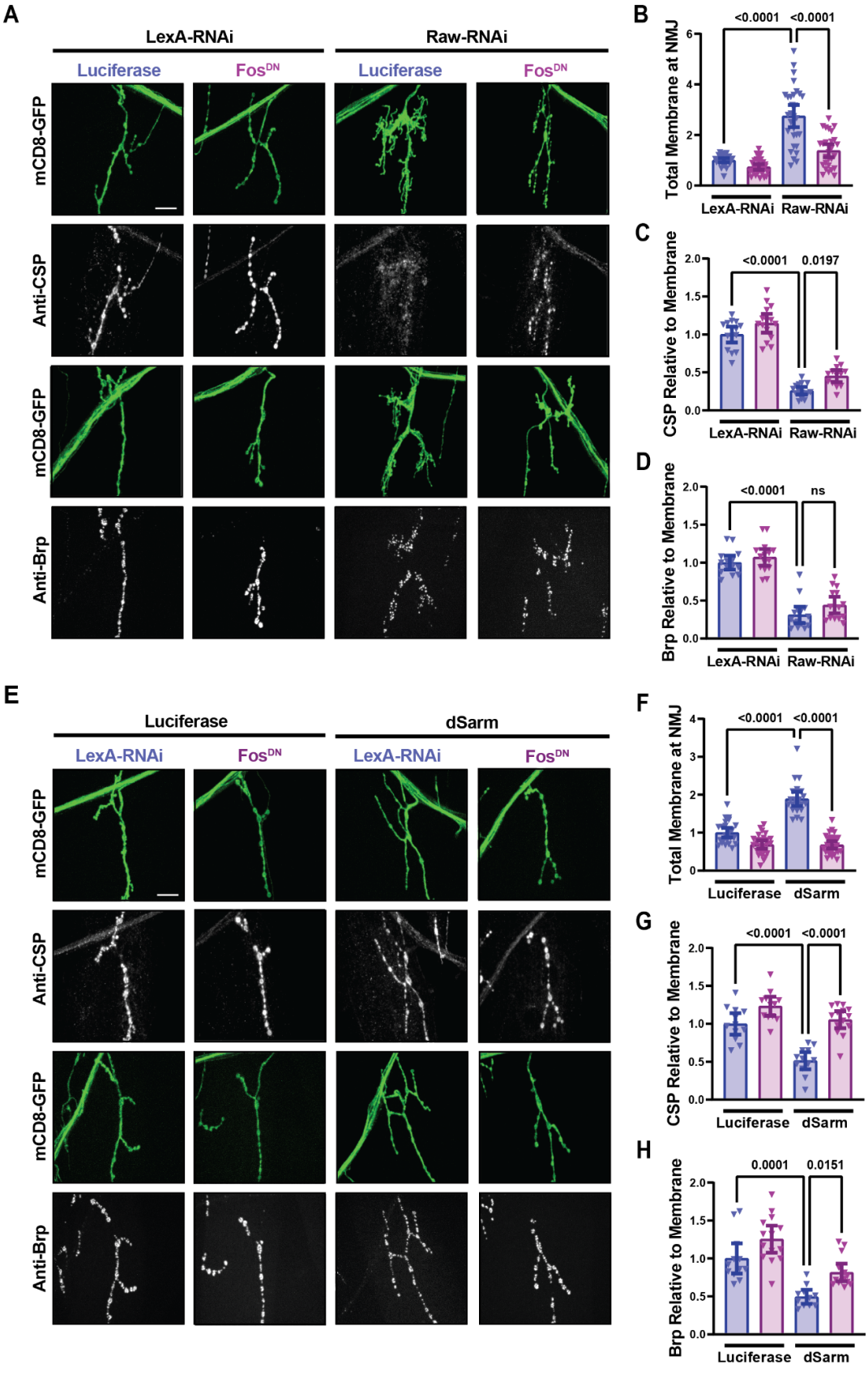

A
LexA-RNAi
Raw-RNAi
Luciferase
Fos^DN
Luciferase
Fos^DN
mCD8-GFP
Anti-CSP
mCD8-GFP
Anti-Brp
B
<0.0001
<0.0001
Total Membrane at NMJ
LexA-RNAi
Raw-RNAi
C
<0.0001
0.0197
CSP Relative to Membrane
LexA-RNAi
Raw-RNAi
D
<0.0001
ns
Brp Relative to Membrane
LexA-RNAi
Raw-RNAi
E
Luciferase
dSarm
LexA-RNAi
Fos^DN
LexA-RNAi
Fos^DN
mCD8-GFP
Anti-CSP
mCD8-GFP
Anti-Brp
F
<0.0001
<0.0001
Total Membrane at NMJ
Luciferase
dSarm
G
<0.0001
<0.0001
CSP Relative to Membrane
Luciferase
dSarm
H
0.0001
0.0151
Brp Relative to Membrane
Luciferase
dSarm

Figure 2-2: Raw and dSarm Regulate NMJ Structure

A-D) Knockdown of *raw* results in overgrowth and altered structure at the Muscle 4 neuromuscular junction (NMJ), as well as reduction in the relative levels of Cysteine-String Protein (CSP) and Brushpilot (Brp); the overgrowth (measured as total membrane marker intensity at the NMJ) shows a strong dependence on Fos, while changes in both CSP and Brp show little to no Fos dependence. **E-H**) Overexpression of dSarm results in overgrowth at the NMJ (matching similar previous findings (McLaughlin et al. 2016)) and relative reductions in CSP and Brp. In contrast to the findings with *raw* knockdown, all three of the measured phenotypes for dSarm overexpression show strong dependence on Fos. The driver for all panels is D42-Gal4, with Dcr2 expressed for RNAi efficiency. All scale bars are 20 μm.

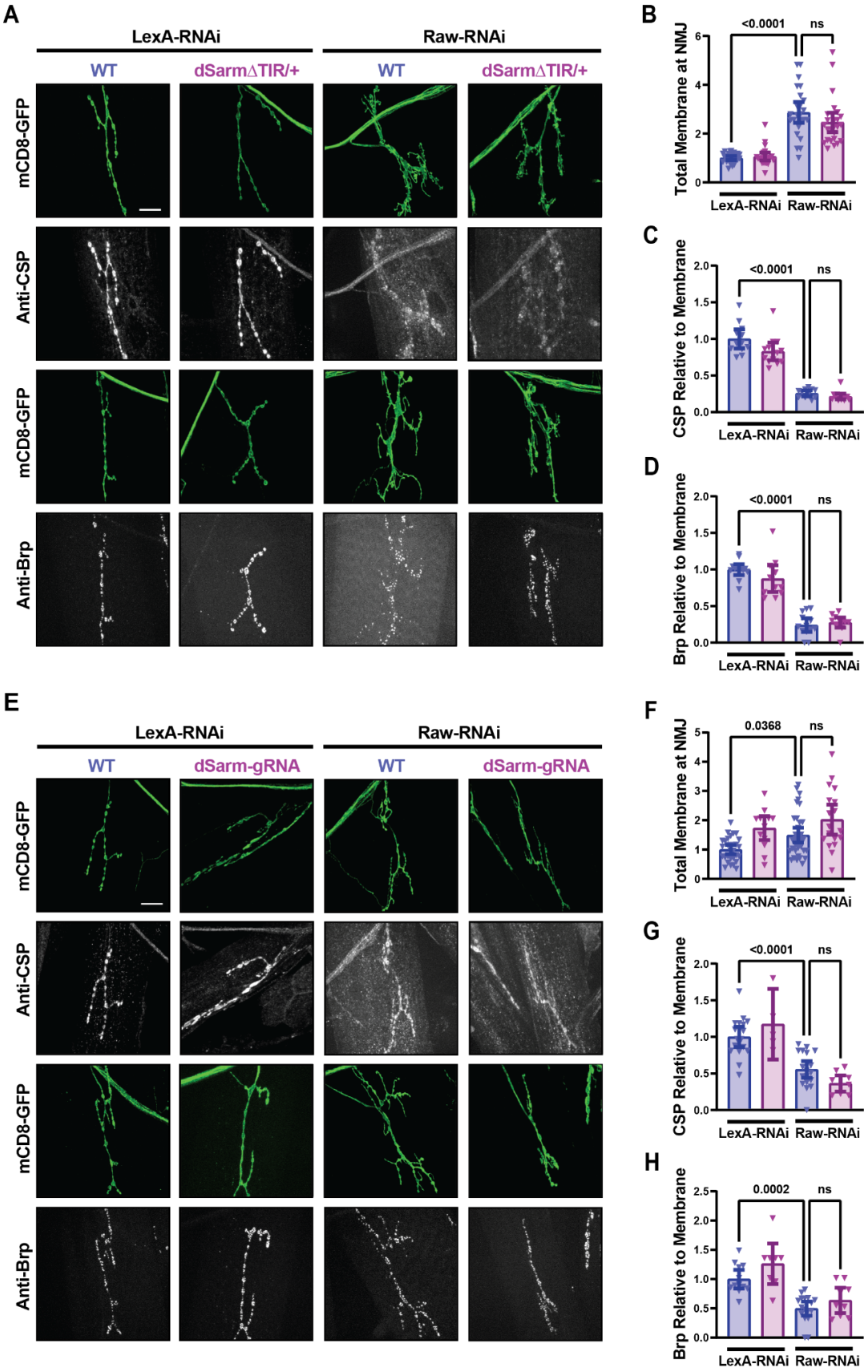

A
LexA-RNAi
Raw-RNAi
WT
dSarmΔTIR/+
WT
dSarmΔTIR/+
mCD8-GFP
Anti-CSP
mCD8-GFP
Anti-Brp
B
<0.0001
ns
Total Membrane at NMJ
LexA-RNAi
Raw-RNAi
C
<0.0001
ns
CSP Relative to Membrane
LexA-RNAi
Raw-RNAi
D
<0.0001
ns
Brp Relative to Membrane
LexA-RNAi
Raw-RNAi
E
LexA-RNAi
Raw-RNAi
WT
dSarm-gRNA
WT
dSarm-gRNA
mCD8-GFP
Anti-CSP
mCD8-GFP
Anti-Brp
F
0.0368
ns
Total Membrane at NMJ
LexA-RNAi
Raw-RNAi
G
<0.0001
ns
CSP Relative to Membrane
LexA-RNAi
Raw-RNAi
H
0.0002
ns
Brp Relative to Membrane
LexA-RNAi
Raw-RNAi

Figure 2-3: dSarm is Not Downstream of Raw in Regulating the NMJ
A-D) The overgrowth and relative reductions of CSP and Brp following *raw* knockdown are not rescued by a dSarm LOF mutant heterozygote (which is sufficient for delaying degeneration, Figure 1J). **E-H**) Knockout of dSarm shows no rescue of *raw*-RNAi NMJ phenotypes. The driver for all panels is D42-Gal4, with Dcr2 expressed for RNAi efficiency. Cas9 is expressed in panels E-H for gRNA-mediated gene knockout. All scale bars are 20 μm.

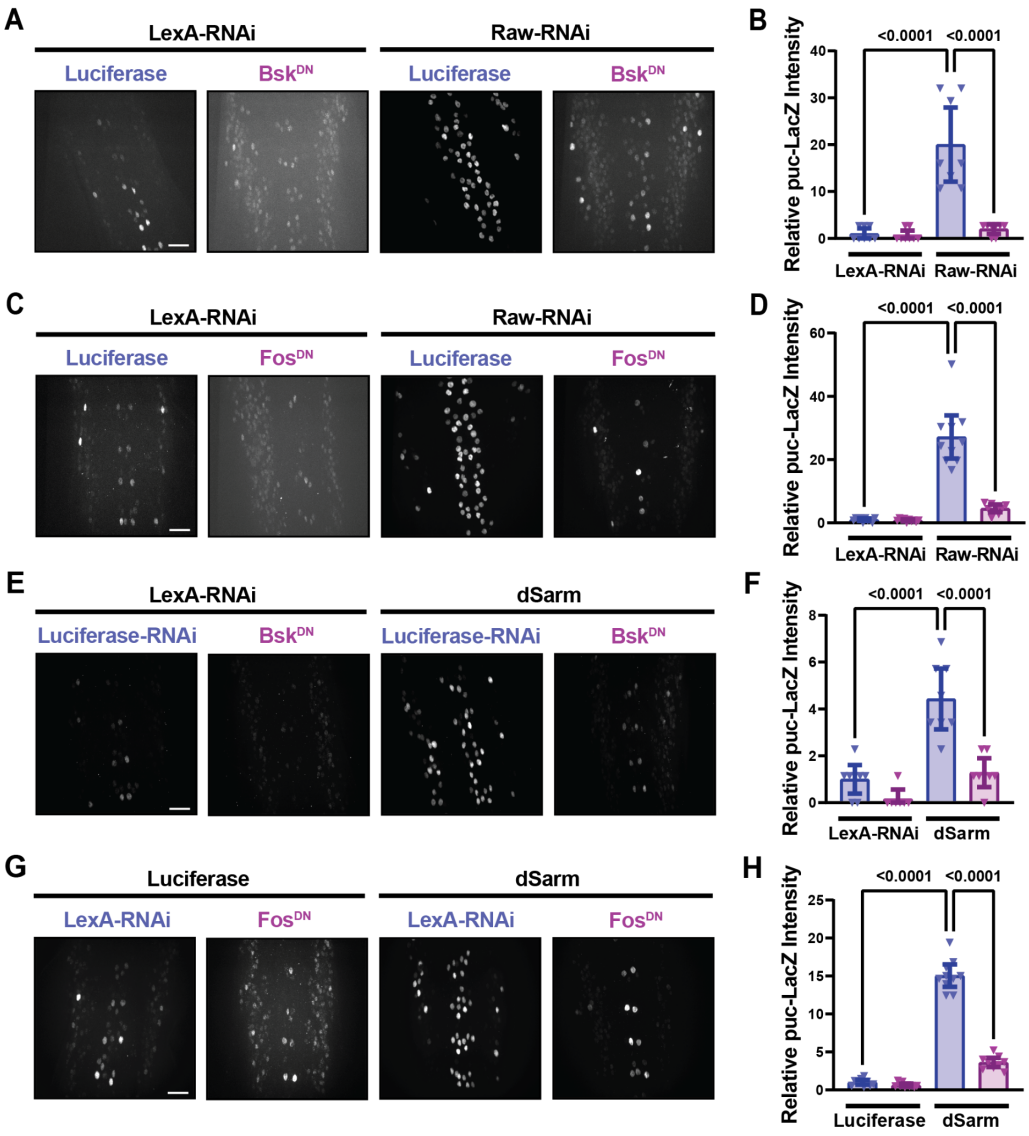


Figure 2-4: Raw and dSarm Have Opposing Effects on Kinase Signaling
A-D) Knockdown of *raw* causes activation of the *puc*-LacZ transgenic transcriptional reporter for JNK (Bsk) signaling, dependent on both Bsk and its target transcription factor Fos. **E-H**) dSarm::GFP overexpression results in activation of *puc*-LacZ expression, which is suppressed by dominant-negative constructs of Bsk and Fos. The driver for all panels is BG380-Gal4, with Dcr2 expressed to aid RNAi efficiency. All scale bars are 20 μm.

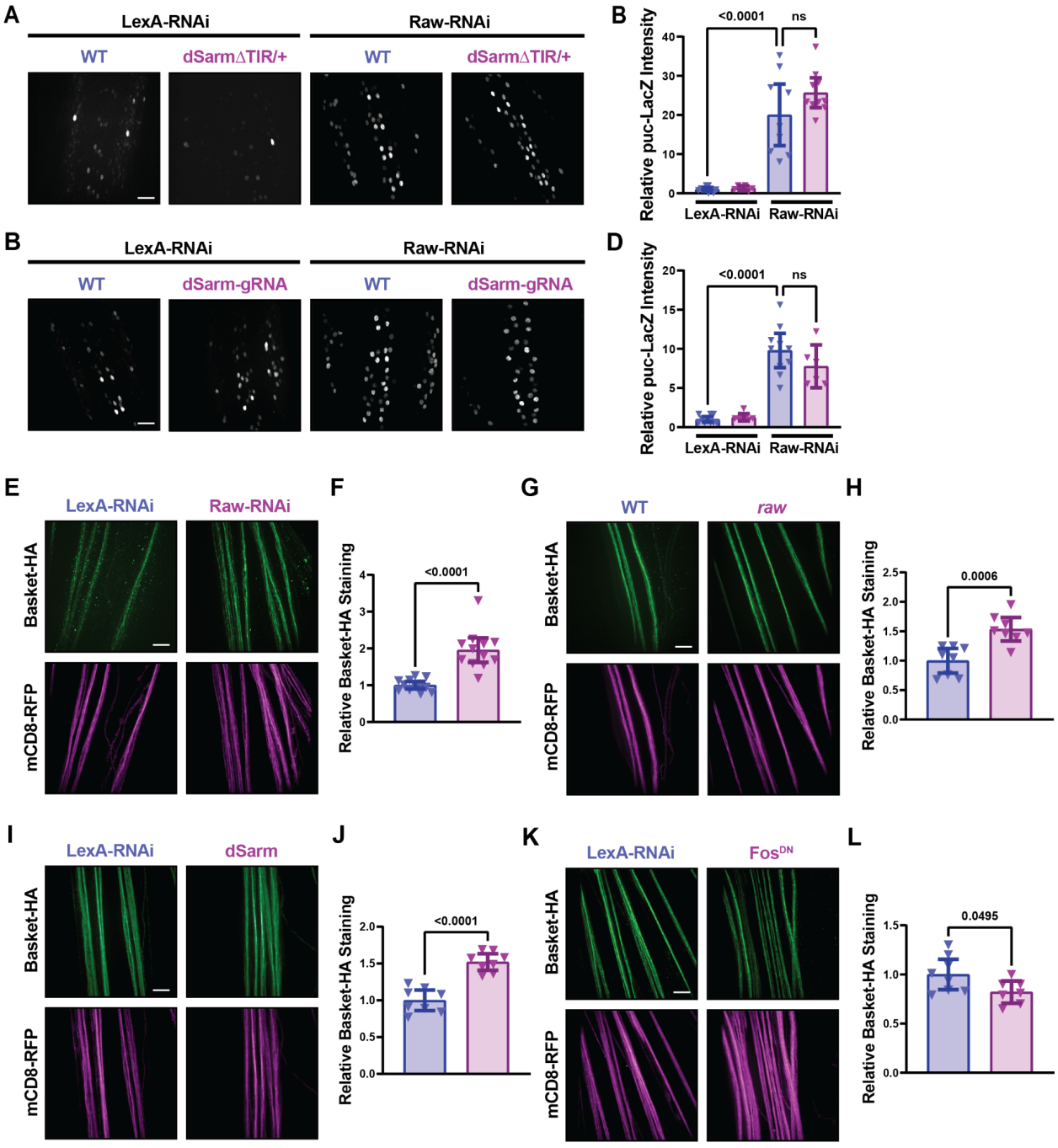


Figure 2-5: Raw Does Not Act via dSarm in Nuclear JNK Signaling

A-D) Activation of the *puc*-LacZ transcriptional reporter for JNK signaling by knockdown of *raw* is not rescued by either a LOF dSarm mutant or knockout of the dSarm locus. **E-H**) Both *raw* knockdown and a $raw^{dcp\text{-}1}/raw^{134.47}$ mutant show increased levels of a UAS-expressed Bsk-HA construct in the nerves, relative to the co-expressed membrane marker mCD8-RFP. **I-J**) dSarm::GFP overexpression also results in elevated Bsk-HA levels in the same assay. **K-L**) Expression of dominant-negative Fos causes a very

slight decrease in expressed Bsk-HA levels. The driver in panels A-D is BG380-Gal4 and the driver in panels E-L is D42-Gal4. Dcr2 is expressed for RNAi efficiency in all panels except G-J. All scale bars are 20 μm.

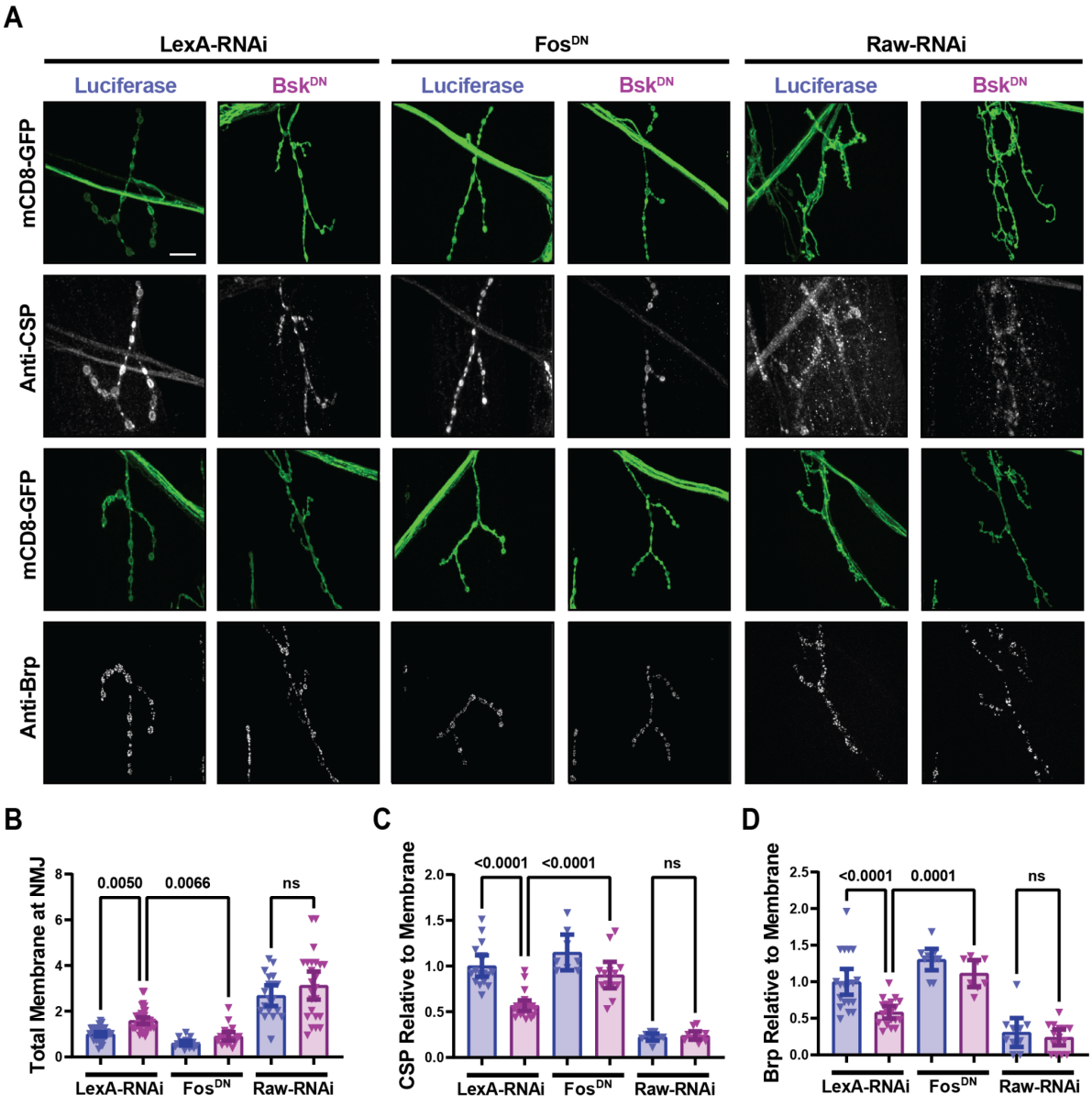


Figure 2-6: JNK Inhibition Phenocopies Raw Loss at the NMJ

A-D) Expression of dominant-negative Bsk does not rescue the overgrowth or CSP/Brp reductions seen in a *raw* knockdown, while Bsk^{DN} alone shows NMJ overgrowth and reductions of these same presynaptic proteins, in a Fos-dependent manner. The driver for all panels is D42-Gal4, with Dcr2 for RNAi efficiency. The scale bar is 20 μm.

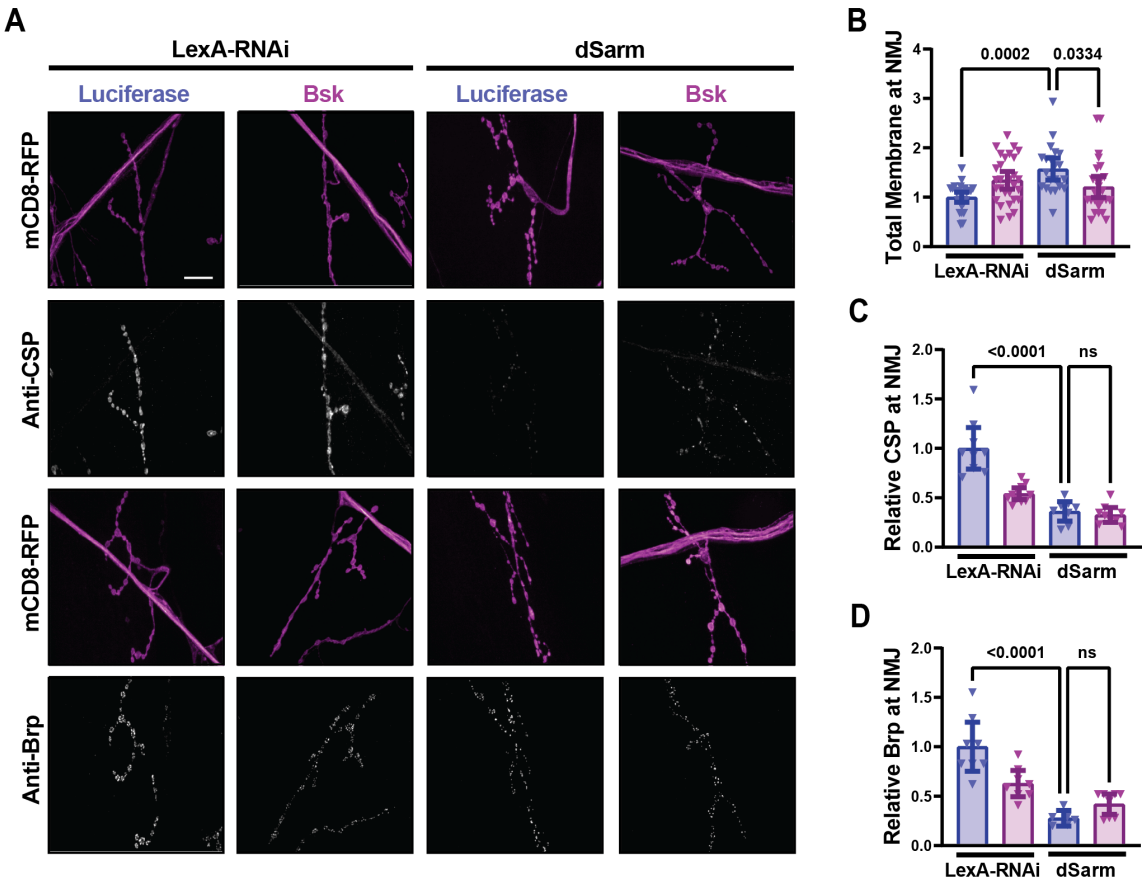


Figure 2-7: JNK Rescues Overgrowth Caused by dSarm Overexpression

A-D) Expression of Bsk-HA rescues the NMJ overgrowth of dSarm expression, but not the relative decreases of CSP or Brp. Bsk-HA expression alone gives similar phenotypes at the NMJ as dominant-negative Bsk (Figure 6). D42-Gal4 is the driver in all panels, with Dcr2 expressed for RNAi efficiency. The scale bar is 20 μm.

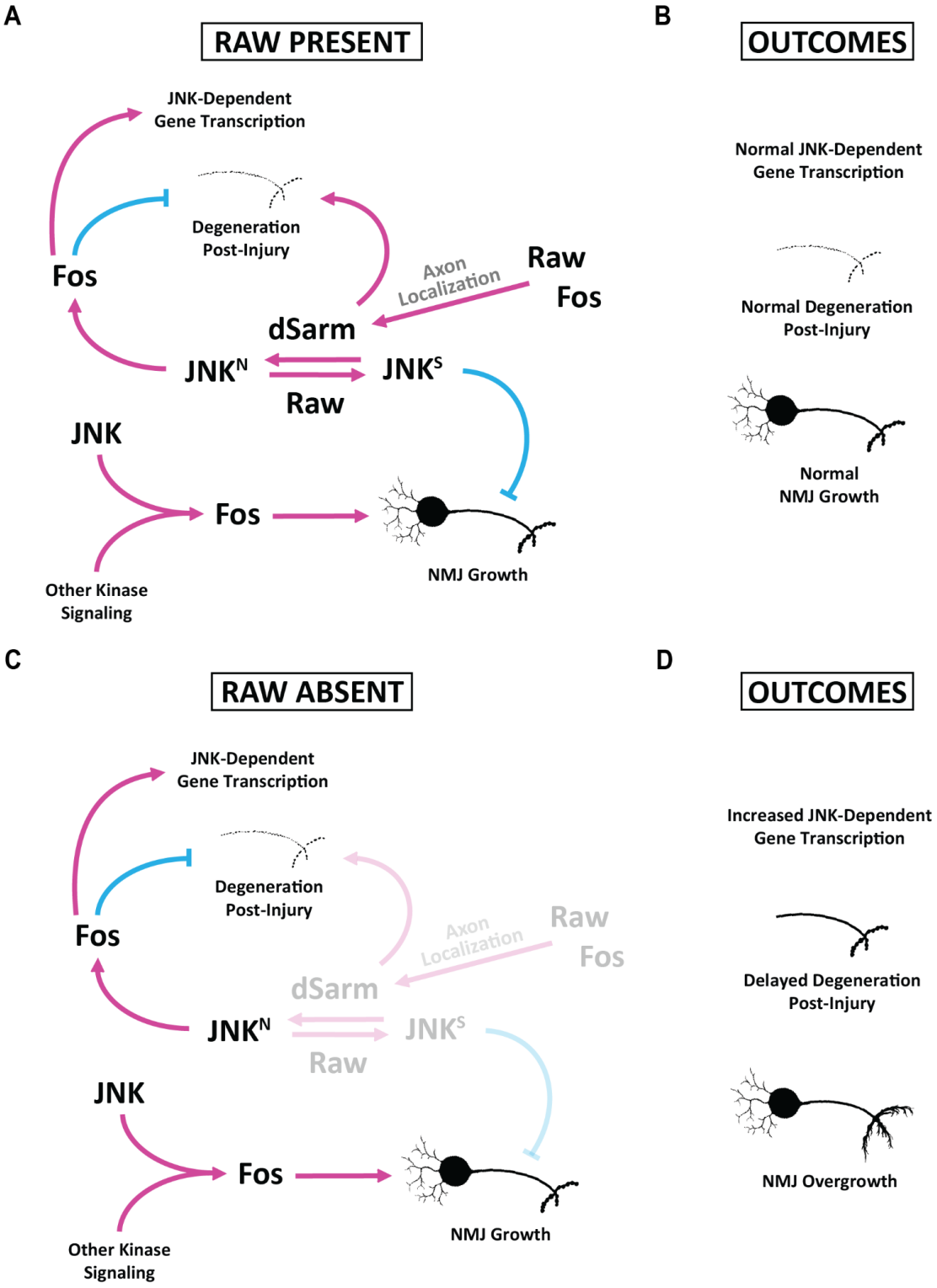

A
RAW PRESENT
JNK-Dependent
Gene Transcription
Degeneration
Post-Injury
Fos
Axon
Localization
Raw
Fos
dSarm
JNK^N
JNK^S
Raw
JNK
Fos
Other Kinase
Signaling
NMJ Growth
B
OUTCOMES
Normal JNK-Dependent
Gene Transcription
Normal Degeneration
Post-Injury
Normal
NMJ Growth
C
RAW ABSENT
JNK-Dependent
Gene Transcription
Degeneration
Post-Injury
Fos
Axon
Localization
Raw
Fos
dSarm
JNK^N
JNK^S
Raw
JNK
Fos
Other Kinase
Signaling
NMJ Growth
D
OUTCOMES
Increased JNK-Dependent
Gene Transcription
Delayed Degeneration
Post-Injury
NMJ Overgrowth

Figure 2-8: Model of Raw and dSarm in Their Regulation of JNK Signaling
A) dSarm and Raw have opposing influences in shifting JNK (Bsk) between a nuclear role ‘N’, which involves activation of gene transcription and delayed degeneration post-injury, and a synaptic role ‘S’ which inhibits synaptic growth. dSarm has the additional effect of promoting degeneration through its TIR activity, while Raw and Fos are both involved in promoting axon localization of dSarm. The Fos-dependent pathway responsible for NMJ overgrowth appears to act in opposition of inhibitory JNK activity, given the data found in this study **B**) With all these components acting in concert, the expected outcomes in the cell are normal levels of JNK-dependent gene transcription, axon degeneration following injury, and appropriately contained growth at the NMJ. **C-D**) When Raw is removed, nuclear JNK signaling is hyperactivated, resulting in increased transcription of gene targets and delayed axon degeneration, dependent on Fos. Given our findings that dSarm or Homo-TIR expression can restore or induce degeneration in Raw-deficient animals, and that dSarm levels are decreased in the axons following *raw* knockdown, it is likely that loss of local dSarm activity contributes to this delayed degeneration, in combination with protective JNK signaling. This would also explain why dominant-negative Fos expression decreases dSarm in the axons, but does not delay injury-induced degeneration, as it also blocks the JNK signaling pathway required for that effect. At the NMJ, loss of Raw leads to a loss of inhibitory JNK activity restraining synaptic growth, so that a Fos-dependent pathway promotes growth beyond normal levels.

Chapter 3: Metabolic Interference Delays Wallerian Degeneration

Abstract

Both axon integrity and axon degeneration are closely tied to energy metabolism. Given the known role of the prodegenerative Sterile Alpha and TIR Motif-Containing Protein 1 (Sarm1) in driving the destruction of NAD+, a key electron carrier for ATP synthesis, it is expected that metabolic disruption has the potential to promote degeneration. Surprisingly, we find that genetic manipulations expected to reduce ATP synthesis in *Drosophila* motoneurons lead to prolonged integrity and impaired axonal degeneration following peripheral nerve injury. RNAi knockdown of pyruvate kinase (Pyk), a key regulator of glycolysis, as well as knockdown of a key regulatory subunit of ATP synthase resulted in an extension of the lag phase phase between injury and axonal fragmentation. Both genetic manipulations also lead to induction of *puc*-lacZ JNK reporter expression, suggesting the activation of a JNK signaling pathway and downstream transcriptional response. However, the protective phenotype of Pyk and ATP-SynG knockdowns does not require other known mediators of JNK signaling, including the Fos transcription factor. Moreover, while this induction of nuclear signaling requires the MAPKKK Wallenda, this same kinase is not required for the degeneration defect of Pyk and ATP-SynG depleted axons. These findings imply that the mechanisms of axonal degeneration are intimately linked to mechanisms that maintain energy

homeostasis in axons and identify unanticipated methods to prolong the integrity of damaged axons.

Introduction: Energy, Metabolism, and Axon Degeneration

One of the core themes of axon degeneration is regulation of local energy supplies (M. P. Coleman and Höke 2020). This can be seen in the cleavage of the electron carrier NAD+ by the pro-degenerative enzyme Sarm1, which leads to a rundown of ATP (Osterloh et al. 2012; Gerdts et al. 2015). While Sarm1 is receiving much current attention, there are many metabolic pathways in the cell that impact energy availability. One such pathway is glycolysis, which is implicated in supporting neuronal energy supplies (Dienel 2019; Tang 2020). However, the importance of glycolysis in neurons under healthy conditions remains unclear (Volkenhoff et al. 2015). Whether or not the breakdown of glucose is required for survival in neurons, it still presents a potential target for disrupting energy maintenance in injury-induced degeneration. This is supported by recent studies in mouse and rat which point towards a link between diabetes and Wallerian degeneration (Y. S. Chen, Chung, and Chung 2010; Zeman et al. 2021).

We set out to test whether modest manipulations of cellular energy could phenocopy aspects of Sarm1 activation, by genetic manipulations of the glycolysis and ATP synthesis. Surprisingly, we found that genetic manipulations expected to impair ATP

synthesis, including knockdown of Pyruvate Kinase (Pyk) and knockdown of ATP-Synthase Subunit S (ATPsynG), prolonged the integrity of injured axons and delayed the initiation of Wallerian degeneration. In addition, both manipulations activate the Wallenda (Wnd) and Jun N-Terminal Kinase (JNK) Mitogen-Activated Protein Kinases (MAPK), leading to Fos-dependent signaling in the nucleus. However, this pathway shows little to no responsibility for the delayed degeneration seen in *pyk* and *ATPsynG* knockdowns. Our findings uncovered an additional facet to Wnd as a mediator of stress response signaling and expand the understanding of how general metabolic disruption can influence axon degeneration.

Materials and Methods

Injuries. Prior to injury, early 3rd instar larvae were placed in 1xPBS on ice for 20 min. Larvae were then transferred to an inverted petri dish where their nerves were pinched with a pair of forceps on their ventral side. Injured larvae (and uninjured controls) were then transferred to a petri dish containing melted fly food and kept at 29°C until dissection.

Injury-Induced Degeneration Scoring. Axons were visualized by expression of GFP- or RFP-tagged mCD8. Scoring was done blinded and reported sample sizes are the total number of axon pairs scored, from at least 5 animals. The scoring was done using this scale: Completely Intact (0%), Continuous with Varicosities (33%), Partially Degenerated and Partially Continuous (66%), and Fully Degenerated (100%).

Live Imaging. Larvae were incubated on ice in 1xPBS, before being transferred to a raised dissection plate. Dissections were done in HL3 containing 0.45 mM calcium. After placing a cover slip on the larval filet, the nerves corresponding to segments 5-7 of the larvae were imaged. Nerves were stamped with five 50uM ROIs and 9 similar readings were made of the area surround the nerves for background readings. The values of all stamps were averaged for a single larve and the background was subtracted. For the conditions using the pHusion pH reporter, final data reported are the ratio of GFP signal to RFP signal relative to the ratio in the control.

Puc-LacZ JNK Signaling Reporter. 3d instar larvae containing the *puc*-LacZ reporter (Ring and Arias 1993) (Ring and Arias 1993)

Neuromuscular Junction Quantifications. Up to three Muscle 4 NMJs from segments 3-5 were imaged per animal. ROIs were manually drawn around the synapse and total intensity of the membrane marker and any synaptic stains was summed. The ROIs were then dragged to empty regions of the muscle for background readings which were subtracts from the NMJ sums. All plotted values are individual NMJs and are relative to the control mean (either total membrane marker fluorescence summed or the ratio of synaptic stain to the membrane marker, depending on the measurement).

Tagged Protein Quantifications. Nerves corresponding to segments 5-7 of the animal were imaged and five 50 μm circular ROIs were stamped in each nerve (for 15 total reads) and 9 total background reads were made from the empty space surrounding the

nerves. The mean sum of the nerve reads was calculated, with the mean background subtracted, and a single average value is plotted per larva, relative to the normalized control. Values are the ratio of signal from any tagged protein relative to the membrane marker intensity.

Dissections and Staining. Prior to dissection, larvae were placed in 1xPBS on ice for 20 min, then were transferred to a dissection dish in an ice bath. Following dissection, larvae filets were fixed with 4% paraformaldehyde at RT for 20 min, then were washed three times with 1xPBS. Next, the filets were blocked for one hour in 5% normal goat serum (NGS) diluted in 1xPBS with 0.25% Triton X (1xPBST), then were stained with antibodies suspended in 5% NGS in 1xPBST. Primary staining was done at 4 ° C overnight and secondary staining was done at RT for 2 hours. After staining, larvae were washed three times with 1xPBST (10 min each), then were mounted on glass slides using Prolong Diamond mounting media and were given at least 24 hours to set before imaging.

Microscopy. For each experiment, imaging for all conditions/genotypes for a single repeat was done at the same time, to limit variation due to equipment variability. All images were taken on a spinning disk confocal (Improvision) using a C9100-50 EMCCD camera (Hamamatsu), with a Nipkow CSU Scanner (Yokogawa) and an Axio Observer (Zeiss). All conditions for a given experimental batch were imaged with the same lazer and capture settings and at the same time to limit noise due to equipment variation. The Volocity software was used for all quantifications and imaging. Primary antibodies used

in this study: Mouse anti-β-Galactosidase (DSHB 40-1A), Mouse anti-Futsch (DSHB 22C10), Mouse anti-CSP (DSHB AB49), Rat anti-mCD8 (Invitrogen MCD0800). Secondary and conjugated antibodies used in this study: 488 Rabbit anti-GFP (Fisher), Cy3 anti-Rat (Fisher), 568 anti-Mouse (Fisher).

Larvae Rearing. All larvae were reared on yeast-glucose media at 29°C.

Fly Stocks. W118 [WT], UAS-*lexA*-RNAi [Control RNAi] (BDSC 67947), UAS-*luciferase*-RNAi [Control RNAi] (BDSC 31603), UAS-Luciferase [Control Protein] (BDSC 35788), UAS-*pyk*-RNAi (BDSC 35218), UAS-*ATPsynG*-RNAi (VDRC 107311), UAS-*pyk*-sgRNA (BDSC 78770), *QUAS*-gRNA [Control gRNA] (BDSC 67539), UAS-dSarm::GFP (Osterloh et al. 2012). UAS-Fos^{DN} (II) (BDSC 7214), UAS-Fos^{DN} (III) (BDSC 7215), UAS-pHusion-Ras (Stawarski et al. 2020), *puc*-LacZ (Martín-Blanco et al. 1998), M12-Gal4 (Ritzenthaler, Suzuki, and Chiba 2000), D42-Gal4 (Sanyal 2009), BG380-Gal4 (Budnik et al. 1996), UAS-Cas9 (BDSC 58985), UAS-Dcr2 (BDSC 24650).

Results

Knockdown of PyK or ATPsynG Delays Degeneration Post-Injury

Neurons are required to support neurite extensions that can span vast distances and require constant resources and energy to remain functional. Recent advances in axon degeneration have revealed that key molecules in the degenerative mechanism functionally regulate the electron carrier NAD+. These include the NAD-synthesizing protein NMNAT and NAD-destroying enzyme Sarm1 (M. P. Coleman and Höke 2020).

Both NAD+ biosynthesis and breakdown pathways are linked to the regulation and execution of axonal degeneration. This, coupled with the fact that NAD+ is a key electron carrier for ATP synthesis, raises the question of whether ATP loss regulates the activation of axonal degeneration machinery in addition to being a downstream consequence of degenerative signaling.

To manipulate energy levels to a less extreme extent than Sarm1 activation, we made use of *pyk* and *ATPsynG* knockdowns to interfere with glycolysis and ATP synthesis, respectively. Recent findings have indicated that interference with glycolysis does not induce spontaneous degeneration (Volkenhoff et al. 2015), which allows us to examine these conditions in an injury model. Our original expectations were that we might see enhanced degeneration by interfering with energy and metabolism prior to axon injury, which had the potential to speed ATP rundown, a known feature of Sarm1-dependent degeneration. To our surprise, these knockdowns had the opposite effect and instead slowed degeneration post-injury (**Figure 3-1**).

Energetic Enzyme Knockdowns Activate Kinase Signaling

Previous studies in *Drosophila* have shown that a conditioning injury activates a protective response that prolongs the integrity of axons following a second injury, dependent on the Wallenda (Wnd) MAPKKK and downstream Fos transcription factor (Xiong and Collins 2012). Consistent with a model of resiliency through transcriptional response, expression of the *puc*-lacZ transcriptional reporter for JNK-Fos signaling in *Drosophila* correlated with axonal resilience in previous studies (Xiong et al. 2010;

Xiong and Collins 2012; Hao et al. 2019). We therefore hypothesized that impairment of ATP synthesis may lead to a similar protective response, especially given the role of DLK/Wnd in stress signaling (Jin and Zheng 2019). We found both *pyk*-RNAi and *ATPsynG*-RNAi manipulations led to activation of *puc*-lacZ reporter (**Figure 3-2**). Induction of *puc*-lacZ was rescued in neurons co-expressing RNAi to disrupt Wnd, and was also rescued in neurons co-expressing Fos^{DN} to inhibit Fos (**Figure 3-2**). We conclude that both manipulations to ATP synthesis lead to activation of the Wnd injury signaling pathway.

While activation of Wnd signaling has been previously shown to cause a delay in the degeneration of injured axons (Xiong and Collins 2012), we found that the degeneration delays caused by *pyk*-RNAi and *ATPsynG*-RNAi did not require Wnd, nor were they significantly affected by co-expression of Fos^{DN} (**Figure 3-1**). We interpret that the mechanism of protection by *pyk*-RNAi (and *ATPsynG*-RNAi) is likely independent of the actions of Wnd and Fos.

Delayed Degeneration in PyK Knockdown is Rescued by dSarm Expression

Following our findings that impaired degeneration of axons depleted for Raw could be rescued by overexpression of dSarm, we asked whether dSarm overexpression could similarly rescue the impaired degeneration of *pyk*-RNAi depleted axons (using a dSarm::GFP line, (Osterloh et al. 2012)). Indeed, we found that overexpression of dSarm fully rescues delayed degeneration post-injury in a *pyk* knockdown (**Figure 3-1**). However, unlike our findings with Raw (**Figure 2-1**), loss of Pyk does not alter

overexpressed dSarm::GFP levels in axons (**Figure 3-3C).** Loss of ATPsynG also had no impact on the axon levels of dSarm::GFP. These data suggest that if dSarm is part of the mechanism by which these energetic knockdowns delay degeneration, then it is through some mechanism other than localization to the axon.

Depletion of Pyk Alters Intracellular pH

Acidity has recently been shown to be an activating factor for Sarm1 (Y. J. Zhao et al. 2021). While this protein is known for its degenerative activity, in *C elegans* it has recently been shown to have protective potential in delaying degeneration caused by mitochondria loss (Ding et al. 2022). To test whether the *pyk*-RNAi manipulation altered intracellular pH, we expressed the genetically encoded pH reporter pHusion in Drosophila motoneuron axons (**Figure 3-3**). The reporter revealed a drop in pH, seen as diminished GFP signal in the *pyk* knockdown (**Figure 3-3H**). This result raises the question of how acidity may be related to the delayed degeneration in these axons, to be addressed in future work.

Pyk Knockdown Alters NMJ Structure

Multiple regulators of axon degeneration also have roles in neuromuscular junction (NMJ) development, including Highwire (Hiw) (Wan et al. 2000), Wnd (Collins et al. 2006), Sarm1 (McLaughlin et al. 2016), and Raw (**Figure 2-2**). We examined the NMJs in a *pyk* knockdown and found that there is a decrease in membrane marker intensity, but no significant change in relative Cysteine-String Protein (CSP) levels (**Figure 3-4**). These NMJs have enlarged boutons which are linked by very thin membrane

connections. We also have qualitative observations that Futsch levels are decreased when PyK is knocked out by Cas9 via a gRNA. These data contrast with our data on Raw, whose loss also results in delayed degeneration but causes overgrowth, rather than a reduction membrane marker amount (**Chapter 2**). This builds on our findings that regulation of degeneration and the synapse can vary drastically between regulators, as some promote both degeneration and growth (dSarm, Pyk), some promote degeneration and inhibit growth (Raw, Hiw), and some inhibit degeneration and promote growth (Wnd) (Wan et al. 2000; Xiong and Collins 2012; Osterloh et al. 2012; McLaughlin et al. 2016; Hao et al. 2019; Collins et al. 2006).

Discussion

Energy capture and manipulation is a central aspect of biology. However, when it comes to death processes, the line between living and dead cellular extensions can become hard to define. This is especially true following events like Sarm1 activation, which rapidly drive down NAD+ and ATP levels prior to degeneration. Under these circumstances, it can be difficult to determine when exactly an axon stops being a cellular process and simply becomes a collection of molecules no longer actively metabolizing. Use of energetic interference in the study of axon degeneration can help us understand how the cell responds to, and uses, changes in energy availability in the process of axon destruction.

Our findings that knockdown of enzymes required for glycolysis and ATP synthesis result in slowed Wallerian degeneration suggest that interference with energy

metabolism may not always be a promoting factor in degeneration. Instead, these manipulations may slow degeneration though pre-injury local energy deficits in the axon or possibly by a stress response from the cell. Manipulations that improve energy efficiency can inhibit degeneration in models of neurodegenerative disorders, though many of these cases involve increased ATP (Mattson and Liu 2002). It remains possible that interference with metabolism prior to injury may force the axon to adapt to low-energy conditions, which could make it resistant to the energetic rundown of Wallerian degeneration. This would be distinct from the understanding of NAD+ and ATP loss documented in degenerating axons (Gerdts et al. 2015). It is also possible that altered metabolism in the axon prior to injury may interfere with some aspect of the degenerative machinery used post-injury. Research on the connection between diabetes and Wallerian degeneration point towards the polyol pathway (which converts glucose to fructose) as a potential promoter of Wallerian degeneration, possibly through reactive oxygen species (ROS) production (Y. S. Chen, Chung, and Chung 2010; Zeman et al. 2021).

Glia may also be important in this delayed degeneration, given their role of supporting neurons through nutrient supply (Volkenhoff et al. 2015). If intra-neuronal energy supplies are hampered, this could be recruiting support from nearby glia prior to injury, which may then be slowing degeneration post-injury. This theory is supported by the finding that the addition of schwann cells to cultured axons results in metabolic coupling and greatly delays degeneration post-injury (Babetto, Wong, and Beirowski 2020). The

impact of glia on the delayed Wallerian degeneration following energetic knockdown is an important next question.

More detailed investigation into axonal ATP levels in these manipulations is also needed to understand how available energy may be impacted. Because of our data indicating a drop in pH with *pyk* knockdown, most ATP sensors would be difficult to implement, given their sensitivity to acidity. However, ATP levels could be determined biochemically if these experiments were done in cell culture, which would offer helpful data for understanding the impact of *pyk* and *ATPsynG* knockdowns in severed axons. Given recent findings that tie acidity with Sarm1 activation (Y. J. Zhao et al. 2021), and Sarm1 signaling with delayed degeneration (Ding et al. 2022), it is also possible that the drop in pH following Pyk loss is what slows degeneration. We note that prior studies of Wallerian degeneration have not paid much attention to intracellular pH, including whether it changes in degenerating axons, when it does so, and its role in the degeneration process. With a robust genetic reporter in hand this is a ripe topic for future study.

Figures

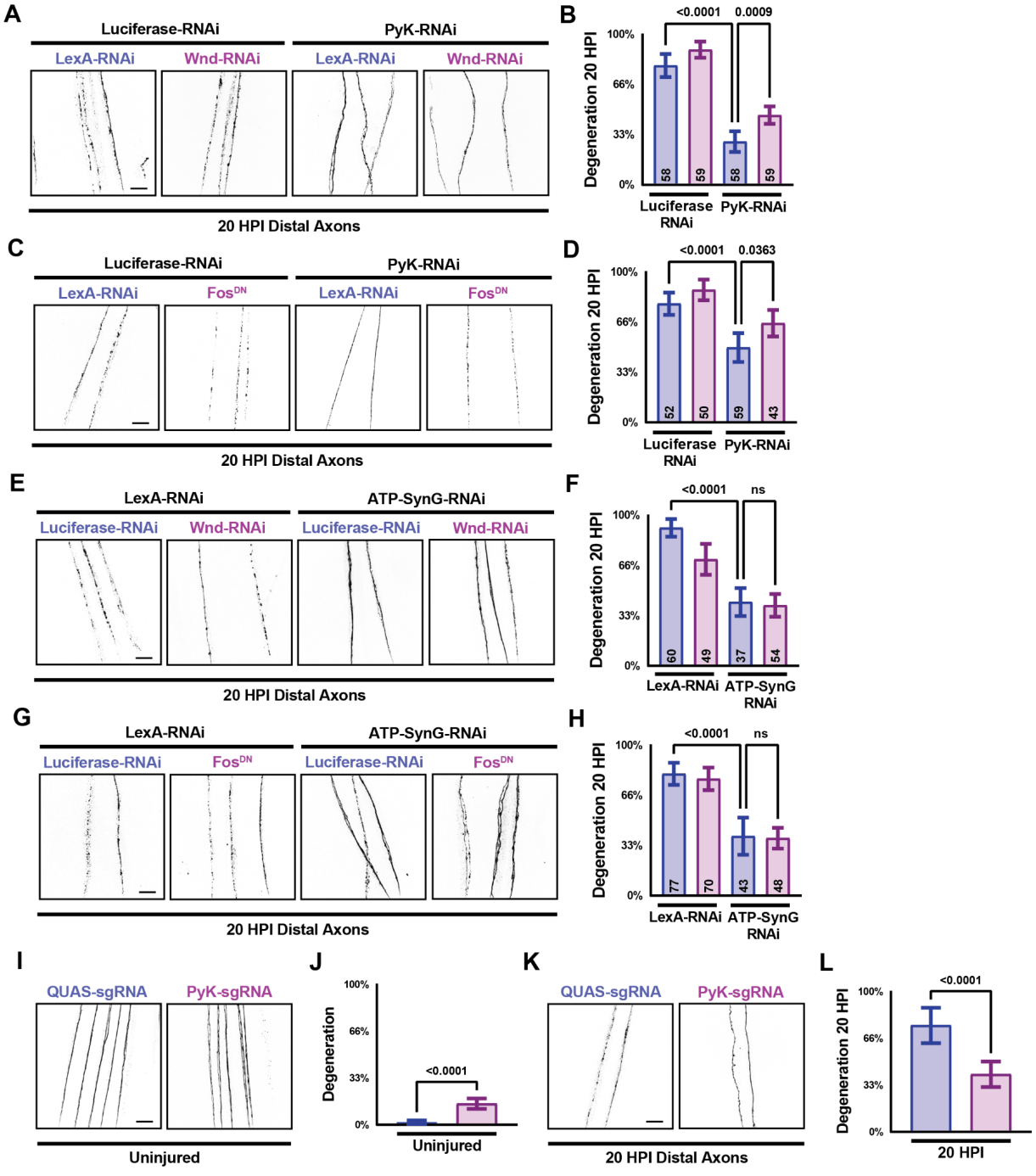


Figure 3-1: Energetic Knockdowns Delay Injury-Induced Degeneration
A-B) Loss of the glycolysis enzyme Pyruvate Kinase (PyK) delays degeneration in distal injured motoneuron axons 20 hours post-injury (HPI). The delay shows only slight dependence on Wallenda (Wnd) kinase (**A-B**) and the transcription factor Fos (**C-D**).

E-F) Similar to *pyk* knockdown, loss of ATP-Synthase Subunit G (ATP-SynG) delays degeneration post-injury. However, this delay shows no dependence on either Wnd (**E-F**) or Fos (**G-H**). Cas9-mediated knockout of *pyk* results in early signs of axon degeneration in uninjured animals (**I-J**), but delays degeneration post-injury similar to the RNAi-mediated knockdown of *pyk* (**K-L**). The driver for all conditions is M12-Gal4. Dcr2 is expressed for RNAi efficiency in panels A-H. All scale bars are 20 μm.

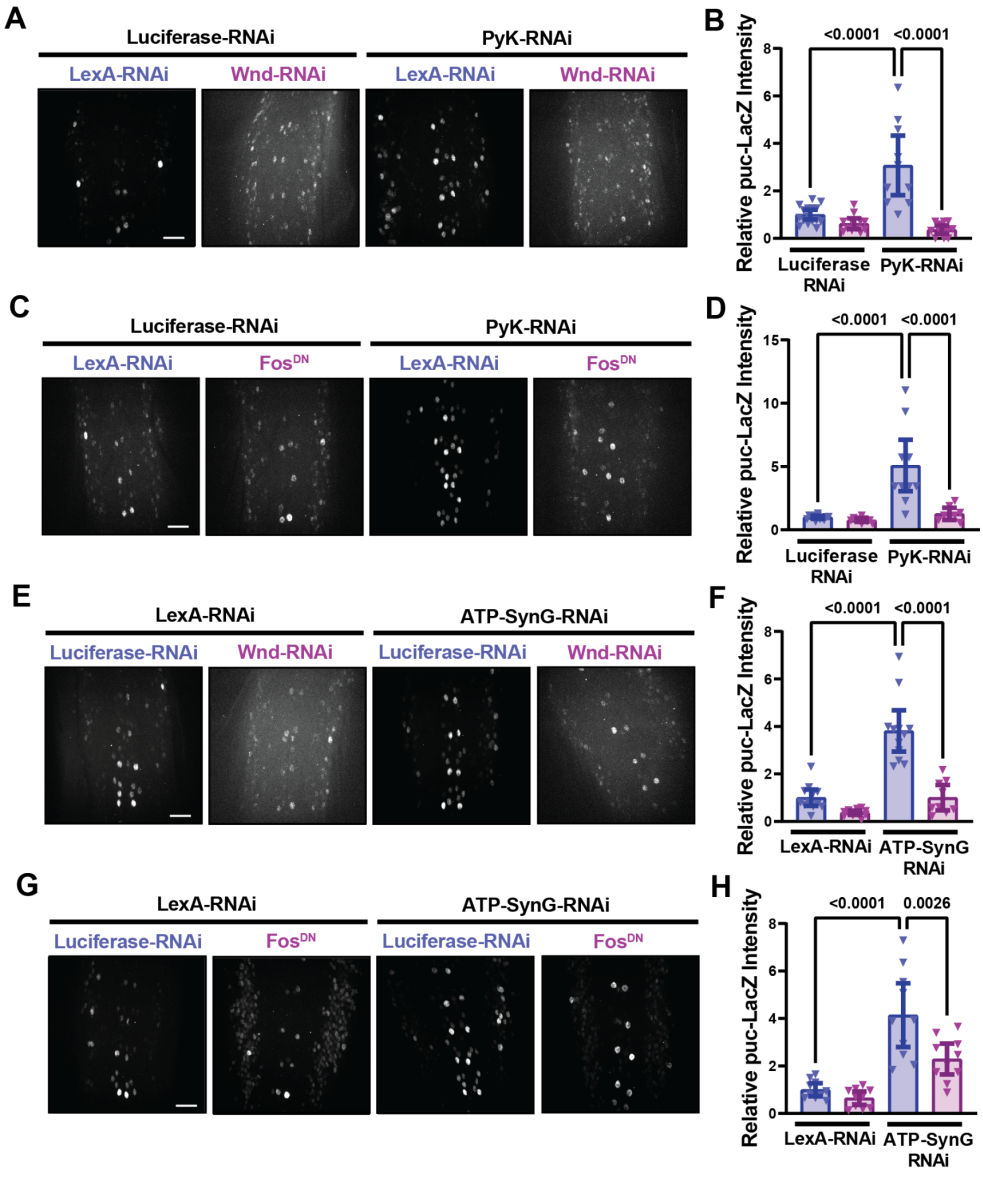


Figure 3-2: Energetic Knockdowns Activate JNK Reporter

A-B) Knockdown of *pyk* leads to increased transcription of the JNK target Puckered (Puc) in the Ventral Nerve Cord (VNC) midline motoneurons. The activation is both Wnd (**A-B**) and Fos (**C-D**) dependent. Likewise, depletion of *ATPsynG* also leads to Wnd-dependent activation of the *puc*-LacZ reporter (**E-F**), though the rescue with knockdown of Fos is partial (**G-H**). BG380-Gal4 is used as the driver for all panels and expresses Dcr2 for RNAi efficiency. All scale bars are 20 μm.

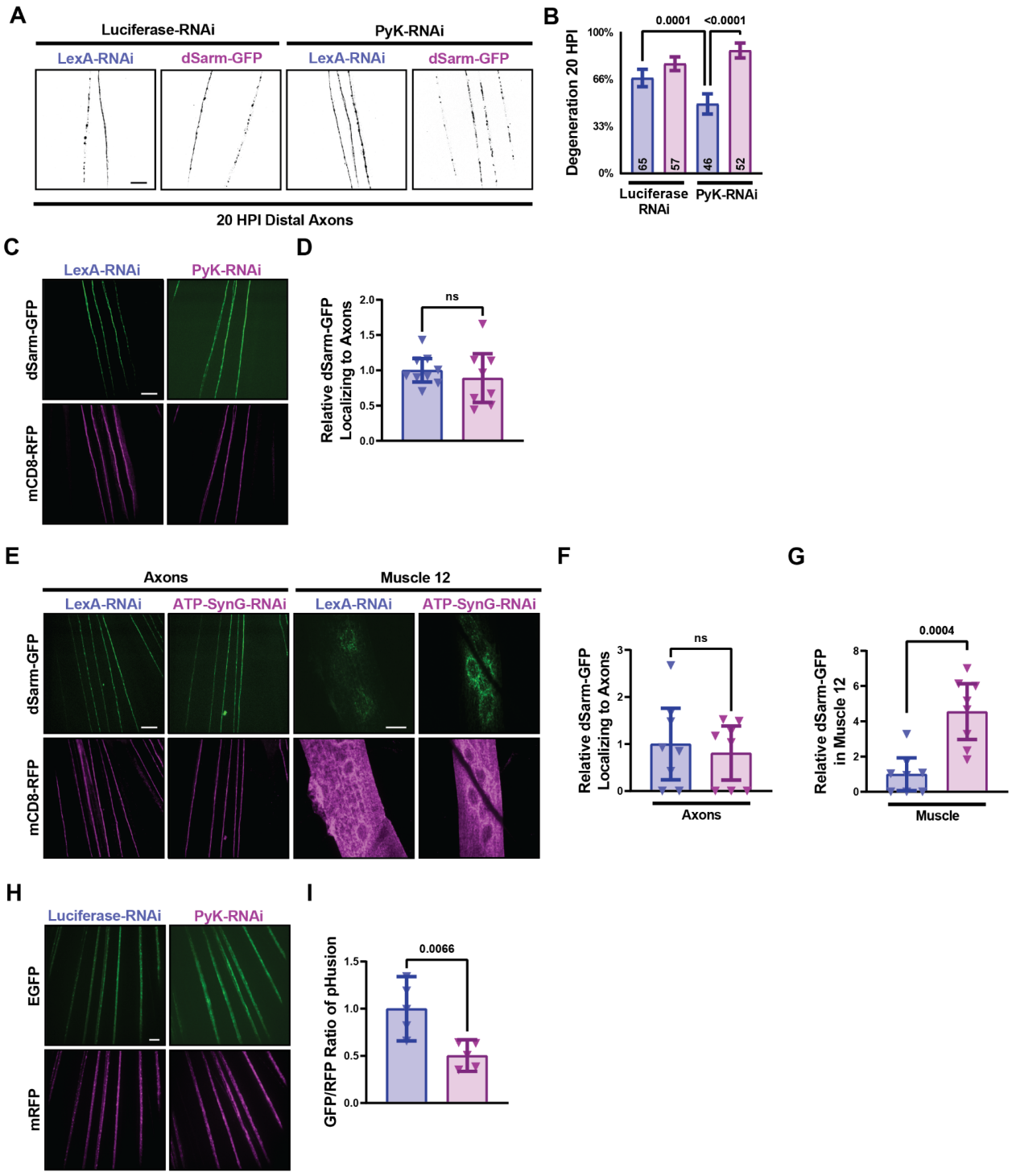


Figure 3-3: dSarm Localization in Energetic Knockdowns

A-B) Overexpression of dSarm fully rescues degeneration 20 HPI in a *pyk* knockdown. **C-D**) Knockdown of *pyk* shows no change in the relative amount of dSarm::GFP localizing to axons compared to the cell body. **E-G**) Knockdown of *ATP-SynG* does not change axon localization of dSarm::GFP, but does increase the amount of dSarm::GFP

found around nuclei in the muscle. **H-I**) Knockdown of *pyk* decreases the live-imaged GFP/RFP ratio of the pHusion pH reporter in the nerves. The driver for panels A-G is M12-Gal4 and the driver for panels H-I is D42-Gal4. Dcr2 is expressed in all panels for RNAi efficiency. All scale bars are 20 μm.

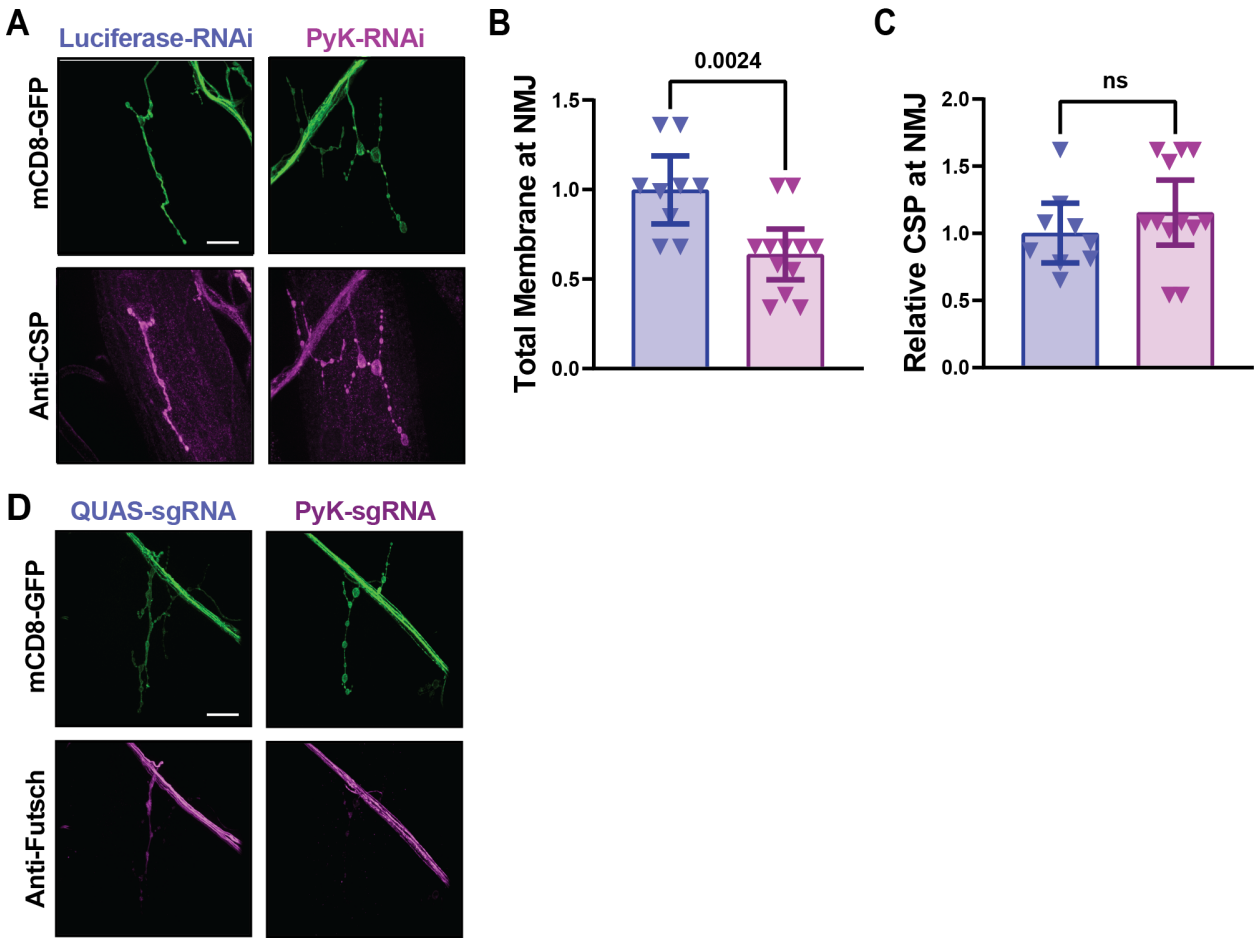


Figure 3-4: PyK Loss Impacts NMJ Structure

A-C) Knockdown of *pyk* leads to reduction of the total neuronal membrane marker at the NMJ, but does not impact the relative level of Cysteine String Protein (CSP). **D**) Qualitative observation that Cas9-mediated knockout of PyK also impacts NMJ structure and appears to alter Futsch. D42-Gal4 is the driver for all panels, with Dcr2 expressed for RNAi efficiency. All scale bars are 20 μm.

Chapter 4: Raw, dSarm, and Metabolism in Degeneration and NMJ Structure

Abstract

Raw is a negative regulator of Jun N-Terminal Kinase (JNK) across multiple tissues and contexts. Despite the numerous examples of Raw inhibiting JNK, the mechanism of how Raw suppresses JNK signaling is still unclear. In this chapter we discuss the exploration of this relationship in *Drosophila* motoneurons, which led to several interesting observations. These serve as puzzle pieces which do not yet fit together into a unifying model. We find evidence that Mitogen Activated Protein Kinase (MAPK) signaling regulated by Wallenda (Wnd) and JNK Interacting Protein 1 (JIP1) does not contribute to the delayed degeneration in a *raw* knockdown, but instead appear to promote degeneration in this context. In a separate series of experiments, we note that the constitutively active TIR domains, which lead to sponteaneous degeneration of uninjured motoneuron axons, also lead to activation of Wnd signaling. In this case, either knockdown or pharmacological inhibition of Wnd causes a slight delay in TIR-induced axon loss. These results complement our findings with Raw and Wnd to further suggest a role for Wnd in promoting (rather than only inhibiting) axon loss. Future work will build on these findings to clarify Raw's mechanism(s) for its multiple roles in motoneurons.

Introduction

Since the discovery that Wallerian degeneration is an active process requiring cellular machinery, the questions of how this machinery is regulated has been examined from

multiple angles, spanning transport, kinase cascades, metabolism, ion flux, and other cell autonomous and non-autonomous mechanisms. One particularly interesting signaling pathway is regulated by the Mitogen-Activated Protein Kinase Kinase Kinase (MAPKKK) Wallenda (Wnd), which influences neuromuscular junction (NMJ) development (Wan et al. 2000; Collins et al. 2006), axonal injury signaling (Xiong et al. 2010), and axonal degeneration (Xiong et al. 2012). In this thesis work, we have found additional molecular links between axonal degeneration and synapse development, both of which are regulated by the NADase dSarm and the transmembrane protein Raw. Raw is a known negative regulator of JNK signaling (Bates, Higley, and Letsou 2008), while both dSarm and Wnd are previously known as positive regulators of JNK signaling (Yang et al. 2015; Collins et al. 2006). Misregulation of Wnd leads to synaptic overgrowth and protection from degeneration (Xiong and Collins 2012; Collins et al. 2006), while dSarm promotes both degeneration and NMJ growth (Osterloh et al. 2012; McLaughlin et al. 2016). From the striking phenotypic similarities for neurons either overexpressing Wnd or dSarm, or depleted/disrupted for Raw, we hypothesized that Wnd, Raw, and dSarm regulate a common signaling pathway.

Materials and Methods

Nerve Crush Injuries. Early 3rd instar larvae we placed on ice in 1xPBS for 20 min, then were transferred to an inverted petri dish. Using a pair of forceps, each larva was pinched on the ventral side just posterior of the ventral nerve cord (VNC) to crush several nerves and severe the axons within. After injury, both injured and uninjured

larvae were transferred to petri dishes containing yeast-glucose food and were kept at 29°C until dissection.

Injury-Induced Degeneration Scoring (Axons). Axons were labeled with mCD8 tagged with a fluorophore (GFP or RFP) for visualization. Scoring was done blinded in fixed and stained filets using the following scale: Completely intact (0%), Continuous with varicosities (33%), Partially fragmented and partially continuous (66%), and Fully degenerated (100%). Reported sample sizes are total number of axon pairs scored per each condition (from at least 5 animals).

Injury-Induced Degeneration Scoring (NMJs). NMJs in fixed and stained animals were stained with HRP and Futsch antibodies to label the neuronal membrane and cytoskeleton, respectively. Scoring was done on NMJs corresponding to fully damaged nerves, using the following scale: Completely Intact - HRP and Futsch Staining Fully Intact (0%); Partially Degenerated - HRP Partially Fragmented and Futsch Partially Lost (50%); Completely Degenerated - Full Fragmentation of HRP and Complete Loss of Futsch (100%). The reported sample size is the total number of NMJs scored per condition, from at least 8 animals.

Homo-TIR-Induced Degeneration and Clearance Scoring. Homo-TIR was co-expressed with the mCD8-RFP membrane marker by Gal4. Axon pairs from the 10 nerves corresponding to larval segments 3-7 were scored (10 axon pairs total). The following

scale was used: Continuous or mostly continuous (0%), Fully degenerated (50%), and Cleared (100%).

Drug Treatment: For DLKi treatment (DLK/Wnd small molecule inhibitor, GNE 3511), larvae were raised on instant fly food with either only vehicle (DMSO) or dissolved drug (50 μM final concentration) mixed in their food.

Puc-LacZ JNK Signaling Genetic Reporter. Larva with the *puc*-LacZ transgene (Ring and Arias 1993) were dissected and fixed, then stained against β-galactosidase. Larvae ventral nerve cords (VNC) were imaged and 8-10 midline motoneuron nuclei from neurons that innervate segments 4-7 were stamped with 50 μm circular ROIs, with 12 background readings made in the surround tissues. The mean of the ROI sums was calculated, with the background subtracted. One average value is reported per larvae, relative to the normalized control.

Tagged Protein Measurements (Nerves). Expressed proteins in nerves were quantified by stamping nerves that innervate segments 5-7 of the larval body immediately posterior to the ventral nerve cord (VNC), using five 50 μm circular ROIs (15 readings total). Nine background readings were made in the empty area around the nerves. The mean of the sums of the nerve readings was calculated, with the background readings subtracted. Each reported value is the average from one larva, relative to the normalized control, of the levels of tagged protein relative to the membrane marker.

Tagged Protein Quantifications (Motoneuron Cell Bodies and Axons). Both the cell bodies and the axons (immediately dorsal to the VNC) of motoneuron pairs innervating Muscles 26, 27, and 29 were imaged. Cell body ROIs were made manually around motoneuron pairs innervating segments 5-7 of the larva, then were dragged into empty regions around the VNC for background measurements. The background was then subtracted from the cell body sums, before calculating the ratio of tagged protein to the membrane marker. Next, the axons were measure using five 20 μm circular ROIs per axon pair (15 total), with nine background readings made in the empty space around the axons. The mean of the sums was calculated for the axon readings, with background subtracted. The ratio was then calculated for the amount of tagged protein relative to the membrane marker in both the axons and the cell bodies, then the ratio of the axon ratio to the cell body ratio was calculated. The ratio of the ratios is plotted relative to the normalized control. Each value is one larva.

NMJ Quantifications. Muscle 4 NMJs from segments 3-5 were manually outlined with ROIs and the total membrane marker intensity at the synapse was summed, along with any synaptic protein stains. The ROIs were then dragged to empty areas of the muscle for background readings, which were subtracted from the NMJ sums. Between 1-3 NMJs were captured from at least 5 total animals per condition. Plotted values are individual NMJ values (total membrane marker intensity or ratio of synaptic protein staining to the membrane marker, relative to the normalized control).

Microscopy. Images were captured using a spinning disk confocal from Improvision, with a C9100-50 EMCCD camera from Hamamatsu, a Nipwow CSU scanner from Yokogawa, and an Axio Observer from Zeiss. All conditions for a given batch of an experiment were imaged using the same settings and during the same session to limit noise due to equipment variation. All imaging and quantification were done using the Volocity software.

Dissections and Staining. Prior to dissection, larvae were placed in 1xPBS on ice for 20 min. Larvae were dissected in 1xPBS, then were fixed in 5% paraformaldehyde for 20 min, followed by three 1xPBS washes. Next, the larval filets were blocked with 5% normal goat serum (NGS) diluted in 1xPBS with 0.25% Triton X (1xPBST) for one hour. Staining was done with antibodies suspended in 5% NGS in 1xPBST overnight at 4 ° C (primary antibodies) or for two hours at RT (secondary and conjugated antibodies). Both between antibodies and before mounting samples were washed three time with 1xPBST for 10 min each. Following the last wash, the filets were mounted on glass slides using Prolong Diamond mounting media and were given at least 24 hours to set before imaging. Primary antibodies used in this study: Mouse anti-β-Galactosidase (DSHB 40-1A), Mouse anti-Futsch (DSHB 22C10), Mouse anti-GFP (Invitrogen 3E6), Mouse anti-Brp (DSHB NC82), Mouse anti-CSP (DSHB AB49), Rat anti-mCD8 (Invitrogen MCD0800), Rabbit anti-HA (Cell Signaling 3724s). Secondary and conjugated antibodies used in this study: Cy3 anti-HRP (Jackson), 488 Rabbit anti-GFP (Fisher), Cy3 anti-Rat (Fisher), 568 anti-Mouse (Fisher), 488 anti-Mouse (Fisher), 647 anti-Rabbit (Jackson).

Larva Rearing. All larvae were raised on yeast-glucose food at 29°C

Fly Stocks: W118 [WT], UAS-Luciferase [Control Protein] (BDSC 35788), UAS-*luciferase*-RNAi [Control RNAi] (BDSC 31603), UAS-*lexA*-RNAi [Control RNAi] (BDSC 67947), UAS-Homo-TIR (gift from DiAntonio lab), UAS-*raw*-RNAi (VDRC 101255), UAS-*dSarm*-RNAi (VDRC 105369), UAS-*jip1*-RNAi (VDRC 50007), UAS-Ask1-RNAi (BDSC 35331), UAS-*miro*-RNAi (gift from Bellen lab), UAS-*wnd*-RNAi (BDSC 35369), UAS-Shg (BDSC 65589), $raw^{dcp\text{-}1}$ (Hao et al. 2019), $raw^{134.47}$ (Jemc et al. 2012), UAS-dSarm (BDSC 17144). UAS-Fos^{DN} (II) (BDSC 7214), UAS-Bsk-HA (F003890), UAS-Wnd (Collins et al. 2006), UAS-NMNAT (Zhai et al. 2006), UAS-GFP-Raw (Lee et al. 2015), UAS-Trc-Myr (Lee et al. 2015), *puc*-LacZ (Martín-Blanco et al. 1998), BG380-Gal4 (Budnik et al. 1996), D42-Gal4 (Sanyal 2009), M12-Gal4 (Ritzenthaler, Suzuki, and Chiba 2000), UAS-Dcr2 (BDSC 24650).

Results

Evidence of Multiple JNK Pathways in Raw-Deficient Animals

This chapter probes the relationship of Raw with JNK signaling, including the arm(s) of JNK signaling regulated by the Wnd MAP3K kinase. Given the role of Wnd in injury signaling (Xiong et al. 2010), it has the potential to intersect with protective JNK signaling seen in Raw-deficient animals. In this study, we find that not only does Wnd not rescue degeneration in a *raw* knockdown, but the delayed degeneration seen with *raw*-RNAi actually enhanced by co-knockdown of *wnd* (**Figure 4-1A**). This result is also

supported by a co-knockdown of *raw* and JNK-Interacting Protein 1 (JIP1), a MAPK scaffolding protein, which shows an enhanced delay in degeneration compared to *raw* knockdown alone (**Figure 4-1E**). The fact that neither *wnd* knockdown nor *jip1* knockdown shows an impact on degeneration on their own suggests that signaling through these proteins may actually be activated following Raw loss and inhibit some aspect of the protective signaling effect seen in Raw-deficient animals. These results are surprising because prior studies have shown that Wnd signaling regulates a protective pathway in *Drosophila* motoneurons (Xiong and Collins 2012); our current findings suggest a role for Wnd in promoting degeneration in the context of Raw loss, which follows observations in injured olfactory receptor neurons (ORNs) (Miller et al. 2009). This result is also supported by co-knockdown of *raw* and *jip1* causing an increase in JNK signaling shown by the Puc-LacZ transcriptional reporter (Ring and Arias 1993), compared to *raw*-RNAi alone (**Figure 4-1C**). JIP1 is a MAPK scaffold required for some aspects of JNK signaling, but not all, suggesting that its loss in this condition may be shifting JNK activity from some role(s) to others (Davis 2000; Dickens et al. 1997; Whitmarsh et al. 2001).

Continuing with the search of what mediates Raw loss-induced JNK activation, we next tested knockdown of the Ask1 MAPKKK. Ask1 is regulated by the Sarm1 homologue TIR-1 in *C. elegans* (Chuang and Bargmann 2005; Ding et al. 2022), and we have seen multiple parallels between Raw and dSarm JNK regulation (**Chapter 2**). We find that while expression of an *ask1*-RNAi causes a very slight delay in injury-induced degeneration on its own, it has no impact on the delayed degeneration seen with a *raw*

knockdown (**Figure 4-1G**). Future work can expand the scope of JNK regulator candidates to uncover Raw's mechanism in restraining this signaling.

Differences in JNK Signaling Between Raw and Injury

After examining potential upstream candidates for JNK activation, we then examined Bsk (JNK) more closely. Given that manipulations of both Wnd and Raw are capable of delaying degeneration (Xiong and Collins 2012; Hao et al. 2019), and that knockdown of Raw causes elevated levels of Bsk in the nerves (**Figure 2-5E**), we examined both overexpression of Wnd and *raw* knockdown in motoneuron pairs. We found that both resulted in increased levels of Bsk (**Figure 4-2I,K**). We also tested if the activated *puc*-LacZ reporter expression following nerve injury is suppressed by Raw overexpression, the way it is by *wnd* knockdown (Xiong et al. 2010), but found no decrease in activation (**Figure 4-2E**). This indicates that while loss of Raw results in activation of JNK signaling to this reporter, it is not sufficient to block activation JNK signaling in some contexts. Given that none of the JNK activators we tested were required for Raw loss-induced JNK activation, we next examined an inhibitor of JNK—the Puckered (Puc) phosphatase. We first examined how overexpression of this protein would impact activation of the *puc*-LacZ reporter and found that it strongly suppresses activation due to *raw*-RNAi (**Figure 4-2A**). A caveat of this assay is that we expressed the same protein that is transcribed by the *puc*-LacZ JNK reporter being used for the readout, making it possible that this is an artifact that we would not see if we were to create reporters for other potential JNK targets. However, when we examine injury-induced activation of the *puc*-LacZ reporter, we find that expression of Puc has no

impact on the activation, which indicates that there is at least some difference between how Raw loss and injury lead to activation of this JNK-dependent gene.

Investigating Additional Candidates for Raw's Mechanism

One of the few studies to examine Raw in neurons described a role for the protein in dendrite patterning (Lee et al. 2015). The authors found that Raw physically interacted with the Tricornered (Trc) kinase in dendrite patterning and took part in integrin signaling to regulate cell adhesion. Trc has also been linked to synapse development (Natarajan et al. 2015), which given our findings that Raw regulates the neuromuscular junction (NMJ) (**Figure 2-2A**), made Trc an ideal candidate for Raw's mechanism. We made use of a myristoylated Trc line (Trc-Myr) that has dominant negative effect (Lee et al. 2015) to test for rescue of Raw's phenotypes in both degeneration and synapse development. However, we found that expression of Trc-Myr strongly inhibits Gal4 expression in these cells, which prevented us from making use of UAS-driven *raw*-RNAi (and therefore prevented us from testing for rescue of NMJ overgrowth, as the $raw^{dcp\text{-}1}/raw^{134.47}$ mutant combination does not visibly impact NMJ growth compared to *raw*-RNAi). We therefore tested if Trc inhibition could rescue degeneration post-injury in a *raw* mutant. We found that there was no significant increase in degeneration when Trc-Myr was expressed in a *raw* knockdown (**Figure 4-3A**), indicating that Raw likely does not act through Trc to regulate degeneration. Given findings from the Parrish lab involving both Trc and integrins (Lee et al. 2015), as well as data linking Raw to regulation of adhesion in dendrite pruning (Rui et al. 2020), we also examined co-expression of the *Drosophila* homologue of E-Cadherin (Shotgun, Shg) with *raw*-RNAi and found similar negative

results (**Figure 4-3C**). From these data, we interpret that Raw may regulate degeneration through a mechanism distinct from its regulation of dendrite patterning.

Potential Connections Between Raw and Mitochondria

Following the important relationship of energy metabolism in axonal degeneration, we asked whether Raw and JNK signaling regulate the localization of mitochondria in axons. Previous unpublished work in the Collins lab has found that Wnd signaling and conditioning injury stimulate the transport of mitochondria into axons. Similar observations were also observed in *C. elegans*, where mitochondria translocate to injured axons to support regeneration (dependent on the *C. elegans* homologue of Wnd, DLK-1) (Han, Baig, and Hammarlund 2016). We made qualitative observations that loss of Raw has a similar effect on mitochondria as injury, and that labeled mitochondria post-injury look similar in both wildtype (degenerating) and *raw*-RNAi (intact) conditions (**Figure 4-3E**). This is seen as a decrease in brightness of the labeling and seemingly fewer labeled mitochondria. Given that Sarm1 has a mitochondrial localization sequence (though loss of this sequence does not disrupt its ability to promote degeneration, (Gerdts et al. 2013)), and that interference with mitochondrial transport in flies can delay injury-induced degeneration (Xin Xiong and Catherine Collins, personal communication), we probed for a possible relationship between the mitochondrial Rho (Miro) GTPase (required for mitochondrial transport to the axon, (Zinsmaier 2021)) and Raw. We found that co-knockdown of *miro* and *raw* results in increased *puc*-LacZ activation compared to *raw* knockdown alone (**Figure 4-3F**). *Miro*-RNAi alone produces a small activation of the JNK signaling reporter (**Figure 4-4A**). Despite their additive

affect on the JNK reporter, co-knockdown of these proteins shows the same degree of delayed degeneration post-injury as either knockdown alone (**Figure 4-3H**). Additionally, in contrast to a *raw* knockdown, the delayed degeneration seen with *miro*-RNAi shows no dependence on Fos (**Figure 4-4C**). Given some of the parallels, it is possible that there is a mechanistic connection between Raw and mitochondria when it comes to degeneration, though important distinctions exist between the two.

TIR-Induced Degeneration Delayed by Wnd Inhibition and Dicer2 Expression

One of the first studies to examine Sarm1 demonstrated that it is required for activated MAPK signaling post-injury and that genetic disruption of all three mammalian JNK isoforms inhibited TIR-induced degeneration (Yang et al. 2015). Signaling through JNK has also been positioned upstream of Sarm1 in another context, where JNK-dependent phosphorylation of Sarm1 enhances its NADase activity for inhibiting mitochondrial respiration. (Murata et al. 2018). It is possible that MAPK signaling is important both upstream and downstream of Sarm1 activation because it is part of a feed-forward loop. Given the feed-forward nature of Sarm1's breakdown of NAD+ leading to more Sarm1 activity due to increased NMN/NAD+ ratio (M. D. Figley et al. 2021; Waller and Collins 2021), this mechanism would be logical for regulation of degeneration, especially when we consider JNK's role in turnover of NMNAT (Walker et al. 2017). We find some small evidence in support of this cyclic theory, as either knockdown of Wnd (**Figure 4-5E**) or Wnd inhibitor treatment (**Figure 4-5G**) result in slight delays of TIR-induced degeneration. While this delay may be small, we also made a surprising discovery that the Dicer2 (Dcr2) protein delayed TIR-induced degeneration much more strongly than

the Wnd manipulations (**Figure 4-5A**). We usually co-express Dcr2 with our RNAis to boost knockdown efficiency, as Dcr2 is involved in processing both endogenous and engineered short interfering RNA (siRNA) (Paturi and Deshmukh 2021). The finding that Dcr2 can delay this potent form of degeneration was unexpected, and given the many siRNAs used to regulate the genome, could be the basis of its own complete study. Here, we did a brief investigation into this phenotype by testing for a dependence on Fos, given that the delayed degeneration of a *raw* mutant, Wnd overexpression, and following a conditioning lesion all show some level of Fos dependence (Hao et al. 2019; Xiong and Collins 2012). In contrast to these findings, we found that the delay in TIR-induced degeneration caused by Dcr2 is not dependent on Fos (**Figure 4-5C**). This leaves open the question of how (and if) altered gene expression may be impacting TIR-dependent degeneration. Examining translational data following Dcr2 expression in these motoneurons has the potential to yield candidate regulators and/or downstream effectors of dSarm's degenerative mechanism.

dSarm Overexpression Does Not Rescue NMJ Overgrowth in Raw Loss

When we first probed for an interaction between Raw and dSarm at the synapse, we looked to see if dSarm overexpression could rescue the altered synaptic protein levels in a *raw* knockdown. We know now that both dSarm overexpression and *raw*-RNAi give similar phenotypes for Cysteine String-Protein (CSP) and Brushpilot (Brp) staining. However, given that Bsk (JNK) overexpression can rescue NMJ overgrowth of dSarm overexpression, despite both manipulations alone causing overgrowth, it is still worth considering if dSarm and Raw could be accomplishing a similar phenotype through

opposite effects. In contrast to the rescue of dSarm by Bsk, we found that overexpression of dSarm has no impact on the decreased levels of CSP and Brp in a *raw* knockdown, compared to Raw alone (**Figure 4-6A**). We also examined the effect of *dSarm* knockdown at the NMJ, using an RNAi line we confirmed delays degeneration as expected (**Figure 4-6I**). We found that there is no significant NMJ overgrowth, but there is a small decrease in both CSP and Brp relative levels (**Figure 4-6D**). While these effects are very mild, they do follow with our findings that either Bsk activation or inhibition can lead to similar effects on CSP and Brp (**Chapter 2**). This suggests a model where it isn't only overactivation of JNK signaling that leads to overgrowth, but that either too much or too little activity can give similar outcomes. This may indicate that JNK activity must be within some specific range to ensure constrained NMJ growth and appropriate levels of synaptic proteins. We do not see any impact on baseline activation of the Puc-LacZ reporter by *dSarm* knockdown which further supports the idea that the impact of JNK at the synapse in dSarm manipulations may be a local one, rather than one mediated by nuclear activity (**Figure 2-8**).

Discussion

The mechanism of Sarm1-driven degeneration is of great importance for both basic research and medical interventions. Given our findings supporting a role for Raw in regulation of dSarm (**Chapter 2**), uncovering how Raw regulates both dSarm and JNK signaling could uncover important features of dSarm in its role in signaling, NMJ development, and degeneration. We found evidence in manipulations of both Raw and dSarm that Wnd may be promoting degeneration to some degree. This is seen by the

enhancement of delayed degeneration in a *raw* knockdown when either Wnd or the MAPK adaptor JIP1 are knocked down, and in the slight delay of TIR-induced degeneration following treatment of a Wnd inhibitor or RNAi expression. Given the role of Wnd and its homologues in stress signaling (Jin and Zheng 2019), Wnd may be acting as a mediator of a general stress pathway activated in response to Raw and dSarm manipulations. This follows with our findings that metabolic knockdowns lead to Wnd activation (**Chapter 3**). Another possible facet to a general stress response could be the delayed TIR-induced degeneration seen with overexpression of Dcr2, which could be enhancing a protective response to TIR activation by endogenous siRNA regulation. Stress responses could also explain the effect that *raw* knockdown has on mitochondria, given their similar appearance to mitochondria in degenerating NMJs. As we have found no clear evidence that the delayed degeneration of Raw has a connection to mitochondrial transport, it is possible that mitochondria are altered in response to the phenotypes in Raw-deficient animals, rather than as part of the mechanism. A clear future goal is identifying the method Raw uses to regulate JNK signaling, to determine how direct effects of this pathway can be defined against the myriad changes in the cell that may occur as responses to the signaling. A fuller understanding of how Bsk (JNK) itself is conditionally regulated by Raw will better separate (or connect) the effects it has on nuclear signaling, NMJ development, and degeneration.

Figures

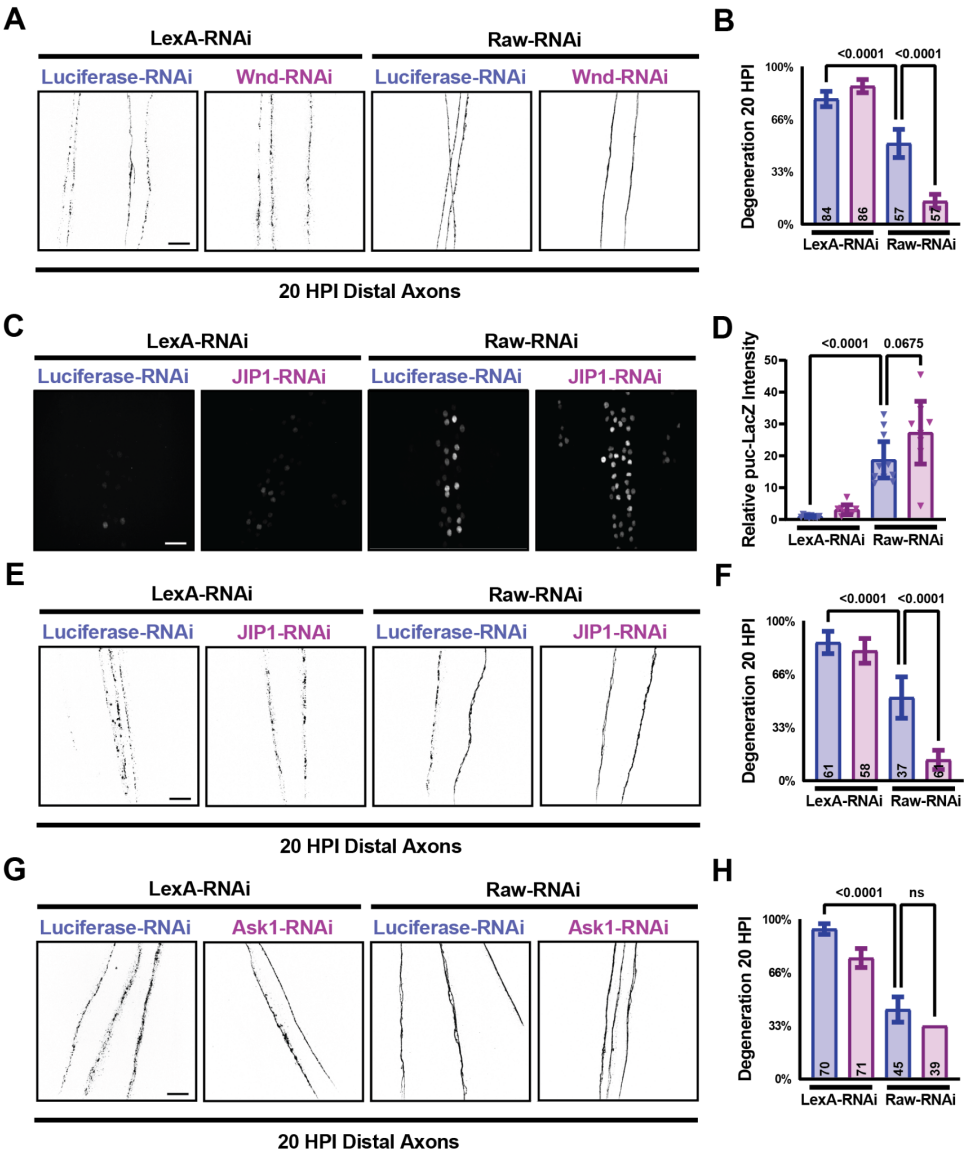


Figure 4-1: Raw and Additional JNK Regulators

A-B) Knockdown of *wnd* kinase in combination with a *raw* knockdown shows a greater delay in degeneration than a Raw knockdown alone, despite *wnd*-RNAi having no effect on degeneration alone. **C-D**) Knockdown of MAPK scaffold protein JNK-Interacting Protein 1 (JIP1) results in an increase of *puc*-LacZ reporter activation in a *raw* knockdown that nears significance. **E-F**) Knockdown of *jip1* results in an even greater delay in post-injury degeneration in a *raw* knockdown, though *jip1* knockdown alone shows no delay in degeneration. **G-H**) Knockdown *of ask1* shows no effect on the delayed degeneration of a *raw* knockdown. Knockdown of *ask1* alone shows a very

slight delay in degeneration. The driver for panels C-D is BG380-Gal4 and for panels A-B,E-F is M12-Gal4. Axons were labeled with mCD8-GFP in panels A-B,E-F. Dcr2 was expressed in all conditions to aid RNAi efficiency. All scale bars are 20 μm.

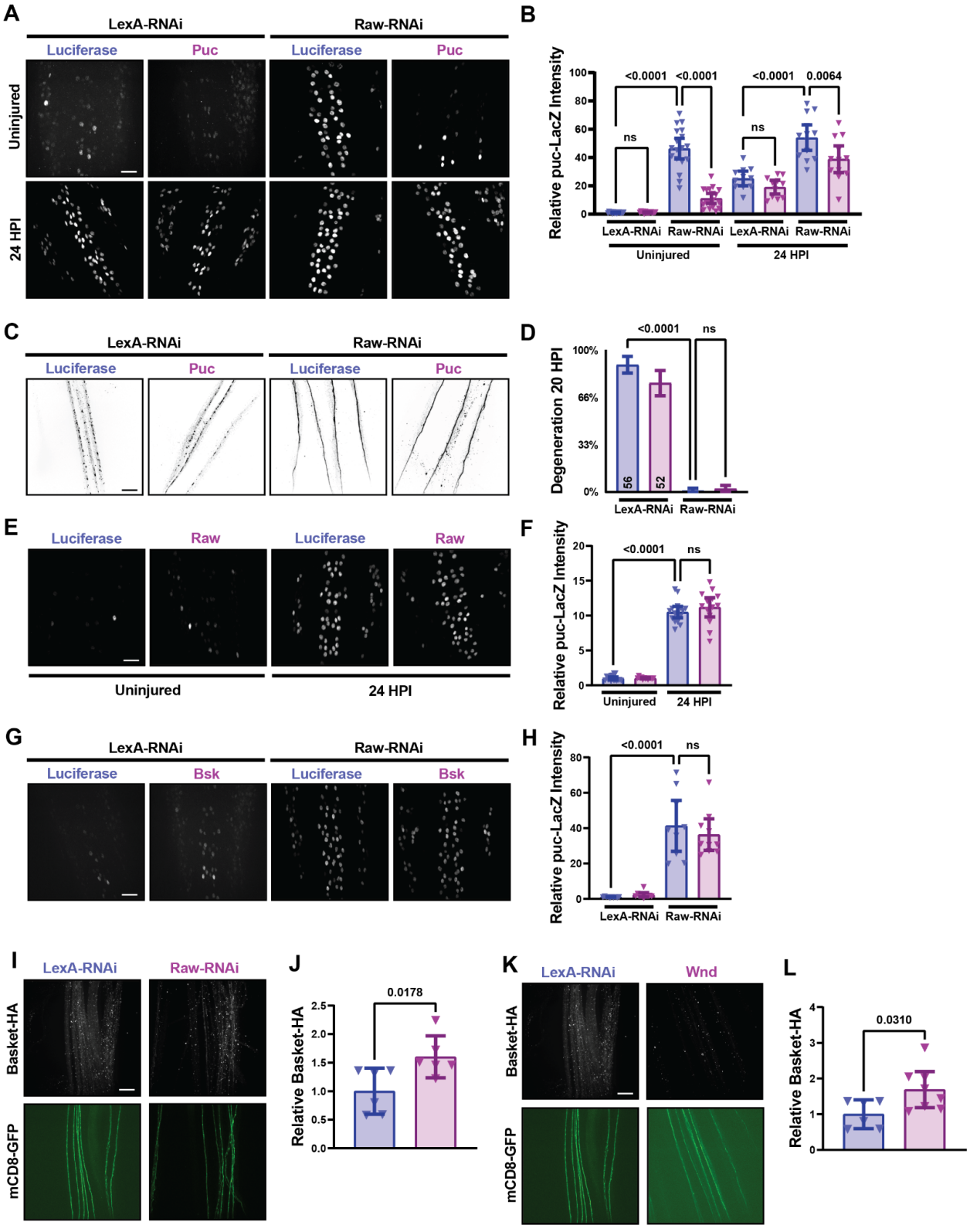


Figure 4-2: Raw and Injury-Activated Kinase Regulation

A-B) Overexpression of the phosphatase Puc strongly inhibits activation of the *puc*-LacZ gene reporter for JNK signaling in a *raw* knockdown, but does not impact activation due to injury. **C-D**) Puc overexpression shows no rescue of post-injury delayed degeneration in motoneuron axons seen with *raw* knockdown. **E-F**) Overexpression of Raw does not inhibit injury-induced *puc*-LacZ transcription activation. **G-H**) Overexpression of Bsk shows no effect on activation of the *puc*-LacZ reporter upon *raw* knockdown. **I-L**) Both *raw* knockdown and Wnd overexpression increase relative overexpressed Bsk-HA (JNK) levels in motoneuron axons. The driver in panels A-B, E-F, and G-H is BG380-Gal4 and the driver in panels C-D and I-L is M12-Gal4. Axons are labeled with mCD8-GFP in panels C-D and I-L. Dcr2 is expressed in all conditions for RNAi efficiency.

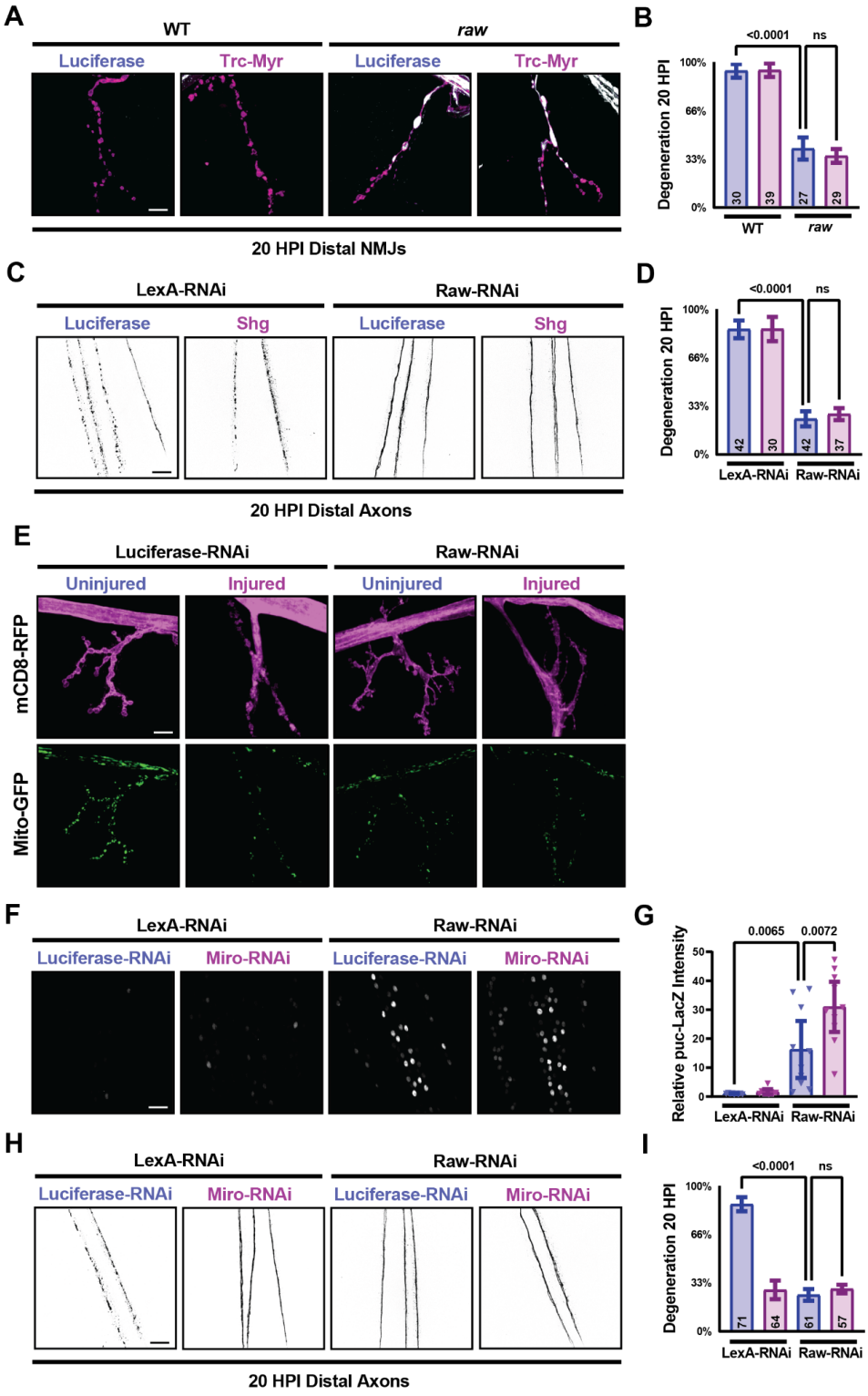


Figure 4-3: Raw Mechanism Candidates

A-B) Overexpression of a dominant-negative Tricornered (Trc) construct shows no rescue of degeneration post-injury in a $raw^{dcp\text{-}1}/raw^{134.47}$ mutant. **C-D**) Overexpression of

Shotgun (Shg) does not rescue the delayed post-injury degeneration of a *raw* knockdown **E**) Qualitative observations that knockdown of *raw* appears to alter mitochondria labeled with Mito-GFP in a similar way to injury in WT. **F-G**) Knockdown of *miro* enhances the *puc*-LacZ reporter activation seen in a *raw* knockdown. **H-I**) Both *raw* knockdown and *miro* knockdown show a similar level of delayed degeneration post-injury, with no additive effect when combined. The driver in panels C-D and H-I is M12-Gal4, the driver in panel D is D42-Gal4, and the driver in panels F-G is BG380-Gal4. Neuronal membranes were labeled with expressed mCD8-GFP (C-D,H-I) or mCD8-RFP (E). Dcr2 was expressed for RNAi efficiency in panels (C-I). All scale bars are 20 μm.

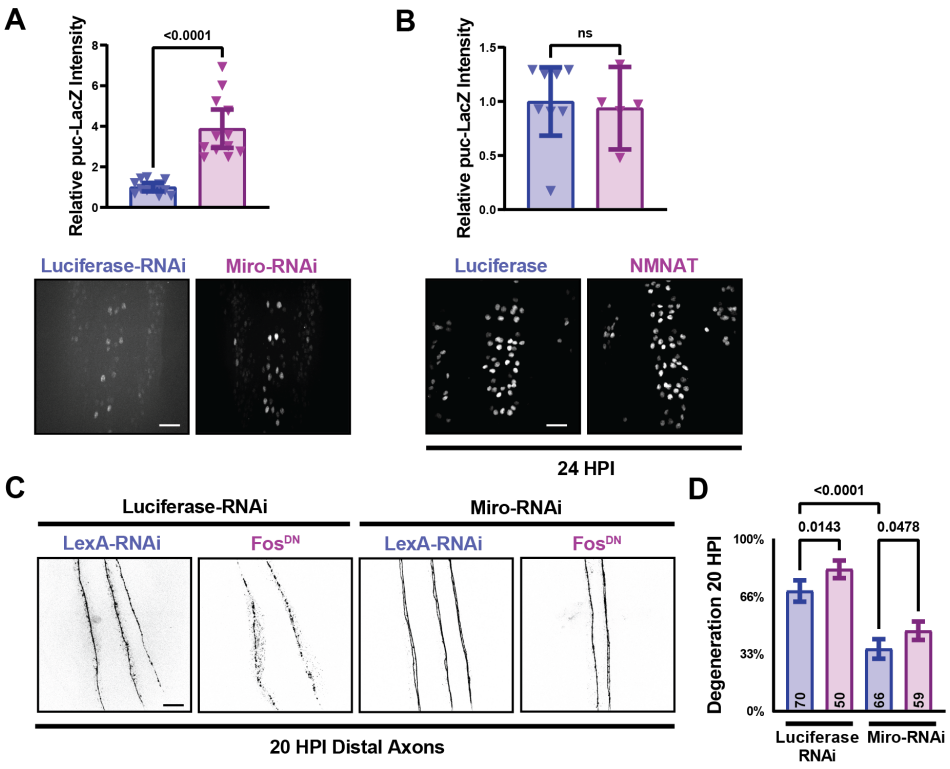


Figure 4-4: Mitochondria, NMNAT, and Injury Signaling
A) Knockdown of *miro* causes *puc*-LacZ reporter activation **B**) Overexpression of Nicotinamide Mononucleotide Adenylyl Transferase (NMNAT) does not block injury-induced *puc*-LacZ reporter activation. **C-D**) The delay in post-injury degeneration of a *miro* knockdown is not rescued by inhibition of Fos, compared to WT. The driver for panels A-B is BG380-Gal4 and the driver for panels C-D is M12-Gal4. Dcr2 is included in all panels to aid RNAi efficiency. All scale bars are 20 μm.

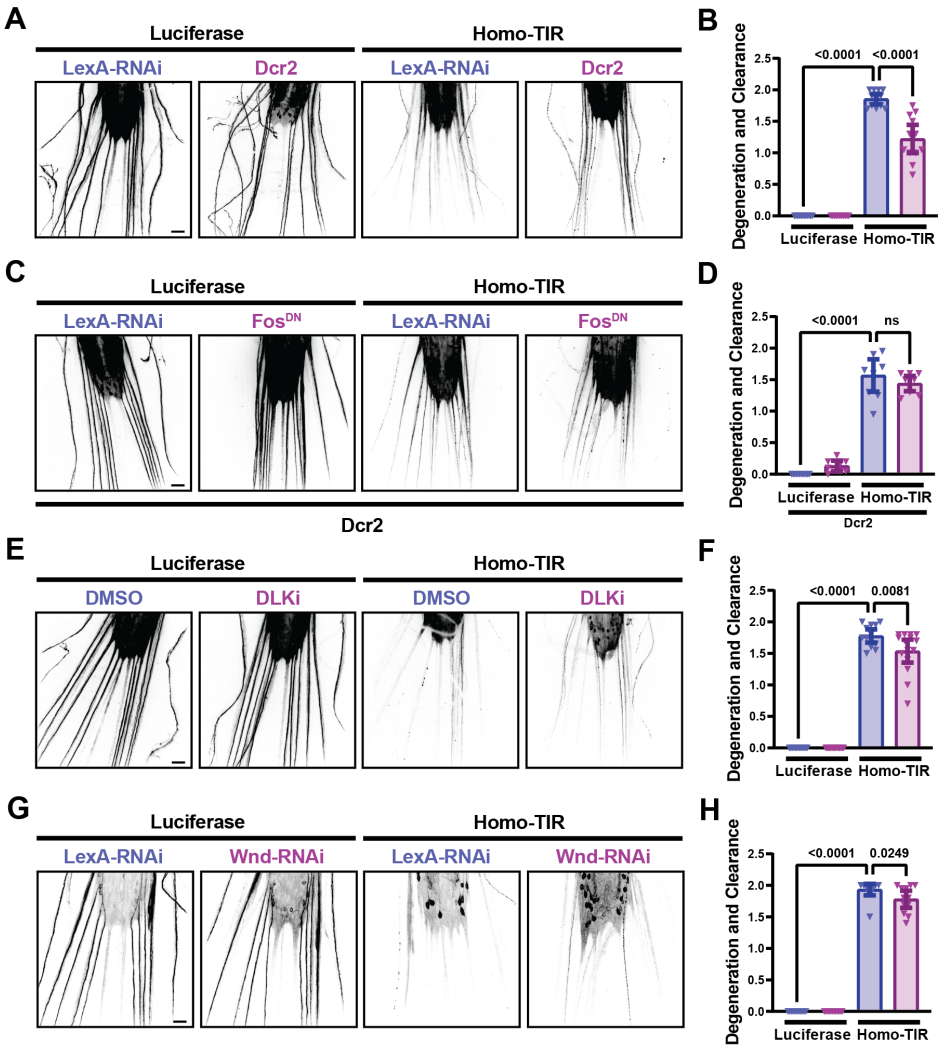
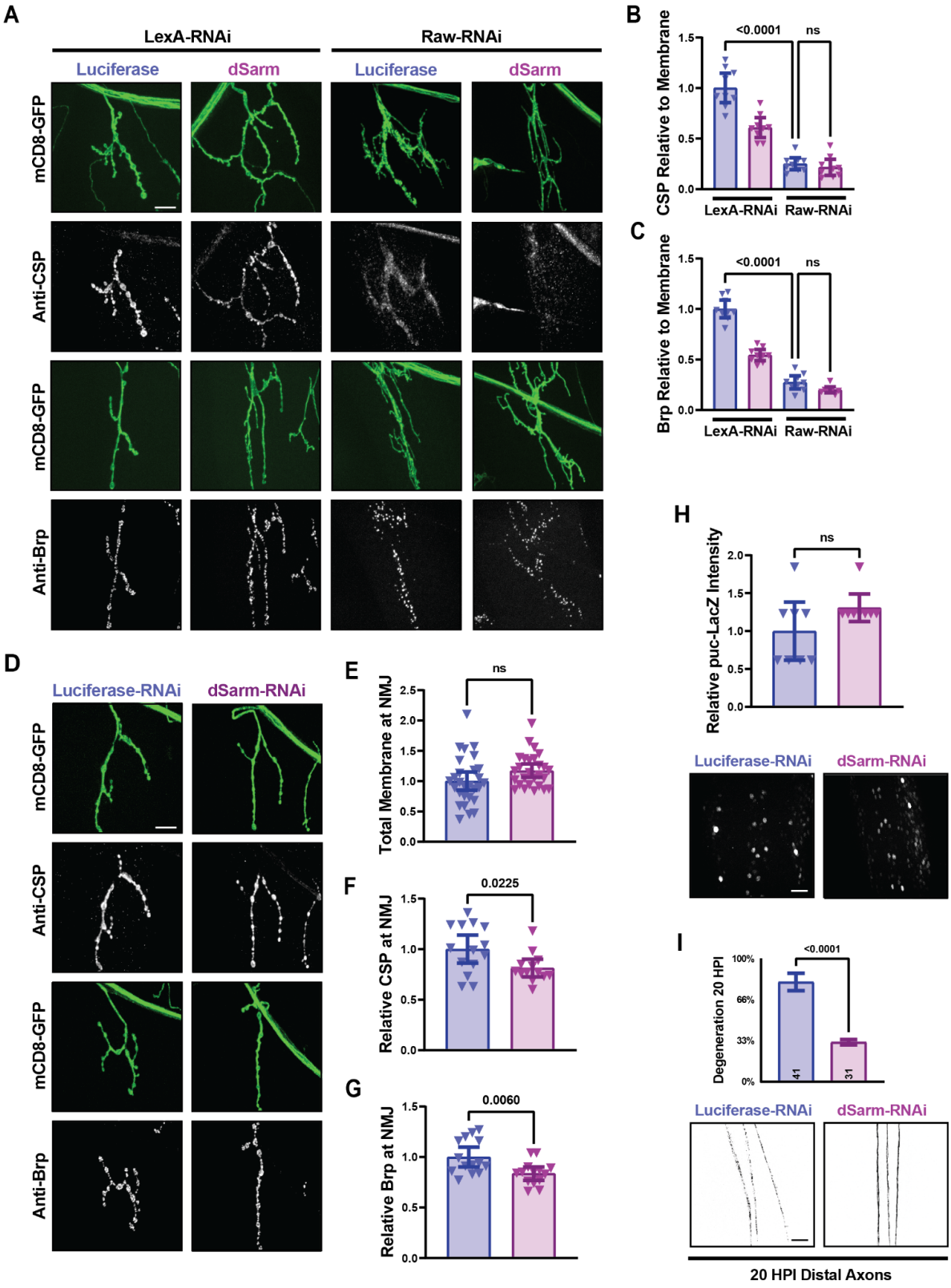


Figure 4-5: Inhibitors of Sarm1-Induced Degeneration

A-B) Overexpression of Dicer 2 (Dcr) delays Homo-TIR-induced degeneration and clearance of axons. **C-D**) The delay in degeneration due to Dcr2 is not rescued by Fos knockdown. A DLK (Wnd) inhibitor (**E-F**) and *wnd* RNAi (**G-H**) both show an extremely small delay in degeneration from Homo-TIR. The driver for all panels is D42-Gal4. Neuron membranes are labeled with mCD8-RFP. All scale bars are 20 μm.


A
LexA-RNAi
Raw-RNAi
Luciferase
dSarm
Luciferase
dSarm
mCD8-GFP
Anti-CSP
mCD8-GFP
Anti-Brp
B
<0.0001
ns
CSP Relative to Membrane
1.5
1.0
0.5
0.0
LexA-RNAi
Raw-RNAi
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<0.0001
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Brp Relative to Membrane
1.5
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0.5
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LexA-RNAi
Raw-RNAi
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Luciferase-RNAi
dSarm-RNAi
mCD8-GFP
Anti-CSP
mCD8-GFP
Anti-Brp
E
ns
Total Membrane at NMJ
2.5
2.0
1.5
1.0
0.5
0.0
F
0.0225
Relative CSP at NMJ
1.5
1.0
0.5
0.0
G
0.0060
Relative Brp at NMJ
1.5
1.0
0.5
0.0
H
ns
Relative puc-LacZ Intensity
2.0
1.5
1.0
0.5
0.0
Luciferase-RNAi
dSarm-RNAi
I
<0.0001
Degeneration 20 HPI
100%
66%
33%
0%
41
31
Luciferase-RNAi
dSarm-RNAi
20 HPI Distal Axons

Figure 4-6: Additional Raw and dSarm NMJ Data
A-C) Overexpression of dSarm does not impact the relative levels of Cysteine String Protein (CSP) or Brushpilot (Brp) in a *raw* knockdown, compared to *raw*-RNAi with a Luciferase control. **D-G**) Knockdown of *dSarm* does not impact total neuronal membrane amount at the NMJ, but does cause a slight decrease in relative levels of both CSP and Brp. **H**) *dSarm* knockdown does not result in activation of the Puc gene reporter for JNK signaling. **I**) Confirmation that this *dSarm*-RNAi successfully delays injury-induced degeneration as expected. The drivers are as follows: panels A-G use D42-Gal4, panel H uses BG380-Gal4, and panel I uses M12-Gal4. For all panels except H mCD8-GFP is expressed to mark neuronal membranes. Dcr2 is expressed in all panels for RNAi efficiency. All scale bars are 20 μm.

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