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Survival mechanisms of peripheral sensory axons

A dissertation presented

by

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to

The Division of Medical Sciences

in partial fulfillment of the requirements

for the degree of

Doctor of Philosophy

in the subject of

Neurobiology

Harvard University

Cambridge, Massachusetts

August 2016

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Survival mechanisms of peripheral sensory axons

Abstract

The ability to receive and process tactile information relies on proper interconnectivity of sensory neuron circuitry. Long peripheral sensory axons are necessary for rapid transmission of sensory information, and are particularly susceptible to degeneration, both during developmental pruning and in response to pathological insults such as injury. While axon degeneration mechanisms have been extensively studied, the opposing mechanisms of axon survival are not well understood. Here we use *in vitro* and *in vivo* methods to investigate intrinsic mechanisms governing axonal survival both during development and in response to chemotoxic injury.

During development, sensory neurons compete for a limited supply of neurotrophic growth factors for survival. The subcellular location of neurotrophin stimulation dictates the survival response, as neurotrophin stimulation at the cell body is insufficient to support axon survival, while neurotrophin stimulation of the growing axonal process supports survival of the whole cell. In these studies we investigate transcriptional and post-translational changes induced by spatially distinct neurotrophin stimulation. Our studies suggest axonal neurotrophin stimulation preferentially upregulates protein synthesis components. These changes will enhance the translational capacity of the cell and may contribute to establishment of axonal connections.

Following initial establishment of sensory circuitry, axon viability must be preserved throughout life to maintain circuit connectivity. Many chemotherapeutic agents can injure long-range axons, causing Chemotherapy-Induced Peripheral Neuropathy (CIPN), a syndrome characterized by impaired tactile sensation and persistent pain. Currently the molecular mechanisms of CIPN are not understood, and there are no available treatments. Here we show that paclitaxel, a chemotherapeutic agent, acts directly

on sensory axons to cause axon degeneration by reductions in IP_3-gated calcium flux and activation of the calcium-dependent protease calpain. Strikingly, Bclw, a Bcl2 family member, binds axonal type 1 IP_3 receptors (IP_3R1) and prevents this degenerative cascade, while other Bcl2 family members are not protective. Paclitaxel treatment selectively reduces expression of Bclw and thereby removes the brakes on this degenerative cascade. Together these data identify a mechanism for CIPN and indicate that selective Bclw-mimetics may provide a preventative therapy for this common disorder. Overall, these studies identify distinct and overlapping mechanisms involved in both developmental and pathological axon survival.

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Acknowledgements

I feel incredibly lucky to have been in the Segal Lab and to have had Dr. Rosalind Segal as my advisor. Students learn to emulate their teachers, and I will consider myself fortunate if I can take with me even a fraction of the passion that Roz has for science and mentorship. I want to thank Roz for her unwavering dedication to her students and to science, and for providing guidance and support every step of the way. For the many hours reading drafts, listening to practice talks, troubleshooting experiments, and deciphering results; for allowing me to try crazy ideas that sometimes worked (and a lot that didn't); and for always being excited about the next scientific finding—thank you, Roz.

I also want to thank Maria Pazyra-Murphy, who has been my partner in investigating Bclw protection against paclitaxel, and who keeps everything running smoothly. For all the good food, good drink, and good times, even when $p > 0.05$, thank you. My sincerest gratitude also to all the members of the Segal Lab, past and present, especially Maria, Sara Fenstermacher, Katharina Cosker, and Ozge Tasdemir-Yilmaz, for the years of scientific discussions, advice, and shared reagents and protocols. I've also been very lucky to have gone through graduate school with an extremely talented cohort of fellow students. Thank you especially to Taralyn Tan, for all the pandas and the coffee dates that kept me sane over the years.

I would like to thank my dissertation advisory committee for guiding me over the years: Dr. Lisa Goodrich, Dr. David Van Vactor, and Dr. Nika Danial. They provided me invaluable advice as my project evolved.

None of this would have been possible without my parents, who have supported me in every aspect of my life and who have always made sure I knew they were proud of me no matter what. Thank you for the hours and hours driving me to Prairie, for the dinner discussions on the psychoactive properties of catnip, and for encouraging me to follow my passion no matter where it led.

And finally, thank you to my soon-to-be husband Aram, who is my first and longest collaborator. Thank you for all the scientific discussions, the practice talks, the rides to lab, the late nights with ready meals, for keeping things in perspective, for the love and support, and for just completely getting it.

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Glossary of Terms

Apaf-1 (Apoptotic protease activating factor 1): A key constituent of the apoptotic machinery that binds cytochrome c and subsequently activates caspase-9.

Bclw (Bcl2-like protein 2): A pro-survival Bcl2 family member that binds pro-apoptotic Bcl2 family members to prevent initiation of the axonal apoptotic caspase cascade[1,2].

Calpains: A family of calcium-activated cysteine proteases that degrade cytoskeletal components and are activated in both developmental and pathological axon degeneration[3].

Calpastatin: An endogenous calpain inhibitor, which is degraded during developmental and pathological axon degeneration[3].

Caspases (*c*ysteine-*asp*artic prote*ases*): A family of cysteine proteases essential for cell body apoptosis and developmental axon degeneration. Caspases are first synthesized as inactivate pro-caspases, which are activated upon cleavage.

DLK (dual leucine zipper kinase): A mitogen-activated protein kinase kinase kinase involved in axon degeneration and regeneration[4-9]. Its *Drosophila* ortholog is Wallenda.

DR6 (death receptor 6): A tumor necrosis factor (TNF) receptor whose activation induces apoptosis and axon degeneration[10,11]

IMPA1 (myo-inositol monophosphatase-1): An enzyme involved in synthesis of myo-inositol and therefore essential for phosphophatidylinositol signaling pathways such as neurotrophin signaling.

IP_3R1 (inositol 1,4,5-trisphosphate receptor type 1): A calcium channel that mediates IP_3-gated calcium efflux from the endoplasmic reticulum and is required for pathological axon degeneration[12].

Lamin B2: A nuclear intermediate filament protein that also localizes to axons and is essential for axon maintenance and survival[13,14].

Neurotrophins: A family of secreted growth factors that promote axonal and neuronal survival by binding to transmembrane tropomyosin-receptor-kinase (Trk) receptors.

NMNAT (nicotinamide mononucleotide adenylyltransferase): A family of NAD^+ biosynthetic enzymes involved in axon maintenance, and a component of the Wld^S fusion gene.

NAD^+ (nicotinamide adenine dinucleotide): An essential coenzyme for cellular metabolism and signaling, which functions in ATP-producing redox reactions and as a substrate for several protein-modifying enzymes[15].

mPTP (mitochondrial permeability transition pore): A protein pore formed in the inner membrane of the mitochondria under pathological conditions, allowing mitochondrial release of calcium stores and reactive oxygen species.

SARM1 (sterile α-motif-containing and armadillo-motif containing protein): A Toll-like receptor essential for axon degeneration by activation of a MAP kinase cascade and/or NAD^+ depletion[16-19]. Its *Drosophila* ortholog is dSARM.

SCG10 (superior cervical ganglion 10): A microtubule destabilizing protein that promotes axonal health. It is degraded in a JNK-dependent manner following axon injury[20].

UPS (Ubiquitin Proteasome System): The major non-lysosomal mechanism by which the cell degrades proteins. Proteins are targeted for degradation by the small protein tag ubiqituin via ubiquitin ligase enzymes, and tagged proteins are subsequently degraded by the proteasome complex.

Wallerian degeneration: A form of axon degeneration resulting from focal nerve transection wherein the axon distal to the injury site swells and fragments.

Wld^S (*Wallerian degeneration slow* protein): A chimeric fusion gene of the NAD^+ biosynthetic enzyme NMNAT1 and the ubiquitination factor E4B, whose expression delays various forms of pathological axon degeneration.

XIAP (X-linked inhibitor of apoptosis protein): An inhibitor of apoptosis protein that binds to and inhibits caspases to prevent initiation of cell body apoptosis and the axonal apoptotic cascade[21,22].

Chapter 1

Introduction

Portions of this Chapter are excerpted from Trends in Neurosciences, 37/10, Sarah E. Pease and Rosalind A. Segal, Preserve and protect: maintaining axons within functional circuits, 572–582, 2014, with permission from Elsevier.

1.1 Establishing and maintaining circuits:

Functional neural circuits depend on proper interconnections formed by long range axonal projections. As the fundamental connective unit of neural circuits, axons must be protected and maintained in the face of multiple potential threats. Axonal maintenance is particularly important because most neurons cannot be replaced and must therefore be preserved throughout the life of the organism. Axon degeneration is a broad term applied to various modes of axon death, with distinct instigators but similar final morphology of axon fragmentation. Though much progress has been made toward elucidating the mechanisms underlying axonal degeneration, a complete understanding requires study of the mechanisms opposing degeneration. Axon regeneration, the recovery and regrowth of axons following acute or chronic trauma, is not the opposite of axon degeneration, but rather a response to it. In reality the opposite of axon degeneration is the process of axonal survival.

It was classically thought that axons degenerate as a result of cell body death, due to a lack of support from the cell body. This theory was first challenged by the discovery of the *Wallerian degeneration slow* (Wld^S) mutant mouse (see Glossary), where neuronal expression of the Wld^S fusion gene delays degeneration of severed axons for weeks. More recent studies have provided direct evidence for active axonal death mechanisms, such as the pro-degeneration molecule dSARM/SARM1[16,17], as well as for pro-survival mechanisms, such as the Bcl2 family member Bclw (Bcl2l2)[1,2,23]. Thus, it is now apparent that the axonal compartment relies on distinctive pathways for survival and degeneration, and these exhibit similarities to and differences from cell body survival and death mechanisms[2,24-30]. In this review, we first examine mechanisms of developmental axon survival and pruning. We then discuss pathways promoting lifelong axonal maintenance and health, and the opposing degenerative processes triggered by injury and disease. Recent reviews have addressed axon regeneration[31,32] and dendritic degeneration[28].

1.2 Developmental axon preservation:

A common theme in neural development is overproduction followed by elimination and refinement. This mechanism allows for great flexibility in potential circuit configuration[24]. In both the central and peripheral nervous systems, neurons initially extend excess axonal connections, and refinement into a mature circuit requires coordinated pruning of inappropriate connections and preservation of appropriate connections. Pruning must therefore be induced in a selective subset of axons while the remaining axons are protected and maintained. Further, the scale of axonal elimination must be closely regulated. Pruning can remove segments as small as axon terminals or as large as whole axons, and can even include subsequent apoptosis of the cell body.

Extracellular cues

Extracellular cues from other neurons within a circuit or from nearby glial or target cells often determine which axons will initiate intracellular axon pro-survival pathways and which will be removed. Critical cues that have been identified include network activation and secretion of growth factors. During early postnatal development of the neuromuscular junction (NMJ), muscle cells are initially innervated by multiple motor neuron terminal arbors. These overlapping inputs compete for survival in an activity-dependent manner. Inputs delivering stronger and more correlated activity are strengthened, and the remaining inputs are eliminated, such that each muscle cell is ultimately innervated by a single motor neuron[24]. A similar activity-dependent mechanism is used in the developing cerebellum to select for survival of a single climbing fiber input onto a single Purkinje cell[33]. Activity regulated mechanisms including changes in transcription as well as cytoskeletal and morphological adaptation, enable maintenance of axons connected within a functional circuit.

Neurotrophins, nerve growth factor (NGF), brain derived growth factor (BDNF), and neurotrophin 3 and 4 (NT3 and NT4), constitute the most well recognized growth factor family that promotes axonal and neuronal survival. In the peripheral nervous system, survival of sympathetic and sensory neurons depends on successful competition for a limited supply of target-derived neurotrophins. Furthermore, local stimulation with neurotrophins regulates axonal growth, branching, and terminal arborization[25,34-37]. Neurotrophins secreted by target cells bind to tropomyosin-receptor-kinase (Trk) receptors located on innervating axon terminals and initiate both local and retrograde signaling events in the axon. This paradigm has been studied *in vitro* through the use of various compartmented culture platforms that spatially and fluidically isolate cell bodies and distal axons, and so replicate the separation between axon terminals and cell bodies that occurs within normal neuronal circuits. In these compartmented culture platforms, cell bodies and axons can be independently deprived of or stimulated with neurotrophins, and changes within cell bodies and axons can be assayed separately. In pioneering studies using sympathetic neurons grown in compartmented cultures, Campenot first demonstrated that local axonal neurotrophin stimulation, a correlate of *in vivo* target-derived neurotrophin stimulation, is required to promote axonal survival, whereas cell body survival is supported by either somatic or axonal neurotrophin stimulation[38].

Inhibitors of axonal apoptosis

Until recently, the involvement of the apoptotic cascade in developmental axon degeneration was largely discounted[39]. Seminal work from several groups has since described an apoptotic caspase cascade within axons that is induced by neurotrophin withdrawal, and identified anti-apoptotic proteins that promote developmental axon survival by inhibiting this specialized cascade (Figure 1.1).

Anti-apoptotic Bcl2 family members Bcl2, $Bclx_L$ (also known as Bcl2l1), and Bclw (Bcl2l2) avert somatic apoptosis by binding and sequestering pro-apoptotic Bcl2 family members, thus preventing

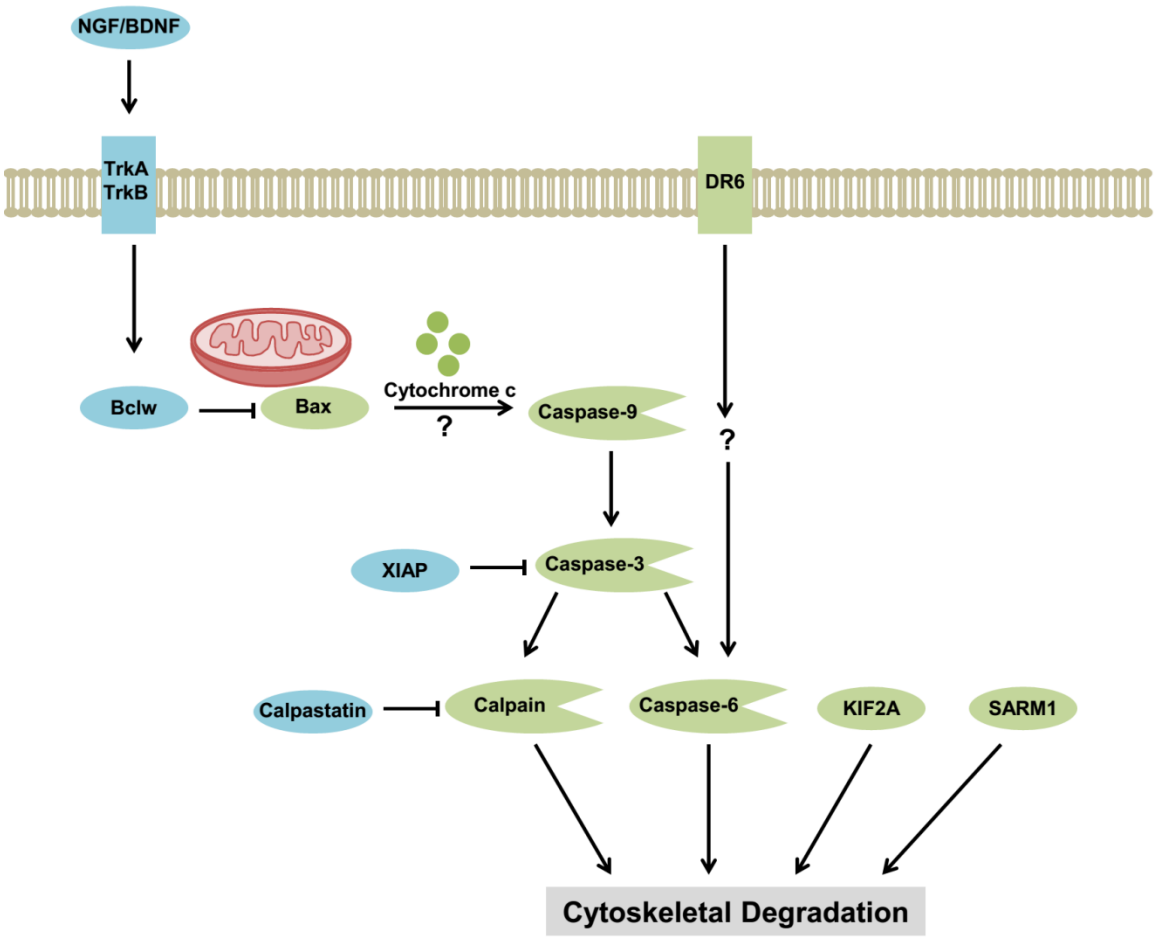


Figure 1.1. Developmental axon survival and degeneration pathways. Following trophic withdrawal, parallel pro-degenerative cascades converge on a common pathway of cytoskeletal degradation to induce axon degeneration. Pro-survival molecules (blue) actively inhibit pro-degenerative molecules (green). The neurotrophins NGF and BDNF stimulate TrkA and TrkB receptors on the growing axon and induce axonal expression of the anti-apoptotic Bcl2 family member Bclw. Bclw inhibits the pro-apoptotic Bcl2 family member Bax, preventing activation of the axonal apoptotic cascade[2,23]. The endogenous inhibitors XIAP and calpastatin also inhibit the degenerative proteases caspase-3 and calpain respectively, preventing downstream cytoskeletal degradation[3,21,22]. In the absence of neurotrophins, Bax elicits mitochondrial release of cytochrome c and activation of the protease caspase-9 by an unknown mechanism[10,22]. Caspase-9 cleaves and activates caspase-3, which itself activates caspase-6 and the calcium-sensitive protease calpain[3,10,21,30,40,41]. In addition, the receptor DR6 can initiate activation of caspase-6[10,11]. The proteins KIF2A[42] and SARM1[16] also induce cytoskeletal degradation in the absence of trophic support, though it is unknown how they are regulated. Abbreviations: Bclw, Bcl2-like protein 2; BDNF, brain derived growth factor; DR6, death receptor 6; KIF2A, Kinesin superfamily protein 2A; NGF, nerve growth factor; TrkA, tropomyosin-receptor-kinase A receptor; TrkB, tropomyosin-receptor-kinase B receptor; SARM1, sterile α-motif-containing and armadillo-motif containing protein; XIAP, X-linked inhibitor of apoptosis protein.

mitochondrial release of cytochrome c and subsequent activation of caspases. Of these closely related family members, only Bclw mRNA and protein are enriched in axons[1,2]. Bclw expression can be detected in late embryonic and early postnatal development as axon terminals reach their peripheral targets, and expression continues throughout adulthood[1]. Moreover, target-derived neurotrophins selectively stimulate a pathway that relies on the MAP kinase ERK5 to induce transcription of *bclw* and of a set of retrograde response genes[23,43]. Bclw binds and inhibits the pro-apoptotic Bcl2 family member Bax, thus preventing changes in the mitochondrial membrane potential, cytochrome c release, and subsequent initiation of the axonal caspase cascade[2]. Genetic studies indicate Bclw functions within the axon to promote axon survival and prevent degeneration[2,23]. In addition to the local protective function of Bclw, a recent study suggests Bclw and $Bclx_L$ function within the cell body to inhibit the pro-apoptotic Bcl2 family member Puma and prevent transmission of an anterograde pro-degenerative signal into axons[44].

In contrast to Bcl2 proteins, the inhibitor of apoptosis (IAP) proteins prevent initiation of the apoptotic cascade by direct inhibition of caspases, as they bind to activated, cleaved caspase-3[21,45]. Thus, X-linked inhibitor of apoptosis protein (XIAP) provides a second mode of control that restrains axonal caspase activity and subsequent axonal pruning. Cultured sensory neurons lacking XIAP degenerate more rapidly when deprived of neurotrophin, and embryonic *XIAP*$^{-/-}$ mice show decreased epidermal sensory innervation without a concomitant loss of cell bodies[21,22]. Furthermore, *XIAP*$^{-/-}$ neurons grown in compartmented cultures exhibit increased somatic levels of active caspase-3 when neurotrophins are withdrawn only from axons, suggesting a role for XIAP in spatially restricting apoptotic cascade activation to the axon[22].

The caspase cascade activated in axonal degeneration involves an initial and essential catalytic function for caspase-3, followed by activation of caspase-6[3,10,21,30,40,41]. The roles of these two caspases in axonal degeneration have been demonstrated by analysis of genetic models. The calcium-activated calpain family constitutes a second set of proteases implicated in both developmental and pathological

axon degeneration. In neurotrophin-supported axons, calpastatin inhibits calpain activation. Upon neurotrophin deprivation, calpastatin is degraded by activated caspase-3, allowing downstream activation of calpain and subsequent cleavage of calpain targets such as neurofilaments[3].

Although primarily regarded as mediating pathological axon degeneration, there is also some evidence that an NAD^+-sensitive pathway operates in parallel with the caspase cascade during axon pruning[30,46]. Combined treatment with NAD^+ and caspase inhibitors completely protects wildtype axons from neurotrophin withdrawal, while the individual inhibitors alone only exert incomplete protective effects[30]. These results suggest that NAD^+ can promote survival of neurotrophin-deprived axons, but it is not yet known whether an NAD^+-sensitive pathway is endogenously activated during axon pruning, and how it might interact with the axonal apoptotic cascade.

Regulating protein levels

Regulation of axonal degenerative cascades requires precisely controlled localization and quantities of protective factors. New axonal proteins are supplied by anterograde microtubule-dependent transport as well as by local translation. Inhibition of axonal protein synthesis with local cycloheximide treatment abolishes the axon survival effect of neurotrophins, suggesting that neurotrophins rely in part on locally synthesized factors to mediate axon survival[2]. Two such factors have been recently identified: Bclw and IMPA1 (myo-inositol monophosphatase-1). As indicated previously, Bclw plays a role in local inhibition of the caspase cascade, and also regulates mitochondrial morphology and function[2]. IMPA1 is a key enzyme of the inositol cycle and therefore necessary for proper induction of phosphoinositide pathways triggered by neurotrophin signaling[47]. Given the thousands of mRNAs identified in embryonic axons, it is likely additional protective factors are locally translated to stabilize axons connected within a circuit[48].

Protein turnover is frequently regulated by the Ubiquitin Proteasome System (UPS). Global inhibition of the proteasome with pharmacological agents or genetic mutations have yielded conflicting results, with some studies finding it prevents axon pruning[3,49] and others finding it accelerates pruning[22]. One mechanism by which proteasome inhibition may be protective is by preventing degradation of pro-survival molecules. Axonal XIAP and calpastatin are both degraded upon neurotrophin withdrawal, resulting in loss of axon viability[3,21]. XIAP is degraded by the UPS, thus releasing caspase-3 and allowing induction of the axonal apoptotic cascade[21,30] and activation of calpain[3]. Conversely, the UPS may also promote axonal survival by degrading pro-apoptotic proteins.

1.3 Developmental axon pruning:

Axons or axonal segments that are not selected to survive can be eliminated by three distinct mechanisms (Figure 1.2A-B). The most well studied is axon degeneration, which culminates in cytoskeletal degradation, axon fragmentation, and removal of debris by glia and possibly epidermal cells (Figure 1.2A)[24,25,28,29,50,51]. In contrast, both axon retraction (Figure 1.2B) and axosome shedding (Figure 1.2C) have only been observed during small-scale pruning of synapses or terminal branches. Axon retraction is the pulling back and absorption of small segments of axon[24,25], and axosome shedding involves axon tip swelling and shedding of membrane-bound axonal remnants called axosomes[24,50,52]. While axon remnants are absorbed by the neuron itself during retraction, axosomes are engulfed by adjacent cells, such as the surrounding Schwann cells[52].

Extracellular signaling

Some cells in a circuit promote axon survival, while others actively induce axon pruning. In sympathetic neurons, competition results in the more active axons eliminating their competitors. NGF-TrkA and activity dependent signaling cascades maintain the winning axons. Meanwhile, the winning

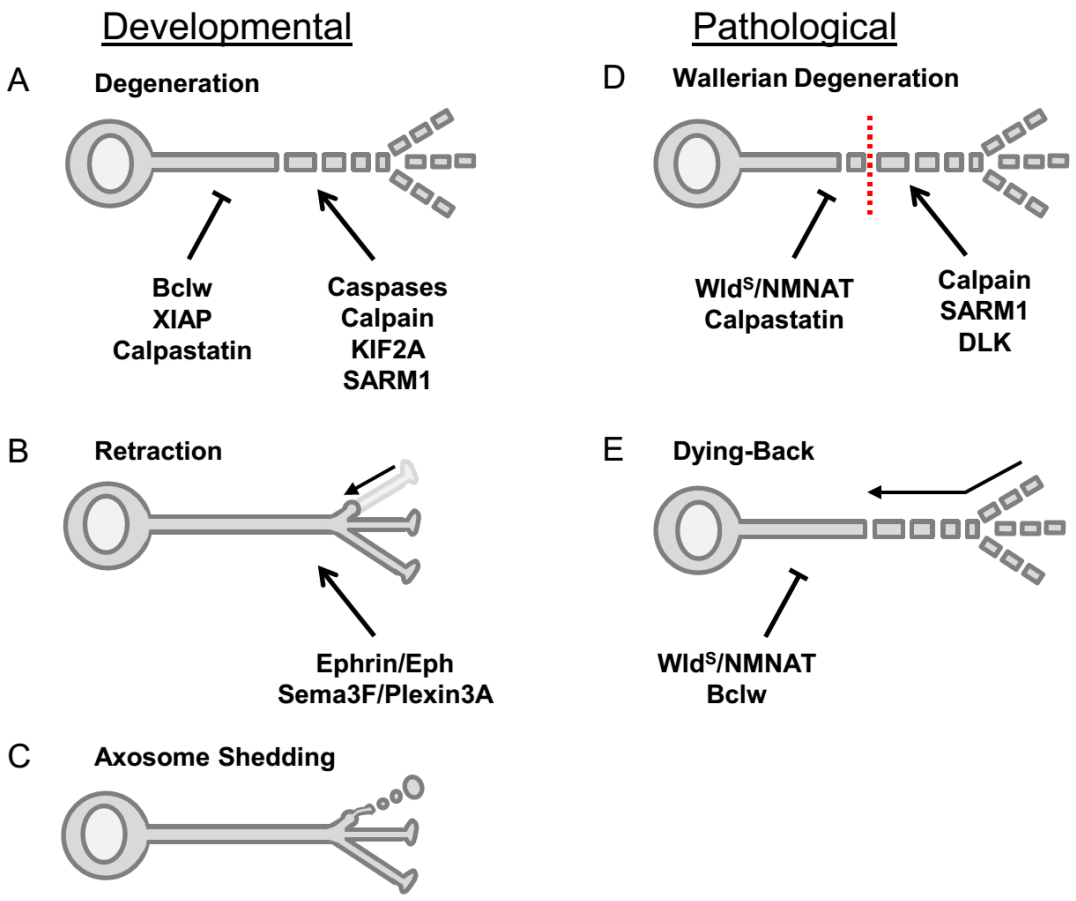


Figure 1.2. Modes and mechanisms of axon elimination. Schematics of neurons during developmental pruning **(A-C)** and pathological axon loss **(D-E)**. **(A)** Developmental axon degeneration, as occurs with trophic deprivation, results in cytoskeletal degradation and axon fragmentation. Degeneration is prevented by Bclw, XIAP, and/or calpastatin and driven by caspases, calpain, KIF2A, and SARM1[1-3,10,16,21-23,30,40,41]. **(B)** Retraction involves pulling back and absorption of small axon segments, such as axon terminal branches. It can be driven by Ephrin/Eph[53,54] or Sema3F/Plexin3A[55] signaling pathways. **(C)** Axosome shedding also removes small axon segments, with swelling of the axon tip and shedding of membrane-bound axon remnants called axosomes. The underlying molecular mechanisms are still unknown[52]. **(D)** Wallerian degeneration is a type of pathological axon elimination induced by axon severing (indicated by a dotted red line). Following a latency phase, the axon distal to the injury site undergoes swelling, cytoskeletal breakdown, and fragmentation. Various NMNAT isoforms, the Wld^S fusion protein, and calpastatin prevent degeneration, while SARM1, DLK, and calpain drive degeneration[3,5,16,17]. **(E)** In many neurodegenerative diseases axons are eliminated by a dying-back process that involves axon swelling and fragmentation beginning distally and propagating in a proximal direction. Various NMNAT isoforms, the Wld^S fusion protein, and Bclw can oppose this process[1]. Abbreviations: Bclw, Bcl2-like protein 2; DLK, duel leucine zipper kinase; KIF2A, Kinesin superfamily protein 2A; NMNAT, nicotinamide mononucleotide adenylyltransferase; Sema3F, semaphorin 3F; SARM1, sterile α-motif-containing and armadillo-motif containing protein; Wld^S, Wallerian degeneration slow protein; XIAP, X-linked inhibitor of apoptosis protein.

axons release proBDNF, which binds to p75 neurotrophin receptor (p75NTR) in losing axons and induces axon degeneration[56]. A similar mechanism of p75NTR- mediated axon degeneration and Trk-mediated axon survival is critical for correct circuit connectivity in adult septal cholinergic neurons[57]. Several guidance molecules secreted in target regions are also known to induce axon pruning. These include Semaphorin 3F and its receptor Plexin3A, which is involved in pruning of hippocampal mossy fiber collaterals[55], and Ephrins and their receptor Ephs, which mediate RGC topographical mapping[24] and axon retraction[53,54].

Luo and colleagues demonstrated that ecdysone hormone signaling instigates axon pruning of mushroom body (MB) γ neuron during *Drosophila* metamorphosis. Ecdysone hormone stimulates ecdysone receptor B1 (EcR-B1) expressed selectively on γ neurons and induces axon pruning[58]. Glial cells control neuronal expression of EcR-B1 by secreting the TGF-β ligand myoglianin, activating TGF-β signaling pathways in γ neurons[59,60].

Neurotrophin deprivation engages additional pathways to mediate axonal destruction. In particular, TNF family receptors such as DR6 contribute to axonal degeneration via downstream activation of caspase-6[10,11]. This cascade may be particularly relevant in Alzheimer's disease.

Intracellular signaling

Several intracellular signaling mechanisms play a role in axon pruning. As discussed, the apoptotic cascade mediates developmental axon degeneration and is controlled by both negative (XIAP, Bclw) and positive (TNF-receptor DR6) regulators. Neurotrophin withdrawal pathways converge on the pro-apoptotic Bcl2 family member Bax, which causes cytochrome c release from mitochondria[10,22]. Interestingly, while cytochrome c binds Apaf-1 to activate caspase-9 in cell soma apoptosis, apparently Apaf-1 is not required for axon degeneration, and so the mechanism for activation of caspase-9 in axons is not yet clear[22]. Following activation of caspase-9, caspase-3 is cleaved and activated, and caspase-3

directly activates caspase-6 and indirectly activates calpain via calpastatin cleavage[3,41]. Together these results suggest that there are both similarities and distinctions between the axonal and somatic apoptotic cascades that will be important to decipher. Furthermore, while the apoptotic machinery is involved in some types of developmental axon degeneration (neurotrophin deprivation-induced degeneration, retinocollicular axon pruning)[3,10,21,30,40,41], it does not appear to be involved in others (MB γ neuron pruning)[49]. These findings suggest there may be multiple mechanisms of developmental axon degeneration that converge on a common pathway of cytoskeletal breakdown.

Developmental axon degeneration can be initiated by the mammalian Toll receptor adaptor SARM1 [16]. SARM1 is activated in parallel with the caspase cascade during neurotrophin deprivation and appears to function primarily at an early stage of degeneration[16]. SARM1 mediates injury-induced axon degeneration by activating a mitogen-activated protein kinase (MAPK) cascade[18] and/or by causing axonal depletion of the metabolic oxidizing agent NAD^+ [16,61], although it is unknown if SARM1 functions by the same mechanisms to initiate developmental degeneration. In multiple systems, the Toll interleukin-1 receptor and sterile α motif domains within SARM1 are necessary for its destructive function[16]. Like the caspase cascade, the Drosophila ortholog dSARM does not appear to play a role in MB γ neuron axon pruning[17], but both SARM1 and dSARM promote injury-induced axon degeneration[16,17].

Cytoskeletal breakdown is a common, late feature of axon degeneration and does not seem to be involved in the more restricted processes of axosome shedding or axon retraction. Recent advances have provided some insight into the mechanisms of cytoskeletal breakdown during degeneration. The Kinesin superfamily protein 2A (KIF2A), a microtubule depolymerizing protein, is a key executor of microtubule breakdown and axonal degeneration during neurotrophin withdrawal-induced axon pruning, and so mice lacking KIF2A exhibit delayed degeneration of sensory neurons innervating the

skin[42]. In the future it will be important to ascertain how KIF2A is regulated to selectively depolymerize the microtubule cytoskeleton in degenerating axons.

1.4 Lifelong axon maintenance:

Numerous mechanisms control the health and homeostasis of axons throughout life, and oppose stressors such as excitation and aging. Injury and disease induce axon degeneration both by compromising maintenance mechanisms and promoting active self-destruction pathways. Expression of the *Wallerian degeneration slow* (Wld^S) mutant protein, a chimeric fusion of the NAD^+ biosynthetic enzyme NMNAT1 and a fragment of the ubiquitination factor E4B (UBE4B), delays axonal degeneration induced by numerous pathological insults[15,62,63] (see Conforti et. al. 2014 for an extensive summary of the effects of Wld^S/NMNAT on various axon pathologies[64]). Study of the Wld^S protein and its constituents has provided great insight into mechanisms of axonal viability that rely on the interrelated processes of metabolic homeostasis, calcium buffering, axonal transport, and protein synthesis.

Metabolic maintenance

Proper metabolism is integral to axon viability and functionality. Axons require large amounts of energy and metabolites to support membrane depolarization and synaptic transmission[65], and thus the NAD^+ biosynthetic NMNAT enzymes are essential in axon maintenance[15,62,63]. Mammalian NMNAT isoforms include the nuclear NMNAT1, the golgi-associated NMNAT2, and the mitochondrial NMNAT3[63]. Wld^S is a chimeric version of NMNAT1, and its ability to delay axon degeneration requires NAD^+ synthetic activity in the cytoplasm, and possibly in the axon[66,67]. While overexpression of either NMNAT2[68,69] or NMNAT3[67] delays degeneration of injured axons, only endogenous NMNAT2 is required for maintenance of healthy axons[68,70]. Wld^S may confer its protection by directly substituting for the more labile NMNAT2, as NMNAT2 is rapidly degraded following injury while Wld^S is degraded more

slowly[68,70]. Furthermore, mutant forms of NMNAT2 with prolonged half-life exhibit a level of axon protection comparable to Wld^{S} [70].

While NMNAT enzymatic activity is necessary for axon viability, it is less clear whether this effect requires the enzymatic product NAD^{+} [66,67]. Injured axons exhibit a decrease in NAD^{+} prior to morphological degradation, and degeneration is mitigated *in vitro* by exogenous NAD^{+} [71,72]. In addition, direct activation of SARM1 causes rapid depletion of NAD^{+}, and axon degeneration is prevented by increased $^{NAD+}$ synthesis[19]. However, protection by exogenous NAD^{+} requires supra-physiological levels[66,71,73,74], increasing cellular NAD^{+} levels by inhibiting NAD^{+}-consuming enzymes does not protect injured axons[75], nor does the inhibition of NAD^{+} biosynthesis abolish Wld^{S}-mediated protection[66]. Furthermore, Wld^{S} does not detectably increase overall cellular levels of NAD^{+} [75,76]. A possible explanation that reconciles these disparate results is that Wld^{S} and NMNATs induce a local increase in NAD^{+}, perhaps at mitochondria, rather than increasing total cytoplasmic content of NAD^{+} [29,67]. This hypothesis is supported by evidence that cytoplasmic Wld^{S} copurifies with mitochondria[66,67,77].

If NAD^{+} is a pro-survival factor, how does it act to protect axons? Axonal energy is primarily supplied by mitochondria, which use NAD^{+} to produce ATP. Axonal injury decreases both NAD^{+} and ATP levels even before morphological degeneration[71]. Exogenous application of NAD^{+} or expression of Wld^{S} sustains both NAD^{+} and ATP levels and delays subsequent degeneration[71]. In addition, Wld^{S} mitochondria exhibit enhanced ability to generate ATP, suggesting that NMNAT/Wld^{S} may therefore protect axons by maintaining mitochondrial bioenergetics (Figure 1.3)[67].

An alternative or additional explanation is that NMNAT2 protects axons by consuming NMN, the substrate used to synthesize NAD^{+} [61]. Although inhibition of NMN biosynthesis leads to only a short-term delay in axon degeneration[75,78], NMN-consuming enzymes strongly delays axon degeneration to a similar degree as Wld^{S} [78].

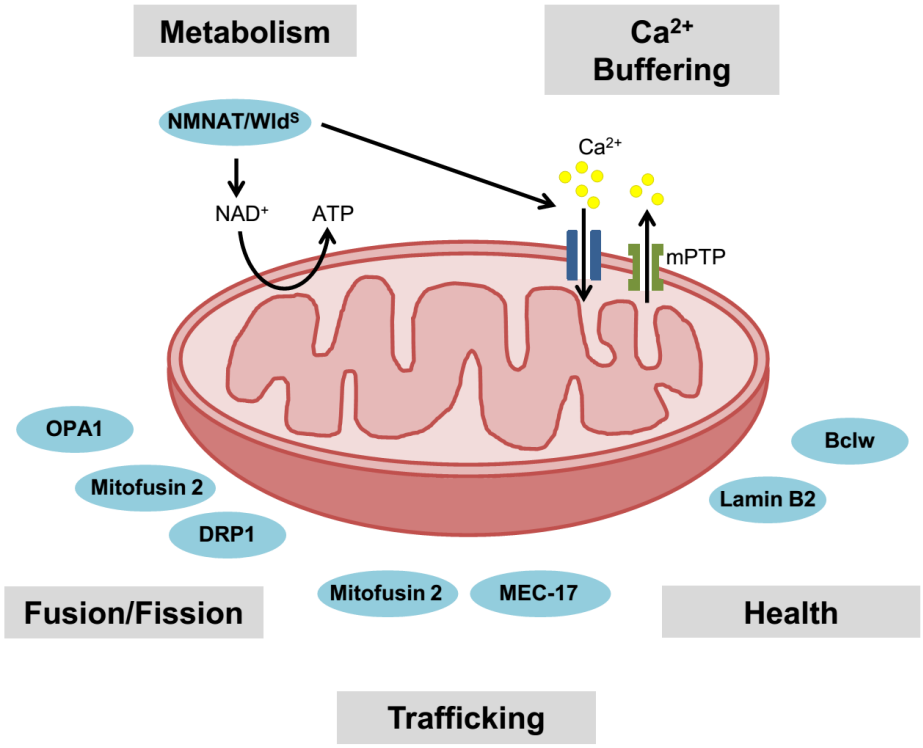


Figure 1.3. Mitochondria are central to axon health and homeostasis. The interdependent mitochondrial processes of metabolism and calcium buffering maintain axonal health and are impaired by injury and disease. Wld^S, a chimeric fusion of the enzyme NMNAT1 and the ubiquitination factor E4B, delays axonal degeneration induced by injury and disease. NMNAT/Wld^S produces NAD^+, which is used by mitochondria to produce ATP. NMNAT/Wld^S may promote axonal survival by preventing injury-induced loss of NAD^+ and ATP[71]. Innate mitochondrial calcium buffering maintains calcium homeostasis and is overwhelmed by axon injury, resulting in formation of the mPTP and increased axoplasmic calcium levels[29,79-81]. NMNAT/Wld^S increases the calcium buffering capacity of axonal mitochondria[77], possibly by increasing ATP synthesis[71]. Several processes are necessary for mitochondrial function and localization, including fusion and fission, mitochondrial trafficking, and maintenance of mitochondrial health by the proteins Lamin B2[13] and Bclw[1]. Mutations in the α tubulin acetyltransferase MEC-17 and the fusion and fission proteins OPA1, Mitofusin2, and DRP1 are implicated in axon degenerative diseases[82-84]. Abbreviations: ATP, adenosine triphosphate; Ca^{2+}, calcium ion; DRP1, GTPase dynamin-related protein 1; mPTP, mitochondrial permeability transition pore; NAD^+, nicotinamide adenine dinucleotide; NMNAT, nicotinamide mononucleotide adenylyltransferase; OPA1, optic atrophy 1; Wld^S, Wallerian degeneration slow protein.

Calcium homeostasis

Calcium, an important regulator of synaptic transmission, mitochondrial transport and function, and of diverse signaling cascades[65,85], also activates pro-degenerative axonal components[3]. Traumatic injury elevates intra-axonal calcium by inducing both extracellular calcium influx[86-88] and intracellular calcium efflux via endoplasmic reticulum IP_3 and ryanodine channels[12], and thereby promotes axon degeneration[29,79,80]. The endoplasmic reticulum and mitochondria normally control calcium levels by cytosolic calcium uptake[89,90]. However, excess intracellular calcium levels can cause mitochondrial overloading, formation of the mitochondrial permeability transition pore (mPTP), and subsequent release of calcium from intra-mitochondrial stores (Figure 1.3)[85]. Therefore, inhibiting mPTP activation or decreasing intracellular calcium prevents degeneration of injured axons[81].

NMNAT/Wld^S functions upstream of increased intra-axonal calcium, since inhibition of mPTP does not further delay degeneration of Wld^S axons[81] and exogenous calcium treatment abolishes Wld^S-mediated axon protection[91]. A recent study demonstrated that Wld^S suppresses injury-induced axonal calcium elevation, and that Wld^S mitochondria have increased calcium buffering capacity[77]. Given that mitochondrial ATP production, membrane potential, and calcium buffering are highly interrelated[85], it is possible that NMNAT/Wld^S increases mitochondrial calcium buffering as a result of increasing ATP synthesis[77]. However, detailed temporal and spatial examinations of mitochondrial ATP and calcium levels are needed to test this theory.

Mitochondrial quality control and localization

Given the importance of mitochondria in metabolism and calcium buffering, maintaining a population of healthy mitochondria is essential for axon viability. Numerous mechanisms promote mitochondrial health and remove dysfunctional mitochondria; impairment of these processes is central to many neurodegenerative diseases (Figure 1.3)[92].

Several proteins contribute to the health of axonal mitochondria. Bclw is necessary for axonal maintenance in mature animals, in addition to its function in development[1]. Mice lacking Bclw exhibit elongated axonal mitochondria together with adult-onset degeneration of peripheral sensory axons[1]. Axonal Lamin B2, a nuclear skeleton protein, is essential for axon viability in *Xenopus* retinal ganglion cells[13]. In the absence of Lamin B2, axonal mitochondria are elongated and exhibit impaired membrane potential[13]. As previously discussed, NMNAT/Wld^S promotes mitochondrial function, enhancing mitochondrial ATP synthesis[67], calcium buffering capacity[77], and motility[77,93]. Mitochondrial fusion and fission are opposing processes whose balance maintains mitochondrial morphology and function[94]. Mutations in components controlling fusion and fission are responsible for several axon degenerative diseases. Notably, mutations affecting the mitochondrial fusion protein optic atrophy 1 (OPA1) or the mitochondrial fission protein GTPase dynamin-related protein 1 (DRP1) result in atrophy of optic nerve axons[82,83]. In addition, mutation of the mitochondrial fusion protein Mitofusin 2 suppresses both mitochondrial fusion and transport and causes Charcot-Marie Tooth Disease[84].

Axonal transport both delivers new mitochondria and removes dysfunctional mitochondria, and so disruptions in mitochondrial transport contribute to multiple neurodegenerative diseases[92,95]. Mitochondria are actively transported to regions with high energetic demands, such as synapses, in order to maintain proper circuit connectivity[92,95]. Mitophagy, or mitochondrial autophagy, which removes damaged mitochondria from axons, also relies on axonal transport[92,96]. While axon injury causes cessation of mitochondrial movement, Wld^S prevents mitochondrial stalling after injury and increases basal mitochondrial motility in uninjured axons[77]. The ability of Wld^S to enhance mitochondrial motility correlates with improved calcium buffering capacity by mitochondria[77]. Similarly, cytoplasmically-targeted NMNAT1 partially protects against chemotoxic injury-induced deficits in mitochondrial transport[97]. Mitochondrial motility is essential for Wld^S or NMNAT1 to protect axons after injury, highlighting the essential role of mitochondrial transport in axon viability[77,97]. Mitochondrial

transport is closely tied to cytoskeletal balance, as demonstrated by a recent study in *C. elegans* showing that loss of the α-tubulin acetyltransferase MEC-17 causes microtubule destabilization, reduced axonal mitochondria numbers, and spontaneous axon degeneration[98].

Cytoskeletal stability

The axonal cytoskeleton consists of microtubules, actin, and neurofilaments. Microtubules, the main highway for long-range retrograde and anterograde transport, are in a constant state of dynamic instability, with continuous depolymerization and repolymerization[99]. Pharmacological hyperstabilization[100] or destabilization[101] of axonal microtubules causes degeneration. Furthermore, injured[102], MEC-17 mutant[98], or neurotrophin-deprived[42] axons, which typically undergo microtubule destabilization, can be protected by the microtubule stabilizing agent paclitaxel. Thus, paclitaxel, which is toxic to healthy axons[100], may act to restore cytoskeletal balance in degenerating axons. These results suggest that carefully balanced microtubule stability is critical for axon health (Figure 1.4).

Cytoskeletal stability is regulated by several protective or degenerative molecules. For example, superior cervical ganglion 10 (SCG10) is a microtubule destabilizing protein that promotes axonal health. SCG10 is rapidly degraded following axon injury, and maintaining SCG10 levels preserves mitochondrial motility and delays axon degeneration[20]. While SCG10 knockdown does not cause spontaneous axon degeneration, it does accelerate injury-induced degeneration[20]. Conversely, inhibition of microtubule destabilizing proteins can protect axons with pathological microtubule instability. *Progressive motor neuronopathy* (*pmn*) mice have a mutation in the tubulin chaperone *Tbce* gene that impairs microtubule polymerization and causes motor axon degeneration[103]. The transcription factor STAT3 locally inhibits axonal stathmin, a microtubule destabilizing protein in the same family as SCG10, and rescues axonal pathology in cultured *pmn* motor neurons[104]. Microtubule stabilizing proteins have also been shown to

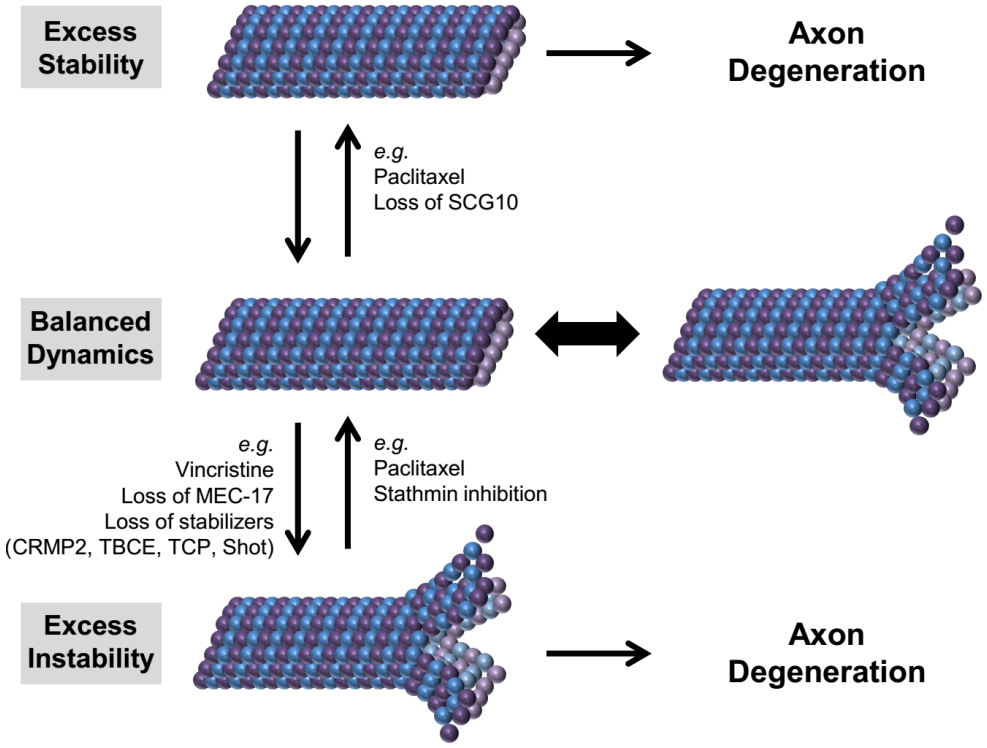


Figure 1.4. Balanced cytoskeletal dynamics are essential for axon maintenance. Microtubules are in a constant state of depolymerization and polymerization. Genetic and pharmacological insults that either stabilize microtubules (such as the chemotherapeutic paclitaxel or loss of the microtubule destabilizing protein SCG10) or destabilize microtubules (such as the chemotherapeutic vincristine, loss of MEC-17, or loss of microtubule stabilizing proteins CRMP2, TBCE, TCP, or Shot) cause axon degeneration[4,20,100,101,103,105]. Axons with pathologically destabilized microtubules can be protected by treatment with the microtubule stabilizing agent paclitaxel[42,98,102] or inhibition of the microtubule destabilizing protein stathmin[104]. Abbreviations: CRMP2, collapsin response mediator protein 2; SCG10, superior cervical ganglion 10; TBCE, tubulin-specific chaperone E; TCP, T-complex protein; Shot, short stop.

be necessary for axon health. The collapsin response mediator protein 2 (CRMP2) promotes microtubule stability. Axon injury causes CRMP2 degradation, and maintaining CRMP2 levels delays degeneration[105].

Accumulating evidence suggests that balanced cytoskeletal stability regulates the pro-degeneration molecule duel leucine zipper kinase (DLK) and its *Drosophila* ortholog Wallenda. Genetic loss of the cytoskeletal stabilizing protein spectraplakin short stop (Shot) or the tubulin chaperone TCP1 leads to cytoskeletal instability and DLK activation[4]. DLK/Wallenda is activated by SARM1[18] and mediates a degenerative response by activating the c-Jun N-terminal kinase (JNK)[5,18]. Notably, DLK function is context dependent and can promote both axon regeneration[6-9] and presynaptic bouton development[106].

Maintaining protein levels

As in developmental axon preservation, axonal maintenance requires supply, localization, and turnover of protein. Axonal transport and local translation provide maintenance factors to the axon. Conversely, UPS-mediated protein degradation promotes axon degeneration by reducing levels of several axonal maintenance factors[107-110]. While local translation is required for mitochondrial function and axon viability[111], only the protective factor Lamin B2 is known to be translated locally in mature axons[13]. In contrast, the maintenance factors NMNAT2 and SCG10 are rapidly degraded in axons by the UPS and must be constantly renewed by anterograde transport from the cell body[68,70].

1.5 Pathological axon degeneration:

Axons damaged by injury or disease must be actively eliminated. In some cases, removal of damaged axons enables axon regrowth and maintains neural circuitry, such as following lesions of peripheral axons. However, when axon trauma is more widespread or axons are not capable of regenerating, pathological axon degeneration compromises neural circuit functionality. Pathological

axon degeneration results from a variety of axon insults, including nerve transection and chemotoxic trauma, and can also occur in neurodegenerative diseases.

Nerve transection

Following nerve transection, axons undergo Wallerian degeneration (Figure 1.2D). During an initial latency stage, the injured axon remains intact and electrically functional; this is followed by a rapid degenerative phase involving swelling, cytoskeletal degradation, and axon fragmentation. Because the precise timing and location of injury can be controlled, nerve transection is commonly used as a simple model of axon degenerative diseases. In Wallerian degeneration, transport is interrupted by the physical severing of the axon, resulting in a loss of labile pro-survival factors such as NMNAT2[68,70,109,110] and SCG10[20]. Axonal ATP levels rapidly decline[71], axonal mitochondria stall[77], and the innate calcium buffering capabilities of the axon fail[29,79,80]. Future studies will be needed to define the exact temporal and causal relationships among these events.

Chemotherapy-induced peripheral neuropathy

In addition to mechanical trauma, axons can also be injured by exposure to toxic chemical substances. One of the most frequent causes of chemotoxic axon injury is chemotherapy-induced peripheral neuropathy (CIPN), a common neurological side-effect of cancer chemotherapy treatment. CIPN presents as pain, tingling, or numbness in the hands and feet, resulting from degeneration of peripheral sensory or motor axons[112-115]. CIPN is caused by multiple types of cytotoxic chemotherapeutic agents, including vinca alkaloids, taxanes, platinum drugs, and proteasome inhibitors. These agents mediate anti-tumor effects by distinct molecular mechanisms. For example, vinca alkaloids and taxanes alter microtubule dynamics and thereby interfere with cancer cell mitosis. While vinca alkaloids such as vicnristine destabilize microtubules, taxanes such as paclitaxel stabilize them, and both agents cause

peripheral axon degeneration (Figure 1.4). Conversely, chemotherapeutics such as bortezomib cause cancer cell apoptosis by inhibiting the proteasome. Regardless of the type of chemotherapeutic, these agents preferentially cause degeneration of peripheral axons innervating the distal extremities. These axons are thought to be most affected because they are the longest axons in the body, although it is not known why longer axons would be more vulnerable to injury. The molecular mechanisms of CIPN are not well understood, and the exact pathology differs by type of chemotherapeutic agent. However, CIPN typically involves axonal mitochondrial dysfunction, impaired axonal calcium signaling, and axon loss in a distal to proximal pattern, described as a dying-back degeneration[112-115] (Figure 1.2E).

Axon degenerative diseases

Many neurodegenerative also exhibit axon loss, with a distal to proximal gradient of swelling and fragmenting that resembles Wallerian degeneration, described as a dying-back axonopathy (Figure 1.2E). Impaired axonal mitochondrial integrity, function, and transport are common pathologies of neurodegenerative diseases, including Alzheimer's Disease, Huntington's Disease, Parkinson's Disease, Amyotrophic Lateral Sclerosis, and others[92].

1.6 Concluding remarks and future directions:

The establishment and functionality of neural networks requires precise control of axon survival and elimination in development and throughout life. Recent studies describe core mechanisms that preserve connections between cells in a circuit and eliminate surplus or damaged connections. The mechanisms that govern axon survival and elimination have similarities to and differences from cell soma viability and death. In particular, interdependent mitochondrial and cytoskeletal processes are central to axon survival, and impairment of these processes by injury or disease leads to pathological axon degeneration.

The field of axonal survival and death has been very active recently, including studies across multiple organisms and multiple types of neurons. These studies have benefited from improved *in vivo* methodologies as well as improved spatially compartmented culture systems that will now enable future studies to address the critical questions that remain. A major question is the degree of overlap between developmental and mature axon survival pathways as well as their opposing developmental and pathological axon destruction mechanisms. In identifying key players in these processes, recent studies have already defined both commonalities and differences. Another major question is whether pro-degeneration factors or other pro-survival factors are translated locally in the axon. Despite the abundance of mRNAs in both developing and mature axons[48], few pro-survival factors are known to be locally synthesized. Investigations into these questions and others will elucidate mechanisms that maintain healthy axons within neural networks.

1.7 Specific aims and overview of dissertation:

This dissertation describes our investigations into the molecular mechanisms of peripheral sensory neuron axon health and survival. Using *in vitro* and *in vivo* models, we explore protective mechanisms both during development and in response to pathological axon injury by the chemotherapeutic agent paclitaxel. In Chapter 2, I detail work demonstrating that Bclw, a Bcl2 family protein required for developmental axon survival, is involved in promoting axon viability in response to chemotoxic injury, and I describe a unique mechanism for this action. In Chapter 3, I test the hypothesis that target-derived neurotrophic support uniquely upregulates translation machinery components within the cell. Our studies defining axon viability mechanisms that may be compromised during injury could inform future therapeutic approaches to allow axons to resist degeneration and thus prevent neurodegenerative syndromes such as chemotherapy-induced peripheral neuropathy.

Chapter 2

Bclw plays a specialized role in preventing chemotherapy-induced axon degeneration

The body of work in this Chapter forms the manuscript: Pease, S.E.*, Pazyra-Murphy, M.F.*, Wachter F., Barclay, L.A., Walensky, L.D., and Segal, R.A. Suppression of IP_3R1 degeneration by the Bclw BH4 domain prevents chemo-induced neuropathy. 2016. (manuscript in preparation)

The work described in this Chapter constitutes a collaborative project between M.F.P.-M. and me. I participated in the design, performance, and/or analysis of all experiments, and wrote the text. I performed and analyzed TMRE intensity assays, calpain assays, calcium imaging, and BH4 pulldowns. Degeneration assays, neurotrophin deprivation, and Bcl2 family alteration experiments were performed and analyzed by M.F.P.-M. *in vivo* experiments were performed and analyzed collaboratively by M.F.P.-M and me. F.W. performed and analyzed the lipsosomal release assay and contributed BH4 reagents and experimental advice. L.A.B. contributed BH4 reagents and experimental advice. All research and writing was performed under the guidance of R.A.S. and L.D.W.

2.1 Introduction:

Axons that span long distances enable rapid communication within a neural circuit, but they are particularly vulnerable to degeneration. Axonal degeneration is a key feature of diverse neuropathological disorders and the developmental process of axonal pruning. Molecular cascades governing pathological degeneration exhibit both commonalities with and differences from developmental axon degenerative cascades[29,116]. Bcl2 family members have been implicated in developmental axon pruning[1,2,10,14,23,30,40,41], but it is not yet known if pathological axon degeneration is regulated by these components. Pathological axon degeneration often involves a SARM-MAP kinase pathway and aberrant calcium signaling[12,18,29]. Both pathological and developmental degeneration involve changes in mitochondrial function and activation of calpain proteases[1,3,12,92,117,118]. In addition to functioning as critical upstream regulators of the apoptotic pathway, Bcl2 family members also regulate mitochondrial morphology and calcium homeostasis, making them potential regulators of both degenerative cascades[119]. Therefore, identification of Bcl2 family members involved in axon degeneration could provide a molecular handle to understand and prevent diverse degenerative cascades.

In oncology, cytotoxic chemotherapies cause axon injury and neurological impairment, resulting in chemotherapy-induced peripheral neuropathy (CIPN)[120]. Patients with CIPN experience pain, tingling, numbness, and/or impaired motor function, due to degeneration of long peripheral sensory or motor neuron axons[112-115]. Chemotherapeutic drugs that cause CIPN include platinum drugs, vinca alkaloids, and taxanes such as paclitaxel. Paclitaxel, a microtubule stabilizing agent that has been used to treat more than a million patients with breast, ovarian, lung or other cancers, causes a primarily sensory neuropathy and affects up to 30% of patients[115,120,121]. It has been suggested that paclitaxel elicits a degenerative cascade similar to that observed after mechanical injury[115,122,123]. However, the molecular mechanism for paclitaxel-induced degeneration is not understood, and there are currently no

treatments available for this common disorder. As CIPN often necessitates reducing the dose of chemotherapeutic drugs, it both limits the ability to treat many cancers and also causes severe disability for cancer survivors.

Here we establish *in vitro* and *in vivo* systems to analyze the pathological effects of paclitaxel on dorsal root ganglia (DRG) sensory neurons. Our data indicate that paclitaxel acts directly on axons of sensory neurons to initiate axon degeneration and alters inositol 1,4,5-trisphosphate receptor (IP_3R) calcium release properties. We show that paclitaxel treatment also selectively reduces axonal expression of Bclw, a Bcl2 family member that binds IP_3R1 and puts the brakes on this axonal degeneration cascade. Together these findings identify a mechanism for CIPN and demonstrate that the underlying axonal degenerative process partially overlaps with critical cascades triggered by developmental pruning or traumatic injury. Importantly, these studies suggest that Bclw-mimetics may provide a clinically useful preventative therapy for CIPN and other disorders of axonal degeneration.

2.2 Results:

Paclitaxel initiates an IP_3R1-dependent axon degenerative cascade

It is not yet known whether paclitaxel acts directly on axons of sensory neurons to cause degeneration, or whether degeneration represents an indirect effect of paclitaxel toxicity on other components in the sensory circuitry[100,124-129]. To determine the site of action, we introduced paclitaxel (30 nM) into the media surrounding either cell body or distal axon compartments of E15 DRG sensory neurons in compartmented cultures (Figure 2.1A-B), and analyzed paclitaxel-induced axonal fragmentation, a direct readout of degeneration[75]. We find that paclitaxel added to axons increases axon degeneration, while paclitaxel added to cell bodies has no effect (Figure 2.2A-B). Notably, paclitaxel treatment of either subcellular compartment did not induce cell body apoptosis as assessed

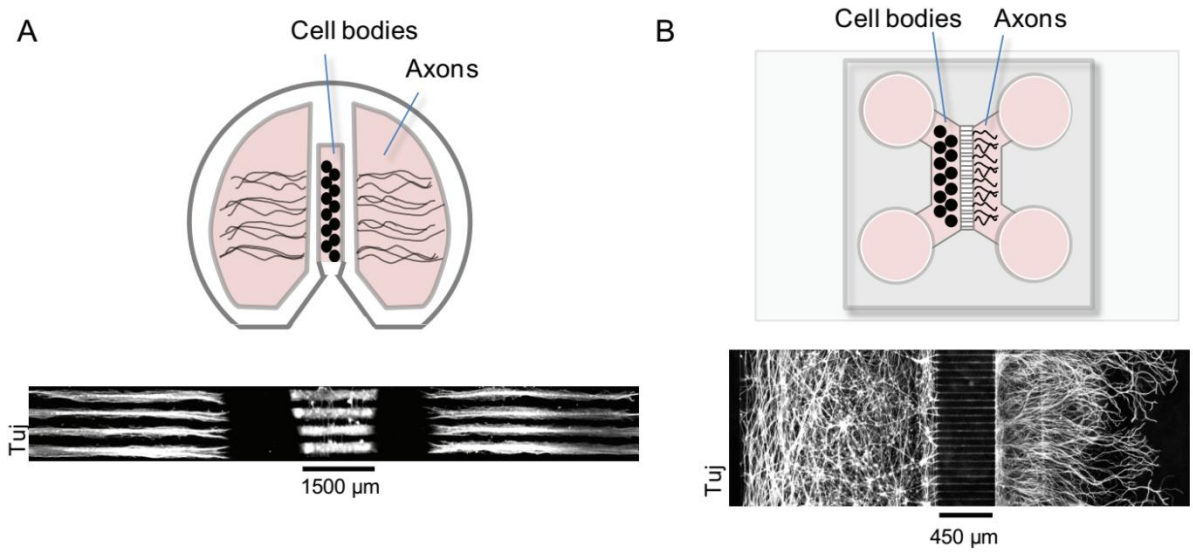


Figure 2.1. Compartmented culture systems for separation of DRG sensory neuron cell bodies and axons. **(A)** Compartmented (Campenot) cultures used for biochemical and degeneration assays; E15 DRG neurons immunostained for Tuj1; scale bar = 1500 µm. **(B)** Microfluidic devices used for high resolution live and fixed imaging, plated on a glass coverslip; E15 DRG neurons immunostained for Tuj1; scale bar = 450 µm.

by nuclear condensation (Figure 2.2C-D). These results indicate that paclitaxel acts locally on sensory neuron axons to induce fragmentation and degeneration.

An early, initiating stage of injury-induced degeneration is calcium deregulation, which is followed by mitochondrial dysfunction and activation of calpain proteases[12,29,81]. Therefore we examined these known molecular indicators of pathological degeneration in response to paclitaxel applied to axons. Paclitaxel treatment reduced axonal mitochondrial membrane potential as assessed using the voltage-sensitive dye TMRE (Figure 2.3A), and increased calpain activity in a dose-dependent manner (Figure 2.3B). Furthermore, the calpain inhibitor III (20 µM to axons) prevents paclitaxel-induced degeneration, indicating that local activation of the calcium-dependent protease calpain is required for axon fragmentation in this system (Figure 2.3C).

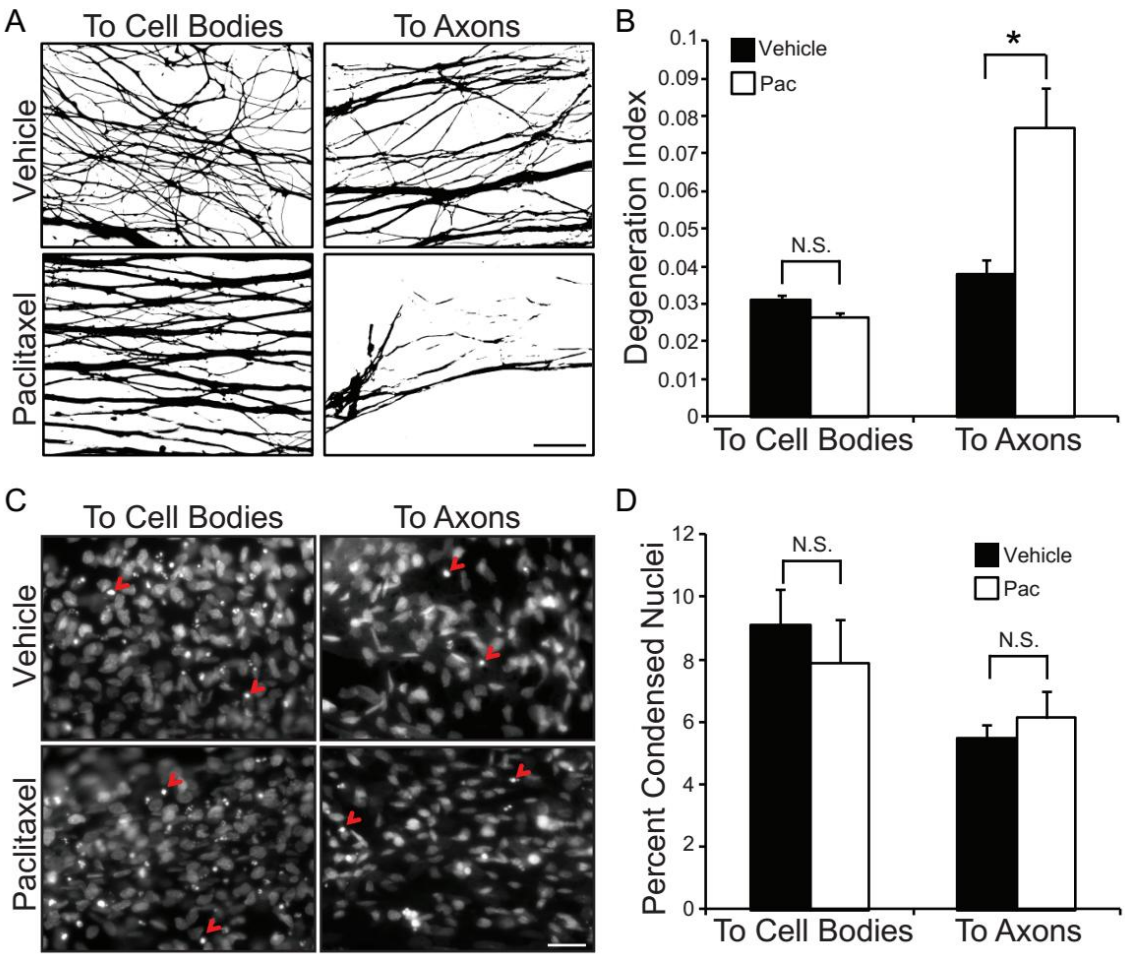


Figure 2.2. Paclitaxel acts locally to induce axon degeneration without cell body apoptosis. **(A)** Binarized Tuj1 immunostaining of axons of E15 DRG neurons grown in compartmented cultures treated with 30 nM paclitaxel or DMSO vehicle control for 24 hours to cell body (left) or distal axon (right) compartments; scale bar = 40 µm. **(B)** Quantification of axonal degeneration: ratio of area of fragmented axons to total axon area (degeneration index); *p<0.05 by Student's *t*-test; n = 3; data represent mean + SEM. **(C)** DAPI images of cell body compartments from (A); scale bar = 10 µm, red arrowheads indicate apoptotic nuclei. **(D)** Quantification of apoptotic/total nuclei after addition of paclitaxel or vehicle to cell bodies or distal axons; *p<0.05 by Student's *t*-test; n = 3; data represent mean + SEM.

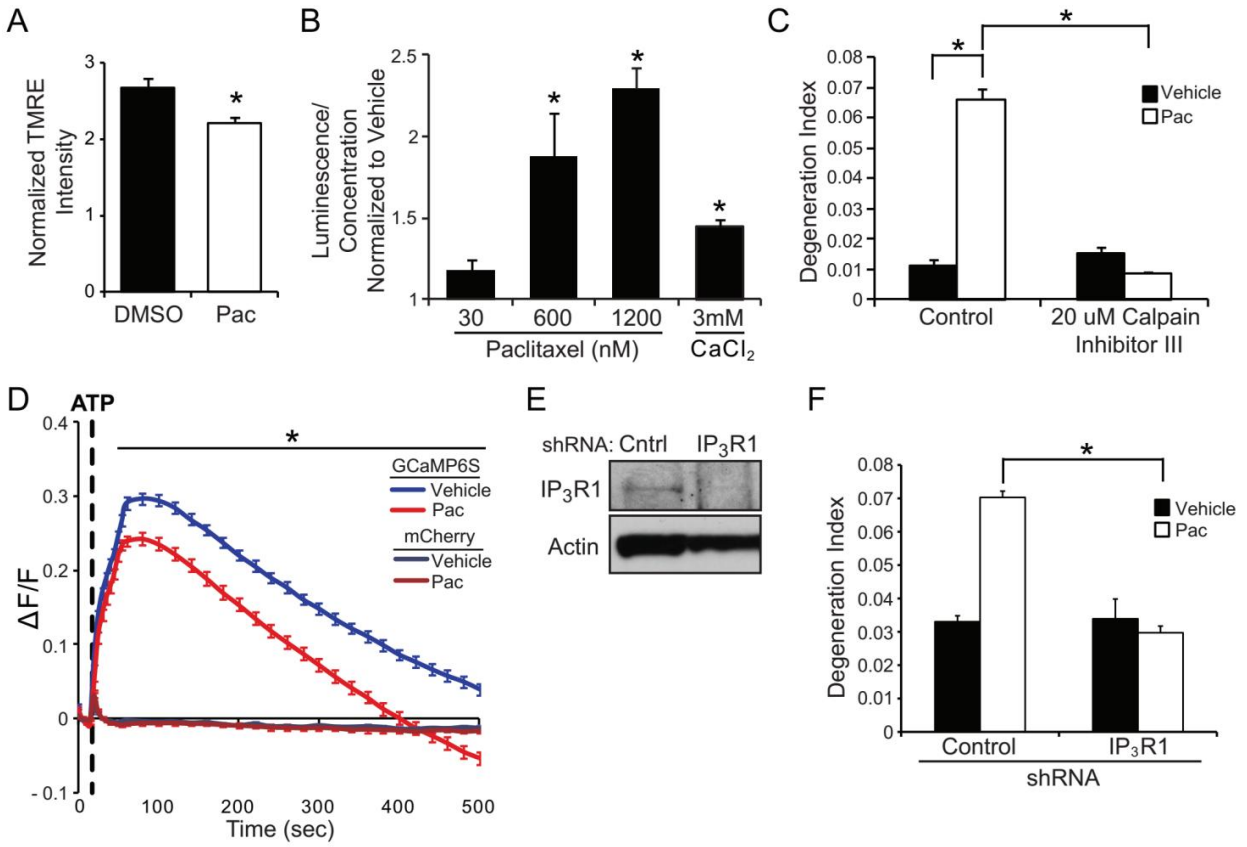


Figure 2.3. Paclitaxel activates a conserved pathological degenerative cascade requiring IP_3R1.
(A) Fluorescence intensity (Fm/Fc) of the voltage-sensitive dye TMRE in axons of DRG neurons in microfluidic cultures; 60 nM paclitaxel (Pac) or vehicle control added to distal axon compartment for 24 hours; *p<0.001 by Student's *t*-test; n = 80 mitochondria across 3 experiments; data represent mean + SEM. **(B)** Luminescence generated by calpain activity from DRG neurons treated for 48 hours with 30 nM, 600 nM, or 1.2 µM paclitaxel or for 24 hours with 3 mM calcium chloride. Data normalized to vehicle control; *p<0.05 by *z*-test; n = 3; data represent mean + SEM. **(C)** Degeneration index of axons treated with paclitaxel or vehicle control in the absence or presence of 20 µM calpain inhibitor III to axons; *p<0.05 by one-way ANOVA with Bonferroni correction; n = 3; data represent mean + SEM. **(D)** Change in fluorescence intensity (ΔF/F) of GCaMP6s or mCherry signal from DRG neurons stimulated with 200 µM ATP (zero extracellular calcium media + 10 mM EGTA); 600 nM paclitaxel or vehicle control added to culture for 24 hours; *p<0.05 by two-way ANOVA with Bonferroni correction; n = 119 (Paclitaxel) or 156 (vehicle) cell bodies from 3 cultures across 3 experiments; data represent mean ± SEM. **(E)** Western blot showing IP_3R1 levels of DRG sensory neurons after infection with lentivirus expressing IP_3R1-targeting shRNA or RFP-targeting control shRNA (Cntrl), with actin loading control. **(F)** Degeneration index after 24-hours of paclitaxel or vehicle added to axons following lentiviral infection with shRNA targeting IP_3R1 or control shRNA; *p<0.05 by one-way ANOVA with Bonferroni correction; n = 3; data represent mean + SEM.

Calcium deregulation via the type 1 inositol 1,4,5-trisphosphate receptor (IP_3R1) is implicated in injury-induced axon degeneration[12]. To determine whether paclitaxel alters IP_3R functionality, DRG neurons expressing the calcium indicator GCaMP6s were stimulated with 200 μM ATP to trigger IP_3 production and calcium release from the endoplasmic reticulum. Paclitaxel treatment significantly reduced ATP-evoked IP_3R calcium release (Figure 2.3D), consistent with previous reports[130]. Importantly, knockdown of IP_3R1 (Figure 2.3E) prevents paclitaxel-induced degeneration (Figure 2.3F). Together these results indicate that paclitaxel treatment activates a conserved pathological axon degenerative cascade dependent on IP_3R1.

Bclw prevents paclitaxel-induced degeneration

IP_3R function can be regulated by Bcl2 family proteins[131-135]. To determine whether anti-apoptotic Bcl2 family members prevent paclitaxel-induced degeneration, we used protein transfection in compartmented cultures to introduce His-tagged recombinant Bclw (Bcl2l2), Bcl2, or $Bclx_L$ (Bcl2l1) protein selectively into axons (Figure 2.4A)[2]. Transfected axonal Bclw completely prevented paclitaxel-induced axon degeneration (Figure 2.4B-C). While both Bclw and $Bclx_L$ can regulate developmental neurotrophin-dependent axon survival[2,14,44], we find that neither $Bclx_L$ nor Bcl2 prevents paclitaxel-induced degeneration (Figure 2.4B-C). Furthermore, selective introduction of Bclw, Bcl2, or $Bclx_L$ protein into cell bodies all failed to inhibit paclitaxel-induced degeneration (Figure 2.4D). Together these data identify Bclw as a specialized Bcl2 family member that acts locally in axons to prevent degeneration.

The BH4 domain of Bclw is sufficient to prevent degeneration

Bclw, Bcl2, and $Bclx_L$ exhibit highly conserved structures with four characteristic Bcl2 homology (BH) domains. In particular, BH4 domains bind and regulate several critical targets, including

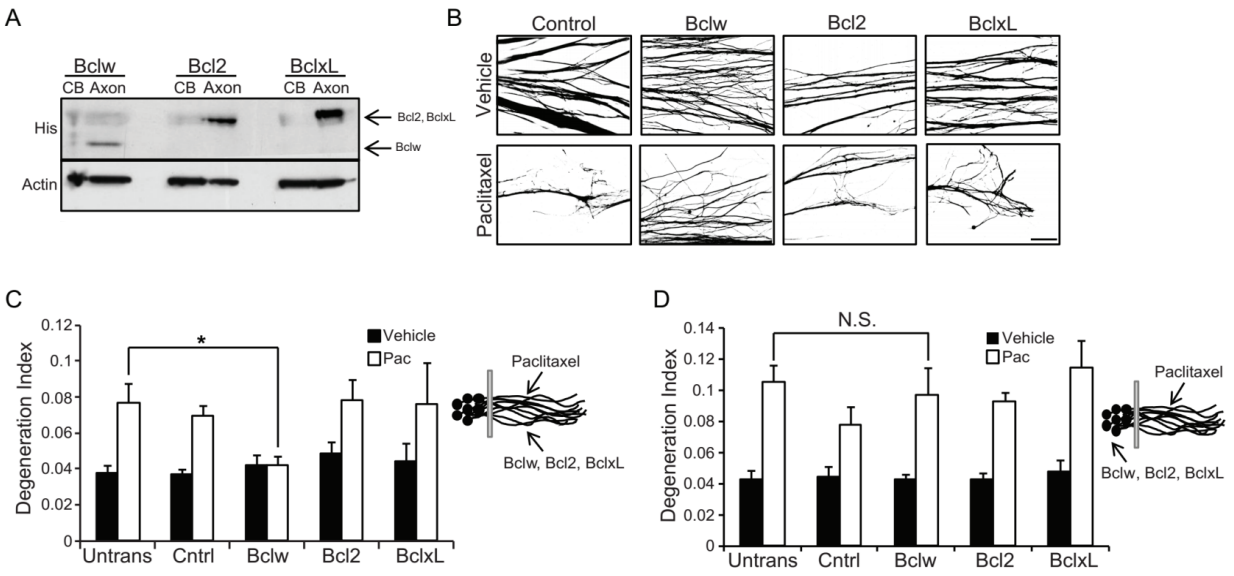


Figure 2.4. Axonal Bclw prevents paclitaxel-induced degeneration. (A) Western blot showing cell body and axon lysate from compartmented cultures; axons transfected with full-length His-tagged Bclw, Bcl2, or $Bclx_L$ proteins. **(B)** Binarized Tuj1 immunostaining of axons treated with paclitaxel or vehicle after protein transfection with Bclw, Bcl2, $Bclx_L$ or β-galactosidase control protein into axons; scale bar = 40 µm. **(C)** Degeneration index of (B) and untransfected (Untrans) control; *p<0.05 by one-way ANOVA with Bonferroni correction; n = 4; data represent mean + SEM. **(D)** Degeneration index of axons treated with paclitaxel or vehicle after protein transfection of Bclw, Bcl2, $Bclx_L$ or β-galactosidase control protein into cell bodies; n = 4; data represent mean + SEM.

IP_3Rs[134,136-142]. We generated a stabilized alpha-helix of Bcl2 domain (SAHB) modeled after the BH4 domain (aa 12-31) of Bclw (called Bclw BH4 $SAHB_A$). SAHBs are alpha-helical hydrocarbon stapled peptides that are bioactive, protease-resistant, and cell-permeable[138,143]. Similar to the previously generated Bcl2 BH4 $SAHB_A$[138], Bclw BH4 $SAHB_A$ inhibited BIM BH3 SAHB-induced, Bax-mediated membrane poration of ANTS/DPX encapsulated liposomes in a dose-dependent manner (Figure 2.5A). We transfected FITC-tagged BH4 peptides of Bclw, Bcl2, or $Bclx_L$ or a vehicle control into axons of compartmented cultures and then treated the axons with paclitaxel for 24 hours (Figure 2.5B). As observed with full-length Bclw, Bclw BH4 $SAHB_A$ prevented paclitaxel-induced axon degeneration. In contrast, Bcl2 BH4 $SAHB_A$ and $Bclx_L$ BH4 $SAHB_A$ did not do so (Figure 2.5C). Importantly, equivalent intracellular bioactivity of these peptides in sensory neurons was confirmed using a neurotrophin-deprivation assay; transfection of Bclw BH4, Bcl2 BH4, or $Bclx_L$ BH4 SAHB peptides into cell bodies equally prevented apoptosis triggered by 24 hours of neurotrophin deprivation (Figure 2.5D). Together these results indicate that the BH4 domain of Bclw is sufficient to prevent axon degeneration, a function that is not conserved by the BH4 domains of Bcl2 and $Bclx_L$.

Bclw regulates IP_3R1 to prevent axon degeneration

The surprisingly specialized role of Bclw in preventing paclitaxel-induced degeneration suggests Bclw may interact with different molecular targets in axons than do Bcl2 and $Bclx_L$. As the BH4 domain of Bclw is sufficient to prevent paclitaxel-induced degeneration, we examined co-precipitation of the biotinylated BH4 SAHB peptides with IP_3R1. Surprisingly, in axon lysates, IP_3R1 co-precipitated preferentially with Bclw BH4 $SAHB_A$ (Figure 2.6A-B), while in cell body lysates, IP_3R1 co-precipitated similarly with all three SAHBs (Figure 2.6C-D). Importantly, biotinylated SAHBs all equally co-precipitated the pro-apoptotic Bcl2 family member Bax from both cell bodies and axons, and do not co-precipitate a negative control protein (tyrosyl-tRNA synthetase, YARS; Figure 2.6A-D).

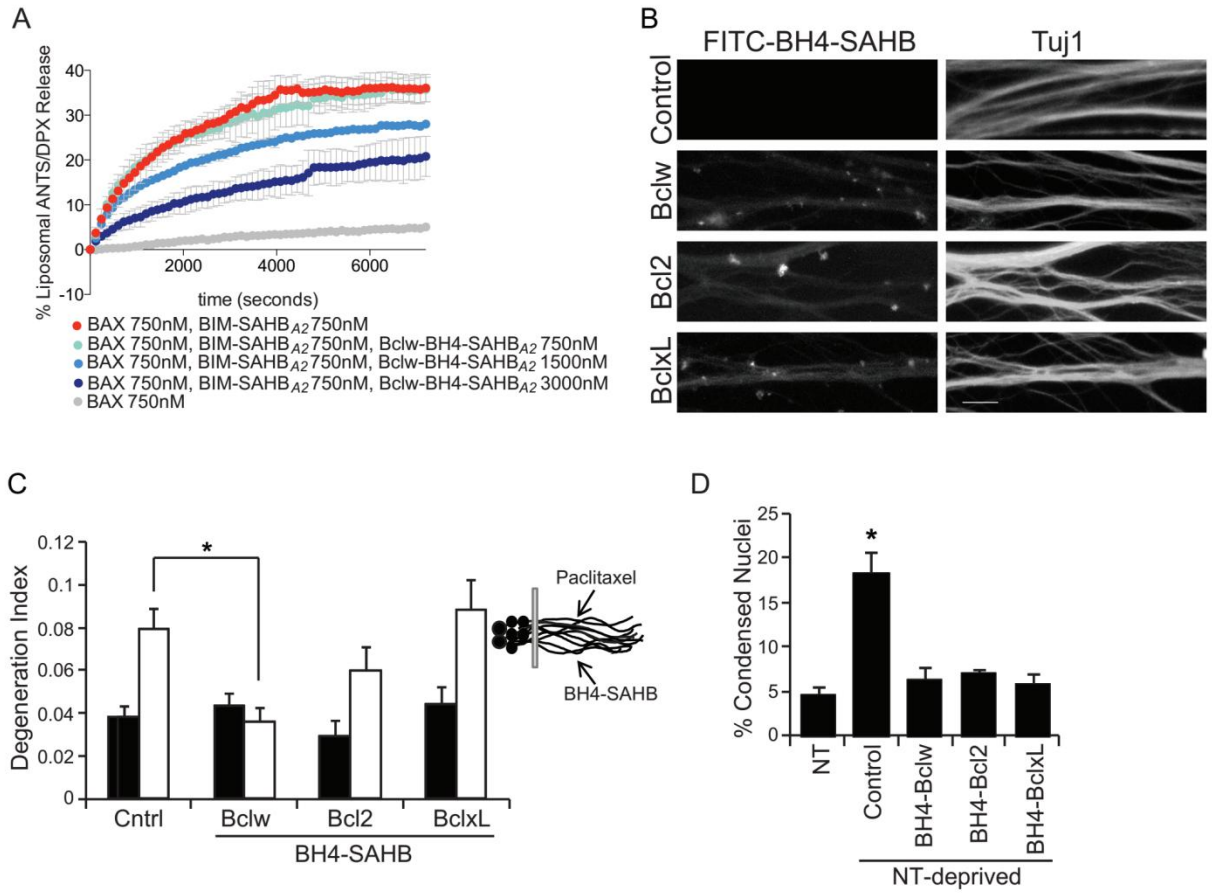


Figure 2.5. Bclw prevents paclitaxel-induced degeneration through its BH4 domain. (A) Percent release of ANTS/DPX encapsulated liposomes in the presence of Bax, Bim SAHB$_{A2}$, and/or Bclw BH4 SAHB$_{A}$ as indicated; n = 3; data represent mean ± SD. **(B)** Axons from compartmented cultures after protein transfection with FITC-BH4-SAHB peptides of Bclw, Bcl2, or Bclx$_{L}$ showing FITC signal (left) and Tuj1 signal (right); scale bar = 20 µm. **(C)** Degeneration index of axons treated with paclitaxel or vehicle after protein transfection of FITC-BH4-SAHB-Bclw, Bcl2, or Bclx$_{L}$ into axons; *p<0.05 by one-way ANOVA with Bonferroni correction; n = 4; data represent mean + SEM. **(D)** Apoptotic/total DAPI-stained nuclei of neurons maintained in neurotrophins (NT) or deprived of neurotrophins, cell bodies were transfected with FITC-BH4 SAHB peptides of Bclw, Bcl2, or Bclx$_{L}$; *p<0.05 by one-way ANOVA with Bonferroni correction; n = 4; data represent mean + SEM.

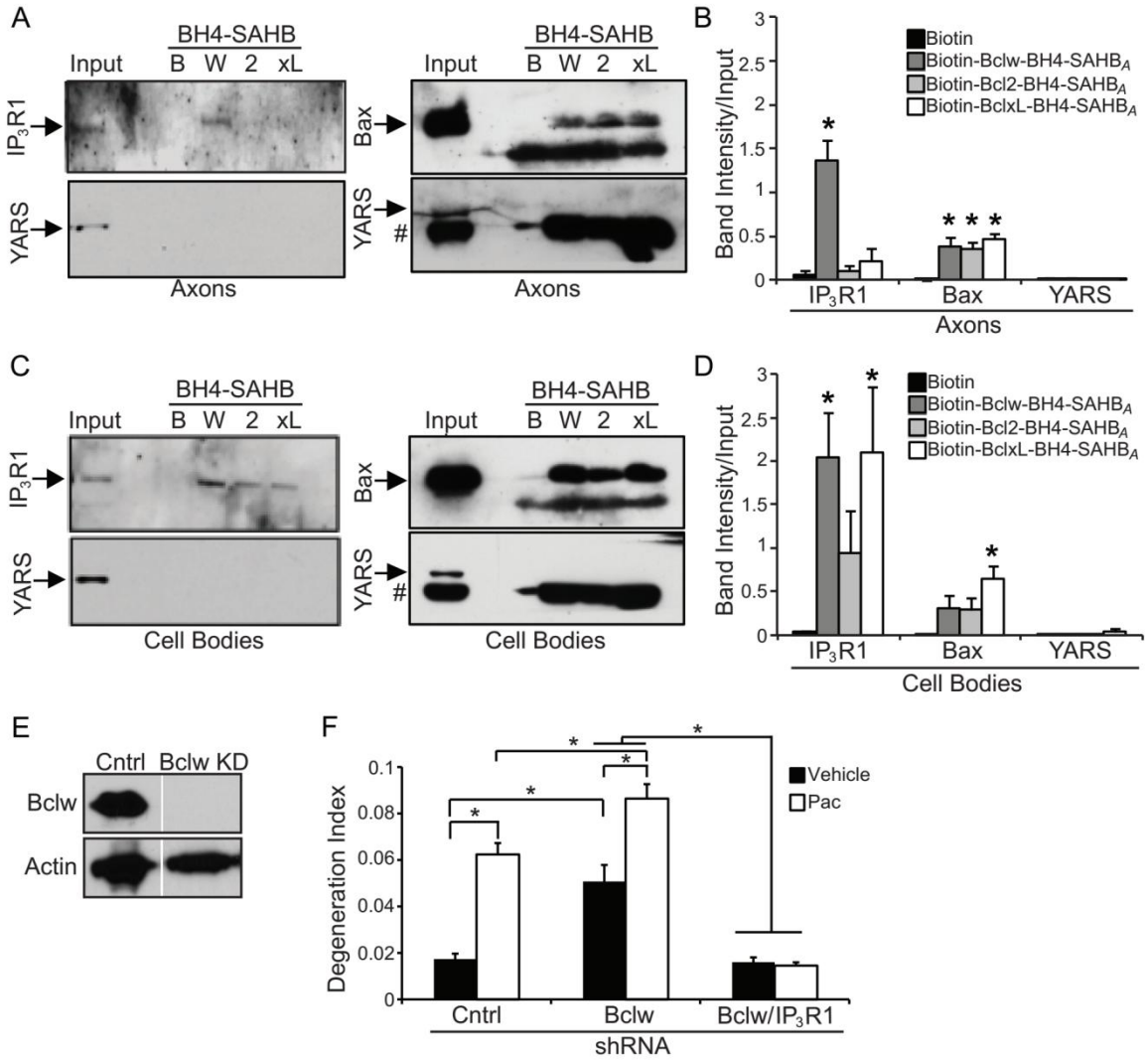


Figure 2.6. Bclw binds IP_3R1 to prevent axon degeneration. (A-D) Western blot of NeutrAvidin pulldowns from axon **(A)** or cell body **(C)** lysate incubated with biotinylated Bclw BH4 $SAHB_A$ (W), Bcl2 BH4 $SAHB_A$ (2), or $Bclx_L$ BH4 $SAHB_A$ (x_L) or biotin control (B). Western blots probed for IP_3R1, Bax, or YARS protein. # shows nonspecific band. **(B)** Quantified band intensity relative to input band from (A). **(D)** Quantified band intensity relative to input band from (C); *p<0.05 relative to biotin control by one-way ANOVA with Dunnett's multiple comparison test; n = 4-6; data represent mean + SEM. **(E)** Western blot showing Bclw levels of DRG sensory neurons after lentiviral knockdown with an shRNA targeting Bclw or RFP-targeting control shRNA (Cntrl), with actin loading control; samples run on same gel. **(F)** Degeneration index after 24-hour addition of paclitaxel or vehicle to axons following lentiviral infection with control shRNA or shRNAs targeting Bclw and/or IP_3R1; *p<0.05 by one-way ANOVA with Bonferroni correction; n = 3-6; data represent mean + SEM.

To determine if Bclw and IP_3R1 function in the same pathway to regulate axon degeneration, neurons were infected with shRNAs targeting Bclw and/or IP_3R1 (Figure 2.3E and Figure 2.6E). Acute knockdown of Bclw increases spontaneous axonal degeneration, and paclitaxel treatment further augments degeneration (Figure 2.6F). Axonal degeneration is greater in paclitaxel treated, Bclw-deficient neurons than in paclitaxel-treated Bclw-expressing neurons or in untreated, Bclw-deficient neurons. Thus endogenous Bclw usually acts as a brake on the degenerative cascade activated by paclitaxel (Figure 2.6F). We find that IP_3R1 knockdown completely prevents the increased axon degeneration caused by Bclw knockdown, both in the absence and presence of paclitaxel (Figure 2.6F). These data indicate that Bclw functions upstream of IP_3R1 to prevent a degenerative cascade.

Paclitaxel specifically reduces axonal Bclw levels

The protective role of Bclw suggests paclitaxel could cause degeneration by disrupting normal Bclw protection of axons. We therefore examined expression of the anti-apoptotic components Bclw, Bcl2, or $Bclx_L$ after paclitaxel treatment. Strikingly, paclitaxel treatment reduced Bclw mRNA and protein levels selectively in axons (Figure 2.7A-C). In contrast, paclitaxel treatment did not alter Bcl2 or $Bclx_L$ mRNA or protein in axons or in cell bodies (Figure 2.7D-I). Together these data showing paclitaxel reduces axonal Bclw levels with no effect on closely related Bcl2 family members indicate that paclitaxel specifically decreases Bclw to induce axon degeneration.

Loss of Bclw exacerbates paclitaxel-induced neuropathy *in vivo*

If paclitaxel causes axon degeneration in part by reducing Bclw levels, then mice lacking Bclw should be more susceptible to paclitaxel toxicity. To examine the role of Bclw in paclitaxel-induced

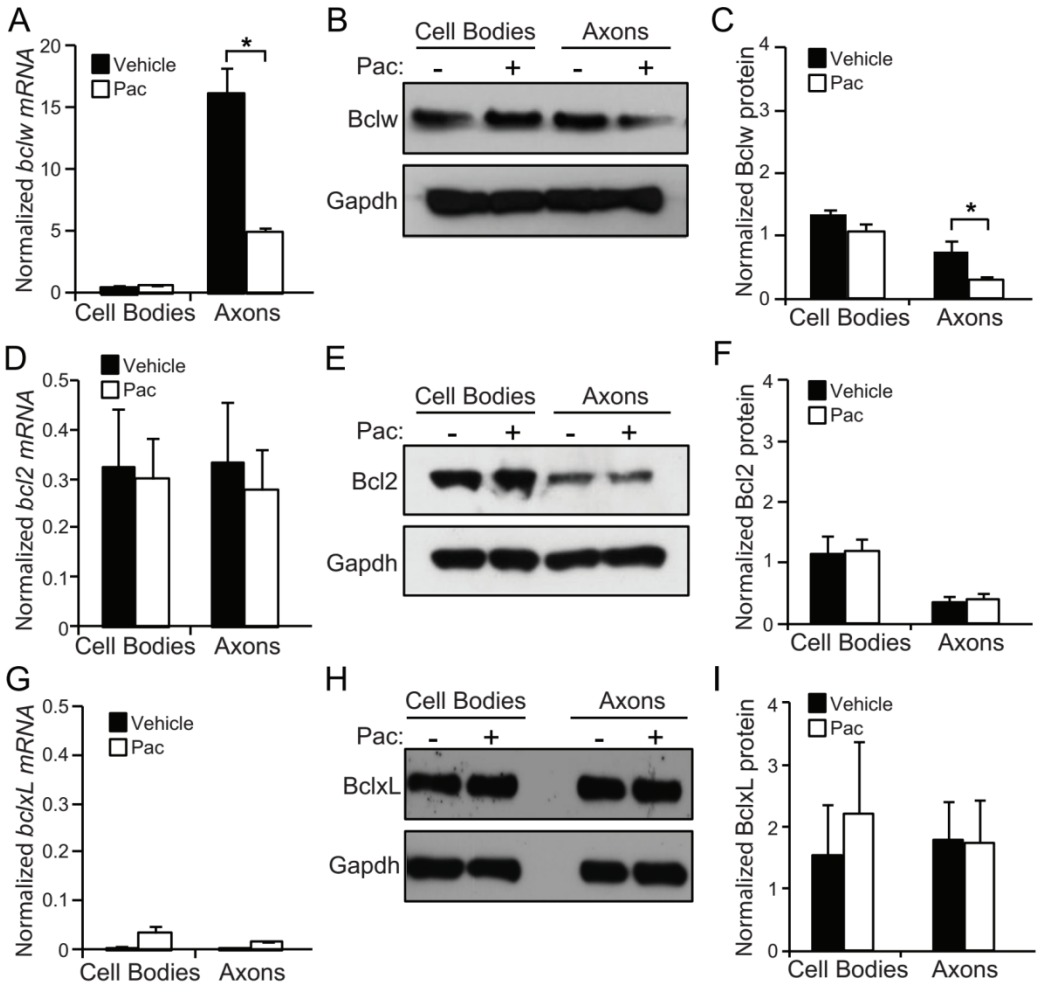


Figure 2.7. Paclitaxel reduces axonal Bclw mRNA and protein levels. *bclw* **(A)**, *bcl2* **(D)** and *bclx*$_L$ **(G)** mRNA analyzed by qRT-PCR from cell body or axon lysate of compartmented cultures after 24 hours of paclitaxel or vehicle treatment to axons. Data normalized to *gapdh*; *p<0.05 by one-way ANOVA with Bonferroni correction; n = 3; data represent mean + SEM. Western blot of protein for Bclw **(B, C)**, Bcl2 **(E, F)**, and Bclx$_L$ **(H, I)** and quantification from cell body or distal axon lysate of compartmented cultures after 24 hours of paclitaxel treatment to axons; data normalized to GAPDH; *p<0.05 by one-way ANOVA with Bonferroni correction; n = 3; data represent mean + SEM.

degeneration *in vivo*, we injected 2 month old *bclw*+/+ and *bclw*-/- mice with paclitaxel (4 mg/kg every other day for 8 days). While *bclw*+/+ mice develop mild thermal hyperalgesia after paclitaxel treatment, as measured by a 50° hot-plate test, *bclw*-/- mice develop a more severe thermal pain sensitivity (Figure 2.8A). Both *bclw*+/+ and *bclw*-/- mice develop severe mechanical allodynia, as assessed by responses to von Frey filaments (Figure 2.8B). In patients, paclitaxel causes a primarily sensory neuropathy with little to no motor symptoms; similarly, paclitaxel-treated mice have normal motor function as assessed in an accelerating RotaRod test (Figure 2.8C). We examined intraepidermal nerve fibers of the hindpaw after paclitaxel treatment, and found that *bclw*-/- mice treated with paclitaxel exhibit a greater reduction in intraepidermal nerve fiber number in both thick (dermal papillae-containing; Figure 2.8D-E) and thin (non-dermal papillae containing; Figure 2.8F-G) skin than exhibited by *bclw*+/+ mice. Together these results indicate that reduced expression of *bclw* enhances paclitaxel-induced degeneration *in vivo* as assessed by both behavior and histopathology (Figure 2.8A-G), and suggest that patients with low levels of axonal Bclw may be more susceptible to this disorder.

2.3 Discussion:

In this study we establish a model system for studying CIPN and demonstrate that paclitaxel exerts a direct pro-degenerative effect on sensory axons by deregulation of a Bclw-IP_3R-calpain cascade. While previous studies indicated importance of Bcl2 interactions with Bax in regulating axon degeneration[2,10,22,30,41], we demonstrate a novel axon survival mechanism involving Bclw interaction with IP_3R1. We show that paclitaxel alters IP_3R-mediated ER calcium release, and activates downstream calpain proteases to cause axon degeneration. We find that paclitaxel specifically reduces axonal Bclw levels, and that axonal Bclw prevents paclitaxel-induced degeneration. Bclw has a unique ability to protect axons from degeneration, as neither Bcl2 nor $Bclx_L$ prevents paclitaxel-induced degeneration, and paclitaxel causes no alterations in expression of either of these closely related factors. Strikingly, the

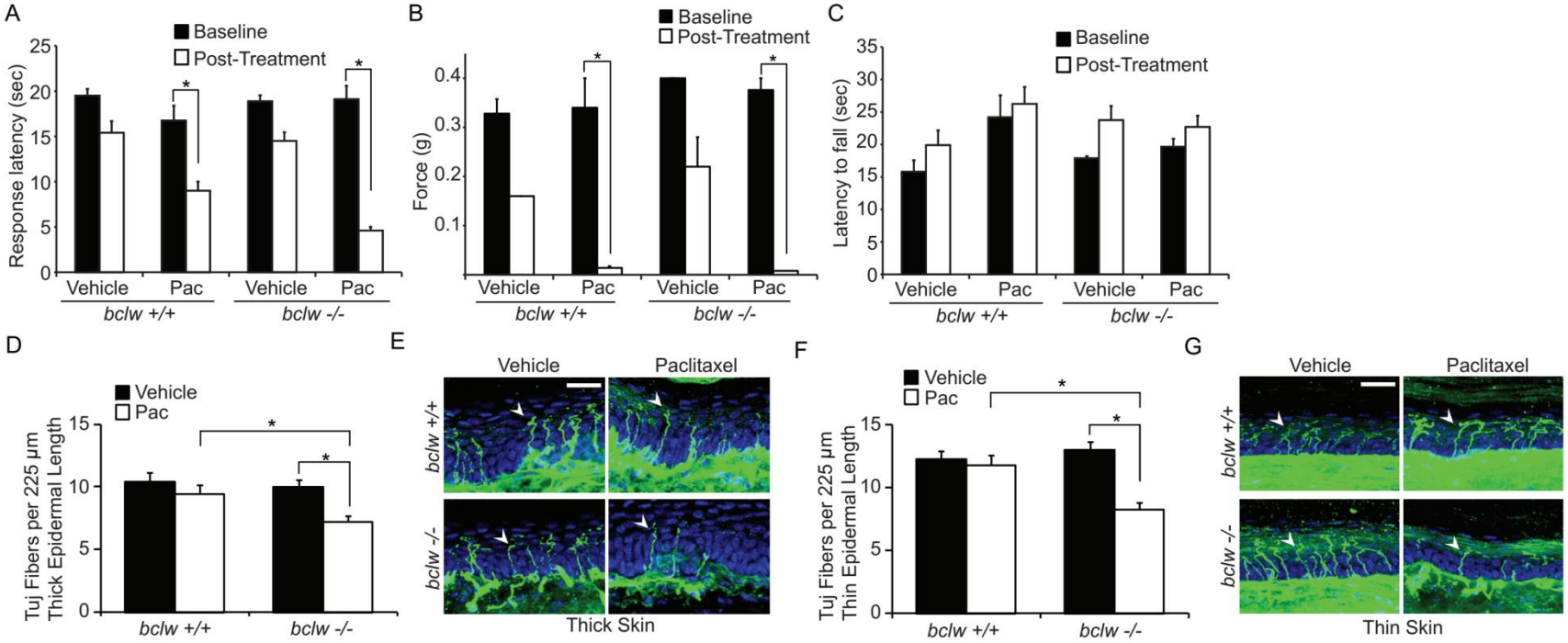


Figure 2.8. Loss of Bclw exacerbates paclitaxel-induced neuropathy *in vivo*. (A-C) Behavioral assessments of mice treated with paclitaxel or vehicle control before (Baseline) and ten days after the final injection (Post Treatment); n = 4-5 mice; data represent mean + SEM. **(A)** Thermal pain threshold measured as time to lick or withdraw hindpaw on a 50° hot-plate. Both *bclw*+/+ and *bclw*-/- exhibit paclitaxel-induced changes in thermal pain threshold (*p<0.05 by one-way ANOVA with Bonferroni correction). The paclitaxel post treatment *bclw*-/- differs from *bclw*+/+ (p<0.05 by Student's *t*-test with Bonferroni correction). **(B)** Mechanical pain threshold determined from response to von Frey; (*p<0.05 by one-way ANOVA with Bonferroni correction. **(C)** Latency to fall off an accelerating Rotarod. **(D-G)** Quantification and representative images of Tuj1 positive sensory fibers (green, arrowheads) entering the epidermis per 225 µm epidermal length in thick **(D-E)** and thin **(F-G)** skin, DAPI counterstain (blue); scale bar = 25 µm, *p<0.05 by one-way ANOVA with Bonferroni correction; n = 3-4 mice with 8-15 images per mouse; data represent mean + SEM.

BH4 domain of Bclw is sufficient for these protective effects, and this BH4 domain selectively interacts with axonal IP_3R1. Together, these data indicate that paclitaxel induces neuropathy by reducing axonal Bclw, thus deregulating IP_3R1 and permitting activation of IP_3R1-dependent degeneration.

Paclitaxel causes defects in sensory neuron calcium dynamics[130,144-148], a critical regulator of pathological axon degeneration (Figure 2.3D). While it is well accepted that a rise in axonal calcium is necessary and sufficient for axon degeneration, the source of this calcium is not clear. Early studies suggested a role for extracellular calcium influx[86-88]; however, more recent studies suggest that calcium efflux from intracellular calcium stores can initiate axon degeneration following mechanical or toxic injury[12]. We find that IP_3R1 is required for paclitaxel-induced degeneration, and that paclitaxel alters IP_3R activity. These data implicating axonal IP_3R1 in paclitaxel-induced degeneration suggest that intracellular stores initiate the pro-degenerative calcium signaling.

Paclitaxel regulates both mRNA and protein levels of axonal Bclw as well as affecting IP_3R1. These data suggest that paclitaxel alters anterograde transport of *bclw* mRNA into axons and/or *bclw* mRNA degradation within axons, resulting in decreased levels of this important axonal protein component. Consistent with the former hypothesis, paclitaxel impairs axonal transport[115,149-152]. Bclw has a specialized protective role in paclitaxel-induced degeneration of sensory axons that is not shared by the closely related Bcl2 or $Bclx_L$ proteins. This is particularly surprisingly because $Bclx_L$, as well as Bclw, were recently shown to promote neurotrophin-dependent sensory axon survival[2,44]. During developmental axon degeneration caused by neurotrophin-deprivation, both Bclw and $Bclx_L$ promote axon survival by binding and inhibiting the anti-apoptotic Bcl2 family member Bax[2,10,22,30,41]. In contrast, in paclitaxel-induced axon degeneration, Bclw functions as a specialized upstream regulator of axonal IP_3R1. Thus, unlike closely related family members, Bclw has a distinctive ability to interact with IP_3R1 in axons, and exhibits a unique ability to protect axons from paclitaxel toxicity.

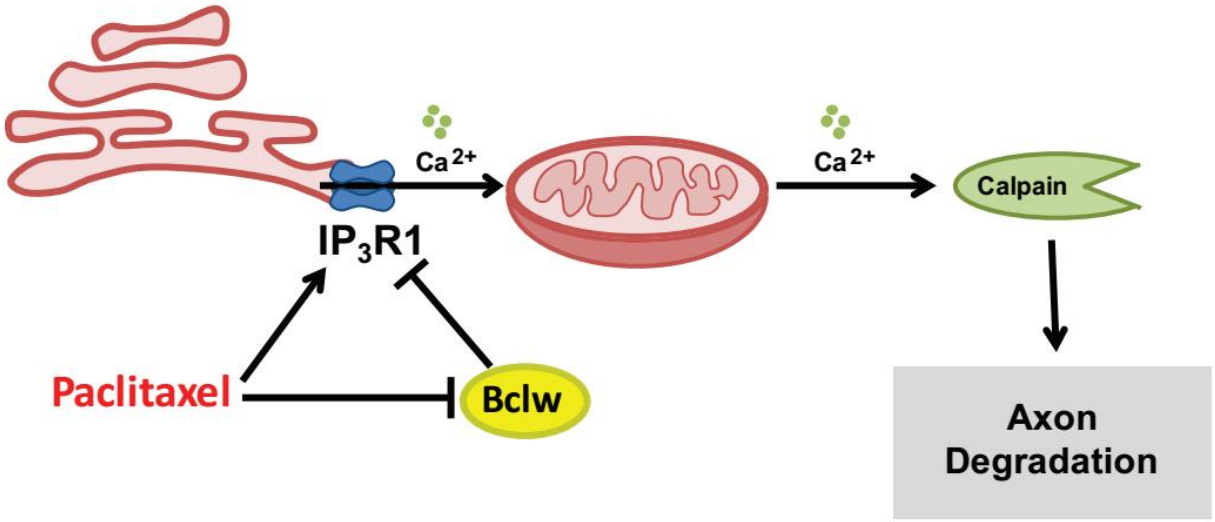


Figure 2.9. Model of paclitaxel-induced axon degeneration. Model showing mechanism of paclitaxel-induced degeneration, wherein paclitaxel reduces axonal Bclw levels and causes activation of an IP_3R1-dependent degeneration cascade characterized by mitochondrial dysfunction and calpain protease activation.

Based on these studies we propose a model in which paclitaxel attenuates Bclw levels and thereby removes the brakes on an axonal degenerative cascade (Figure 2.9). As paclitaxel enhances axon degeneration even in the absence of Bclw (Figure 2.6F and Figure 2.8A-G), paclitaxel may also initiate an additional pro-degenerative signal. Strikingly knockdown of IP_3R1 completely prevents paclitaxel-induced degeneration either in the presence or absence of Bclw, indicating that IP_3R1 functions as a convergence point of these two pathways. Thus paclitaxel both inhibits a Bclw-dependent brake on degeneration and simultaneously activates an IP_3R1-mediated accelerator of the degenerative cascade.

Despite the high incidence of CIPN and paclitaxel-induced neuropathy, there are no approved methods to prevent or reverse the neuropathy[153-155]. Current treatment options therefore are to reduce or discontinue chemotherapy, and to provide non-specific symptomatic pain relief. Here we show that a stapled peptide of the Bclw BH4 domain binds IP_3R1 and prevents paclitaxel-induced degeneration, suggesting the BH4 domain of Bclw may be a promising template for a Bclw- mimetic drug that targets IP_3R1 and so prevent axonal degeneration. IP_3R1 is required for axon degeneration induced by other

chemotherapeutic agents[12], and alterations in calcium are implicated in axon degeneration due to diverse causes[92]. Therefore, reduction in Bclw and consequent activation of IP_3R1-dependent degeneration may represent a conserved mechanism for many neurodegenerative syndromes, and Bclw-mimetics may provide a broad therapeutic approach for these prevalent disorders.

2.4. Methods:

All experimental procedures were done in accordance with the National Institutes of Health guidelines and were approved by the Dana-Farber Cancer Institutional Animal Care and Use Committee.

Animal use. Timed pregnant Sprague-Dawley rats were purchased from Charles River. *bclw-/-* mice were a generous gift from Grant MacGregor (University of California, Irvine, CA)[156]. Genotyping for the wild-type *bclw* gene and/or lacZ gene were performed by Transnetyx using the Bclw targeting sequence GCTCTGAACCTCCCCATGACTTAAATCCGTTGCTCTTTCT-TGGCCCTGCCCAGTGCCTCTGAGCATTTCACCTATCTCAGGAGC and the lacZ sequence CGATCGTAATCACCCGAGTGTGATCATCTGGTCGCTGGGGAATGAGTCAGGCCACG-G. *bclw-/-* mice were maintained on a C57BL/6JEiJ background[157].

Cell culture. Compartmented chamber (Campenot) cultures were prepared as described previously[158] with modifications. Briefly, DRGs from embryonic day 15 (E15) rats of either sex were dissected and trypsinized. DRGs (1.2×10^5 cells) were plated in the center compartment of a Teflon divider[38] (Camp10, Tyler Research) affixed to a p35 culture dish coated with growth factor reduced Matrigel basement membrane (1:45 in DMEM; BD Biosciences). Cultures were maintained in media consisting of Neurobasal (Invitrogen) with 2% B27 supplement (Invitrogen), 1% penicillin-streptomycin, 1% GlutaMAX (Life Technologies), 0.08% glucose, and 0.3 μM cytosine arabinoside (AraC) at 37°C, 7.5% CO^2; BDNF +

NGF (PeproTech) were added to the cell body compartment at a concentration of 10 ng/mL and to the axon compartment at a concentration of 100 ng/mL for 2d. On day 3, media was replaced and the AraC was omitted. On day 5 neurotrophins were removed from the cell body compartment and reduced to 1 ng/mL in the axon compartments for 3-5 days. For paclitaxel treatment experiments, nM paclitaxel (Sigma-Aldrich) or vehicle control (0.1% DMSO) was added to either cell body or distal axon compartments on day 7 for 24 hours. For calpain inhibition experiments, 20 µM calpain inhibitor III (VWR) and paclitaxel were added simultaneously to distal axon compartment.

Microfluidic chambers cultures were prepared as described previously[158], with modifications. Briefly, 3 x 10^4 E15 DRG neurons (4 µL volume) were plated into one channel of a microfluidic device (Xona Microfluidics, SND450) affixed to a PDL/laminin-coated cover glass (Fisherbrand Microscope Cover Glass; 24 x 40-1.5). Cells were plated in Neurobasal media (described above) with 0.3 µM AraC; 50 ng/mL NGF + BDNF was added to cell body wells, and 100 ng/mL was added to axon wells. On day 2, cell body neurotrophins were reduced to 10 ng/mL. On day 4 or 5, cell body neurotrophins were reduced to 1 ng/mL and axon neurotrophins were reduced to 10 ng/mL, and cultures were maintained for 1-3 more days.

Mass cultures consisting of 3 x 10^5 E15 DRG neurons were grown on Matrigel-coated p35 culture dishes in neurotrophin-enriched (100 ng/mL NGF + BDNF) media with 0.3 µg/mL AraC. On day 3, neurotrophins were reduced to 10 ng/mL and cultures maintained for 3-6 more days.

Axonal degeneration assay. Compartmented chamber cultures were fixed at room temperature with 4% PFA diluted 1:2 in media for 10 min, then undiluted 4% PFA for an additional 20 min. Cultures were permeabilized with 0.1% Triton X-100 for 10 minutes, blocked in 3% BSA and 0.1% Triton X-100 for 1 hour at room temperature, and incubated with mouse anti-Tuj1 (1:400; clone Tuj1; Covance) overnight at 4°C. Cultures were then incubated with goat anti-mouse AlexaFluor (1:1000; Invitrogen) for 1 hour at

room temperature and counterstained with DAPI. Images of distal axon tips were obtained using a 40x air objective, and axonal degeneration was quantified as a degeneration index, as previously described[2,75]. Apoptosis analysis was carried out on the same cultures by taking images in the cell body compartment and counting total and condensed nuclei in NIH ImageJ software by an observer blind to condition.

Western blotting. For analysis of Bcl2 family proteins, cell bodies and axons of E15 DRGs in compartmented cultures were lysed in nonionic detergent, lysates were separated by SDS-Page and probed with the following antibodies: anti-Bclw (1:1000; clone 31H4; Cell Signaling Technology), anti-Bcl2 (1:1000; Abcam), anti-$Bclx_L$ (1:1000; Cell Signaling Technology), and anti-GAPDH (1:2000; clone 14C10; Cell Signaling Technology). Bands were visualized with secondary antibodies conjugated to HRP (1:10,000; Bio-Rad) and SuperSignal chemiluminescent substrate signal. Using NIH ImageJ software, protein levels were quantified and levels of protein were normalized to GAPDH.

Quantitative reverse transcription-PCR. RNA was extracted from DRG neurons in compartmented cultures using TRIzol (Invitrogen) according to manufacturer's protocol. Reverse transcription (RT) was performed using the cDNA archive kit (Applied Biosystems) according to the manufacturer's protocol, and quantitative real-time RT-PCR was performed using Taqman Gene expression assays (Applied Biosystems) to analyze expression of *bclw* (Rn00821025_g1), *bcl2* (Rn99999125_m1), and $bclx_L$ (Rn00580568_g1). Data were normalized to *gapdh* (glyceraldehyde-3-phosphate dehydrogenase; Applied Biosystems) for each sample.

Calpain protease activity luminescence assay. DRG neurons in mass cultures were treated with paclitaxel (30 nM, 600 nM, or 1.2 μM for 48h), calcium chloride (3 mM for 24h), or vehicle control (0.1% DMSO for

48h). Cultures were harvested in 1x Passive Lysis Buffer (Promega). Lysate was spun 10,000 x g for 5 minutes at 4°C, and 50 µL supernatant was combined with 50 µL Calpain-Glo Reagent (Promega) in a 96 well plate. Plate was shaken briefly and incubated 40 minutes in the dark; luminescence intensity was measured with a microplate reader and normalized to protein concentration.

Tetramethylrhodamine ethyl ester measurements of mitochondrial membrane potential. E15 DRG neurons in microfluidic devices were labeled using tetramethylrhodamine ethyl ester (TMRE) (Invitrogen). TMRE was applied (10 nM) for 20 min at 37°C, then cells were rinsed with phenol-free media and imaged live using a 60x oil 1.4NA objective. Fluorescence intensity of axonal mitochondria was measured by dividing the fluorescence intensity of each mitochondria (Fm) by the background fluorescence intensity of a nearby cytoplasmic region (Fc). Fluorescence intensity was measured using NIH ImageJ software by an observer blind to condition.

Calcium imaging. E15 DRG neurons in microfluidic devices were infected at 1 day *in vitro* (DIV) with AAV9-GCaMP6s (AAV9.CAG.GCaMP6s.WPRE.SV40) and AAV9-mCherry (AAV9.CB7.CI.mCherry.WPRE.rBG) for 24 hours (Penn Vector Core, courtesy of Vivek Jayaraman, Rex A. Kerr, Douglas S. Kim, Loren L. Looger, and Karel Svoboda from the GENIE Project, Janelia Farm Research Campus, Howard Hughes Medical Institute). After 4-5 days, 600 nM paclitaxel or vehicle control (0.1% DMSO) were added to both cell body and axon compartments for 24 hours. Cultures were incubated 15 minutes (37°C, 7.5% CO^2) in calcium-free media (130 mM NaCl, 4.7 mM KCl, 2.3 mM $MgSO_4$, 1.2 mM KH_2PO_4, 10 mM EGTA, 20 mM HEPES pH 7.3, 5 mM Glucose), then imaged with a Nikon Ti-E at 37°C (60x oil 1.4NA objective) while cells bodies were stimulated with 200 µM ATP (Sigma-Aldrich) in calcium-free media. For two fields of view, GCaMP6s and mCherry channels were acquired every 5 seconds for 60 seconds, then every 20 seconds for 440 seconds. ATP was added after 15 seconds of baseline

measurements. Images were analyzed using NIH ImageJ software. For each individual cell at each timepoint, a mask was generated from mCherry channel; GCaMP6s and mCherry fluorescence intensities were measured; and background fluorescence was subtracted. The change in fluorescence (ΔF/F) was calculated per cell according to the equation [(F - F0)/F0)] where F0 is the average baseline fluorescence at time 0-15 seconds and F is the recorded fluorescence for a given experimental time point. 119 (Paclitaxel) and 156 (vehicle) neurons were measured across 3 independent experiments with 3 cultures per experiment.

Stapled peptide generation. Stapled peptides were synthesized, derivatized at the N-terminus with FITC-bALA or biotin-bAla, purified by LC-MS to > 95% purity, and quantified by amino acid analysis according to our established methods[138,159]. Lyophilized SAHBs were reconstituted in 100% DMSO and diluted into aqueous buffers for experimentation. The following peptide sequences were used, where X = pentenyl alanine and where B = norleucine, replacing a cysteine:

Bclw BH4 $SAHB_A$	ALVADFVGYKLRXKGYXBGA
Bcl2 BH4 $SAHB_A$	EIVBKYIHYKLSXRGYXWDA
$Bclx_L$ BH4 $SAHB_A$	RELVVDFLSYKLSXKGYXWSQ
BIM BH3 $SAHB_{A2}$	EIWIAQELRXIGDXFNAYYA

Liposomal release assay. Large unilamellar vesicles with lipid composition resembling the mitochondrial outer membrane were generated and entrapped with ANTS and DPX as described[160,161]. Recombinant Bax was generated in BL-21 DE3 E. Coli and then purified by chitin affinity and size exclusion chromatography as described[162,163]. For measurement of BIM BH3 $SAHB_{A2}$-induced activation of BAX, recombinant, full-length BAX protein, BIM_{A2} BH3 $SAHB_{A2}$ (aa 145–164), and Bclw BH4 $SAHB_A$ were added

at the indicated concentrations to liposomes (5 μL) to a final volume of 30 μL in 384-well plate format. ANTS dequenching due to DPX dissociation (F) upon liposomal release was measured over a period of 7200 seconds with a Tecan M1000 plate reader (excitation and emission wavelengths of 355 and 520 nm, respectively). Plates were re-read following lysis with 1% Triton X-100 to determine maximal release (F100). Percent ANTS/DPX release was calculated according to the equation [(F - F0)/(F100 - F0)] where F0 is the baseline fluorescence at time zero and F is the recorded fluorescence for a given experimental time point.

Protein and peptide introduction. Recombinant His-tagged Bclw, Bcl2, and $Bclx_L$ proteins (R&D Systems) or control β-Galactosidase protein were introduced into cell bodies or axons of compartmented chamber cultures as described previously[2]. Briefly, 1 μg/μL protein was introduced into cultures using 2 μL Chariot reagent (Active Motif). To confirm expression of His-tagged proteins, cell bodies and axons were washed and then lysed in nonionic detergent, and protein lysates were separated as above and probed with the following antibodies: anti-His (1:1000; Novagen) and anti-pan-Actin (1:1000; Cell Signaling Technology). FITC-SAHB peptides (Bclw BH4 $SAHB_A$, Bcl2 BH4 $SAHB_A$, and $Bclx_L$ BH4 $SAHB_A$; stock solutions 1 mM in DMSO) were introduced into cell bodies or axons using 350 ng peptide and 2 μL of Chariot reagent diluted 1:10 in water. Control was no peptide with 2 μL of 1:10 diluted Chariot. To confirm peptide uptake, cultures were processed for axonal degeneration assay as above, and FITC immunofluorescence was examined with a 40X air objective. Paclitaxel was added to cultures 1 hour after protein or peptide transfection.

Neurotrophin-deprivation. FITC-SAHBs (Bclw, Bcl2, and $Bclx_L$ or no peptide control) were transfected into the cell body compartment of compartmented cultures as described above. One hour later, both cell body and axon compartments were changed into media without NGF + BDNF for 24 hours. A non-

transfected control culture was maintained in media with normal levels of NGF + BDNF. Cultures were fixed and incubated with DAPI (1:1000). Images were taken with a 40x air objective, and an observer blind to condition counted the number of total and condensed nuclei using NIH ImageJ software.

Biotinylated SAHB pulldowns. DRGs were grown in compartmented chamber cultures for 7-8 days, and cell bodies and axons were separately harvested in lysis buffer containing 1% CHAPS detergent, 150 mM NaCl, 50 mM Tris pH 7.4, 1 mM DTT, 500 mM NaF, 100 mM PMSF, 200 mM Na_3VO_3, and EDTA-free cOmplete Mini protease inhibitor cocktail (Sigma-Aldrich). Lysate was pre-cleared 2 hours at 4°C with High Capacity Neutravidin Agarose Beads (1:20 in lysate; Thermo Scientific). For each pulldown, 200-600 µg of precleared lysate was incubated overnight at 4°C with biotin alone or with biotinylated-BH4 SAHB peptides of Bclw, Bcl2, or $Bclx_L$ to a final peptide or biotin concentration of 20 µM. The next day, Neutravidin beads were added (1:14) to lysate for 2 h at 4°C. Lysate was removed, beads were washed on ice with cold PBS + protease inhibitor cocktail, and sample was eluted by boiling for 5 minutes in non-ionic lysis buffer, sample buffer, and reducing agent. Protein was resolved with either 4-12% Bis-Tris SDS-Page (Bax, YARS, IP_3R1) or 3-8% Tris-Acetate SDS-Page (YARS, IP_3R1) and probed with the following antibodies at 1:1000: anti-Bax (Cell Signaling), anti-YARS (tyrosyl tRNA synthetase; clone EPR9927; Abcam), and anti-IP_3R1 (Thermo Scientific). A 10% input lane from the original cell body or axon lysate was run alongside pulldown lysate. Band intensity was quantified as described above, and each pulldown intensity was normalized to the input intensity.

shRNA lentiviral knockdown. Lentiviral particles were generated using shRNA constructs targeting Bclw (TRCN0000321174; Sigma-Aldrich) and IP_3R1 (TRCN0000321161; Sigma-Aldrich) and validated for protein knockdown in mass cultures. For degeneration assays, 50 µL lentivirus was added to cell bodies of compartmented cultures for 24 hours, then virus was removed, media containing puromycin (1

μg/mL; Sigma-Aldrich) was added, and cultures were allowed to grow for 5 days prior to paclitaxel treatment.

Paclitaxel treatment and behavioral testing. 2 month old age-matched *bclw*-/- and *bclw*+/+ mice (17-30 g) of either sex were injected intraperitoneally (IP) with 4 mg/kg paclitaxel (Bristol-Myers Squibb) every other day for 8 days (4 total injections). Paclitaxel was prepared as 1 part 6 mg/mL paclitaxel stock solution diluted in vehicle (1:1 v/v Cremophor EL [EMD Millipore] and dehydrated ethanol) and 2 parts sterile saline and injected at 10 μL/g. Control mice were injected with 1 part vehicle and 2 parts saline. At 6 months of age *bclw-/-* mice have altered noxious sensation; however 2-3 month old *bclw-/-* mice exhibit normal motor function and noxious sensation[1]. For three days prior to the baseline testing, mice were weighed, trained on an automated RotaRod apparatus (4 rpm for 1 minute without falling), and habituated in von Frey cages. The next two days, baseline behavioral performance was assessed and averaged. The first paclitaxel injection was given three days later, and mice were weighed and behaviorally tested 10 days after the final injection. To assay motor function, mice were placed on the RotaRod with a ramp of 4-40 rpm and 0.4 rpm/sec acceleration, and latency to fall was measured. Noxious mechanosensation threshold was assayed as described previously[1] using von Frey filaments (0.008-1.4g). Withdrawal threshold was determined to be the applied force at which the animal withdrew the stimulated paw on at least 2 of 10 applications. Noxious thermal sensation threshold was assayed as described previously[1] using a 50°C hotplate and measuring latency to flick or lick the hindpaw. Mouse behavior was assessed by an experimenter blind to genotype and condition.

Epidermal footpad innervation. Footpad tissue from hindpaws of *bclw-/-* and *bclw+/+* mice was harvested, fixed, and sectioned as described previously[1,2]. Briefly, mice were euthanized with isoflurane 11 days after final paclitaxel injection and footpad tissue was removed and divided into thick (dermal

papillae containing) and thin (non-dermal papillae containing) skin. Footpads were fixed in Zamboni's fixative overnight at 4°C, cryopreserved in 30% sucrose overnight at 4°C, frozen, and sectioned into 30 µm floating sections. Sections were blocked in 10% normal goat serum with 0.1% Triton X-100 in PBS 1 hour at room temperature and incubated with anti-Tuj1 (1:300; Covance) overnight at 4°C. Sections were then incubated with goat anti-mouse AlexaFluor 488 (1:200; Invitrogen) and DAPI (1:1000) for 2 hours at room temperature and mounted on gelatin-coated slides. Epidermal images were acquired on a Nikon Ni-E C2 confocal with a 40x 1.3NA oil objective as 30-35 µm z-stacks (1 µm step size) and converted into a maximum intensity projection image. Intraepidermal nerve fiber density was determined to be the number of Tuj1-positive fibers penetrating ≥10 µm into the epidermis, normalized to the measured epidermal length (225-450 µm per image) and displayed as number of Tuj1-positive fibers per 225 µm. Images were acquired and quantified in NIH ImageJ by an experimenter blind to condition and genotype.

Statistics. Data are expressed as mean $\pm$ SEM. To assess statistical significance, data were analyzed by unpaired two-tailed Student's *t* test. For multiple comparisons, data were analyzed by one-way ANOVA with *post hoc* Bonferroni or Dunnett correction. Significance was placed at $p < 0.05$ unless otherwise indicated.

Chapter 3

Neurotrophin regulation of sensory neuron translation machinery

The work described in this Chapter includes collaborative contributions: all TRAP-related experiments were done under the joint guidance of Rosalind Segal and Myriam Heiman (MIT), and *in vivo* TRAP purification was done together with Ozge Tasdemir-Yilmaz (Figure 3.5E). I carried out experiments and analyzed data generated in all figures.

3.1 Introduction:

The establishment of properly wired neuronal circuitry depends on precise temporal and spatial extracellular signals that support neuronal guidance, growth, survival, maintenance, and functionality. Neurotrophins are a family of secreted trophic factor proteins important for all of these functions in both the central and peripheral nervous systems. Neurotrophins include nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4 (NT-4), each of which binds to specific high-affinity receptor tyrosine kinases (Trk receptors). Neurotrophin activation of Trk receptors induces receptor autophosphorylation followed by initiation of various signal transduction cascades. The type of signaling cascades and subsequent cellular responses elicited by neurotrophins are specified by the type, location, and timing of receptor activation[164,165]. While upstream signaling cascades initiated by neurotrophin stimulation have been studied in detail, the transcriptional mechanisms mediating subcellular responses such as axon survival are not well understood.

During neuronal development, dorsal root ganglia (DRG) sensory neurons are initially produced in excess, and surplus neurons are removed in order to match the number of neurons to the size of the target field. A model addressing the mechanism by which the appropriate number of neurons is preserved is described by the neurotrophin hypothesis. This hypothesis posits that developing sensory neurons compete for a limited supply of neurotrophins secreted by target cells, such as in the skin, and only neurons that innervate the target and receive neurotrophins survive (Figure 3.1A). Developing sensory neurons can also receive neurotrophins from other sources, including from DRG neurons within the ganglia in a paracrine or autocrine fashion[166,167], or from intermediate target regions on the way to the final peripheral target[168-171]. However, retrograde signaling initiated by target-derived neurotrophin stimulation is required for a variety of specific cellular responses. For example, neurotrophin stimulation of sensory neurons at the axon supports survival of the whole cell, whereas stimulation at the cell body

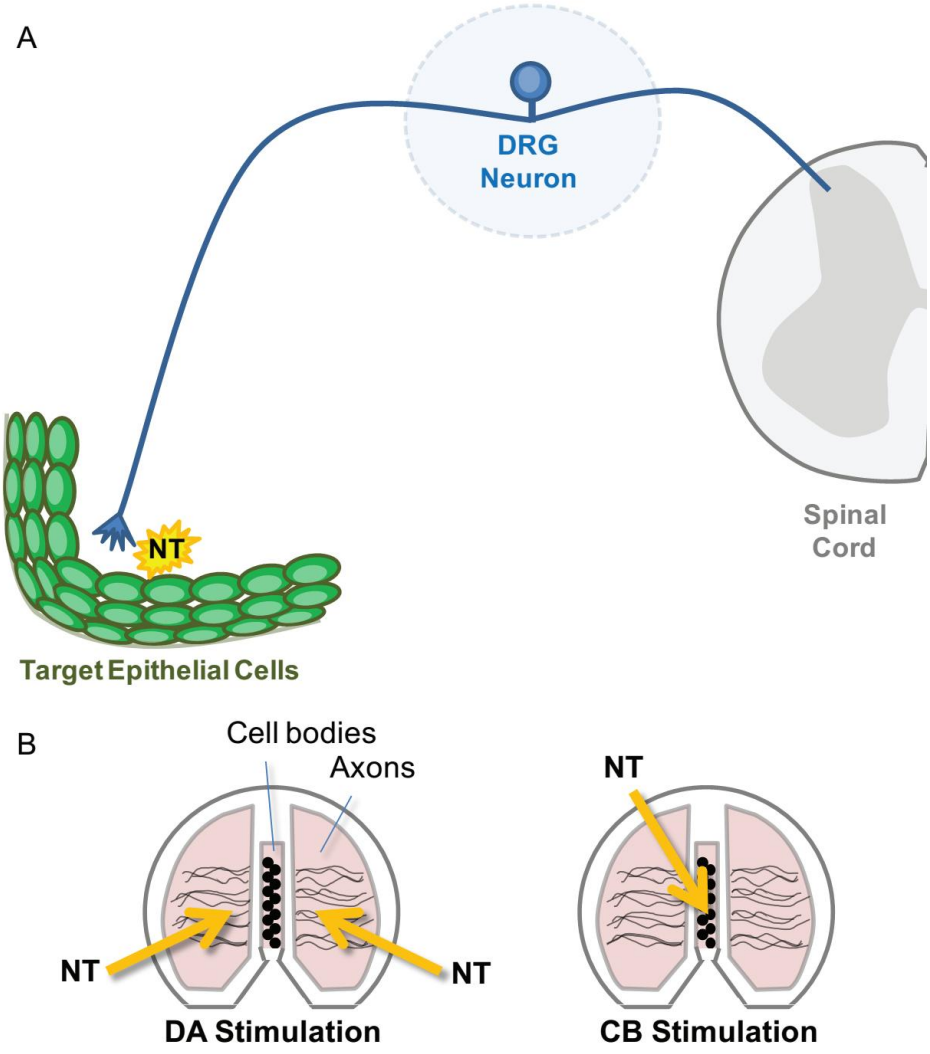


Figure 3.1. Neurotrophic support of DRG sensory neurons in development. (A) Neurotrophins (NTs) secreted by peripheral targets such as epithelial cells (green) activate Trk receptors on DRG sensory neuron axons (blue) and initiate a retrograde survival response in DRG cell bodies within the dorsal root ganglia (dotted line). **(B)** NT stimulation of distal axons (left) or cell bodies (right) of sensory neurons in compartmented cultures.

supports cell body but not axonal survival[38,43,172,173]. These findings and others suggest that target-derived neurotrophin signaling initiates discrete molecular cascades and cellular responses, and that there may be distinct but overlapping mechanisms that promote cell body and axonal survival. It is still unclear how spatially distinct neurotrophin stimulation coordinates transcription, translation, and transport throughout the cell to activate differential molecular cascades and promote compartment-specific survival.

In these studies we investigate the effects of spatially distinct neurotrophin stimulation on the sensory neuron transcriptome. We find that axonal neurotrophin stimulation with NGF and BDNF, which is analogous to target-derived neurotrophic support, selectively upregulates translation machinery transcripts including mRNAs encoding ribosomal proteins, elongation and initiation factors, and tRNA synthetases, as well as ribosomal RNAs (rRNAs). In addition to long-term transcriptional changes in translation machinery components, axonal neurotrophin stimulation induces translation-promoting phosphorylation of protein synthesis regulators S6 and 4E-BP1. Finally, we establish an *in vitro* and *in vivo* method to examine the translatome within the subcellular compartments of the axon and the cell body. These studies suggest a novel mechanism by which survival-promoting cues may act through ribosome biogenesis to alter the translational state of the neuron.

3.2 Results:

Transcriptome profiling of axonal versus cell body neurotrophin stimulation

To investigate the differential effects of cell body (CB) versus distal axon (DA) neurotrophin stimulation, we used a compartmented culture system to treat E15 rat DRG cell body or distal axon compartments with neurotrophins (NGF and BDNF) or vehicle control for 2 hours, then purified cell body RNA and examined changes in the transcriptome via Affymetrix microarray analysis from one biological replicate of 12-16 cultures pooled per condition (Figure 3.2A). Normalized gene expression values were

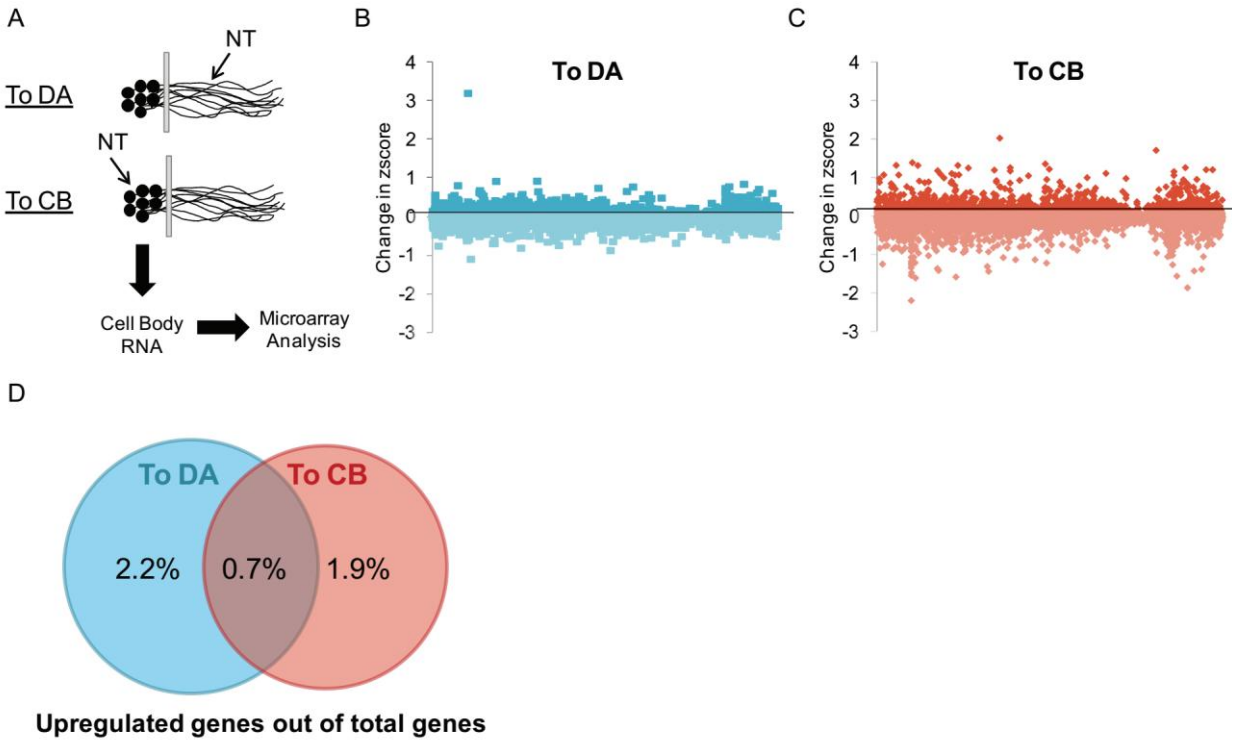


Figure 3.2. **Transcriptome analysis of sensory neurons after acute axonal or somatic neurotrophin stimulation. (A)** Experimental setup showing conditions "To DA" where neurotrophins ("NT") were added selectively to distal axons and "To CB" where NT were added selectively to cell bodies, then cell body RNA was purified and analyzed by microarray analysis. **(B-C)** NT-induced change in zscore of cell body RNA expression values after axonal **(B)** or cell body **(C)** NT stimulation. X axis is unique genes, and dotted line denotes 2 fold standard deviation. All values above the dotted line were considered upregulated. **(D)** Percentage of genes upregulated out of total assayed genes by axonal NT stimulation (blue), cell body NT stimulation (red), or by either axonal or cell body stimulation (purple).

converted into zscores relative to the total transcriptome expression, and genes were considered upregulated if the difference between the neurotrophin stimulation condition and the control condition were positive and exceeded twice the standard deviation of change for that condition (dotted line; Figure 3.2B-C). A similar number of genes were upregulated by axonal or cell body stimulation: 2.2% of the 24,992 total assayed genes were significantly upregulated only by distal axon stimulation, 1.9% only by cell body stimulation, and 0.7% were upregulated by either cell body or distal axon stimulation

(Figure 3.2D). The immediate early gene *cfos* was successfully induced by both types of neurotrophin stimulation.

Gene ontology (GO) analysis of biological processes was performed with PANTHER GO slim to determine if certain functional gene categories were upregulated selectively by axonal neurotrophin stimulation (Figure 3.3A). Metabolic process genes were the most abundant upregulated gene category for all types of neurotrophin stimulation (Figure 3.3A). Axonal neurotrophin stimulation preferentially upregulated protein metabolic process genes (Figure 3.3B), particularly genes associated with translation. This GO category of translation included genes encoding ribosomal proteins, tRNA synthetases, elongation factors, and initiation factors, which were preferentially upregulated by axonal neurotrophin stimulation. 7.4% of distal axon neurotrophin upregulated genes were translation-associated (40 total genes), while fewer translation genes were upregulated by cell body stimulation (1.3% of upregulated genes, 6 total genes) or by either cell body or distal axon stimulation (2.2% of upregulated genes, 4 total genes; Figure 3.3C). When these upregulated translation genes were subdivided into types of genes (Figure 3.3D and Table 3.1), there was an enrichment of ribosomal protein genes upregulated selectively by distal axon stimulation (26 ribosomal protein genes). All types of neurotrophin stimulation upregulated a small number of elongation factors, initiation factors, initiation factor kinases, and/or tRNA synthetases. Thus, there are both unique and overlapping factors induced by neurotrophin stimulation of cell bodies and distal axons. The preferential increase in translation genes with distal axon stimulation, particularly ribosomal protein genes, suggests neurotrophin stimulation of axons may initiate a long-term transcriptional program that increases the translational capacity of the cell and/or axon.

Axonal neurotrophin stimulation activates somatic translation machinery

To further investigate whether neurotrophin signaling stimulates translation machinery, we

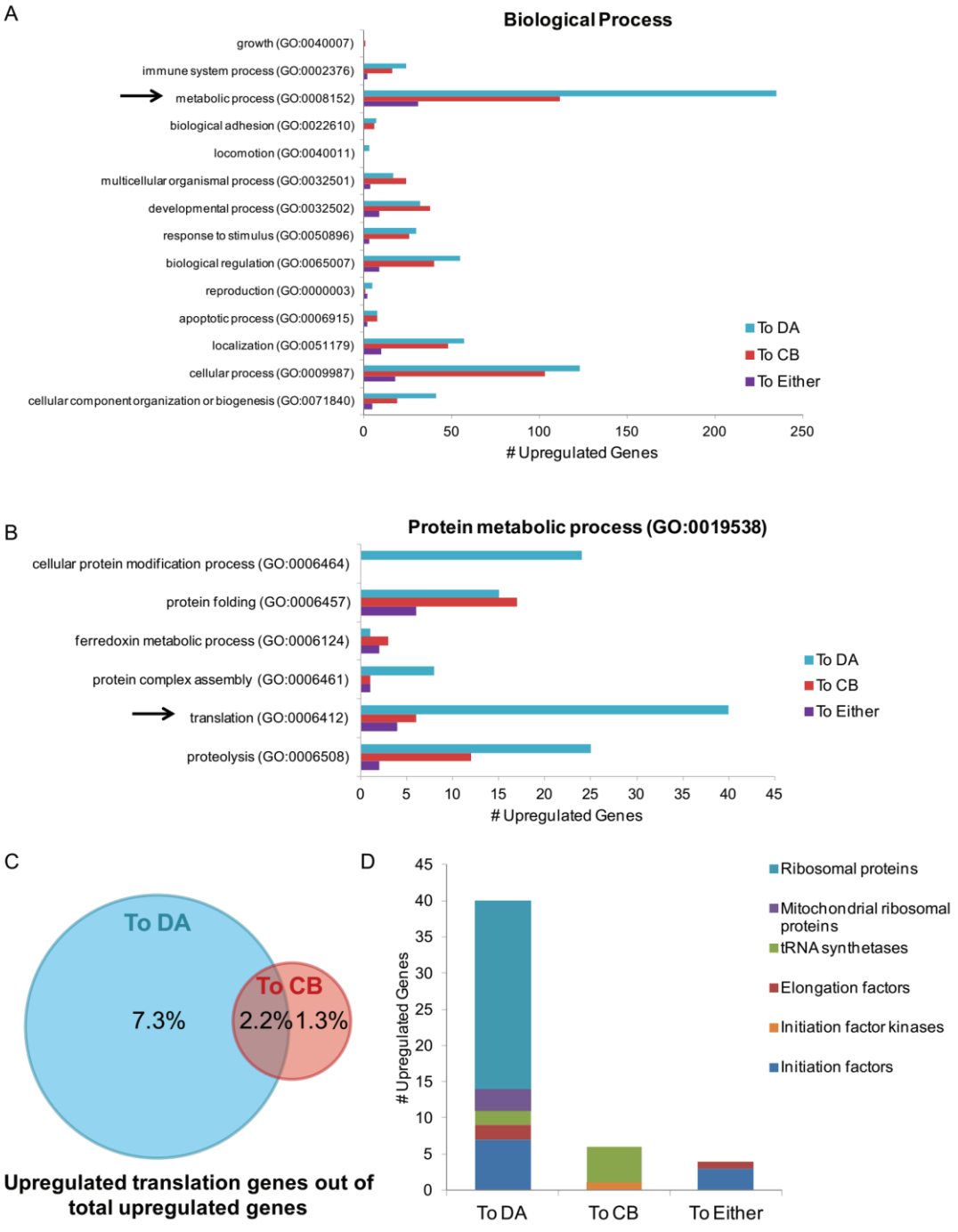


Figure 3.3. **Axonal neurotrophin stimulation preferentially upregulates translation genes. (A)** Gene ontology (GO) analysis showing genes sorted by biological process upregulated by axonal NT, cell body NT, or by either axonal or cell body NT stimulation. Arrow indicates category that includes protein metabolic process GO term shown in (B). **(B)** Upregulated genes from Protein metabolic process GO term. Arrow indicates Translation GO term shown in (C) and (D). **(C)** Percentage of upregulated translation genes (GO term) out of total upregulated genes. (**D)** Number of upregulated translation genes broken down into type of gene.

Table 3.1. Translation genes upregulated by neurotrophin stimulation of axons or cell bodies.

	Upregulated Translation Genes (GO:0006412)
To DA	**Ribosomal proteins:** L29, L21, L24, S17, L37, L5, S10, L31, L22, L22l2, L4, S7, S8, L9, S21, L23A, L7A, S23, S3, L35A, L27, S3A, L35, S20, L15, L23 **Mitochondrial ribosomal proteins**: Mrpl13, Mrpl18, Mrpl41 **tRNA synthetases:** Nars, Rars **Eukaryotic translation elongation factors:** Eef1a1, Eef1b2 **Eukaryotic translation initiation factors:** Eif3k, Eif2s3, Eif3e, Eif1a, Eif4g1, Eif4e, Eif2s1
To CB	**tRNA synthetases:** Iars, Sars, Cars, Yars, Mars **Eukaryotic translation initiation factor kinase:** Eif2ak1
To Either	**Eukaryotic translation elongation factors:** Eef1e1 **Eukaryotic translation initiation factors:** Eif1a, Eif2s2, Eif3c

examined alterations in translation machinery due to long-term transcriptional changes and more short-term post-translational modifications. The increase in transcripts encoding ribosomal protein in response to axonal neurotrophin stimulation (Figure 3.3D) suggests neurotrophins may induce formation of new ribosomes, the large ribonucleoprotein complex that catalyzes protein synthesis. Eukaryotic ribosome biogenesis is a highly complex process involving coordination of hundreds of reactions spanning the nucleolus, nucleus, and cytoplasm. The process is initiated in the nucleolus by RNA Polymerase 1 (Pol1)-mediated transcription of 45S pre-ribosomal RNA (rRNA). Transcription of rRNA drives ribosome biogenesis and has been used as a read-out for the rate of ribosome production[174-178]. Many of the signaling cascades that regulate ribosome biogenesis are downstream targets of neurotrophin signaling, including mTOR, ERK, and MYC signaling pathways[174,179]. We therefore examined levels of small ribosomal subunit-associated 18S rRNA and large ribosomal subunit-associated

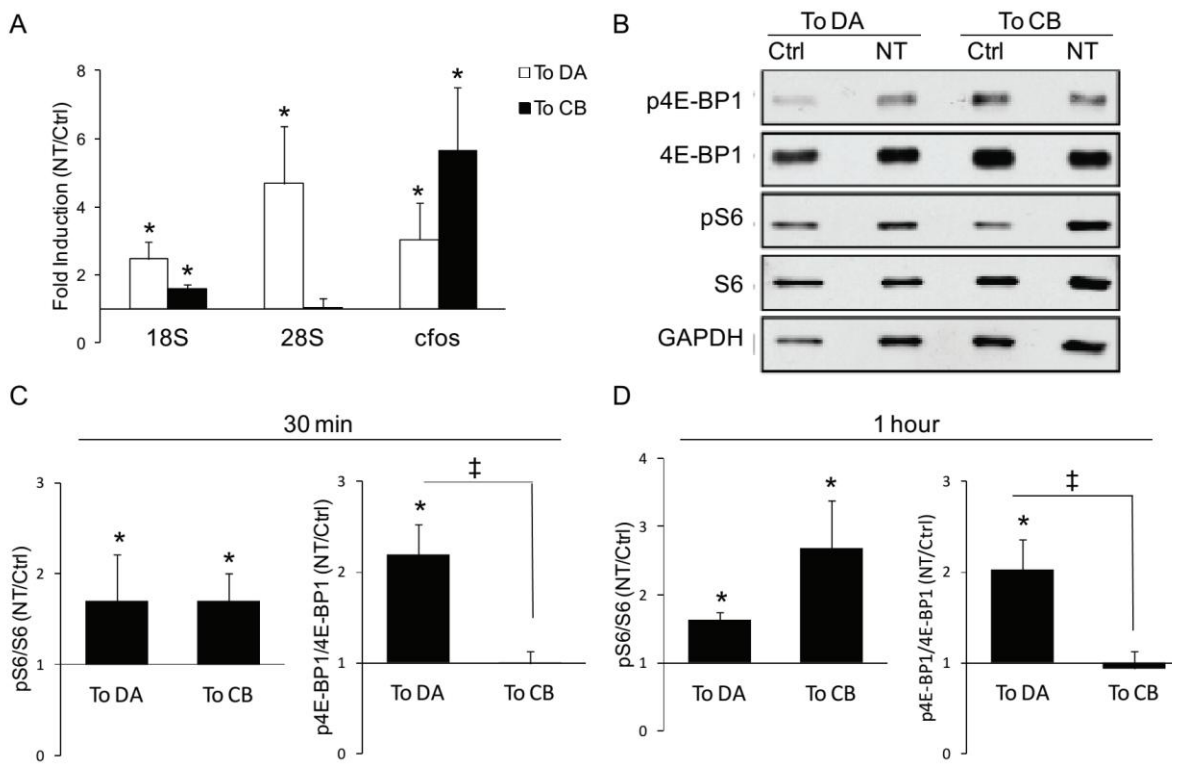


Figure 3.4. Axonal neurotrophin stimulation acutely activates translation machinery. (A) NT/Control levels of 18S rRNA, 28S rRNA, or *cfos* mRNA by qRT-PCR after 1 hour axonal or cell body neurotrophin stimulation; * $p < 0.05$ greater than 1 by ztest; n = 4-5 experiments; data represent mean + SEM. **(B)** Phosphorylated and total S6, 4E-BP1, and loading control GAPDH after 1 hour neurotrophin stimulation to axons (left) or cell body (right). **(C-D)** Quantified phosphorylated protein/total protein band intensity of NT/Control conditions for S6 (left) and 4E-BP1 (right) after 30 minutes **(C)** or 1 hour **(D)** neurotrophin stimulation; * $p < 0.05$ greater than 1 by ztest; ‡$p < 0.05$ by Student's *t*-test; n = 3 experiments; data are mean + SEM.

28S rRNA by quantitative real-time PCR analysis after 1 hour axonal or cell body neurotrophin stimulation. Both 18S and 28S rRNAs were rapidly increased by axonal neurotrophin stimulation, while cell body stimulation caused a small increase in 18S and no increase in 28S rRNA (Figure 3.4A). The immediate early gene *cfos* is induced by both types of neurotrophin stimulation (Figure 3.4A). Together with the transcriptome analysis, these data suggest axonal neurotrophin stimulation may preferentially induce ribosome biogenesis.

In addition to transcriptional and translational events that may increase the number of available ribosomes, the amount of cellular protein synthesis is controlled acutely by post-translational modifications of the translation machinery or associated regulators. For example, protein synthesis is increased by mTOR-mediated phosphorylation of the small ribosomal subunit protein S6 or of the translation initiation factor inhibitor 4E-binding protein 1 (4E-BP1)[180]. Neurotrophins are a well known regulator of the mTOR pathway[180,181]. We therefore examined levels of total and phosphorylated S6 and 4E-BP1 proteins after 30 minutes or 1 hour of axonal or cell body neurotrophin stimulation. 4E-BP1 phosphorylation was preferentially increased by axonal neurotrophin stimulation at both time points, while S6 phosphorylation was similarly increased by both cell body and axonal neurotrophin stimulation (Figure 3.4B-D). These results suggest axonal neurotrophin stimulation may differentially and rapidly regulate the translational capacity of the cell through unique post-translational modifications.

Establishment of a method for *in vivo* sensory axon translatome profiling

The cell body has traditionally been regarded as the sole source of protein synthesis in the cell. This soma-centric view of translation was challenged by the discovery of translation machinery first in dendrites and more recently in axons. There is now strong and accumulating evidence that protein synthesis occurs in axons, and numerous studies have identified ribosomes, selective subsets of mRNAs, and other translation machinery components within embryonic and adult axons[48,182-190]. Axoplasmic protein synthesis is important for many axonal functions, including axon regeneration, growth, branching, maintenance, growth cone dynamics, and viability[13,111,191-196]. Numerous studies have identified specific mRNAs whose axonal localization and/or translation is altered by neurotrophins, and data from our lab shows that axonal translation is required for neurotrophin-mediated axonal survival[2,47,183,187,194,195]. Although much research has been done on the regulation of axonal mRNA *in vitro*, the same methods cannot be applied *in vivo* due to the presence of contaminating non-sensory

neuron cells. In these studies we sought to establish use of the Translating Ribosome Affinity Purification (TRAP) methodology for *in vivo* purification of ribosome-associated mRNAs from DRG sensory neuron cell bodies and axons[197-199]. By expressing an eGFP-tagged version of the large ribosomal subunit protein RPL10a selectively in sensory neurons, ribosome-associated mRNAs can be immunoprecipitated and purified from sensory neuron axons of the sciatic nerve without contamination from surrounding Schwann cells, perineural fibroblasts, and motor neuron axons.

We first tested the TRAP methodology in cultured DRG sensory neurons by infecting mass cultured E15 rat sensory neurons with an AAV9 virus expressing eGFP-L10a. After 3 days, cells were lysed and TRAP was performed. Bioanalyzer analysis showed RNA purified from infected cultures was high quality, containing both 18S and 28S rRNAs, while RNA from uninfected control cultures lacked 18S and 28S peaks (Figure 3.5A). To determine if purified RNAs represented ribosome-associated and therefore actively translated RNAs, cells were stimulated with NGF and BDNF for 2 hours and qRT-PCR analysis was performed on TRAP-purified RNAs. We found that *cfos,* a transcript whose translation is known to be upregulated by cell body neurotrophin stimulation, was significantly induced, while there was no significant increase in *bclw* mRNA, whose translation is upregulated selectively by distal axon stimulation and not by cell body stimulation (Figure 3.5B)[23]. These data suggest purification of ribosome-associated mRNAs from DRG sensory neurons is a reliable method for examining actively translated mRNAs.

We then utilized a Cre-Lox system to express eGFP-L10a selectively in DRG sensory neurons for *in vivo* profiling. We used mice expressing Cre recombinase under the promoter for Nav1.8, a sodium channel expressed in small diameter DRG sensory neurons, including NGF-dependent C fibers[200] . In these studies, we examined the sciatic nerve (axons) and the lumbar L3-L5 DRGs (cell bodies), the DRGs whose peripheral axons contribute to the sciatic nerve (Figure 3.5C). Endogenous eGFP signal was readily detectable in a subset of sensory neurons in wholemounted L3-L5 DRGs from Cre positive floxed-

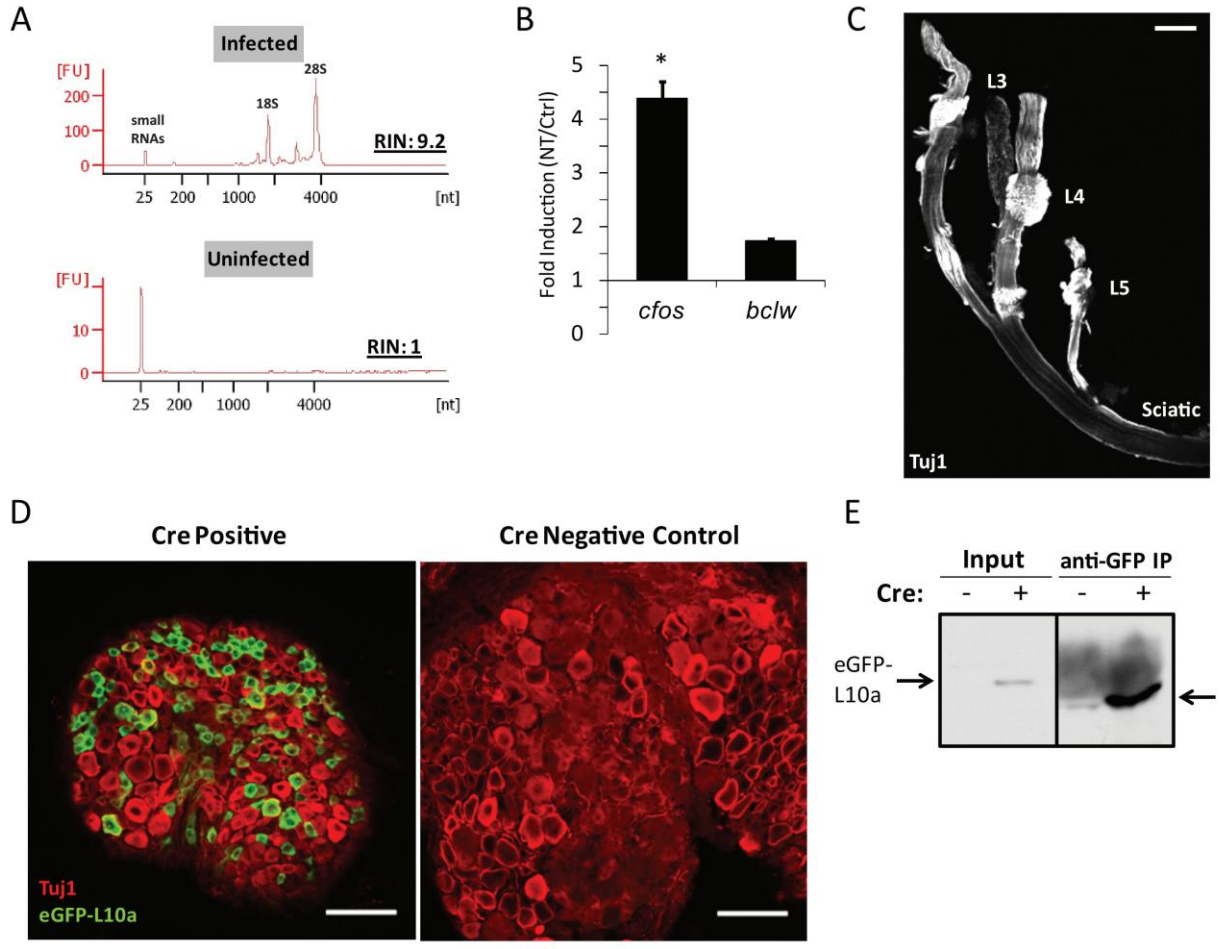


Figure 3.5. TRAP purification of DRG sensory neurons. (A) Bioanalyzer electropherogram of polysomal RNA from cultured DRG neurons infected with AAV9-eGFP-L10a (top) or uninfected control (bottom), showing RNA integrity number (RIN). **(B)** NT/Control levels of polysomal RNA transcripts *cfos* and *bclw* purified from cultured DRG neurons infected with AAV9-eGFP-L10a and stimulated 2 hours with neurotrophins; * $p < 0.001$ by ztest; n = 2 experiments; data represent mean + SEM. **(C)** Adult mouse wholemount sciatic nerve and attached L3-L5 DRGs immunostained with anti-Tuj1; scale bar 1 mm. **(D)** L3-L5 wholemount DRGs from P15 mice immunostained with anti-Tuj1 (red) and expressing eGFP-L10a ribosomal protein (green); DRGs from flx-eGFP-L10a mice expressing Cre under NaV1.8 promoter (left) or Cre negative (right); scale bar = 100 µm. **(E)** Anti-GFP immunoprecipitation (IP) of eGFP-L10a protein from Cre positive (+) or Cre negative (-) L3-L5 DRGs of P15 flx-eGFP-L10a mice, probed with anti-GFP antibody. Input and IP were run on same gel.

eGFP-L10a mice at postnatal day 15 (P15) and absent in L3-L5 DRGs from Cre negative littermates (Figure 3.5D). Importantly, eGFP signal was only present in Tuj-positive neurons and absent from contaminating cell types such as satellite glia or fibroblasts. In addition, standard anti-GFP immunopurification from L3-L5 DRGs showed successful pulldown of eGFP-L10a protein from Cre positive P15 mice (Figure 3.5E). These findings suggest that the Nav1.8-Cre/flx-eGFP-L10a mouse is a viable tool for examining the sensory neuron and axon translatome. Ongoing studies are applying TRAP methodologies to purify ribosome-associated RNAs from L3-L5 DRGs and sciatic nerves from neonatal Nav1.8-Cre/floxed-eGFP-L10a mice. An initial purification of sciatic nerves showed a 5.54 fold enrichment of *β-actin*, one of the most abundant locally translated axonal transcripts; however, substantial nonspecific background in Cre negative controls prohibited identification of other axonally translated transcripts.

3.3. Discussion:

In these studies we investigated how spatially distinct neurotrophin stimulation regulates gene expression on multiple levels. Using a compartmentalized culture system to recapitulate *in vivo* spatial separation of sensory neuron cell bodies and axons, we examined transcriptional and post-translational changes selectively induced by cell body or axon neurotrophin stimulation. We find that neurotrophin stimulation of distal axons, an *in vitro* correlate of target-derived neurotrophin stimulation, preferentially upregulates and activates translation machinery components. Distal axon stimulation increases transcription of rRNAs and ribosomal protein transcripts, as well as causes acute post-translation modifications that promote protein synthesis. Together our studies suggest that axonal neurotrophin support preferentially upregulates the translational capacity of the cell, which may be a mechanism by which axonal neurotrophin stimulation promotes axon survival.

Our studies suggest that neurotrophin activation of axonal Trk receptors preferentially stimulates new ribosome biogenesis. However, it is unclear how a global increase in cellular translation could lead to the specific cellular response of axonal survival. We postulate that newly made ribosomes may be transported to the axon to promote local protein synthesis of pro-survival components such as *bclw* and *laminb2*[14]. This hypothesis extends previous findings that BDNF stimulates new ribosome biogenesis in hippocampal neurons to support neurite outgrowth, possibly through ribosomal localization to dendrites[174,176]. A second possibility is that specificity could be conferred by parallel and complementary changes, such as upregulation of pro-survival mRNAs or alterations in mRNA silencing and transport by RNA-binding proteins.

Here we show that target-derived neurotrophin stimulation also acutely regulates translation machinery through post-translational modifications. Phosphorylation of the small ribosomal subunit protein S6 enhances global mRNA translation[180]. We find that both axonal and cell body neurotrophin stimulation increase S6 phosphorylation (Figure 3.4B-D), suggesting a common mechanism of enhancing global protein synthesis. In contrast, phosphorylation of 4E-BP has a strong effect on translation of subsets of mRNAs[180,201-204], as 4E-BP is a translational repressor that binds and inhibits the initiation factor eIF4E, the limiting initiation factor for cap-dependent translation. Phosphorylation of 4E-BP causes release and derepression of eIF4E, permitting its association with the eIF4F complex and initiation of translation at the 5' untranslated region (UTR) cap of mRNAs[204]. Unlike phosphorylated S6, eIF4E promotes translation of specific pools of mRNAs[180,201-204]. eIF4E-sensitive mRNAs have long and highly structured 5'UTRs and encode targets governing cell survival, proliferation, and reactive oxygen species regulation[180,201,205]. A recent study defined a cysteine-rich motif within the 5'UTR of numerous eIF4E-sensitive mRNAs, and identified genes such as *laminb1* as eIF4E targets[205]. We find that retrograde neurotrophin signaling preferentially increases 4E-BP1 phosphorylation (Figure 3.4B-D). This result

identifies a mechanism by which target-derived neurotrophins may selectively upregulate translation of specific pro-survival mRNAs with long 5'UTRs.

In addition to cell survival, axonal neurotrophin stimulation also promotes a variety of other cellular functions, including axon guidance, growth, and branching. It is therefore also likely that selective enhancement of whole cell or axon-specific translation supports a general upregulation of proteins required for multiple cellular responses.

Here we establish a method to study the *in vivo* axonal translation of peripheral sensory neurons. While multiple studies have used genome-wide analyses to profile axonal mRNAs[48,206] or proteomics-based techniques to catalog locally synthesized proteins[183], these studies were limited to *in vitro* systems and had significant technical caveats. Ribosome purification was only very recently used to profile the *in vivo* axonal translatome of retinal ganglion cells[207], confirming the feasibility of this approach. TRAP purification of ribosome-associated mRNAS from the sciatic nerve would allow examination of a variety of questions, including how the sensory neuron axonal translatome changes across development and adulthood, how it differs among sensory neuron subtypes, and how it is modulated by acute or chronic exposure to neurotrophins via injection or genetic methods.

3.4 Methods:

All experimental procedures were done in accordance with the National Institutes of Health guidelines and were approved by the Dana-Farber Cancer Institutional Animal Care and Use Committee.

Animal use. Timed pregnant Sprague-Dawley rats were purchased from Charles River. NaV1.8-Cre mice[200] were a generous gift from David Ginty (Harvard University) and Qiufu Ma (Harvard University). flx-eGFP-L10a mice[208] were obtained from Jackson Laboratory (Stock number 024750; B6;129S4-Gt(ROSA)26Sortm9(EGFP/Rpl10a)Amc/J).

Cell culture. Compartmented chamber (Campenot) cultures were prepared as described previously[158] with modifications. Briefly, DRGs from embryonic day 15 (E15) rats of either sex were dissected and trypsinized. DRGs (1 x 10^5 cells) were plated in the center compartment of a Teflon divider[38] (Camp10, Tyler Research) affixed to a p35 culture dish coated with 0.71 mg/mL of collagen I (BD Biosciences). Cultures were maintained in media consisting of DMEM (Invitrogen) with 5% horse serum, 1% penicillin-streptomycin, and 0.3 μM cytosine arabinoside (AraC) at 37°C, 7.5% CO^2; BDNF + NGF (PeproTech) were added to the cell body compartment at a concentration of 10 ng/mL and to the axon compartment at a concentration of 100 ng/mL for 2d. On day 3, media was replaced and the AraC was omitted. On day 5 neurotrophins were removed from the cell body compartment and reduced to 1 ng/mL in the axon compartments for 3-5 days. For neurotrophin stimulation experiments, media was changed to DMEM without serum or neurotrophins for 2 hours, then neurotrophins (100 ng/mL NGF and 100 ng/mL BDNF) or vehicle (100 ng/mL BSA) were added for indicated time to either cell body compartment or axon compartments.

Mass cultures consisting of 3 x 10^5 E15 DRG neurons were grown on growth factor reduced Matrigel (1:45 in DMEM, BD Biosciences) coated p35 culture dishes in neurotrophin-enriched (100 ng/mL NGF + BDNF) media with 0.3 μg/mL AraC. On day 3, neurotrophins were reduced to 10 ng/mL) and cultures maintained for 3-6 more days.

Microarray analysis. DIV8 compartmented chamber cultures were maintained in DMEM with no NGF or BDNF for 2 hours and then stimulated for 2 hours with either vehicle (100 ng/mL BSA in PBS) or neurotrophins (100 ng/mL NGF [Preprotech] and 100 ng/mL BDNF [Peprotech]) at either the cell body compartment or the axon compartments. Cell body compartment RNA was collected in Trizol, extracted with Trizol/Chloroform method, DNAse treated, and analyzed using Affymetrix Rat 230 2.0 Array by the

Microarray Core Facility at Dana-Farber Cancer Institute. Raw .cel files were normalized and modeled in dChip Software using a file with representative brightness, and outputted intensity values were exported to Excel where zscores were calculated relative to total transcriptome intensity of that condition. The change induced by stimulation was calculated as the difference of: (zscore of neurotrophin stimulation - zscore of control stimulation). Genes were considered significantly upregulated in a given condition if the change was positive and greater than twice the standard deviation of change for that condition. Gene ontology (GO) analysis was performed using PANTHER GO slim (http://www.pantherdb.org)[209]. Translation gene categories from Figure 3.3D (e.g. ribosomal proteins or elongation factors) were manually identified. Data are from one biological replicate, with 12-16 cultures pooled per condition.

Translating ribosome affinity purification (TRAP). Cell lysis and TRAP protocol were performed as described previously[197] with modifications. Briefly, GFP-coated MyOne Streptavidin T1 Dynabeads (Invitrogen) were prepared by incubating (per IP) 120 µg biotinylated protein L (Pierce) and 300 uL beads for 35 minutes at room temperature. Beads were then washed with 3% BSA in PBS, incubated for 1 hour at room temperature in 0.15 M KCL buffer (20 mM HEPES-KOH pH 7.4, 5 mM MgCl2, 150 mM KCl, 1% NP-40) with high affinity monoclonal anti-GFP antibodies HtzGFP_04 (clone 19F7) and HtzGFP_02 (clone 19C8; Memorial Sloan-Kettering; 50 ug each per IP), and then washed in 0.15M KCl buffer + 3% BSA. E15 DRGs were grown as mass cultures and infected with AAV9-eGFP-L10a (supplied by Myriam Heiman, MIT) on DIV1. On DIV5, neurotrophins were removed from media for 2 hours, and then vehicle control (100ng/mL BSA in PBS) or neurotrophins (100ng/ml NGF and BDNF) were added for 2 hours. After stimulation, cycloheximide (Calbiochem; 100 µg/mL) was added to media for 15 minutes, then cells were washed with PBS + 100 ug/mL cycloheximide and lysed in buffer containing 20 mM HEPES-KOH pH 7.4, 150 mM KCl, 5 mM MgCl2, 1% NP-40, EDTA-free protease inhibitor, 0.5 mM DTT,

100 ug/mL cycloheximide, 20 U/µL rRNAsin (Fisher), 20 U/µL Superasin (Ambion). A post-nuclear superanatant was prepared by 2,000 x g centrifugation for 10 minutes at 4°C. 1/9 sample volume of DHPC (Avanti; 300 mM stock) was added to supernatant and the sample was incubated on ice for 5 minutes. A post-mitochondrial supernatant was then prepared by 16,000 x g centrifugation for 10 minutes at 4°C, and used for anti-GFP immunoprecipitation. Per IP, 200 µL of anti-GFP beads were combined with post-mitochondrial supernatant overnight at 4°C. After incubation, beads were washed in 0.35 M KCl buffer (20 mM HEPES-KOH pH 7.4, 5 mM MgCl2, 350 mM KCl, 1% NP-40, 0.5 mM DTT, 100µg/mL cycloheximide) and eluted in 100 uL Lysis Buffer with B-ME from RNeasy Micro kit (Qiagen). RNA cleanup and on-column DNase treatment as performed as per manufacturer's instructions. Purified RNA was analyzed by Agilent Pico Bioanalyzer for RNA integrity number (RIN) and assessed by qRT-PCR.

Quantitative reverse transcription-PCR. RNA was extracted from DRG neurons in compartmented cultures using TRIzol (Invitrogen) according to manufacturer's protocol. Reverse transcription (RT) was performed using the cDNA archive kit (Applied Biosystems) according to the manufacturer's protocol, and quantitative real-time RT-PCR was performed using Taqman Gene expression assays (Applied Biosystems) to analyze expression of 18s rRNA (Rn03928990_g1), 28s rRNA (Rn03034784_g1), *cfos* (Rn02105452_S1), and *bclw* (Rn00821025_g1). Note that 28s rRNA probe has possible cross-reactivity with the rRNA promoter binding protein ribin, a gene identified by our microarray analysis as upregulated by both cell body and axon neurotrophin stimulation; however, by qRT-PCR the 28s rRNA probe is only induced by axonal neurotrophin stimulation. Data were normalized to *gapdh* (glyceraldehyde-3-phosphate dehydrogenase; Applied Biosystems) for rRNA studies and to total RNA concentration for TRAP studies.

Protein analysis. For studies of phosphorylated translation machinery, compartmented cultures were lysed in nonionic detergent with phosphatase inhibitors (500 mM NaF, 100 mM PMSF, 200 mM Na_3VO_3). or eGFP-L10a immunoprecipitation, L3-L5 DRGs from P15 Cre positive or Cre negative flx-eGFP-L10a/NaV1.8Cre mice were homogenized in RIPA lysis buffer and incubated overnight at 4°C with anti-GFP-coated MyOne Streptavidin T1 Dynabeads (Invitrogen; 200 µL beads per IP) prepared as described above. The next day beads were washed in 0.35M KCl solution + 3% BSA, then eluted by boiling in sample buffer, RIPA lysis buffer, and reducing agent. Lysates were separated by 4-12% Bis-Tris SDS-Page (Thermo Fisher Scientific), and probed with the following antibodies: anti-S6 ribosomal protein (1:1000; clone 54D2; Cell Signaling), anti-phospho-S6 ribosomal protein Ser235/236 (1:1000; Cell Signaling), anti-4E-BP1 (1:1000; Cell Signaling), anti-phospho-4E-BP1 (1:1000; Cell Signaling), anti-GFP (1:1000, Abcam). Bands were visualized with secondary antibodies conjugated to HRP (1:10,000; Bio-Rad) and chemiluminescent substrate signal. Using NIH ImageJ software, protein levels were quantified and levels of protein were normalized to GAPDH.

DRG wholemount immunostaining. L3-L5 DRGs and the attached peripheral and central nerves from P15 Cre positive or Cre negative flx-eGFP-L10a/NaV1.8Cre mice were dissected and fixed overnight in 4% PFA at 4°C. DRGs were then permeabilized 1 hour at room temperature in 0.5% TritonX-100 in PBS, blocked for 4 hours at room temperature in 5% BSA + 0.1% TritonX-100, and then incubated for four days at 4°C with anti-Tuj1 (1:300; Covance) and anti-GFP (1:1000; Abcam). DRGs were washed in 0.1% TritonX-100 in PBS three times for 15 minutes each at room temperature, then washed overnight at 4°C. The next day DRGs were incubated 2 hours at room temperature with donkey anti-rabbit AlexaFluor 647 (1:500; Invitrogen) and goat anti-mouse AlexaFluor 568 (1:500; Invitrogen), counterstained with DAPI, and mounted. DRGs were imaged using a Nikon Ni-E Confocal with a 20x 0.75NA objective, and eGFP-

L10a expression was confirmed by both anti-GFP signal (647 nm excitation) and eGFP-L10a fluorescence (488 nm excitation). Images shown are single z-planes with endogenous eGFP-L10a signal.

Statistics. Data are expressed as mean ± SEM. To assess statistical significance, data were analyzed by ztests or unpaired two-tailed Student's *t* test. For multiple comparisons, data were analyzed by one-way ANOVA with *post hoc* Bonferroni correction. Significance was placed at $p < 0.05$ unless otherwise indicated.

Chapter 4

Discussion

Dorsal root ganglia sensory neurons are highly polarized cells with distinct cell body and axonal subcellular compartments. The axon of these neurons can be up to a meter in length in humans, more than 10,000 times the diameter of the cell body[210]. This extreme polarization poses a unique challenge to integrate signals received at distant parts of the cell, respond in a timely and spatially appropriate manner, and maintain the health and functionality of a vast axonal compartment. In this dissertation I describe investigations into the mechanisms critical for axon survival of sensory neurons. The broad goal of these studies was to define molecular mechanisms responsible for protecting axons both during development and in response to injury. In Chapter 3, I describe a novel mechanism by which retrograde neurotrophin signaling enhances the translational capacity of the neuron. The ability of neurotrophins to promote protein synthesis machinery may enable specific axon survival or growth responses. In Chapter 2, we examined neurotoxicity of the common chemotherapeutic agent paclitaxel and defined a role for the pro-survival protein Bclw in preventing paclitaxel-induced axon degeneration. These studies illustrate how spatially restricted events within the axon, such as Bclw regulation of axonal IP_3R1 or activation of axonal Trk receptors, can promote and maintain axon viability. In addition, we establish tools such as local expression of the Bclw BH4 SAHB peptide or TRAP methodologies that can be used to further probe mechanisms of axonal survival.

A major question we sought to address in these studies is how spatially distinct extracellular cues can mediate differential cellular responses. Neurotrophin stimulation at the axonal process initiates both local signaling cascades and a retrograde signal that travels to the remote cell body. This retrograde signal is transmitted by endosome-trafficked activated Trk receptors and promotes axonal survival[165,211]. In contrast, local neurotrophin stimulation at the cell body is incapable of supporting axon survival[38]. This differential response ensures that only axons that successfully innervate their target regions survive. Previous studies have shown that axonal neurotrophin stimulation engages unique signaling pathways in the cell body, as cell body stimulation activates the mitogen-activated protein

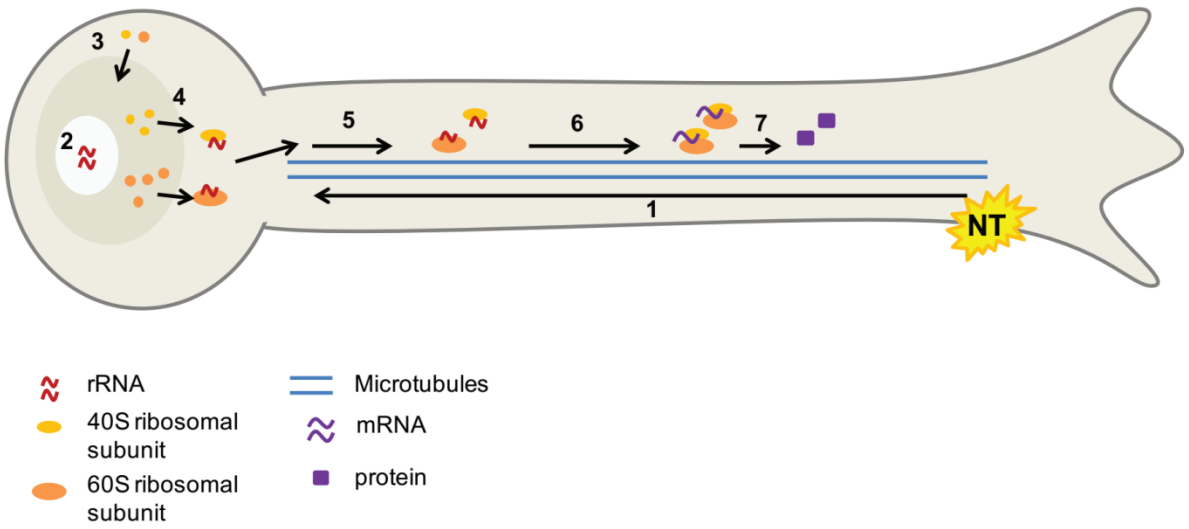


Figure 4.1. Model of retrograde neurotrophin-induced localization of newly made ribosomes to axons. Target-derived neurotrophins (NT) activate axonal Trk receptors, which are internalized and retrogradely trafficked to the cell body (1), triggering rRNA synthesis in the nucleolus (2) and transcription, translation, and nuclear import of ribosomal proteins (3). rRNA and ribosomal proteins are assembled into small (40S) and large (60S) ribosomal subunits in the nucleolus and nucleus and then exported into the cytoplasm[212,213] (4). Ribosomal subunits and/or ribosomal proteins are transported anterogradely into the axon (5) where they assemble and translate axonal mRNAs into protein (7).

(MAP) kinase ERK1/2 while axonal stimulation activates both ERK1/2 and ERK5[43]. Our studies suggest further response specificity is conferred by axonal neurotrophin stimulation preferentially increasing translation machinery components at both the transcriptional and post-translational levels. We propose that this increase supports axonal growth and survival by increasing the translation capacity of the cell and/or axonal compartment.

Our data suggest retrograde neurotrophin stimulation may initiate new ribosome biogenesis. These newly made ribosomes may be trafficked anterogradely into the axon to promote local protein synthesis within the axon (Figure 4.1). Alternatively, individual ribosomal subunit proteins, not yet fully assembled into the large and small ribosomal subunits, may be localized to axons. These individual ribosomal subunit proteins could then be inserted locally into axonal ribosomes, either as a repair

mechanism[214,215] or as a way to create functionally specialized ribosomes with unique protein compositions or modifications[213,215-217].

Intra-axonal protein synthesis solves several challenges created by the extreme polarity of neurons. One major challenge is the need for appropriate levels and localization of proteins responsible for the structural and functional integrity of the axon. Local protein synthesis within the axon provides a fast and highly localized method for the axon to dynamically regulate its protein composition. Extracellular or intracellular cues can induce mRNA localization to axons, as occurs with neurotrophin-regulated axonal recruitment of pro-survival *bclw* and *laminb2* mRNAs[14]. Alternatively, these signals can utilize mRNA already present in the axon by releasing translationally repressed mRNAs[215] or translation machinery tethered to the plasma membrane[189]. Many studies have demonstrated a functional requirement for intra-axonal translation of specific mRNAs. For example, neurotrophin-dependent axon survival requires local translation of *bclw*[2]; netrin 1- or BDNF-dependent growth cone attraction requires local translation of *β-actin*[196,218]; and NGF-dependent axonal outgrowth requires local translation of *par3*[219]. Our hypothesis that retrograde neurotrophin signaling may recruit ribosomes or ribosomal proteins to axons could provide a mechanism by which target-derived neurotrophins enhance intra-axonal translation, perhaps in a highly localized manner, and thereby support axonal survival and growth. TRAP-based profiling of the *in vivo* sensory axon translatome would provide further insight into the dynamic regulation of axonal translation in an intact circuit from development through adulthood.

A major limitation of these experiments is that levels of translation machinery may or may not correlate with enhanced translational capacity. Further studies should directly address whether target-derived neurotrophins preferentially increase protein synthesis and whether this effect is global or localized to the axon. Additionally, while our focus was primarily on axonal survival mechanisms, it is likely that the observed changes in translation machinery contribute to other cellular mechanisms such as axon growth, axon branching, and cell body survival. Future experiments will be required to

demonstrate that a neurotrophin-dependent increase in translational capacity is required for any of these cellular functions.

Together our findings suggest retrograde neurotrophin signaling regulates sensory neuron translation at multiple levels. We propose a model wherein a retrograde neurotrophin signal activates mTOR (mechanistic target of rapamycin) pathways in the cell body via Ras/ERK and/or PI3K (phosphatidylinositol 3-kinase) cascades[165,180] and thereby promotes translation machinery (Figure 4.2). Activated mTOR phosphorylates 4E-BP, thus derepressing eIF4E-dependent translation, and phosphorylates S6 to increase global translation[180]. Retrograde neurotrophin signaling also initiates a more long-term transcriptional response, where mTOR signaling increases rRNA synthesis through the transcription initiation factor IA (TIF-IA)[180,220]. In parallel, Ras/ERK/Myc[179,221-223], mTOR[224,225], or another signaling cascade increases transcription of genes encoding translation machinery components, which would then be translated and assembled with rRNA into new ribosomes. Altogether we propose this cascade of events would increase translation of components necessary for the target-derived neurotrophin-dependent responses of axon growth and survival.

Importantly, our studies identify Bclw as a critical overlapping component between axon survival pathways in both development and pathology. Previous work from our lab and others has shown that Bclw is required for neurotrophin-dependent axon survival[2,14,44]. However, in a developmental context, Bclw appears to function by inhibiting Bax and thereby preventing subsequent activation of the caspase cascade. The closely related component $Bclx_L$ also promotes neurotrophin-dependent axon survival by this mechanism[44]. In contrast, we find that only Bclw can prevent paclitaxel-induced degeneration. We instead identify a novel and unique protective mechanism for Bclw, where Bclw acts as a brake on calpain-mediated pathological axon degeneration by interactions with the endoplasmic reticulum channel IP_3R1.

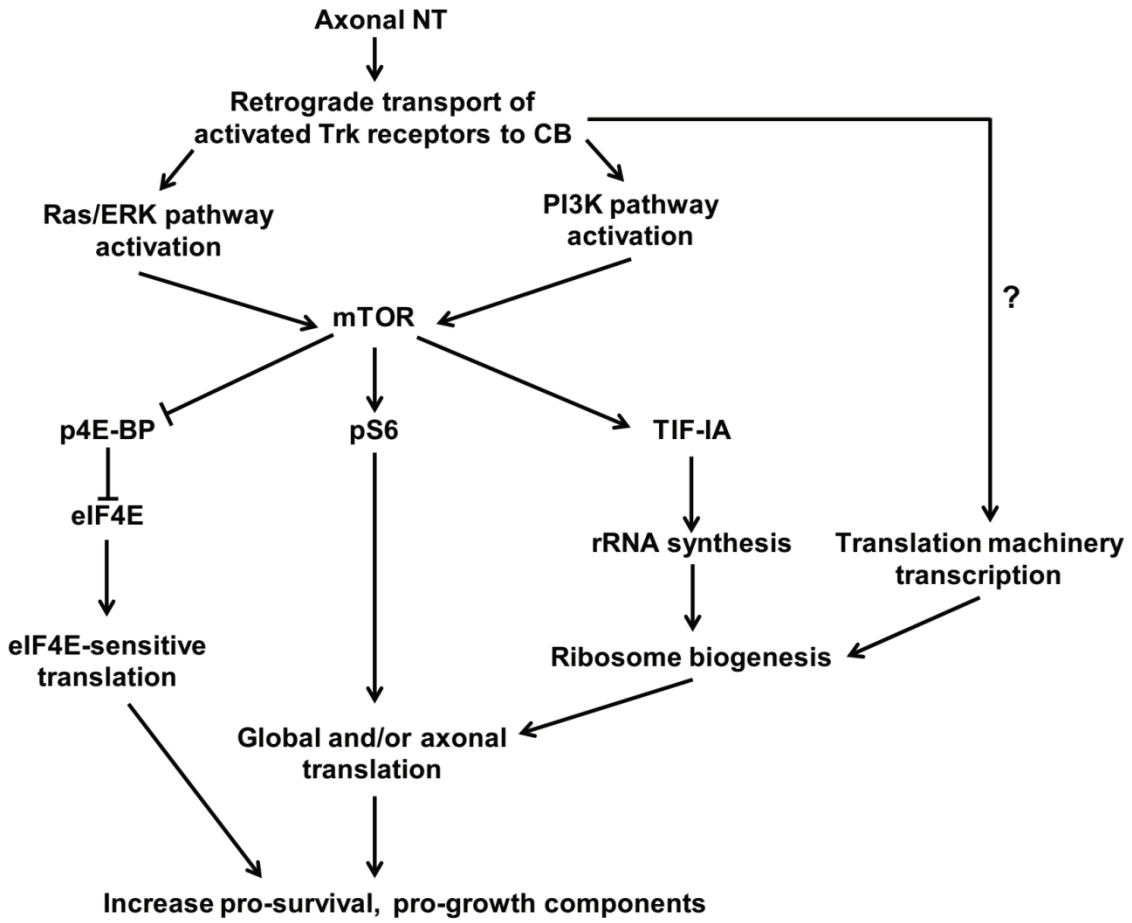


Figure 4.2. Model of retrograde neurotrophin-induced upregulation of translation. Axonal neurotrophins cause retrograde trafficking of endocytosed and activated Trk receptors[211], to the cell body, activating mTOR via Ras/ERK or PI3K pathways[165,180]. The mTOR complex then phosphorylates 4E-BP and derepresses eIF4E-dependent translation; phosphorylates S6 to increase translation; and activates rRNA synthesis via TIF-IA[180,220]. In parallel, there is increased transcription of genes encoding translation machinery components, which may facilitate new ribosome synthesis to increase global and/or axonal translation, and ultimately there is increased translation of pro-survival and pro-growth components.

Our studies show that paclitaxel induces degeneration by reducing endogenous levels of axonal Bclw. While we have described the downstream degenerative consequences of this reduction, further studies will be required to identify the mechanism by which paclitaxel reduces axonal Bclw. As this regulation is reflected in both the mRNA and protein levels of axonal Bclw, and as our lab has shown that Bclw is locally synthesized in axons[2], we propose that the primary effect of paclitaxel is to reduce *bclw* mRNA in axons and that decreased protein levels is a secondary consequence of the reduced mRNA. Paclitaxel could reduce axonal *bclw* mRNA by altering either the anterograde transport of *bclw* mRNA into axons or the local stability of *bclw* mRNA in axons. It is likely that paclitaxel alters appropriate transport of *bclw* (Figure 4.3), as paclitaxel been shown to impair axonal transport[115,149-152]. In addition, the half-life of *bclw* mRNA in cultured DRG neurons is 5.15 hours, which is consistent with the idea that anterograde transport of new *bclw* mRNA would be required within the 24 hour paclitaxel treatment period to replenish degraded mRNAs[2]. Alternatively, impairment of axonal *bclw* by paclitaxel could occur by modulation of components and signals required for axonal *bclw* localization such as the RNA-binding protein SFPQ[14]. JNK, a kinase known to be activated by axonal injury[18] and to interact with SFPQ[226] could phosphorylate SFPQ and alter its ability to bind *bclw* and transport it to axons. This hypothesis is particularly intriguing because it could explain the specific reduction in *bclw* mRNA relative to other axonal mRNAs such as *β-actin*.

In these studies we establish clinically relevant models of paclitaxel-induced axon degeneration both *in vitro* and *in vivo*. The effective cytotoxic dose of paclitaxel on cultured human cancer cells lines is less than 50 nM, with higher concentrations actually causing an increase in cancer cell survival[227]. In addition, paclitaxel steady state plasma concentrations of ovarian cancer patients 2-24 hours after treatment ranges from 21-860 nM[228]. While some studies on cultured monolayer cells use micromolar concentrations of paclitaxel, we used a low concentration of paclitaxel (30-60 nM) *in vitro* to better approximate clinical exposure. A wide range of *in vivo* dosages have been used in mouse models and

cause different phenotypes. Low paclitaxel dosages (8-18 mg/kg cumulative dose) cause loss of only the nerve endings in the epidermis and sensory abnormalities without motor deficits[113,145,229-231]. Conversely, high paclitaxel dosages (24-180 mg/kg cumulative dose) cause loss of axons within the sciatic nerve, both sensory and motor behavioral effects, and general health impairments such as weight loss and increased mortality[122,123,232-235]. The low dosage paclitaxel rodent model better recapitulates symptoms seen in patients, where paclitaxel primarily causes a sensory and not motor neuropathy[115]. We therefore treated mice with a low, repeated dose regimen of 4 mg/kg paclitaxel every other day for a week, for a cumulative dose of 16 mg/kg. With this regimen, mice exhibited sensory and not motor deficits and did not exhibit weight loss or increased mortality (data not shown).

While our studies demonstrate that paclitaxel causes deficits in somatic IP_3-gated calcium flux and activates calpain proteases, we have a limited understanding of the pro-degenerative calcium changes caused by paclitaxel. Experiments were performed to examine potential changes in axonal calcium signaling, including alterations in resting axoplasmic calcium at multiple durations of paclitaxel treatment, changes in ATP-induced IP_3R functionality in axons, and changes in axonal ER calcium content via thapsigargin treatment. No significant deficits were observed in axons with the tested parameters (data not shown). However, paclitaxel-induced changes in axonal calcium signaling could be difficult to detect with the methods used. Alternatively, paclitaxel could cause changes in calcium flux at contact sites between ER and mitochondria, which would not be detected by cytoplasmic calcium imaging methods.

Our identification of Bclw-IP_3R1 interactions as critical for preventing paclitaxel-induced degeneration evokes the larger question of how this mechanism relates to other identified components of the pathological axon degenerative cascade. The requirement of IP_3R1 for axon degeneration induced by mechanical[12], chemotoxic[12] (Figure 2.3), and ischemic injuries[236] suggests Bclw may be capable of preventing axon degeneration caused by diverse insults. We propose a model integrating our findings

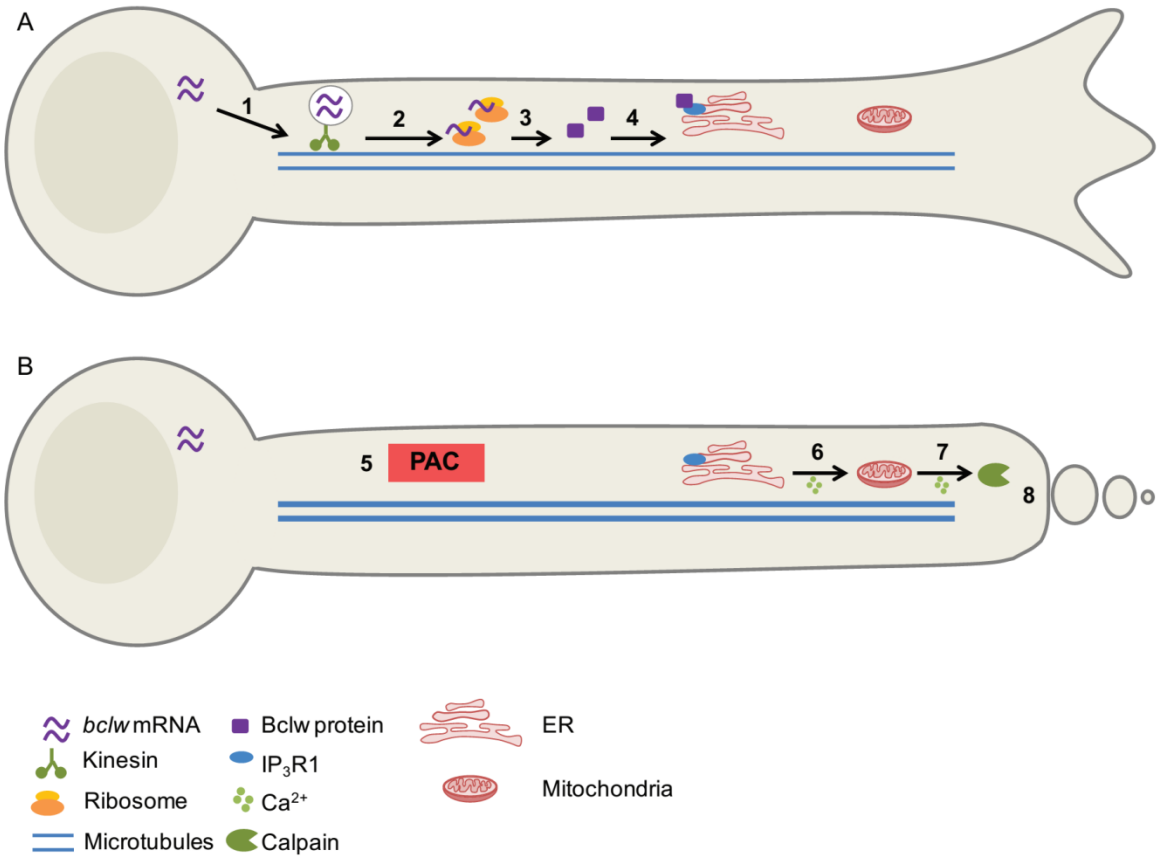


Figure 4.3. Model of paclitaxel-induced loss of axonal Bclw resulting in axon degeneration. (A) Under healthy conditions, *bclw* mRNA is transcribed in the cell body and packaged into RNA granules (1), then trafficked out into the axon (2) where it is locally translated into Bclw protein (3). Bclw protein binds and regulates IP_3R1 on axonal endoplasmic reticulum (ER; 4). **(B)** Paclitaxel treatment of axons (5) impairs *bclw* transport out to axons, reducing Bclw mRNA and protein levels, causing deregulation of IP_3R1 and pro-degenerative calcium signaling (6) that impairs mitochondrial function and calcium buffering (7) and ultimately activates calpain proteases that mediate axon fragmentation (8).

with the current view of SARM1/MAPK/NMNAT interactions (Figure 4.4). Injury activates a MAPK cascade via SARM1, which involves kinases DLK and JNK and results in ATP depletion[18]. NMNAT or the fusion protein Wld^S, which converts NMN into NAD^+, inhibits activation of this MAPK cascade[18], but injury leads to NMNAT degradation[68] via the Phr1 ubiquitin ligase[110,237]. Loss of NMNAT results in NAD^+ depletion and reduced ATP levels[68], and consequent NMN accumulation also activates SARM1[61,78,238]. We propose that JNK-mediated phosphorylation of the *bclw*-binding protein SFPQ[14] may prevent appropriate trafficking of *bclw* to axons, thereby deregulating IP_3R1-gated ER calcium signaling, causing mitochondrial impairment and further intracellular calcium release, and subsequently activating the calcium-dependent protease calpain. Ultimately, these convergent and parallel pathways result in calpain-mediated axon degeneration.

Together the experiments presented in this dissertation identify specific mechanisms governing axon viability. These findings have important implications for understanding the molecular mechanisms of axon survival both during development and under pathological conditions, and also suggest a potential preventative therapy for paclitaxel-induced axon loss.

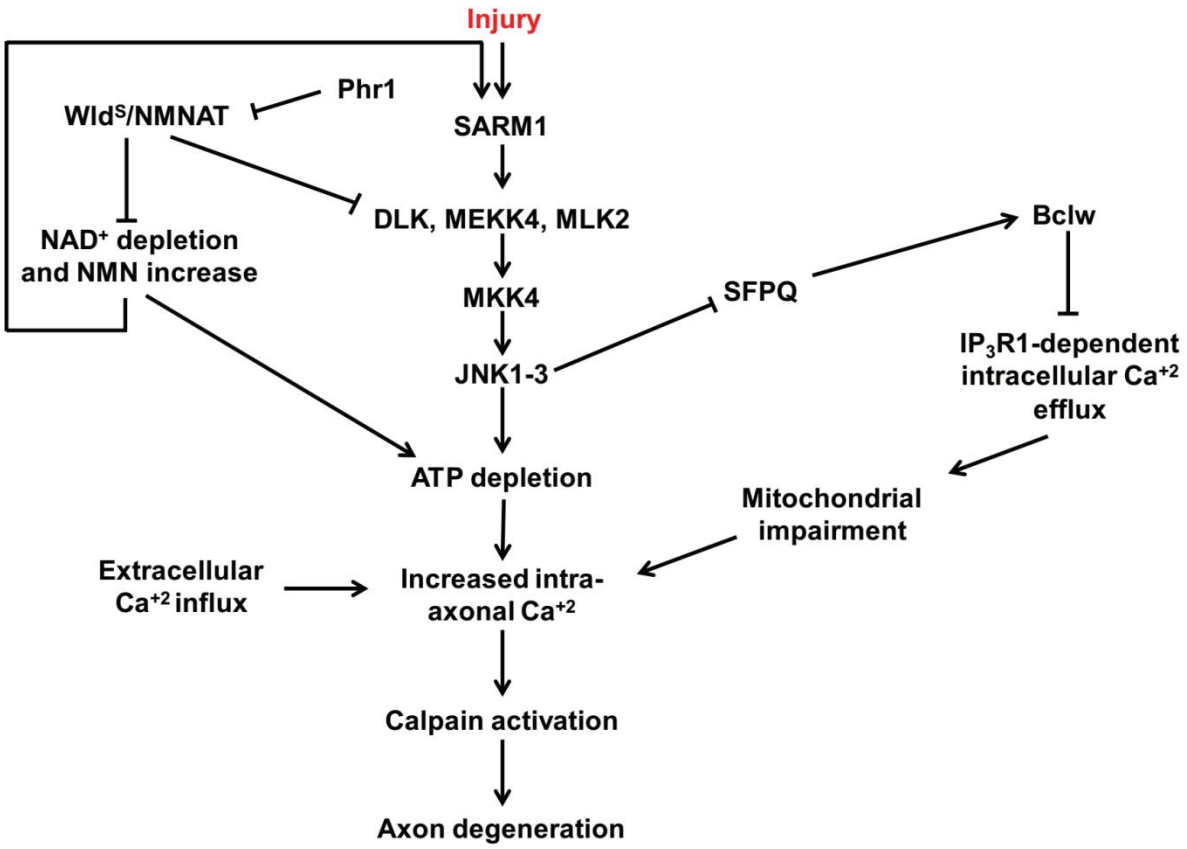


Figure 4.4. Model of pathological axon degeneration. Injury activates a MAPK cascade via SARM1[18] and decreases NMNAT levels[68] via the Phr1 ubiquitin ligase[110,237]. Decreased NMNAT causes a reduction in NAD^+ [71], which contributes to ATP depletion, and an accumulation of NMN, which contributes to SARM1 activation in a mechanism dependent on extracellular calcium influx[61,78,238]. NMNAT or the fusion protein Wld^S opposes activation of the MAPK cascade, which involves activation of JNK and ATP depletion[18]. JNK has been shown to interact with SFPQ[226], the RNA-binding protein that facilitates transport of *bclw* mRNA to axons[14]. Potential JNK phosphorylation of SFPQ could impair SFPQ binding to *bclw* and thereby reduce axonal Bclw levels. Loss of Bclw deregulates IP_3R1 at the ER and leads to pro-degenerative calcium dynamics that impair mitochondrial function and may induce further calcium efflux via the mitochondrial permeability transition pore (mPTP)[81]. Together with extracellular calcium influx[29,86-88], intracellular calcium efflux[12] increases intra-axonal calcium levels and activates the calcium-dependent protease calpain, which cleaves cytoskeletal proteins and causes fragmentation of the injured axon[29].

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