Coordination of Axon Degeneration in the Peripheral Nervous System

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Abstract

Neuronal axon degeneration is a hallmark of all neurodegenerative disorders including Alzheimer's, Luo Gehrig's, and Huntington's disease, as well as neural injury (*e.g.* stroke, traumatic brain injury and spinal cord injury). Moreover, loss of axons and synapses via degeneration is thought to be a main cause of cognitive decline, movement disorders, and paralysis associated with these pathologies. Despite their importance as therapeutic targets, very little is known about the mechanisms underlying axon loss. Beyond pathological situations, axon degeneration is also an important aspect of nervous system refinement during development. One particular aspect of developmental degeneration that has yet to be discovered in injury-induced degeneration, is the ability to respond to axon derived extrinsic factors. My thesis work addressed this question and establishes that axon degeneration is promoted by TNFR family members and is also coordinated via extrinsic factors.

Recent studies have revealed that members of the Tumor Necrosis Factor Receptor (TNFR) superfamily are required for developmental degeneration in Peripheral Nervous System (PNS) and Central Nervous System (CNS). However, the involvement of a cell surface receptor such as a TNFR family member has not been described previously for Wallerian Degeneration. The pathways underlying Wallerian degeneration have also long been obscured in part because of a series of long held and incorrect assumptions including that axon degeneration occurs in an axon autonomous manner.

In this thesis, I sought to determine whether the TNFR family members are involved in injury-induced degeneration. Using an in vitro microfluidic injury paradigm we assessed the neurons lacking various TNFR family members. We found that loss of TNFR family member Death Receptor 6 (DR6) delay Wallerian degeneration. I also discuss my in vivo findings, which employ electron microscopy to study the degeneration of transected sciatic nerves in different TNFR family member knockout mice. Further, I show that DR6 feeds in to the same axon degeneration downstream pathway by phosphorylation of MAPK member JNK. Additionally, I demonstrate that abberant myelination in DR6 knockout mice and full protection of axon degeneration in $DR6^{-/-}$;$p75NTR^{-/-}$ mice, indicating uncoupling of axon and myelin degeneration processes. This work demonstrates the first described receptor involved in Wallerian degeneration and it's downstream pathway. Furthermore, this finding indicates that there are other signaling pathways, which would involve these cell surface receptors to coordinate axon degneration.

To identify the coordination factors in Wallerian degeneration, I employed a conditioned media experiment with injured axons. My experiments demonstrate that calcium, secreted from injured axons, functions as an extrinsic factor, which coordinates axonal degeneration of neighbors in a DR6-dependent autocrine/paracrine manner. Interestingly, in the event of an injury, the calcium stores in the axons (ER and mitochondria) release calcium to the cytoplasm, which in turn gets released to the outside milieu. Taken together, these findings suggest that injured nerves coordinate their degeneration via calcium release and death receptor signaling.

In conclusion, this work establishes the notion that DR6 promotes Wallerian degeneration and MAPK/JNK signaling is involved in the downstream pathway. Furthermore, axon degeneration is coordinated by extrinsic calcium via DR6. However, several open questions remains to be addressed such as, the mechanism of calcium entry and release, the upstream/downstream components of Wallerian Degeneration pathway with respect to DR6 and other factors/ligands that affect DR6.

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Chapter 1

Introduction

Elimination of axons, which are incorrectly targeted or diseased, is a common feature in nervous system development, trauma and neurodegenerative disease. During nervous system development, an excess of neurons are born and many more branches are created than are required. At later stages of development these excess neurons and axon branches are removed leaving the "strongest" connections intact. Several distinct modes of axon degeneration are apparent from basic morphological changes in the axons such as axon retraction, granular fragmentation of axon branches, which are then cleared by local phagocytes. The mechanism for the pruning process has been the subject of investigation for decades. Both pathological and developmental degeneration share morphological characteristics including beading and blebbing of axonal membranes as well as fragmentation and breakdown of the cytoskeleton (Martin et al., 1988; Raff et al., 2002). Consistent with this idea, neurodegenerative diseases such as Alzheimer's, Parkinson's, Amyotrophic Lateral Sclerosis and Multiple Sclerosis (MS) display axon loss morphologically indistinct from developmental degeneration (Coleman and Perry, 2002; Luo and O'Leary, 2005). Preservation of axonal structure has become an important therapeutic target for these disorders (Coleman and Perry, 2002; De Vos et al., 2008; Luo and O'Leary, 2005; Wang and Barres, 2012).

Pathological axon degeneration occurs after an insult to the neurons such as an injury

(spinal cord injury, traumatic brain injury), exposure to toxic substances (Aβ, alpha-synuclein) to cause neurodegenerative diseases, or chemotherapies like vincristine. Given the similarity in morphological breakdown, it stands to reason that these distinct degeneration etiologies have convergent pathways, an understanding of which would be essential for rationalizing therapeutic solutions for neurodegenerative diseases.

Developmental Axon degeneration

Unlike Wallerian degeneration, developmental degeneration has been a subject of many studies. Relevant to this proposal, studies have been carried out to confirm that developmental axon pruning involves the death receptor p75-NGFR and one of its ligands Brain-Derived Neurotrophic Factor (BDNF)(Singh *et al.*, 2008; Park *et al.*, 2010). Developmental cell death and axonal loss appears to be driven by a competition between antagonistic trophic and "punishment" signaling pathways, which promote axon stabilization or degeneration, respectively (Deppmann et al., 2008; Singh et al., 2008). Interestingly, the punishment pathways appear to rely on autocrine/paracrine mechanisms allowing "strong" axons to expedite the elimination of weaker neighbors. Highly related Tumor Necrosis Factor Receptor Super Family (TNFRSF) members, p75 neurotrophin receptor (p75NTR), Tumor Necrosis Factor Receptor 1a (TNFR1a) and Death Receptor 6 (DR6), have been implicated in this extrinsic degeneration program in the peripheral nervous system (PNS) (Olsen et al., 2014; Park et al., 2010; Singh et al., 2008; Vilar et al., 2009; Wheeler et al., 2014). For example, mice deficient in p75NTR or its ligand, brain derived neurotrophic factor (BDNF), display hyper-innervation of sympathetic target organs suggesting that p75NTR and BDNF are essential for axonal pruning in vitro

and in vivo (Lee et al., 1994; Singh et al., 2008). Finally, similar to p75NTR, DR6 has been shown to promote axon degeneration in developmental paradigms (Olsen et al., 2014). The notion of paracrine mechanisms being employed in injury contexts has gone unexplored perhaps because of the long-standing assumption that these processes are axon-autonomous.

The molecular pathways underlying local axon degeneration in development is well documented (Figure 1). Trophic factor deprivation (NGF deprivation) in sympathetic and nociceptive sensory neurons leads to the activation of tumor necrosis factor (TNF) receptor family members such as DR6 and p75NTR and this receptor signaling leads to the upregulation and release of pro-apoptotic Bax (Bcl2-associated X protein) from mitochondria, which triggers the activation of initiator caspase 9 (Casp9) and effector caspases 3 and 6. Bcl-w (B cell lymphoma w) prevents pruning by inhibiting Bax (Cusack *et al.*, 2013). The role of cytochrome c (Cyt c) release from mitochondria in local axon degeneration remains unclear. Recently, Tessier-Lavigne and colleagues showed that the cell body also plays a role in gating axonal degeneration via caspase activation, despite the evidence for axonal apoptotic machinery in development (Simon *et al.*, 2016). They showed that loss of trophic support initiates a retrograde activation of JNK signaling (loss of AKT) in order to increase the expression of an anterograde pro-degenerative factor Puma in the cell body. Puma, overcomes the inhibition by pro-survival factors Bcl-xL and Bcl-w and initiates axon degeneration indicating an important role for cell body in developmental axon degeneration (Simon *et al.*, 2016).

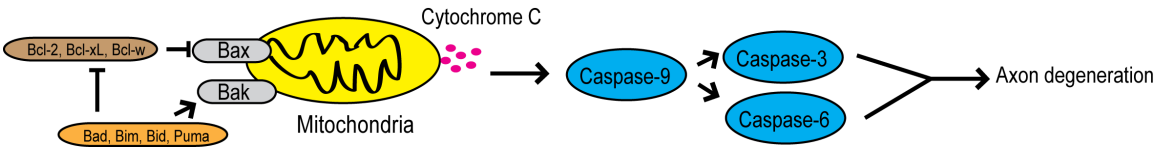

Figure 1: Axon degeneration in trophic deprivation. When neurons are deprived of trophic factor NGF, the mitochondrial pro-apoptotic pathway is activated (Bad, Bim, Bid & Puma) and anti-apoptotic members are inhibited (Bcl-2, Bcl-xL, Bcl-w). Activation of Bax releases Cytochrome-c and activates downstream initiator and effector caspases to lead to axon degeneration.

Wallerian Degeneration

Wallerian Degeneration (WD) is defined as the degeneration of the axon distal to the site of sever or crush. This term was coined after Augustus Waller's seminal observation of the progressive degeneration of a severed frog nerve in 1850 (Waller, 1850). This type of degeneration is conserved from insects to vertebrates and a variety of insults such as injury from trauma, toxic, inflammatory and hereditary syndromes result in classic WD pathways. Axonal events such as granular disintegration of the axonal cytoskeleton, fragmentation and degeneration of myelin are characteristic to this type of degeneration (Raff, Whitmore and Finn, 2002; Coleman and Freeman, 2010). Furthermore, the clearance of the axonal and myelin debris is also a part of this process and is being extensively studied. Controversy of whether the latter process is actually a part of "Wallerian Degenereration" remains unresolved (Vargas and Barres, 2007). While many groups attribute the clearance event as a part of Wallerian degeneration, demyelination and axon degeneration occur so rapidly and it has not been studied extensively to

determine whether they are mechanistically the same process. Furthermore, the Wallerian Degeneration slow mutants reported to date have historically spared not only the axons but also myelin from degenerating, leading to the belief that demyelination and axon degeneration are not two distinct processes.

A great deal of work has examined the degeneration of axons after injury. Progressive fragmentation and loss of axons associated with nerve injury (WD) was once thought to be the result of passive wasting away (Waller, 1850). However, the seminal finding that a spontaneous mutation in mice, referred to as slow Wallerian degeneration (*Wld*^{s}), results in injured axons that degenerate 10 times slower than wild-type animals, suggests that this process is actively regulated (Lunn *et al.*, 1989; Brown, Lunn and Perry, 1992; Araki, Sasaki and Milbrandt, 2004; Hoopfer *et al.*, 2006). This protection in Wld^{s} is due to an autosomal dominant fusion protein comprised of full-length nicotinamide mononucleotide adenylyltransferase-1 (NMNAT1) and a 70 amino acid portion of the ubiquitin ligase (Ube4b), which confers a neomorphic gain of function mutant (Conforti *et al.*, 2007). The resultant chimeric protein localizes primarily in the nucleus and to a lesser extent in axons, possibly increasing nicotinamide adenine dinucleotide (NAD^{+}) levels and thus acting as a molecular modulator to delay axon degeneration after injury (Wang *et al.*, 2015). More recently, the first loss of function mutant was discovered, sterile alpha and armadillo motif 1 (Sarm1), where injury-induced axon degeneration is blocked as robustly as in the *Wld*^{s} mice (Osterloh *et al.*, 2012). Indeed, these pathways appear to be related since Sarm1 initiates the local axon degeneration process by breaking down NAD^{+} after injury (Gerdts *et al.*, 2015). Although Sarm1 was first described as a

toll-like receptor (TLR) adaptor protein in the context of provoking immune responses, a receptor has yet to be linked to Sarm1 in the context of injury-induced axon degeneration (Mink *et al.*, 2001; Gohda, Matsumura and Inoue, 2004).

Wallerian Degeneration Signaling Pathway

The discovery of Wallerian Degeneration Slow mouse (*Wld*^{S}) suggested that the degeneration of injured axons is not simply due to loss of trophic support from the cell body, but a pathway triggered by intrinsic signals in the axons. Furthermore, this gain of function mutation *Wld*^{S}, can suppress axon degeneration in other neurodegeneration modalities such as traumatic brain injury, peripheral nerve injury, Glaucoma, Hypoxic ischaemic injury and some Parkinson's disease models suggests that a common mechanism might regulate axon degeneration in these different conditions(Conforti, Gilley and Coleman, 2014).

Although different etiologies of axon degeneration show similar morphological characteristics including membrane blebbing and cytoskeletal fragmentation, Wallerian degeneration does not involve the classic apoptotic pathway. Previous reports show that BAX, caspases and necroptotic pathway components are not involved in injury-induced axon degeneration (Finn *et al.*, 2000; Simon *et al.*, 2012; Yang *et al.*, 2015).

Tessier-Lavigne and colleagues recently showed a systematic investigation of MAPK family members in pathological axon degeneration (Yang *et al.*, 2015). They showed that as an early degenerative response, Sarm1 activates the MAPK pathway, which in turn causes an energy deficit in the axon before activating calpains and breaking down axonal

structures. Furthermore, they showed that NMNAT1 and AKT signaling could inhibit this pathway, which leads to JNK activation (Yang *et al.*, 2015) (Figure 2).

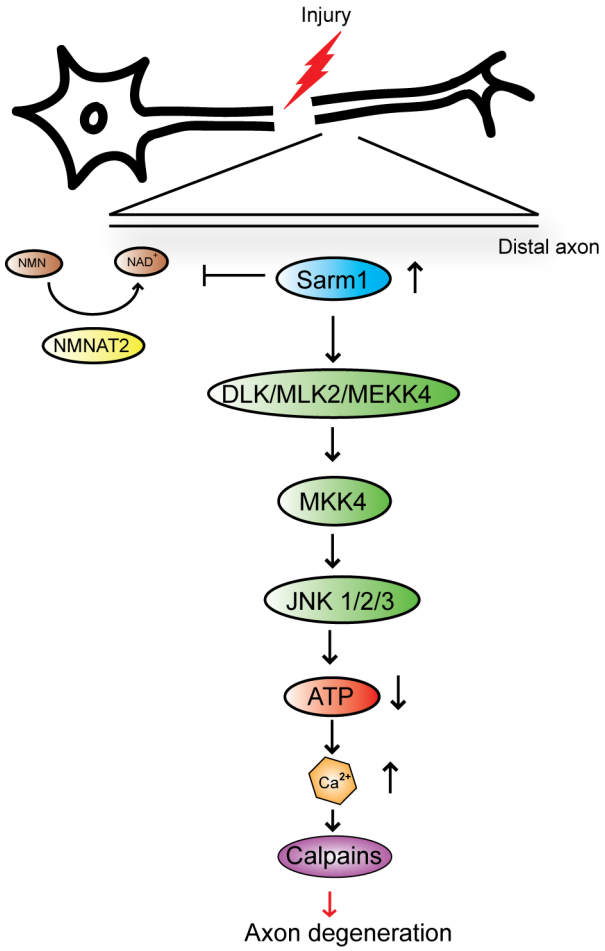

Figure 2: Molecular mechanism of axon degeneration after injury. Distinct upstream signals activate the axon executioner protein Sarm1 to further converge in to MAPK cascade. The eventual energy deficit leads to calpain activation and morphological disintergration.

Tumor Necrosis Factor Receptor (TNFR) Family members in axon degeneration

Developmental cell death and axonal loss appears to be driven by a competition between

antagonistic trophic and "punishment" signaling pathways, which promote axon stabilization or degeneration, respectively (Deppmann *et al.*, 2008; Singh *et al.*, 2008). Interestingly, the punishment pathways appear to rely on autocrine/paracrine mechanisms allowing "strong" axons to expedite the elimination of weaker neighbors. Highly related Tumor Necrosis Factor Receptor Super Family (TNFRSF) members, p75 neurotrophin receptor (p75NTR), Tumor Necrosis Factor Receptor 1a (TNFR1a) and Death Receptor 6 (DR6) have been implicated in this extrinsic degeneration program in the peripheral nervous system (PNS) (Singh *et al.*, 2008; Vilar *et al.*, 2009; Park *et al.*, 2010; Olsen *et al.*, 2014; Wheeler *et al.*, 2014). More generally, all TNFR family members are death/cytokine receptors that bind a diversity of ligand types. Some of the TNFR family members that are implicated in axon degeneration, are discussed below.

TNFR1a

The founding member of TNFR super family and the prototypic death receptor, TNFR1, has been implicated in developmental as well as injury-induced axon degeneration. TNFR1, which possesses a death domain bind to homotrimeric TNF-α and involves recruitment of adapter signaling molecules TRADD (TNFR-associated death domain), FADD, and RIP (receptor interacting protein). TNFR1 is a constitutively expressed receptor and is detected in rat sciatic nerves, dorsal root ganglion cells as well as Schwann cells (George, Buehl and Sommer, 2005).

Developmentally, TNFR1 plays a dual role by suppressing TrkA in nociceptive neurons as well as enhancing Ret signaling in peptidergic and nonpeptidergic nociceptors,

respectively (Wheeler *et al.*, 2014). Specifically, TrkA positive peptidergic nociceptors require forward signaling of TNFα -TNFR1 to suppress neurite growth, survival, excitability, and differentiation mediated by TrkA-NGF. Reverse signaling of TNFR1-TNFα has been shown to affect the neurite growth and excitability of Ret positive nonpeptidergic nociceptors (Wheeler *et al.*, 2014). Although the exact role is unclear, it has also been shown that the expression levels of TNFR1 is elevated following sciatic nerve unilateral crush or chronic constriction injury (CCI) suggesting a role for TNFR1, possibly as a death receptor in Wallerian degeneration (George, Buehl and Sommer, 2005).

p75NTR

p75 neurotrophin receptor (p75NTR) is the first identified and one of the most studied TNFR family members. p75NTR was identified simultaneously by Eric Shooter's group and Moses Chao's group (Chao, 1994). Expression analyses revealed that p75NTR is a widely expressed receptor in the developing nervous system showing robust expression in sympathetic, sensory, and motor neurons, as well as in most parts of the brain such as cerebral cortex, cerebellum, hippocampus and the basal forebrain. Interestingly, p75NTR is also expressed in glial cells (radial glial cells, neural stem cells and peripheral/central glial cells) (Ibáñez and Simi, 2012). As the organism reaches adulthood, the expression of p75NTR is diminished and switched off, retaining low expression only in a few types of neurons such as sensory neurons, spinal cord motor neurons and basal cholinergic neurons (Ibáñez and Simi, 2012). P75NTR has the typical ligand binding tandem arrays of cysteine rich domains in the extracellular side of the receptor common to all TNFR

super family members and an intracellular death domain making it a death receptor. p75NTR is a promiscuous receptor as it binds to all the neurotrophins and their pro-forms known with similar affinity (NGF, BDNF, NT3/4). Upon ligand binding, p75NTR can initiate a pro-apoptotic or a pro-survival signal depending on the ligand/co-receptor (Gentry, Barker and Carter, 2004). When p75NTR interacts with TrkA receptor it activates a pro-survival signal through activating PI3K and MAPK signaling pathways and activating NF-kB signaling (Gentry, Barker and Carter, 2004). In contrast, p75NTR can bind to BDNF and transmit pro-apoptosis signals through JNK and caspases(Gentry, Barker and Carter, 2004; Singh *et al.*, 2008; Ibáñez and Simi, 2012). Similar other functions have also been elucidated for p75NTR such as decreasing synaptic function (Ibáñez and Simi, 2012). Furthermore, p75NTR could bind to other ligands emananting from myelin such as Myelin-associated glycoprotein (MAG) and Nogo, to activate the small GTPase RhoA. Interestingly, p75NTR also acts as a co-receptor to Nogo receptor and Lingo-1 inducing activity of RhoA, in order to lead to growth cone collapse and inhibition of axon growth, which is directly relevant to regeneration after injury (Ibáñez and Simi, 2012).

p75NTR is involved in developmental axon pruning, which is required to achieve a functional circuit. Both incorrectly targeted, and weakly trophic axons undergo the process of developmental axon degeneration. Early studies show a reduction of apoptosis and a delay of sympathetic neuronal attrition in in *p75NTR*$^{-/-}$ mice, indicating a role for p75NTR in apoptosis (Bamji *et al.*,1998). With further experiments, Singh and others explained the mechanism as “winning” axons transducing high NGF-TrkA signals,

leading to upregulation and paracrine secretion of BDNF by strongly trophic axons. Secreted BDNF then binds to p75-NGFR and commences a degeneration-signaling program in "losing," or weakly trophic, axons (Singh *et al*, 2008). The same group later showed that p75NTR is involved in regulating neural connectivity in the adult brain by driving myelin-mediated axon degeneration (Park *et al.*, 2010). Specifically, p75NTR induced degeneration of septal cholinergic axons from aberrantly growing in to myelinated tracks *in vivo* and *in vitro*. They further elucidated that this type of myelin-dependent p75NTR driven axon degeneration could be rescued by increasing TrkA-NGF signaling as well as elevation of cellular cyclic AMP, suggesting a competition between TrkA and p75NTR (Park *et al.*, 2010).

The function of p75NTR in apoptosis is further clarified, as it has been shown that proNGF binds to both Sortilin and p75NTR. The pro-domain of NGF binds with Sortilin and the mature domain binds with p75NTR, thereby signaling to the JNK pathway, which ultimately leads to cell death (Nykjaer *et al*, 2004). In some cell types p75NTRR works in concert with TrkA to promote survival. Co-expression of p75NTR with TrkA induces the responsiveness of TrkA to NGF and promotes survival and differentiation of the sympathetic neurons (Nykjaer *et al*, 2005). Thus, p75NTR shifts TrkA's affinity to NGF and prevents it binding to NT-3. This mechanism occurs to reduce axonal growth and to promote NGF induced neuronal survival and differentiation in the maturing sympathetic nervous system (Nykjaer, Willnow and Petersen, 2005) . Therefore, p75NTR plays dual roles by facilitating TRK- mediated neuronal survival and also as a pro-apoptotic activator, depending on the molecules that p75NTR partnering with, such as co-receptors

and ligands.

p75NTR in injury

Several studies describe the upregulation of p75NTR in axon injury and in neurodegenerative disease models. In neural injury and stress situations such as peripheral nerve crush/transection, sciatic nerve transection, optic nerve axotomy, ischemic injury, hypertoxicity and spinal cord injury, surrounding tissue display elevated p75NTR expression suggesting a destructive role for p75NTR during these situations (Ibáñez and Simi, 2012). Consistent with the injury/stress situations, disease models of Alzheimer's disease, multiple sclerosis, seizures and glaucoma also show increased p75NTR expression in affected neurons/supporting glial cells (Ibáñez and Simi, 2012). Collectively, these data suggested that p75NTR plays a pathogenic role in neural injury and diseases, as opposed to the protective role played in development. Although the above mentioned correlative data exist for a destructive role for p75NTR in injury, a careful analysis of p75NTR's role in injury as well as in disease has yet to be done.

Death Receptor 6 (DR6)

DR6 (TNFRSF21) is also a type I transmembrane protein and a member of the TNFR super family with a cysteine rich extracellular domains and a cytoplasmic death domain(Pan *et al.*, 1998). Vishva Dixit's group first identified and characterized DR6 in 1998 and explained how DR6 could activate both NFkB and JNK(Pan *et al.*, 1998). DR6 is capable of inducing apoptosis in mammalian cells and induce lymphocyte development(Nikolaev *et al.*, 2009). DR6 is considered an orphan receptor as its ligand

remains unknown. Furthermore, the activators of its downstream Jun-N-terminal kinase pathway is also unknown. DR6 does not appear to be required for embryonic or immune lineage development as *DR6*$^{-/-}$ mice are viable, fertile and no gross pathology is observed in any of the major organs. Although, a slight elevation is observed in the number of T-cells in the thymus and peripheral blood of DR6-/- mice, T-cell numbers in the spleen and lymph nodes are comparable with wild type mice. DR6 is reported to regulate $CD4^+$ T-Cell Proliferation, Th Cell differentiation, B-cell expansion and humoral response indicating that DR6 is critical for adaptive immunity(Benschop, Wei and Na, 2009) .

DR6 in axon degeneration

The first implication of DR6 in axon degeneration came from Marc Tessier-Lavigne's group showing DR6 activating a caspase dependent axon self destruction program(Nikolaev *et al.*, 2009). They showed that, while the cell body has a Bax and caspase-3 dependent apoptosis, NGF deprived axons undergo caspase-6 dependent degeneration process in development (Nikolaev *et al.*, 2009). Following NGF deprivation, the N-terminus of the amyloid precursor protein (APP) was shown to act as the ligand for DR6. However, the same group and others later questioned the previous finding, and Tessier-Lavigne's group followed up showing that β-secretase activity is not required for DR6 activation and the more C terminal part of APP could act as a ligand for DR6 (Olsen *et al.*, 2014). Therefore, the notion of APP acting as a ligand for DR6 has been amended(Olsen *et al.*, 2014).

In light of the APP-DR6 finding, Milbrandt and colleagues showed that the N-APP-

dependent axonal degeneration pathway involves the function of Nmnat-1, which acts downstream of Caspase-6 after trophic factor withdrawal (Vohra et al., 2010). APP cleavage and Caspase 6 activation was not observed in injury/toxin mediated degeneration, but was observed in degeneration induced by disrupting axonal transport, indicating a role in specific disease-associated degeneration(Vohra *et al.*, 2010).

DR6 structure

Due to the recent interest of DR6 and APP interaction, two groups independently showed the crystal structure of DR6 and APP(Kuester *et al.*, 2011; Xu *et al.*, 2015). Than and colleagues prepared a construct containing the extracellular cysteine rich domains (CRD) of DR6 and solved its crystal structure. They used a bacterial expression system and a three-step chromatographic purification protocol to obtain milligram-amounts of highly pure human DR6-CRD, and demonstrated that it is stably folded and contained all nine disulfide bridges (Kuester *et al.*, 2011). Furthermore, the final structure revealed that, similar to other TNFR family members, DR6 also contained one crystallographic, antiparallel contact point which could be interpreted as an intermolecular interaction suggesting potential physiological relevance(Kuester *et al.*, 2011). However, DR6 did not show any preligand assemble domain (PLAD), which is common to some TNFR family members (e.g. Fas, TNFR-1, TNFR-2) that existed as preassembled oligomers(Chan, 2007; Kuester *et al.*, 2011). DR6 resembled the other TNFR family members by having four CRDs and formed an overall tube-like rigid structure. Than and colleagues predicted several possible interactions of DR6 and APP, involving a trimeric receptor complex binding to a trimeric ligand, a 2:2 receptor: ligand interaction and two receptor, two

ligand (DR6 and APP) bound perpendicularly to each other(Kuester *et al.*, 2011). However, based on Nikoleav et al 2009 finding, they predicted that a direct involvement of full length APP is unlikely, but could have possible short heparin binding of dimeric APP with DR6 to form a complex structure, whose biological significance could be determined in the future(Kuester *et al.*, 2011).

In 2015, the same group who explained the involvement of DR6 and APP in trophic factor withdrawal induced degeneration, explained the crystal structure of DR6 ectodomain in complex with the E2 domain of APP(Xu *et al.*, 2015). Consistent with previous studies, they found that, DR6 contains four CRD modules and each CRD were stabilized by two or three pairs of disulfide bonds. Mostly, the CRDs were composed of loops and strands of various lengths and a kink between the second and the third CRD is present while he third CRD is stabilized by a magnesium ion. They predicted that the DR6-APP interface is a relatively small area and the H1 and H2 helices of APP E2 domain is interacting with the first CRD of DR6. Consistent with Kuester et al, 2011, they showed that the linker between the second and the third CRDs is flexible, allowing a conformational change upon APP binding and a ~20° shift making DR6 move closer to APP (Xu *et al.*, 2015). Structure based mutagenesis of key residues in APP also showed their effects on binding to DR6. Although their results were fairly consistent with the previous literature, Nikolov and colleagues proposed a 1:1 stoichiometric binding of APP to DR6 via cis interactions. They further proposed that the two DR6 molecules are being brought together by dimeric APP, which in turn positions the C-terminal death domains of DR6 to trigger downstream signaling (Xu *et al.*, 2015) (Figure 3). Although this work suggested a cell autonomous function of DR6 and APP in axon and synapse pruning, it is

imperative to confirm it with further investigation.

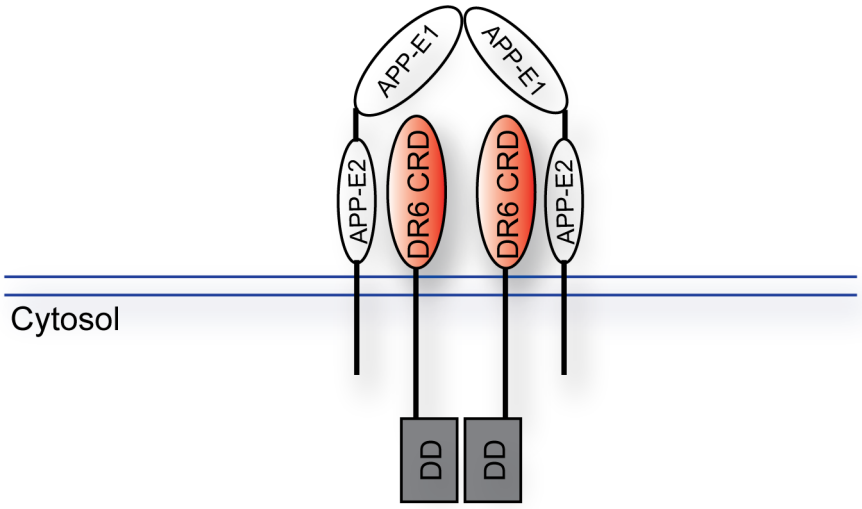

Figure 3: Proposed DR6-APP crystal structure. This DR6/APP signaling complex occurs at the neuronal surface and binding of APP dimerization and activation of DR6.

Wallerian degeneration slow - Wld^{s}

The discovery of slow Wallerian Degeneration mouse (Wld^{s}) fundamentally changed the understanding of WD, as it was previously thought to be an axon autonomous, passive wasting away of the axonal material after being severed from the cell body. From the studies of the Wld^{s} mouse, it is now known that Wld^{s} is a chimeric fusion protein comprised of NAD^{+} biosynthetic enzyme, NMNAT1 (nicotinamide mononucleotide adenyltransferase) and a fragment of the ubiquitination factor UBE4B, liked via a 18 amino acid linker region with an unknown function (Conforti, Gilley and Coleman, 2014). Expression of Wld^{s} in neurons is sufficient to suppress the granular disintegration of motor, sympathetic, sensory and axons of multiple layers of CNS neurons(Conforti, Gilley and Coleman, 2014). The protection rendered by Wld^{s} is evolutionary conserved

from insects to mice (Conforti, Gilley and Coleman, 2014).

NMNAT proteins

The NMNAT protein has three mammalian paralogs, namely, NMNAT1, NMNAT2 and NMNAT3. While NMNAT1 and NMNAT3 reside in the cell body, NMNAT2 is trafficked anterogradely in the axoplasm. Unlike NMNAT1 and NMNAT3, NMNAT2 is a labile protein due to constitutive proteasomal degradation. This degradation leads to a depletion of axonal NMNAT2, where NMNAT1 would substitute for its function (Gerdts *et al.*, 2016). Knocking down NMNAT2 in cultured neurons induces axon degeneration in the absence of injury suggesting that NMNAT2 acts as a survival factor in the axon (Gilley and Coleman, 2010). How exactly NMNAT1, NMNAT2 or their product, NAD^+ exert the axon protection after injury is still unclear.

As NMNAT2 is a critical enzyme on axon protection after injury, the function of NAD^+ in the context of injury has gained attention of late. NAD^+ is a ubiquitous metabolite exerting critical roles in energy metabolism and cell signaling (Gerdts *et al.*, 2016). The major precursor of NAD^+ in the mammals is Nicotinamide (Nam). Nam gets converted to nicotinamide mononucleotide (NMN) by the NAMPT (nicotinamide phosphoribosyltransferase) enzyme and later NMNAT enzymes (NMNAT 1,2 & 3) convert NMN to NAD^+ (Figure 4). Although NMNAT plays a critical role in WD, increased NAD^+ levels in the axon do not protect it from injury-induced degeneration (Mack *et al.*, 2001). Furthermore, accumulation of NMN did not trigger axon

degeneration either suggesting that, SARM1 dependent NAD^+ consumption is central to axon degeneration after injury (Sasaki *et al.*, 2016). With this evidence, SARM1 is believed to be the central executioner of the axonal degeneration pathway during injury and recently the same group showed evidence that thee SARM1 Toll/Interleukin-1 Receptor (TIR) Domain possesses intrinsic NADase activity (Essuman *et al.*, 2017).

Figure 4: Diagram of mammalian nicotinamide adenine dinucleotide (NAD+) biosynthesis pathway. Nicotinamide (Nam) is the major NAD+ precursor in mammals. It is converted to nicotinamide mononucleotide (NMN) by the NAMPT (nicotinamide phosphoribosyltransferase) enzyme, and then to NAD+ by the NMNAT (nicotinamide mononucleotide adenylytransferase) enzymes. There are three isoforms of NMNAT in mammalian cells; NMNAT1, 2, and 3.

SARM1

Sterile a/Armadillo/Toll-Interleukin receptor 1 (SARM1), was first described to delay injury-induced axon degeneration in mice and in Drosophila by Marc Freeman's group in 2012 (Osterloh *et al.*, 2012). Sarm1 is a toll receptor adaptor protein and loss of Sarm1 blocks axon degeneration after injury for weeks as well as traumatic brain injury and vincristine induced axon degeneration (Osterloh *et al.*, 2012; Gerdts *et al.*, 2015, 2016). Examining Sarm1 protein has revealed 3 major regions, (1) an auto-inhibitory N terminus (Nterm) comprised of multiple ARMs, (2) tandem SAM domains that mediate Sarm1-

Sarm1 binding (SAMx2), and (3) a TIR domain that triggers axon degeneration upon multimerization. Multimers of Sarm1 are auto-inhibited and inactive in uninjured axons. With an injury, an unknown signal triggers Sarm1 activation, by releasing the auto-inhibition in order for TIR domains to interact (Gerdts *et al.*, 2013, 2016).

Sarm1 is required for NAD^+ depletion after an injury to the axon and the dimerization of the TIR1 domain is sufficient to induce this NAD^+ depletion in the axon in order to lead to axon degeneration(Gerdts *et al.*, 2015). Recently, Sarm1, specifically its TIR domain, was shown to possess intrinsic enzymatic activity to cleave NAD^+, establishing Sarm1 as a central executioner in the axon degeneration pathway in injury (Essuman *et al.*, 2017).

The exact regulation of Sarm1 mediated axon degeneration is still not clear. To this end, various localization studies have been carried out to find details about resting and activated Sarm1 function. One antibody generated against Sarm1 show punctate staining in neurites and another show a more uniform distribution with some enrichment to mitochondria suggesting mitochondria as a potential site of action for Sarm1(Freeman, 2014). However, deletion of the mitochondrial localization motifs did not prevent injury-induced axon degeneration suggesting that further careful investigation of Sarm1 is necessary (Gerdts *et al.*, 2013; Freeman, 2014).

Mitochondrial dysfunction is the underlying cause for most neurodegenerative diseases and peripheral neuropathies as mitochondria play a vital role in producing ATP, metabolites and Reactive Oxygen Species (ROS). Mitochondria also play an important

role in the regulation and execution of Wallerian degeneration as evidenced by blocking mitochondrial permeability transition pore (mPTP) extending axon survival in transected sciatic nerves (Barrientos *et al.*, 2011). Common features in Wallerian degeneration are also loss of mitochondrial potential and mitochondrial swelling after an injury, which would eventually lead to axon membrane fragmentation. Interestingly, it has been reported that mitochondrial dysfunction induces Sarm1 dependent axonal death; by blocking formation of the mPTP, which induces depolarization of mitochondria, ATP depletion, calcium influx, and the accumulation of ROS (Summers, DiAntonio and Milbrandt, 2014). Loss of Sarm1 also protects axons form increased ROS, indicating that Sarm1 could be functioning downstream of ROS release (Summers, DiAntonio and Milbrandt, 2014).

Molecular mechanism of Wallerian Degeneration

Axon degeneration during development and in injury closely resembles one another in morphological changes and several downstream events. Further insight in to these morphological events, reveled that they could be related at various points in the downstream pathway. The most prominent observation is that disintegration of the microtubule network always preceded neurofilament breakdown during both developmental and Wallerian degeneration(Watts, Hoopfer and Luo, 2003; Zhai *et al.*, 2003). Additionally, activation of the Ubiquitin Proteasome System (UPS) is required for axon degeneration in drosophila mushroom body neurons as well as mouse sympathetic axons, as inhibiting UPS prevented axon degeneration in both paradigms (Watts, Hoopfer and Luo, 2003; Zhai *et al.*, 2003; MacInnis and Campenot, 2005; Hoopfer *et al.*, 2006).

Furthermore, both developmental pruning and Wallerian degeneration use glial cells to clear the axon debris via the cell corpse engulfment receptor Draper on glia, which supposedly detects the not yet fully described "eat me" signal released during axon degeneration (Watts, Hoopfer and Luo, 2003; Hoopfer *et al.*, 2006; MacDonald *et al.*, 2006).

However, it should be noted that axon degeneration during development and in injury, bear their dissimilarities as well. While developmental axon degeneration is a tightly regulated and timed event, which occurs in early development, Wallerian degeneration occurs only after an injury. In developmental axon pruning, an entire incorrectly targeted axon arbor could be eliminated, while Wallerian degeneration only removes the distal nerve/axon segment from the site of injury. Studies of Wallerian degeneration slow mice (Wld^{s}) showed that Wallerian degeneration is not just a passive wasting away of the axon once it is disconnected from the cell body, but an axon autonomous active process (Hoopfer *et al.*, 2006). Furthermore, they showed that the *Wld*^{s} gene does not protect axon degeneration in NGF deprivation, like it does in injury (Hoopfer *et al.*, 2006). Interestingly, canonical members of apoptotic pathways, like Bax and caspase 3/6/9 do not prevent or delay axon degeneration after injury, although Calpastatin, an inhibitor of ion selective proteases like calpains, are involved in both developmental pruning and in injury (Deckwerth *et al.*, 1996; Whitmore *et al.*, 2003; Simon *et al.*, 2012; Cusack *et al.*, 2013; Yang *et al.*, 2013). These findings suggest that these two processes are significantly different from each other, but at different points share multiple steps converging on similar downstream pathways to execute axon degeneration.

As Sarm1 was discovered as the central executioner in Wallerian degeneration, MAPK signaling, which is linked to sarm1, also received attention as downstream pathway components. Genetic analyses reveled that MAPKKK member DLK is a weak regulator in Wallerian degeneration and DLK acts as the signal in the proximal stump initiating axon regeneration (Ghosh-Roy *et al.*, 2010; Ghosh *et al.*, 2011; Stone *et al.*, 2014). Furthermore, Tessier-Lavigne's lab showed that the MAPK cascade is the central downstream pathway for axon degeneration in injury. They showed that three MAPKKKs (MEKK4/ MLK2/DLK), two MAPKKs (MKK4/MKK7), three MAPKs (JNK1/ JNK2/JNK3), and the scaffold protein JIP3 are the key components regulating axon degeneration after injury (Yang *et al.*, 2015) (Figure 2).

One of the steps towards understanding the downstream pathway of Wallerian degeneration came from Marc Tessier-Lavigne's lab, where they explained involvement of Calpastatin and MAPK pathway in axon degeneration (Yang *et al.*, 2013, 2015). Calpains are calcium sensitive proteases and have long been implicated in axon degeneration *in vitro, in vivo*, as well as in neurodegenerative diseases (George, Glass and Griffin, 1995; Ma *et al.*, 2013; Yang *et al.*, 2013). However, calpain is not only regulated by intracellular calcium, but also by endogenous proteinaceous inhibitor Calpastatin (Yang *et al.*, 2013). Calpastatin functions as a suicide inhibitor where it is inhibited by activated calcium and is rapidly depleted after axon injury. However, similar results were shown in NGF deprivation indicating Calpain and calpastatin play similar downstream roles in several modes of axon degeneration (Yang *et al.*, 2013). As cytosolic calcium overload is also required to activate calpain, it is important to focus on

calcium entry/release in various degeneration paradigms, which disturbs regular calcium homeostasis.

Calcium Signaling in Wallerian Degeneration

Neurons use calcium signaling in a very disparate array of functions from development and maintenance to death (Mattson, 2007). Tight and precise control as well as flexibility of this second messenger is vital in coordinating its functionality. As calcium signaling affects a broad array of functions in neurons such as synaptic plasticity and apoptosis, it is evident that dysregulation of calcium homeostasis in neurons leads to detrimental effects. Perturbations to intracellular calcium have been implicated in many neurodegenerative diseases including AD (Berridge, 2014). As an example, the amount of free and protein bound calcium, and the activity of calcium dependent proteases is increased in neurons with neurofibrillary tangles in AD patient brains (Mattson, 2007). Whether calcium concentration alterations precede Aβ accumulation or vice versa is still debatable. Nevertheless, it has been shown that Aβ affected neurons have elevated calcium levels and disrupted calcium homeostasis(Mattson, 2000, 2007). As Bacskai and colleagues further illustrated, in aged APP mice, 20% of the dendrites and axons showed elevated levels of baseline calcium (Kuchibhotla *et al.*, 2008).

Similar to neurodegenerative diseases, calcium dysregulation contributes to axonal degeneration in developmental pruning as well as in injury (Villegas *et al.*, 2014; Vargas *et al.*, 2015). In the initial stages of injury-induced degeneration, calcium is reported to aid in sealing the damaged axonal membranes immediately after injury (Vargas *et al.*,

2015). Intriguingly, calcium plays an important role in promoting axon degeneration because blocking intracellular calcium stores, voltage gated calcium channels and chelating intracellular calcium, delay axons degeneration while increasing intracellular calcium accelerates axon degeneration (George, Glass and Griffin, 1995; Vargas *et al.*, 2015). Calcium also promotes axon degeneration by activating the protease calpain, which breaks down neurofilaments (Yang *et al.*, 2013). Studying the temporal and spatial distribution of calcium in injured axons in zebrafish has revealed that there are two distinct phases of calcium influx during axon degeneration (Vargas *et al.*, 2015). The initial transient calcium wave occurs immediately after axotomy at the injury site and disrupts the mitochondria near the injury site. The second, more prominent calcium wave occurs just before the fragmentation and affected most of the mitochondria in the axon. While expressing the protective Wld^s protein does not alter the initial wave of calcium, the second wave was abolished by Wld^s expression. Furthermore, chelating the second wave of calcium only was sufficient to protect axons from fragmenting indicating that the terminal calcium wave plays a key role in axon degeneration after injury (Vargas *et al.*, 2015).

Chapter 2

Death Receptor 6 (DR6) promotes peripheral axon degeneration after axotomy.

Authors

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Summary

Axon degeneration during development is required to sculpt a functional nervous system and is also a hallmark of pathological insult such as injury (Luo and O'Leary, 2005; Singh *et al.*, 2008). Despite similar morphological characteristics, very little overlap in molecular mechanisms have been reported between pathological and developmental degeneration (Martin *et al.*, 1988; Raff, Whitmore and Finn, 2002; Park *et al.*, 2010). In the peripheral nervous system (PNS) developmental axon pruning relies on receptor mediated extrinsic degeneration mechanisms to determine which axons are maintained or degenerated (Park *et al.*, 2010; Olsen *et al.*, 2014; Wheeler *et al.*, 2014). Receptors have not been implicated in axotomy-induced axon degeneration; instead axon autonomous, intrinsic mechanisms are thought to be the primary driver for this type of axon disintegration(Coleman *et al.*, 1998; Osterloh *et al.*, 2012; Babetto *et al.*, 2013). Here we survey the role of neuronally expressed, paralogous Tumor Necrosis Factor Receptor Super Family (TNFRSF) members in axotomy-induced degeneration. We find that an orphan receptor, Death Receptor 6 (DR6), is required to drive axon degeneration after axotomy in sympathetic and sensory neurons cultured in microfluidic devices. We sought to validate these *in vitro* findings *in vivo* using a transected sciatic nerve model. Consistent with the *in vitro* findings, $DR6^{-/-}$ animals displayed preserved axons up to 4 weeks after injury. In contrast to phenotypes observed in Wld^{s} and $Sarm1^{-/-}$ mice,

preserved axons in *DR6*$^{-/-}$ animals display profound myelin remodeling. This indicates that deterioration of axons and myelin after axotomy are mechanistically distinct processes. Finally, we find that JNK signaling after injury requires DR6, suggesting a link between this novel extrinsic pathway and the axon autonomous, intrinsic pathways that have become established for Wallerian degeneration.

Results and Discussion

In vitro comparison of trophic withdrawal-induced and axotomy-induced degeneration rates of peripheral axons

We first sought to compare axon degeneration rates in trophic factor withdrawal versus axotomy paradigms *in vitro*. To model these scenarios we used sympathetic neuron cultures to perform trophic deprivation or axotomy (enucleation), respectively (Cusack *et al.*, 2013; Yang, Robby M. Weimer, *et al.*, 2013) For both paradigms, we established sympathetic neurons from postnatal day (P) P0-P3 wild-type mice in microfluidic devices as previously described (Suo *et al.*, 2014a). These devices allow for neurons of interest to be compartmentalized such that cell bodies and axons reside in different compartments of the device (Park *et al.*, 2006). In the trophic withdrawal degeneration scenario, both soma and axonal compartments are deprived of NGF using a neutralizing anti-NGF antibody (Figure S1A). Trophic factor withdrawal is a widely used model to mimic a "die back" type of axon degeneration observed during developmental axon pruning as well as in neurodegenerative disorders (Pease and Segal, 2014; Gerdts *et al.*, 2016). For the

axotomy paradigm neurons established in microfluidic devices (maintained in 45ng/mL NGF for 5 days *in vitro* (DIV)) are enucleated mechanically using an aspirating pipette leaving the axon architecture intact (Figure S1B, Supplemental movie). NGF deprived neurons and enucleated axons are incubated at 37°C with 5% CO2 for indicated periods of time followed by immunostaining for βIII-tubulin and manual quantification of axon degeneration as previously described (Zhai *et al.*, 2003; Singh *et al.*, 2008). NGF deprived axons from wild-type mice began undergoing degeneration at ~18 hours, showed ~50% degeneration at 20 hours and reached maximal degeneration (defined as greater than or equal to 80%) by ~24 hours, consistent with previous findings (Martin *et al.*, 1988; Deckwerth *et al.*, 1996). In contrast, injured axons underwent degeneration starting at 1.5 hours, reached ~50% degeneration around 2.5 hours and showed maximal degeneration by 8 hours (Figure S1C,D). This is a slightly faster rate of degeneration than reported in previous studies, which we attribute to the properties of axotomy in microfluidic devices (*i.e.* smaller media volume) (Sasaki and Milbrandt, 2010; Yang, Robby M. Weimer, *et al.*, 2013). Although there is an 18 hour difference in latent phases between trophic withdrawal and axotomy-induced degeneration, the kinetics of the catastrophic degeneration phase are quite similar taking no more than ~2-4 hours to go from baseline to maximal degeneration (above 80%) (Martin *et al.*, 2010) (Figure S1C). For the axotomy paradigm, the presence of NGF in the medium or the age of the neurons does not change the rate of axon degeneration (Figure S1D, E).

Highly related receptors P75NTR, DR6, TNFR1a are enriched in the nervous system

To determine which TNFR family members to focus on we determined the phylogenetic relationship and expression profile of all TNFR family members. We generated a phylogenic tree using full-length TNFR family rat amino acid sequences (FigS2A)(Dereeper *et al.*, 2008). We next sought to examine the expression pattern of TNFR family members using cDNA from the superior cervical ganglia (SCG) and brain of P0 rats and mice, via RT-PCR (Figure S2B-D). p75NTR, TNFR1 and DR6, which are in the same phylogenetic clade, display robust expression in the SCG of both mice and rats. TNFR1 and DR6 also show high expression in the brain, whereas p75NTR does not, which is consistent with previous reports (Bibel and Barde, 2000; Locksley, Killeen and Lenardo, 2001; Gentry, Barker and Carter, 2004; Tam *et al.*, 2012). This expression pattern is similar to what we've reported for mouse sensory neurons (Wheeler *et al.*, 2014). The expression of these receptors is also known to be dynamic in response to insult with p75NTR and TNFR1 increasing their expression after nerve damage [26,27]. While DR6, p75NTR and TNFR1a have all been implicated in influencing axon growth and/or degeneration during development they have not been examined broadly in the context of axotomy-induced degeneration (Ferri, Moore and Bisby, 1998; Ferri and Bisby, 1999; Barker *et al.*, 2001; Beattie *et al.*, 2002; Park *et al.*, 2010; Olsen *et al.*, 2014; Wheeler *et al.*, 2014).

p75NTR and DR6 promote axon degeneration induced by trophic deprivation

We first sought to examine the relative contribution of DR6, p75NTR and TNFR1a in trophic withdrawal induced degeneration as described in Figure S1. To this end, we established sympathetic neuron cultures in microfluidic devices from wild-type, *DR6*$^{-/-}$, *p75NTR*$^{-/-}$ and*TNFR1a*$^{-/-}$ mice and deprived these cultures of NGF (in the presence of anti-NGF) for up to 72 hours prior to quantification of degeneration (Figure 1A, Figure S3A). Consistent with previous findings, wild-type axons reach maximal degeneration by 24hrs post-NGF deprivation, while sympathetic neurons from *p75NTR*$^{-/-}$ and *DR6*$^{-/-}$ mice displayed 25% and 50% axon degeneration, respectively (Figure 1A,B) (Singh, 2008; Olsen *et al.*, 2014). *p75NTR*$^{-/-}$*;DR6*$^{-/-}$ axons showed a greater delay in degeneration at 24 hours compared to either *DR6*$^{-/-}$ or *p75NTR*$^{-/-}$, however a detailed time course reveals that both *p75NTR*$^{-/-}$ and *p75NTR*$^{-/-}$*;DR6*$^{-/-}$ neurons enter the rapid catastrophic phase of degeneration shortly after 24hrs (Figure 1A,B and S3A). We also examined neurons from *Wld*^{s} and *Sarm1*$^{-/-}$ mice, which are resistant to axotomy-induced degeneration (Beirowski *et al.*, 2005; Osterloh *et al.*, 2012). Consistent with previous observations, neurons from *Wld*^{s} mice display a modest reduction in the rate of trophic withdrawal-induced degeneration similar to what we observe for *DR6*$^{-/-}$ neurons whereas *Sarm1*$^{-/-}$ showed no protection (Hoopfer *et al.*, 2006; Osterloh *et al.*, 2012). Together, these data indicate that in sympathetic neurons p75NTR has a greater contribution to trophic withdrawal-induced degeneration than DR6 and that the metabolic intermediates induced

by Wlds or NMNAT1 are partially protective in degeneration induced by trophic withdrawal. Furthermore, our results indicate that p75NTR and DR6 work in parallel to promote degeneration in response to trophic withdrawal.

DR6 is required for axotomy-induced axon degeneration *in vitro*

We next examined the role of death receptors in axotomy-induced degeneration as described in Fig. S1B, using neurons from *DR6*$^{-/-}$, *p75NTR*$^{-/-}$, *p75NTR*$^{-/-}$*;DR6*$^{-/-}$,*TNFR1a*$^{-/-}$ mice and compared them to known delayed models *(Wld*^{s} and *Sarm1*$^{-/-}$)(Fig. 2A). Wild-type, *p75NTR*$^{-/-}$, and *TNFR1a*$^{-/-}$ axons rapidly disintegrated reaching maximal degeneration by 8 hours post-axotomy (Figure 2A, B and S3B). Remarkably, injured *DR6*$^{-/-}$ axons remained intact up to 24 hours post-axotomy and the full time course revealed that the kinetics of degeneration in *DR6*$^{-/-}$ axons after injury are comparable to axons from *Wld*^{s} and *Sarm1*$^{-/-}$ mice (Figure 2A,B and S3B). Interestingly, axons from *p75NTR*$^{-/-}$*;DR6*$^{-/-}$ mice degenerate faster than those from *DR6*$^{-/-}$ mice suggesting that loss of *DR6* unmasks an anti-degenerative role for p75NTR. Because these are pure populations of sympathetic axons, these data suggest that DR6 drives axotomy-induced degeneration in an axon autonomous manner. We next performed dorsal root ganglia (DRG) neuron cultures isolated from E14.5 wild-type, *p75NTR*$^{-/-}$ and *DR6*$^{-/-}$ mice. WT and *p75NTR*$^{-/-}$ sensory axons show maximal degeneration by 8 hours after axotomy while *DR6*$^{-/-}$ sensory axons displayed minimal degeneration at this time (Figure S3C,D). We also examined a later step in degeneration, loss of the calpain substrate neurofilament-M (NF-M)(Yang *et al.*, 2015). In wild type neurons, NF-M starts to disappear by 8 hours of axotomy while *DR6*$^{-/-}$ axons retained NF-M staining 24 hours after axotomy (Figure

S3E). Together, these results suggest that DR6 is required for axotomy-induced degeneration in PNS neurons grown *in vitro*.

DR6 is required for Wallerian degeneration *in vivo*

We next sought to examine the role of DR6 in promoting axon degeneration *in vivo* using a sciatic nerve axotomy (SNA) model (Figure S4A). In contrast to the pure population of sympathetic or sensory axons that we used for our *in vitro* model, the sciatic nerve contains mixed populations of sensory, sympathetic, and motor axons as well as glia. Therefore, we can examine whether DR6 is universally required for peripheral axon degeneration *in vivo*. The right sciatic nerve from wild-type, $DR6^{-/-}$, Wld^{S} and $Sarm1^{-/-}$ mice was transected and we assessed the integrity of axons 2 and 4 weeks post lesion. We first assessed the integrity of axons within the sciatic nerve with and without injury using light and transmission electron microscopy approaches. In our light microscopy approach we visualized myelinated nerve fibers by staining semi-thin cross sections (0.5-2µm) of the distal sciatic nerve with toluidine blue. For electron microscopy we examined ultrathin sections (80-100nm) allowing visualization of myelinated and unmyelinated fibers (Figure 3A,B). Similar to previous findings, wild-type nerves exhibited a complete breakdown of the axonal structure 14 days after SNA (Beirowski *et al.*, 2005; Conforti *et al.*, 2007). Consistent with our *in vitro* data, $DR6^{-/-}$ mice displayed spared large and small diameter axons 2 and 4 weeks after SNA (Figure 3B,C; Figure S4 G,H). Importantly, at 38.5% (n=13) the penetrance of this rescue phenotype is relatively low compared to Wld^{S}

and *Sarm1*$^{-/-}$ animals, which display 80% and 100% penetrance, respectively (Figure 3 and Figure S4).

Immediately apparent from electron micrographs is that spared axons in injured nerves from *DR6*$^{-/-}$ mice display aberrant myelination profiles (Figure 3B, C i-iii). *Wld*^{s} and *Sarm1*$^{-/-}$ animals, which display intact axons and myelin sheaths after injury, showed no dramatic changes between the groups of axons with varying myelin sheath thickness before and after injury. *DR6*$^{-/-}$ mice show a significant increase of the percentage of axons bearing thin (0-0.3μm) myelin sheaths after injury, which was not observed in *Wld*^{s} and *Sarm1*$^{-/-}$ animals (Figure 3D). These thinly myelinated injured *DR6*$^{-/-}$ axons show remarkably preserved axonal neuro-filaments, Schwann cells and myelin similar to *Wld*^{s} and *Sarm1*$^{-/-}$ animals (Figure 3C iv)(Osterloh *et al.*, 2012). We also examined the composition of axons in these mutant mice based on diameter. Prior to injury, *DR6*$^{-/-}$ axons are larger than wild type axons and do not significantly change their diameter in response to injury (Figure 3C i,ii,iii 3000x images, Figure S4I). We further characterized this phenotype by quantifying the ratio of axon to fiber diameter (G-ratio) with and without injury (Figure S4B-F)(Keilhoff, Fansa and Wolf, 2002; Michailov *et al.*, 2004). Relative to other genotypes examined, *DR6*$^{-/-}$ animals displayed a broad distribution of G-ratios and axon diameters, which is consistent with variable myelin thicknesses (Figure S4F). Taken together, these data imply that the process of axon degeneration and myelin remodeling after injury are mechanistically distinct.

DR6 links to intrinsic pathways of Wallerian degeneration

A great deal of progress has been made in the last several years delineating the axon autonomous intrinsic pathway that promotes Wallerian degeneration. Upon axotomy, it is known that the adaptor protein Sarm1 is activated and NAD^+ is depleted (Gerdts *et al.*, 2016; Sasaki *et al.*, 2016). Sarm1 also activates the MAPK pathway (MKK4 and JNK), which is upstream of disrupted axonal energy homeostasis (Weston and Davis, 2007; Gilley and Coleman, 2010; Yang *et al.*, 2015; Sasaki *et al.*, 2016). We sought to determine whether DR6 feeds into this axon autonomous, intrinsic pathway. To this end, we harvested injured distal sciatic nerves 30 minutes after transection and assessed the activation/phosphorylation (Thr183/Tyr185) of JNK. In nerves from wild-type mice, p-JNK levels increased 4-fold 30 minutes after transection, representing an early signaling response prior to physical breakdown of axonal cytoskeletal components (Figure 3E, F)(Yang *et al.*, 2015). Remarkably, injured distal sciatic nerves from *DR6*$^{-/-}$ or *Sarm1*$^{-/-}$ animals did not show a significant increase in JNK phosphorylation (Figure 3E,F). These data suggest that DR6 signaling following injury feeds into known "intrinsic" degenerative pathways (Figure 4). The mechanism by which it links to Sarm1, NAD depletion and other pro-degenerative signaling cascades remains an open question.

Conclusions

In this study we examined the role of TNFR family members in promoting axon degeneration in response to troph ic withdrawal or axotomy. As previously reported, p75NTR and DR6 initiate axon degeneration in trophic deprivation paradigms, which is used to model developmental axon die back (Ferri, Moore and Bisby, 1998; Ferri and Bisby, 1999; Olsen *et al.*, 2014). Remarkably, in an *in vitro* axotomy paradigm, loss of *DR6* but not *p75NTR* delays axon degeneration with kinetics similar to axons derived from Wld^S or $Sarm1^{-/-}$ mice. We next asked whether this also occurs *in vivo*. Indeed, loss of DR6 rescues axonal degeneration 2 and 4 weeks after sciatic nerve injury, with moderate penetrance. While axons remained intact in these mice, we also observe a dramatic loss in myelin thickness, which is not observed in other mutants known to rescue axotomy-induced degeneration. To our knowledge this is the first example of a receptor-mediated mechanism driving Wallerian degeneration.

In vitro, $p75NTR^{-/-};DR6^{-/-}$ axons do not display as long of a rescue period after axotomy as $DR6^{-/-}$ axons (Figure 2A,B). This reveals a surprising anti-degenerative role for p75NTR in the context of axotomy-induced degeneration, which is in contrast to its pro-degenerative role in trophic withdrawal. This coupled with the known roles for TNFR family member in suppressing axon regeneration somewhat complicates their role in functional recovery after nerve injury (Ferri, Moore and Bisby, 1998; Ferri and Bisby, 1999; George, Buehl and Sommer, 2005; Song *et al.*, 2006) .

Sciatic nerves from uninjured *DR6*$^{-/-}$ mice showed larger axon diameters but normal myelin thicknesses compared to wild type (Figure S4I). 2 weeks after nerve transection, we observe thin myelin sheaths as well as aberrant wrapping in *DR6*$^{-/-}$ mice, which may suggest a persistent cycle of demyelination and remyelination similar to what has been observed in MS and chemical demyelination paradigms (Figure 3C,D, Figure S4G,H) (Kornek *et al.*, 2000; Coman *et al.*, 2006; Song *et al.*, 2006). Injured nerves from *Wld*^{S}, *Sarm1*$^{-/-}$ and *Phr1*$^{-/-}$ animals do not show this phenotype (Figure 3C,D) (Beirowski *et al.*, 2005; Osterloh *et al.*, 2012; Babetto *et al.*, 2013). How do we reconcile the differences between *DR6*$^{-/-}$ mice and other mutants that rescue Wallerian degeneration? One possibility is that activation of *Sarm1* or depletion of NAD+ is required for disassembly programs in both axons and glia, whereas activation of DR6 may only be required in axons. Alternatively, all of these factors may work exclusively in axons. In this scenario activation of Sarm1 or Phr1 as well as NAD^+ depletion may be upstream of the release of a putative demyelination factor, whereas DR6 is either downstream or dispensable for this process. It is unlikely that this putative demyelinating cue is simply axonal debris, since injured nerves still lose myelin even though axons remain intact in the absence of *DR6.*

Our results showing that DR6 promotes axon degeneration after axotomy implies an extrinsic mechanism that may be similar to what has been observed for trophic factor deprivation [1]. Given that this putative ligand would be axon derived, this may be a mechanism for axons to coordinate their degeneration, which could allow macrophages and glia to remove debris efficiently. The ligand for DR6 has remained elusive and it is

still widely considered to be an orphan receptor (Pan *et al.*, 1998; Xu *et al.*, 2015). In 2009, it was suggested that an N-terminal fragment of amyloid precursor protein (APP) may serve as a ligand for DR6 (Nikolaev, McLaughlin, O'Leary, *et al.*, 2009). However, the relationship between APP and DR6 has since been amended (Olsen *et al.*, 2014). While it is clear that in several instances APP and DR6 are in the same genetic pathway, it may not be as a ligand receptor pair (Olsen *et al.*, 2014). A recent crystal structure reveals that the way in which DR6 and APP interact is consistent with a co-receptor relationship (Olsen *et al.*, 2014; Xu *et al.*, 2015). It will be important to examine the role of APP in axotomy-induced degeneration in the future. If APP is indeed acting as a co-receptor it will be critical to continue seeking ligands for DR6 in the context of axotomy-induced degeneration. Whether this putative ligand also promotes demyelination, presumably through a different receptor, must also be examined.

DR6 downstream signaling has gone largely uncharacterized, in part due to lack of a ligand. Because of the robustness of phenocopy between *Wld^{S}, Sarm1$^{-/-}$* and *DR6$^{-/-}$* mice (Figure 3A,B & Figure S4D-F), it's tempting to speculate that they may be in the same pathway to promote axotomy-induced degeneration. Although first described as a Toll like receptor adaptor protein, a primary receptor for Sarm1 in axotomy-induced degeneration has yet to be identified. It is known that axonal injury results in the release of autoinhibition by the N-terminal domain of Sarm1 although the precise mechanism that triggers this remains obscure (Belinda *et al.*, 2008; Summers *et al.*, 2016). Nevertheless, it is apparent that activation of Sarm1 downstream of axonal injury leads to JNK activation (Shin *et al.*, 2012; Yang *et al.*, 2015; Gerdts *et al.*, 2016). Our finding that

DR6 is also required for JNK activation after injury suggests that this receptor may link with known components of the intrinsic pathway. Further studies will be required to determine how death receptor signaling in axotomy induced axon degeneration links to Sarm1 activation, NAD^+ depletion and other previously described pathways.

Author contributions

K.K.G, R.E.P and M.S.K conducted and analyzed all experiments unless otherwise indicated. I.C. carried out timed mating and isolation of DRGs from E14 mouse embryos and SCG isolation from p0-p3 postnatal mice. A.J.S. provided support for biochemistry experiments. K. E. carried out the determination of TNFR phylogenetic tree and performed RT-PCR experiments. C.H. provided advice and equipment for microfluidic chambers design and preparation. A.E. provided advice and training for *in vivo* experiments and the electron microscope. K.K.G. and C.D.D. planned the experiments and wrote the manuscript with input from co-authors. C.D.D. supervised the project.

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Competing Financial Interests

The authors declare no competing financial interests.

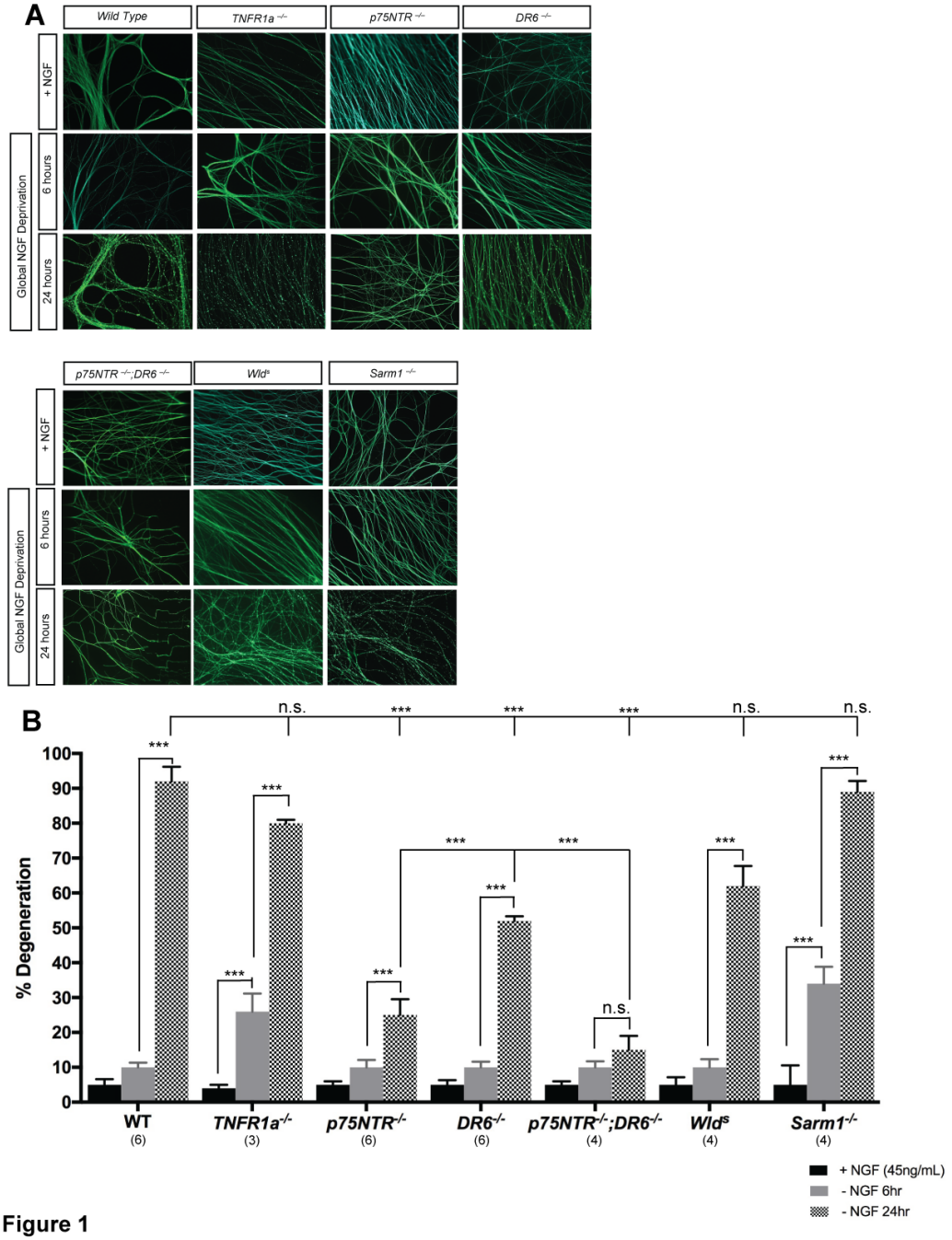


Figure 1

Figure 1. **DR6 and p75NTR are required for trophic factor deprivation-induced axon degeneration *in vitro*.**

A. Representative images of βIII-tubulin immuno-stained distal sympathetic axons before and after global NGF deprivation for 0, 6 and 24 hours for indicated genotypes.

B. Quantification of degeneration in **A** with n indicated in parentheses.

See also Supplementary Fig.1.

Here, and throughout, values are represented as mean ± SEM. n.s.= not significant; ***p < 0.001. n=3 for each time point and genotype, unless otherwise specified. For each repeat at least 100 axons are scored for degeneration. Significance determined by unpaired two tailed t test.

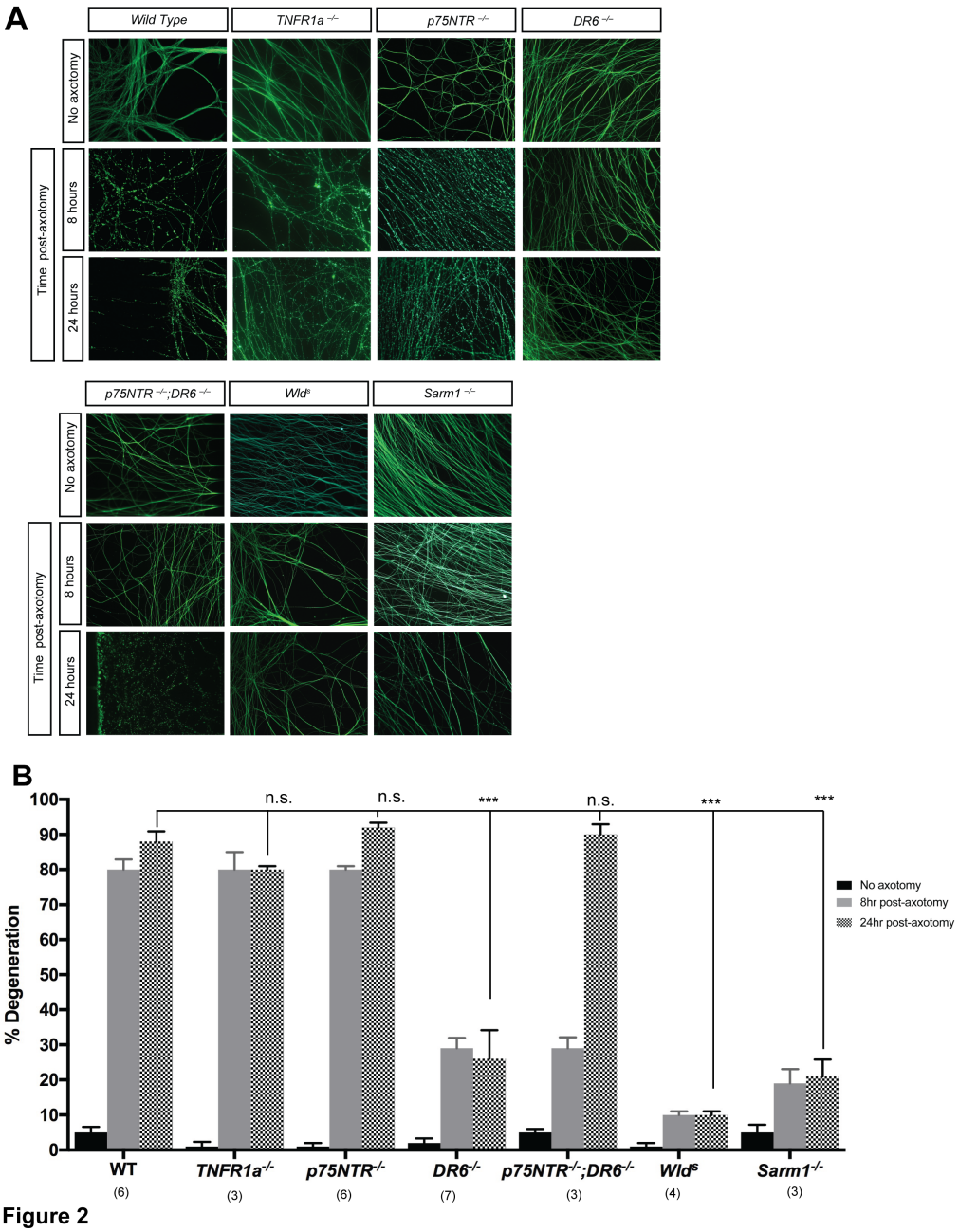


Figure 2

Figure 2: **DR6, but not p75NTR or TNFR1a is required for axotomy-induced axon degeneration *in vitro.***

A. Representative images of βIII-tubulin immuno-stained distal sympathetic axons before and after axotomy for 0,8 and 24 hours for indicated genotypes.

B. Quantification of degeneration in **A** for indicated times after axotomy with n indicated in parentheses.

See also Supplementary Fig.2 and 3.

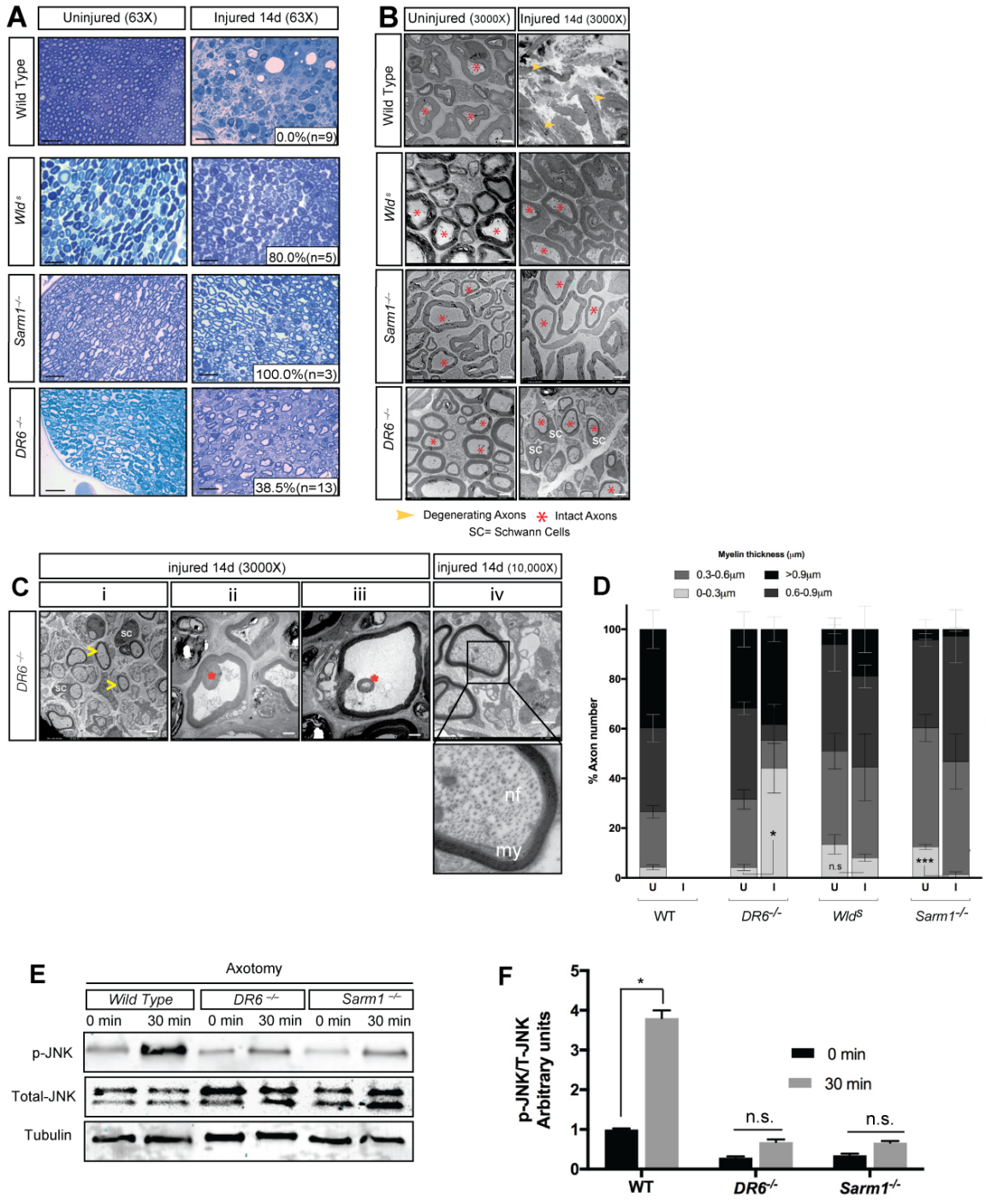


Figure 3

Figure 3: **DR6 is required for axotomy-induced nerve degeneration *in vivo*.**

A. Wild type, *DR6*$^{-/-}$, *Wld*^{s} and *Sarm1*$^{-/-}$ distal sciatic nerves were sectioned (0.5-2μm) and stained with toluidine blue to visualize myelin sheaths before and 14 days after sciatic nerve transection. Penetrance of phenotype is in the bottom right corner of the images with the number of animals examined in parentheses. Penetrance represents the percentage of animals that displayed rescued axon degeneration after Sciatic Nerve Axotomy (SNA). Scale bar = 20 μm.

B. Representative electron micrographs of cross sections (80-100 nm) from wild type, *DR6*$^{-/-}$,*Wld*^{s} and *Sarm1*$^{-/-}$ distal sciatic nerves (before and 14 days after sciatic nerve transection) showing intact (red stars) degenerating axons (yellow arrows) and Schwann cells (SC). In contrast to **A.** small diameter unmyelinated axons and remak bundles can be observed. Scale bar = 2 μm.

C. Representative electron micrographs of cross sections (80-100 nm) from *DR6*$^{-/-}$ injured nerves showing thin myelin sheaths (yellow arrow heads), thick/aberrant myelin sheaths (red stars) (i-iii 3000X). High magnification electron micrograph of thin myelin bearing axon, showing intact myelin (my) and neurofilaments (nf) (iv & inset). Scale bars – 3000X= 2 μm, 10,000X = 1.5μm.

D. Percentage of injured and uninjured axons binned by myelin thickness in Wild Type, *DR6*$^{-/-}$, *Wld*^{s} and *Sarm1*$^{-/-}$ mice before and after injury. * $P < 0.05$. 200 or more axons were measured in each mouse nerve, n=3 for each genotype.

E. Immunoblot analysis of distal injured sciatic nerve segment at 0 minutes and 30 minutes post transection. n=4 mice for each time and genotype. Level of p-JNK is not elevated in *Sarm1*$^{-/-}$ and *DR6*$^{-/-}$ injured nerves compared to WT injured nerves.

F. Quantification of **E**.

See also Supplementary Fig.4.

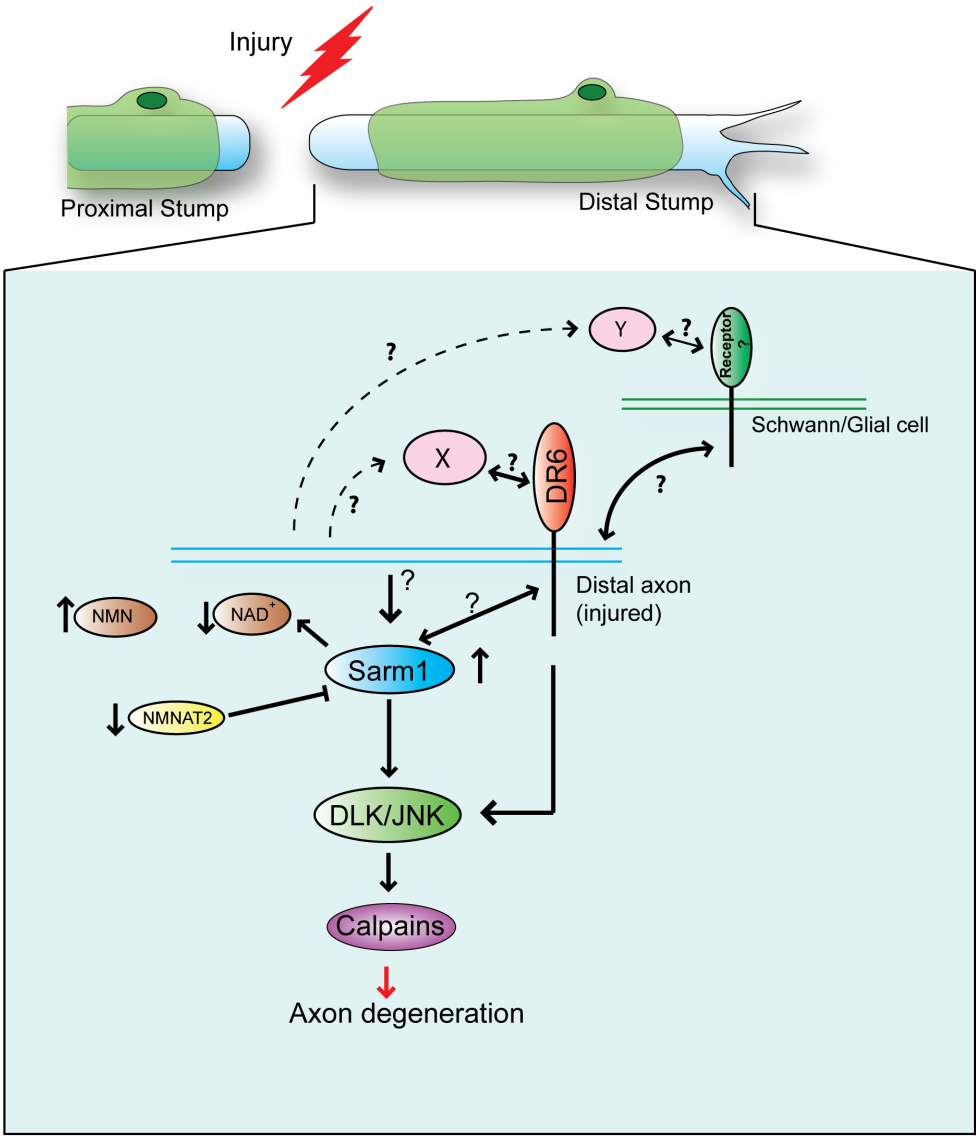
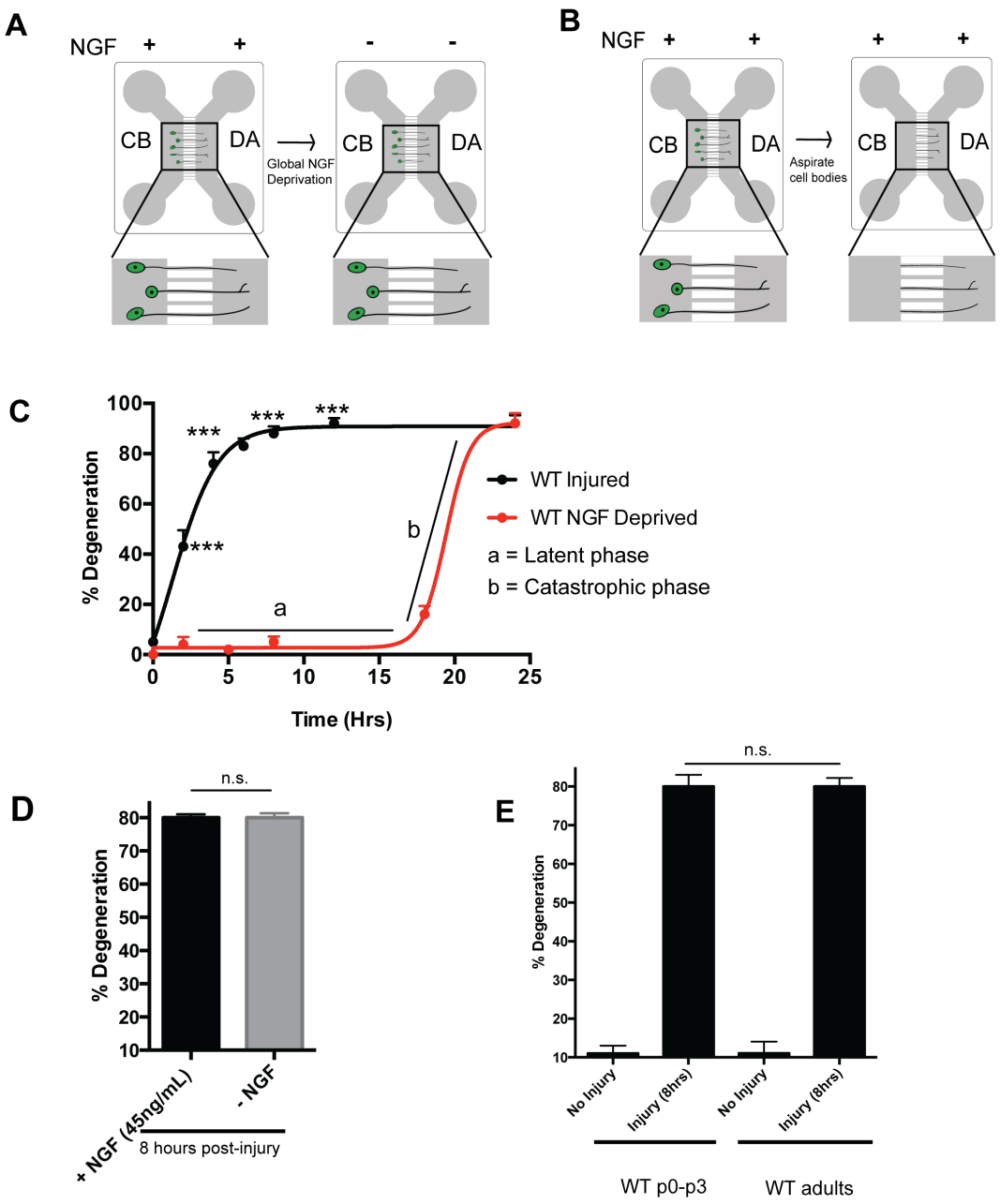


Figure 4: **Proposed model for DR6 regulation of Wallerian degeneration signaling pathway.**

Loss of *DR6* rescues injured distal axons from axon degeneration indicating an essential rol e for DR6 in axotomy-induced degeneration. The signaling pathway leading to axon degeneration after injury involves activation of Sarm1, a drop in NAD^+ levels and subsequent activation JNK and Calpain. Our *in vitro* and *in vivo* results indicate that after injury, DR6 acts as a receptor that leads to downstream activation of JNK.



Supplementary Figure 1

Supplementary Figure 1: Related to Figure 1: **Trophic factor withdrawal and injury *in vitro* show similar catastrophic axon degeneration kinetics but different latencies.**

A. Schematic illustration of NGF deprivation paradigm *in vitro*.

Sympathetic neurons from P0-P3 mice are established with 45ng/ml NGF in microfluidic devices, which allow separation of cell bodies and axons. Neurons are globally deprived of NGF using anti-NGF antibody for indicated times. (CB= Cell bodies, DA = Distal Axons)

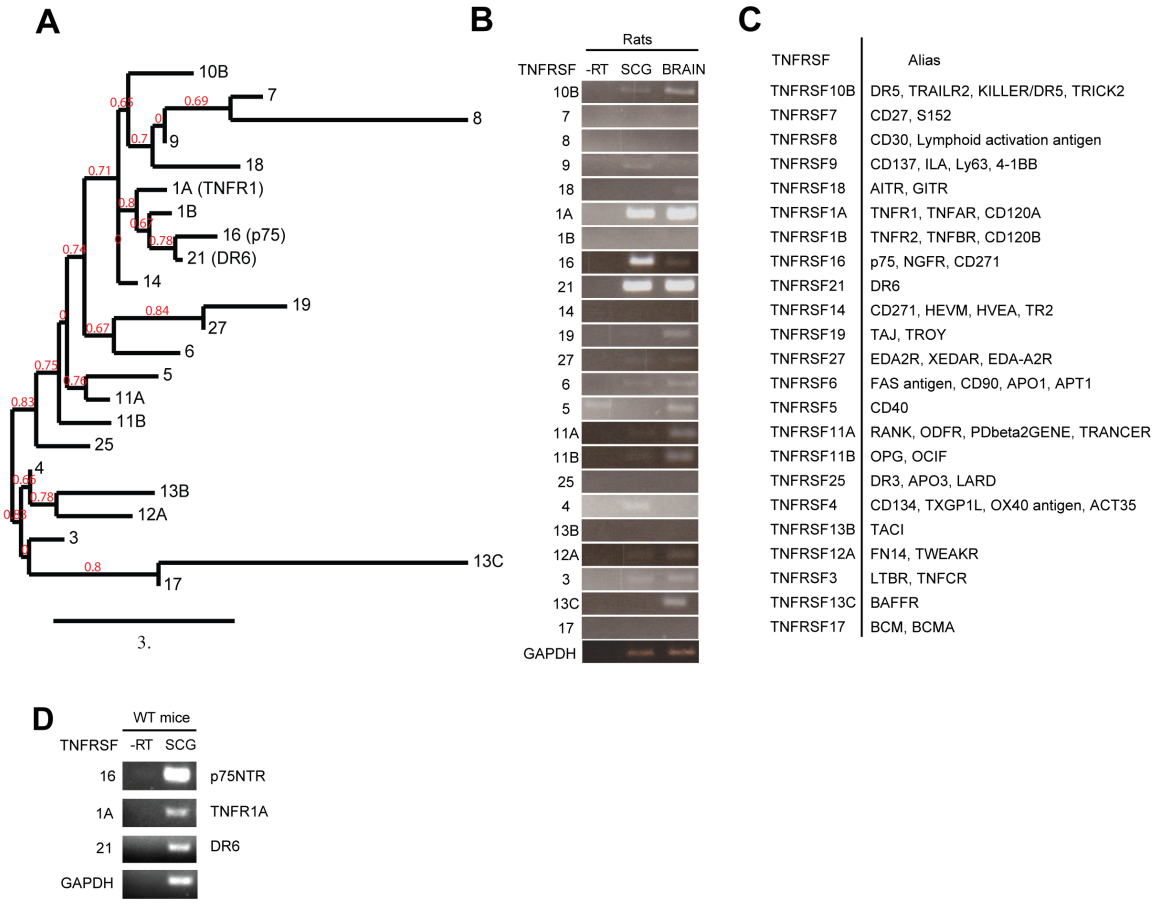
B. Schematic illustration for injury paradigm *in vitro*. Sympathetic neurons from P0-P3 mice are established with 45ng/ml NGF in microfluidic devices and the neurons are enucleated by aspiration.

C. Quantification of the percentage of axon degeneration after NGF deprivation and injury as a function of time**.** The latent and the catastrophic phases are indicated.

D. Extent of axon degeneration with and without NGF, 8 hours after injury.

E. Percentage of axon degeneration after 8 hours of injury in wild-type P0-P3 neurons and adult neurons. n=3 for each time point and genotype.

Here, and throughout, values are represented as mean ± SEM. n.s.= not significant; ***$p < 0.001$. n=3 independent experiments for each time point and genotype, unless otherwise specified. Maximum degeneration is defined as ≥80%. Significance determined by unpaired two tailed t test.



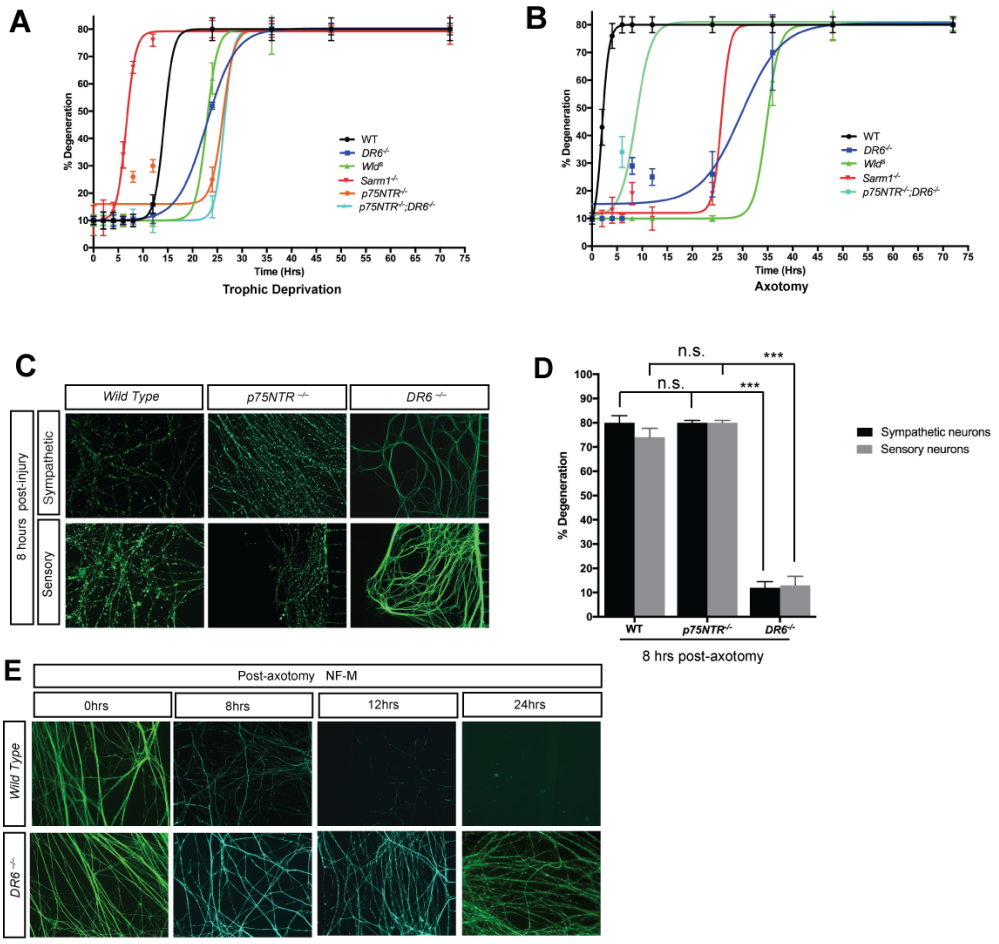
Supplementary Figure 2: Related to Figure 2: **Expression analysis of TNFR superfamily members in rats and mice.**

A. Phylogenetic tree of TNFR super family members generated using their amino acid sequences.

B. RT-PCR of TNFR super family members in rat. The expression of TNFR super family members was analyzed using mRNA isolated from the superior cervical ganglia (SCG) or brain of P0 rats, via reverse transcriptase polymerase chain reaction (RT-PCR). Each experiment was performed at least 3 times (n=3).

C. TNFR superfamily member aliases.

D. RT-PCR of p75NTR, TNFR and DR6 in mouse sympathetic neurons. The expression of TNFR super family members was analyzed using mRNA isolated from the superior cervical ganglia (SCG) of P0 mice, via reverse transcriptase polymerase chain reaction (RT-PCR). Each experiment was performed at least 3 times (n=3).



Supplementary Figure 3: Related to Figure 2: **DR6 promotes axon degeneration in trophic deprivation and axotomy.**

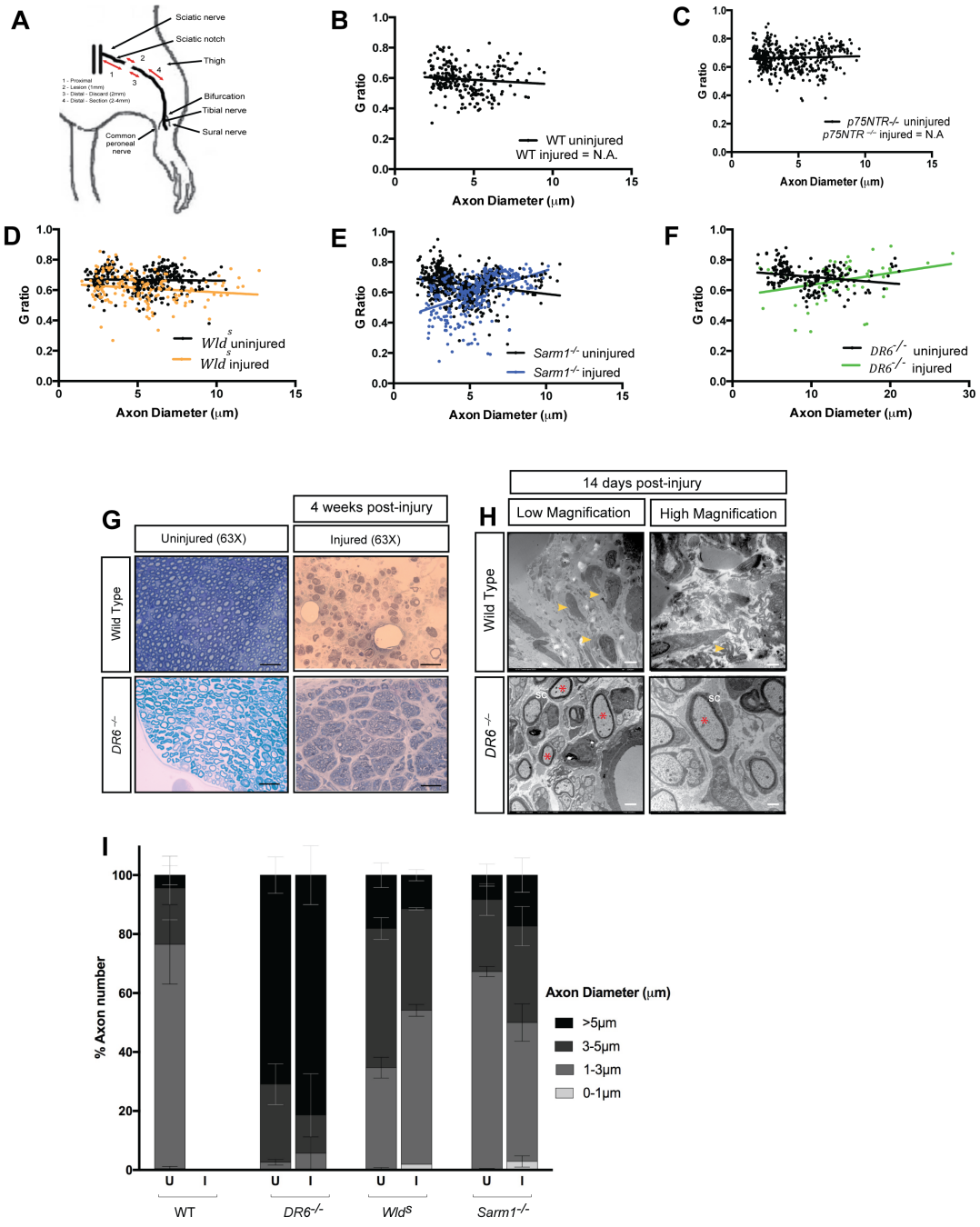
A. Percent distal axon degeneration after global NGF deprivation over time (hours) for wild type, *DR6*$^{-/-}$, *p75NTR*$^{-/-}$, *p75NTR*$^{-/-}$*;DR6*$^{-/-}$, *Wld*^{s} and *Sarm1*$^{-/-}$.

B. Percent distal axon degeneration after axotomy over time (hours) for wild type, *DR6*$^{-/-}$, *p75NTR*$^{-/-}$*;DR6*$^{-/-}$ *Wld*^{s} and *Sarm1*$^{-/-}$.

C. Representative images of βIII-tubulin immuno-stained distal sympathetic and sensory axons for Wild Type, *DR6*$^{-/-}$ and *p75NTR*$^{-/-}$ 8 hours after axotomy. Experiment is repeated 3 times.

D. Quantification of degeneration in **C**. n=3 for each time point and genotype.

E. Representative images of Neurofilament-M immuno-stained distal sympathetic Wild Type and *DR6*$^{-/-}$ 0,8, 12 ad 24 hours after axotomy. n=3 for each time point and genotype.



Supplementary Figure 4

Supplementary Figure 4: Related to Figure 3: **Sciatic Nerve Axotomy (SNA) in WT and *DR6*$^{-/-}$.**

A. Schematic representation of Sciatic Nerve Axotomy paradigm. Numbered red arrows indicate the region of the nerve used for experiments.

B. G ratio vs. Axon Diameter (μm) graph for Wild Type mice injured and uninjured sciatic nerves.

C. G ratio vs. Axon Diameter (μm) graph for *p75NTR*$^{-/-}$ mice injured and uninjured sciatic nerves.

D. G ratio vs. Axon Diameter (μm) graph for *Wld*^{s} mice injured and uninjured sciatic nerves.

E. G ratio vs. Axon Diameter (μm) graph for *Sarm1*$^{-/-}$ mice injured and uninjured sciatic nerves.

F. G ratio vs. Axon Diameter (μm) graph for *DR6*$^{-/-}$ mice injured and uninjured sciatic nerves.

G. Toluidine Blue stained cross sections of uninjured and injured Wild Type (n=3) and *DR6*$^{-/-}$ (n=3, penetrance of segmented myelination phenotype 33.3%), distal sciatic nerves 4 weeks after SNI. Scale bar = 20 μm.

H. Additional representative electron micrographs of cross sections from WT and *DR6*$^{-/-}$ distal sciatic nerves (2 weeks after injury) showing intact (red stars) degenerated axons (yellow arrows) and Schwann cells (SC) with higher magnifications (3000X and 6000X). Scale bars = 2μm (3000X) and 1μm (6000X). n≥3.

I. Percentage of injured and uninjured axons binned by axon diameter in Wild Type, *DR6*$^{-/-}$, *Wld*^{S} and *Sarm1*$^{-/-}$ mice before and after injury. 200 or more axons were measured in each mouse nerve, n=3 for each genotype.

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Chapter 3

Wallerian degeneration is coordinated by axon-derived calcium.

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Key words

Calcium, Wallerian Degeneration, NGF deprivation, DR6

Introduction

Axon degeneration is a hallmark of pathological insult, such as injury or disease, and often precedes loss of neuronal function. Since the 1850s it has been appreciated that such degeneration occurs across species, from worms to humans, however, we still know very little about the mechanisms governing axon degeneration (Waller, 1850; Wang and Barres, 2012). Axon degeneration occurs in two major contexts: (1) developmental and (2) pathological (Raff, Whitmore and Finn, 2002). Models of developmental degeneration include local trophic deprivation and branch elimination, which results in a degenerative process called pruning. Axons that are not properly connected to a target (*e.g.* muscle, skin, postsynaptic neuron(s)) undergo degeneration/die back as a result of deprivation from target-derived neurotrophic factors (Martin *et al.*, 1988; Raff, Whitmore and Finn, 2002; Luo and O'Leary, 2005; Singh *et al.*, 2008; Cusack *et al.*, 2013). In pathological axon degeneration including *neurodegenerative disorders* like Alzheimer's, Parkinson's, Amyotrophic lateral sclerosis (ALS) and Multiple Sclerosis (MS) display axon loss morphologically indistinct from axon loss in developmental degeneration (Coleman and Perry, 2002; Luo and O'Leary, 2005). In injury-induced/Wallerian degeneration acute insults, such as spinal cord injury, trigger axon loss similar to the above processes often resulting in permanent loss of sensory, sympathetic and/or motor function (Vargas and Barres, 2007). Of all the forms of degeneration described, least is known about the molecular mechanisms governing injury-induced degeneration. Whether developmental and pathological degeneration share similar molecular mechanisms remains unclear.

Wallerian degeneration was historically thought to be the result of passive wasting away of axons after an injury. We now believe that this process is actively regulated because of the finding of a spontaneous neomorphic gain of function mutation in mice known as *Wld(s)* that displays degeneration 10 times slower than wild-type neurons after injury (Lunn *et al.*, 1989; Brown, Lunn and Perry, 1992). More recently, Freeman and colleagues found the first loss of function mutant, sterile alpha and armadillo motif 1 (Sarm1), where Wallerian degeneration is blocked as robustly as observed in the *Wld(s)* mice (Osterloh *et al.,* 2012). Furthermore, other reported loss of function mutations have been shown to show remarkable protection from degeneration in transected axons in mutant mice (*e.g.* Phr1 Ubiquitin Ligase and DR6) (Babetto *et al*, 2013; Gamage et al., 2017). Importantly, molecular programs underlying Wallerian degeneration appear to be molecularly distinct from cellular apoptosis (Deckwerth *et al*, 1994, Finn *et al*, 2000, Whitmore *et al*, 2003). For example, caspases have yet to be implicated as a requirement for Wallerian degeneration (Finn *et al.*, 2000). Instead, another calcium sensitive protease, Calpain, has been implicated in degeneration, which is known to be tightly controlled by an inhibitor, Calpastatin (Yang *et al,* 2013). Importantly the source and timing of calcium required to activate Calpain has yet to be described. On the other hand, Sarm1 appears to be upstream of calpain in injury-induced degeneration events. It is thought to work by directly breaking down nicotinamide adenine dinucleotide (NAD^+) after injury via its Toll/Interleukin 1 Receptor (TIR) domain (Gerdts *et al,* 2015) (Essuman *et al.*, 2017).

Receptors have until recently not been implicated in injury-induced degeneration, although their role is well known in developmental paradigms. In developmental cell death and axonal loss paradigms it is established that "punishment" signaling pathways mediated by TNFR family members promote degeneration in the absence of a trophic cue (Deppmann *et al.*, 2008; Singh *et al.*, 2008). Interestingly, the punishment pathways appear to rely on autocrine/paracrine mechanisms allowing "strong" axons to expedite the elimination of weaker neighbors. Highly related Tumor Necrosis Factor Receptor Super Family (TNFRSF) members, p75 neurotrophin receptor (p75NTR), Tumor Necrosis Factor Receptor 1a (TNFR1a) and Death Receptor 6 (DR6), have been implicated in this extrinsic degeneration program in the peripheral nervous system (PNS) (Singh *et al.*, 2008; Vilar *et al.*, 2009; Park *et al.*, 2010; Olsen *et al.*, 2014; Wheeler *et al.*, 2014). The requirement of a receptor exists in injury contexts as well. Recently, we showed that Death Receptor 6 (DR6), is required to promote Wallerian degeneration *in vitro* and *in vivo* (Gamage *et al.*, 2017). This was the first implication of a receptor being required for Wallerian degeneration. Implicit in this finding is the notion that there may be an extrinsic, cue to promote degeneration.

Injury induced degeneration occurs in 3 stages: 1) a brief period of local degeneration in close proximity to the lesion site, immediately after injury, followed by 2) a latent phase which lasts for 2 hours *in vitro* and up to 48 hours *in vivo*, and finally 3) a rapid and near synchronous "catastrophic" disintegration of the cytoskeleton in injured axons (Rosenberg *et al*., 2012; Beirowski *et al*., 2005). While latent phases vary between developmental (18 hours *in vitro*) and injury-induced degeneration (2 hours *in vitro*), the

kinetics of rapid, catastrophic disintegration are similar, which may suggest not only shared molecular mechanisms but also coordination between susceptible axons (Kerschensteiner *et al*., 2005, Coleman *et al*., 2005).

The role of intra-axonal calcium has received attention of late for its role in promoting axon degeneration presumably via activation of Calpain (Avery *et al.*, 2012; Vargas *et al.*, 2015). Importantly though, Griffin and colleagues found that extracellular calcium is necessary and sufficient for injury induced axon degeneration (George, Glass and Griffin, 1995). However, their study did not clearly elucidate the source of extracellular calcium and whether it could fluctuate as a function of injury. We asked the question: is there a role for inter-axonal calciuim signaling in Wallerian degeneration?

We provide evidence that extracellular calcium is released from injured axons and that this is both necessary and sufficient to promote axon degeneration in both developmental and injury paradigms. We show that after injury and trophic deprivation, conditioned media surrounding degenerating axons contains elevated levels of calcium, at time points preceding the catastrophic phases of degeneration regardless of etiology (*i.e.* trophic deprivation and injury). Finally, we assessed the role of NMNAT1/2, Sarm1 and DR6 in the release and transduction of extracellular calcium. Taken together, we show that calcium released from injured axons functions as an extrinsic factor capable of promoting the degeneration of neighboring axons. These results implicate inter-axonal calcium signaling as a common molecular mechanism between several modes of axon degeneration.

Results

Extrinsic cues are capable of coordinating degeneration

Different axon degeneration modalities (*e.g.* injury, developmental, pathological) display different latent phases prior to rapid, catastrophic degeneration (Fig.1a) (Beirowski *et al.*, 2005; Rosenberg *et al.*, 2012). How do axons that degenerate in response to distinct triggers (*e.g.* NGF withdrawal or enucleation) disintegrate in near unison? Our recent discovery of a receptor required for injury-induced degeneration led us to hypothesize the existence of an extrinsic degeneration coordinating factor (DCF) (Gamage *et al.*, 2017b). To test this prediction, we performed conditioned media experiments to determine if a factor derived from degenerating axons can hasten degeneration of uninjured axons. We plated sympathetic neurons in microfluidic devices and collected conditioned media (CM) surrounding the distal axons, which were subjected to NGF deprivation (24 hours) or injury (8 hours) as degenerative triggers. Prior to these manipulations all the neurons were subjected to NGF deprivation for 12 hours in order to predispose them to degenerate (Figure 1b). We then added these CM as well as control CM (uninjured) to intact sympathetic neurons established in microfluidic devices and deprived of NGF for 12 hours, a time 12 hours prior to onset of degeneration induced by NGF withdrawal. Neurons treated with uninjured CM (UCM – axons are intact with their cell bodies, but NGF deprived for 12 hours) for 5 hours displayed minimal degeneration, which is consistent with the kinetics of normal developmental degeneration time course (Fig. 1a) (Cusack *et al.*, 2013; Gamage *et al.*, 2017b). However, we found that neurons deprived of

NGF but treated with injured axon conditioned media (ICM) or NGF deprived CM (NDCM) displayed maximal degeneration by 5 hours after addition (Fig. 1c). Taken together, these data indicate the existence of a Degeneration Coordinating Factor (DCF) secreted from degenerating axons is capable of hastening the degeneration of uninjured, NGF-deprived axons.

CM induced axon degeneration is blocked by trophic signaling.

Must axons be primed to respond to the putative extrinsic coordinating factor? To address this, we applied ICM to uninjured recipient axons bathed in NGF, which would model healthy neighboring neurons. Under these conditions ICM loses efficacy in promoting degeneration (Fig. 2a). We next sought to determine which NGF-TrkA dependent pathways conferred protection from ICM dependent degeneration. We pre-treated recipient axons bathed in NGF with PD98059 (50μM) or LY294002 (50μM) to inhibit classic TrkA dependent pathways, MAPK/ERK or PI3K/AKT, respectively. Blocking PI3K but not MAPK prevented the ability of NGF to protect against ICM mediated degeneration (Fig. 2a). Taken together, these data suggest that trophic PI3K signaling determines whether degeneration of uninjured axons are susceptible to the degeneration promoting effects of ICM.

The pro-degeneration bioactivity in CM from degenerating axons does not appear to be proteinacious.

We next sought to characterize the biochemical nature of the prodegeneration bioactivity in ICM using size fractionation, temperature cycling, or protease treatment (Fig.3a). We first examined the size of the factor by fractionating the ICM using a Centricon spin concentrator with a 10kDa cut off. When the flow through fraction was applied to uninjured recipient neurons, this fraction retained its ability to promote degeneration, indicating that the DCF is smaller than 10kDa (Fig.3 a,b). We next sought to determine whether the DCF is proteinacious. To inactivate proteins in the ICM we used temperature cycling by boiling or freeze/thaw as well as digestion by trypsin or proteinase k prior to the bioassay described in Figure 1B (Fig.3 c,d). ICM retains pro-degeneration activity after each of these treatments indicating that the bioactivity may not be derived from a protein.

Calcium is necessary and sufficient for CM prodegeneration bioactivity

The above experiments suggest that the prodegeneration bioactivity from ICM is under 10KDa and not a protein. We next turned our attention to calcium based on a report from Griffin and colleagues who found that extracellular calcium is necessary and sufficient for injury induced axon degeneration (George, Glass and Griffin, 1995). To determine if calcium is required for ICM bioactivity, we used chelex resin to deplete calcium from injured ICM (Supplementary Fig.1a). This treatment ablated the prodegeneration bioactivity of ICM (George, Glass and Griffin, 1995) (Fig. 4a and b). Because Chelex

resin chelates several ions beyond calcium, we next used EGTA, which specifically chelates calcium. Adding 6mM EGTA to ICM also suppressed its ability to hasten degeneration in recipient axons (Fig. 4a and b). Similarly, chelating calcium in NDCM blocked its prodegeneration activity (Fig. 4c & d).

To determine whether calcium is necessary to induce axon degeneration after injury, we used calcium free DMEM media in our injury degeneration paradigm. Injured wild type axons maintained in DMEM with 1.8mM calcium, showed classic degeneration signs like beading, blebbing and fragmentation around 4 hours after injury and by 6 hours most of the axons were fully degenerated (Fig. 5a & b). Remarkably, when calcium free media was used, axons showed no sign of degeneration by 6 hours after injury indicating calcium is necessary for the Wallerian degeneration pathway to function (Fig. 5a & b). Taken together, these experiments suggest that Calcium in the ICM is required for prodegeneration bioactivity. Similarly, incubation with calcium free media delayed axon degeneration following NGF withdrawal at 24 hours and 48 hours, suggesting that calcium is also necessary in developmental paradigms of axon degeneration (Fig 5c,d).

Calcium is released from degenerating axons

We next sought to determine whether extracellular calcium levels fluctuate after injury or NGF deprivation. To this end we compared the levels of free calcium in UCM, ICM and NDCM using a spectrophotometric Fluo-4 assay (Fig. 6a). ICM displayed an approximately 50% increase in calcium (2.4mM) over UCM (1.6mM) within one hour

after injury along with a dramatic reduction of intra-axonal calcium (Fig.S1b). Interestingly, the calcium concentration in UCM is lower than the calcium concentration in DMEM (1.8mM). We attribute this reduction to uptake and buffering of calcium by axons, as previously reported (Schlaepfer and Bunge, 1973; Arundine and Tymianski, 2003; Ganitkevich, 2003; M. a. Avery *et al.*, 2012). Chelating the intracellular calcium with BAPTA-AM added 1 hour before injury also reduced the amount of calcium released to extracellular space (Fig.6b). Collectively, these data suggest that axons release calcium shortly after injury. Similarly, in the trophic withdrawal paradigm extracellular calcium levels were elevated at 24 and 36 hours after removal of NGF (Fig.6c).

Extracellular calcium is derived from axonal ER and mitochondria

Intracellular calcium is buffered and stored by a contiguous network of Endoplasmic Reticulum (ER) and mitochondria (Mattson, 2007; Vargas *et al.*, 2015). At rest, the cytosolic calcium concentration is in the low nanomolar range (50-300nM), but these levels are capable of rising to low micromolar range after neuronal activation or activation of particular signaling pathways (LaFerla, 2002). The lumen of the ER contains 100-500μM of calcium and mitochondria also serves as a calcium store (Mattson, 2007). To deplete ER calcium, we treated neurons with thapsigargin to block SERCA pumps for 12 hours prior to injury, resulting in reduced calcium release from injured axons (Fig. 6d). We next asked whether extracellular calcium may be derived from mitochondria, previous studies have shown that Mitochondrial Permeability Transition Pore (mPTP) mediates axon degeneration and is capable of releasing calcium

(Barrientos *et al.*, 2011). We blocked the mPTP with cyclosporine A to observe changes of calcium release after injury and remarkably it also prevented calcium from releasing to the extracellular space (Fig. 6e). Taken together, these data suggest that elevated extracellular calcium during degeneration originates from the ER and mitochondria.

Evaluation of extracellular calcium levels in Wallerian degeneration deficient mutants

We next set out to measure the post-injury extracellular calcium levels in the Wallerian degeneration slow mutants: *DR6*$^{-/-}$, *Wld*^{s} and *Sarm1*$^{-/-}$. We measured calcium levels of ICM collected 1 hour after injury from wild-type and mutant axons. ICM from *DR6*$^{-/-}$ and *Sarm1*$^{-/-}$ neurons showed significantly elevated calcium levels after 1 hour of injury, suggesting the calcium release is upstream of DR6 and Sarm1 (Fig.7a). Remarkably, injured *Wld*^{s} axons showed no difference in extracellular calcium levels before or after the injury (Fig. 7a).

Calcium derived degeneration activity requires DR6.

Since calcium is required for the pro-degenerative activity of ICM, we next examined whether extracellular Ca^{2+} is sufficient to induce degeneration of NGF deprived axons. We applied increasing concentrations of $CaCl_2$ in DMEM (1.8mM calcium) to uninjured axons for 5 hours (Fig. 7c & d). We observed that a total calcium concentration as low as 1.9mM was sufficient to hasten degeneration in wild type axons. However, *DR6*$^{-/-}$ axons were resistant to at least 3.6 mM calcium in the same assay (Fig. 7c & d). Taken together,

these data indicate that calcium from injured axons is necessary and sufficient to induce degeneration of uninjured axons via DR6.

Discussion

In this study, we examined whether extrinsic axon derived factors are capable of promoting degeneration in both injury and developmental scenarios. We found that conditioned media from degenerating axons is capable of hastening axon degeneration of uninjured axons. The release of calcium to the extracellular space occurred after one hour of injury or 18 hours of trophic deprivation. Previous studies have explicitly shown the calcium dynamics inside the axons following an injury, explaining two stereotyped calcium waves taking place, and the ER as the source of calcium (Villegas *et al.*, 2014; Vargas *et al.*, 2015). Although calcium dynamics have been investigated, the fate or role of the calcium following injury, over time is not well understood. We observe elevation of calcium in media bathing axons after one hour of injury and 24 hours of NGF deprivation, which is prior to observing any visible morphological changes at 4-5 hours and 20-24 hours in culture, respectively. Additionally, this timing is coincidence with catastrophic degeneration in Wallerian degeneration as well as NGF deprivation, (Gamage *et al.*, 2017). Biochemical analysis of the CM revealed that the putative factor is small, not a protein and its activity can be blocked by NGF-TrkA-PI3K signaling. Chelating calcium within CM using chelex beads or EGTA suggests that this prodegenerative activity requires calcium. Moreover, calcium free media blocks Wallerain degeneration indicating necessity, which is consistent with previous reports (George, Glass and Griffin, 1995). Can injured axons release calcium into the

extracellular space? Spectrophotometric measurements of conditioned media revealed that calcium is increased from 1.6mM to 2.4 mM in the media surrounding injured axons relative to uninjured axons (Figure 6a). This release requires intracellular ER and mitochondria stores using the mPTP. While some mutants deficient in Wallerian Degeneration *DR6*$^{-/-}$ and *Sarm1*$^{-/-}$ retain their ability to release calcium after injury, *Wld*^{s} axons are incapable of this. This also suggests that calcium release could be downstream of Wld^{s} but upstream of DR6 and Sarm1. However, this is the first implication of calcium being released to the outside of the axon once it's injured and could be of conflicting evidence to the notion of axon intrinsic degeneration and coordination.

Our finding that calcium is a key degeneration promoting factor in CM is consistent with work from Griffin and colleagues who showed that extracellular calcium is required for axon degeneration after injury (George, Glass and Griffin, 1995). However, in that study they did not define a physiological source of calcium or a receptor that mediates its effect, whereas here we find that it is the injured axon itself that is the source of extracellular calcium and it requires DR6 to elicit degeneration.

Is increased extracellular calcium working through a cell surface receptor? From these data it is still unclear, however we can now say that calcium at least indirectly operates through DR6. The mechanism of direct or indirect interaction requires further scrutiny. Titration of exogenous calcium on wild type and *DR6*$^{-/-}$ axons lead us to conclude that higher levels of extracellular calcium (above 3.3mM total) can promote degeneration independent of DR6 (Fig. 5 e & f). This DR6 independent mechanism may occur via

reversal of Na^{+}-Ca^{2+} exchanger as shown previously in damaged axons, which would be sufficient to induce calpain dependent degeneration (George et al., 1995; LoPachin and Lehning, 1997; Ma, 2013; Ma et al., 2013; Yang et al., 2013). Furthermore, direct or indirect activation of DR6 may open channels that would allow influx of calcium. It is known that Wild Type axons show two functionally distinct calcium waves after an injury and *Wld*^{s} axons show increased mitochondrial flux in order to exert enhanced Ca^{2+} buffering and axon preservation after an injury(M. A. Avery *et al.*, 2012; Vargas *et al.*, 2015). How the loss of Sarm1 and DR6 affect the calcium dynamics should be carefully analyzed in the future.

How is calcium released given that it must go against the electrochemical gradient? The extracellular milieu contains ~1.8mM compared to the axoplasm at 50-300nM (LaFerla, 2002). One possible calcium efflux method would be to use the plasma membrane Calcium ATPase pump, which would require ATP to function. But, recent reports show that as the axon degenerates it experiences a rapid drop of ATP, making the axon energy deficient and precludes use of ATP to pump out calcium (Yang *et al.*, 2015). Other possibilities for calcium efflux after axonal injury are; 1) ER-mitochondrial contact sites at the plasma membrane – Previous reports have shown that in order to regulate the calcium signaling, ER and mitochondria could form a largely interconnected dynamic network (Rizzuto *et al.*, 1998). These reports are in agreement with our calcium release data suggesting that such a connected network could be modulating cytosolic calcium signal and its release. Therefore, it is possible that this type of apposition of sites could be formed with the plasma membrane allowing easy release of microdomains of high

calcium in that network, to the outside milieu. 2) Autophagosome/lysosome transport of extra calcium – Autophagy is a tightly regulated intracellular degradation/recycling system aiding cellular homeostasis. Although the exact role is unclear, enhanced autophagy is a feature commonly seen in many modes of axon degeneration such as trophic deprivation, chemical toxicity and neurodegenerative diseases (Yang *et al.*, 2013). There is also emerging evidence for autophagosome formation at the ER-mitochondria contact sites, therefore, the idea of autophagosomes or lysosomes transporting extra calcium to be released to the outside of the cell may be worth investigating (Hamasaki *et al.*, 2013). 3) Calcium permeable pores – There is compelling evidence that nanomolar levels $A\beta_{1-42}$ as well as prion proteins could form calcium permeable pores in cell membranes that may allow calcium influx (LaFerla, 2002). If an injury signal is also capable of making such calcium permeable pores, allowing a similar response, which is axon degeneration, it is likely that the electrochemical gradient is overcome to release calcium outside the axon. Therefore, a thorough investigation of calcium release mechanisms at the event of a damage/injury is vital to gain a better understanding of coordinated axon degeneration.

Our previous finding that receptors such as DR6 are involved in Wallerian degeneration has broad implications for nervous system injury. We suggest that this represents communication between injured axons and neighboring axons that are competent to undergo degeneration. Release of calcium from axons may represent a mechanism by which axons coordinate each other's degeneration after injury or in developmental paradigms. This challenges nearly two centuries of dogma that axons autonomously self-

destruct after insult. Perhaps coordination between susceptible axons is intuitive given that the kinetics of rapid, catastrophic disintegration are similar regardless of triggering events (Coleman, 2005; Kerschensteiner *et al.*, 2005; Martin *et al.*, 2010; Rosenberg *et al.*, 2012). The notion of paracrine signaling coordinating injury-induced axon degeneration may represent a clue into the basis of the "bystander" degeneration of uninjured axons surrounding sites of injury (Stirling *et al.*, 2014). This idea may provide an explanation for why the majority of neuron loss after ischemia, stroke, traumatic brain injury (TBI), and sciatic nerve injury (SCI) is outside of the injured region (Perry and Anthony, 1999; Stirling *et al.*, 2014). This effect has been largely attributed to inflammation, but it seems possible that calcium and inflammatory cues could be working in concert. It is interesting to note that DR6 belongs to a family of receptors, TNFRSF classically defined as responding to inflammatory ligands (Pfeffer, 2003; Whitney *et al.*, 2009; Amor *et al.*, 2010). Future studies will specifically examine the role of TNFR family members and secreted extrinsic factors on neurons and glia in promoting bystander degeneration in the context of traumatic injury and stroke. Finally, our results indicate a novel concept of coordination of axon degeneration via calcium, which is DR6 dependent. We show that extracellular calcium is necessary and sufficient to induce degeneration in healthy uninjured axons. Our data showing that calcium is released from axons after injury points toward possible therapeutic intervention to alleviate bystander degeneration after traumatic brain or spinal cord injury. It is also worth noting that calcium is not just released inside the axon to activate proteases like calpain, but it bears

a more complex and important role in coordinating the degeneration of an insulted group of neurons.

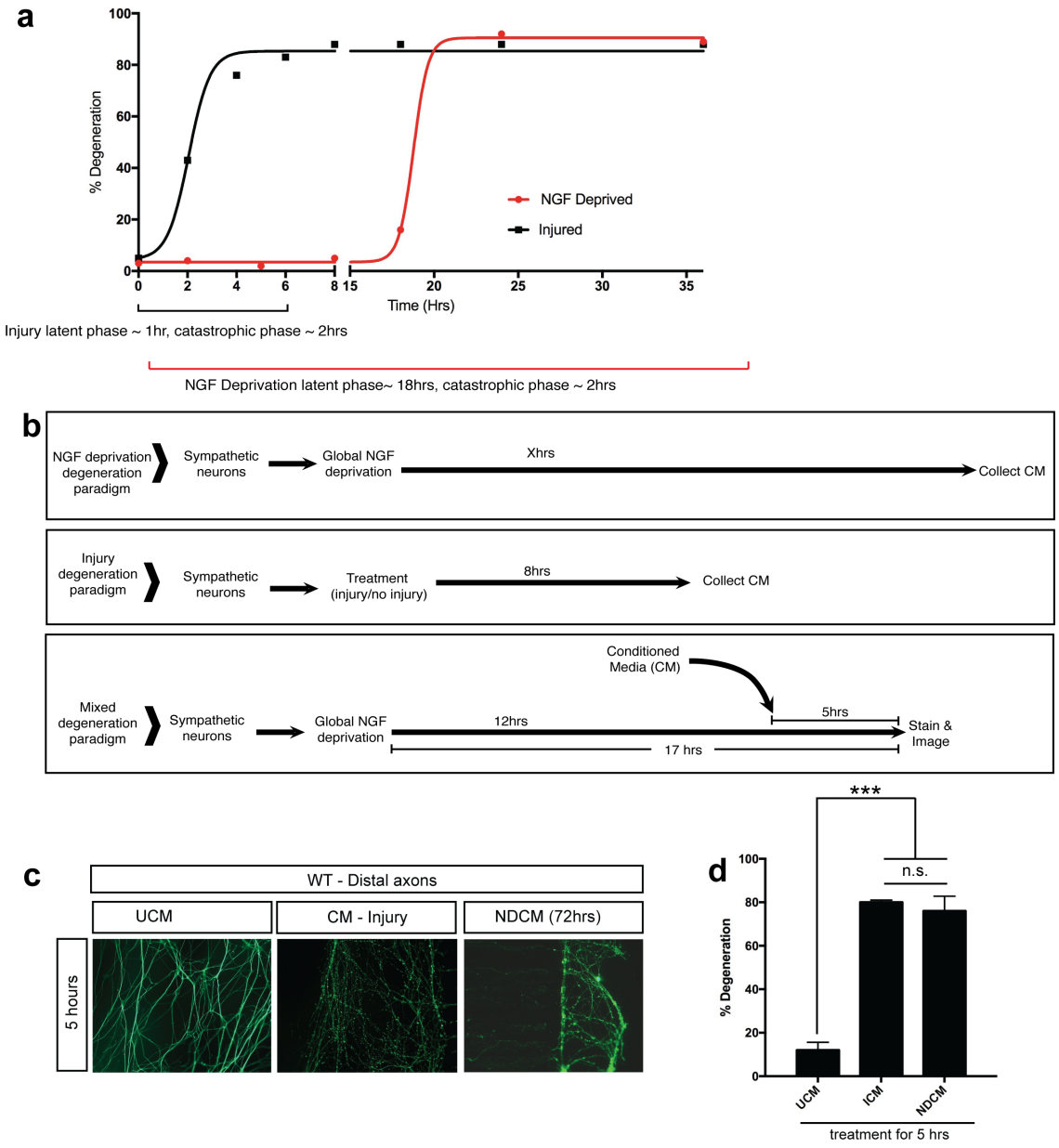


Figure 1. **Trophic factor withdrawal and injury in vitro show similar catastrophic axon degeneration kinetics but different latencies.**
a. Latent and Catastrophic phases of injury and NGF deprivation.
b. Axon degeneration paradigms used in this paper. NGF Deprivation induced degeneration paradigm – WT Sympathetic neurons are globally NGF deprived and the conditioned media surrounding the axons (NDCM) is collected.
Injury Degeneration Paradigm - WT Sympathetic neurons are injured and the conditioned media (ICM) surrounding the axons is collected.
Mixed Degeneration Paradigm - WT Sympathetic neurons are globally NGF deprived for 12 hours and the conditioned media (NDCM or ICM) is applied to the axons for 5 hours.
c. Representative images of βIII-tubulin immuno-stained distal sympathetic axons after treatment with ICM and NDCM for 5 hours.
d. Quantification of c.
Here, and throughout, values are represented as mean ± SEM. n.s.= not significant; ***$p < 0.001$. n=3 for each time point and genotype, unless otherwise specified. For each repeat at least 100 axons are scored for degeneration. Significance determined by unpaired two tailed t test.

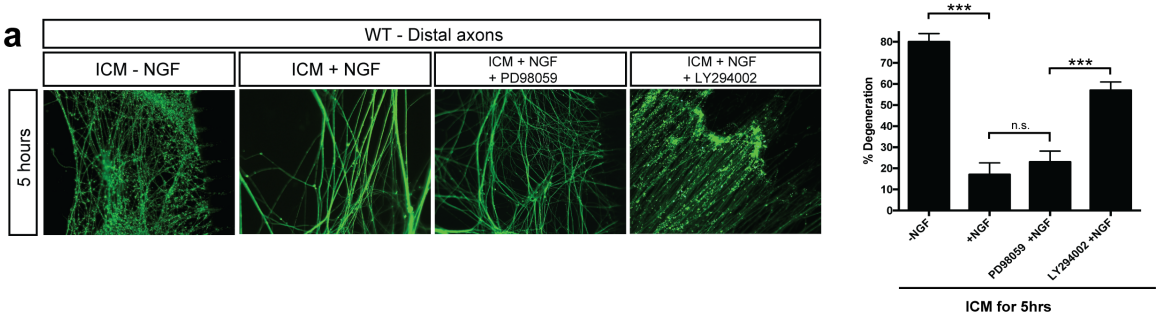


Figure 2. Trophic PI3K signaling determines the susceptibility of axons to ICM.

a. ICM applied to uninjured wild-type neurons in the presence or absence of NGF (45ng/ml) for 5 hours. Inhibitors of MAPK/ERK (PD98059- 50μM) and PI3K/AKT (LY294002- 50μM) pathways were added to ICM supplemented with NGF (45ng/ml) and applied to recipient wild type axons for 5 hours.

b. Quantification of b.

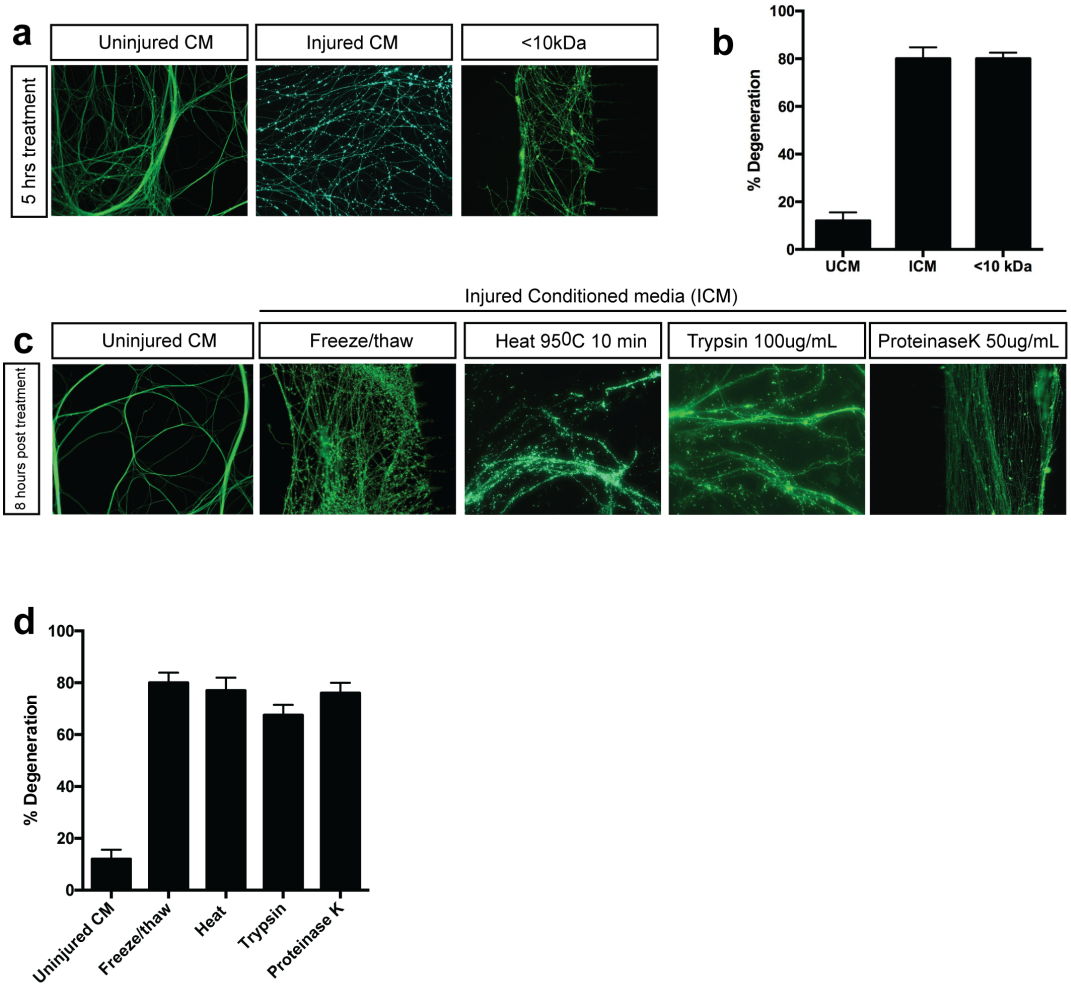


Figure 3. Putative degeneration coordinating factor in ICM is not a protein.

a. ICM was size fractionated using a 10kDa filter and the collected eluate was applied to NGF deprived uninjured neurons. Representative images of distal axons reveal significant degeneration in the axons that received <10KDa ICM fraction.
b. Quantification of degeneration in b.
c. Stability of the ICM is determined by subjecting ICM to a freeze/thaw cycle, heat (950C for 10 minutes), Trypsin treatment (100µg/mL, 370C for 30 minutes) and Proteinase K treatment (50µg/mL, 370C for 30 minutes) and applied to recipient axons for 5 hours. Representative images are shown.
d. Quantification of degeneration in c.

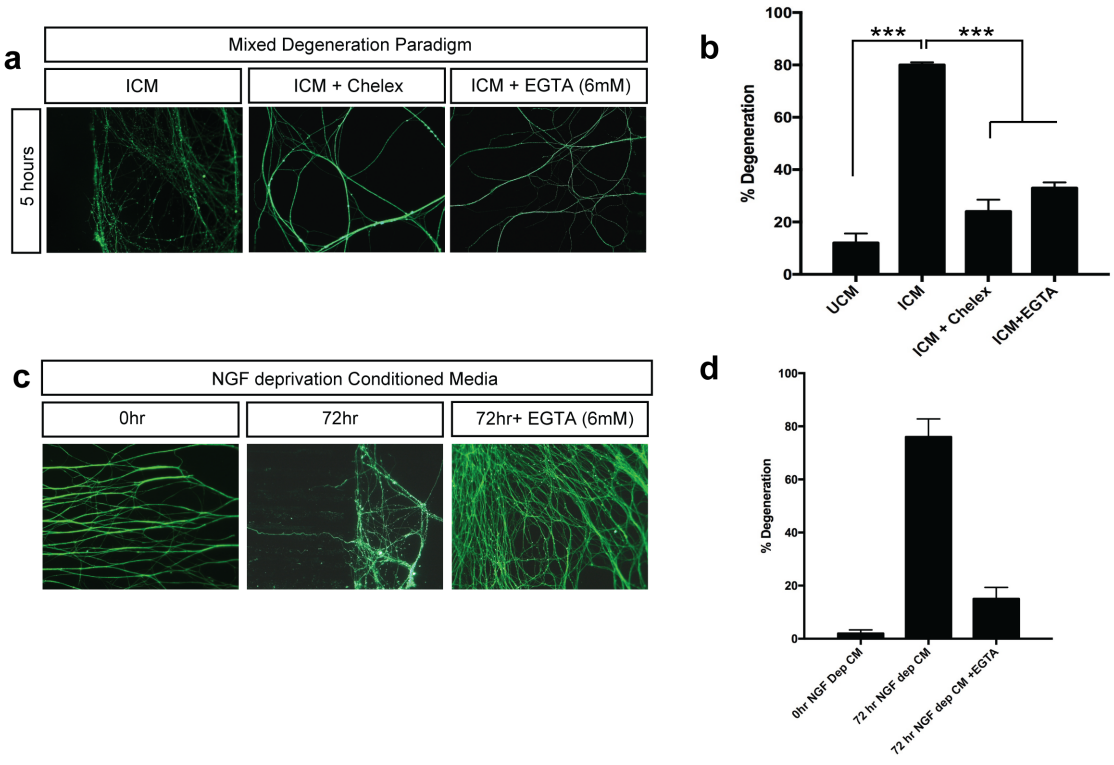
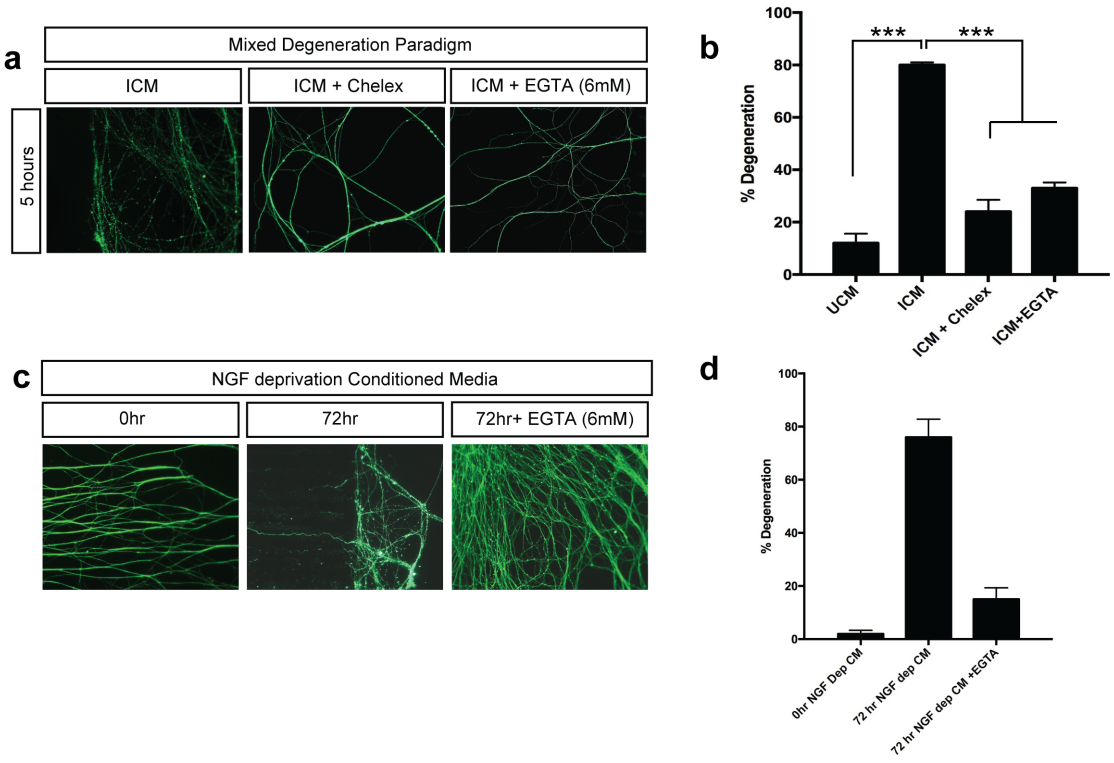


Figure 4. Calcium is sufficient for CM-induced axon degeneration in injury and in NGF deprivation.

a. Calcium was depleted from ICM using Chelex beads or 6mM EGTA and applied to uninjured NGF deprived sympathetic neurons for 5 hours followed by visualization by immunostaining for βIII-tubulin.
b. Quantification of a.
c. Calcium was depleted from NDCM using 6mM EGTA and applied healthy sympathetic neurons for 5 hours followed by visualization by immunostaining for βIII-tubulin.
d. Quantification of c.

Figure 4



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d. Quantification of c.

Figure 4

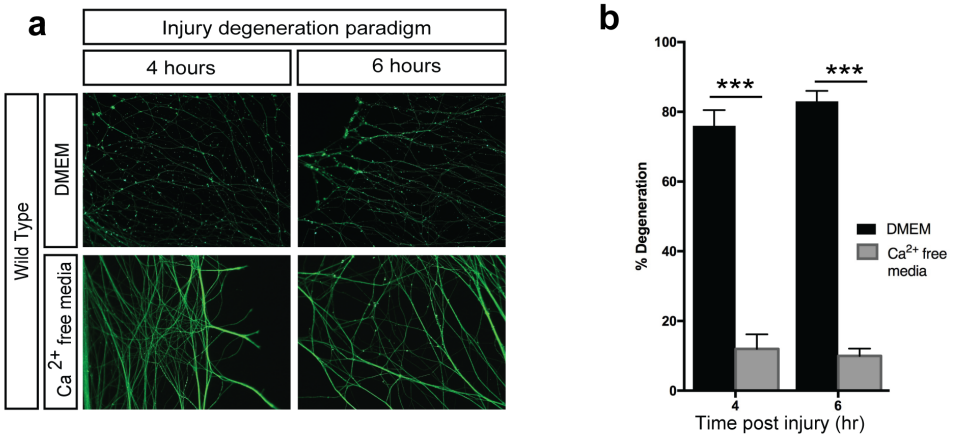


Figure 5. Calcium is necessary for CM-induced axon degeneration in injury and in NGF deprivation.

a. Representative images of βIII-tubulin immuno-stained distal sympathetic axons after 4 and 6 hours of injury in media with calcium (DMEM) and calcium free media.
b. Quantification of a.

Figure 5

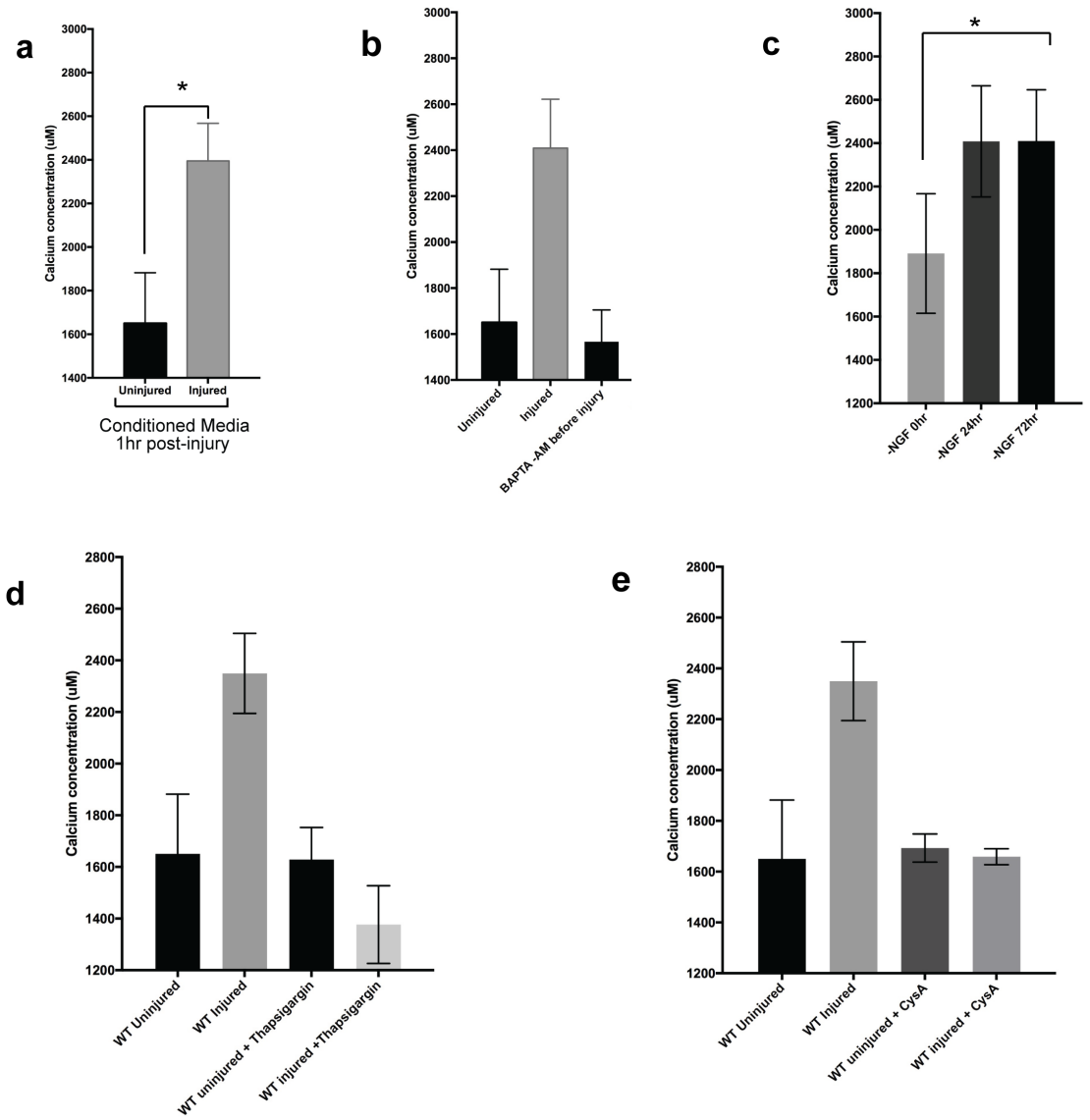


Figure 6. Conditioned media contains elevated concentrations of Calcium.

a. Measurements of Ca2+ concentration using a spectrophotometric assay with fluo-4 calcium indicator of uninjured and injured conditioned media.
b. Calcium measurements in the ICM with treatment of BAPTA-AM before (1hr) the injury.
c. Calcium measurements in the NDCM for 0, 24 and 72 hours of NGF deprivation.
d. Calcium measurements in the ICM with overnight treatment of Thapsigargin to deplete ER calcium.
e. Calcium measurements in the ICM with overnight treatment of Cyclosporin A to block mitochondrial mPTP.

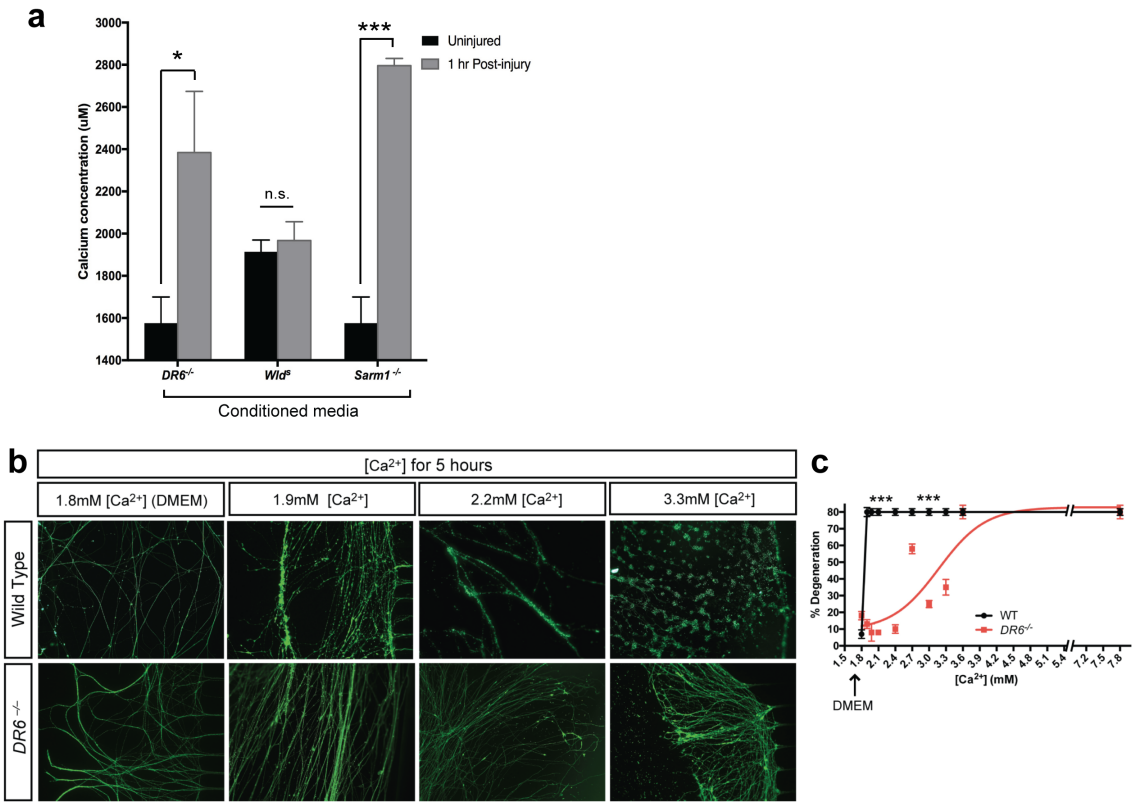
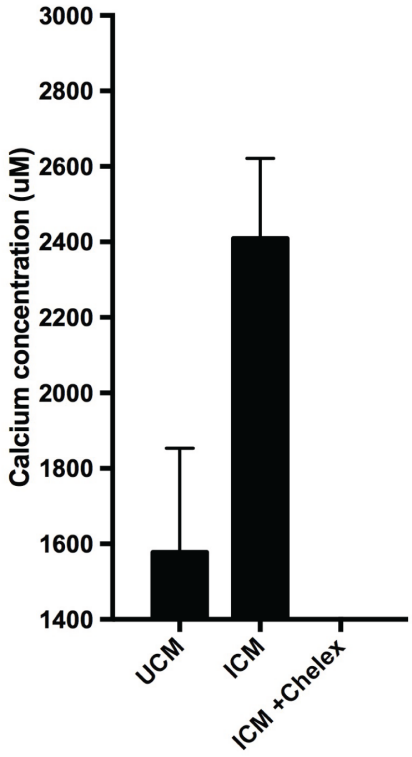


Figure 7. Calcium concentration is variable in Wallerian Degeneration mutants and loss of DR6 blocks exogenous Calcium mediated axon degneration.

a. Calcium measurements of Wallerian degeneration mutant neurons 1 hour after injury..
b. Exogenous calcium on WT and DR6 mutant nurons for 5 hours.
c. Quantification of b.

a



Supplementary Figure 1

a. Calcium concentration of uninjured CM, Injured CM and Injured CM passed through with chelex beads.

Chapter 4

Discussion

The molecular mechanisms underlying axon degeneration has been a black box for decades. Although some downstream pathways such as MAPK and Calpain have emerged in recent years, the initiator of the signaling has not been described (Yang, Robby M Weimer, *et al.*, 2013; Yang *et al.*, 2015). Our findings show that the TNFR family member, DR6 may represent this long sought degeneration-initiating factor. This finding marks the first receptor discovered as required for axon degeneration and is only the second loss of function mutation to delay Wallerian Degeneration as robustly as the Wld(s) mutation (Sarm was the first LOF described to do this). As DR6 has been previously shown to be involved in axon degeneration in trophic factor deprivation, this finding implies that there are common mechanisms governing axon degeneration in development and injury, with a few exceptions in the downstream pathway. Is it possible the DR6 is a common link between all forms of degeneration? To address this we will ask the following questions, 1) is DR6 performing the same pro-degenerative role in central nervous system injury e.g. Traumatic brain injury (TBI) or stroke? 2) Is DR6 activated by toxic proteins in neurodegenerative disease like Aβ in AD or α-synuclein in PD?

The notion of a receptor being involved in degeneration also suggests the coordination between neighboring axons during an injury condition. In **Chapter 3** we show that after injury or NGF deprivation, calcium is released to the outside of axons and signals to

neighboring axons via DR6. Thus, several questions remain such as the mechanisms of calcium release from the injured axons, calcium entry to the neighboring neurons and the roles of extracellular calcium in axon degeneration of the neighboring neurons. However, it is already known that the calcium sensitive protease is activated when extracellular calcium enters axons(Ma, 2013). It would be interesting to tease apart the timing of calcium entry and calpain activation in the context of TBI, specifically employing live brain cell imaging in wild type as well as degeneration slow genotypes. Taken together, these data suggest that a common mechanism involving DR6 and calcium govern the coordination of axon degeneration in injury and in development.

DR6 is the first receptor implicated in Wallerian Degeneration.

Although there has been much attention on Wallerian degeneration molecular mechanisms, the concept of receptors involved in this process has been novel until our finding of DR6 being the receptor involved in WD (Gamage *et al.*, 2017a). Perhaps this shouldn't be surprising given that DR6 has been previously implicated in modulating developmental axon degeneration(Nikolaev *et al.*, 2009; Olsen *et al.*, 2014). However, the findings on DR6 binding to APP in order to activate downstream caspase cascade resulting in axon pruning has been controversial. Genetic deletion of APP and DR6 did not reflect the protection rendered by antibodies, which was later found to be exhibiting off target effects (Olsen *et al.*, 2014). The genetic deletion studies showed only modest protection of APP knockouts compared to BAX and/or Caspase-3 knockouts, which showed more protection even at 72 hours following trophic factor deprivation(Olsen *et*

al., 2014). Although a careful analysis of the function of APP has not been carried out yet, we have verified the role of DR6 in trophic factor deprivation induced axon degeneration. Loss of DR6 shows a modest protection from axon degeneration following trophic deprivation indicating that other receptors (e.g. p75NTR) play a significant role in developmental axon degeneration (Gamage *et al.*, 2017).

A receptor responsible for initiating axon degeneration has implications for nervous system injury, neurodegenerative diseases and peripheral neuropathies. Until our finding, WD was thought to be an axonal intrinsic program and the degeneration signal was thought to be acting on the adaptor protein Sarm1(Gerdts *et al.*, 2013). DR6 is considered an orphan receptor as no ligand binding to it has been found yet (Klíma *et al.*, 2009). Moreover, how classic TNFR downstream signaling links to the previously described WD intrinsic pathway remains unclear.

In light of this finding, we must next focus on defining a ligand/co-receptor for DR6, which could represent an extrinsic degeneration signal, which triggers the WD molecular pathway. This would be a very important finding because; pharmacological intervention for blocking the production, trafficking and/or release of this degeneration signal could serve to delay axon degeneration in directly affected axons as well as in bystanders in an injury situation. If the axon degeneration in the damaged area were slowed, regeneration of the proximal stumps of the nerve would be more efficient. Previous studies have shown that robust repair of the damaged PNS nerves employ “Bands of Bungner” due to the rapid changes that Schwann Cells (SC) undergo during the repairing process (Stoll,

Jander and Myers, 2002). These de-differentiated SCs provide structural guidance and growth factors for the regenerating axons to find their correct targets (Vargas and Barres, 2007). Similarly, it can be speculated that delayed degeneration of the distal stump of a damaged nerve could also provide structural guidance to the regenerating nerve. Furthermore, the possibility of reconnection of the distal and proximal stumps after a crush/transection should be investigated in mouse/rat nerves compared to the previous studies in lower organisms like worms (Wang and Jin, 2011). These phenomena are of particular importance, specifically in the PNS, which possesses the ability to regenerate and regrow its damaged axons as a result of better and efficient clearance of the debris, unlike the CNS (Vargas and Barres, 2007). Therefore, finding means of slowing down the degeneration of damaged nerves, could serve the purpose of aiding regeneration, which could be of therapeutic interest.

Calcium signaling in neurodegeneration

Basal levels of cytoplasmic calcium is maintained in the nanomolar range by various calcium ATPases in the plasma membrane, SR/ER Calcium ATPases and Na+/Ca2+ exchanger (Mattson, 2007). The most prominent sources of calcium in the cytoplasm are ER and mitochondria have been recently shown to be associated with neurodegenerative diseases(Mattson, 2007). Mitochondria and ER have also been shown to physically and functionally tightly interconnected. Recent evidence has revealed that the impairment in their communication might represent a common feature in different neurodegenerative diseases(Mattson *et al.*, 2000; Court and Coleman, 2012). The existing evidence for ER

and mitochondria contact sites show that they are crucial for calcium signaling because, upon releasing calcium from the ER, the mitochondrial uniporter takes up the high local spikes in cytoplasmic calcium(Rizzuto *et al.*, 1998; Calì, Ottolini and Brini, 2013; Hamasaki *et al.*, 2013). This interaction is crucial and bi-functional to the cell as alterations in ER– mitochondria juxtaposition determines mitochondrial dysfunctions and compromise lipid metabolism, protein synthesis, and folding. However, this physical link between ER and mitochondria is crucial for the accumulation of calcium in the mitochondria and has been shown to play a key role in calcium dyshomeostasis during neurodegenerative diseases. In particular, it is clearly established that a constitutive InsP3R-mediated ER-mitochondria calcium transfer is important to sustain mitochondrial respiration and normal cell bioenergetics (Calì, Ottolini and Brini, 2013). Proteins involved in Parkinson's disease (α-synuclein, Parkin) have been also shown to increase the ER-mitochondria contact sites to induce calcium accumulation in the mitochondria, suggesting a calcium mediated mitochondrial dysfunction during these diseases (Calì, Ottolini and Brini, 2013). Furthermore, studies on familial AD mutant PS1 and APP expressing cells and in fibroblasts from patients with the familial and sporadic forms of AD, have shown increased ER-mitochondria connections, explaining the calcium overload in the neurons during AD (Calì, Ottolini and Brini, 2013). There is also evidence that in ALS, VAPB protein localizes to mitochondria associated ER-membranes to interact with mitochondrial outer membrane proteins and regulate mitochondrial calcium uptake (Calì, Ottolini and Brini, 2013).

All of the above evidence point to the importance of ER and mitochondria interaction to sustain many cellular functions, involving calcium homeostasis. As dysregulation of these ER-mitochondria contact sites and impaired calcium transfer is shared by many neurodegenerative disease situations, it is imperative that this phenomenon is investigated in an injury context. High-resolution calcium imaging would be essential in determining the behavior of the calcium waves and their fate after an injury. Although it is now established that there are two waves of calcium, flushing through the axon following an injury, the function of these calcium waves are yet to be described (Vargas *et al.*, 2015). Current evidence suggests that a major role for calcium is to activate the protease calpain. How else should this substantial flow of calcium after injury be investigated? In light of previous data on calcium overload in neurons of AD brains and our findings of extracellular calcium elevation after injury, several questions remain (Kastanenka *et al.*, 2016). 1. Is the neuron responding to the injury and calcium overload in its axoplasm, by calcium efflux to the extracellular milieu? 2. Is the role of calcium released outside the axon, to induce degeneration in the neighboring compromised axons/neurons? 3. Is calcium inducing any cleavage events of various receptors/ligands or co-factors to activate death receptors (DR6) or executioner proteins like Sarm1? These questions could be answered by scrutinizing the molecular mechanisms induced by calcium in damaged as well as neighboring neurons. Live imaging of calcium dynamics and genetic deletions of calcium sensitive proteins specifically in bystander neurons would also be instrumental in delineating these roles.

Neurons modulate calcium signals inside the cells by releasing calcium from the internal stores or by regulating the influx of calcium from the extracellular environment. Why do we observe elevated levels of calcium shortly after injury? It is known that the axonal calcium is elevated above the normal range (50-300nM) following axotomy (LaFerla, 2002; Villegas *et al.*, 2014). Adding more calcium using ionophores increases the damage to the axon and this process could be blocked using chelating agents (George, Glass and Griffin, 1995). Calcium dynamic studies on zebrafish has revelaed two waves of calcium inside the axon following axotomy, first a transient calcium wave originated from the injury site and the second more prominent calcium wave which affected the mitochondria along the axon (Vargas *et al.*, 2015). These observations point to the importance of calcium release from the internal stores following axon injury, however, none of the studies explained the fate of the released calcium within the axon. Our data suggest that once these calcium waves occur, the axon releases it to the outside environment.

Downstream signaling of Wallerian Degeneration

Tessier-Lavigne's group elucidated the downstream signaling pathway of Wallerian degeneration by showing the involvement of MAPK pathway components and Calpain (Yang, Robby M. Weimer, *et al.*, 2013; Yang *et al.*, 2015). Although it is clear that activation of Sarm1 leads to activation of MEKK4/DLK and Sarm1 acts as an NADase, the link between NAD^+ and MAPK pathway is still unclear (Summers *et al.*, 2016). Furthermore, from our results, it is apparent that the calcium release from damaged axons precedes any morphological changes in the axon. However, activation of the calcium

sensitive calpain protease takes place as one of the final steps in the pathway, which presumably does not occur immediately after an injury(Ma *et al.*, 2013; Yang, Robby M. Weimer, *et al.*, 2013). What molecular mechanisms underlie this early calcium release and how it activates the Sarm1-MAPK pathway should be further studied. More importantly, the link between Sarm1 and DR6 also should be thoroughly investigated in order to achieve a full understanding of the downstream pathway. It is possible that Sarm1 and DR6 could physically interact, which we are currently investigating.

Role of Schwann cells in Wallerian Degeneration

Schwann cells (SCs) play an important role in axon regeneration by dedifferentiating in to more immature-like stage in the injured nerves (Jessen and Mirsky, 2008). Following an injury, the axons lose contact with the cell body, leading to activation of specific intracellular signaling molecules that drive the dedifferentiation program to establish a favorable environment for axon degeneration (Jessen and Mirsky, 2008; Woodhoo *et al.*, 2009; Arthur-Farraj *et al.*, 2012). However, little is known about the events that occur prior to Schwann cell dedifferentiation and the identity of the that signals trigger SC dedifferentiation. Studies on Schwann cell contribution to any of the Wallerian degeneration slow mutant mice such as Wld^{s} and $Sarm1^{-/-}$ also have not been well documented yet. Jessen and Mirsky's group reported that over-expression of c-Jun in crushed Wld^{s} nerves could induce regeneration suggesting defective Schwann cell activation in those nerves(Arthur-Farraj *et al.*, 2012). Although, neither Wlds nor Sarm1 has been conditionally manipulated (expressed/knocked out) in SCs to study their exact roles in injured SCs.

Our studies on injured DR6 mutant nerves shows intact axons and aberrant myelination indicate that the SCs and axons receive different triggers after an injury. This is the first implication of uncoupling of axon degeneration and demyelination. The data are still not clear on whether axons detect the injury signal first and SCs proceed to detect it or vice versa. If axons signal to the SCs to degenerate after an injury, we should observe simultaneous protection of axons as well as SCs in injured DR6 mutant nerves as DR6 is the primary receptor of the injury signal. However, our results show a possible regenerative Schwann cell behavior after an injury in DR6 mutant sciatic nerves. Therefore, it is unlikely that injury signal detection only occurs in damaged axons, but it occurs in Schwann cells as well. However, expression of DR6 is not reported in Schwann cells, indicating that SCs might have their own injury detection mechanism. Interestingly, *p75NTR*$^{-/-}$*;DR6*$^{-/-}$ nerves show complete protection from injury induced axon degeneration as well as demyelination, suggesting that p75NTR may be playing a role in SC demyelination as previously reported (Appendix 1) (Cosgaya, Chan and Shooter, 2002; Vargas and Barres, 2007). Careful investigation must be carried out to delineate the different mechanisms of axon and SC response to peripheral nerve injury. For example if we knock out p75NTR specifically in SCs in a DR6 knockout background, it is possible that injured nerves would show intact axons and remyelinating SCs confirming that p75NTR is responsible for dedifferentiating SCs following an injury. More interestingly, if we conditionally knocked out p75NTR in a wild type background, would we observe intact axons as well as SCs? This type of investigation would also be instrumental in answering the question whether SCs receive the demyelination signal from axons after an

injury or both axons and SCs simultaneously receive the injury signal. Furthermore, it would be interesting to study how SCs respond to changes of calcium level outside the axons and whether calcium acts as a messenger to induce SC demyelination and dedifferentiation. Therefore, our finding of the uncoupling of axon degeneration and demyelination after an injury is a stepping-stone for a thorough investigation of the autonomy of SC response to peripheral injury.

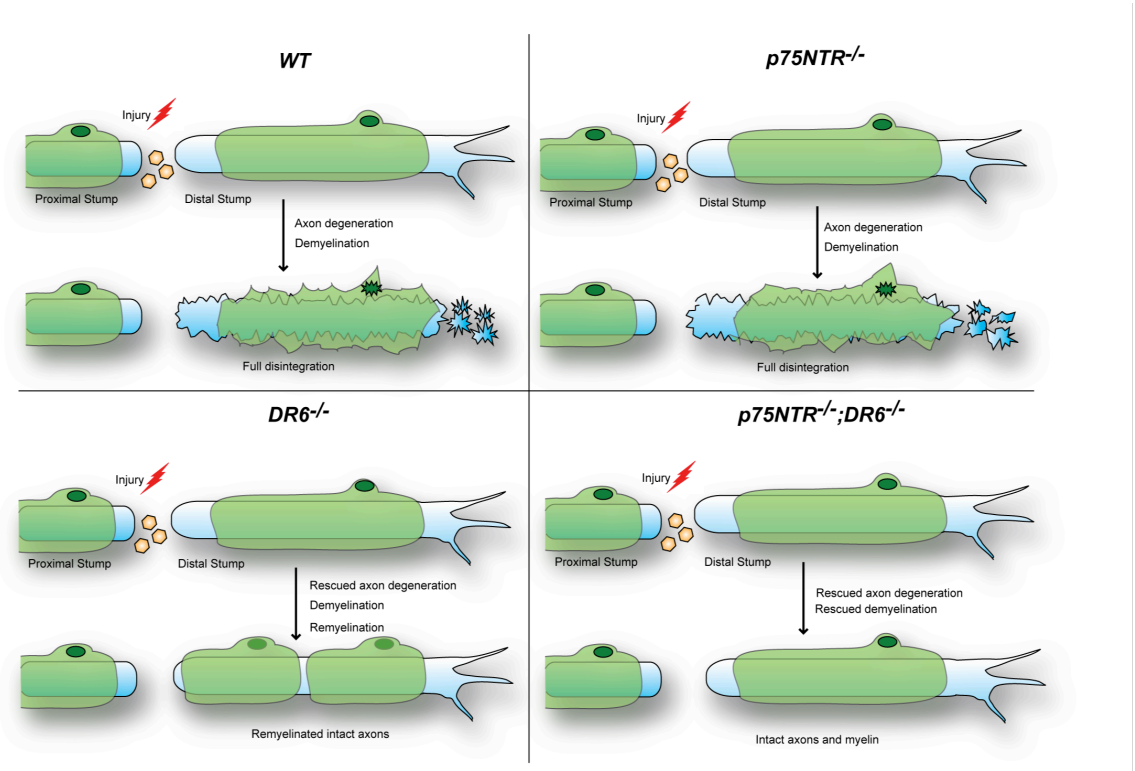


Figure 1: Model for the roles of DR6 and p75NTR in injury-induced axon degeneration. Loss of *DR6* rescues injured distal axons from axon degeneration, and shows demyelination followed by remyelination. Wild type and *p75NTR* mutant axons are not protected from either axon degeneration or demyelination after injury, indicating an essential role for DR6 in injury-induced degeneration. In the absence of both *DR6* and *p75NTR,* axon degeneration and demyelination are abrogated. Therefore, it can be

speculated that DR6 plays an exclusive role in injury-induced degeneration on the axon while p75NTR has a role in demyelination, presumably on glia. Beyond the hierarchal role of TNFR family members in nerve disassembly this also suggests that loss of axons triggers loss of myelin and Schwann cells.

DR6 in the CNS

As the role of DR6 in WD of the PNS neurons are evident from our studies, the next important question to answer would be whether DR6 plays the same role in initiating axon degeneration in the CNS. It would be important to investigate how a receptor like DR6 is involved in disease conditions like Alzheimer's . Although it was previously reported that N-APP is the ligand for DR6, those results have been amended with further investigations (Nikolaev *et al.*, 2009; Olsen *et al.*, 2014). However, the notion that APP could be functioning as a ligand or a co-receptor should be investigated more as it has been shown that DR6 and APP act in a cell autonomous manner in the same pathway to control axon pruning in development in vivo (Olsen *et al.*, 2014). Further evidence for interaction between DR6 and APP have been shown by other groups by crystal structure analyses of DR6 and APP (Kuester *et al.*, 2011; Xu *et al.*, 2015). Based on these findings, it's proposed that DR6 activation occurs through APP induced dimerization in order to lead to synaptic elimination and axon pruning. In other words, two DR6 molecules are bound to a dimeric APP molecule (APP E2) at the neuronal surface, leading to a 1:1 stoichiometric ratio. This association positions the C-terminal death domains of adjacent DR6 molecules in close proximity to trigger the activation of downstream signaling (Xu *et al.*, 2015).

Members of the TNFR family have been shown to hetero-multimerize with its other family members as well as other receptor family members. For instance, p75NTR has been shown to bind to Nogo-66 (NgR1) receptor and inhibit axon regeneration (Tam *et al.*, 2012). Furthermore, another member of the TNFR family, TROY, has been shown to physically interact with NgR1 as well as DR6, via coimmunopreciptaion studies (Tam *et al.*, 2012). This suggests that DR6 is a receptor which, homo- or hetero-dimerize with itself and other receptors in order to activate its downstream signaling pathways. The interactions DR6 has in the PNS and in the CNS, during axonal injury and diseases, should be vigorously studies in order to find therapeutic solutions. In parallel to finding a co-receptor, a search for the ligand should also be continued, as the possibility of DR6 working through a ligand, shouldn't be dismissed yet.

All of these questions would be very interesting to address in order to delineate the molecular mechanism of coordinated axon degeneration. If the mechanisms for injury-induced axon degeneration were revealed, it would be possible to apply similar approaches in disease situations, which would be of greater therapeutic interest. Especially, in the instance of Traumatic Brain Injury (TBI), the structure and stability of microtubules may render them especially vulnerable to mechanical disruption, shown by a reduction in the number of axon microtubules after trauma (Tang-Schomer *et al.*, 2010). This loss of microtubules is thought to be mediated by post-traumatic influx of calcium in to axons(Tang-Schomer *et al.*, 2010). Therefore, widespread axon swelling and degeneration could be originating from a localized mechanical trauma, which produces

cues to evolve axon pathology after trauma. We believe that by finding the molecular mechanism behind coordination of axon degeneration, would serve means of therapy for TBI.

Chapter 5

Materials and Methods

Mice

All experiments were carried out in compliance with the Association for Assessment of Laboratory Animal Care policies and approved by the University of Virginia Animal Care and Use Committee. All mice are on a C57BL/6J.129S mixed background except for Wld^S mice, which are FVB/NJ background. $p75NTR^{-/-}$,$TNFR1a^{-/-}$,Wld^S and $Sarm1^{-/-}$ animals were purchased from the Jackson labs. $DR6^{-/-}$ animals were a generous gift from the Genentech. $p75NTR^{-/-}$; $DR6^{-/-}$ double knockout animals were generated at Mendelian ratios by breeding the homozygous single knockout lines together for several generations to obtain the double homozygous state. Sprague Dawley rats were purchased from Harlan.

Primary Sympathetic neuronal cultures

Sympathetic neuron cultures were established as described previously(Deppmann *et al.*, 2008). Briefly, neurons were obtained by dissociation of P0-P3 mouse superior cervical ganglia. These neurons (from each litter of pups) were plated in compartmentalized microfluidic devices in DMEM supplemented with 10% FBS, penicillin/streptomycin (1U/mL), and 45 ng/mL of NGF purified from mouse salivary glands. Glia are removed from cultures using either 5 μM cytosine arabinofuranoside (Ara-C) (Sigma) for 48-72 hours. For NGF deprivation, cultures were rinsed three times with medium lacking NGF

and then maintained in NGF- deficient media containing a neutralizing antibody (50 mg/ml anti NGF antibody, Millipore) through designated time points at 37^0C (Cusack *et al.*, 2013).

Primary sensory neuronal cultures

DRGs from either E14.5 animals were dissected into DMEM/F12 supplemented with 10% FBS and penicillin/streptomycin (1 U/mL) on ice. E14.5 DRGs were processed in the same enzymes at 10% of the concentration for 30 minutes per enzymatic digestion. DRGs were then triturated with 23 and/or 27 gauge needles and plated into compartmentalized cell cultures on poly-D- lysine (50 μg/mL) and laminin (1μg/mL) coated coverslips. 45ng/mL of NGF purified from mouse salivary glands and 5μM cytosine ß(D arabinofuranoside hydrochloride (AraC) (Sigma) were added to all dissociated. Cells were maintained at 37°C (10% CO2) in a humidified cell culture incubator.

Phylogenetic analysis and PCR primers

Amino acid sequences of all rat TNFR family members were analyzed on Phylogeny.fr. The platform performed sequence alignment by MUSCLE (v3.7), and reconstituted the phylogenetic tree using the maximam likelihood method on the PhyML (v3.0 aLRT). Then the platform performed the graphical representation and editing of the phylogenetic tree using TreeDyn (198.3)(Dereeper *et al.*, 2008). PCR was performed using standard

reactions with cDNA materials synthesized from mRNA isolated from P0 rat SCG or brain. The following sequences were used as primers:

TNFRSF1A- F	ACCAAGTGCCACAAAGGAAC
TNFRSF1A- R	CTGGAAATGCGTCTCACTCA
TNFRSF1B- F	AAATGCAAGCACAGATGCAG
TNFRSF1B- R	CAGCAGACCCAGAGTTGTCA
LTBR- F	GAGCCCTAAACATGGCAGAG
LTBR- R	CTGCCCTTCTCACTGTCCTC
TNFRSF4- F	CTTGTACCTGCTCCGAAAGG
TNFRSF4- R	AGGATATGGGCTGTCTGTGC
CD40- R	TCTGAGCCCTGGAACTGTTT
CD40- F	TATTACTGCGGACCCCTGAC
FAS- F	ACCTGGTGACCCTGAATCTG
FAS- R	TGATACCAGCACTGGAGCAG
CD27- F	TGTGCAGCTCCGACTGTATC
CD27- R	GGCAGCTGTAAGGACAAAGC
TNFRSF8- F	TGCAGAGAAGTGGGTCAGTG
TNFRSF8- R	GTGGCTCTGGAGGTTCTCTG
TNFRSF9- F	CTGGTTCTCTGTGCCCAAAT
TNFRSF9- R	AGTGCTTCTCGGTTTCCTGA
TNFRSF10B- F	AAACCAGGCAGCTTTGAAGA

TNFRSF10B- R	AGCTGGGTTGTTTCCATTTG
TNFRSF11A- F	GCCAGCAAGAAGTGTGTGAA
TNFRSF11A- R	CCGGTCCGTGTACTCATCTT
TNFRSF11B- F	TGGGAATGAAGATCCTCCAG
TNFRSF11B- R	GAGGAAGGAAAGGGCCTATG
TNFRSF12A- F	CACTGATCCAGTGAGGAGCA
TNFRSF12A- R	CTCTCTGTCTGCCCCAGAAC
TNFRSF13B- F	GGCCGGATAACTTAGGAAGG
TNFRSF13B- R	TGGGAAGTGGCTCTCCTCTA
TNFRSF13C- F	GTGGGTCTGGTGAGTCTGGT
TNFRSF13C- R	TTGAATGGAGGCCAGTTAGG
TNFRSF14- F	CAGCTAGATCGGCCTACCAC
TNFRSF14- R	GCTGTTCCACAGCATGAGAA
NGFR-RT F	TTGCTTGCTGTTGGAATGAG
NGFR-RT R	AGCTCCTGGGGAGGAAAATA
TNFRSF17- F	ACTAAGAGCAGGGCTGGTGA
TNFRSF17- R	CTTGCCATAGTCACCCGTTT
TNFRSF18- F	CTGTGCCATGGGTACCTTCT
TNFRSF18- R	AAGCAGCCACACTAGGAGGA
TNFRSF19- R	TCAATCCCGAAAATGAAAGC
TNFRSF19- F	GTCCTTTGAGCATCCTGAGC
TNFRSF21- F	CTCGCGGTACCTTCTCTGAC

TNFRSF21- R	CGTGTGCTCAGGATGAGAAA
TNFRSF25- F	GTGCTGAGGACCTTCGTAGC
TNFRSF25- R	GCCCCTTCTGGTATTTCTCC
EDA2R- F	GGCCAACTGCACAAATACCT
EDA2R- R	TCCTACCAGTGCGACAAGTG

Fabrication and use of microfluidic devices

Microfluidic devices were generated as described previously (Park *et al.*, 2006; Suo *et al.*, 2014b).These chambers were affixed to coverglass coated with poly-D-lysine (50 µg/mL) and laminin (1µg/mL).

Axotomy experiments in vitro

Neurons from each litter of pups were allowed to project their axons to the axonal chamber (3-6 DIV) after plating. After the axons had grown into the axonal chamber, neurons were enucleated by aspirated 6mL of 1X PBS through the cell body chamber leaving the axons intact in their respective chamber. Unless otherwise indicated, both compartments were replaced with DMEM supplemented with 10% FBS, penicillin/streptomycin (1U/mL) without NGF and incubated at 37^0C and 5% CO_2 for indicated times.

Immunocytochemistry

Immunocytochemistry was carried out as previously described (Singh *et al.*, 2008).

Briefly, at indicated times, axons were fixed in 4% paraformaldehyde (w/v)/ phosphate-buffered saline (PBS) at room temperature for 20 minutes and blocked/permeabilized (5% goat serum, 0.05% Triton-x-100 in PBS) for 30 minutes at room temperature. Axons were then incubated overnight at 4°C with primary antibody diluted in blocking buffer. Cells were then washed 3x with 1x PBS and incubated with fluorescent secondary antibody for 1 hour at room temperature. Cells were again washed with 1x PBS 3 times and images using a fluorescent inverted microscope. The antibodies used in this study are mouse anti-Tuj1 (1:1000,Covance), Neurofilament-M (1:15, 2H3- DSHB-Iowa) and goat anti-mouse Alexa 488 (1:800, Life Technologies). All *in vitro* experiments were performed in triplicate with at least two microfluidic devices used for each condition.

Image Processing and analysis

Axon degeneration in culture was quantified from β-III Tubulin stained fluorescence images by counting the number of individual axons at the leading edge that had at least three beads/blebs as described(Zhai *et al.*, 2003). A blinded investigator counted ten representative pictures of the axons, in two microfluidic chambers per condition/time point. On each image 10 50μm boxes were randomly assigned to single axons. The investigator took care not to box bundles of axons, which may confound analysis. Then the number of boxes, which had 3 or more beads/blebs were counted and categorized as degenerating axons. Equal to or more than 80% degeneration was considered maximum degeneration and equal to or less than 10% degeneration of axons was considered as minimum degeneration. The percentage of the total number of degenerating axons was calculated using Microsoft Excel. At least 300 total axons were counted for each

condition. The standard error of the mean was considered as error. Each experiment was repeated 3 times (n) with separate litters of mouse pups of the same genotype.

Mouse surgery

All surgical and experimental procedures in mice were performed in compliance with the protocols approved by the Animal Care and Use Committee of University of Virginia. Mice were anesthetized with isoflurane, eye lubrication provided, fur removed over one side of the right side back/hindlimb region and the area cleaned with 3 alternating wipes of iodine then alcohol. Prior to incision, bupivacaine was applied locally (0.005 mL of a 0.25% solution) to prevent pain at the surgery site. An incision through the skin was made over the dorsal hindlimb region. Blunt dissection through the muscle allowed access to the sciatic nerve and was transected as close to the thigh with a pair of sterile surgical scissors, and 1- to 2-mm of nerve segment was removed to prevent the regeneration of axons into the distal stump (visual confirmation that ends did not rejoin was also performed prior to harvest). The gluteal muscles were then brought back into their original anatomical position, and the overlying skin was re-approximated by surgical staples or sutures.

Light and electron microscopy

Mice were euthanized by intracardial perfusion after indicated time of surgery and the sciatic nerve segments 3-6mm distal to the lesion were fixed with 4% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M PBS, pH 7.4 for 72 hours at 4°C. Nerves were post-fixed in 1% Osmium Tetroxide and 5% Uranyl Acetate followed by sequential

dehydrations with EtOH and embedding in EPON 812 (Electron Microscopy Sciences) along the axis of a resin block. A Leica Ultramicrotome was used to cut resin embedded nerves: The first 2mm of the nerve was discarded, transverse semi-thin sections (0.5-2μm) were cut with a diamond knife, and mounted on subbed slides for light microscopy. These slides were stained with 0.5% Toluidine Blue and imaged with a Leica Microscope with 1.6X-63X objectives, and a Leica MC170 digital camera. Ultrathin sections (50-80nm) were also cut from within the same nerve segment for electron microscopy. These were collected on copper grids and imaged via a JEOL 1010 Transmission Electron Microscope that is equipped with a 16Mpixel CCD camera (SIA). Axonal and myelin diameters were measured via Image Pro Plus (Mediacy) software.

Quantification of G-ratio

The G-ratio of axons from the sciatic nerve of mice was determined as previously reported(Michailov et al.,2004). Image Pro Plus was used for digital tracing. The myelinated axon area and diameter were measured by digitally tracing the inner and outer layers of the myelinated fiber. The G-ratio was calculated by dividing the inner diameter of the axon (without myelin) by the diameter of the total fiber (including myelin). The G-ratio data are displayed as a scatter plot against axon diameter.

Immunoblots

The biochemical analysis of sciatic nerves was carried out after euthanizing the animals at indicated time points post-surgery. A 10-mm segment of the nerve distal to the transection site was harvested, including the injury site, and immediately homogenized in

200 µL Urea / SDS buffer [50 mM Tris-Cl (pH 6.8), 8.0 M urea, 10 % (w/v) SDS, 10 mM sodium EDTA, and 50 mM DTT]. Injured nerve samples from mice for each time point were processed and contralateral nerve samples were used as controls. After heating at 95^0C for 10 min, a 10µg aliquot of each nerve homogenate was subjected to 4 - 15 % gradient Tris-glycine SDS-PAGE (Bio-Rad), and immunoblotted using the primary antibodies, rabbit p-JNK (Thr183/Tyr185 #9255), rabbit total JNK (#9252) from Cell Signaling Technology and AA2 for total tubulin.

Conditioned media experiments

Established compartmentalized neuron cultures were deprived of NGF by washing with NGF free, serum free media 3 times and incubating at 37^0C with a neutralizing anti-NGF antibody for 12 hours. The cell bodies were then removed by aspiration similar to injury experiments, and conditioned media was collected from the axonal chamber of the device after incubation at 37^0C and 5% CO_2 for 8 hours. A volume of ~150µL of ICM was pooled per device from more than 20 devices per experiment. The media in the wells of the device on the axonal side was also collected. Importantly, fresh serum free/NGF free media is added to distal axons before incubation so as not to confound analysis with factors secreted during normal growth. Injured and uninjured conditioned media was applied on non-injured axons, which were grown in the presence or absence of NGF deprived for 12 hours.

Analysis of size and stability of ICM

ICM was subjected to a freeze/thaw cycle (- 80^0C 1-2 days and thaw), heat (95^0C for 10 minutes), Trypsin 100µg/mL for 30 minutes (in serum free culture media) at 37^0C and Proteinase K (50µg/mL) for 60 minutes at 37^0C each prior to applying to uninjured axons.

SDS-PAGE and Silver staining

ICM was collected at indicated time points, filtered, resolved in Biorad 4-15% Tris-Glycine polyacrylamide gels and silver stained to visualize proteins. Silver stain was performed as previously described by Mann and colleagues (Shevchenko *et al.*, 1996).

Statistical Analysis

Statistical analysis was performed in graphpad prism software as indicated in figure legends. Error bars represent standard error of the mean. Sample number (n) was defined as the number of times each experiment was carried out, which contained at least 3 replicates.

Appendix 1

p75NTR is required for injury-induced demyelination in vivo

Implicit within the observation that *DR6*$^{-/-}$ nerves are remyelinated is the notion that remyelination is preceded by complete demyelination. Therefore, we predicted that ablating a pro-demyelination signal in conjunction with loss of DR6 would result in injured nerves that are completely rescued. As a candidate for this demyelination activity, we examined another TNFR family member, p75NTR, which has been implicated in promoting death of Schwann cells after SNA (Cragnolini and Friedman, 2008; Jung etal., 2011; Lemke and Chao, 1988; Soilu-Hänninen et al., 1999). Remarkably, *p75NTR*$^{-/-}$*;DR6*$^{-/-}$ mice displayed a complete rescue of axon and myelin degeneration at both 2 and 4 weeks after transection, while *p75NTR*$^{-/-}$ nerves exhibited complete breakdown of the axonal structure 14 days after SNA as previously observed (Fig. 1f & g, Figure 2a,b &d) (Ferri et al., 1998). This phenotype is as penetrant as observed in *Wld*^{s} and *Sarm1*$^{-/-}$ mice. *p75NTR*$^{-/-}$*;DR6*$^{-/-}$ mice showed a similar distribution of G-ratios before and after transection, comparable to *Wld*^{s} (Fig. 1h). Finally, *p75NTR*$^{-/-}$*;DR6*$^{-/-}$ mice showed comparable axon numbers and myelin thicknesses with and without injury, indicating a minimal effect on axonal architecture after the transection (Figure 2c-e). These findings are consistent with the notion that DR6 initiates degeneration on the axon, which then may signal for p75NTR to induce disassembly and clearance of myelin by an as yet unknown mechanism.

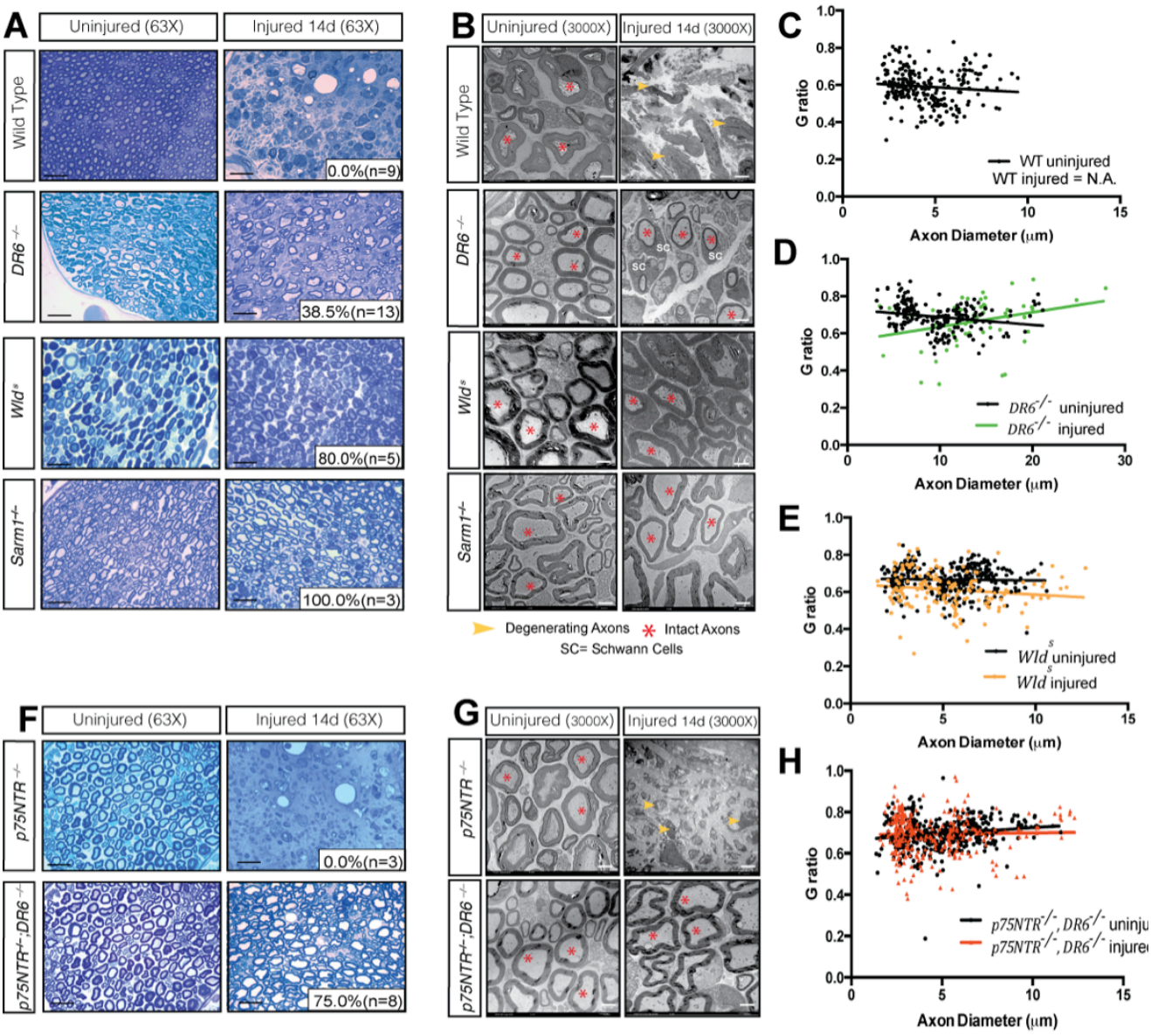


Figure 1: **DR6 and p75NTR are required for injury-induced nerve degeneration *in vivo*.**

A. Wild type, *DR6*$^{-/-}$, *Wld*^{s} and *Sarm1*$^{-/-}$ distal sciatic nerves were sectioned (0.5-2μm) and stained with toluidine blue to visualize myelin sheaths before and 14 days after sciatic nerve transection. Penetrance of phenotype is in the bottom

right corner of the images with n in parentheses. Penetrance represents the percentage of animals that displayed rescued sciatic nerve degeneration after injury. Scale bar = 20 μm.

B. Representative electron micrographs of cross sections (80-100 nm) from wild type, *DR6*$^{-/-}$,*Wld*^{s} and *Sarm1*$^{-/-}$ distal sciatic nerves (before and 14 days after sciatic nerve transection) showing intact (red stars) degenerating axons (yellow arrows) and Schwann cells (SC). In contrast to **A.** small diameter unmyelinated axons and remak bundles can be observed. Scale bar = 2 μm.

C. G ratio vs. Axon Diameter (μm) graph for Wild Type mice injured and uninjured sciatic nerves.

D. G ratio vs. Axon Diameter (μm) graph for *DR6*$^{-/-}$ mice injured and uninjured sciatic nerves.

E. G ratio vs. Axon Diameter (μm) graph for *Wld*^{s} mice injured and uninjured sciatic nerves.

F. Toluidine Blue stained cross sections of *p75NTR*$^{-/-}$ and *p75NTR*$^{-/-}$*;DR6*$^{-/-}$ distal sciatic nerves (before and 14 days after sciatic nerve transection). Penetrance of phenotype in the bottom right corner of the images with n in parentheses. Scale bar = 20 μm.

G. Representative electron micrographs of cross sections from *p75NTR*$^{-/-}$ and *p75NTR*$^{-/-}$*;DR6*$^{-/-}$ double knockout distal sciatic nerves (before and 14 days after sciatic nerve transection) showing intact (red stars) and degenerating axons (yellow arrows). Scale bar = 2 μm.

H. G ratio vs. Axon Diameter (μm) graph for *p75NTR*$^{-/-}$*;DR6*$^{-/-}$ mice injured and uninjured sciatic nerves.

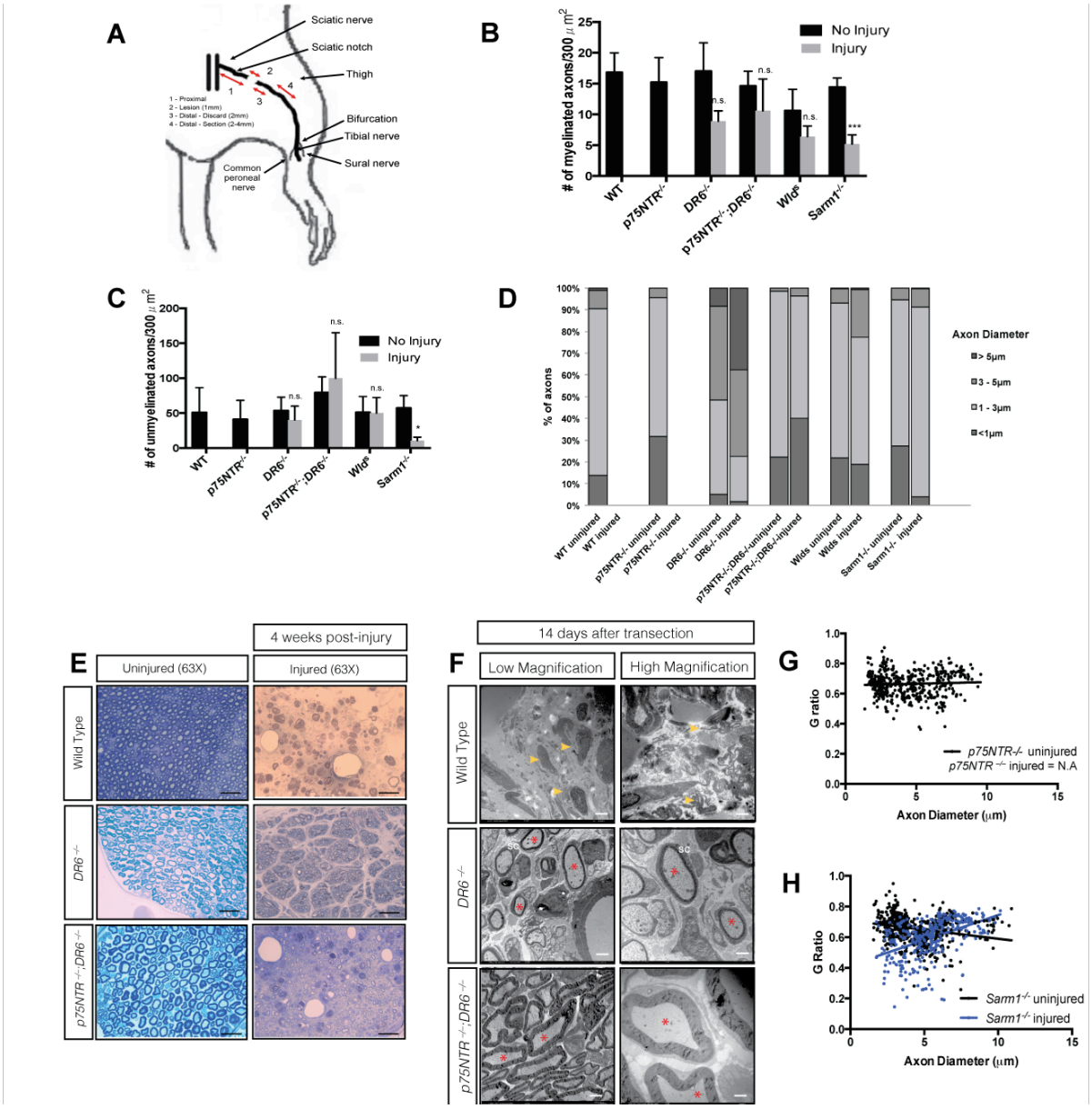


Figure 2 . **Further analysis of axon and myelin properties after Sciatic Nerve Axotomy (SNA).**

a. Schematic representation of Sciatic Nerve Axotomy paradigm. Numbered red arrows indicate the region of the nerve used for experiments.

b. Number of myelinated axons per field (area of 300 μm^2) with or without transection for Wild Type, *p75NTR*$^{-/-}$, *DR6*$^{-/-}$, *p75NGFR*$^{-/-}$*;DR6*$^{-/-}$, *Wld*^{s} and *Sarm1*$^{-/-}$mice.

c. Number of unmyelinated axons per field (area of 300 μm^2) with or without transection for Wild Type, *p75NTR*$^{-/-}$, *DR6*$^{-/-}$, *p75NGFR*$^{-/-}$*;DR6*$^{-/-}$, *Wld*^{s} and *Sarm1*$^{-/-}$mice.

d. Percentage of injured and uninjured axons binned by diameter in Wild Type, *p75NTR*$^{-/-}$, *DR6*$^{-/-}$, *p75NGFR*$^{-/-}$*;DR6*$^{-/-}$, *Wld*^{s} and *Sarm1*$^{-/-}$ mice.

e. Toluidine Blue stained cross sections of uninjured and injured Wild Type (n=3), *DR6*$^{-/-}$(n=3, penetrance of segmented myelination phenotype 33.3%), *p75NTR*$^{-/-}$*;DR6*$^{-/-}$ (n=3 , penetrance for preserved axon phenotype 100%) double knockout distal sciatic nerves 4 weeks after SNI. Scale bar = 20 μm.

f. Additional representative electron micrographs of cross sections from WT, *DR6*$^{-/-}$ and *p75NTR*$^{-/-}$*;DR6*$^{-/-}$ distal sciatic nerves (2 weeks after injury) showing intact (red stars) degenerating axons (yellow arrows) and Schwann cells (SC) with higher magnifications (3000X and 6000X). Scale bars = 2μm (3000X) and 1μm (6000X). n≥3.

g. G ratio vs. Axon Diameter (μm) graph for *p75NTR*$^{-/-}$ mice injured and uninjured sciatic nerves.

h. G ratio vs. Axon Diameter (μm) graph for *Sarm1*$^{-/-}$ mice injured and uninjured sciatic nerves.

i. *DR6*$^{-/-}$ transected nerves show thin myelin (yellow arrow heads) and overly myelinated axons (red asterisks) 14 days after transection. **i**. Thinly myelinated small diameter axons. SC=Schwann Cells. **ii**. & **iii**. Large diameter axons with thick myelin. **iv**. & **v**. Large myelinated axons with over/ double myelination.

This remarkable phenotype of *p75NTR*$^{-/-}$*;DR6*$^{-/-}$ mice showing complete rescue from axon degeneration and demyelination, indicates a role for p75NTR in myelin degeneration and/or Schwann cell function, which is consistent with previous findings (Ferri and Bisby, 1999; Cosgaya, Chan and Shooter, 2002; Nykjaer, Willnow and Petersen, 2005) (Figure 1F-H). Future work will examine the hierarchical relationship between axon autonomous and non-autonomous TNFR family dependent degeneration pathways.

Future experiments

1. Co-culturing Schwann cells and sympathetic neurons to investigate the demyelination/remyelination *in vitro.*
    a. WT neurons with WT Schwann cells.
    b. WT neurons and *p75NTR*$^{-/-}$ Schwann cells.
    c. DR6-/- neurons with WT Schwann cells.
    d. DR6-/- neurons with *p75NTR*$^{-/-}$ Schwann cells.

2. More time points on *DR6*$^{-/-}$ and *DR6*$^{-/-}$*;p75NTR*$^{-/-}$ sciatic nerve axotomy. E.g. 3 days, 7 days, 4 weeks, 8 weeks

3. Functional recovery of *DR6*$^{-/-}$ and *DR6*$^{-/-}$*;p75NTR*$^{-/-}$ sciatic nerve axotomized mice at 4-8 weeks after treatment. E.g. Walking track analysis.

4. *P75NTR fl/fl; P0 Cre* Sciatic nerve axotomy.

5. *DR6*$^{-/-}$, *P75NTR fl/fl; P0 Cre* Sciatic nerve axotomy.

Appendix 2

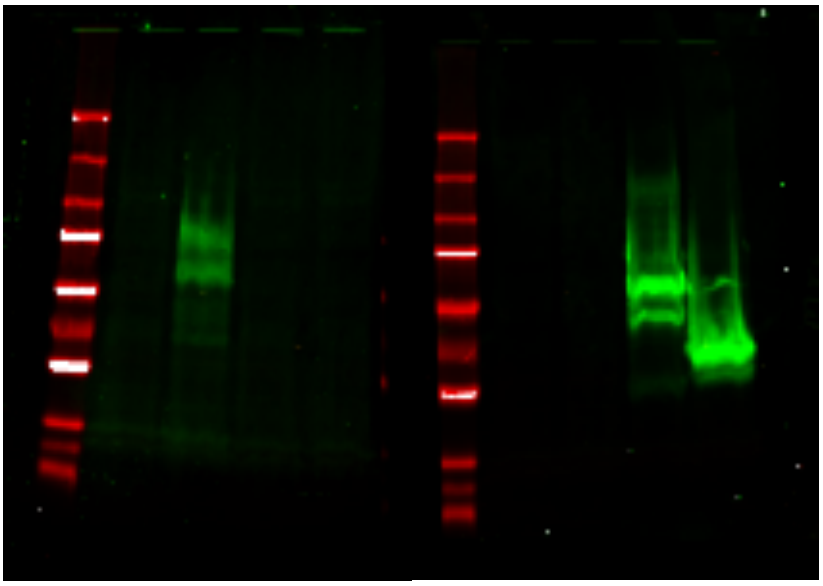
Interactions of DR6 with Sarm1

Sterile Alpha and Toll/Interleukin Receptor (TIR) motif-containing protein 1, Sarm1, is an evolutionary conserved executioner protein in the Wallerian degeneration cascade. Axon injury activates Sarm1 by releasing the auto-inhibition of Sarm1, leading to loss of NAD^+(Summers *et al.*, 2016). However, the mechanism of action or binding partners of Sarm1 have not been elucidated. Here, we sought to investigate the association of Sarm1 with the cell surface receptor DR6. We used co-immunoprecipitation (Co-IP) methods of Sarm1 and DR6 constructs expressed in HEK293 cells.

The clones used for the Co-IP experiments are listed in Table 1.

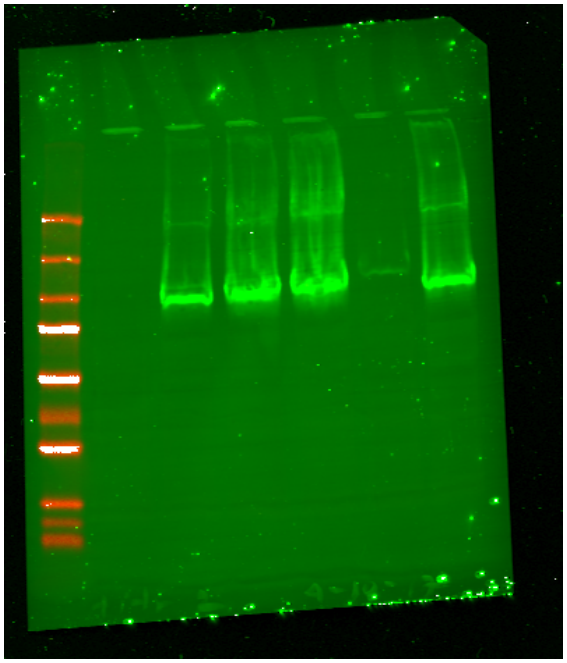
- Test of the Expression of the 3 new Clones for Co-IP DR6-Ecto-HA, 6Xmyc-Sarm-Autoreulatory Domain, and 6Xmyc-Sarm-TIR Domain.
- Below are the three new clones expressed in HEKs. The DR6-Ecto-HA is supposed to be 42 kDa but it runs high, maybe 55-75 kDa. On the right hand side, the myc clones should be 54 kDa for Auto and that runs at about 60kDa, and 28 kDa for TIR and that runs at about 37 kDa or maybe slightly below.

1 2 3 4 5 1 2 3 4 5



Anti-HA Anti-myc (9E10)

- First attempt of co-expressing DR6 and Sarm1.

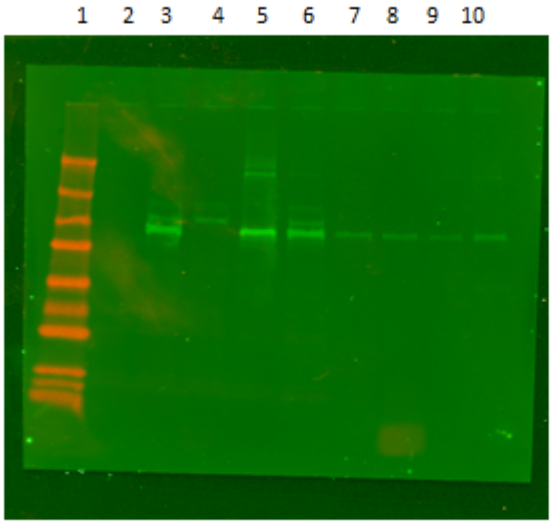


Well	Sarm-1	DR6BirA(In)	mCherry	Comment

	(µg)	(µg)	(µg)	
1	0.0	0.0	0.0	Negative control
2	0.06(0.63)	2.94(29.37)	0.0	DR6 >>>Sarm
3	0.13(1.25)	2.87(28.75)	0.0	DR6 >> Sarm
4	0.25 (2.5)	2.75(27.5)	0.0	DR6 > Sarm
5	0.0(0)	3.00 (30)	0.0	Just DR6 (and Cherry control, not seen)
6	2.00 (20)	0.0	1.0 (1)	Just Sarm; expect 90kDa + mCherry

- Co-IP trial 1

A co-IP between Sarm-1 and DR6 (as DR6BirA(In)HA) in HEK cells at 30 hours post-transfection.



9E10 anti-myc overnight at 4C with 800 nm goat anti-mouse secondary 1:25000 for 1.5 hours.

Lane 1 - standards.

Lane 2 - untransfected.

Lane 3 - the mix of both Sarm and DR6BirA(In)HA.

Lane 4 -DR6BirA(In)HA alone.

Lane 5 - Sarm-1 alone.

Lane 6- the whole cell lysate before 16K spin.

Lane 7 - SNT above 16K spin.

Lane 8 -Input (16K spin that was protein A absorbed)

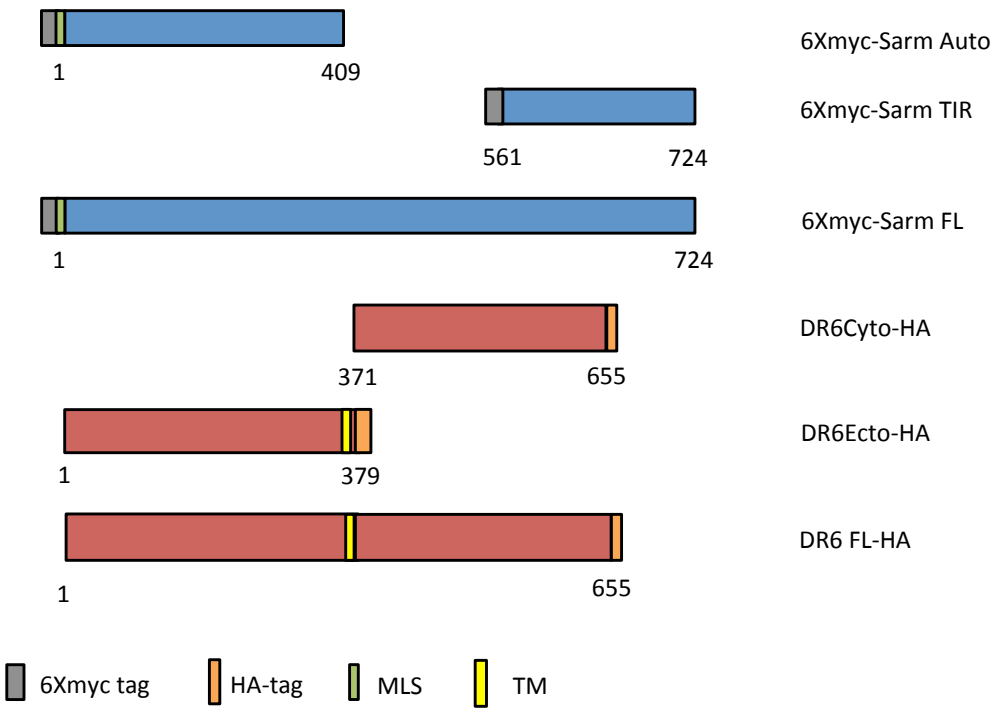
Lane 9 - “above beads”

Lane 10 - dissociated off beads.

While it seems that following lysis we lose DR6BirA, we ought to make sure

- DR6BirA is degraded and this is not because of proteases being active without neutralizing. More trials with EGTA/EDTA needed.
- The DR6BirA could in part co-precipitate with the fraction of Sarm that goes into the pellet at 16K.
- Does Sarm1 presence induce the degradation of DR6BirA? The extract containing DR6BirA alone without co-transfecting Sarm should be lysed and analyzed for Sarm1 degrading DR6 (Sarm and DR6BirA can coexist in cells together at least until lysed).

New Constructs to repeat the Co-IP



MLS = mitochondrial localization signal

TM = transmembrane domain

FL = full length

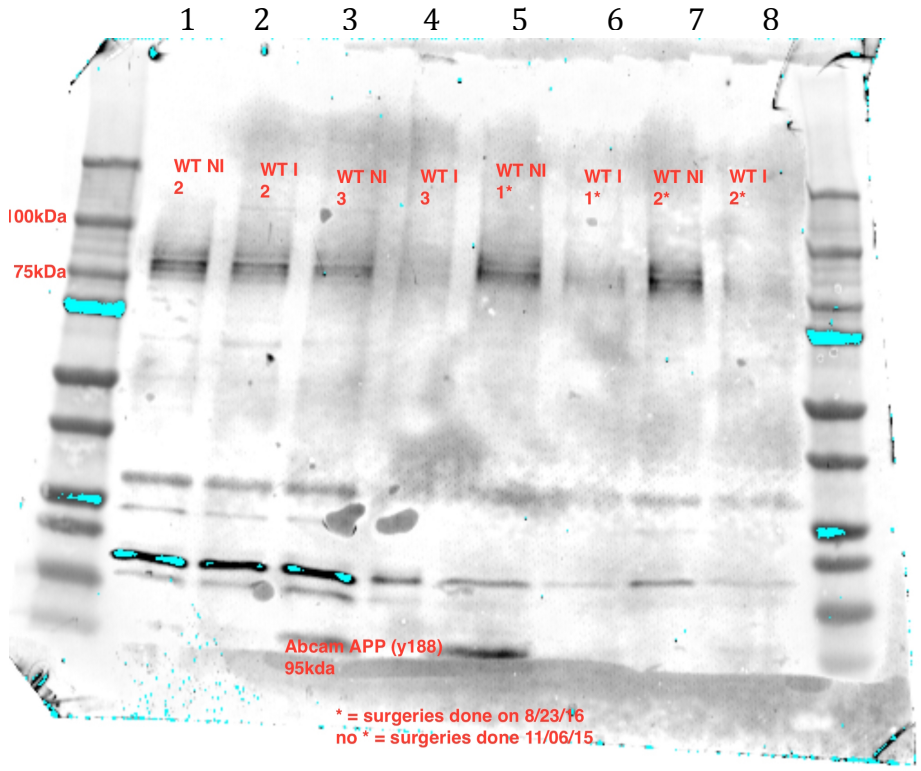
Appendix 3

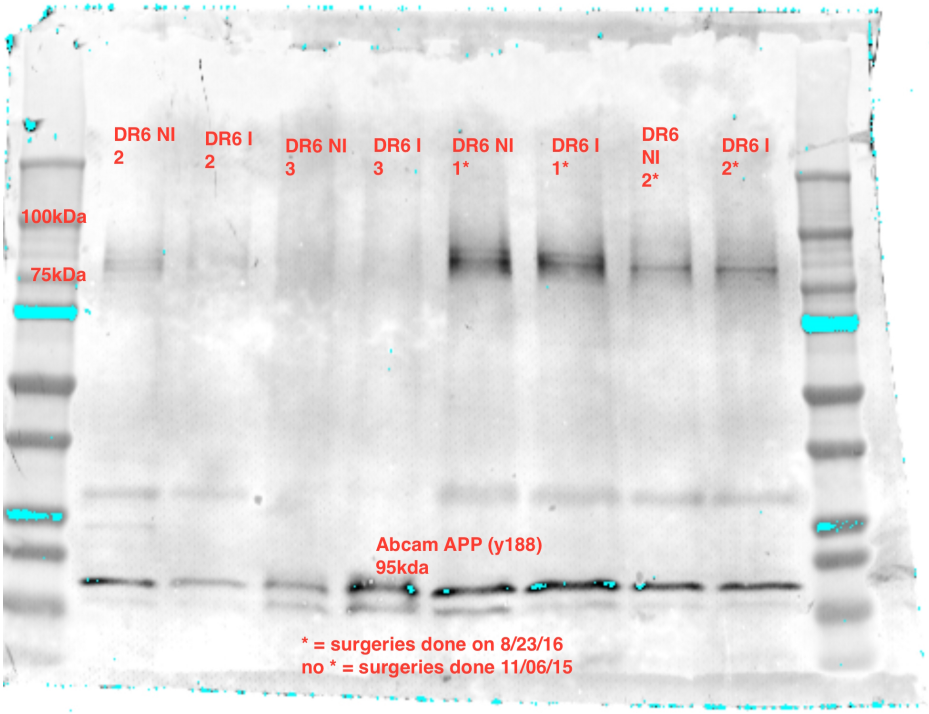
Possible Role for DR6 in Alzheimer's disease

Alzheimer's disease is marked by neuronal and axon degeneration, which is a common morphological change observed in both developmental axon degeneration and injury-induced axon degeneration. Previous reports have shown the involvement of Amyloid precursor protein (APP) cleavage in activation of DR6 and caspase 6 in NGF deprivation (Nikolaev, McLaughlin, O'Leary, *et al.*, 2009). Although the evidence of APP being the ligand for DR6 has been amended, the notion that APP could be playing a role in axon degeneration is indisputable. Other studies reveal that Neurturin deprivation, dynactin 1 (Dctn1) dysfunction have both shown activation of APP/caspase 6 pathway, indicating the importance of APP in axon degeneration in development as well as in diseases (Vohra *et al.*, 2010). Further reports about a physical interaction between APP and DR6 also indicate a possible link between these two proteins in the Wallerian degeneration pathway (Kuester *et al.*, 2011; Olsen *et al.*, 2014).

Wallerian degeneration signaling pathway involves activation of MAPK members, including JNK. Phosphorylation of JNK preceded Wallerian degeneration, and is absent in Wallerian degeneration slow mutants, e.g. DR6 and Sarm1 (Gamage *et al.*, 2017). During my sciatic nerve transection experiments in WT and $DR6^{-/-}$ mice, I observed, a

defect in APP cleavage in distal sciatic nerve lysates from *DR6*$^{-/-}$ mice. This is in contrast to lysates from WT mice which display rapid APP cleavage after injury.


1 2 3 4 5 6 7 8
WT NI 2
WT I 2
WT NI 3
WT I 3
WT NI 1*
WT I 1*
WT NI 2*
WT I 2*
100kDa
75kDa
Abcam APP (y188) 95kda
* = surgeries done on 8/23/16
no * = surgeries done 11/06/15


DR6 NI 2
DR6 I 2
DR6 NI 3
DR6 I 3
DR6 NI 1*
DR6 I 1*
DR6 NI 2*
DR6 I 2*
100kDa
75kDa
Abcam APP (y188) 95kda
* = surgeries done on 8/23/16
no * = surgeries done 11/06/15

Above western blots show the cleavage of APP is different in WT and *DR6*$^{-/-}$ sciatic nerve lysates before and after injury (lanes 5-8 both blots). While WT loses the 95kDa band of APP in injured sciatic nerves, *DR6*$^{-/-}$ seems to retain the APP 95kDa band after injury. This indicates that APP cleavage may play a role in axon degeneration, which is blocked in the absence of DR6. We plan to further investigate this by carrying out in vitro and in vivo injury-induced axon degeneration studies in APP mutant mice.

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