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Death Receptor 6 promotes Wallerian degeneration in peripheral axons

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Graphical abstract

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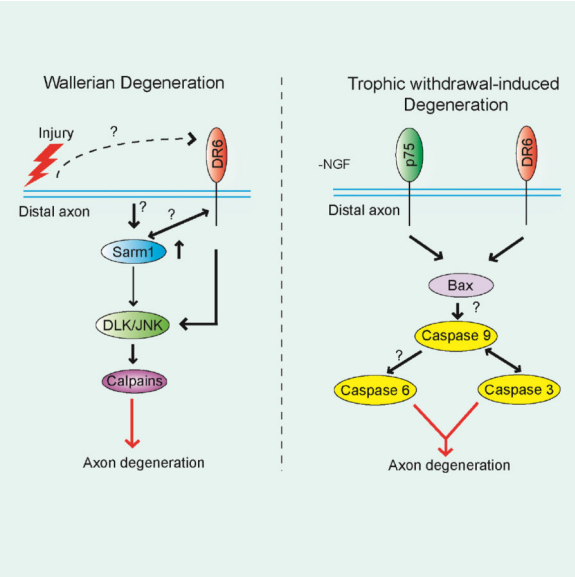
Author contributions

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.

Competing Financial Interests

The authors declare no competing financial interests.



Keywords

DR6; Wallerian Degeneration; NGF deprivation; Axotomy

Summary

Axon degeneration during development is required to sculpt a functional nervous system and is also a hallmark of pathological insult such as injury [1, 2]. Despite similar morphological characteristics, very little overlap in molecular mechanisms have been reported between pathological and developmental degeneration [3–5]. In the peripheral nervous system (PNS) developmental axon pruning relies on receptor mediated extrinsic degeneration mechanisms to determine which axons are maintained or degenerated [5–7]. Receptors have not been implicated in Wallerian axon degeneration; instead axon autonomous, intrinsic mechanisms are thought to be the primary driver for this type of axon disintegration[8–10]. Here we survey the role of neuronally expressed, paralogous Tumor Necrosis Factor Receptor Super Family (TNFRSF) members in Wallerian 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

In vitro comparison of trophic withdrawal-induced and Wallerian 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 [11,12] For both paradigms, we established sympathetic neurons from postnatal day (P) P0-P3 wildtype mice in microfluidic devices as previously described [13]. These devices allow for neurons of interest to be compartmentalized such that cell bodies and axons reside in different compartments of the device [14]. 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 [15]. 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, Movie S1). NGF deprived neurons and enucleated axons are incubated at 37°C with 5% CO2 for indicat ed periods of time followed by immunostaining for βIII-tubulin and manual quantification of axon degeneration as previously described [1, 16]. 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 [3, 17]. 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, which we attribute to the properties of axotomy in microfluidic devices (*i.e.* smaller media volume) [12,18]. Although there is an 18 hour difference in latent phases between trophic withdrawal and Wallerian 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%) (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)[19]. 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 [20,21]. This expression pattern is similar to what we’ve reported for mouse sensory neurons [6]. 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 Wallerian degeneration [5–7,24–26].

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) [1, 7]. *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 Wallerian degeneration [9, 27]. 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 [9, 28]. 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 Wallerian degeneration *in vitro*

We next examined the role of death receptors in Wallerian 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 Wallerian 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)[29]. 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 Wallerian 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 [27,30]. 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)[9]. 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)[31,32]. 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 [15,33]. Sarm1 also

activates the MAPK pathway (MKK4 and JNK), which is upstream of disrupted axonal energy homeostasis [29,33,34]. 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)[29]. 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).

Discussion

In this study we examined the role of TNFR family members in promoting axon degeneration in response to trophic 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 [7, 25, 26]. 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. Importantly, degeneration using explants occurs slower, than what we observe in dissociated neurons grown in microfluidic devices [9, 12]. 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. We attribute the difference in degeneration kinetics between *in vivo* and *in vitro* paradigms to the presence or absence of supporting cells (*i.e.* glia), respectively. 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 Wallerian degeneration. To our knowledge this is the first example of a receptormediated 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 Wallerian degeneration, which is in contrast to its prodegenerative 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 [22,25,26,35].

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) [35]. Injured nerves from *Wld*^{S}, *Sarm1*$^{-/-}$ and *Phr1*$^{-/-}$ animals do not show this phenotype (Figure 3C,D) [9, 10, 27]. 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 [36,37]. In 2009, it was suggested that an N-terminal fragment of amyloid precursor protein (APP) may serve as a ligand for DR6 [38]. However, the relationship between APP and DR6 has since been amended [7]. 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 [7]. A recent crystal structure reveals that the way in which DR6 and APP interact is consistent with a co-receptor relationship [7, 37]. If APP is indeed acting as a co-receptor it will be critical to continue seeking ligands for DR6 in the context of Wallerian degeneration.

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 Wallerian degeneration. Although first described as a Toll like receptor adaptor protein, a primary receptor for Sarm1 in Wallerian 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 [39]. Nevertheless, it is apparent that activation of Sarm1 downstream of axonal injury leads to JNK activation [15,29,40]. 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 Wallerian degeneration links to Sarm1 activation, NAD^+ depletion and other previously described pathways.

Experimental Procedures

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. Experimental procedures can be found in the Supplemental experimental procedures.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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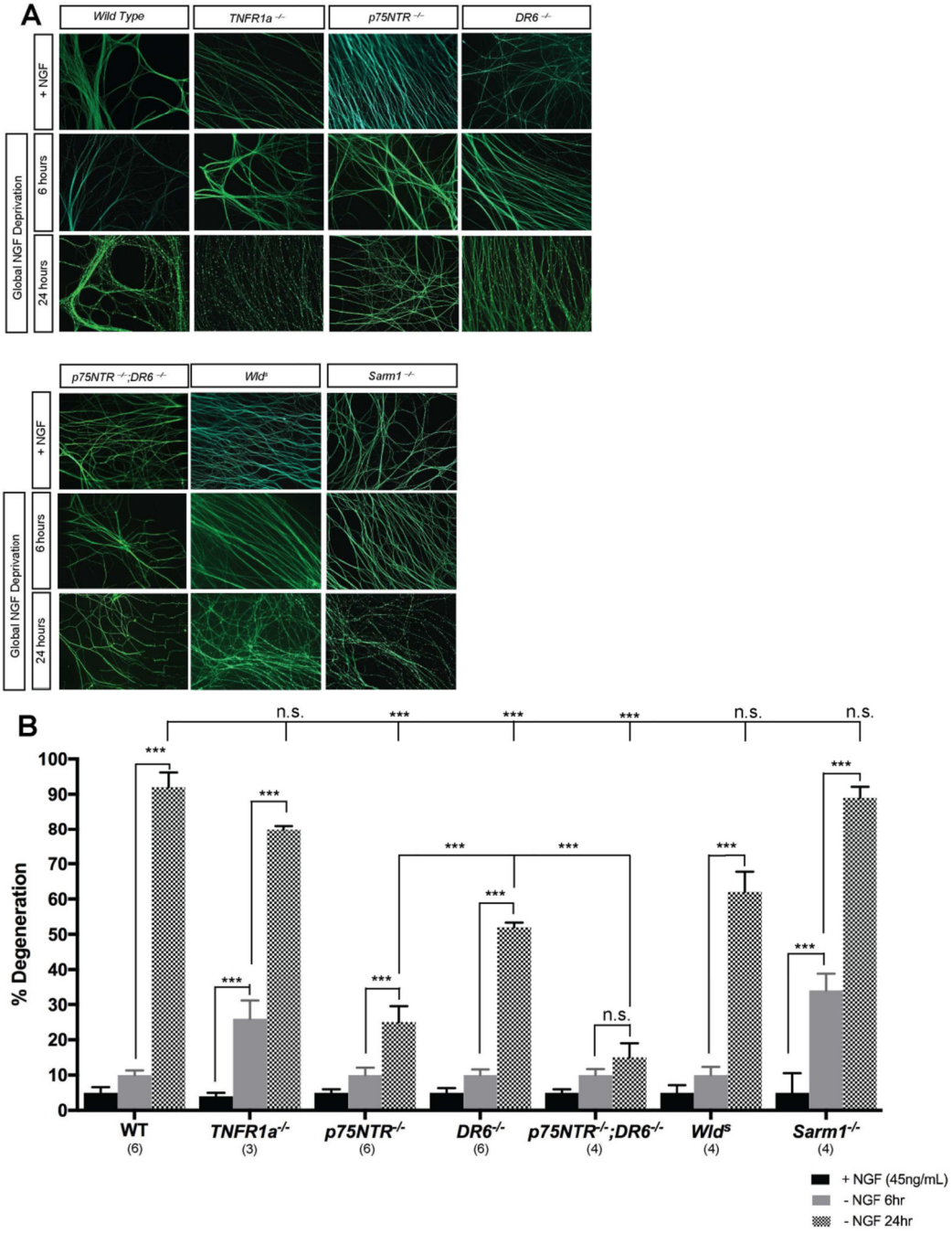

Figure 1. DR6 and p75NTR are required for trophic factor deprivation-induced axon degeneration *in vitro*

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 Figure S1. 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.

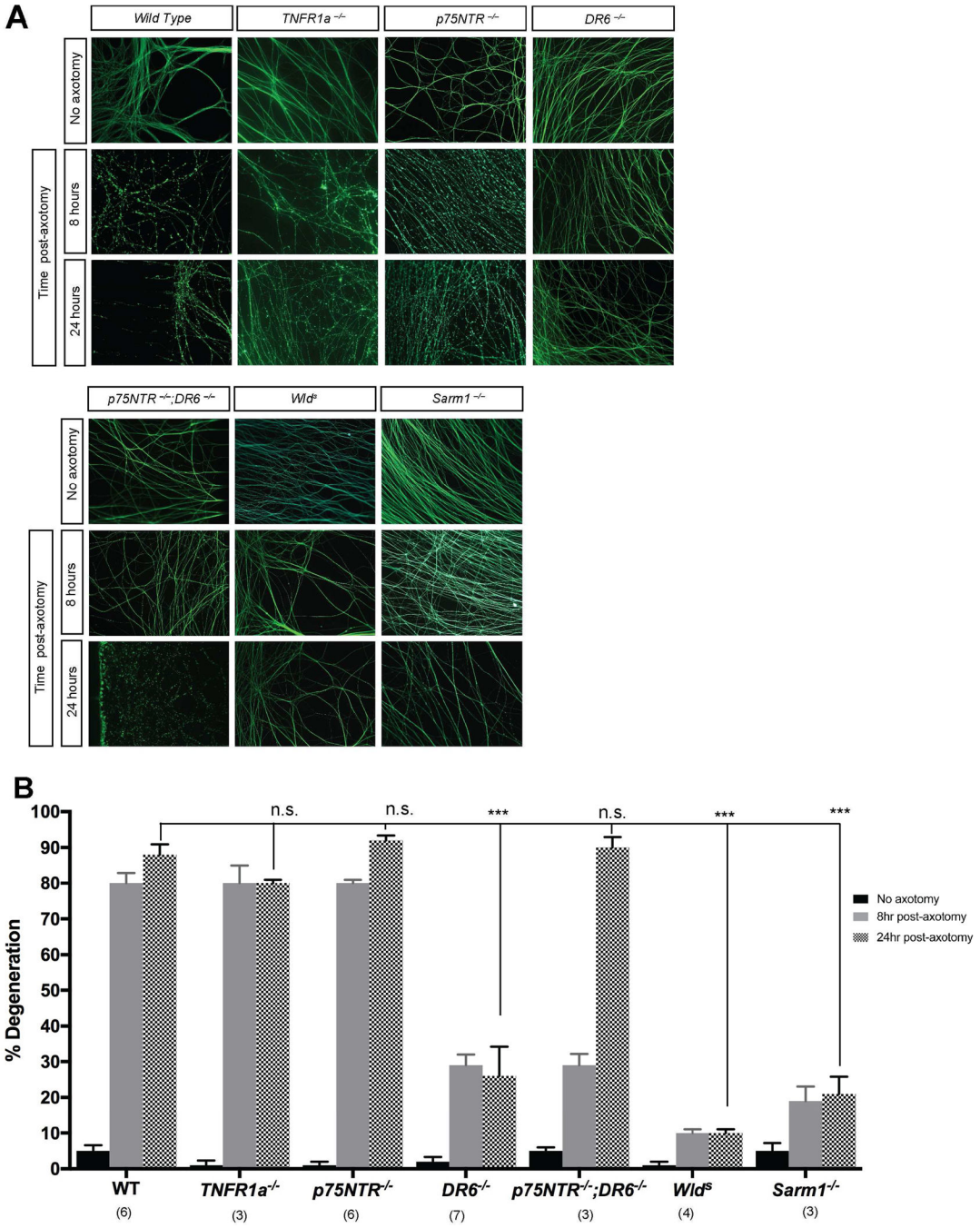

Figure 2. DR6, but not p75NTR or TNFR1a is required for Wallerian axon degeneration *in vitro*

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 Figure S2, S3 and Movie S1.

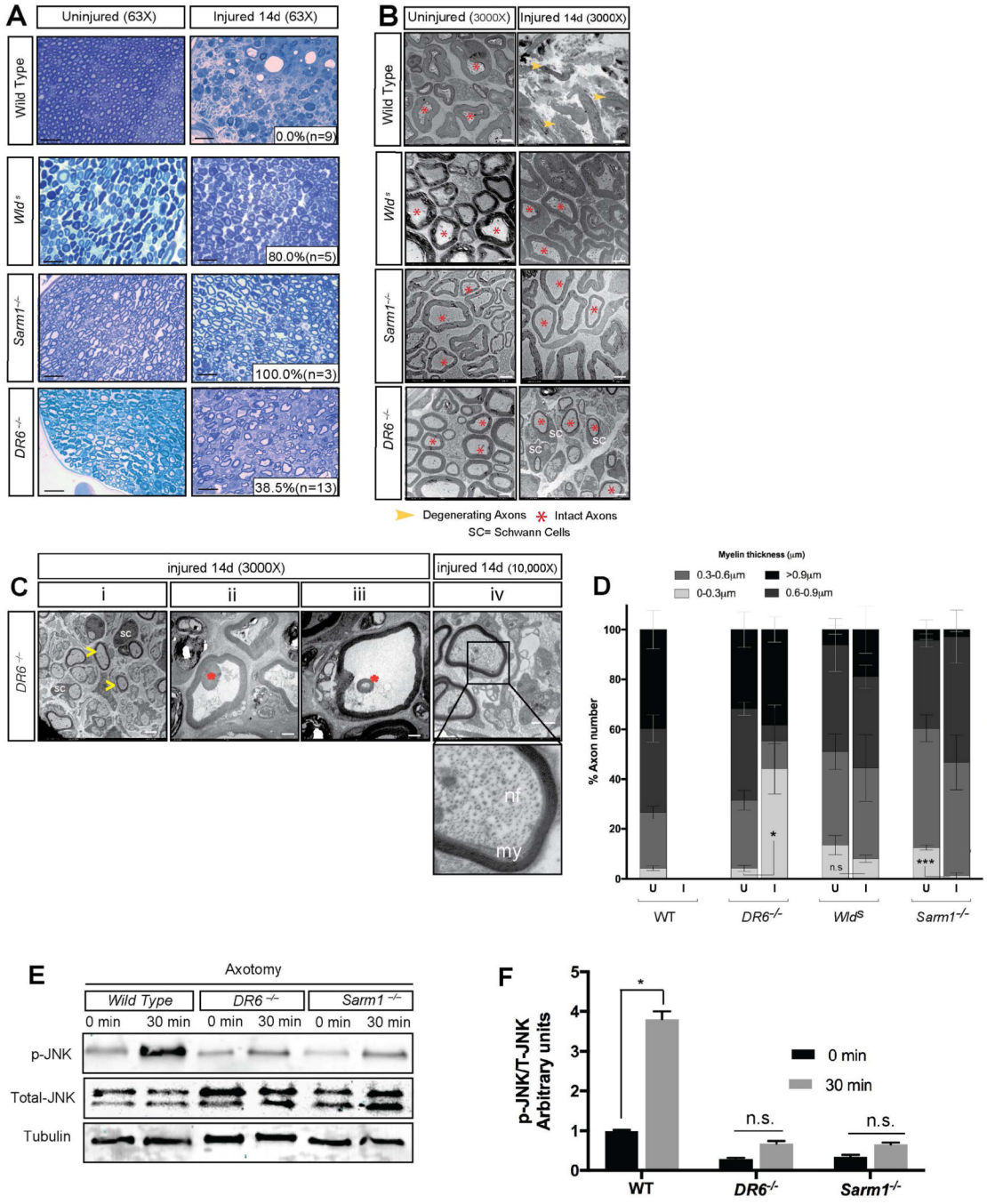

Figure 3. DR6 and p75NTR are required for Wallerian nerve degeneration *in vivo*

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 Figure S4.

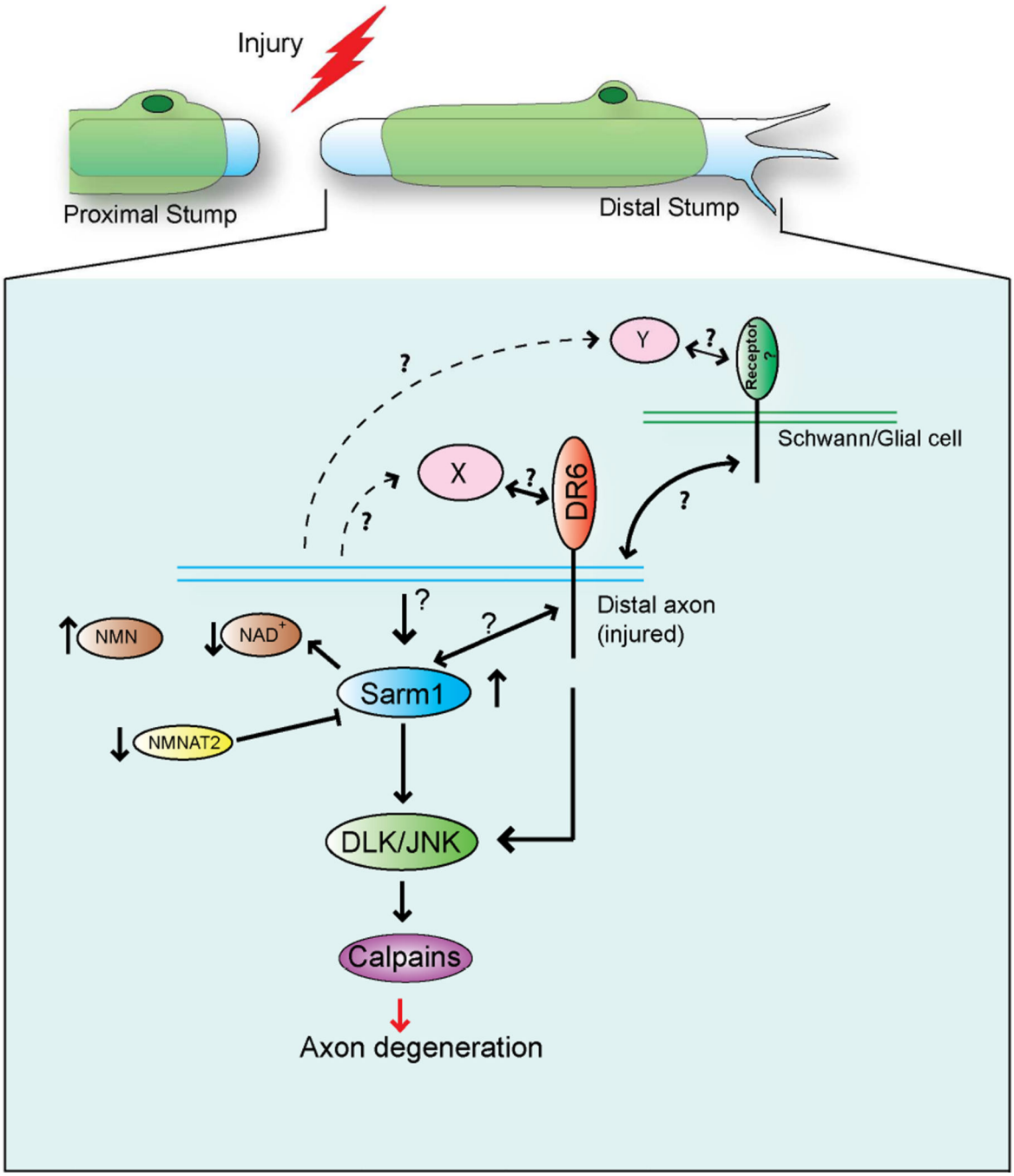

Figure 4. Proposed model for DR6 regulation of Wallerian degeneration signaling pathway

Loss of *DR6* rescues injured distal axons from axon degeneration indicating an essential role for DR6 in Wallerian 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.