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dSarm/Sarm1 Is Required for Activation of an Injury-Induced Axon Death Pathway

Jeannette M. Osterloh¹, Jing Yang², Timothy M. Rooney¹, A. Nicole Fox¹, Robert Adalbert⁴, Eric H. Powell³, Amy E. Sheehan¹, Michelle A. Avery¹, Rachel Hackett^{1,*}, Mary A. Logan^{1,†}, Jennifer M. MacDonald¹, Jennifer S. Ziegenfuss¹, Stefan Milde⁴, Ying-Ju Hou⁵, Carl Nathan⁵, Aihao Ding⁵, Robert H. Brown Jr⁶, Laura Conforti⁷, Michael Coleman⁴, Marc Tessier-Lavigne², Stephan Züchner³, and Marc R. Freeman^{1,‡,*}

¹Department of Neurobiology, University of Massachusetts Medical School, Worcester, MA 01605, USA

²Laboratory of Brain Development and Repair, The Rockefeller University, New York, NY 10065, USA

³John P. Hussman Institute for Human Genomics, Dr. John T. Macdonald Foundation Department of Human Genetics, University of Miami Miller School of Medicine, Miami, FL, 33136, USA

⁴Babraham Institute, Cambridge CB22 3AT, UK

⁵Department of Microbiology and Immunology, Weill Medical College of Cornell University, New York, NY 10021, USA

⁶Department of Neurology, University of Massachusetts Medical School, Worcester, MA 01605, USA

⁷School of Biomedical Sciences, University of Nottingham Medical School Queen's Medical Centre, Nottingham, NG7 2UH, UK

Abstract

Axonal and synaptic degeneration is a hallmark of peripheral neuropathy, brain injury, and neurodegenerative disease. Axonal degeneration has been proposed to be mediated by an active autodestruction program, akin to apoptotic cell death; however, loss-of-function mutations capable of potentially blocking axon self-destruction have not been described. Here, we show that loss of the *Drosophila* Toll receptor adaptor dSarm (sterile α /Armadillo/Toll-Interleukin receptor homology domain protein) cell-autonomously suppresses Wallerian degeneration for weeks after axotomy. Severed mouse *Sarm1* null axons exhibit remarkable long-term survival both in vivo and in vitro,

‡To whom correspondence should be addressed. marc.freeman@umassmed.edu.

*Howard Hughes Medical Institute, Chevy Chase, MD 20815–6789, USA.

†Present address: Jungers Center for Neurosciences Research, Department of Neurology, Oregon Health & Science University, Portland, OR 97239, USA.

Supplementary Materials

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Materials and Methods

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indicating that Sarm1 prodegenerative signaling is conserved in mammals. Our results provide direct evidence that axons actively promote their own destruction after injury and identify dSarm/Sarm1 as a member of an ancient axon death signaling pathway.

When axons are severed, the portion of the axon distal to the injury site undergoes extensive fragmentation. This process, termed Wallerian degeneration, was traditionally thought to result from passive wasting away of axons due to a lack of cell body–derived nutrients (1). However, this notion was challenged by the *slow Wallerian degeneration* (*Wld^S*) mutant mouse in which the distal portion of severed axons remained morphologically intact for weeks after axotomy (2, 3). The long-term survival of severed *Wld^{S+}* axons raised the intriguing possibility that Wallerian degeneration might be driven by an active molecular program akin to apoptotic cell death (4, 5). However, the *Wld^S* phenotype results from a gain of function, likely neomorphic mutation that leads to neuronal overexpression of a chimeric fusion protein containing the nicotinamide adenine dinucleotide (NAD⁺) biosynthetic enzyme *Nmnat1* (6, 7). Despite its ability to inhibit axonal degeneration, the gain-of-function nature of the *Wld^S* phenotype does not provide direct evidence supporting the existence of an axon death signaling pathway and may be unrelated to normal *Nmnat1* function. Importantly, mutants reported to suppress Wallerian degeneration, such as *wnd/DLK*, delay the clearance of degenerating axons in *Drosophila* for only ~1 to 2 days, and mouse axons for several hours (8). This is quite weak suppression when compared with *Wld^S*. Thus, the existence of axon death pathways in Wallerian degeneration has remained largely speculative.

Wallerian degeneration appears to be molecularly distinct from apoptosis, because potent genetic or chemical inhibitors of cell death do not block axonal disintegration (9–11). We revisited this question and performed a comprehensive screen of existing mutants and dominant negative constructs for *Drosophila* genes affecting apoptosis, autophagy, or other defined cell degradative pathways, but these failed to suppress Wallerian degeneration (table S1).

If Wallerian degeneration is indeed an active process, then loss-of-function mutants that exhibit-*Wld^S*-like protection of severed axons should exist. We therefore performed an F₂ forward genetic screen in *Drosophila* for mutants that exhibited long-term survival of severed axons (fig. S1). Because genes required for Wallerian degeneration may be lethal when mutated, we designed our screen to allow for characterization of both viable and lethal mutants through mosaic analysis with a repressible cell marker (MARCM) clonal analysis (12). In control animals, severed olfactory receptor neuron (ORN) axons degenerated and were completely cleared from the antennal lobe 7 days after axotomy. We identified three lines, *I(3)896*, *I(3)4621*, and *I(3)4705*, in which severed homozygous mutant axons generated by MARCM remained intact 1 week after axotomy (Fig. 1A). Although the number of uninjured axons is slightly reduced in each mutant, 100% of green fluorescent protein (GFP)–labeled axons exhibited long-term preservation after injury (table S2). Mutant axons remained fully intact 30 days after injury (Fig. 1B), and a significant but reduced number remained even 50 days after injury (Fig. 1C). *I(3)896*, *I(3)4621*, and *I(3)4705* therefore provide axonal preservation that rivals that of *Wld^S* in *Drosophila*, and lasts

throughout the life span of the fly. Neuroprotection in these mutants extended to synapses: Synaptobrevin punctae localized to synaptic terminals even 30 days after axotomy (fig. S2, D and E). Neuronal morphology appeared normal in mutant animals (Fig. 1 and fig. S2B), indicating that these mutations do not grossly affect neuronal development.

We next asked whether *I(3)896* was broadly required for neuron pruning or apoptotic cell death. We examined dendritic and axonal pruning in MARCM clones in *Drosophila* mushroom body γ neurons during metamorphosis (13). In both control and *I(3)896* animals, mushroom body γ neuron axons and dendrites were pruned normally (Fig. 1D). During early embryogenesis, dMP2 neurons are present in each segment, but by late embryogenesis, all but the posterior three pairs undergo developmentally programmed apoptosis (14). We found that dMP2 neurons were generated normally in *I(3)4621* animals, and the appropriate subset of neurons underwent apoptosis (Fig. 1E). Finally, we expressed the proapoptotic gene *hid* in the *Drosophila* visual system (15) to induce widespread apoptotic death in cells of the developing eye disc. We found that *I(3)896* mutant clones failed to suppress activation of cell death (Fig. 1F).

These mutants were all recessive (fig. S2A) in axon degenerative phenotypes and fell into a single lethal complementation group; therefore, each line represented an independently isolated lethal mutation in the same gene. To identify the gene mutated in *I(3)896*, *I(3)4621*, and *I(3)4705*, we took two complementary approaches. First, we mapped the lethality of each mutant using small chromosome deficiencies and identified a single gene at cytogenetic region 66B (Fig. 2A). We also used next-generation sequencing technology to resequence mutant genomes to a read depth of 70 \times (table S3). We identified a single gene affected in all three mutants, which resided in cytogenetic region 66B: *ect4*, which we refer to as *dsarm* (*Drosophila* sterile alpha and Armadillo motif). The *dsarm* gene encodes a protein with an Armadillo/HEAT (ARM) domain, two sterile alpha motifs (SAM), and a Toll/interleukin-1 receptor homology (TIR) domain. Each identified *dsarm* allele contained a unique premature stop codon in *dsarm* open reading frame (Fig. 2B and table S4). From these data we conclude that *I(3)896*, *I(3)4621*, and *I(3)4705* are loss-of-function alleles of *dsarm*. Consistent with this interpretation, expression of full-length *dsarm* cDNA using the postmitotic *OR22a-Gal4* driver in *I(3)896* mutant clones was sufficient to fully revert the suppression of axonal degeneration observed in *dsarm* mutants (Fig. 2C). In addition, we rescued both the lethality and suppression of Wallerian degeneration phenotypes of *I(3)896/I(3)4621* and *I(3)896/Df(3L)BSC795* trans-heterozygous animals with a *BAC* clone containing the *dsarm* gene (fig. S2C). Together, these data indicate that dSarm function is necessary in postmitotic neurons to drive axonal destruction after axotomy.

Based on RNA in situ hybridizations to embryos, larval brains, and adult brains, reverse transcription polymerase chain reaction from dissected neural tissues, and analysis of a *dsarm-Gal4* driver line, *dsarm* is widely expressed in the *Drosophila* nervous system (fig. S3, A to E). These data raise the possibility that dSarm may be broadly required to promote Wallerian degeneration in the nervous system.

We next assayed Wallerian degeneration in null mutants for the mouse ortholog of *dsarm*, *Sarm1*. We grew 5-day cultures of superior cervical ganglia (SCG) from wild-type, *Sarm1*^{-/-}

(16) and *Wld^S* mice, severed axons, and scored axonal integrity over the next week. *Sarm1^{-/-}* SCG explants exhibited robust protection from degeneration up to 72 hours after axotomy, similar to what is observed with *Wld^S* SCG neurons, whereas wild-type axons degenerated within 8 hours (Fig. 3, A and B). This robust protection was also seen in cultured *Sarm1^{-/-}* cortical neuron axons (Fig. 3, C and D, and fig. S5C) and dorsal root ganglia (DRG) (Fig. 3, E and F, and fig. S5D). Notably, *Sarm1^{-/-}* DRG explants were not protected from nerve growth factor (NGF) deprivation (fig. S5, A and B), a model for developmental axon pruning (4, 13, 17, 18), suggesting that Sarm1 protection is specific to injury-induced axon degeneration.

To determine whether Sarm1 is required for Wallerian degeneration in vivo, we performed sciatic nerve lesions of the right hind limb of *Sarm1^{-/-}* mice and controls. Whereas *Sarm1^{+/-}* controls exhibited a dramatic breakdown of the axon and myelin sheath within 3 days of injury, 61.2% of lesioned *Sarm1^{-/-}* axons were protected from degeneration at least 14 days after injury ($P=0.002$) (Fig. 4A). Ultrastructural analysis revealed a remarkable preservation of the Schwann cell and myelin sheath, axonal neurofilaments, and axonal mitochondria at this time point (Fig. 4B and fig. S4). Western blots of sciatic nerve with antibodies to neurofilament-M (NF-M), α -Internexin, and β -tubulin class III (TUJI) confirmed that the axonal cytoskeleton was robustly preserved in severed *Sarm1^{-/-}* axons (Fig. 4D). We further note preservation of the NF-M signal by immunofluorescent staining nerves in *Sarm1^{-/-}* mutants (fig. S6, A and E).

Synaptic integrity was scored by colocalization of presynaptic marker (NF-M/synaptophysin) with the postsynaptic acetylcholine receptor (AChR) in tibialis anterior muscles after sciatic nerve transection. In wild-type animals, motor end plate denervation was complete by 2 days after axotomy. However, in *Sarm1^{-/-}* animals, most synaptic terminals were partially innervated even at 6 days after injury (Fig. 4C and fig. S5D). Consistent with a strong preservation of nerve integrity, macrophage/monocyte infiltration of lesioned nerves was suppressed in *Sarm1* knockout animals (fig. S6, B, C, and F). Taken together, our results indicate that *Sarm1^{-/-}* mutations strongly preserve sciatic nerves in vivo from initial axonal cytoskeletal breakdown to recruitment of macrophages for myelin clearance.

To help define potential sites of action, we assayed localization of dSarm and Sarm1 in neurons. Expression of dSarm::GFP in *Drosophila* larval neurons resulted in punctate localization in neuronal cell bodies and broad localization to neurites in vivo (fig. S7, A and B). Immunostaining with antibodies to Sarm1 of in vitro cultured mouse neurons showed a similar pattern, and we note that endogenous Sarm1 did not show preferential localization with a mitochondrial marker (fig. S7, C and D), although mitochondrial localization has been reported for overexpressed Sarm1::GFP (16). Thus, dSarm/Sarm1 appears to be broadly localized in the axonal compartment.

Our analysis of dSarm/Sarm1 provides evidence that Wallerian degeneration is driven by an ancient, conserved axonal death program. Intriguingly, its *Caenorhabditis elegans* homolog, Tir-1, was recently implicated in nonapoptotic cell death (19), suggesting mechanistic links between these nonapoptotic degenerative mechanisms. Influx of extracellular calcium is

known to be necessary and sufficient to trigger Wallerian degeneration (20). Tir-1 functions downstream of the Ca²⁺-CaM kinase signaling pathway in *C. elegans* (21, 22); therefore, dSarm/Sarm1 may respond directly to axonal calcium increases after axotomy. *Sarm1*^{-/-} neurons in slice cultures exhibit reduced cell death in response to oxygen/glucose deprivation (18); whether this is an indirect result or reflects axonal preservation remains untested. Pharmacological inhibition of Sarm1 may represent a promising therapeutic avenue for patients with axonal loss, particularly if Sarm1 deletion is shown to benefit animal models of neurodegenerative disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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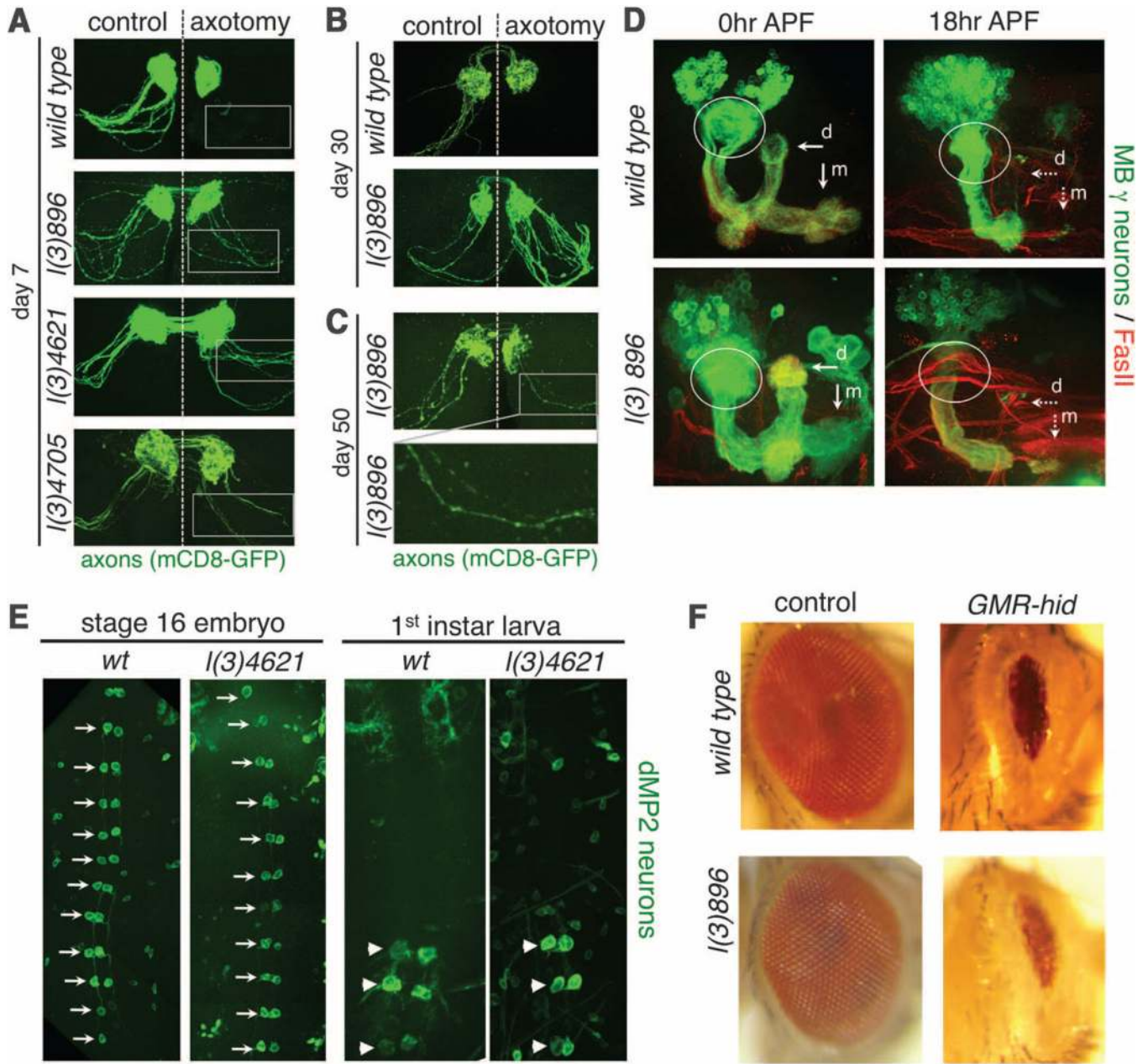


Fig. 1. Identification of mutations that suppress Wallerian degeneration. **(A)** ORN MARCM clones in control, *I(3)896*, *I(3)4621*, and *I(3)4705*. Right, axotomy; left, uninjured control. $n \geq 15$ brains. **(B)** Control and *I(3)896* brains 30 days after injury. $n \geq 10$ brains. **(C)** *I(3)896* clones 50 days after injury. $n = 11$ brains. **(D)** MARCM clones in mushroom body (MB) γ neurons in control and *I(3)896* backgrounds at the indicated developmental stages. dorsal (d) and medial (m) axonal branches (arrows), and dendrites (circled). $n \geq 15$ brains for all. **(E)** dMMP2 neurons with *GFP*. Ventral views (anterior up) of stage 16 embryos (left) and first-instar larvae. dMMP2 neurons before (arrows) and after (arrowheads) segment-specific apoptosis. n

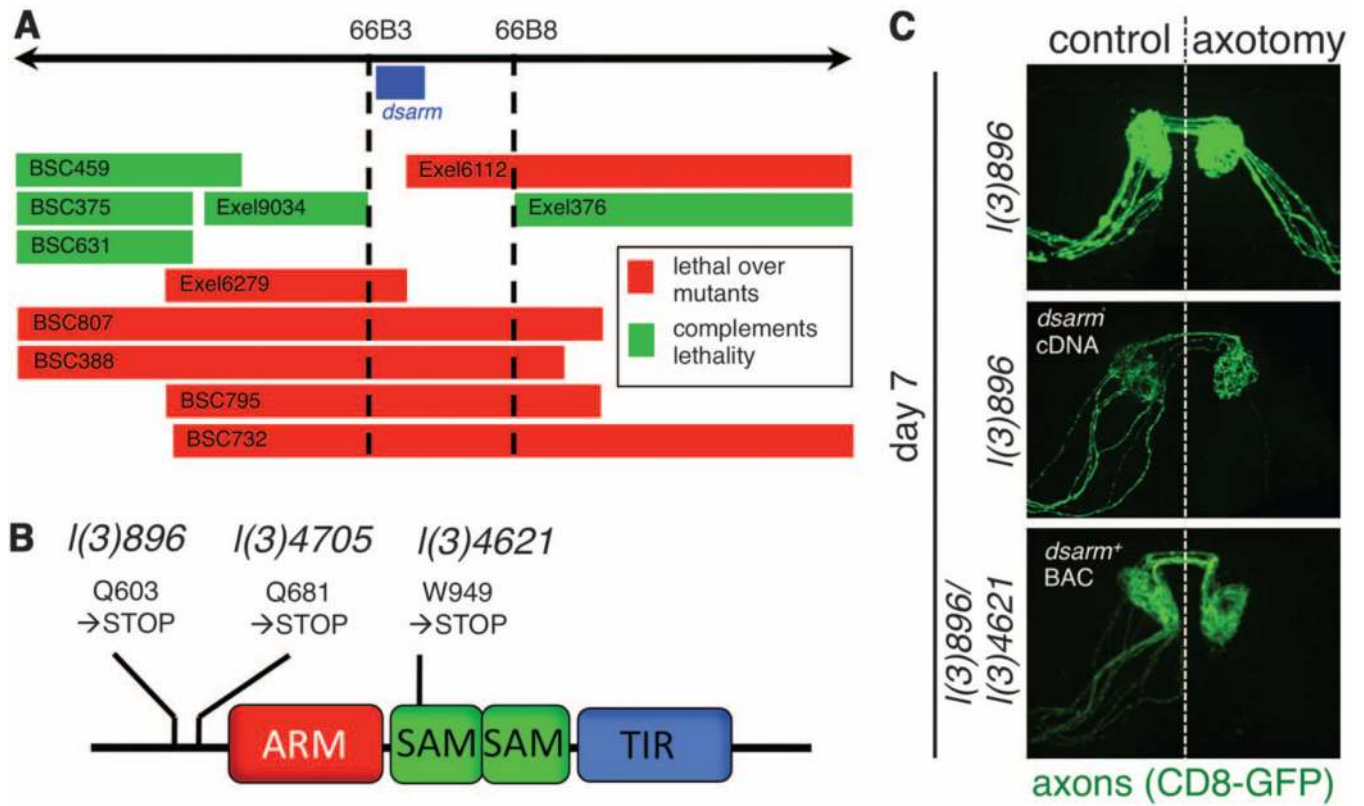
≥ 20 flies at each time point. **(F)** Wild-type and *I(3)896* mutant clones with (right) or without (left) ectopic expression of *hid*. $n \geq 20$ flies.

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**Fig. 2.**

Mutations in *dsarm* block Wallerian degeneration. (A) The lethality of *I(3)896*, *I(3)4621*, and *I(3)4705* was mapped to region 66B using Exel or Bloomington Stock Center deficiencies. (B) *Dsarm* protein domains, positions, and effect of predicted point mutations. (C) *UAS-dsarm* in *I(3)896* mutant clones or *dsarm*⁺ bacterial artificial chromosome rescue axonal degeneration defects in *I(3)896/I(3)4621* flies. *n* = 12 flies.

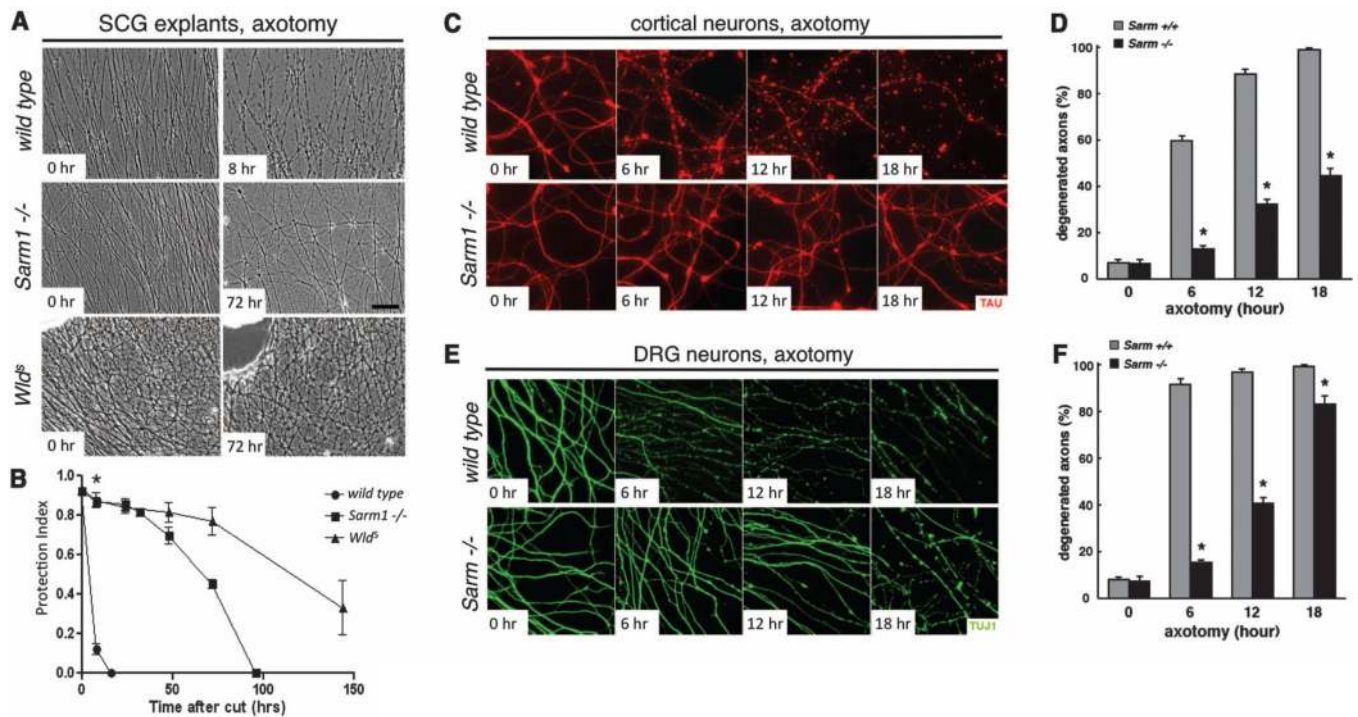


Fig. 3. *Sarm1*^{-/-} neurons in primary culture are protected from Wallerian degeneration. (A) Phase contrast images of SCG explant cultures from wild-type (top), *Sarm1*^{-/-} (middle), and *Wld^S*-expressing (bottom) animals at the indicated time after axotomy. (B) Quantification from (A). Mean T SEM, **P* < 0.01. (C) Axon preservation at the indicated time points in cortical neuron cultures from E16.5 mouse embryos. α-Tau, red. (D) Quantification from (C). Mean T SEM, **P* < 0.01. (E) Axon preservation at the indicated time points in DRG cultures from E13.5 mouse embryos. α-TUJI, green. (F) Quantification from (E). Mean T SEM, **P* < 0.01.

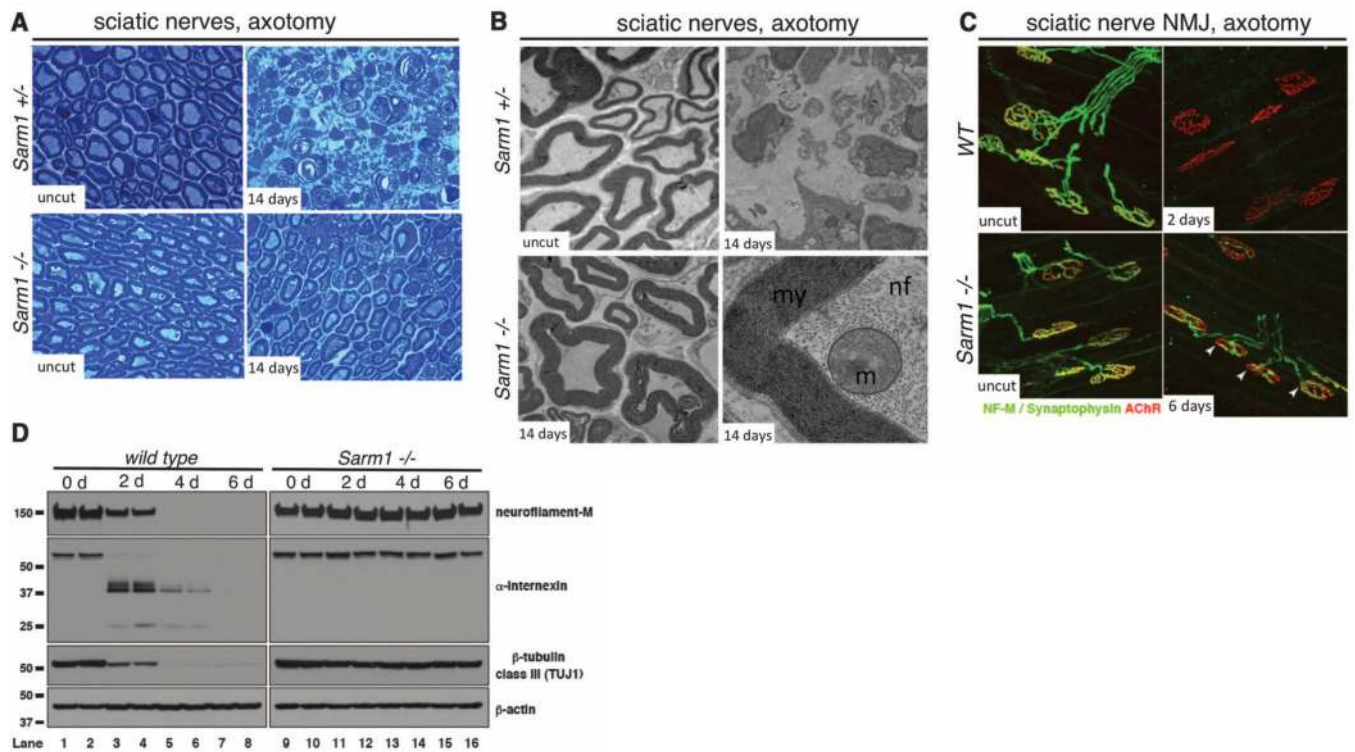


Fig. 4. *Sarm1* is required for Wallerian degeneration in mice in vivo. **(A)** Sciatic nerve distal to the injury site stained with Toluidine blue. Time points and genotypes as indicated. **(B)** Ultrastructural analysis of *Sarm1*^{+/-} and *Sarm1*^{-/-} axons before or 14 days after axotomy. Higher magnification image (lower right) is shown in its entirety in fig. S4. my, myelin; nf, neurofilaments; m, mitochondrion. **(C)** Neuromuscular junction (NMJ) preservation at tibialis anterior muscles. red, AChR (postsynapse/muscle); green, NF-M/synaptophysin (presynapse). Arrows, motor end plates. **(D)** Immunoblot analysis of distal injured nerve segment. $n = 4$ mice at each time point and genotype.