Sarm1 Deletion, but Not WldS, Confers Lifelong Rescue in a Mouse Model of Severe Axonopathy

Highlights
- Rescue of an axonopathy model by Sarm1 deletion or WldS compared in an aging study
- Young adult NMNAT2-deficient mice rescued by WldS develop a hindlimb motor defect
- NMNAT2-deficient mice rescued by Sarm1 deletion are overtly normal up to 24 months
- SARM1 depletion/inhibition may have analytical and therapeutic advantages over WLDS

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In Brief
Both Sarm1 deletion and WldS prevent axonopathy and perinatal lethality in NMNAT2-deficient mice. Gilley et al. report that those rescued by WldS develop hindlimb motor problems as young adults, whereas Sarm1 deletion allows survival to 24 months with no overt defect. These findings have important analytical and therapeutic implications.
**Sam1 Deletion, but Not WldS, Confers Lifelong Rescue in a Mouse Model of Severe Axonopathy**

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**SUMMARY**

Studies with the WldS mutant mouse have shown that axon and synapse pathology in several models of neurodegenerative diseases are mechanistically related to injury-induced axon degeneration (Wallerian degeneration). Crucially, an absence of SARM1 delays Wallerian degeneration as robustly as WldS, but their relative capacities to confer long-term protection against related, non-injury axonopathy and/or synaptopathy have not been directly compared. While Sarm1 deletion or WldS can rescue perinatal lethality and widespread Wallerian-like axonopathy in young NMNAT2-deficient mice, we report that an absence of SARM1 enables these mice to survive into old age with no overt phenotype, whereas those rescued by WldS invariably develop a progressive neuromuscular defect in their hindlimbs from around 3 months of age. We therefore propose Sarm1 deletion as a more reliable tool than WldS for investigating Wallerian-like mechanisms in disease models and suggest that SARM1 blockade may have greater therapeutic potential than WLD5-related strategies.

**INTRODUCTION**

WldS, a spontaneous mutant mouse allele encoding a fusion protein (WLD5) with nicotinamide mononucleotide adenyltransferase (NMNAT) activity, robustly delays injury-induced axon and synapse degeneration (Wallerian degeneration) by locally substituting for loss of the endogenous NMNAT2 isoform (Mack et al., 2001; Gilley and Coleman, 2010; Cohen et al., 2012; Conforti et al., 2014). WldS has been the tool of choice for investigating the molecular basis of axon pathology in animal models of neurodegenerative diseases and has revealed an involvement of Wallerian-like mechanisms in several cases (Conforti et al., 2014). Key steps in this process are thus potential targets for intervention in patients.

Sterile alpha and TIR motif-containing protein 1 (SARM1) acts downstream of NMNAT2 loss to promote axon degeneration (Osterloh et al., 2012; Gerdts et al., 2013; Gilley et al., 2015; Lor-...
However, despite continued silencing of the trapped Nmnat2 alleles in each case (Figure S1A), further aging has revealed clear differences between the lines: Nmnat2<sup>2E<gt>gE</sup>; Sarm<sup>1<gt>gE</sup> mice remarkably survived for up to 2 years with no noticeable behavioral deficiency or phenotype, whereas Nmnat2<sup>2E<gt>gE</sup>; Wld<sup>S</sup>S mice invariably developed a conspicuous, progressive hindlimb defect from around 3–5 months of age. The defect in Nmnat2<sup>2E<gt>gE</sup>; Wld<sup>S</sup>S mice (male and female) first presented as a modest hindlimb gait abnormality during spontaneous locomotion, but this progressively deteriorated, resulting in mice invariantly dragging their hindlimbs regularly during locomotion from around 6 months onward as a result of worsening paraparesis (Movies S1, S2, S3, and S4). Consistent with this, locomotor ability of Nmnat2<sup>2E<gt>gE</sup>; Wld<sup>S</sup>S mice in an accelerating Rotarod task deteriorated rapidly between 4 and 6 months (Figure 1A). Movement became so limited by 10–12 months that it impaired free access to food and water, so Nmnat2<sup>2E<gt>gE</sup>; Wld<sup>S</sup>S mice were not aged further. In contrast, locomotor performance of Nmnat2<sup>2E<gt>gE</sup>; Sarm<sup>1<gt>gE</sup> mice did not decline during the same period (Figure 1A; Movies S5 and S6), and Nmnat2<sup>2E<gt>gE</sup>; Sarm<sup>1<gt>gE</sup> mice still performed as well as Sarm<sup>1<gt>gE</sup> controls up to at least 15 months (Figure 1B).

Deteriorating locomotor function in Nmnat2<sup>2E<gt>gE</sup>; Wld<sup>S</sup>S mice coincided with progressive and widespread wasting of hindlimb muscles (Figure S1B). A specific analysis of gastrocnemius muscle revealed evidence of some muscle fiber atrophy and slightly reduced muscle weight even at 10 weeks in Nmnat2<sup>2E<gt>gE</sup>; Wld<sup>S</sup>S mice, before the onset of overt locomotor dysfunction, but this had progressed to severe muscle fiber atrophy and...
Neuromuscular Denervation in Changes in neuromuscular junction (NMJ) innervation indicated Nmnat2gtE/gtE largely unaffected. In contrast, developmental weight gain in the upper torso and forelimbs, which appeared between 10 weeks and 10–12 months, they failed to gain weight Nmnat2gtE/gtE genic. Motor endplate occupancy in 10-month-old Cell Reports showing normal innervation (Figures 2A and 2B). denervation was extensive, with only around 10% of endplates cle fiber atrophy and weight loss in these mice, by 10 months, 10 weeks, but consistent with the timing of gastrocnemius mus- gastrocnemius was found to be moderately reduced at 10 months. Although denervation was also evident in this muscle, no concurrent loss of muscle fiber atrophy or loss of mass was seen in Nmnat2gtE/gtE;Sarm1−/− mice both performed maximally in locomotor tests, and neither showed signs of neuromuscular denervation at the ages studied. Although a direct comparison with wild-type mice will be needed to establish whether other intrinsic differences in motor function exist in either line, our data suggest that both have broadly normal neuromuscular function. The defect in Nmnat2gtE/gtE;WldS/S mice thus appears to be specific to a declining inability of WLD3 to counter the lack of NMNAT2 in older mice, rather than other intrinsic differences.

Neuromuscular Denervation in Nmnat2gtE/gtE;WldS/S Mice, but Not Nmnat2gtE/gtE;Sarm1−/− Mice

Changes in neuromuscular junction (NMJ) innervation indicated that the muscle defect in Nmnat2gtE/gtE;WldS/S mice is neurogenic. Motor endplate occupancy in Nmnat2gtE/gtE;WldS/S gastrocnemius was found to be moderately reduced at 10 weeks, but consistent with the timing of gastrocnemius muscle fiber atrophy and weight loss in these mice, by 10 months, denervation was extensive, with only around 10% of endplates showing normal innervation (Figures 2A and 2B).

We also investigated motor endplate occupancy in a more distal hindlimb muscle, flexor digitorum brevis (FDB), at 10 months. Although denervation was also evident in this muscle (Figure 3A), it was less severe than in gastrocnemius at the same age. Clear regional variation in the pattern of endplate occupancy was seen in FDB, with discrete zones of normal innervation being found adjacent to zones of complete denervation (Figures 3B and 3C). Isometric tension recordings indicated that this distinctive pattern of NMJ innervation reflects discrete loss of entire motor units (Figures 3D and 3E), with apparently normal function of remaining motor units (Figure S2).

In contrast, and consistent with the lack of locomotor problems, no significant endplate denervation and/or motor unit loss was evident in either gastrocnemius or FDB muscles from 10-month-old Nmnat2gtE/gtE;Sarm1−/− mice (Figures 2, 3A, 3D, 3E, and S2) and innervation remained comparable to Sarm1−/− controls at 24 months, despite modest age-dependent denerva- tion in both (Figure 2A).

WldS/S and Sarm1−/− mice both performed maximally in locomotor tests, and neither showed signs of neuromuscular denervation at the ages studied. Although a direct comparison with wild-type mice was needed to establish whether other intrinsic differences in motor function exist in either line, our data suggest that both have broadly normal neuromuscular function. The defect in Nmnat2gtE/gtE;WldS/S mice thus appears to be specific to a declining inability of WLD3 to counter the lack of NMNAT2 in older mice, rather than other intrinsic differences.

No Loss of Myelinated Tibial Nerve Axons in Either Nmnat2gtE/gtE;WldS/S or Nmnat2gtE/gtE;Sarm1−/− Mice

Despite progressive denervation of motor endplates in hindlimb muscles from Nmnat2gtE/gtE;WldS/S mice, no concurrent loss of myelinated axons was seen in the tibial nerve (Figure 4A) and axons remained morphologically normal (Figure 4B). A gross assessment revealed that most hindlimb muscles became extensively atrophied in these mice (Figure S1B), so significantly reduced numbers of axons would have been expected, even in a mixed nerve such as this, if motor axon loss was the underlying cause. The age-dependent neuromuscular denervation in Nmnat2gtE/gtE;WldS/S muscles thus appears to result from selective loss of the distal ends of motor axons and/or their terminals. This mirrors the age-dependent loss of protection of synapses at NMJs after axotomy in homozygous WldS mice, despite continued protection of the main body of the transected axon (Gillingwater et al., 2002).

Counts of myelinated axons in Nmnat2gtE/gtE;Sarm1−/− tibial nerves remained comparable to those of Sarm1−/− controls, even up to 24 months (Figure 4A), with no age-related axon loss in either group up to this age. We also found no significant axon loss in a separate cohort of wild-type mice (on a related background) up to 24 months (1,514 ± 22 myelinated axons at 1.5 months, compared to 1,473 ± 40 at 24 months). This contrasts a previous study that reported significant loss of myelinated tibial...
nerve axons by 24 months in wild-type mice (Valdez et al., 2010), although this could simply reflect strain differences.

**DISCUSSION**

To date, WldS has been the preferred tool for assessing the involvement of Wallerian-like axon and synapse degeneration in rodent models of neurodegeneration (Conforti et al., 2014). However, the relatively short-term preservation of neuromuscular innervation by WldS in hindlimb muscles of Nmnat2<sup>gtE/gtE</sup> mice raises the possibility that this strategy might have greatly underestimated the involvement of Wallerian-like mechanisms in some models. Likely candidates are wabbler-lethal (Atp8a2<sup>wl/wl</sup>) and gracile axonal dystrophy (Uchl1<sup>gad/gad</sup>) mice, in which WldS robustly protects (proximal) axons but does not rescue neuromuscular dysfunction (Mi et al., 2005; Zhu et al., 2012). Human SOD1<sup>G37R</sup>, SOD1<sup>G85R</sup>, and SOD1<sup>G93A</sup> transgenic mouse models of amyotrophic lateral sclerosis (ALS) are also candidates, although WldS largely fails to protect axons in these models, suggesting that unrelated degenerative mechanisms contribute substantially to disease signs (Vande Velde et al., 2004; Fischer et al., 2005). Because SARM1 deficiency confers longer-lasting preservation of NMJ innervation in NMNAT2-deficient mice than WldS, it could confer a better outcome in these or related models.

We consider that prolonged preservation of Nmnat2<sup>gtE/gtE</sup>; Sarm1<sup>–/–</sup> motor axon terminals, compared to those in Nmnat2<sup>gtE/gtE</sup>; WldS<sup>S</sup> mice, might reflect that local availability of WLD<sup>S</sup>, which is required for protection (Beirowski et al., 2009; Cohen et al., 2012), is likely to be subject to a variety of influences, whereas protection conferred by an absence of SARM1 will be invariant. Global expression of WLD<sup>S</sup> in homozygous WldS<sup>S</sup> mice does not diminish significantly with age up to 12 months (Gillingwater et al., 2002), but we propose that normal changes in physiology, from as young as 2 months of age, could alter the stability, delivery, or activity of WLD<sup>S</sup> in Nmnat2<sup>gtE/gtE</sup>; WldS<sup>S</sup> motor axon terminals, or otherwise alter the local environment, such that it can no longer effectively substitute for the lack of NMNAT2 to promote survival. More widespread NMJ degeneration in gastrocnemius compared to FDB in Nmnat2<sup>gtE/gtE</sup>; WldS<sup>S</sup> mice suggests that changes specific to different muscle or motor unit types are more critical to the loss of
caused by a NMNAT2 deficiency. And can be inhibited by NMNAT activity (Gerdts et al., 2015; Gillingwater et al., 2002). Annulospiral sensory nerve endings in the muscle remained protected in older mice in that context, suggesting a motor-specific defect (Oyebode et al., 2012). Although we have seen qualitative preservation of annulospiral endings in Nmnat2<sup>gE/gE</sup>, Wld<sup>S</sup> FDB at 10 months (not shown), a comprehensive analysis of sensory innervation will be required to determine whether sensory endings in general are better preserved than motor axon terminals.

A model in which sustaining the effective potency of WLD<sup>S</sup> locally is required for its protective effects leaves open the possibility that Sarm1 deletion may be more effective than Wld<sup>S</sup> at rescuing symptoms in models of other types of neurodegenerative disease, not just those with early neuromuscular symptoms. If the disease-causing defect in a given model additionally reduces the activity or concentration of WLD<sup>S</sup> within axons or synapses in some way, then its protective capacity might be diminished. This could apply to disorders of axonal transport, protein synthesis, or protein turnover, among others.

Our findings have therapeutic implications for human disorders. Specifically, they suggest that strategies directed at blockade of SARM1 function have the potential to be more effective than WLD<sup>S</sup>-related therapies in neuromuscular synaptopathies and potentially in a broader group of neurodegenerative disorders. In addition, the remarkable survival and health of Nmnat2<sup>gE/gE</sup>, Sarm1<sup>−/−</sup> mice into old age suggests that even long-term therapeutic interventions based on blocking SARM1 function might be both effective and well tolerated by patients. This study confirms SARM1 as a key regulator of degeneration caused by a NMNAT2 deficiency. Sarm1 deletion appears to block this process indefinitely without affecting long-term survival, despite the predicted substantial reduction in nicotinamide adenine dinucleotide (NAD)-synthesizing capacity (Gilley et al., 2015). SARM1 has been shown to possess NADase activity that promotes injury-induced axon or synapse degeneration and can be inhibited by NMNAT activity (Gerds et al., 2015; Sasaki et al., 2016; Essuman et al., 2017). Therefore, a model in which survival depends on NMNAT-dependent NAD production balancing NAD consumption, including any resulting from constitutive SARM1 NADase activity, is attractively simple. However, NAD consumption in uninjured Sarm1<sup>−/−</sup> axons has been shown to be comparable to wild-type consumption, suggesting that SARM1 NADase activity under normal conditions is minimal (Sasaki et al., 2016). Instead, a situation in which a loss of Nmnat2<sup>gE/gE</sup>, Wld<sup>S</sup> mice have an advanced neuromuscular defect at this age.

**Figure 4. No Loss of Myelinated Tibial Nerve Axons in Either Nmnat2<sup>gE/gE</sup>, Wld<sup>S</sup> or Nmnat2<sup>gE/gE</sup>, Sarm1<sup>−/−</sup> Mice**

(A) Numbers of myelinated axons in tibial nerve (mid-calf level) from mice of the indicated genotypes and ages (Wld<sup>S</sup> control groups include some Nmnat2<sup>gE/gE</sup>, Wld<sup>S</sup> mice that were indistinguishable from Wld<sup>S</sup> control mice). Individual values (n = 3–8, as shown, male and female) and means ± SEM are plotted. No statistically significant differences were identified between groups (one-way ANOVA with Tukey’s multiple comparisons). (B) FluoroMyelin red-stained tibial nerve cross-sections from 10-month-old Wld<sup>S</sup> and Nmnat2<sup>gE/gE</sup>, Wld<sup>S</sup> mice (representative of n = 5 each genotype). No structural differences are evident, even though Nmnat2<sup>gE/gE</sup>, Wld<sup>S</sup> mice have an advanced neuromuscular defect at this age.

**EXPERIMENTAL PROCEDURES**

**Mouse Breeding and Maintenance**

Animal work was performed in accordance with the 1986 Animals (Scientific Procedures) Act under Project License PPL 70/7620 following an appropriate ethical review process at the Babraham Institute. Genotyping for the Nmnat2<sup>gE/gE</sup>, Wld<sup>S</sup>, and Sarm1 knockout alleles was performed as described previously (Gilley et al., 2013, 2015). Littermates were used where possible. The ages and genders of mice used in individual experiments are described in the figure legends.

**RT-PCR**

Semi-quantitative endpoint RT-PCR was used to confirm Nmnat2 gene silencing in the brains of Nmnat2<sup>gE/gE</sup>, Wld<sup>S</sup> and Nmnat2<sup>gE/gE</sup>, Sarm1<sup>−/−</sup> mice aged 10–12 months essentially as described previously (Gilley et al., 2013).
Accelerating Rotarod Task
Locomotor performance was tested on an accelerating Rotarod (Ugo Basile, Model 7650, Varese, Italy). Mice were familiarized with the apparatus (two 5 min runs at 10 rpm) one day before testing. At each test age, mice performed three 5 min trials (3 to 30 rpm) separated by 30 min rests. Latency to fall (max 300 s) was recorded. Only involuntary falls were scored. Mice dismounting voluntarily were placed back onto the apparatus once, but the run was excluded from the analysis if repeated. Best trial performance was used for statistical analyses.

H&E Staining
Transverse cryosections of gastrocnemius muscles snap frozen in liquid nitrogen-chilled isopentane (8 μm thickness) or fixed in 4% paraformaldehyde (20 μm thickness) were stained with H&E as previously described (Gilley et al., 2013). Images were captured using a MicroPublisher camera (Qimaging) on an Olympus BX50 microscope (20× objective). Staining of snap-frozen muscle sections was optimal for visualization of muscle structure without the artifactual muscle fiber separation seen on fixed sections.

NMJ Innervation
Innervation of NMJs in gastrocnemius and FDB muscles was assessed by immunofluorescent staining. Staining was performed essentially as described previously (Krieger et al., 2013) on whole-mount muscles or longitudinal cryosections (60 μm thickness). Confocal z stack series were acquired using Olympus FV1000 or Leica SPE scanning laser confocal microscopes (20× or 40× objectives). Multiple z stack series were acquired for each muscle, and z projections were generated for analysis. Endplate occupancy was determined by assessing the extent of overlap or direct abuttal of Jil1-tubulin staining (axon terminal) with α-bungarotoxin staining (endplate). Endplates were scored as denervated when essentially none of the endplate (less than ~5%) was deemed to be occupied by the axon terminal, fully innervated with complete (greater than ~95%) occupancy, and partially innervated with intermediate occupancy (observer determined, scored blind). Original z stack series were examined to exclude chance overlap of proximal axon segments and endplates in non-adjacent focal planes.

Isometric Muscle Tension Recordings
Force measurements for FDB muscle were made from FDB muscle-tibial nerve preparations as described previously (Beirowski et al., 2009), except that the proximal tendon was connected to a MLT0202 (0–25 g) isometric force transducer (AD Instruments, Oxford, UK) and the tibial nerve was stimulated using 0.1–0.2 ms pulses of up to 10 V using a Digitimer DS2 isolated stimulator (Digitimer, Welwyn Garden City, UK) triggered via a Powerlab 26T interface. Tension responses were digitized at 1 kHz using Chart 7 or Scope 4 software (all ADInstruments).

Counts of Myelinated Tibial Nerve Axons
Transverse sections (20 μm) of fixed calf (from mid-way between knee and ankle) were stained with FluoroMyelin red according to the manufacturer’s instructions (Life Technologies). Images of tibial nerve were captured on an Olympus FX1000 point scanning confocal microscope imaging system (40× objective). Axon counts (inferred from numbers of myelin sheaths) were performed blind using the multi-point selection tool in ImageJ.

Statistical Analysis
Appropriate statistical testing of data was performed using Prism (GraphPad Software, La Jolla, USA). Tests are described in the figure legends. A p value < 0.05 was considered significant.

DATA AND SOFTWARE AVAILABILITY
Source data for graphs can be found in the the University of Cambridge Repository (Apollo) at https://doi.org/10.17863/CAM.13389.

SUPPLEMENTAL INFORMATION
Supplemental Information includes two figures, one table, and six movies and can be found with this article online at https://doi.org/10.1016/j.celrep.2017.09.027.

AUTHOR CONTRIBUTIONS

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