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Deletion of Sarm1 gene is neuroprotective in two models of peripheral neuropathy

Elliot Turkiew, Debbie Falconer, Nicole Reed, and Ahmet Höke

Department of Neurology and Neuroscience, Johns Hopkins School of Medicine, Baltimore, MD 21205

Abstract

Distal axon degeneration seen in many peripheral neuropathies is likely to share common molecular mechanisms with Wallerian degeneration. Although several studies in mouse models of peripheral neuropathy showed prevention of axon degeneration in the slow Wallerian degeneration (Wlds) mouse, the role of a recently identified player in Wallerian degeneration, Sarm1, has not been explored extensively. In this study we show that mice lacking the Sarm1 gene are resistant to distal axonal degeneration in a model of chemotherapy induced peripheral neuropathy caused by paclitaxel and a model of high fat diet induced putative metabolic neuropathy. This study extends the role of Sarm1 to axon degeneration seen in peripheral neuropathies and identifies it as a likely target for therapeutic development.

Keywords

Wallerian degeneration; distal axon degeneration; peripheral neuropathy; Wlds; NMNAT2; Sarm1; paclitaxel; diabetic neuropathy

Introduction

Peripheral neuropathy is a common neurodegenerative disorder that affects quality of life of many patients. Although there are many causes of peripheral neuropathy, diabetes and toxic neuropathy due to chemotherapy are among the most common causes (Grisold, et al., 2012; Juster-Switlyk and Smith, 2016). Currently there are no effective medications available that prevent axon degeneration (apart from strict blood glucose control in diabetic patients) or promote regeneration. Although the exact underlying molecular mechanisms of axon degeneration across various chemotherapy agents and diabetes are likely to be varied, they all do share some common features with Wallerian degeneration that is seen after traumatic injury to the peripheral nerves (Cashman and Hoke, 2015).

Corresponding author: Ahmet Höke MD, PhD, 855 N. Wolfe St., Baltimore, MD 21205, Tel: 410-955-2227, Fax: 410-502-5459, ahoke@jhmi.edu.

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In recent years, there have been two significant advances made in unlocking the underlying molecular mechanisms of Wallerian degeneration. First was the identification of a spontaneous mutation that arose in a colony of mice resulted in delayed Wallerian degeneration when peripheral axons were crushed or cut (Lunn, et al., 1989; Perry, et al., 1990). Subsequent nerve transfer experiments showed that this was an intrinsic property of axons (Glass, et al., 1993) and a distinct mechanism that was unaffected by blocking apoptotic pathways in the neuronal cell body (Deckwerth and Johnson, 1994). Later termed Wallerian Degeneration Slow (*Wlds*), this mutation is due to a fusion protein consisting of the first 70 amino acids of a ubiquitination factor (Ube4B) and the full sequence of an enzyme involved in synthesis of NAD⁺ (Nmnat1) (Mack, et al., 2001). Later studies confirmed the pivotal role of NMMNAT and its isoforms in mediating this axon protection (reviewed in (Coleman and Freeman, 2010)). In addition to delaying Wallerian degeneration, *Wlds* mutation protects axons against paclitaxel induced peripheral neuropathy (Wang, et al., 2002), suggesting a shared pathway between Wallerian degeneration and distal axonal degeneration induced by paclitaxel.

The second advance was identification of another molecule, Sarm1 that played a key role in Wallerian degeneration. Although *Wlds* mutation arose spontaneously, Marc Freeman and colleagues took a different approach and, using the forward genetic screen power of drosophila, showed that deletion of dSarm/Sarm1 delays Wallerian degeneration (Osterloh, et al., 2012). The relevance of Sarm1 was validated by an RNAi approach as well (Gerdtz, et al., 2013). How Sarm1 mediates axon degeneration is unknown, but a recent study by Millbrandt's group suggests that local activation of Sarm1 in axons triggers axon degeneration via destruction of NAD⁺ (Gerdtz, et al., 2015) and a previous study showed that Sarm1 is required to mediate cell death and axon degeneration induced by depolarization of the mitochondrial membrane (Summers, et al., 2014). Whether Sarm1 plays a similar role in axon degeneration seen in metabolic or toxic neuropathies remains to be seen. In this study, we examined the development of neuropathy induced by paclitaxel or metabolic syndrome in wild type and Sarm1 knockout mice.

Methods and Materials

Experiments involving animals were approved by the Johns Hopkins University Animal Care and Use Committee, and carried out according to relevant guidelines and regulations in a facility, which is accredited by the American Association of the Accreditation of Laboratory Animal Care. Sarm1 knockout (KO) mice (Szretter, et al., 2009) were obtained from a colony bred at the University of Massachusetts (courtesy of Dr. Marc Freeman) and bred in house. Genotyping was confirmed for each mouse using the protocols described in the original paper that generated the Sarm1 KO mouse (Szretter, et al., 2009). Wild-type control C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). For both studies only male mice aged 10–16 weeks were used.

Paclitaxel model of peripheral neuropathy

Paclitaxel was purchased from Sigma-Aldrich (St Louis, MO), dissolved in Cremophor EL/ethanol (50/50 v/v) for a final concentration of 7.5mg/ml and stored at 4°C. Paclitaxel was

administered by tail vein injection to both Sarm1 KO mice and wild-type animals at a dose of 25 mg/kg/dose once daily on days 1, 3 and 5 of the study as previously described (Melli, et al., 2006; Zhu, et al., 2013). Control groups received vehicle only. The injections were given at the same time each day using standard rodent injection techniques. Two weeks following the final injection, the mice were euthanized by isoflurane inhalation and tissues were harvested for histopathological evaluation.

High fat diet model of metabolic neuropathy

Mice were fed a High Fat Diet (HFD) (Research Diets, New Brunswick, NJ; catalogue # D12492) containing 60% fat for 8 weeks. The diet was replaced on a weekly basis to ensure the freshness of the food. Before being placed on the HFD, mice received the normal rodent fed. Baseline glucose levels were measured in each mouse before starting the HFD. For fasting glucose testing, mice were prevented from eating anything for 6 hours (no bedding, no food) but were allowed access to water during this period. In order to perform the blood glucose test, mice were placed in a veterinarian-approved restrainer with the tail exposed. The tail was pricked with a sterile needle and the blood glucose level was measured twice using a glucose meter (OneTouch Ultra2, LifeScan) and the average was recorded. Fasting glucose measurements were performed once a week for 8 weeks. Mice were also weighed at baseline and once a week along with glucose testing. At the end of 8 weeks, the mice were euthanized by isoflurane inhalation and tissues were harvested for histopathological evaluation.

Thermal sensation testing

Thermal sensation was evaluated using IITC Plantar Analgesia Meter 400 (Woodland Hills, CA). For the paclitaxel model, measurements were made 2 days before the first injection and 2 days before euthanasia. Similarly, for the HFD model of diabetic neuropathy, measurements were done at baseline before the mice were placed on the HFD and at the end of the study 2 days before they were harvested. Each subject was placed inside a containment box and allowed to acclimate to its surroundings for 15 minutes. The light source was then switched on, emitting heat onto the plantar surface of the subject's left foot. The time taken for the mouse to lift this paw was measured 6 times with a 10 minute rest period between each reading. An average was taken of the readings for each day, and the performance data recorded before and after the treatment was compared.

Evoked nerve conduction studies

Nerve conduction studies using PowerLab (ADI Instruments, CO) were carried out under isoflurane anesthesia the day before the first injection (baseline) and repeated one day before the animals were euthanized (end-point) for the paclitaxel treated mice. In the HFD groups, the baseline measurements were done 1 day before the mice were placed on the HFD and terminal experiments were conducted the day before euthanasia and tissue harvesting after 8 weeks on the HFD. Evoked sensory responses were recorded from the tail as previously described (Zhu, et al., 2013). Briefly, mice were anesthetized using isoflurane inhalation and placed over a warming blanket with a temperature probe to maintain the tail temperature consistent during the recording. Recording electrodes were placed at the base of the tail and the stimulating electrodes were placed 5 cm distally. Orthodromic stimulation was carried

and responses from 20 stimulations were averaged. Sensory nerve action potential (SNAP) amplitude and conduction velocity were calculated.

Epidermal Nerve Fiber Analysis and Sural Nerve Morphometry

Evaluation of intraepidermal innervation was performed using immunohistochemical staining for pgp 9.5, a pan-axonal marker, according to standard protocols (Griffin, et al., 2001) as described before (Melli, et al., 2006). Briefly, 2 mm punch biopsies were done in the medial footpads of the left hind limbs and fixed in PLP fixative. After cryosectioning, the slides were stained with anti-pgp 9.5 antibody (Biogenesis, catalogue # 7863-0504). Intraepidermal nerve fibers were counted in 4–6 sections for each animal and averaged. In addition, left hind limb sural nerves were harvested and post-fixed in 4% paraformaldehyde-3% glutaraldehyde in Sorenson's buffer overnight. Afterwards the nerves were embedded in plastic and stained with toluidine blue as described previously (Hoke, et al., 2003). Images were captured for nerve morphometry using unbiased sampling methods with a stereological set up and axon counts, axon and fiber diameters, myelin thicknesses and G-ratios were calculated using ImageJ 1.46r software.

Statistical analysis

The in vivo studies were carried out with $n=4-8$ in each group. ANOVA with correction for multiple comparisons was used for statistical analysis using Prism v.6 (GraphPad).

Results

Sarm1 deletion partially protects against paclitaxel's neurotoxicity

When administered to WT mice, paclitaxel causes a sensory neuropathy that predominantly affects distal epidermal innervation (Melli, et al., 2006). In this study, we used Sarm1 KO mice and age- and gender-matched wild-type (WT) mice from the same genetic background (C57BL/6) to induce a length-dependent sensory neuropathy by paclitaxel. As seen in Figure 1, paclitaxel caused decreased withdrawal response to thermal stimulation in the WT mice but this thermal hypoalgesia was largely prevented in the Sarm1 KO mice. All groups had mean withdrawal latency of 1.66 – 1.74 seconds at baseline and were comparable to each other without any statistically significant difference. In comparison, a decline in the tail sensory nerve action potential (SNAP) amplitude was not prevented in the Sarm1 KO mice (Figure 2). It is interesting to note that at baseline, Sarm1 KO mice had lower SNAP amplitudes compared to WT mice (mean 25.45 μ V in Sarm1 KO mice versus 35.91 μ V in WT mice). However, both groups of animals showed similar decline in SNAP amplitudes when paclitaxel was administered. Although there was a trend to decreased conduction velocity in paclitaxel-treated mice, the differences were not statistically different from the vehicle control mice. These findings indicate dissociation between the effects of Sarm1 deletion on small unmyelinated versus large myelinated sensory fibers. In fact, when we evaluated the epidermal innervation in hind paws 2 weeks after paclitaxel or vehicle administration, we found that WT mice treated with paclitaxel had a reduction in epidermal nerve fiber (IENF) density but the Sarm1 KO mice had preserved epidermal nerve fibers consistent with the observation on thermal sensation (Figure 3). In order to evaluate the large fiber populations we examined the nerve morphometry of the sural nerves, but there were no

obvious morphological abnormalities and no differences among the groups (Supplemental Figure 1); consistent with what others have found in mice or rats treated with lower doses of paclitaxel (Carozzi, et al., 2010; Siau, et al., 2006).

Neuropathy induced by high fat diet is abrogated in Sarm1 KO mice

Mice that are fed a diet where a majority of calories are from fat develop dyslipidemia and evidence of peripheral neuropathy (Obrosova, et al., 2007; Vincent, et al., 2009). In a previous cohort of WT mice we had measured lipid profiles and showed that compared to normal diet, mice fed HFD for 4 weeks developed elevated levels of total cholesterol (172 mg/dL vs 99.8 mg/dL), triglycerides (134.1 mg/dL vs. 97.8 mg/dL) and low density lipoprotein (45.7 mg/dL vs. 20.2 mg/dL) (n=6 per group). These high fat diet (HFD) fed mice are used as a model small fiber sensory neuropathy seen in patients with metabolic syndrome and pre-diabetes. We used the same model in WT and Sarm1 KO mice and asked if Sarm1 protected mice against development of metabolic syndrome, pre-diabetes and peripheral neuropathy. As seen in Figure 4, both WT and Sarm1 mice fed HFD gained weight at a faster rate than their counterparts fed a normal diet. However, only the WT mice developed pre-diabetes as defined by fasting glucose levels of higher than 200 mg/dL. When we compared the thermal withdrawal latency at the end of 8 weeks of HFD to thermal latencies obtained at baseline, we found that the WT mice developed thermal hypoalgesia because the withdrawal latency was prolonged (Figure 5). However, the Sarm1 KO mice did not show a difference in thermal withdrawal latency whether they were on normal diet or HFD. We also evaluated the tail sensory nerve evoked responses and found that small reduction in SNAP amplitudes seen in WT HFD-fed mice was not observed in Sarm1 KO (Figure 6). There were no significant differences among the groups in terms of conduction velocity. The findings in thermal sensitivity correlated with preservation of IENF densities in Sarm1 KO mice. WT mice that were fed HFD developed small fiber sensory neuropathy with loss of epidermal fibers (Figure 7) but the Sarm1 KO mice had similar densities of IENFs whether they were fed normal diet or HFD. Similar to what we observed in our paclitaxel study, sural nerve morphometry did not show any significant degeneration of large fibers, changes in axon density or myelin thickness (Supplemental Figure 2).

Discussion

Our study shows that mice lacking the Sarm1 gene are resistant to distal degeneration of small fiber sensory fibers seen in a model of putative metabolic neuropathy and a model of chemotherapy induced peripheral neuropathy (CIPN). In addition to what had been described in the literature with traumatic axon degeneration (Osterloh, et al., 2012) and models of vincristine-induced neurotoxicity (Geisler, et al., 2016; Gerdts, et al., 2013), these results suggest an important role for Sarm1 in axon degeneration induced by both toxic and metabolic insults.

Unlike the traumatic injury models used in the initial manuscripts evaluating the role of Sarm1, the two models of peripheral neuropathy used in this study are relatively mild and affect only sensory axons and predominantly unmyelinated sensory fibers. The neuroprotection seen in the putative metabolic neuropathy model was complete with

preservation of thermal sensitivity, tail SNAP amplitude and epidermal innervation indicating an effect on both unmyelinated and myelinated axon degeneration. In the paclitaxel model of CIPN, there was partial preservation of the thermal sensitivity and epidermal innervation but there was no effect on decrease in tail SNAP amplitudes, suggesting that molecular pathway(s) of axon degeneration involving Sarm1 may not be applicable to all modes of axon degeneration. One drawback to reaching a conclusion about the myelinated axons in the paclitaxel model is that we did not see any evidence of large myelinated axon degeneration in the sural nerve even in wild type mice and our attempts at trying to collect and do nerve morphometry of the tail sensory nerves were not successful. These nerves are too small to be isolated individually so we attempted to embed them in plastic with the whole tail, including the bones. Unfortunately the decalcification process distorted the nerve structures so much that reliable nerve morphometry could not be evaluated. It is possible that the reason we did not see any changes in the sural nerve morphometry is that tail sensory nerves are longer than the sural nerve and may have experienced very distal axonal degeneration, hence the reduction in SNAP amplitudes, but same did not happen in the shorter sural nerves. Alternatively, paclitaxel could have caused axonal dysfunction leading to reduced SNAP amplitudes in these long nerves without causing axonal degeneration. It is premature to conclude one way or another whether Sarm1 deletion protects against large fiber degeneration as robustly as it does against small unmyelinated fiber degeneration.

So, how could Sarm1 deletion provide protection against axon degeneration by such different means as traumatic, toxic and metabolic insults? A recent review highlights the roles NMNAT (a key mediator of axon protection in the Wlds mouse), Sarm1, and DLK (Dual leucine zipper kinase) play in Wallerian degeneration induced by axon transection (Gerdtts, et al., 2016). The genetic mechanism by which axon degeneration is prevented in the Wlds mouse is ectopic expression of NMNAT1 protein in the axon (Araki, et al., 2004). Furthermore, overexpression of NMNAT2 (the axonal form of NMNAT) is sufficient to prevent axon degeneration, and reduction in levels of NMNAT2 in axons is sufficient to induce axon degeneration (Gilley, et al., 2013; Gilley and Coleman, 2010). Enzymatic activity of NMNAT is required for axon protection (Sasaki, et al., 2009), indicating a role for NAD⁺, and exogenous supplementation of NAD⁺ is sufficient to delay Wallerian degeneration (Sasaki, et al., 2006; Wang, et al., 2005). However, supplementation of NAD⁺ in a rat model of inherited neuropathy did not alter rate of axonal degeneration (Meyer zu Horste, et al., 2011), but the same study found that NAD⁺ supplementation had a small delaying effect on traumatic axon degeneration, complicating the picture as to whether exogenous NAD⁺ supplementation can have a therapeutic role in slowly progressing neuropathies.

How does NMNAT activity link to axon degeneration seen in peripheral neuropathies and neurodegeneration? There is very limited insight from published work in the field, but a recent study (Slivicki, et al., 2016) showed that embryonic cortical neurons isolated from mice lacking NMNAT2 had reduced levels of survival when exposed to paclitaxel and vincristine, another commonly used chemotherapy agent that frequently causes peripheral neuropathy. When the mice that were heterozygous (homozygous KO mice die at birth) for NMNAT2 gene were exposed to vincristine during adulthood, they exhibited an exaggerated

pain behavior. Unfortunately, this study did not examine whether there was an impact on actual axon degeneration distally in NMNAT2 heterozygous mice exposed to vincristine, so we do not really know whether reduced levels of NMNAT2 predisposes to axon degeneration in toxic neuropathies or if the effect seen in this study was due to some other change in the pain pathways. Another study using a mouse model of human tauopathy found that transcription of NMNAT2 was down-regulated prior to neurodegeneration and that overexpression of NMNAT2 or NMNAT1 was sufficient to reduce neurodegeneration (Ljungberg, et al., 2012). Although we have not examined the levels of NMNAT2 in our paclitaxel or diabetic neuropathy models, it would be interesting to see if NMNAT2 levels are reduced prior to distal axonal degeneration and whether a potential reduction is prevented in the Sarm1 KO mice. Unfortunately, this would be a technically difficult study to do because the distal axon degeneration we see in these models of mild sensory polyneuropathy do not affect the majority of fibers in a nerve, and a likely small reduction in NMNAT2 levels prior to axon degeneration may not be appreciated with preserved levels in remaining healthy fibers. Perhaps a transgenic mouse with genetically labeled NMNAT2 expressed only in unmyelinated axons can be used in the future to see if levels of NMNAT2 decline in the distal axons prior to degeneration.

The link between *Wlds*, therefore NMNAT activity, and role of Sarm1 in axonal degeneration is complex. Earlier studies showed that axonal NAD⁺ levels decline rapidly after axotomy (Wang, et al., 2005), and in a recent study Gerdt and colleagues show that this NAD⁺ decrease is dependent on Sarm1 activity (Gerdt, et al., 2015). Furthermore, embryonic lethality of NMNAT2 homozygous deletion is rescued by genetic deletion of Sarm1 (Gilley, et al., 2015), suggesting that the two molecules are key players in the same pathway. Although Sarm1 activation leads to NAD⁺ depletion and correlates with axonal degeneration, an alternative hypothesis is that nicotinamide mononucleotide (NMN, precursor of NAD⁺), accumulates after injury and leads to axonal degeneration (Di Stefano, et al., 2015). Lowering levels of NMN in axons prevents degeneration even if NAD⁺ levels are decreased, and expression of *Wlds* gene prevents NMN rise and protects axons from degeneration. This point is controversial though as others have shown that axonal degeneration occurs independent of levels of NMN (Sasaki, et al., 2016).

These observations on the role of Sarm1 in axonal health are supported by other studies examining the role of Sarm1 in other neuronal populations. An earlier study showed that Sarm1 was highly expressed in the nervous system and upregulated in central nervous system neurons after hypoxia (Kim, et al., 2007). Furthermore, death of hippocampal neurons after oxygen and glucose deprivation was prevented in neurons isolated from Sarm1 KO mice. We do not know if Sarm1 levels are regulated in models of toxic or metabolic neuropathies, but our study clearly shows that genetic deletion of Sarm1 prevents distal sensory axonal degeneration induced by paclitaxel and metabolic syndrome.

Although a previous study showed that *Wlds* protects against development of diabetes, diabetic retinopathy, and diabetic neuropathy in a streptozotocin model of Type 1 diabetes (Zhu, et al., 2011), this study did not clearly demonstrate prevention of neuropathy independent of the effect of reduced glucose levels in the diabetic mice. The primary effect of *Wlds* transgene was to preserve the pancreatic islet cells from the degeneration induced

by streptozotocin so that the mice did not develop diabetes. Studies examining the development of neuropathy in models of metabolic syndrome and type 2 diabetes will need to be carried out to see if Wlds (or overexpression of NMNAT2) protects against diabetic or pre-diabetic neuropathy. Similarly, the observation that Sarm1 KO mice did not develop hyperglycemia is very interesting and point to a possible diversion of hyperglycemia and metabolic syndrome due to HFD. Although it would have been ideal to collect blood samples at the end of the study to look for evidence of metabolic syndrome, this was not carried out in this study. A larger study with detailed evaluation of metabolic syndrome markers is underway as well as examination of expression of Sarm1 in other tissues such as muscle, liver, and pancreas. In future studies it will be important to determine whether or not SARM KO mice develop metabolic syndrome in response to high fat diet.

In summary, our findings add to a growing body of literature suggesting that Wallerian degeneration and distal axonal degeneration seen in metabolic and toxic neuropathies share common pathways, and that drugs aimed at promoting NMNAT2 activity or inhibiting Sarm1 activation may have therapeutic utility in peripheral neuropathies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Thermal withdrawal latency is increased in WT mice treated with paclitaxel but this increase was not seen in Sarm1 KO mice (n=5 mice for WT-vehicle group, n=6 mice for WT-paclitaxel group, n=8 for Sarm1-vehicle and -paclitaxel groups; * p<0.05 compared to vehicle control and ** p<0.05 compared to WT-paclitaxel group; error bars denote SEM).

Figure 2.

The decline in SNAP amplitude caused by paclitaxel was not prevented in Sarm1 KO mice (A). There was no difference among the groups in terms of changes in evoked sensory response conduction velocity (B) (n=5 mice for WT-vehicle group, n=6 mice for WT-paclitaxel group, n=8 for Sarm1-vehicle and -paclitaxel groups; * $p < 0.05$ compared to vehicle control; error bars denote SEM).

Figure 3.

Epidermal innervation density was markedly reduced in the paclitaxel treated WT mice but not in Sarm1 KO mice. Panels in (A) are representative images of pgp 9.5 stained plantar footpads (bar = 20 μ m). (B) shows quantitation of epidermal nerve fibers (n=5 mice for WT-vehicle group, n=6 mice for WT-paclitaxel group, n=8 for Sarm1-vehicle and -paclitaxel groups; * p<0.05 compared to vehicle control and ** p<0.05 compared to WT-paclitaxel group; error bars denote SEM).

Figure 4.

Weekly weights (A) and fasting blood glucose levels (B) of WT and Sarm1 KO mice placed on high fat diet (HFD). Both WT and Sarm1 mice gained excess weight on HFD by the 7th week (A) but fasting glucose levels were elevated only in the WT-HFD group (B). (n=4 mice for WT-ND and WT-HFD groups, n=8 mice for Sarm1 KO-ND and Sarm1 KO-HFD groups; * p<0.05 compared to normal diet (ND) control; error bars denote SEM).

Figure 5.

Thermal withdrawal latency is increased in WT mice placed on HFD but this increase was not seen in Sarm1 KO mice (n=4 mice for WT-ND and WT-HFD groups, n=8 mice for Sarm1 KO-ND and Sarm1 KO-HFD groups; * $p < 0.05$ compared to ND control and ** $p < 0.05$ compared to WT-HFD group; error bars denote SEM).

Figure 6.

The decline in SNAP amplitude caused by HFD in WT mice was not seen in Sarm1 KO mice placed on HFD (A). There was no difference among the groups in terms of changes in evoked sensory response conduction velocity (B) (n=4 mice for WT-ND and WT-HFD groups, n=8 mice for Sarm1 KO-ND and Sarm1 KO-HFD groups; * p<0.05 compared to ND control and ** p<0.05 compared to WT-HFD group; error bars denote SEM).

Figure 7.

Epidermal innervation density was markedly reduced in WT mice placed on HFD but not in Sarm1 KO mice. Panels in (A) are representative images of pgp 9.5 stained plantar footpads (bar = 20 μ m). (B) shows quantitation of epidermal nerve fibers (n=4 mice for WT-ND and WT-HFD groups, n=8 mice for Sarm1 KO-ND and Sarm1 KO-HFD groups; * $p<0.05$ compared to ND control and ** $p<0.05$ compared to WT-HFD group; error bars denote SEM).