The neuroprotective factor *Wld^s* fails to mitigate distal axonal and neuromuscular junction (NMJ) defects in mouse models of spinal muscular atrophy

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Abstract

Spinal muscular atrophy (SMA) is a common autosomal recessive neurodegenerative disorder in humans. Amongst the earliest signs of neurodegeneration are severe and progressive defects of the neuromuscular synapse. These defects, characterized by poor terminal arborization and immature motor endplates, presumably result in a loss of functional synapses. The slow Wallerian degeneration (*Wld^s*) mutation in rodents has been shown to have a protective effect on mouse models of motor neuron disease by retarding axonal die-back and preventing neuromuscular synapse loss. In this study we tested the effects of the *Wld^s* mutation on the disease phenotype of SMA model mice. Consistent with previous reports, the mutation slows axon and neuromuscular synapse loss following nerve injury in wild-type as well as in SMA mice. However, the synaptic defects found in severely affected SMA patients and model mice persist in the double (Wld^s;SMA) mutants. No delay in disease onset was observed and survival was not significantly altered. Finally, *Wld^s* had no effect on the striking phrenic nerve projection defects that we discovered in SMA model mice. Our results indicate that the reported protective effects of *Wld^s* are insufficient to mitigate the neuromuscular phenotype due to reduced SMN protein, and that the mechanisms responsible for distal defects of the motor unit in SMA are unlikely to be similar to those causing neurodegeneration in genetic mutants such as the *pmn* mouse which is partially rescued by the Wld^s protein.

Keywords

Spinal muscular atrophy; Neuromuscular junctions; Wallerian degeneration

There is a growing recognition that defects in the distal segment of a neuron are often a first step in the degenerative process that characterizes neuronal loss due to injury, toxins or as a result of genetic perturbations [3]. There is also an increasing awareness that the mechanisms underlying axonal and synaptic loss may be distinct and separate from those involved in the degeneration of the cyton [7]. This compartmentalization of the neurodegenerative process explains, in part, emerging efforts to stem neuronal degeneration by preventing synapse and axon loss [5,6,8,16,22,23]. Amongst the many neurodegenerative diseases wherein such a

strategy may be tested and prove to be effective, spinal muscular atrophy (SMA) stands out for a number of reasons. First, it is a disorder that affects the spinal motor neurons, cells with long axons terminating at neuromuscular synapses. Second, the distal ends of the affected cells lie in the periphery making them relatively accessible to potential therapies compared to cells affected in diseases of the CNS. Third, there is strong evidence that denervation, a process involving a loss of functional synapses, is a key aspect of disease pathology in SMA. Finally, the genetics of the disease have been clearly defined allowing the generation of SMA model mice in which to determine whether protecting axons and synapses mitigates the disease phenotype.

SMA is a pediatric neuromuscular disorder caused by mutations in the *SMN1* gene [12]. An almost identical copy gene, *SMN2*, fails to compensate due to a single exonic nucleotide change that disrupts splicing, resulting in reduced levels of the full-length (FL) SMN transcript [17]. Consequently, patients express reduced levels of the multifunctional SMN protein (reviewed in [20]). It is unclear why this ubiquitously expressed protein selectively affects the neuromuscular system. Introducing the human *SMN2* into mice lacking murine *Smn* (*SMN2;Smn$^{-/-}$*) results in animals with a severe form of the human disease [18]. Additional mutant *SMN* transgenes are capable of modulating the disease phenotype in *SMN2;Smn$^{-/-}$* mice [11,19]. We previously demonstrated that neuromuscular weakness in SMA mice appears well before the loss of motor neurons in the spinal cord [9]. We further showed that there is a direct correlation between the onset of weakness and the appearance of defects at the distal end of the motor unit. These defects which are most obvious at the neuromuscular junction (NMJ) involve the pre-synaptic as well as post-synaptic specializations and became apparent as early as post-natal day 2 (P2). Pre-synaptic abnormalities include swellings of neurofilament (NF) protein in pre-terminal axons and nerve terminals, poor terminal arborization and numerous retraction bulb-like structures suggesting denervation. Post-synaptic abnormalities are defined by acetylcholine receptor (AChR) clusters that express significantly higher levels of the fetal (γ) subunit of the receptor than age-matched Controls and fail to mature into the characteristic "pretzels" found in wild-type animals. The severity of these defects presumably results in a loss of functional neuromuscular synapses. Evidence from a more severe animal model of SMA suggests that denervation and synapse loss become apparent as early as embryonic day 18.5 (E18.5) [14]. If these defects contribute to the SMA phenotype and eventually lead to spinal motor neuron loss, protecting the distal end of the motor unit may be beneficial.

Wld^s (Slow Wallerian degeneration) is a chimeric mutant gene in mice that encodes nicotinamide mononucleotide adenylyltransferase (*Nmnat1*; a NAD$^+$-synthesizing enzyme) fused in-frame via a novel 18-amino sequence to the N-terminus of the ubiquitination enzyme *Ube4b* [2]. *Wld^s* mice are indistinguishable from their wild-type littermates but exhibit delayed Wallerian degeneration [13]. In particular, distal axons and neuromuscular synapses of these mice remain morphologically and functionally intact for as long as 2 weeks following axotomy, a finding consistent with delayed atrophy of denervated *Wld^s* muscle [1]. The *Wld^s* gene has also been shown to retard axon and NMJ loss in dying-back axonopathies [5,6,23]. Although molecular mechanisms to explain these effects are unclear, the observations suggest a clear rationale for testing the effect of *Wld^s* on other neuropathies. In this study, we assessed the phenotypic and cellular consequences of expressing the *Wld^s* gene in mouse models of SMA. In contrast to other models of neurodegeneration in which *Wld^s* provided a significant neuroprotective effect, we found that the chimeric gene did not alter the SMA phenotype. This was reflected in a persistence of distal axonal and NMJ defects in the double (Wld^s;SMA) mutants. Our analysis also revealed a novel and striking axonal phenotype in the diaphragms of severely affected SMA mice which is not rescued by *Wld^s*. The new phenotype suggests that insufficient SMN protein may impair appropriate signaling between the nerve and its target muscle and could explain early mortality in the most severe instances of the disease.

Wld^s mice were obtained from Harlan, Bicester, UK and back-crossed to FVB/N mice (Jackson Labs, Bar Harbor, ME, USA) over 10 successive generations. The Wld^s gene was identified by PCR using the following primers: 5′-CGTTGGCTCTAAGGACAGCAC-3′; 5′-CTGCAGCCCCCACCCCTT-3′ while SMA carriers were genotyped as previously described [11,18]. RNA expression of the Wld^s gene was assessed by RT-PCR using a 5′-TGTAGGTCAACACCACCAAC-3′ forward primer and a 5′-TTCCCACGTATCCACTT CCA-3′ reverse primer.

To determine whether the Wld^s phenotype was maintained on the FVB/N strain background, 2-month-old animals with or without the gene were anesthetized with 2.5% avertin (8 μl/g), the right sciatic nerve exposed and transected before suturing the wound. Five or ten days later, the animals were euthanized and the distal stump of the transected nerve dissected out. The contralateral nerve served as a Control. Nerve segments were processed for Toluidene blue staining as previously described [9] to examine individual axons. Transections in neonates were carried out following anesthesia on wet ice. Two days later, the mice were euthanized and relevant muscles isolated to analyze NMJs and intramuscular nerves by immunohistochemistry as described elsewhere [9]. To examine the effect of Wld^s on E18.5 SMA embryos, timed pregnancies were set up and fetal gastrocnemius, intercostal and diaphragm muscles harvested for NMJ analysis. Analysis of the phrenic nerve was carried out on the distal 600 μm portion the sternal branch. AChR clusters and nerves were visualized using labeled α-bungarotoxin (Molecular Probes, Eugene, OR, USA) and anti-NF-160 kDa antibody (Chemicon, Temecula, CA, USA) respectively. Terminal axon lengths and endplate band width were measured using SPOT advanced image analysis software (Diagnostic Instruments, Starling Heights, MI, USA). Differences in means between samples were calculated using the two-tailed Student's t-test and all data presented as mean ± S.E.M. Kaplan–Meier curves were used to determine survival. All animal work was carried out according to institutional guidelines.

To determine the effect of the Wld^s gene on the SMA phenotype, Wld^s;SMA double mutant mice ($Wld^{s+/-}$;$SMN2^{+/+}$;$SMN\Delta7^{+/-}$;$Smn^{-/-}$) were generated. Since Wld^s(C57BL/Ola) and SMA carriers (FVB/N) are on different genetic backgrounds which can mask genuine Wld^s effects in their mixed background progeny, we first derived congenic FVB/N Wld^s mice. Next, we undertook to ensure that the originally reported phenotype was preserved on the new genetic background. Two-month-old mice with or without the Wld^s gene were subjected to sciatic nerve transection and the number of intact axons distal to the lesion were quantified 5 and 10 days later. Wallerian degeneration was significantly delayed in Wld^s mice but not wild-type littermates (Fig. S1A and B) indicating that strain differences do not abolish the Wld^s phenotype. Furthermore, as expected and in keeping with previous reports, the Wld^s gene is ubiquitously expressed and at relatively high levels in neural tissue (Fig. S1C). Next, we introduced that Wld^s gene into SMA carrier mice and then crossed these animals ($Wld^{s+/-}$;$SMN2^{+/+}$;$Smn^{+/-}$) to $SMN2^{+/+}$;$SMN\Delta7^{+/+}$;$Smn^{+/-}$ mice to generate SMA mutants with or without the Wld^s gene. At birth, SMA mice were significantly smaller than Control littermates irrespective of whether they carried the Wld^s gene or not (Fig. 1A). Additionally, there was no difference in weight between SMA and Wld^s;SMA pups (SMA, 1.20 ± 0.02 g; Wld^s;SMA, 1.23 ± 0.02 g; Control, 1.37 ± 0.01 g; $P < 0.05$ for Control vs. SMA and Control vs. Wld^s;SMA; $P > 0.37$ for SMA vs. Wld^s;SMA, $n \geq 10$ for each group). Disease progression in SMA mice with and without the Wld^s gene was virtually identical (Fig. 1B) and there was no significant difference in survival (SMA, 6.1 ± 0.6 days; Wld^s;SMA, 7.2 ± 0.9 days; $P > 0.3$; $n \geq 10$; also see Fig. 1C). These results indicate that the Wld^s gene neither delays disease onset nor enhances survival in SMA model mice.

Previously we showed that the earliest neuromuscular defects in SMA mice are observed at the NMJ and that the onset of these defects parallels the onset of disease symptoms [9]. To

determine if *Wld^s* has any effect on this cellular phenotype, we examined the distal axons and NMJs in the gastrocnemius muscles of SMA, Wld^s;SMA and Control littermates. To simultaneously rule out the possibility that reduced SMN abolishes the delayed Wallerian degeneration effect in *Wld^s* transgenic mice, we transected the right sciatic nerves of the animals at P3 while leaving the contralateral side intact. Distal nerves and NMJs were examined 48 h later at P5. Consistent with our previous findings, an examination of the NMJs in the non-axotomized muscle of SMA mice revealed severe synaptic defects characterized by NF aggregates in the nerve terminals and pre-terminal axons, poor terminal arborization and motor end-plates of reduced size (Fig. 2A and B). In comparison, few if any NMJs in Control littermates with or without *Wld^s* exhibited these abnormalities. An analysis of the same (non-axotomized) NMJs in muscle from Wld^s;SMA mice revealed defects strikingly similar to those in SMA mutants lacking the *Wld^s* gene (Fig. 2A and B). Together, these results indicate first, that *Wld^s* is unable to attenuate the NMJ defects resulting from reduced SMN protein and second, that the chimeric gene does not on its own cause abnormalities of the neuromuscular synapse.

We next examined NMJs of the gastrocnemius innervated by the injured nerve in the various groups of mice. As expected, distal axons were relatively well preserved and AChR clusters remained innervated in Control mice carrying the *Wld^s* gene. In contrast, the vast majority of endplates in non-*Wld^s* Control littermates were devoid of nerve endings and there was clear evidence of extensive Wallerian degeneration of the distal axons (Fig. 2A and B). Interestingly, our findings in the mutant animals mirrored those in the Controls with widespread denervation of NMJs and degeneration of axons in SMA but not Wld^s;SMA mice. Furthermore, the characteristic dispersal of AChRs following neonatal transection [25] was suppressed in Controls as well as in SMA mice carrying the *Wld^s* gene (Fig. S2). Collectively, these results indicate that the Wld^s phenotype is maintained in FVB/N neonates including animals expressing greatly reduced levels of the SMN protein.

To validate our findings described above, we tested the effect of *Wld^s* on a different but related mouse model of SMA [18]. These mice carry only the human *SMN2* transgene on a murine *Smn* null background (*SMN2;Smn^{−/−}*). On a pure FVB/N background they are more severely affected than previously reported [18]. Indeed, death frequently occurs at P0 (S. Kariya and U. Monani, unpublished observation). Given the severity of this phenotype, we examined the effect of *Wld^s* in E18.5 mutant embryos. Timed pregnancies were set up to generate Wld^s;SMA progeny (*Wlds^{+/+};SMN2^{+/+};Smn^{−/−}*) on the one hand, and SMA (*SMN2^{+/+};Smn^{−/−}*) mutants on the other. An examination of the embryos revealed no appreciable difference in gross morphology or body weight between the various genotypes (Table S1). We next asked if SMA mutant embryos displayed NMJ defects similar to those described above and whether *Wld^s* had a mitigating effect on the abnormalities. An analysis of the distal gastrocnemius muscle indicated no abnormalities in the SMA mutants. We therefore examined the more proximally located diaphragm which was previously shown to exhibit NMJ defects early in the disease [9]. Interestingly, we found that NF varicosities at the nerve terminals of the phrenic nerve axons are indeed present in mutant embryos at E18.5 (Fig. S3). To further characterize nerve defect in this muscle, we quantified the number of motor end-plates in a defined area of the sternal branch of the phrenic nerve, the mean distance of these endplates from the nerve trunk, the average number of terminal axons on either side of the nerve trunk and mean length of these axons from the nerve trunk. The parameters were first compared between SMA mutants and Controls (*SMN2^{+/+};Smn^{+/+}*). No differences were found between the numbers of axon terminals or endplates and the distances of the endplates from the nerve trunk (data not shown). However, we found that the average length of the axon terminals from the nerve trunk was ~50% longer in the mutants than in Control littermates (Fig. 3A and B). *Wld^s* had no effect on this novel phenotype which was absent in the two other muscles (gastrocnemius and intercostals) that we examined (data not shown). Nor were the NF varicosities reduced in the

double mutants, a result consistent with our findings in the *Wld^s*;*SMN2;SMNΔ7;Smn*$^{-/-}$ mice. Taken together, this data confirms our conclusions that *Wld^s* fails to attenuate the distal axonal and NMJ defects seen in SMA mice.

Defects in the distal axons and at the NMJs are amongst the first signs of motor neuron pathology in SMA model mice [9]. These defects likely result in a loss of functional neuromuscular synapses which in turn lead to motor neuron cell death. The *Wld^s* mutation in mice has been shown not only to delay Wallerian degeneration following axotomy but also to protect axons and synapses from degenerating due to toxins or genetic mutations. In this study, we assessed whether the protective effects of *Wld^s* benefit mice with SMA. We conclude that although Wallerian degeneration is delayed in Wld^s;SMA mice following nerve injury, the *Wld^s* mutation does not attenuate the SMA phenotype or the NMJ defects characteristic of this motor neuron disease. Similar findings were reported [21] while this manuscript was being prepared although the authors used a single mouse model—the originally reported mixed background *SMN2*$^{+/+}$;*SMNΔ7*$^{+/-}$;*Smn*$^{-/-}$ animals [11] and examined the proximal rather than distal compartments of the motor neuron. The lack of any protective effect ought not to be completely unexpected. While *Wld^s* has been beneficial in a number of mouse models of neurodegeneration, there are prominent examples where the effect has either been modest [6, 16] or completely lacking [26]. Even in instances where it was demonstrated to be of benefit, the underlying mechanisms are not clear. Attempts to understand the mechanisms underlying *Wld^s*-mediated protection have implicated numerous proteins involved in regulating mitochondrial stability and degeneration [27]. Additionally, RNA microarray analyses have revealed changes in axonal transport genes in *Wld^s* mice [24]. Non-somatic mitochondrial defects [10] and axonal transport perturbations [4] lie at the basis of many neurodegenerative diseases. If *Wld^s* does indeed alter synaptic mitochondrial responses and/or axonal transport to confer its neuroprotective effects, it is possible that neither process underlies motor neuron disease in SMA. However, concrete evidence of this will require further investigation.

Perhaps the more interesting outcome of this study was the striking phrenic nerve projection defects we discovered in the most severely affected SMA mice. Axon pathfinding defects have been previously described in a fish model of SMA [15] but were not found in SMA mice [14]. Our findings are in contrast to those recently reported [14]. One explanation for the different results is the severity of the phenotypes of the mice used in the two studies, the fully congenic mutants being considerably more severe [9]. Indeed, we have previously shown that in E18.5 SMA mutants carrying the *SMNΔ7* transgene which reduces SMA severity, there were no signs of phrenic nerve projection defects [9]. This is consistent with the hypothesis that SMN has numerous functions that are affected in a hierarchical fashion depending on SMN levels. Our data showing an increase in mean length of the phrenic nerve axons but no significant increase in the distance of the motor endplates from the nerve trunk (endplate band width) suggests that the axons overshoot the AChR clusters, implying either an inability of the muscle to signal appropriately and/or a failure of the nerve to respond to cues secreted by the muscle. Although it is unclear which, if either, mechanism serves to explain our findings, the phenotype may be indicative of a novel function of the SMN protein in muscle and/or nerve. Furthermore, it could account for pre-natal or early neonatal death in the most severely affected SMA model mice and human patients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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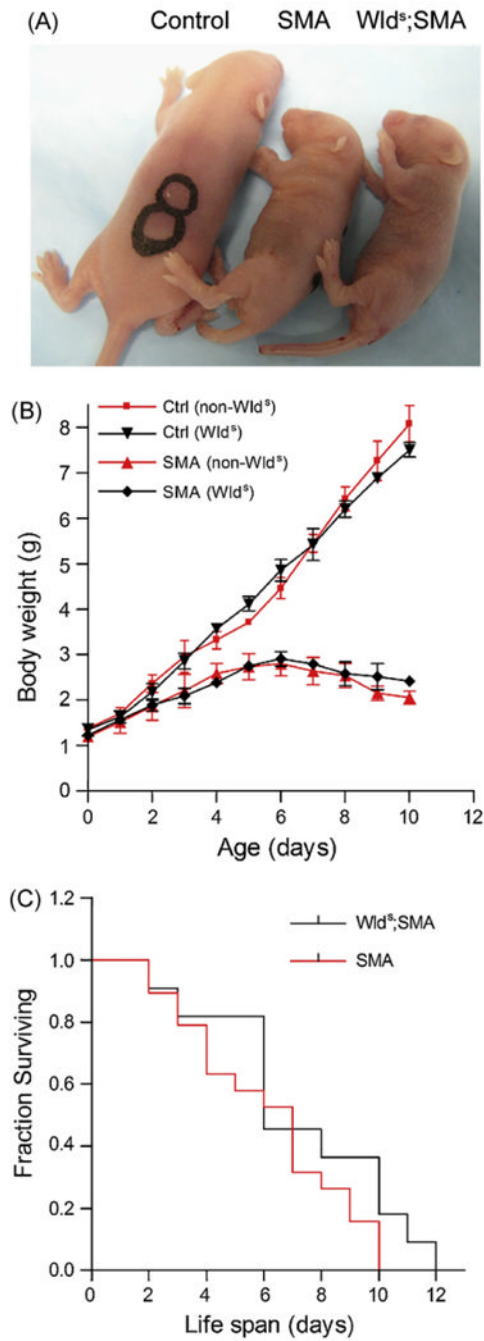
Fig. 1.
The effect of the *Wld^s* gene on the SMA phenotype. (A) Gross morphology at P5 and (B) body weights of Control (*SMN2^{+/+}*;*SMNΔ7^{+/−}*;*Smn^{+/+}*) and SMA mice with or without *Wld^s* indicating that the chimeric gene has no effect on the overt phenotype or disease progression in mutant animals. (C) Kaplan–Meier survival curve analysis indicates that the presence of the *Wld^s* gene in SMA mice does not significantly alter survival. The log-rank test was used to statistically assess survival in the two groups.
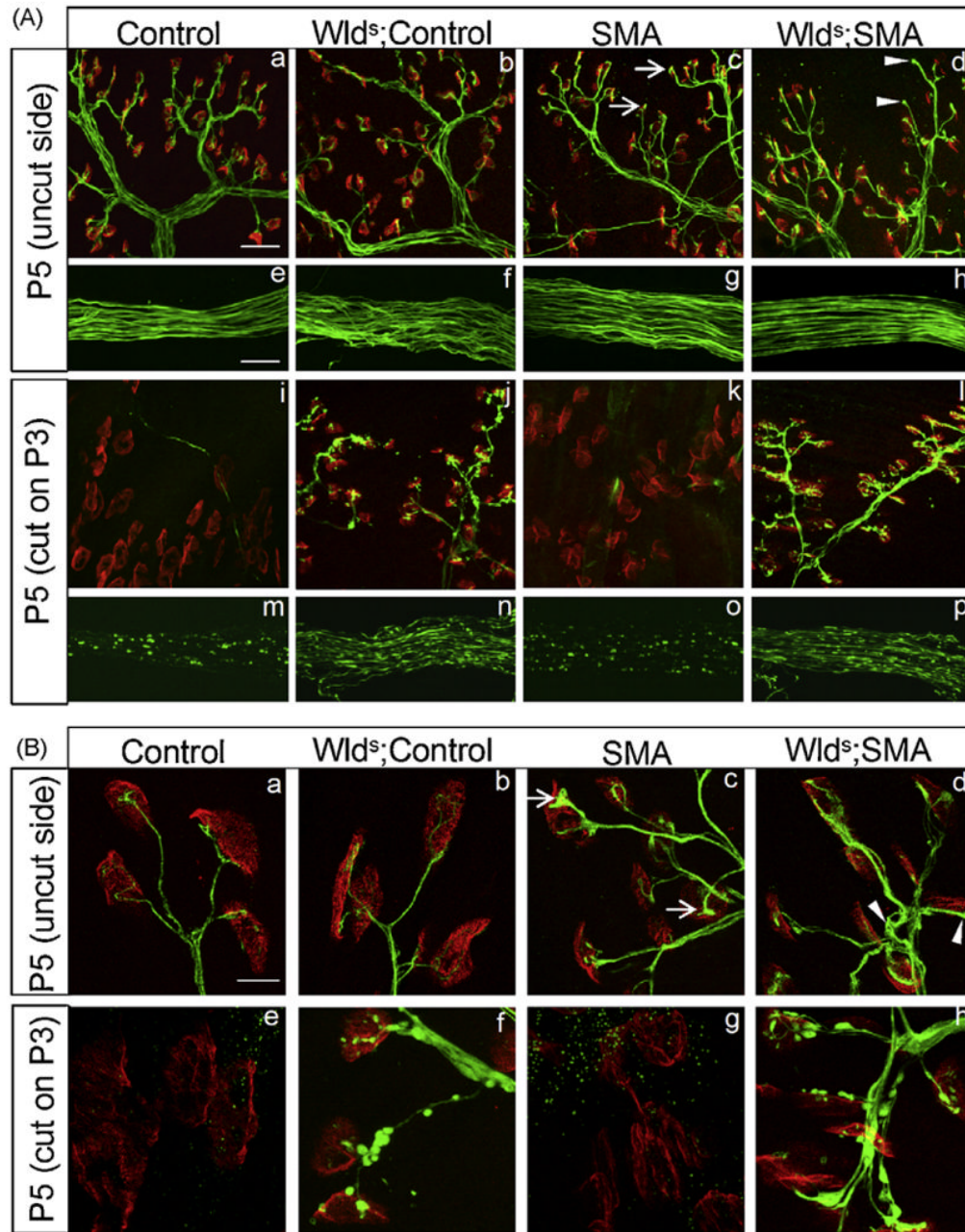
Fig. 2.
The *Wld^s* gene does not mitigate NMJ defects in SMA mice despite delaying Wallerian degeneration. (A) NMJs in the gastrocnemius muscle of Control ($SMN2^{+/+}$;$SMN\Delta7^{+/-}$;$Smn^{+/+}$) and SMA mice with or without *Wld^s* were immunohistologically analyzed following transection of the sciatic nerve. The contralateral nerve was left intact. NMJ defects as assessed by NF aggregates in pre-terminal axons and nerve terminals, poor terminal arborization and AChR clusters of reduced complexity seen in SMA mice (c, arrows) persisted in Wld^s;SMA mice (d, arrowheads). Unoccupied AChR clusters were not significantly increased in either group of mutants. Following transection, severe Wallerian degeneration, characterized by fragmented axons (m and o) and denervated endplates (i and k), was seen in mice lacking *Wld^s* while Control (j and n) as well as SMA (l and p) mice carrying the mutation

were protected. (B) Higher magnification images indicate NF swellings in SMA (c, arrows) as well as in Wld^s;SMA (d, arrowheads) mice. Synpase loss following transection in mice lacking *Wld^s* (e and g) is marked by enlarged AChR clusters. Scale bars: a–d, i–l = 40 μm and e–h, m–p = 25 μm.
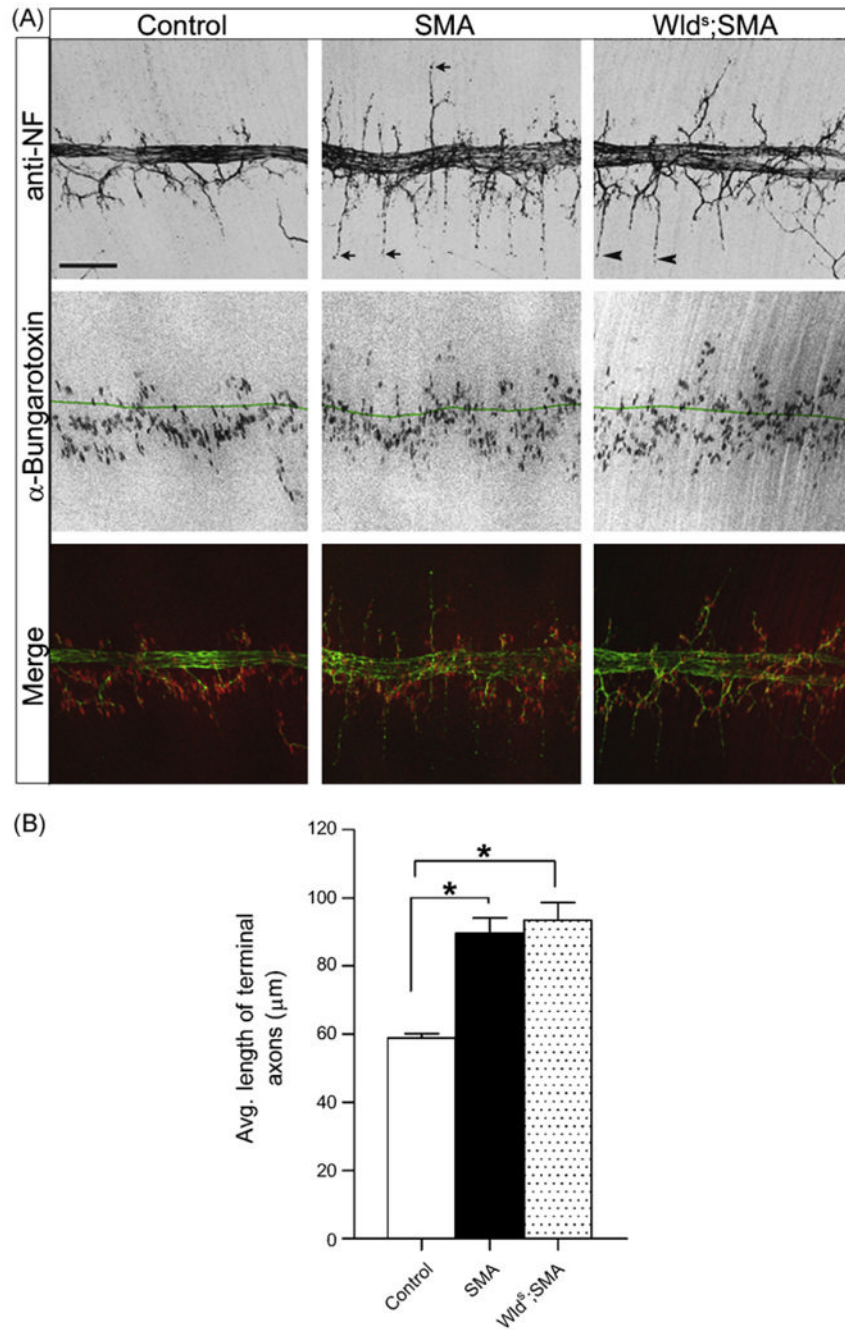
Fig. 3.
Axon projection defects in severe SMA. (A) Phrenic nerve axons in *SMN2*$^{+/+}$;*Smn*$^{-/-}$ E18.5 embryos with or without *Wld^s* project past AChR clusters (arrows in SMA; arrowheads in Wld^s;SMA) and are significantly longer than those in Control (*SMN2*$^{+/+}$;*Smn*$^{+/+}$) littermates. Note: green line in middle row panels represents the midline of the nerve trunk. Note: green line in middle panel represents nerve trunk midline. (B) Quantification of increased length of mutant axon terminals in SMA and Wld^s;SMA mice. *Significant difference with $P < 0.001$, Student's *t*-test, $n \geq 3$. Scale bar= 120 μm.