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The Wallerian degeneration slow (*Wld*^{s}) gene does not attenuate disease in a mouse model of spinal muscular atrophy

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ABSTRACT

Spinal muscular atrophy (SMA) is a severe neuromuscular disease characterized by loss of spinal α-motor neurons, resulting in the paralysis of skeletal muscle. SMA is caused by deficiency of survival motor neuron (SMN) protein levels. Recent evidence has highlighted an axon-specific role for SMN protein, raising the possibility that axon degeneration may be an early event in SMA pathogenesis. The Wallerian degeneration slow (*Wld*^{s}) gene is a spontaneous dominant mutation in mice that delays axon degeneration by approximately 2–3 weeks. We set out to examine the effect of *Wld*^{s} on the phenotype of a mouse model of SMA. We found that *Wld*^{s} does not alter the SMA phenotype, indicating that Wallerian degeneration does not directly contribute to the pathogenesis of SMA development.

Spinal muscular atrophy (SMA) is an autosomal recessive genetic disease, and is the leading genetic cause of death in infants [1]. SMA is a severe neuromuscular disease characterized by loss of spinal α-motor neurons, resulting in the paralysis of skeletal muscle. SMA is caused by deficiency of survival motor neuron (SMN) protein levels. SMN is a ubiquitously expressed protein that has a well-described role in RNA metabolism [2]. It is not currently known why low levels of this ubiquitously expressed protein lead to specific loss of α-motor neurons; however, recent work has revealed a compelling connection between SMA development and aberrant pre-mRNA splicing [3,4].

Recent research has elucidated one function of SMN protein in the axons of motor neurons: SMN is required for efficient transport of β-actin mRNA to the growth cones of developing axons [5]. Consequently, motor neurons isolated from SMA mouse embryos have decreased axon outgrowth due to the very low levels of this vital protein [5]. Further research has strengthened the argument for an axon-specific role of SMN protein. It is well established that β-actin G/F ratios are important for axon growth [6]. Recent data show that Plastin-3, an actin bundling protein, is a potent modifier of the SMA phenotype, evincing the direct link between SMN protein and axonal functioning [7].

The Wallerian degeneration slow (*Wld*^{s}) gene is a spontaneous dominant mutation in mice that delays axon degeneration by approximately 2–3 weeks [8]. Since axon degeneration is a common feature of many neurodegenerative diseases, *Wld*^{s} or one of its downstream mediators may be of therapeutic benefit [9]. Although the *Wld*^{s} mutation delays the onset of symptoms in several mouse models of neurodegeneration [10–12], it provides only modest protection for some, and none for others [13–15]. We set out to examine the effect of *Wld*^{s} on the phenotype of a mouse model of SMA. We found that *Wld*^{s} does not alter the SMA phenotype, indicating that Wallerian degeneration does not directly contribute to the pathogenesis of SMA development.

Materials and methods

Animal breeding. All animal protocols were approved by the University of Missouri Animal Care and Use Committee. SMA carrier mice [16] were purchased from Jackson Labs (Stock # 5025), and *Wld*^{s} mice were originally obtained from Harlan-Olac, Bicester, UK. SMA carrier mice were interbred with *Wld*^{s} mice to produce the following experimental genotypes: [*SMNΔ7+/+*; *SMN2+/+*; *Smn−/−*] and [*Wld*^{s}*+*; *SMNΔ7*; *SMN2*; *Smn−/−*]. Mice were housed in microisolator cages on a 12-h light/dark cycle and were given

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food and water *ad libitum*. The *Wld*^{S} gene was detected by either conventional PCR or qPCR according to published protocols [17,18]. SMN was genotyped using the following primers 5′-TCTG TGTTCGTGCGTGGTGACTTT-3′ and 5′-CCCACCACCTAAGAAAGCCTC AAT-3′ for the WT allele and 5′-CCAACTTAATCGCCTTGCAGCACA-3′ and 5′-AAGCGAGTGGCAACATGGAAATCG-3′ for the knockout allele.

Ventral horn: morphological analysis. Mice were transcardially perfused with 4% paraformaldehyde, 2% glutaraldehyde, and 2% paraformaldehyde in 0.1 M cacodylate buffer. Lumbar spinal cords were dissected and post-fixed overnight. Ten serial sections were taken at 150 μm intervals (5 μm thick) from paraffin embedded lumbar spinal cords [19]. Tissue was stained with Cresyl violet, and images were taken using an Axio Imager microscope (Zeiss). Ventral horn cells that contained distinct nucleolus and darkly stained cytoplasm were outlined using Multigauge software (Fig. 4A) (Fujifilm). Only ventral horn cells with a cross-sectional area $\geqslant 250\ \mu m^2$ were counted [20]. Cross sections of L5 ventral roots were analyzed for three mice per genotype. Axonal diameters were measured using Axio image software (Zeiss). Entire roots were imaged, imaging thresholds were selected individually, and the cross-sectional area of each axon was calculated and reported as a diameter of a circle of equivalent area. Axon diameters were grouped into 0.5 μm bins.

Results and discussion

Sciatic nerve protected by Wld^S *in SMA animals*

Recently, the cellular basis for SMA development in the SMA mouse models has been an area of increasing interest (for review

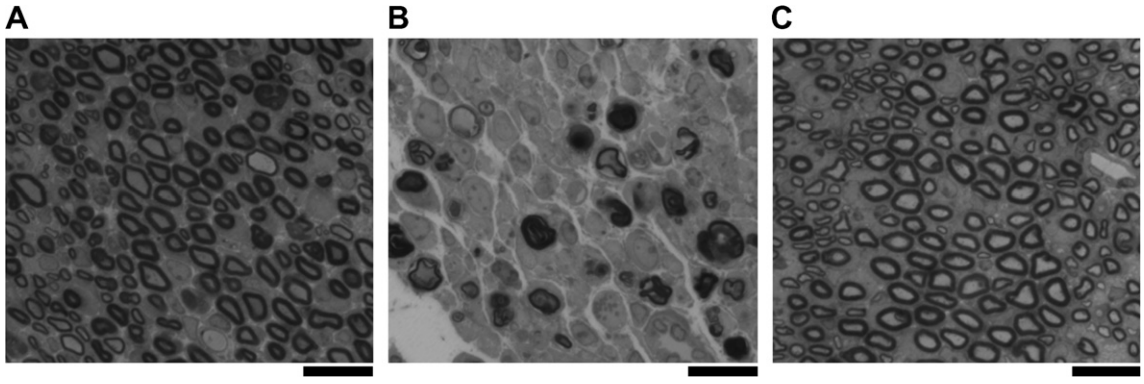

Fig. 1. *Wlds* delays axon degeneration in the SMA mouse genetic background. Distal sciatic nerve stumps from mice carrying *Wld*^{S} (A), and WT (B) 72 h after transaction. Uninjured sciatic nerve (C) is included as control. Bar = 25 μm.

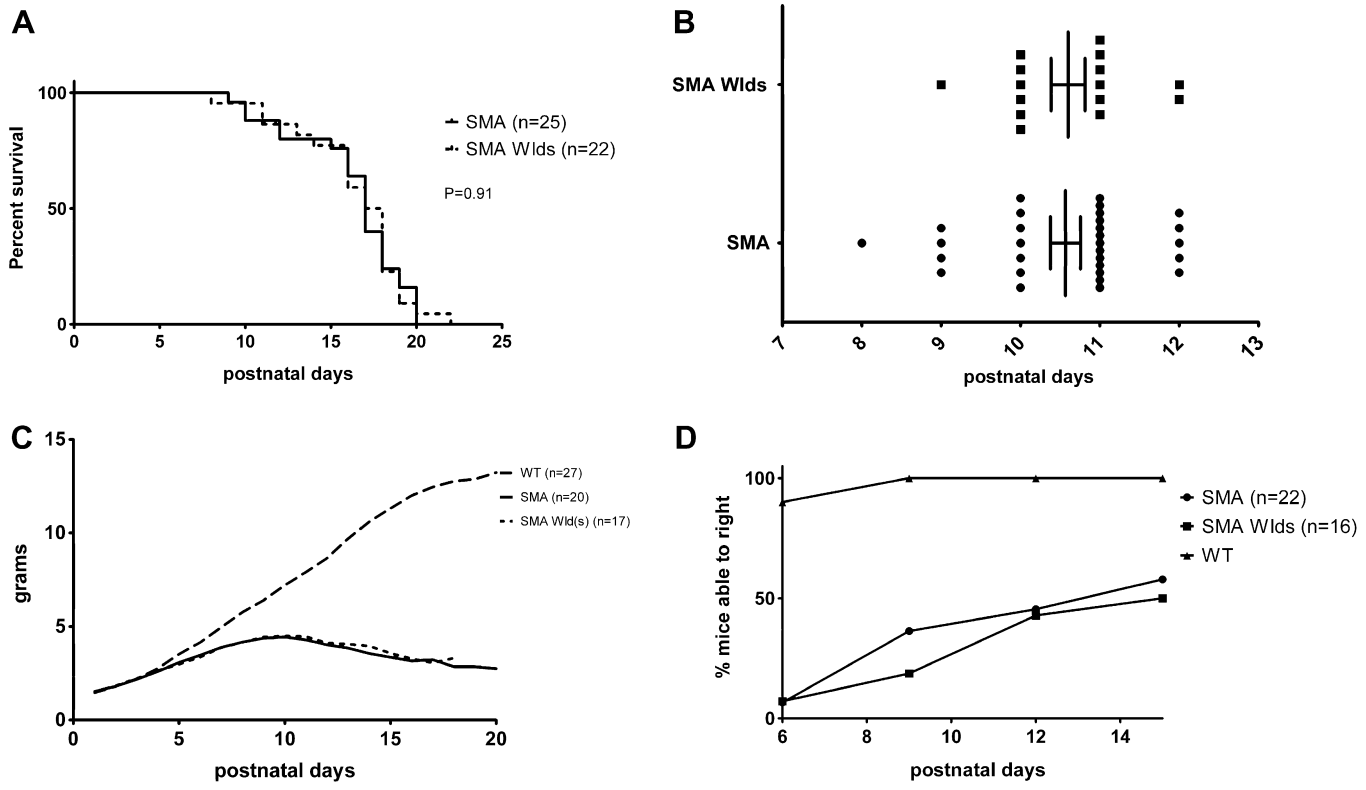

Fig. 2. *Wlds* does not slow the progression of SMA. Kaplan Meier survival curves for SMA and SMA *Wlds* mice (A). The onset of weight loss was plotted such that each symbol represents one mouse at the postnatal day it began to loose weight (SMA = circles, SMA *Wlds* = squares) (B). Total body weight was measured daily for SMA, SMA *Wlds*, and WT littermates (C). The ability of the SMA and SMA *Wlds* mice to right themselves from a prone position was also monitored (D).

see [19]). A variety of animal models of neurodegenerative diseases have been used to examine the effects of *Wld*^{S} gene expression to determine whether Wallerian degeneration contributes to SMA pathogenesis development [10,11,13–15,21,22]. To determine whether Wallerian degeneration contributes to the SMA phenotype, we used the well-described SMA mouse model referred to as "Δ7" [16]. This model was selected because the phenotype is well detailed and has been used extensively in therapeutic analyses [19,23,24]. To confirm that the *Wld*^{S} gene was expressed in our mice, cDNAs were generated from SMA;*Wld*^{S} brain tissue and used to amplify the *Wld*^{S} chimeric cDNA using gene-specific primers [8]. The sequence of the *Wld*^{S} product was intact and identical to previously published reports [25], GenBank AF260924 (data not shown). To confirm the *Wld*^{S} gene could delay axon degeneration in a relatively early postnatal time period and in the genetic background of the SMA mouse model, the sciatic nerve of p12 pups was surgically severed, and the distal stumps were examined after 72 h. WT sciatic nerves displayed extensive breakdown of myelin sheaths, whereas the sciatic nerves from SMA *Wld*^{S} and *Wld*^{S} mice were remarkably protected from the hallmark signs of Wallerian degeneration (Fig. 1A, B, and C). These results were consistent with previous reports describing *Wld*^{S}-mediated protection in neonatal animals [26] and in a variety of other models of neurodegenerative disease [10,11,14,15].

Wld^S *does not delay onset or progression of SMA*

Having established that the *Wld*^{S} gene protected axons in the SMA context, we next examined whether *Wld*^{S} expression altered disease progression of the SMA-Δ7 mouse model. SMA-Δ7 mice have a mean lifespan of approximately 16 days. Similarly, the SMA-Δ7;*Wld*^{S} animals displayed a nearly identical life span, demonstrating that *Wld*^{S} does not significantly extend survival of the SMA animals ($P = 0.91$) (Fig. 2A). Although our SMA-Δ7 colony exhibits a modestly longer median survival compared to the initial description of these animals [16], the analysis of more than 300 animals has clarified this modest, but reproducible extension of life span (data not shown).

The onset of weight loss is the first detectable and most sensitive means of identifying a change in disease progression in this model of SMA [23]. No significant difference was found in the onset of weight loss between SMA mice with or without the *Wld*^{S} gene (Fig. 2B). Furthermore, there was no statistically significant difference observed in the total body weight of the mice at any time point (Fig. 2C). This may suggest that presence of the *Wld*^{S} gene does not act to preserve gross muscle mass. Additionally, an established measure of gross motor function in the SMA-Δ7 mice is their ability to right from a prone position; SMA mice are unable to right themselves efficiently when placed on their backs [23]. Consistent with the rest of the phenotypic data, there was no statistically significant difference in the righting ability between the SMA and SMA;*Wld*^{S} animals (Fig. 2D). To further confirm these findings, the *Wld*^{S} gene was bred to homozygousity on the SMA background, with no alteration of the SMA phenotype (data not shown). This was largely expected, as the gene has been previously shown to function in a hemizygous state [10,22].

Ventral root analysis

SMA is a disease of the motor unit. Therefore, in p12 mice, we examined the L5 ventral roots, which contain a pure population of motor axons. By this time point, the SMA mice display clear hind limb weakness. Therefore, it was anticipated that axon numbers would be decreased in this region in our mouse model. However, we found no significant difference between the numbers of axons in the SMA mice and the WT mice (Fig. 3A). This is consistent with a recent report that found no significant loss of motor axons in the L4 ventral root [27]. Furthermore, we saw no difference in the number of axons between SMA with or without the *Wld*^{S} gene. The average diameters of the axons from the three types of mice were not significantly different (Fig. 3B). The presence of these motor axons does not necessarily imply functionality; however, this finding may provide insight as to why the *Wld*^{S} gene fails to slow the progression of SMA. The functional protection of *Wld*^{S} occurs solely in degenerative neurons. At a relatively late stage of disease, SMA mice still retain normal numbers of non-degenerative motor axons. This finding also indicates that axon degeneration does not correlate with the progression of paralysis in this mouse model of SMA.

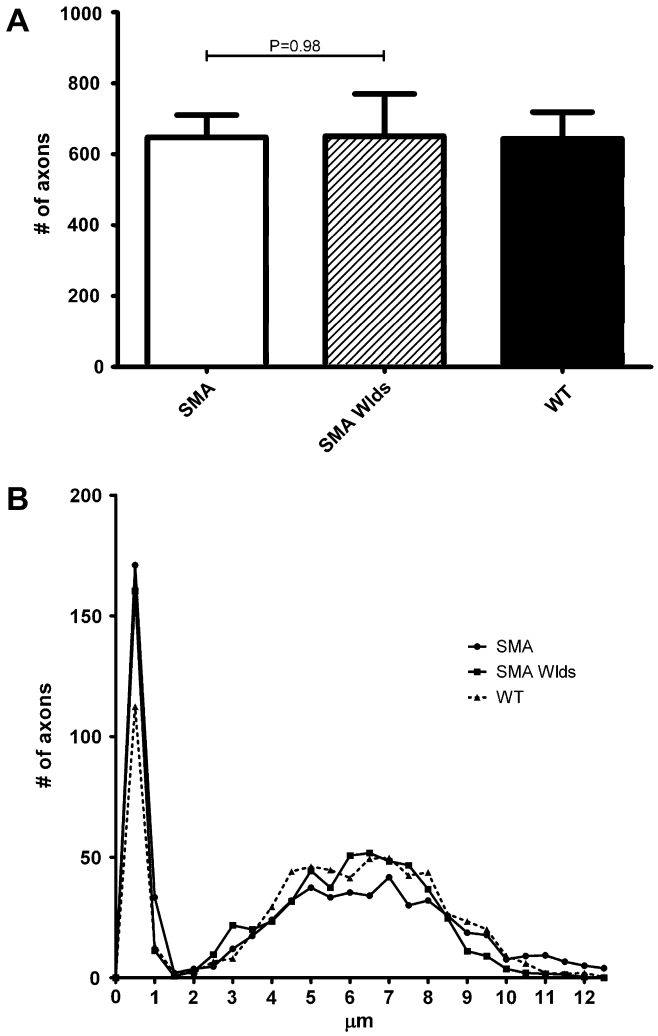

Fig. 3. Ventral root axon analysis. The average number (A) and diameter of L5 ventral root axons were measured ($N = 3$ for each genotype).

Ventral horn analysis

We examined lumbar ventral horn cells in the SMA mice and WT controls and observed no statistically significant numerical difference among the SMA, SMA;*Wld*^{S}, or the WT mice (Fig. 4B). However, a clear trend emerged of fewer ventral horn cells in the SMA mice than in the SMA;*Wld*^{S} or WT mice. These results are consistent with previously reported data showing that the SMA-Δ7 mouse model exhibits a modest loss of lumbar motor neurons by postnatal day 9 [16]. Our data further support the conclusion that

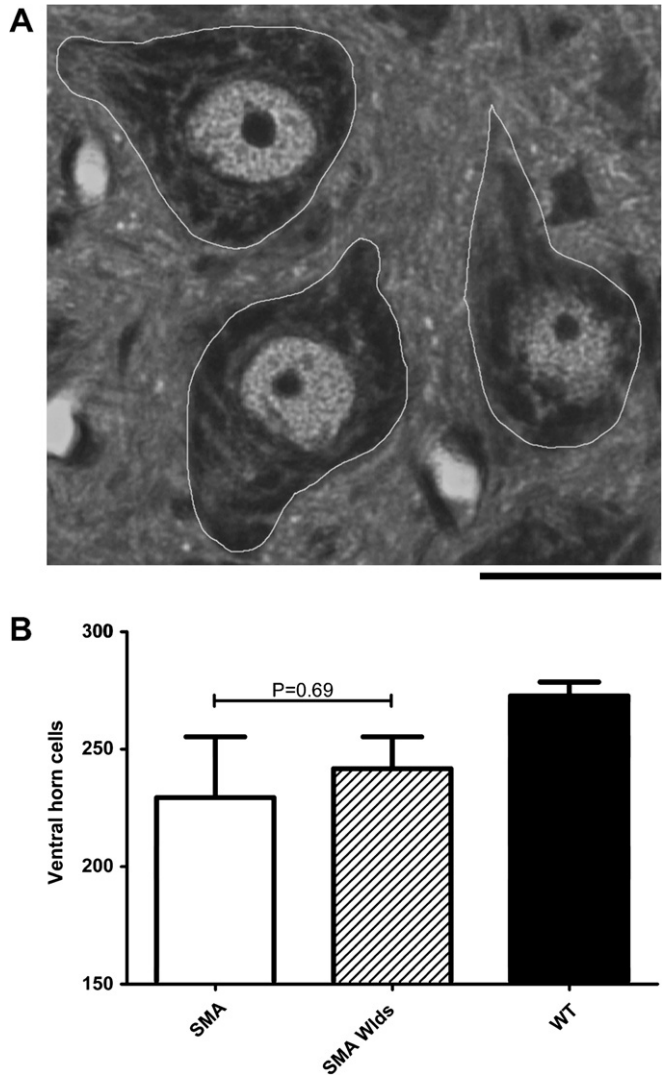

Fig. 4. Ventral horn cell body count. The cross-sectional area of ventral horn cells (A) was measured and cell bodies greater than or equal to 250 μm^2 were included (B).

SMA mice retain non-degenerative motor neuron cell bodies and axons, despite having significant functional weakness.

Collectively, these results demonstrate that the *Wld*^{S} gene was unable to change the onset or progression of disease in a mouse model of SMA. This conclusion is supported by a recent report that did not observe any of the classical markers of Wallerian degeneration, such as myelin debris or plasma membrane breakdown, within the intramuscular nerves of a SMA mouse model [28]. These combined data suggest that Wallerian degeneration likely does not contribute to SMA pathogenesis, thereby reducing the likelihood that therapeutic approaches designed to impede Wallerian degeneration will ameliorate the SMA phenotype.

Acknowledgments

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