IMPAIRMENT OF MOTOR AXON DEVELOPMENT IN SPINAL MUSCULAR ATROPHY

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A thesis submitted to Johns Hopkins University in conformity with the requirements for the degree of Master of Science

Baltimore, Maryland
May, 2014
ABSTRACT

Spinal muscular atrophy (SMA) is an inherited motor neuron disease caused by mutation of the survival of motor neuron 1 (SMN1) gene and deficient expression of SMN protein. In humans, muscle weakness is traditionally thought to be determined by the magnitude of motor neuron degeneration. However, SMA mice show severe muscle weakness without substantial motor neuron loss suggesting that impaired function of motor neurons is the principal determinant of symptoms early in the disease course. Recent studies have also indicated that SMA may not be a motor neuron cell autonomous disease, but rather other cell types or tissues may play contributing role to SMA pathogenesis. In this study, we characterized ventral root (VR) motor axons at the light and electron microscopy level in SMA patient tissues collected at the time of autopsy and in SMA mice. Our results reveal a significant reduction of large myelinated axons, but a surprising, large increase of small unmyelinated axons in both human and mouse SMA VRs, suggesting an early arrest of axonal development. As axonal radial growth is dependent on interactions between axons and Schwann cells, we hypothesized that SMN-deficient Schwann cells contribute to impaired motor axonal growth and to muscle weakness in SMA.

To explore the relative contribution of SMN-deficient motor neurons and Schwann cells to the axonal pathology in SMA, we utilized conditional SMA mouse lines with full-length SMN expression selectively increased in motor neurons (\textit{Chat}^{Cre+} SMA), Schwann cells (\textit{Dhh}^{Cre+} SMA), or both (\textit{Chat}^{Cre+} \textit{Dhh}^{Cre+} SMA). Behavioral data did not show any significant improvements in the motor behavior, survival, or body weight of the \textit{Dhh}^{Cre+} SMA mice compared with the \textit{Dhh}^{Cre-} SMA mice. In contrast,
$ChAT^{Cre+}$ SMA showed a significant improvement in motor function, survival, and body weight, along with improved number of mature motor axons. $ChAT^{Cre+}Dhh^{Cre+}$ SMA mice did not show additional synergistic effect beyond what is observed in the $ChAT^{Cre+}$ SMA mice. These findings suggest that motor axon development is impaired in SMA mice largely due to deficiency of SMN in motor neurons. SMN-deficient Schwann cells do not appear to contribute to impairments of motor axon radial growth in SMA.
ACKNOWLEDGEMENT

This project would not have been possible without the support of many people. I would like to express my special appreciation and thanks to my advisor, Dr. Charlotte Sumner, who has been a tremendous mentor by providing encouragement and guidance. Your advices on both my research as well as my career development have truly been valuable.

With great appreciation, I would also like to acknowledge the help of Jackson Laboratory for providing the conditional mouse models and assisting me in my research. I would especially like to thank Dr. Robert Horner and Dr. Lingling Kong, my thesis reader and mentor for their guidance and help. Special thanks to Dr. Katherine Tiffi, and all the members of Sumner Lab: Dr. Constantin van Outryve d'Ydewalle, Dr. Jeremy Sullivan, David Valdivia, Rhiannon Desideri, Noah Pyles, Adam Miller, Mario Gorz, for their kind support, remarks and advice through the process of developing this master thesis. And finally, thanks to my parents, and my friends who are in the process with me, always offering support and love.
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INTRODUCTION

General overview of Spinal Muscular Atrophy (SMA)

Spinal muscular atrophy (SMA) is the leading inherited cause of infantile mortality with an incidence of 1 in 6000-10,000 live birth and a carrier frequency of 1 in 40-60 (Prior et al., 2010). The disease phenotype varies in severity, and is classified into three subtypes depending on the age of the disease onset and the degree of impaired motor function. SMA type I (Werdnig Hoffman disease) is the most severe type with the patients displaying symptoms before the age of 6 month including weakness of truncal and proximal limb muscles that precludes gaining the ability to sit. SMA type I usually results in death within 2 years of age without respiratory support. SMA type II is the intermediate type with the onset before the age of 18 months. Patients demonstrate the inability to stand or walk without support. Patients with SMA type II usually have an average lifespan of 30-40 years. SMA type III, also known as the (Kugelberg Welander Disease) is the mild type with disease onset after 18 month of age, and the patients are able to stand and walk independently until the progression of the disease (Zerre & Davies, 1999).

Nevertheless, all SMA patients demonstrate a characteristic pattern of muscle weakness that affects the proximal muscles more than distal ones, the lower limbs more than the upper ones and intercostal and axial muscle groups more than the diaphragm (Monani & Vivo, 2013). Patients with SMA type I usually manifest profound hypotonia, and a frog-legged posture due to weak pelvic muscles. Areflexia of the extremities, failure to suckle and swallow efficiently and a bell-shaped torso are also clinical features often observed in infants with severe SMA.
In 1995, the genetic defect underlying SMA was shown to be an autosomal recessively inherited mutation of the ubiquitously expressed survival of motor neuron 1 (SMN1) gene. In 95% of the patients, there are deletion mutations involving at least exon 7 and exon 8 (Lefebvre et al., 1995). Humans have two nearly identical genes located on chromosome 5, 5q13, the telomeric copy of SMN1 and the centromeric copy of SMN2. These two highly similar genes vary at base pair position 840, with a cytosine (C) to thymidine (T) change in SMN2. This nucleotide change resides within an exonic splicing motif and leads to alternative splicing of SMN2 pre-mRNAs that leads to exclusion of exon 7 from approximately 85-90% of SMN2 transcripts (Lorson et al., 1999). The truncated SMN protein produced by shortened SMN2 transcript lacks function and is immediately degraded. Thus, compared with SMN1, much less of the SMN2 transcripts can produce fully functional SMN protein (Schrank et al., 1997). SMA is thus caused by reduced, but not absent expression of SMN protein.

Multiple studies have confirmed an inverse correlation between the severity of SMA and the copy number of the SMN2 gene. Previous studies have concluded that the majority of the type I patients have 1 or 2 copies of SMN2, while most type II patients have 3 SMN2 copies, and type III patients usually have 3 or 4 copies of SMN2 genes (Feldkotter et al., 2002; Mailman et al., 2002; Swoboda et al., 2005; Wirth et al., 2006). Due to this negative correlation between the severity of the SMA and SMN2 copy number, many of the therapeutic strategies are based on improving the function of SMN2 to produce more full-length SMN transcripts. Multiple small molecules have been used as therapeutic interventions to increase the amount of full-length SMN or to correct aberrant splicing and stabilize mRNA. One of these categories of small molecules is the histone
deacetylase (HDAC) inhibitors, which have been shown to enhance the expression of
SMN2 in SMA patient cells and in mouse models of SMA (Avila et al., 2007).

Quinazolines are a family of compounds that inhibit RNA decapping enzymes DcpS and
increase SMN2 expression as well (Singh et al., 2008). Additional approaches to treat
SMA rely on altering the splicing pattern of SMN2 to yield more full length SMN
proteins using antisense oligonucleotides (ASOs). Because ASOs are more specific with
nucleotides designed to bind specifically to a sequence, they potentially have lower risks
for non-specific bindings (Passini et al., 2011).

SMN protein has an essential function in the assembly of small nuclear
ribonucleoprotein (snRNPs) particles in the cytoplasm. In the nucleus, SMN is localized
within a nuclear structure, called “Gems”, or Gemini of coiled bodies, interacting with
RNA-binding proteins (Liu & Dreyfuss, 1996). In the cytoplasm, the SMN complex
functions as an assemblysome in the formation of small nuclear ribonucleic proteins
(snRNP). Thus complete loss of SMN protein expression is embryonic lethal to an
organism, as snRNPs are crucial in the fundamental biological process of pre-mRNA

SMN protein is also involved in many other fundamental housekeeping functions
such as (1) regulating gene expression through interacting with a nuclear transcription
factor (Strasswimmer et al., 1999), (2) forming granules in response to stress (Zou et al.,
2011), (3) having synergistic anti-apoptotic activity with other anti-apoptotic factors
(Iwahashi et al., 1997), (4) transporting specific mRNAs in axons and dendrites of motor
neurons (Pagliardini et al., 2000) and (5) regulating the expression pattern on
cytoskeleton protein (Bowerman et al., 2007). Extensive studies about the function of
SMN protein have revealed its role in RNA metabolism and splicing regulation, suggesting that SMA should be a splicing disease not restricted to just motor neurons (Zhang et al., 2008). Yet it remains unresolved why motor neurons are particularly vulnerable to SMN protein deficiency.

*Common SMA mouse models*

Mouse models remain one of the most important tools to understand the pathogenesis of SMA. Over the years, multiple SMA mouse models have been developed. It is a common goal to create a mouse model of SMA that can recapitulate the disease phenotypes and mechanisms observed in humans. Currently, the two most commonly used mouse models in SMA are: the ‘severe’ model and the ‘SMA Δ7’ model. Unlike humans, mice only have one copy of the Smn gene and homozygous deletion of Smn results in massive embryonic cell death before implantation. So the ‘severe’ model is created with one or two copies of the human SMN2 transgene expressed on the mouse Smn-null background (Smn\(^{-/-}\); SMN2\(^{+/+}\)). This mouse model is indistinguishable from controls at birth but only has a lifespan of 7 days (Monani et al., 2000). Compared to the ‘severe’ model, the ‘SMA Δ7’ model has an additional copy of human SMNΔ7 cDNA (Smn\(^{-/-}\); SMN2\(^{+/+}\); SMNΔ7\(^{+/+}\)) that can extend the lifespan from 6 to 13 days (Le et al., 2005), providing a longer window to study disease mechanisms. These mice are usually born small with neuromuscular defects by 4-5 days old that progress to abnormal gait, hind limb weakness/immobility, and survival of only 13-17 days.
**SMA as a multi-system disorder**

Several recent studies have challenged the notion that SMA is a motor neuron or neuromuscular system-specific disease suggesting that there might be additional cell and tissue types that are also vulnerable to reduced levels of SMN and contribute to SMA pathogenesis. One piece of crucial evidence comes from the experiment in which SMN protein was specifically depleted in the motor neuronal progenitor cells using the Olig2-Cre mice (Park et al., 2010). Unexpectedly, low levels of the SMN protein in the neuronal progenitor cells only resulted in a modest disease phenotype compared to mice that express low levels of SMN protein ubiquitously. Moreover, both the SMA symptoms as well as early electrophysiological abnormalities that were seen in the neonates were alleviated with age. The results of this study suggested that SMN deficiency in motor neurons is insufficient to fully recapitulate the SMA phenotype, and that reduced SMN expression in other tissues or cells may play a role in mediating the disease severity.

Moreover, the reverse experiment also provided surprising results. When SMN protein was selectively increased in the motor neurons of the SMA mice, the disease phenotype was slightly ameliorated with only moderately increased lifespan and motor activity (Martinez et al., 2012). The median survival for mice that has Cre-recombinase expression driven by the expression of choline acetyltransferase (ChAT) promoter, **ChAT**\(^{Cre}\) SMA mice, demonstrated an increase in median survival from 15 days to 23 days. Weights were also significantly increased and motor behavior assays showed reduced righting time latency (Martinez et al., 2012). Interestingly, by increasing the SMN expression in muscle through the **MyoD**\(^{Cre}\) and **Myf5**\(^{Cre}\), the study also reported
significant improvements in the survival, (13 to 19 days in \(\text{MyoD}^{\text{Cre}}\) and 15 to 21 days in \(\text{Myf5}^{\text{Cre}}\) body weights, and motor behaviors (Martinez et al., 2012).

In contrast, when SMN protein expression in rescued early in postnatal development in all cells using a tamoxifen-induced, ubiquitously expressed Cre transgene (Cre-ER), both the progression of the disease and the overt disease phenotype can be substantially improved postnatally in SMA mice. Approximately 50% of Cre-ER positive mice survived to 300 days or more (Lutz et al., 2011), a much effective rescue than the \(\text{ChAT}^{\text{Cre}+}\) SMA mice. Together, these studies strongly suggest that simply restoring SMN just in motor neurons, the disease symptoms are only partially ameliorated. However, increased SMN expression solely in muscle can also independently produce a partial improvement in the SMA disease phenotype. These findings further suggest that SMN protein loss in cells types other than motor neuron contributes to the pathogenesis of SMA.

One treatment strategy that has been used to treat SMA is antisense oligonucleotides targeted to SMN2-derived pre-mRNAs and designed to enhance exon 7 inclusion, produces full-length mature mRNAs thus producing increased levels of normal SMN protein. Recently, Hua et al., (2011) used such an antisense oligonucleotides (ASO) to enhance the SMN expression in SMA mice. The authors demonstrated that delivery of the ASO to either the CNS alone (given intraventricularly) or systemically and to the CNS (by early subcutaneous injection) both achieved robust increased SMN expression in the CNS, but systemic delivery also increased SMN in numerous other tissues. Interestingly, systemic treatment with two subcutaneous injections resulted in a median survival of 108 days from 14 days whereas intracerebroventricular injection resulted in a
meager increase of survival to 16 days. In addition, the mice that were treated systematically with the ASO showed no overt signs of motor dysfunction. These findings further underscore the potential importance of SMN protein expression in cells and tissues of peripheral tissues.

*Schwann cells in spinal muscular atrophy*

Neuroglial cells are a group of non-neuronal cells in close association with neurons that protect and maintain functions of neurons and their axons. In neurodegenerative disease such as amyotrophic lateral sclerosis (ALS), it has been found that modification of astrocytes can contribute to the neuronal damage (Dickenson et al., 2006). More recently, it was discovered that oligodendrocytes are also direct targets of ALS. The oligodendrocytes demonstrate an apoptotic phenotype, which was detected in the ventral grey matter of the spinal cord from mutant SOD1 transgenic mice before actual motor neuron loss became evident (Philips et al., 2013). Moreover, in mutant SOD1 mice, there is an increased differentiation of NG2+ cells into oligodendrocytes. With the degeneration of oligodendrocytes from an early time point and the accelerated turnover of these cells, the ALS mice and human CNS have showed an overt demyelination in the grey matter (Kang et al., 2013). In SMA, Murry et al. (2009) demonstrated a notable change in the myelination pathway gene, myelin protein zero (MPZ), in the spinal cord of the late-symptomatic SMA mice. Given the intimate relationship between motor neuronal axons and myelin, these findings highlight the importance of further investigating whether myelinating oligodendrocytes in the CNS or Schwann cells in the PNS are involved in myelin-related pathology in SMA. SMN
deficiency within the oligodendrocytes or Schwann cells may be highly relevant for the neurodegenerative pathology of SMA.

Very recently, SMN-deficient Schwann cells have been examined in vitro for alterations of strictly regulated gene expression patterns, Schwann cell proliferation, and the interaction between Schwann cells and motor axons during the axonal development. This study from Hunter et al., (2013) demonstrated that there are intrinsic, SMN-dependent defects in Schwann cells in SMA. Using two SMA mouse models, the study reported abnormalities in myelination in intercostal nerves. In the SMA mice, the maturation of the interaction between axons and Schwann cells was highly disrupted. When co-culturing healthy neurons with diseased Schwann cells, the Schwann cells demonstrated a lack of response to the myelination cue and revealed deficient myelination. Thus this study suggests that deficiency of SMN protein results in intrinsic defects in Schwann cells that contribute to SMA pathogenesis.

Axonal development

In the peripheral nervous system, development of axonal caliber is a meticulously controlled process regulated by both intrinsic and extrinsic factors (Griffin & Hoke, 2005). In the initial stage of motor axon development, small and unmyelinated axons are enveloped by the cytoplasm of a single Schwann cell. With the growth of axon caliber, each of these axons gradually will become ensheathed by Schwann cell processes individually. For axons that have >1μm diameter that are destined to be myelinated, they then undergo radial sorting during which Schwann cells and axons establish a strict 1:1
relationship. Once this relationship is established, the axons start to grow rapidly radially and initiate myelination (Appendix I; Webster, 1975).

The process of initiating and terminating myelination is highly dependent on signaling between axons and Schwann cells. One important signaling pathway involves axonal-derived neuregulin I (NRG1) communicating through the ErbB2/3 receptors on Schwann cells (Michailov et al., 2004). One particular isoform of NRG1, NRG1-III can control not only myelination but also the ensheathment of small-diameter axons and their segregation into Remake bundles (Traveggia et al., 2005). During postnatal axonal maturation, Schwann cells have to undergo significant morphological and gene expression changes (Sherman and Brophy, 2005). Some of the important genes for myelin proteins such as myelin protein zero (MPZ) and myelin basic protein (MBP) are expressed at basal levels constitutively in the absence of axons and their level of expression are modulated by axonal contact (LeBlanc & Poduslo, 1990). Notch-Jagged signaling also plays an important role in promoting the generation of Schwann cells from Schwann cell precursors and inhibits myelination process of the Schwann cell (Woodhoo et al., 2009).

**Hypothesis**

The aim of this thesis was to identify and characterize abnormalities motor axon development in SMA and determine the relative contribution of motor neurons and Schwann cells to these abnormalities. We hypothesize that axon development in SMA mice and human is abnormal and that Schwann cells with low SMN protein levels impair the normal process of axon development and myelination.
Through examining the morphological changes in Schwann cells from human patients and different transgenic SMA mice at both the light level and ultralstructural level, we characterized abnormalities of motor axons and Schwann cell. Moreover, using conditional SMA mouse lines in which SMN expression was selectively increased in Schwann cells \((Dhh^{Cre+}\text{SMA})\) or in both Schwann cells and motor neurons \((ChAT^{Cre+}Dhh^{Cre+}\text{SMA})\), we investigated the involvement of SMN-deficient Schwann cells in axon development. The findings of this project will provide more insights into the role of cells types other than the motor neurons, such as the Schwann cells, in SMA pathogenesis. Better understanding about relative contributions of different tissue and cell types to SMA pathogenesis will allow for the development of more effective and precise therapeutic targets in the future.
MATERIAL AND METHODS

Mice

All experiments were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and were approved by Johns Hopkins University and Jackson Laboratory Animal Care Committees. Delta 7 mice (Δ7 mice) was created on a FVB background (Le et al., 2005) (Stock number 005025) and its breeder pairs were obtained from Jackson Laboratories. Dhh\textsuperscript{Cre} (Jackson Laboratory Stock #012929) (Jaegle et al., 2003) were bred to a lacZ-Cre reporter strain, B6.129S4-Gt(ROSA)26Storm1Sor/J (Jackson Laboratory Stock # 003474) to analyze Cre expression patterns in 15.5d post-conception embryos (E15.5) and postnatal day 7 (P7) pups.

SMA mice expressing a Cre-inducible Smn hybrid allele (Smn\textsuperscript{Res}) (Lutz et al., 2011) and two copies of SMN2 and SMN\textDelta7 alleles (Smn\textsuperscript{Res}/SMN2\textsuperscript{+/+}/SMN\textDelta7\textsuperscript{+/+}) were bred to Cre-expressing mice under control of either the desert hedgehog (Dhh) promoter, choline acetyltransferase (ChAT) promoter, or both. Cre-SMA mice were those carrying the inducible Smn allele in a homozygous state (Smn\textsuperscript{Res}/Smn\textsuperscript{Res}/SMN2\textsuperscript{+/+}/SMN\textDelta7\textsuperscript{+/+}) in the absence of Cre, Cre+ SMA mice were those carrying the inducible Smn allele in a homozygous state (Smn\textsuperscript{Res}/Smn\textsuperscript{Res}/SMN2\textsuperscript{+/+}/SMN\textDelta7\textsuperscript{+/+}) with the expression of Cre. Heterozygous (Het) mice were those carrying the inducible Smn allele in a heterozygous state (Smn\textsuperscript{Res}/Smn\textsuperscript{+/+}/SMN2\textsuperscript{+/+}/SMN\textDelta7\textsuperscript{+/+}) in the presence of the Cre, and wild-type (WT) were mice homozygous for the wild-type (WT) allele (Smn\textsuperscript{+/+}/SMN2\textsuperscript{+/+}/SMN\textDelta7\textsuperscript{+/+}) that are not expressing Cre.
Genotyping

Mice were genotyped by PCR using DNA extracted from tail as previously described (Avila et al., 2007). The following primers were used to detect the uninverted $Smn^{Res}$ allele: MUT-F: 5’-GGCAGTTTTAGACTCATGTATCTG-3’ and MUT-R: 5’-ACTTATGGAGATCCCTCGAGATAAC-3’. The product is a 103 base pair product. The inverted form of the $Smn^{Res}$ allele is detected using the following primers: INV-F: 5’-GGCAGTTTTAGACTCATGTATCTG-3’ and INV-R: 5’-GTGTGAGTGAACAAATTCAAGCC-3’ yielding a 190 base pair inverted product and/or a 143 base pair endogenous product (Martinez et al., 2012). In order to test if Cre allele was presented in the $Dhh^{Cre}$ mice, the following primers were utilized: Dhh WT-F: 5’-CTAGGCCACAGAATTGAAAGATCT-3’, Dhh WT-R: 5’-GTAGGTGGAAATTCTAGCATCATCC-3’, Dhh Cre-F: 5’-CCTTCTCTATCTGCCTTGCT-3’ and Dhh Cre-R: 5’-ACGGACAGAAGCATTCTTCCA-3’. To detect the presence of the Cre allele in the $ChAT^{Cre}Dhh^{Cre}$ mice, the following primers were used: ChAT Ires WT-F: 5’-GTGGCAGAAAGCGTGTTG-3’, ChAT Ires WT-R: 5’-AGATAGATAATGAGAGGCT-3’, and ChAT Cre-R: 5’-CCTTCTATCGCCTTTGGACG-3’.

Phenotypic assessment of mice

Body weights of the mice were measured daily starting from the day of birth, which is designated as P1. For righting time of each mouse, the average value of two trials of righting time was determined starting on P1 with maximal time of 30 seconds per
trial as previously described (Avila et al., 2007). In addition, the hind-limb suspension test was performed on mice starting at P2 and ending on P12. Mice were suspended from the hind limbs on the edge of a 50 ml conical tube as described (El-Khodor et al., 2008). Parameters such as average position score, total number of pulls, and total latency of fall time (maximum 30 seconds) were recorded for two trials. A composite score of this test was calculated as described by Heier et al., (2010).

**Quantitative reverse transcription PCR**

RNA was isolated from sciatic nerve using TRIzol reagent (Invitrogen) and converted to cDNA as previously described (Avila et al., 2007). Primers (SMN6m8h) were used to amplify the cDNA product arising from the uninverted and inverted hybrid rescue Smn allele. Exon6m8h: 5’-GGCTACCACACTGGCTACTATATGG-3’, Exon8hR: 5’-CTTCACATTCAGATCTGTCT-3’, and Exon8hprobe: 6FamCATAGACAGCTCTA ATGACACCACATAAAGAAtamra. SMN67m8h were primers used to amplify full-length cDNA products arising from the inverted Smn allele. Exon67F: 5’-GCTACTATATGGGTTCAGACAAAAATAAAAA-3’, Exon8hR: 5’-GCTTCACATTCAGATCTGTCT-3’, and Exon8hprobe: 6FamCATAGAGCAGCTCTAATGACACCACCTAAAGAAtamra. For the endogenous controls, primers that are localized to the mouse exon 1 and 2 region (SMN12m) were used. Exon67F: 5’-GCTACTATATGGGTTCAGACAAAAATAAAAA-3’, Exon8hR: 5’-GCTTCACATTCAGATCTGTCT-3’, and Exon8hprobe: 6FamCATAGAGCAGCTCTAAATGACACCACCTAAAGAAtamra. Other probes used in the experiments were commercially available assays: Jagged1 (Mm00496902_m1,
Applied Biosystems); Notch1 (Mm00435249_m1, Applied Biosystems); MBP (Mm01266402_m1, Applied Biosystems); Erbb3 (Mm01159999_m1, Applied Biosystems); MPZ (Mm00485141_g1, Applied Biosystems);

**Histology**

To determine the expression pattern of Cre in the $Dhh^{Cre}$/ROSA26 mice, P7 mice were sacrificed with brain, skeletal muscle, kidney, testis, sciatic nerve and spinal cord dissected, collected and frozen in O.C.T compound on dry ice immediately after collection. Frozen tissues were sectioned at 10μm of thickness with cryostat, fixed in 0.2% Glutaraldehyde in PBS for 10 minutes on ice, and washed in detergent rinse for 10 minutes. The slides were then immersed in 1mg/ml X-gal staining solution overnight at room temperature. Post-fixed the slides in 4% PFA for 10min the next day and washed in distill water twice for 5 minutes. The slides are then counter-stained with Nuclear Fast Red and washed in distill water for 2 minutes.

**Electron Microscopy**

The $ChAT^{Cre}/Smn^{Res}$ and Δ7 mice were transcardially perfused and postfixed for 24hr with 3% glutaraldehyde (glut)/4% paraformaldehyde (PFA) on P12. Human ventral roots were obtained from autopsy of patients with SMA and age-matched controls. Ventral roots from lumbar level 1 and 5 were dissected and fixed in 2% Glut and 2% PFA for one overnight at 4°C. After the fixation, both human and mice tissues were rinsed with 0.1M Sorensen’s buffer, post-fixed in 2% osmium tetroxide, dehydrated in serial alcohol dilutions, embedded in propylene oxide and Embed 812 plastic (Electron Microscopy
Sciences), and placed in an oven to harden into capsule form. Thick sections (1µm) were cut on an ultramicrotome and multiple sections were placed on Superfrost slides, dried, stained with toluidine blue, and examined under light microscopy. Thin sections were cut at 60-90nm, placed on Formvar grids, and imaged using Libra 120 (Zeiss) electron microscope at 8,000x or higher.

Statistics

All data are expressed as mean ± SEM. Morphological and biochemical data were analyzed using Excel and Graphpad Prism. Statistical significance was determined using either Student’s t test or a two-way ANOVA.
RESULTS

Motor axon abnormalities in human SMA ventral roots

In order to examine the pathology of motor axons in human SMA, ventral roots were dissected from type I SMA patients and age matched controls at the time of autopsy. Roots were fixed in gluteraldehyde, plastic embedded, and semi-thin sections were stained with toluidine blue (Figure. 1A-B). The areas of the ventral roots examined at cervical, thoracic, and lumbar levels showed an approximately 2 fold reduction in area of the lower lumbar roots (Figure. 1C). The number of myelinated axons at all four levels of the spinal cord evident by light microscopy was counted. A significant 2-3 fold reduction in number of myelinated axons was observed in SMA roots at lower lumbar roots compared to controls (Figure. 1D). Despite this marked reduction in axon number, only 2-3% axons in the lower lumbar roots were ongoing active axonal degeneration as defined by the presence of a myelin ovoid (a collapsed myelin sheath at the site of lost axon) (Figure. 1E). This magnitude of degeneration would appear insufficient to account for the degree of axonal loss unless: 1) a massive wave of axonal degeneration occurred in utero with clearance of most axonal debris by neonatal stages or 2) a significant proportion of axons never properly developed into large myelinated axons in SMA ventral roots.

A large population of very small, unmyelinated axons is present in SMA ventral roots

In order to further investigate the pathology of SMA axons, ventral roots were further examined by electron microscopy. Lumbar ventral roots from 5 patients with type I SMA (average age= 357.4 days) were compared to 5 age-matched controls (average
age= 1035.8 days) (Figure. 2A). Myelinated axons were evident in both controls and SMA ventral roots, but the average diameter was reduced in SMA (3900±80 nm) compared to controls (6160±640 nm) (Figure 2B). The G-ratio (defined as the ratio of axon diameter over the axon+myelin sheath diameter) was equivalent in SMA (0.78±0.07) compared to controls (0.79±0.96) (Figure. 2C). Strikingly, while all axons in control ventral roots were large and myelinated, this was only the case for approximately 15% of SMA axons. The vast majority of axons in SMA roots (88.45±3.88%) were very small (<1 µm in diameter) and bundled within the cytoplasm of a single Schwann cells. A great proportion of these small, unmyelinated axons remained unsegregated (80.99±6.09%) with multiple axons encompassed in a single Schwann cell cytoplasm, while a small proportion (7.46±5.04%) did segregate and establish a 1:1 ratio with a Schwann cell (Figure. 2D). These very small, unsegregated axons have the appearance of very immature axons present during fetal stages of development.

In order to further examine the proportion of axons surrounded by a mature myelin sheath versus those lacking an encircling myelin sheath, immunohistochemistry was used to-label myelin using antibodies to myelin basic protein (MBP) and axons with antibodies TuJ1. Ventral roots were visualized using confocal microscopy (Figure. 3A). We found that control and SMA ventral roots had a similar total number of TuJ1 positive axons (Figure 3B). Nonetheless, while controls demonstrated that most TuJ1 positive axons were ensheathed with a ring of myelin, only 20-30% of SMA TuJ1 positive axons showed an encircling rim of MBP staining (Figure. 3B). We also examined the dorsal roots from the same spinal levels, and there were no differences in the percentages of myelinated versus unmyelinated axons (data not shown) consistent with the selective
involvement of motor neurons in this disease. Together, these results indicate that there is an abundant population of small, unmyelinated axons in SMA ventral roots, suggesting impaired motor axon development in human SMA.

Motor axon abnormalities in SMA mice

Given our observations in human ventral roots, we next examined whether similar abnormalities were observed in SMA mouse models. In this experiment, we have utilized a long established SMA mice model, SMAΔ7 mice (Le et al., 2005). In this model, mice contain a SMN cDNA lacking exon 7, a human SMN2 gene, and also a mouse Smn knockout allele. We analyzed the morphology of motor axons in ventral roots of SMAΔ7 mouse model at the spinal lumbar 1 level of the ventral roots (LVs) at P14 with both light (Figure 4) and electron microscopy (Figure 5). At the light level, we identified a significant reduction both in the total area of the SMA L1VRs [WT=9748.39±1004.58 μm² (N=6); SMA=3223.85±127.60 μm² (N=3); P<0.05] and in the number of myelinated axons [WT=384.83±41.02 (N=6); SMA=218.33±7.51 (N=3); P<0.05] in the L1 ventral roots in the Δ7 SMA mouse model (Figure. 4B).

To evaluate the relative contribution of motor neurons and Schwann cells to peripheral axon development, we utilized a conditional SMA mouse line in which full-length SMN expression was selectively increased in motor neurons (ChAT<sup>cre+</sup> SMA). The ChAT<sup>Cre+</sup> SMA mice showed a modest increase in the area of L1VR (7141.67±1268.12 μm², N=3) as well as the number of myelinated axons (276.67±48.52, N=3) at the light level (Figure. 4B).
Examination at the ultrastructural level demonstrated similar changes to those observed in the human autopsy ventral roots (Figure. 5A). The average diameter of the myelinated axons was significantly reduced in the SMA compared with the wild-type (WT) ventral roots (WT=3100.43±131.93 nm; SMA=2338.31±73.99 nm; N=3/group, P<0.01) (Figure 5B). No significant difference in G-ratio of the myelinated axons was observed in the SMA vs. L1VR at P14 (WT=0.73±0.01; SMA=0.73±0.01; N=3/group, P=0.21) (Figure, 5C). In contrast, the percentage of myelinated axons was significantly reduced in the SMA mice (WT=68.26±0.96%; SMA=50.56±4.45%; N=3/group, P<0.05) with corresponding increase in the percentage of the small, unmyelinated axons (WT=31.74±0.96%; SMA=49.43±4.45%; N=3/group, P<0.05). Within the unmyelinated axon group, it was the unsegregated subgroup that was selectively unregulated in SMA (WT=24.99±3.44%; SMA=44.69±2.84%; N=3/group, P<0.05) (Figure 5D). These data indicate that although not as severe as in human tissue, there is a population of immature axons in SMA mouse ventral roots, further suggesting impaired axonal development.

The ChAT\textsuperscript{cre+} SMA mouse ventral roots showed modest increases in myelinated axon diameter (2819.68±96.70 nm, N=3) and percentage of myelinated axons (59.63±15.23%, N=3), and corresponding decrease of the percentage of unmyelinated axons (40.36±15.23%, N=3) (Figure. 5B-D). Interestingly, there was an increase in the proportion of segregated, unmyelinated axons in the ChAT\textsuperscript{cre+} SMA (WT=6.74±3.45%, SMA=4.73±1.68%, ChAT\textsuperscript{cre+} SMA=15.65±4.06%, N=3/group) indicating that increase in SMN expression in motor neurons improves radial outgrowth of motor axons in SMA mice.
Abnormal axon development is not associated with altered expression of Schwann cell genes.

During postnatal myelin development, a gene network associated with the initiation of the myelination should be activated and simultaneously genes that mark earlier stages of the development should be repressed (Huang et al., 2012). Therefore, I have examined the expression profile of a set of genes that might be expressed in immature Schwann cells such as Notch-1 and Jagged-1. I have also examined genes associated with myelination such as myelin basic protein (MBP) and myelin protein zero (MPZ) and genes involved in the neuregulin I type III-ErbB2/B3 signaling pathway. The later was chosen as in the peripheral nervous system, axonal neuregulin 1 type III promotes myelination by activating ErbB2/B3 receptors in the Schwann cells (Flicker et al., 2011).

Sciatic nerves were dissected from the WT mice and SMA mice at P7 and P10 (N=4/group), because myelination events usually have significant changes during this period of postnatal development. No significant differences in the expression level of these genes were detected between SMA and WT sciatic nerves at P7 and P10, but we were able to detect an increase in the transcription level of MBP and MPZ with developmental time for both WT and SMA mice (Figure. 6A-B)

SMN is expressed in a tissue specific manner in conditional mice

To examine if the expression of SMN in Schwann cells would improve motor neuron axon development and SMA disease manifestations, we generated a SMA conditional mouse model that expresses increased levels of SMN selectively in Schwann
cells or selectively in both Schwann cells and motor neurons. SMA mice that express a Cre-inducible \textit{Smn} allele (\textit{Smn}^{Res}) (Lutz et al., 2011) were bred to mice expressing Cre recombinase under the control of either the Desert Hedgehog (Dhh) promoter (Jaegle et al., 2003) or both the Dhh and ChAT promoters (Kanisicak et al., 2009). Dhh is known to be expressed in Schwann cell precursors in developing peripheral nerves (but not in neurons) from embryonic day (E) 12 (Bitgood and McMahon 1995), while ChAT is expressed in motor neurons in the ventral horn of the spinal cord starting at E12.5 (Phelps et al., 1991).

As expected, the lacZ staining of the P7 tissue dissected from the \textit{Dhh}^{Cre+} lacZ mice showed positive staining for \(\beta\)-galactosidase in the cells that are known to have active \textit{Dhh} expression. These tissues are Schwann cells of sciatic nerves, Sertoli cells of testis, and blood vessel endothelial cells in the kidney (Figure 7A, D, and E). As expected, tissues such as spinal cord, brain and muscle show no lacZ staining, confirming the lack of \textit{Dhh} expressions in these tissues (Figure 7B, C, and F).

The Cre-inducible \textit{Smn} allele (\textit{Smn}^{Res}) contains a switch cassette flanked by loxP sites with opposite orientation that was introduced into the endogenous \textit{Smn} mouse gene (Lutz et al., 2011). The switch cassette contains human exon 7 sequence in the unflipped direction and the mouse exon 7 sequence in the opposite orientation. In the absence of Cre recombinase, this cassette includes mouse \textit{Smn} exon 1-6 sequence together with the sequences of human \textit{SMN2} exons 7 and 8 and this produces mainly truncated SMN transcripts (SMN68) (Figure. 8A). With the Cre-mediated recombination, there is a flip of the cassette and the mouse \textit{SMN2} exon 7 is in the correct direction, leading to the production of predominately full-length SMN transcript (SMN678) (Figure 8A).
Expressing the conditional Smn allele (Smn\textsuperscript{Res}) together with Cre recombinase can lead to increased SMN expression in tissue specific patterns. To test for the expression patterns of truncated and full-length hybrid SMN transcript, we evaluated the sciatic nerve and spinal cord tissues isolated at P10 by quantitative reverse transcription PCR (qRT-PCR). A set of primers were used to detect the truncated transcript (SMN68) and the full-length hybrid SMN transcript (SMN678). Primers specific for mouse exon6, 7 and human exon 8 (SMN67m8-h) will detect only full-length hybrid transcripts (SMN678). Primers designed with mouse exon 6 and human exon 8 (SMNm6h8) can detect both truncated (SMN68) and full-length hybrid transcripts SMN678. As an endogenous control, primers specific for mouse exons 1-2 (SMN12m) were used.

The SMN6m8h primers detected SMN68 and/or SMN678 hybrid transcript expression in the sciatic nerve of Cre- and Cre+ SMA mice homozygous for Smn\textsuperscript{Res}. For WT mice, there is no Smn\textsuperscript{Res} allele in its genome, thus no expression can be detected (Figure. 8B). The SMN67m8h primers only detect full-length SMN678 transcript in the sciatic nerve of Dhh\textsuperscript{Cre+} (Figure. 8B).

*Increased SMN expression in sciatic nerves does not improve the disease phenotype of SMA mice*

I also examined the phenotypic outcome of SMA mice with the tissue-specific increase in full-length SMN expression (Figure. 9). Dhh\textsuperscript{Cre+} SMA mice did not demonstrate increased survival compared with Dhh\textsuperscript{Cre-} SMA mice. The median survival of Dhh\textsuperscript{Cre+} SMA mice (N=26) is 17 days, equivalent to that of the Dhh\textsuperscript{Cre-} SMA mice (N=14) (Figure. 9A). In order to further examine whether increased expression of SMN
in both motor neurons and Schwann cells had a synergistic effect on SMA survival and behavior, we generated “double rescued” mice by combining $ChAT^{Cre}$ with the $Dhh^{Cre}$ SMA mice. And the median survival of the $ChAT^{Cre+}Dhh^{Cre+}$ SMA mice is 24 days (N=21), which is comparable to the improvements previously observed in the $ChAT^{Cre+}$ mice (Martinez & Kong et al., 2012), 25 days (N=22) (Figure. 9B). Moreover, little improvement was observed in the body weights for the $Dhh^{Cre+}$ SMA mice (N=24) compared to that of the $Dhh^{Cre-}$ SMA mice (N=14) (Figure. 9C). For the $ChAT^{Cre+}Dhh^{Cre+}$ SMA mice, the improvements in the trend of body weights were similar to the increase in the $ChAT^{Cre+}$ mice, suggesting no further improvement was provided by the $Dhh^{Cre+}/Smn^{Res}$ (Figure. 9D). Together, the absence of improvement in both body weights and survival suggests that there might be no beneficial effect with an increase of SMN expression in the Schwann cell driven by the $Dhh^{Cre+}/Smn^{Res}$.

*Increased SMN expression in sciatic nerves does not improve SMA disease manifestations*

In addition, motor behavior assays showed no significant difference in the righting time latencies between the $Dhh^{Cre-}$ (SMA) mice (N=4) and the $Dhh^{Cre+}$ (N=4). Both lines showed an increase in the righting time latencies in comparison with the WT (N=7) (Figure. 10A). For the tube test scores, the $Dhh^{Cre-}$ (SMA) mice and the $Dhh^{Cre+}$ mice demonstrate a test score trend that are inseparable from P1, but both scored lower than the WT (Figure. 10B).
DISCUSSION

In this study, I characterized motor axonal development in both human and mouse SMA ventral roots and investigated whether SMN-deficient Schwann cells may contribute to SMA disease manifestations. The analysis of SMA ventral roots revealed a large population of small, unmyelinated axons clustered within a single Schwann cell cytoplasm. Early in their development, all axons begin as clusters of very small axons ensheathed by a single Schwann cell cytoplasm, but motor axons ultimately segregate one to one with Schwann cells and grow radially with parallel increases in myelin thickness. The presence of an abundant population of small axons in SMA ventral roots could indicate impaired axonal development that starts during fetal stages. In order to investigate the relative contribution of SMN deficiency in motor neurons and Schwann cells to this axonal developmental defect, we utilized three conditional SMA mouse models with SMN level specifically increased in motor neurons (ChAT\textsuperscript{Cre+} SMA), Schwann cells (Dhh\textsuperscript{Cre+} SMA), and both motor neurons and Schwann cells (ChAT\textsuperscript{Cre+} Dhh\textsuperscript{Cre+} SMA). Dhh\textsuperscript{Cre+} SMA mice did not show improved survival or motor behavior. On the other hand, ChAT\textsuperscript{Cre+} SMA mice demonstrated significant improvements in both the survival and motor behavior. The median of survival of the ChAT\textsuperscript{Cre+} SMA mice increased from 15 days to 23 days, and maximal weights improved by 64.7%. In terms of the motor behaviors, the righting time latency reduced significantly (Martinez & Kong et al., 2012).

The histological study of the ChAT\textsuperscript{Cre+} SMA mice ventral roots demonstrated an increased proportion of myelinated axons as well as a reduction of the unmyelinated unsegregated axons. ChAT\textsuperscript{Cre+} Dhh\textsuperscript{Cre+} SMA did not produce additional beneficial effects
on survival or motor behavior compared to $ChAT^{Cre+}$ mice. Together, our results provide evidence that motor axonal development in SMA is largely dependent on the full-length SMN expression in the motor neurons. Schwann cells deficient of SMN protein appear to contribute a less crucial role in the regulation of motor axon development in SMA.

*Developmental deficits in motor axon development*

In this study, we undertook a detailed and systematic examination of the SMA ventral roots at the light as well as ultrastructural level in both humans and mice. Based on our observations, SMA ventral roots revealed a population of unmyelinated axons that remained unsegregated from the Schwann cells and a comparatively smaller population of unmyelinated, but segregated axons. This observation raises question about the origin of these small unmyelinated motor axons. One possibility is that these unsegregated, unmyelinated axons are the result of attempted regeneration of motor axons due to substantial degeneration. Arguing against this interpretation is that there are very low levels of active degeneration of motor axons, which is seemingly insufficient to account the huge number of small, unmyelinated axons. The other possibility is that these axons represent a population of axons arrested at a particular point of development. This interpretation is compatible with all the axons showing a similar appearance, rather than axons evident a multiple different stages of development or regeneration. In addition, analysis of ventral roots in conditional mice expressing increased SMN in motor neurons ($ChAT^{Cre+}$ SMA) shows a moderate reduction in the percentage of the unsegregated unmyelinated axons and a corresponding increase in the percentage of segregated and
myelinated axons, phenomena consistent with a more advanced stage of axonal development.

The specific role of SMN protein in axon development will need further investigation. In the SMA ventral roots, most the axons appear arrested at a stage prior to radial sorting. The precise arrangement of Schwann cells along developing axons is an important event before myelination. Immature Schwann cells will encircle groups of nerves, and then the individual cells will migrate away from the cohort and initiate the sorting to the periphery of the nerve bundle to establish a 1:1 relationship with the axon. Radial sorting is a process that can only occur after Schwann cells have started to produce basal lamina (Simons & Trotter, 2007). Conditional deletion of different laminin isoforms in mice, such as laminin gamma1, laminin-2 and laminin-8 have shown impairments in radial sorting (Chen & Strickland, 2003; Yang et al., 2005), similar to what we have observed in SMA human and mouse ventral roots. The SMN complex may have a role in axonal transportation of mRNAs and in protein translation. Recent research has suggested that laminin signaling, which regulates local translation of β-actin in growth cone of motor neurons is disturbed in spinal muscular atrophy (Rathod et al., 2012). Thus, it is possible that the reduction in SMN level along the axonal projections in the motor neurons could lead to lack of transportation of the specific laminin mRNAs along the neurites and the misregulation of the mRNA localization, stability as well as translation, leading to the stall in radial sorting of the motor axons.
Intrinsic abnormalities in Schwann cells

A recently published study suggested that low levels of SMN can induce primary, intrinsic changes in Schwann cells possibly impairing myelination, axo-glial interactions and development of the ECM in the peripheral nerve. In vitro studies revealed that Schwann cells isolated from SMA mice could not maintain normal responses to differentiation cues, leading to the abnormal expression of key myelinating proteins such as MBP and MPZ. A neuron/Schwann cell co-culture experiment provided evidence that defects in the affected Schwann cells’ ability to myelinate are due to an intrinsic abnormality (Hunter et al., 2013).

In contrast to the in vitro findings that restoring SMN in Schwann cells can reverse the myelination defects (Hunter et al., 2013), our mice model $Dhh^{Cre+}$ SMA, which showed increased SMN expression specifically in Schwann cells did not provide any improvement in survival, weight, or motor behavior compared to the SMA mice. One possible explanation for the differences is that the increase of SMN level in $Dhh^{Cre+}$ SMA takes place on embryonic day 15.5, which is around the end of the time point when the transition from Schwann cell precursor to Schwann cells is complete (E16). It was previously shown that immature Schwann cells isolated from SMA mice were stable and expressed the normal complement of markers associated with immature (non-myelinating) Schwann cells. Thus it is reasonable to speculate that SMN protein could play a critical role in facilitating the transition from immature to myelinating Schwann cells. During this transition, the expression of many genes is undergoing drastic changes. It is known that SOX10 is the essential transcriptional factor for Schwann cells to differentiate into myelinating cells. Thus given SMN protein’s role in transcription and translation, low
levels of SMN protein during the critical phase of Schwann cell transition might result in the intrinsic abnormalities that cannot be reversed later on. Thus using Dhh as a driver for the Cre-recombinase expression might not be able to provide the increase of SMN early enough for correct the intrinsic abnormalities in SMA affected Schwann cells.

Although there are might be intrinsic defects within Schwann cells deficient of SMN, it remains a question how much the defective Schwann cell contributes to the abnormalities observed in motor axonal development. Axon development is a process influenced by multiple factors and cells, and most importantly the motor neurons. So we utilized another conditional mouse model, $ChAT^{Cre+}Dhh^{Cre+}$ SMA mice, in which the SMN level is increased in both motor neuron and Schwann cells. The survival, weight gain, and motor behavior of $ChAT^{Cre+}Dhh^{Cre+}$ SMA mice did not demonstrate additional improvements in the SMA phenotype compared to the $ChAT^{Cre+}$ SMA. While the morphology study of $Dhh^{Cre+}$ mice L1 VR is ongoing, the improvements in the ventral root area, number of myelinated axons in the ventral roots in $ChAT^{Cre+}$ SMA mice provide strong evidence that Schwann cell with low levels of SMN may not contribute substantially to the motor axon defects in SMA mice. Further exploration for additional factors that might contribute to impairments in the motor axon is necessary.

Consequences of low SMN levels in a wide range of cells and tissues

The SMN complex plays an important role in chaperoning the cytoplasmic assembly of small nuclear ribonucleoprotein particles (snRNPs), the core components of the pre-mRNA splicing machinery. Since SMN is a protein expressed ubiquitously in all cells types and maintains a function crucial to all cell types, it still remains to be
answered why motor neurons are the most affected cell type in the face of reduced SMN levels and if there are other cells and tissues also affected by SMN reduction. Obtaining a better understanding of how SMN deficiency is affecting other cell types is a critical step in putting together the puzzle of SMA pathogenesis as well as resolving the fundamental functions of the SMN protein and its role in transcription, alternative splicing of pre-mRNA, translation, signal transduction, stress granule formation and intracellular trafficking (Seo et al., 2013).

In addition, identifying additional cell types and tissues affected by SMN deficiency will provide more guidance in developing effective treatments for SMA. Generally, therapeutic strategies developed for SMA to date have primarily focused on increasing the amount of full-length SMN2 transcript and SMN protein in motor neurons. Yet it is a challenge to deliver drugs into the nervous system safely and effectively. Therefore, a better understanding of other cell types and tissues affected by low levels of SMN would provide additional therapeutic targets other than motor neurons. Recent studies have begun in revealing the value of systemic delivery of drugs that enhance the SMN protein expression. Valori et al., (2010) reported that a single injection of self-complementary adeno-associated virus serotype 9 (scAAV9) expressing a codon-optimized version of the SMN protein into the facial vein of SMA mice one day after birth can result in a substantial extension of life span in these animals. And Hua et al., (2011) described that peripheral restoration of SMN using antisense oligonucleotides (ASO) can extend the median lifespan by 25 fold compare to intracerebroventricular injections, concluding that the besides motor neuron, liver also plays an important role in SMA pathogenesis.
Future directions

The results of our experiments to date suggest that SMN-deficient Schwann cells do not contribute substantially to SMA pathogenesis because SMA mice expressing increased SMN in Schwann cells do not show overt improvements in survival or motor function. However, we have not yet examined the L1 ventral roots in these mice to see if there is a pathological improvement in axonal development, which does result in gross phenotypic changes. Thus, experiments are ongoing to examine the L1 VRs in Dhh\textsuperscript{Cre+} and Dhh\textsuperscript{Cre+}ChAT\textsuperscript{Cre+} SMA mice. In addition, there is a need for continued investigations to determine what other cell types might contribute to the impaired motor axon development in SMA. We are also examining the effects of ASO treatment on axonal development in SMA treated by either intracerebroventricular (ICV) administration to restore SMN protein in the central nervous system (CNS) or subcutaneous injection to restore SMN protein for widely. Comparing the degree of axonal development can provide further insights into whether it is the affected motor neurons or cells other than Schwann cells that disturb the normal development of motor axon in SMA.

The bidirectional communication between the motor axons and CNS glial cells also greatly contributes to the normal axon development (Sherman & Brophy, 2005). Thus examining the interactions between motor axons and oligodendrocytes in the CNS of SMA mice could provide additional information about the pathogenesis of motor axon development. Through comparing and contrasting the roles of oligodendrocytes (the glial cells that myelinate axons in the CNS) and Schwann cells, we will have more insight into whether affected glial cells contribute to abnormal axon development in SMA. In order to
achieve this goal, we are currently developing $Cnp^{Cre+}$ SMA mice, a conditional mouse model with increased SMN expression in Schwann cells and oligodendrocytes (Lappe-Siefke et al., 2003). These mice will inform us about the involvement of oligodendrocytes in motor neuron maturation and axon development in SMA.

Lastly, partial improvements in the axonal development observed in the $ChAT^{Cre+}$ mice raised an important question on identifying the minimal threshold of SMN protein that is required for cell viability and normal function. Does this minimal threshold vary among different tissues? As the full length SMN proteins expressed in the spinal cord of $ChAT^{Cre+}$ mice still remain much lower than the physiological level, is the SMN level in the motor neurons from SMA mice still remain insufficient to revert their intrinsic abnormalities? Previous studies have shown that as little as 70% of control SMN protein can sufficiently revert disease pathology if introduced during pre-symptomatic period (Foust et al., 2010). By elevating the SMN protein closer to the physiological level in both Schwann cells and motor neurons, can the impaired motor axon developments have more significant improvements? By answering these questions in the future, we hope to further elucidate the function of SMN and the disease mechanism of SMA for the development of efficient treatment for SMA patients.
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D.

![Bar graph showing axon numbers for control and SMA groups in different root regions.](image)

- **X-axis:** Root regions (Cervical roots, Thoracic roots, Upper lumbar roots, Lower Lumbar Roots)
- **Y-axis:** Axon number

E.

![Bar graph showing percentage (%) for control and SMA groups in different root regions.](image)

- **X-axis:** Root regions (Cervical roots, Thoracic roots, Upper lumbar roots, Lower Lumbar Roots)
- **Y-axis:** Percentage (%)

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**Figure 1.** Characterization of abnormalities in motor axons in human SMA ventral roots. Toluidine blue stained sections of 4 month old control L4 ventral root (**A**) and 4 month old SMA L5 ventral roots. The red arrow indicates myelin ovoids, a signature for axon degeneration (**B**). Quantification of the total area of the ventral roots in controls versus SMA cervical, thoracic, upper and lower lumbar ventral roots shows a significant reduction in the total area in the SMA lower lumbar ventral roots (**, P<0.001**) (**C**). Quantification of total myelinated axon number in controls versus SMA cervical, thoracic, upper and lower lumbar ventral roots demonstrate significant differences between control and SMA in the lower lumbar roots (P=0.16) (**D**). Quantification of percent of degenerating axons in control, SMA cervical, thoracic, upper and lower lumbar ventral roots indicates a higher percentage of the degenerating axons in the SMA lower lumbar ventral roots (**E**). [Cervical root: control (N=1), SMA (N=5)], [Thoracic root: control (N=2), SMA (N=4)], [Upper lumbar: control (N=3), SMA (N=4)], [Lower lumbar: control (N=5), SMA (N=6)]
These data are kindly shared by Dr. Lingling Kong.

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Figure 2. Ultrastructure of the human ventral roots shows large population of small, unmyelinated axons. Autopsy ventral roots were collected from 5 patients with type I SMA (average age=357.4 days) and compared with ventral roots from 5 age-matched controls (average age=1035.8 days) by electron microscopy. The white arrow indicates a small, segregated unmyelinated axon. And the white arrow head indicates unsegregated unmyelinated axons. Scale bar = 2μm (A). Axon diameter is significantly decreased in SMA with 3900±80 nm compared to control=6160±640 nm (B). The G ratio of the myelinated axons in both the control and SMA groups remained unchanged indicating appropriate myelin thickness for axon diameter (C). In the control group, 100% of axons were myelinated axons. Only 11.55 ±3.88% is myelinated axons in the SMA group. Approximately 90% of axons remain small and unmyelinated in SMA, and within the number of unmyelinated axons, ~81% of unmyelinated axons remain unsegregated (D).
Figure 3. Immunohistochemical co-labeling of myelin (MBP) and axons (Tuj1) in human ventral roots showed an increase in the number of SMA axons without MBP ensheathment. The white arrow indicates axons that are positive for both MBP and Tuj1, while the white arrow heads shows the axons that are positive for Tuj1, but negative for MBP (A).

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3 These data are contributed by David Valdivia
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In controls, ~88.2% of the Tuj1 positive axons are ensheathed with a ring of MBP positive staining, while in SMA, only ~27.7% of Tuj1 positive axons showed an encircling rim of MBP staining (B).
Figure 4. Analysis of ventral root motor axons in SMA mice shows impaired axon development. Cross sections of toluidine blue stained ventral roots from WT, SMA, and motor neuron specific rescued, \textit{ChAT}^{\text{Cre+}} SMA mice are shown (63X, N=3/group) (A).

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Total ventral root area (right) was significantly reduced in the SMA mice compared to the WT [WT=9748.39±1004.58 μm$^2$ (N=6); SMA=3223.85±127.60 μm$^2$ (N=3); P<0.05]. Myelinated axon number (left) of the SMA L1 VR at P14 showed a significant reduction [WT=384.83±41.02 (N=6); SMA=218.33±7.51 (N=3); P<0.05]. The ChAT$^{cre+}$ SMA showed a modest increase in the area (7141.67±1268.12 μm$^2$, N=3) of L1VR as well as the number of myelinated axons (276.67±48.52, N=3) (B). *, P<0.05 and **, P<0.01 by student t-test.
These data are kindly contributed by Dr. Lingling Kong.

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Figure 5. Analysis of mouse L1 VR morphology at the untralstructural level demonstrates decreased axon diameter in SMA mice. EM images of L1VR from WT, SMA and ChAT$^{\text{Cre}^+}$ SMA mice (8000X). White triangles indicate myelinated axons, while the white arrows point to bundles of unmyelinated axons (A). Quantification of the myelinated axons diameter revealed a significant reduction for the SMA motor axons. Increasing the SMN level specifically in the motor neurons (ChAT$^{\text{Cre}^+}$) resulted in the moderate increase the myelinated axon diameters, but did not complete recovery the size of the axons compared to the control (B). The G-ratio of the myelinated axons did not differ among the control, SMA, and ChAT$^{\text{Cre}^+}$ mice (C). Quantifying the segregated, unsegregated, and the total number of unmyelinated axon per 100 um$^2$ (N=3/group) showed a significant increase in the number of unsegregated unmyelinated axon in the SMA mice. Increasing the SMN level in motor neurons showed a moderate decrease in the number of the unsegregated unmyelinated axon compared to the SMA mice (D).
Figure 6. Expression profile of genes associated with immature Schwann cells and myelin development did not reveal differences between SMA and WT mice. Sciatic nerves from WT and SMA Δ7 mice were harvested at P7 and P10. Jagged-1, Notch1, MBP, ErbB3, MPZ expression levels were analyzed by quantitative RT-PCR using 18s (A) or Gusb (B) as endogenous controls. No significant changes in the transcription level of these genes were observed between SMA and WT at P7 or P10. However, there were significant increases in the expression of myelin associated genes, MBP and MPZ between P7 and P10 for both SMA and WT, reflecting the continuing myelination process in young mice (N=8/group).
Figure 7. Representative images of lacZ staining of P7 tissues in Dhh<sup>Cre</sup> lacZ line (N=2). Scale bar 2mm. Images of lacZ staining of sciatic nerve (A), spinal cord (B), brain (C), testis (D), kidney (E), muscle (F) are shown. As expected, the lacZ staining demonstrated Cre activity in the Schwann cells of sciatic nerve (A), Sertoli cells of testis (B), blood vessel endothelial cells of the kidney (C). Cre recombinase is absent in the motor neurons in the spinal cord (B), neurons in the brain (C), and myofibers in the muscle (F). The weak lacZ activity observed in the muscle should be the Cre activity in the nerves that are innervating the muscle.

5 These images are contributed by our collaborator, Crystal Davis, Cathleen Lutz Lab, Jackson Laboratory
Figure 8. SMN is expressed in a tissue-specific manner in the conditional SMA mice. From the schematic representation of the Smn allele (SmnRes) switch cassette and the potential transcript product of the hybrid allele, it shows that in the absence of Cre recombinase, truncated hybrid transcript (SMN 68) is made containing upstream mouse Smn (exons 1–6), human SMN2 exon 8, and no exon 7 sequence. In the presence of Cre recombinase, a full-length hybrid transcript (SMN 678) containing mouse Smn exons 1–7 and human SMN2 exon 8 sequence may be made.

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The positions of qRT-PCR primers used are indicated with arrows indicating forward and reverse primers and asterisks indicating the positions of the probes. This figure is reproduced from (Martinez & Kong et al., 2012) (A). SMN6m8h primers detected hybrid transcripts in the sciatic nerve of P10 Cre-SMA and Cre+ SMA mice, but not in the WT mice. SMN67m8-h primers detected SMN 678 transcripts in sciatic nerve only in $Dhh^{Cre+}$ mice (B). (N=8/group)

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Figure 9. Increased SMN expression in Schwann cells did not provide improvements in the survival and body weights of SMA mice.

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Survival curves of $Dhh^{Cre}$ mice (A) and $ChAT^{Cre}Dhh^{Cre}$ mice (B) indicated no significant additional improvements in the survival of $ChAT^{Cre+}Dhh^{Cre+}$ SMA mice. Weight curves also demonstrated no significant improvements. Similar to the trend displayed in the SMA mice, the $Dhh^{Cre+}$ mice started to have slower weight increases starting P10 (C).

For $ChAT^{Cre}Dhh^{Cre}$ mice, $ChAT^{Cre+}Dhh^{Cre+}$ (double rescued) mice did not demonstrate additional benefit in weight gain compared to mice with just $ChAT^{Cre+}$. The separation in the trend of weight increase for $ChAT^{Cre+}Dhh^{Cre+}$ and $ChAT^{Cre+}$ started to slow down on P10 compared to WT mice, but they are still increasing at a faster trend than $Dhh^{Cre+}$ and SMA mice (D). $Dhh^{Cre}$ mice [Cre-SMA (N=14), Cre+ SMA mice (N=26)], $ChAT^{Cre}Dhh^{Cre}$ mice [ChAT$^{Cre+}$ SMA (N=22), ChAT$^{Cre+}Dhh^{Cre+}$ SMA (N=21), Dhh$^{Cre+}$ SMA (N=12), and Cre- SMA (N=6)].

7 The behavior and survival data of the $Dhh^{Cre}$ and $ChAT^{Cre}$ are collaboration with Crystal Davis and Cathleen Lutz, Jackson Laboratory.
Figure 10. Motor behavior assays demonstrated no significant differences between the Cre+ SMA and Cre- SMA groups. Both the Cre-SMA and Cre+ SMA have a higher righting tendency than the WT (A).

Figure legend continues
Figure legend continued

The composite score from the tube test showed that both Cre+ SMA and Cre- SMA mice have a trend of lower score than the WT. [$Dhh^{Cre}$ mice: N=4 Cre-SMA, N=4 Cre+ SMA mice, and N=7 WT mice]
Appendix I. Schematic of axonal development from Webster, 1975. Red arrow indicates unmyelinated, clustered axons; orange arrow indicates unmyelinated, ensheathed axons; purple arrow indicates an unmyelinated, segregated axon, and green arrow indicates a myelinated, segregated axon.
CURRICULUM VITAE

Xixi Xu was born in Lanzhou, China. In 2009, after completing her school work at the High School Affiliated to Renmin University of China, Beijing, China, she started her undergraduate education at Johns Hopkins University in Baltimore, Maryland. She received the degree of Bachelor of Science in Molecular and Cellular Biology as well as the degree of Bachelor of Art in Psychology from Johns Hopkins University in May 2013. In September, 2013, she entered the Master’s program in the Department of Biology at Johns Hopkins University.