Neuromuscular denervation and deafferentation but not motor neuron death are disease features in the Smn^{2B/-} mouse model of SMA

Maria J. Carlini^{1,2*}, Marina K. Triplett^{1,2*} and Livio Pellizzoni^{1,2,3*}

1 Center for Motor Neuron Biology and Disease, Columbia University, New York, NY 10032, USA
2 Department of Neurology, Columbia University, New York, NY 10032, USA
3 Department of Pathology and Cell Biology, Columbia University, New York, NY 10032, USA

* These authors contributed equally to this work.

* Corresponding author

E-mail: lp2284@cumc.columbia.edu
Abstract

Spinal muscular atrophy (SMA) is a neurodegenerative disease characterized by loss of motor neurons and skeletal muscle atrophy which is caused by ubiquitous deficiency in the survival motor neuron (SMN) protein. Several cellular defects contribute to sensory-motor circuit pathology in SMA mice, but the underlying mechanisms have often been studied in one mouse model without validation in other available models. Here, we used Smn

2B/− mice to investigate specific behavioral, morphological, and functional aspects of SMA pathology that we previously characterized in the SMNΔ7 model. Smn

2B/− SMA mice on a pure FVB/N background display deficits in body weight gain and muscle strength with onset in the second postnatal week and median survival of 19 days. Morphological analysis revealed severe loss of proprioceptive synapses on the soma of motor neurons and prominent denervation of neuromuscular junctions (NMJs) in axial but not distal muscles. In contrast, no evidence of cell death emerged from analysis of several distinct pools of lumbar motor neurons known to be lost in the disease. Moreover, SMA motor neurons from Smn

2B/− mice showed robust nuclear accumulation of p53 but lack of phosphorylation of serine 18 at its amino-terminal, which selectively marks degenerating motor neurons in the SMNΔ7 mouse model. These results indicate that NMJ denervation and deafferentation, but not motor neuron death, are conserved features of SMA pathology in Smn

2B/− mice.
Introduction

Spinal muscular atrophy (SMA) is an inherited neurodegenerative disease characterized by loss of motor neurons and skeletal muscle atrophy, leading to motor dysfunction, paralysis and eventually death in its most severe form [1,2]. SMA is caused by ubiquitous reduction in the levels of the survival motor neuron (SMN) protein—reflecting homozygous loss of the SMN1 gene but preservation of the nearly identical SMN2 gene [3]. Due to inefficient splicing of exon 7 [4], the SMN2 gene mainly produces an unstable protein isoform (SMNΔ7) and low levels of full-length functional SMN that cannot compensate for the loss of SMN1, leading to the disease [1,2].

Since higher SMN2 copy numbers are associated with reduced disease severity in SMA patients, most therapeutic efforts have focused on increasing expression of SMN through modulation of SMN2 splicing or SMN replacement by gene therapy [5–8]. Importantly, three distinct SMN-inducing therapies have demonstrated efficacy in clinical trials and are currently approved for treatment of SMA patients [9–14]. Nevertheless, these therapies are not a complete cure for the disease, and the development of additional therapeutics that could help address unmet clinical needs of SMA patients is necessary [15,16]. In principle, these novel drugs should target pathogenic events and enhance clinical benefit in combination with SMN-inducing therapies.

Loss of motor neurons is a hallmark of SMA pathology that is widely recognized to have a significant clinical impact on the disease course [1,2]. Genetic studies in mouse models have indicated that neurodegeneration in SMA is primarily an intrinsic, cell-autonomous process induced by SMN deficiency in motor neurons [17,18]. Moreover, motor neurons are the only cell type known to die during disease course, and their loss represents an irreversible pathogenic event that cannot be corrected after it has occurred. Thus, preventing motor neuron degeneration has important clinical implications for SMA therapy.
The availability of distinct mouse models of SMA has been instrumental to the study of disease mechanisms and preclinical evaluation of SMA therapies that are now approved for treatment of patients [19–22]. To date, several non-mutually exclusive mechanisms have been proposed to contribute to motor neuron loss in SMA mice [23–29]. Our previous work has highlighted that motor neurons degenerate through activation of the tumor suppressor p53 in the SMN<sub>Δ7</sub> mouse model of SMA [27]. Importantly, not all motor neurons are equally susceptible to SMN deficiency [30], and we identified at least two distinct pathogenic events that converge on p53 to trigger selective death of vulnerable SMA motor neuron pools [27]: i) upregulation of p53; and ii) phosphorylation of the amino-terminal transcriptional activation domain of p53 including serine 18 (p53<sup>S18</sup>). Mechanistically, we showed that disruption of SMN-dependent alternative splicing of specific exons in the pre-mRNAs of Mdm2 and Mdm4 – two well-established inhibitors of p53’s stability and function – is responsible for nuclear accumulation of p53 in SMA motor neurons [28]. We also showed that U12 splicing-dependent dysregulation of the Stasimon/Tmem41b gene induced by SMN deficiency contributes to the cascade of events leading to p53 phosphorylation and death of motor neurons in SMN<sub>Δ7</sub> SMA mice [29,31]. Lastly, we found that Stasimon dysfunction induces p38 mitogen-activated protein kinase (p38MAPK) activation and that pharmacological inhibition of p38αMAPK reduces p53 phosphorylation and improves motor neuron survival in SMN<sub>Δ7</sub> mice [29], highlighting the neuroprotective effects of p38αMAPK inhibition in SMA mice. However, these as well as other proposed death mechanisms of SMA motor neurons have often been studied only in one mouse model without cross validation in other available models.

This study was designed as part of our efforts to determine whether p53 activation is a shared pathogenic mechanism associated with motor neuron death across different SMA models and to further validate pharmacological inhibition of p38αMAPK as a candidate neuroprotective approach. We used the Smn<sup>2B−/−</sup> mouse model of SMA that harbors a hypomorphic Smn allele (Smn<sup>2B</sup>) with a mutation in the splicing regulatory sequence of exon 7 of the endogenous gene and a knockout Smn allele in a pure FVB/N genetic
background [32]. We performed behavioral and morphological studies of sensory-motor circuit pathology in this mouse model to determine the effects of SMN deficiency on synaptic integrity and motor neuron survival. Consistent with previous studies [32], we found that Smn$^{2B/c}$ SMA mice display reduced weight gain, impaired motor function, and median survival of ~19 days. We also found severe loss of proprioceptive synapses on motor neurons and selective neuromuscular junction (NMJ) denervation of axial but not distal muscles. Surprisingly, however, we found no evidence for motor neuron loss, which correlated with nuclear accumulation of p53 but lack of amino-terminal phosphorylation of p53$^{S18}$ in SMA motor neurons. Collectively, these findings highlight shared and distinct features of SMA pathology in SMN$\Delta 7$ and Smn$^{2B/c}$ mice, which have important implications for guiding the selection of appropriate models for basic and translational studies of specific aspects of SMA pathology in the future. They also indicate that the Smn$^{2B/c}$ model is not well suited for in vivo testing of neuroprotective drugs that specifically target the motor neuron death pathway.
Materials and methods

Mouse lines

All mouse work was performed in accordance with the National Institutes of Health Guidelines on the Care and Use of Animals, complied with all ethical regulations and was approved by the IACUC committee of Columbia University. Mice were housed in a 12h/12h light/dark cycle with access to food and water ad libitum. Heterozygous $Smn^{+/−}$ mice harboring the $Smn^{I^{tm1Msd}}$ knockout allele [33] on a pure FVB/N genetic background were obtained by crossing the $SMN\Delta7$ mouse line FVB.Cg-Grm7Tg(SMN2)89Ahmb $Smn^{I^{tm1Msd}}$ Tg(SMN2*delta7)4299Ahmb/J (Jax stock #005025) with FVB/N mice until removal of the $SMN\Delta7$ and SMN2 transgenes. The $Smn^{2B/2B}$ mice on a pure FVB/N genetic background were previously described [32]. $Smn^{2B/2B}$ mice were crossed with $Smn^{+/−}$ mice to generate $Smn^{2B/+}$ SMA mice and $Smn^{2B/−}$ littermates that were used as normal controls. Equal proportions of mice of both sexes were used and aggregated data are presented because gender-specific differences were not found.

Genotyping

Genotyping was performed from tail DNA using a common forward primer (5’-GATGATTCTGACATTGGGATG-3’) and specific reverse primers (5’-TGGCTTATCTGGAGTTTCACAA-3’) and (5’-GAGTAACAACCCGTCGGATTC-3’) for wild type $Smn$ and $Smn^{I^{tm1Msd}}$ knockout alleles, respectively [34]. The $Smn^{2B}$ allele was genotyped using forward (5’-AACTCCGGGTCTCCTCTTCTC-3’) and reverse (5’-TTTGGCAGACTTTTAGCAGGGC-3’) primers as previously described [32].

Behavioral assays

Mice from all experimental groups were monitored daily for survival and weight from birth to weaning at 21 days. Righting reflex was assessed by placing the mouse on its back and measuring the time it took to turn upright on its four paws (righting time). Cut-off test time was 60 seconds. For each testing session, the
test was repeated three times and the mean of the recorded times was calculated. For the hindlimb suspension test [32], the mouse was suspended by its hindlimbs from the rim of a cylindrical container with cushioning at the bottom. The time it took for a mouse to fall from the rim into the container was recorded with a cut-off time of 60 seconds. Mice able to climb back to the rim of the container during the test were scored as meeting the cut-off time. The test was repeated twice for each testing session and the mean of the recorded times was calculated.

**Antibodies and fluorescent probes**

For western blot analysis, we used an anti-SMN mouse monoclonal antibody (BD Transd Lab, clone 8, #610646; 1:10,000), an anti-Tubulin mouse monoclonal antibody (Sigma, clone DM1A, #T9026; 1:50,000), and a HRP conjugated goat anti-mouse secondary antibody (Jackson #115-035-044; 1:10,000). For spinal cord immunohistochemistry, we used goat anti-ChAT (Millipore #AB144P; 1:100), guinea pig anti-VGlut1 (Covance, custom made; 1:5,000) [18], rabbit anti-p53 (Leica Novocastra #NCL-p53-CM5p; 1:1,000) and rabbit anti-phosphorylated-p53S15 (Cell Signaling #9284, Lot: #15; 1:250) as primary antibodies. For muscle immunohistochemistry, we used guinea pig anti-Synaptophysin 1 (Synaptic Systems #101-004; 1:500), rabbit anti-Neurofilament M (Millipore #AB1987; 1:500), and Alexa Fluor™ 555 conjugated α-bungarotoxin (Invitrogen, #B35451; 1:500). Species-specific secondary antibodies coupled to Cy3 or Cy5 were used as appropriate (Jackson ImmunoResearch Laboratories, Inc; 1:250).

**Protein analysis**

For Western blot analysis, mice were sacrificed and spinal cord collection was performed in a dissection chamber under continuous oxygenation (95%O₂/5%CO₂) in the presence of cold (~12°C) artificial cerebrospinal fluid (aCSF) containing 128.35mM NaCl, 4mM KCl, 0.58mM NaH₂PO₄, 21mM NaHCO₃, 30mM D-Glucose, 1.5mM CaCl₂, and 1mM MgSO₄. Total protein extracts were generated by homogenization of spinal cords in SDS sample buffer (2% SDS, 10% glycerol, 5% β-mercaptoethanol, 60mM Tris-HCl pH 6.8, and bromophenol blue), followed by brief sonication and boiling. Proteins were
quantified using the RC DC\textsuperscript{TM} Protein Assay (Bio-Rad) and analyzed by SDS/PAGE on 12% polyacrylamide gels followed by Western blotting as previously described [35].

Immunohistochemistry

Animals were anesthetized and transcardially perfused with 4% paraformaldehyde (PFA). The spinal cord and skeletal muscles were dissected and post-fixed in 4% PFA for 4 hours. For immunohistochemistry, the spinal cords were briefly washed with PBS, specific lumbar segments were identified by the ventral roots and subsequently embedded in warm 5% agar. Transverse sections (75\textmu m) of the entire spinal segment were obtained with a VT1000 S vibratome (Leica). All the sections were then blocked with 10% normal donkey serum in 0.01M PBS containing 0.4% Triton X-100 (PBS-T; pH 7.4) for 1 hour and incubated overnight at room temperature with different combinations of primary antibodies diluted in PBS-T. The following day, six washing steps of 10 minutes each were done prior to incubation with secondary antibodies for 3 hours in PBS. Another six washing steps were performed before sections were mounted in 30% glycerol/PBS. For NMJ analysis, skeletal muscles were cryoprotected through sequential immersion in 10% and 20% sucrose/0.1M phosphate buffer (PB) for 1 hour at 4°C followed by overnight immersion in 30% sucrose/0.1M PB at 4°C. The following day muscles were frozen embedded in Optimal Cutting Temperature (OCT) compound (Fisher), frozen on dry ice, and stored at -80°C until processing. Longitudinal cryosections (30\textmu m) were collected onto Superfrost Plus glass slides (Fisher) using a CM3050S cryostat (Leica). Sections were washed once with PBS for 5 minutes to remove OCT, blocked for 1 hour with 5% donkey serum in TBS containing 0.2% Triton-X at room temperature and incubated with primary antibodies in blocking buffer overnight at 4°C. Following incubation, sections were washed three times for 10 minutes in TBS containing 0.2% Triton-X and then incubated with tetramethylrhodamine-conjugated \textalpha-bungarotoxin (Invitrogen #T1175, 1:500) and the appropriate secondary antibodies for 1 hour at room temperature, followed by 3 washing steps. Slides were mounted using Fluoromount-G Mounting Medium (SouthernBiotech).
Confocal microscopy and image analysis
All images were collected with an SP5 confocal microscope (Leica) running the LAS AF software (v2.5.2.6939) and analyzed off-line using the Leica LAS X software (v1.9.0.13747). For motor neuron number quantification, 1024 x 1024 pixels images were acquired from all the 75 μm sections of each specific spinal segment using a 20X objective at 3 μm steps in the z-axis and a 200 Hz acquisition rate. Only motor neurons (ChAT+) with a clearly identifiable nucleus were counted to avoid double counting from adjoining sections. For quantification of VGluT1+ synapses, 1024 x 1024 pixels images were acquired from L2 spinal sections (75 μm) using a 40X objective at 0.3 μm steps in the z-axis and a 200 Hz acquisition rate. The total number of VGluT1+ synapses on soma was determined by counting all the corresponding inputs on the surface of each ChAT+ motor neuron cell body. At least 10 motor neurons per mouse were quantified. For NMJ analysis, 1024 x 1024 pixels images were acquired from 30 μm muscle sections using a 20X objective at 2 μm steps in the z-axis and a 200 Hz acquisition rate. Maximum intensity projections of confocal stacks were analyzed and at least 200 randomly selected NMJs per muscle were quantified. NMJs lacking any coverage of the α-bungarotoxin-labeled postsynaptic endplate by the presynaptic markers Synaptophysin and Neurofilament-M were scored as denervated.

Statistical analysis
Statistical analysis was performed by two-tailed unpaired Student's t-test or by two-way ANOVA followed by the Bonferroni’s multiple comparison test as indicated. Comparison of survival curves was performed using the Log-rank (Mantel-Cox) test. GraphPad Prism (v9.3.1) was used for all statistical analyses and P values are indicated as follows: *P<0.05; **P<0.01; ***P<0.001; **** P<0.0001.
Results

Behavioral characterization of Smn^{2B-} SMA mice

Smn^{2B} is a hypomorphic allele harboring a mutation in the splicing regulatory sequence of exon 7 in the mouse Smn gene leading to exon skipping and reduced expression of full-length Smn protein [22]. Accordingly, we first analyzed the levels of Smn expression from the Smn^{2B} allele by Western blot analysis of spinal cord tissue and found that homozygous Smn^{2B/2B} mice express half the levels of Smn protein relative to wild type (Smn^{+/+}) mice at P16 (Fig S1A). A similar reduction in the levels of Smn expression was also found by comparing spinal cords from Smn^{2B/-} SMA mice and control Smn^{2B/+} littermates at P16 (Fig S1B). Thus, the Smn^{2B} allele expresses approximately 25% of the amount of Smn protein relative to the wild type Smn allele in the mouse spinal cord, which is consistent with previous studies [22].

Next, we sought to characterize the disease phenotype of Smn^{2B/-} SMA mice. We monitored daily weight gain from birth to weaning in Smn^{2B/-} SMA mice and Smn^{2B/+} littermates, which were used as normal controls in this study. While both groups displayed a similar weight gain in the first two postnatal weeks, Smn^{2B/-} SMA mice showed a significant and progressive decline in weight relative to Smn^{2B/+} littermates starting at P15 (Fig 1A). The decline in body weight was rapidly followed by death of Smn^{2B/-} SMA mice, which displayed a median lifespan of 19 days (P < 0.0001, Log-rank Mantel-Cox test) (Fig 1B). To analyze motor function, we performed the righting reflex and the hindlimb suspension tests, which are two behavioral assays widely used to monitor disease-related motor phenotypes in mouse models of SMA. Smn^{2B/-} SMA mice showed a comparable ability to acquire the righting reflex relative to controls during early postnatal development (Fig 1C). On the other hand, while Smn^{2B/+} control mice rapidly improved and then maintained their performance in the hindlimb suspension test from P11 onward, Smn^{2B/-} SMA mice not only failed to show improvement but also progressively worsened their performance over time, pointing to compromised hindlimb muscle strength (Fig 1D). Thus, consistent with previous studies [22,32], we found that Smn^{2B/-} SMA mice display impaired weight gain and motor function as well as reduced survival.
Severe loss of proprioceptive synapses on motor neurons of $Smn^{2B/-}$ SMA mice

Spinal sensory-motor circuit dysfunction is one of the earliest pathological features of SMA in mouse models [1]. In particular, the loss of VGluT1$^+$ proprioceptive synapses on the soma and proximal dendrites of motor neurons has been shown to occur early in the disease course [18,30,36,37] as well as independently from motor neuron loss in the $SMN^{A7}$ mouse model of SMA [18,27,28]. Furthermore, the molecular defects induced by Smn deficiency that contribute to deafferentation of SMA motor neurons have recently emerged, including dysregulation of U12 splicing of the Stasimon gene [29,38]. Since most of these studies were performed in the $SMN^{A7}$ mouse model of SMA, we sought to investigate the effects of SMN deficiency on the connectivity of proprioceptive synapses onto motor neurons in $Smn^{2B/-}$ SMA mice. As in our previous studies, we focused on lumbar motor neurons innervating disease-relevant proximal muscles and employed immunohistochemistry and confocal microscopy to quantify the number of VGluT1$^+$ proprioceptive synapses juxtaposed to the soma of ChAT$^+$ motor neurons. (Fig 2A). We found that the number of proprioceptive synapses onto motor neurons of $Smn^{2B/-}$ SMA mice is markedly reduced relative to $Smn^{2B/+}$ mice at P16 (Fig 2B). Thus, Smn deficiency induces severe deafferentation of motor neurons in the $Smn^{2B/-}$ mouse model of SMA.

Loss of neuromuscular junction innervation in axial but not distal muscles of $Smn^{2B/-}$ SMA mice

The loss of neuromuscular junction (NMJ) synapses between motor neurons and skeletal muscle is a key disease feature of SMA pathology with proximal and axial muscles being more affected than distal muscles in both patients and mouse models [39–42]. Therefore, we sought to examine NMJ innervation in the axial muscle quadratus lumborum (QL) and the distal muscle tibialis anterior (TA) in $Smn^{2B/-}$ SMA mice relative to $Smn^{2B/+}$ controls at P16. To do so, we performed NMJ staining using antibodies against Neurofilament-M and Synaptophysin as pre-synaptic markers and α-bungarotoxin as post-synaptic marker of the motor endplate. As expected, 100% of the NMJs were fully innervated in both QL and TA muscles of control mice (Fig 3). Importantly, analysis of the QL muscle from $Smn^{2B/-}$ SMA mice revealed strong NMJ
denervation as many α-bungarotoxin-labeled motor endplates completely lacked pre-synaptic coverage by nerve terminals (Fig 3A). Quantification of this defect showed that approximately 40% of the NMJs in QL muscle are denervated in Smn^{2B/-} SMA mice (Fig 3B). In contrast, nearly all the NMJs in the TA muscle from Smn^{2B/-} SMA mice were innervated (Fig 3D). Taken together, these results demonstrate marked and preferential loss of NMJ innervation from an axial muscle relative to a distal muscle in Smn^{2B/-} SMA mice, which is consistent with the features of neuromuscular pathology in the human disease.

Survival of motor neurons is not affected in Smn^{2B/-} SMA mice

The selective degeneration of specific pools of motor neurons is a hallmark of SMA [1,2]. Reflecting the characteristic profile of differential muscle vulnerability, motor neuron pools innervating proximal and axial muscles are more prominently affected and preferentially lost in the disease. Accordingly, vulnerable pools comprise lumbar motor neurons residing in the L1 and L2 segments of the spinal cord as well as in the L5 medial motor column (MMC), which innervate proximal and axial muscles [18,30,41]. In contrast, L5 lateral motor column (LMC) motor neurons that innervate distal muscles are resistant to death in SMA. We sought to determine whether motor neurons in the Smn^{2B/-} SMA mice displayed a similar profile of differential vulnerability to death induced by SMN deficiency. To do so, we performed immunostaining of all sections from the L1, L2 and L5 segments of the spinal cord with antibodies against ChAT and counted the total number of ChAT⁺ motor neurons in each of these segments from Smn^{2B/-} SMA mice and Smn^{2B/+} control littermates at P16 (Fig. 4A, 4C and 4E). Surprisingly, we found no significant loss of motor neurons in any of the lumbar segments analyzed from the spinal cord of Smn^{1B/-} mice relative to controls (Fig. 4B, 4D, 4F and 4G). This analysis indicates that SMN deficiency does not affect the survival of motor neurons in the Smn^{2B/-} mouse model of SMA at a late symptomatic time point.

SMN deficiency induces p53 accumulation but not serine 18 phosphorylation in motor neurons of Smn^{2B/-} SMA mice
Our previous studies implicated activation of a p53-dependent pathway in the selective death of motor neurons in SMNΔ7 SMA mice [27–29]. Therefore, we sought to investigate this pathway in Smn<sup>2B/−</sup> SMA mice, which do not display significant loss of motor neurons. As for the analysis of motor neuron survival, we focused on the study of the L1, L2 and L5 segments of the spinal cord from Smn<sup>2B/−</sup> SMA mice and Smn<sup>2B/+</sup> controls at P16. First, we performed immunohistochemistry experiments with antibodies against total p53 as well as ChAT to identify motor neurons followed by confocal microscopy. These experiments revealed strong nuclear accumulation of p53 in L1 (Fig 5A and 5B), L2 (Fig S2A and S2B) and L5 LMC and MMC (Fig S3A and S3B) motor neurons from Smn<sup>2B/−</sup> SMA mice but not from control Smn<sup>2B/+</sup> mice. Quantification showed nuclear accumulation of p53 in approximately 40% of vulnerable L1, L2 and L5 MMC motor neurons as well as resistant L5 LMC motor neurons from Smn<sup>2B/−</sup> SMA mice (Fig 5D, Fig S2D, Fig S3E and S3G). Moreover, we found nuclear p53 immunoreactivity in other spinal cells from Smn<sup>2B/−</sup> mice (Fig 5A, Fig S2A and S3A). Thus, SMN deficiency induces robust p53 accumulation in both motor neurons and other spinal cells in the Smn<sup>2B/−</sup> mouse model of SMA.

We previously showed that p53 nuclear accumulation is necessary but not sufficient to induce motor neuron death [27,28], which additionally requires phosphorylation of several serine residues in the amino terminus of p53 [27,29]. We also showed that phosphorylation of serine 18 of p53 (phospho-p53<sup>S18</sup>) selectively marks motor neurons destined to degenerate in SMA mice but is absent from resistant SMA neurons exhibiting p53 accumulation at late stages of disease [27,29]. Therefore, we investigated the expression of phospho-p53<sup>S18</sup> by immunostaining of the L1, L2 and L5 spinal segments from Smn<sup>2B/−</sup> SMA mice and Smn<sup>2B/+</sup> controls at P16. Noteworthy, we did not detect any immunostaining of phospho-p53<sup>S18</sup> in SMA motor neurons from Smn<sup>2B/−</sup> SMA mice (Fig 5C and 5E, Fig S2C and S2E, Fig S3C, S3D, S3F and S3H). Thus, despite strong nuclear accumulation of p53, the absence of detectable expression of phospho-p53<sup>S18</sup> correlates with the lack of motor neuron loss in the Smn<sup>2B/−</sup> mouse model of SMA.
Discussion

Here we carried out behavioral and morphological characterization of SMA pathology in the \( Smn^{2B/-} \) mouse model of SMA. By monitoring the same parameters of sensory-motor circuit pathology and using the same assays we previously employed in our studies of \( SMN\Delta7 \) SMA mice, the study design allows direct comparison of our findings in the two models. Accordingly, we document similar features of synaptic pathology in \( Smn^{2B/-} \) and \( SMN\Delta7 \) SMA mice, including severe loss of proprioceptive synapses on the soma of motor neurons and selective NMJ denervation of axial but not distal muscles. Surprisingly, however, we report the lack of significant loss of lumbar motor neurons at a late symptomatic stage of disease in \( Smn^{2B/-} \) SMA mice that is in stark contrast with findings in \( SMN\Delta7 \) SMA mice. The observed differences in motor neuron survival are consistent with our proposed mechanisms of motor neuron death in \( SMN\Delta7 \) SMA mice implicating both nuclear accumulation and amino-terminal phosphorylation of p53 [27–29], the latter of which does not occur in \( Smn^{2B/-} \) SMA mice. Collectively, these findings highlight shared and distinct features of SMA pathology across mouse models of SMA and indicate that \( Smn^{2B/-} \) SMA mice are suitable for the study of some but not all the aspects of sensory-motor circuit pathology. Moreover, the lack of motor neuron death hinders the use of this model for \textit{in vivo} testing of neuroprotective drugs specifically aimed at targeting motor neuron death.

The results of behavioral analysis of the SMA phenotype in \( Smn^{2B/-} \) mice are well aligned with previous studies in the same model [32]. Accordingly, we found that \( Smn^{2B/-} \) mice display a decline in weight gain at about two weeks of age that is mirrored by signs of progressive muscle weakness as revealed by failure to perform in the hindlimb suspension test. These deficits are compounded by shortened lifespan with a median survival of 19 days. Interestingly, however, \( Smn^{2B/-} \) mice acquire the ability to right themselves in a manner indistinguishable from control littermates, which is very different from the severe impairment in performing this motor function found in \( SMN\Delta7 \) mice [17,30]. The reason for this difference in motor behavior remains to be established but could relate to a later onset in the loss of proprioceptive synapses in
the Smn^{2B/-} mice [37] at a time when the contribution of these synapses to the righting behavior is outweighed by the activity of descending vestibulo-spinal pathways [43,44].

We show here that the number of VGluT1⁺ excitatory synapses of proprioceptive neurons on the soma of motor neurons is severely reduced in Smn^{2B/-} mice. Deafferentation of motor neurons was previously reported as one of earliest synaptic defects occurring in the SMNΔ7 mouse model [30], mainly resulting from the effects of SMN deficiency in proprioceptive neurons [18]. Consistent with our results, loss of proprioceptive synapses on motor neurons was also observed in Smn^{2B/-} mice on a different genetic background (C57BL/6) as well as in the Taiwanese model of SMA [37,45–47]. Thus, motor neuron deafferentation emerges as a conserved pathogenic event across all mouse models of SMA. Through rescue experiments in SMNΔ7 mice, we have previously shown the direct contribution of U12 splicing dysregulation and Stasimon dysfunction in this process [29,31,38]. Other studies using Taiwanese SMA mice implicated deficits in pathways related to UBA1/GARS and Plastin [46,47]. Moreover, activation of the classical complement cascade has been linked to the execution of synaptic elimination of proprioceptive synapses in SMNΔ7 mice [48]. It remains to be established whether these findings can be reconciled into a coherent cascade of events and the same mechanisms are responsible for the loss of central synapses in the different mouse models.

Our analysis of neuromuscular pathology reveals strong loss of NMJ innervation in the axial muscle QL but nearly complete sparing of the distal muscle TA. These findings complement and extend previous studies of NMJ pathology in Smn^{2B/-} mice [22,37,49], indicating that the QL is among the most severely affected muscles in this mouse model. They are also consistent with the preferential susceptibility to NMJ denervation of proximal and axial SMA muscles that is observed across mouse models and appears more pronounced in SMNΔ7 mice [37,40,50,51]. Lastly, the loss of NMJs from the QL muscle without death of
the corresponding innervating motor neurons corroborates the conclusion that these two key pathogenic events are mechanistically uncoupled in SMA [28,29,52–54].

A surprising finding of this study is the lack of motor neuron loss, which is a hallmark of SMA pathology [1,2]. We investigated distinct pools of lumbar motor neurons known to be highly vulnerable in SMNΔ7 mice [18,27,30,41], which include L1 and L2 as well as L5 MMC motor neurons. However, in all instances we found no significant reduction in the total number of spinal motor neurons at a late symptomatic time point (P16) in Smn2B−/− mice, highlighting a marked difference between SMNΔ7 and Smn2B−/− mice. These results disagree with earlier studies reporting loss of motor neurons already at P11 in Smn2B−/− mice on an FVB/N background that are identical to those analyzed here [32]. The reason for the discrepancy is unclear, but one possibility may lie in the accuracy of estimating motor neurons by sampling a subset of sections [32,55,56] as compared to counting the total number of motor neurons in all sections from the entire spinal segment (this study and [18,27–30,37]), the latter of which we consider more reliable. Other potentially confounding elements relate to the methods used for identification of specific spinal segments and the pooling of motor neuron counts obtained from sections spanning multiple segments that differ in the overall number of motor neurons as well as their susceptibility to disease [32,55,56]. Along these lines, we note that significant loss of motor neurons was also reported to occur at P15-P16 in Smn2B−/− mice on the C57BL/6 background [32,55,56], but a recent study using the same experimental approach employed here found only limited loss of L1 motor neurons at P26, which is two days past median survival, but neither at earlier time points nor in other spinal segments [37]. Therefore, although we cannot exclude the possibility that a small loss of motor neurons may occur in Smn2B−/− mice on the FVB/N background at times beyond their median survival, we conclude that motor neuron death is not a disease-relevant feature of SMA pathology in Smn2B−/− mouse models.
To identify a potential reason for the lack of motor neuron loss in Smn<sup>2B−</sup> mice, we investigated the status of p53 expression that we have previously linked to the death pathway in SMND<sup>7</sup> mice [27]. By immunostaining experiments, we found widespread nuclear accumulation of p53 in SMA motor neurons from Smn<sup>2B−</sup> mice at P16. Similar to the situation we reported in SMND<sup>7</sup> mice at late symptomatic stages [27,28], p53 accumulation in Smn<sup>2B−</sup> mice is observed in vulnerable L1, L2 and L5 MMC motor neurons as well as resistant L5 LMC motor neurons and other spinal cells that do not degenerate in the disease. These findings are consistent with the transcriptional upregulation of some p53-regulated genes in motor neurons of Smn<sup>2B−</sup> mice [57]. However, given that p53 induction alone is necessary but not sufficient to drive death of motor neurons in vivo [27,28], we looked for amino-terminal phosphorylation of p53 that we showed to be a prerequisite for activation of the neurodegenerative process [27,29]. Specifically, we investigated the phosphorylation of p53 at serine 18, which we have previously shown to be a p38αMAPK-mediated event that marks vulnerable SMA motor neurons destined to die in SMND<sup>7</sup> mice [29]. Importantly, we found no evidence for p53<sup>S18</sup> phosphorylation in lumbar SMA motor neurons from Smn<sup>2B−</sup> mice on FVB/N background. The absence of this post-translational modification of p53 may explain why motor neuron survival is unaffected in this mouse model and corroborates our proposed model in which convergence of upregulation and amino-terminal phosphorylation of p53 are distinct events necessary for driving motor neuron death [27–29]. Despite contrasting observations [55,56], which may be related to issues of reliability in motor neuron counting described above, this conclusion is also consistent with recent findings that the modest loss of L1 motor neurons in Smn<sup>2B−</sup> mice on C57BL/6 background correlates with the expression of phospho-p53<sup>S18</sup> and is rescued by p53 inhibition [37].

In sum, our work highlights the importance of monitoring the same pathogenic events with the same experimental approaches when comparing sensory-motor circuit pathology in different mouse models of SMA. Together with previous studies [30,36,37,45–47], we identify the loss of proprioceptive synapses on motor neurons as a conserved cellular defect induced by SMN deficiency across mouse models of SMA.
Similarly, NMJ denervation of axial muscles such as the QL displays good conservation across models and accurately reflects the proximo-distal gradient of muscle vulnerability characteristic of SMA patients. In contrast, selective loss of motor neurons unexpectedly emerges as the most distinguishing feature across mouse models of SMA despite being a hallmark of the human disease. While the $SMN\Delta 7$ model shows early onset and progressive death of specific motor neuron pools [27,30], in $Smn^{2B/-}$ mice the same motor neurons are either entirely spared (FVB/N background, this study) or a subset thereof only affected at very late disease stages (C57BL/6 background, [37]). Moreover, no loss of motor neurons has recently been reported in the Taiwanese SMA model [37]. Thus, not all mouse models of SMA are equally poised for the study of every aspect of sensory-motor circuit pathology. In this context, $SMN\Delta 7$ mice rather than $Smn^{2B/-}$ and Taiwanese mice are better suited for in vivo testing of neuroprotective drugs that selectively target the motor neuron death pathway. Collectively, these findings should help guide the selection of the most appropriate mouse models for elucidating specific disease mechanisms and pre-clinical testing of SMN-independent therapies.
Acknowledgements

We are grateful to George Mentis and Christian Simon for comments and critical reading of the manuscript. We thank Rashmi Kothary for providing the Smn^{2B} mouse line. This work was supported by NIH grant R01NS116400 (L.P.) and the NSF Graduate Research Fellowship Program (M.T.). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Author Contributions

L.P. conceived and supervised the study. M.J.C. and M.K.T. performed the experiments. M.J.C., M.K.T. and L.P. analyzed the data. M.J.C. and L.P. wrote the manuscript.

Declaration of Interests

The authors declare no competing interests.
References

1. Tisdale S, Pellizzoni L. Disease Mechanisms and Therapeutic Approaches in Spinal Muscular Atrophy. J Neurosci. 2015; 35:8691–8700. doi:10.1523/JNEUROSCI.0417-15.2015

2. Wirth B, Karakaya M, Kye MJ, Mendoza-Ferreira N. Twenty-Five Years of Spinal Muscular Atrophy Research: From Phenotype to Genotype to Therapy, and What Comes Next. Annu Rev Genomics Hum Genet. 2020; 21:231–261. doi:10.1146/annurev-genom-102319-103602

3. Lefebvre S, Burglen L, Reboullet S, Clermont O, Burlet P, Viollet L, et al. Identification and characterization of a spinal muscular atrophy- determining gene. Cell. 1995; 80:155–165.

4. Lorson CL, Hahnen E, Androphy EJ, Wirth B. A single nucleotide in the SMN gene regulates splicing and is responsible for spinal muscular atrophy. Proc Natl Acad Sci U S A. 1999; 96:6307–6311.

5. Hua Y, Sahashi K, Rigo F, Hung G, Horev G, Bennett CF, et al. Peripheral SMN restoration is essential for long-term rescue of a severe spinal muscular atrophy mouse model. Nature. 2011; 478:123–126. doi:10.1038/nature10485

6. Naryshkin NA, Weetall M, Dakka A, Narasimhan J, Zhao X, Feng Z, et al. Motor neuron disease. SMN2 splicing modifiers improve motor function and longevity in mice with spinal muscular atrophy. Science. 2014; 345:688–693. doi:10.1126/science.1250127

7. Foust KD, Wang X, McGovern VL, Braun L, Bevan AK, Haidet AM, et al. Rescue of the spinal muscular atrophy phenotype in a mouse model by early postnatal delivery of SMN. Nat Biotechnol. 2010; 28:271–274. doi:10.1038/nbt.1610

8. Palacino J, Swalley SE, Song C, Cheung AK, Shu L, Zhang X, et al. SMN2 splice modulators enhance U1-pre-mRNA association and rescue SMA mice. Nat Chem Biol. 2015; 11:511–517. doi:10.1038/nchembio.1837

9. Finkel RS, Chiriboga CA, Vajsar J, Day JW, Montes J, De Vivo DC, et al. Treatment of infantile-onset spinal muscular atrophy with nusinersen: a phase 2, open-label, dose-escalation study. Lancet. 2016; 388:3017–3026. doi:10.1016/S0140-6736(16)31408-8

10. Finkel RS, Mercuri E, Darras BT, Connolly AM, Kuntz NL, Kirschner J, et al. Nusinersen versus Sham Control in Infantile-Onset Spinal Muscular Atrophy. N Engl J Med. 2017; 377:1723–1732. doi:10.1056/NEJMoa1702752

11. Mercuri E, Darras BT, Chiriboga CA, Day JW, Campbell C, Connolly AM, et al. Nusinersen versus Sham Control in Later-Onset Spinal Muscular Atrophy. N Engl J Med. 2018; 378:625–635. doi:10.1056/NEJMoa1710504

12. Mendell JR, Al-Zaidy S, Shell R, Arnold WD, Rodino-Klapac LR, Prior TW, et al. Single-Dose Gene-Replacement Therapy for Spinal Muscular Atrophy. N Engl J Med. 2017; 377:1713–1722. doi:10.1056/NEJMoa1706198

13. Baranello G, Darras BT, Day JW, Deconinck N, Klein A, Masson R, et al. Risdiplam in Type 1 Spinal Muscular Atrophy. N Engl J Med. 2021; 384:915–923. doi:10.1056/nejmoa2009965

14. Darras BT, Masson R, Mazurkiewicz-Beldzińska M, Rose K, Xiong H, Zanoteli E, et al. Risdiplam-Treated Infants with Type 1 Spinal Muscular Atrophy versus Historical Controls. N Engl J Med. 2021; 385:427–435. doi:10.1056/nejmoa2102047

15. Mercuri E, Pera MC, Scoto M, Finkel R, Muntoni F. Spinal muscular atrophy — insights and challenges in the treatment era. Nat Rev Neurol. 2020; 16:706–715. doi:10.1038/s41582-020-00413-4
16. Chaytow H, Faller KME, Huang YT, Gillingwater TH. Spinal muscular atrophy: From approved therapies to future therapeutic targets for personalized medicine. Cell Rep Med. 2021; 2:100346. doi:10.1016/j.xcrm.2021.100346

17. Martinez TL, Kong L, Wang X, Osborne MA, Crowder ME, Van Meergebe JP, et al. Survival motor neuron protein in motor neurons determines synaptic integrity in spinal muscular atrophy. J Neurosci. 2012; 32:8703–8715. doi:10.1523/jneurosci.0204-12.2012

18. Fletcher E V, Simon CM, Pagiazitis JG, Chalif JI, Vukojicic A, Drobc E, et al. Reduced sensory synaptic excitation impairs motor neuron function via Kv2.1 in spinal muscular atrophy. Nat Neurosci. 2017; 20:905–916. doi:10.1038/nn.4561

19. Hsieh-Li HM, Chang JG, Jong YJ, Wu MH, Wang NM, Tsai CH, et al. A mouse model for spinal muscular atrophy. Nat Genet. 2000; 24:66–70. doi:10.1038/71709

20. Monani UR, Sendtner M, Coovert DD, Parsons DW, Andreassi C, Le TT, et al. The human centromeric survival motor neuron gene (SMN2) rescues embryonic lethality in Smn(-/-) mice and results in a mouse with spinal muscular atrophy. Hum Mol Genet. 2000; 9:333–339.

21. Le TT, Pham LT, Butchbach ME, Zhang HL, Monani UR, Coovert DD, et al. SMN Delta7, the major product of the centromeric survival motor neuron (SMN2) gene, extends survival in mice with spinal muscular atrophy and associates with full-length SMN. Hum Mol Genet. 2005; 14:845–857.

22. Bowerman M, Murray LM, Beauvais A, Pinheiro B, Kothary R. A critical smn threshold in mice dictates onset of an intermediate spinal muscular atrophy phenotype associated with a distinct neuromuscular junction pathology. Neuromuscul Disord. 2012; 22:263–276. doi:10.1016/j.nmd.2011.09.007

23. Miller N, Feng Z, Edens BM, Yang B, Shi H, Sze CC, et al. Non-aggregating tau phosphorylation by cyclin-dependent kinase 5 contributes to motor neuron degeneration in spinal muscular atrophy. J Neurosci. 2015; 35:6038–6050. doi:10.1523/JNEUROSCI.3716-14.2015

24. Genabai NK, Ahmad S, Zhang Z, Jiang X, Gabaldon CA, Gangwani L. Genetic inhibition of JNK3 ameliorates spinal muscular atrophy. Hum Mol Genet. 2015; 24:6986–7004. doi:10.1093/hmg/ddv401

25. Kannan A, Jiang X, He L, Ahmad S, Gangwani L. ZPR1 prevents R-loop accumulation, upregulates SMN2 expression and rescues spinal muscular atrophy. Brain. 2020; 143:69–93. doi:10.1093/brain/awz373

26. Hensel N, Cieri F, Santonicola P, Tapken I, Schüning T, Taiana M, et al. Impairment of the neurotrophic signaling hub B-Raf contributes to motoneuron degeneration in spinal muscular atrophy. Proc Natl Acad Sci U S A. 2021; 118: e2007785118. doi:10.1073/pnas.2007785118

27. Simon CM, Dai Y, Van Alstyne M, Koutsioumpa C, Pagiazitis JG, Chalif JI, et al. Converging Mechanisms of p53 Activation Drive Motor Neuron Degeneration in Spinal Muscular Atrophy. Cell Rep. 2017; 21:3767–3780. doi:10.1016/j.celrep.2017.12.003

28. Van Alstyne M, Simon CM, Sardi SP, Shihabuddin LS, Mentis GZ, Pelizzoni L. Dysregulation of Mdm2 and Mdm4 alternative splicing underlies motor neuron death in spinal muscular atrophy. Genes Dev. 2018; 32:1045–1059. doi:10.1101/gad.316059.118

29. Simon CM, Van Alstyne M, Lotti F, Bianchetti E, Tisdale S, Watterson DM, et al. Stasimon Contributes to the Loss of Sensory Synapses and Motor Neuron Death in a Mouse Model of Spinal Muscular Atrophy. Cell Rep. 2019; 29:3885-3901. doi:10.1016/j.celrep.2019.11.058

30. Mentis GZ, Blivis D, Liu W, Drobc E, Crowder ME, Kong L, et al. Early functional impairment of sensory-motor connectivity in a mouse model of spinal muscular atrophy. Neuron. 2011; 69:453–
31. Lotti F, Imlach WL, Saieva L, Beck ES, Hao le T, Li DK, et al. An SMN-Dependent U12 Splicing Event Essential for Motor Circuit Function. Cell. 2012; 151:440–454. doi:10.1016/j.cell.2012.09.012

32. Eshraghi M, McFall E, Gibeault S, Kothary R. Effect of genetic background on the phenotype of the Smn2B/- mouse model of spinal muscular atrophy. Hum Mol Genet. 2016; 25:4494–4506. doi:10.1093/hmg/ddw278

33. Schrank B, Gotz R, Gunnersen JM, UrE JM, Toyka KV, Smith AG, et al. Inactivation of the survival motor neuron gene, a candidate gene for human spinal muscular atrophy, leads to massive cell death in early mouse embryos. Proc Natl Acad Sci U S A. 1997; 94:9920–9925.

34. Van Alstyne M, Tattoli I, Delestrée N, Recinos Y, Workman E, Shihabuddin LS, et al. Gain of toxic function by long-term AAV9-mediated SMN overexpression in the sensorimotor circuit. Nat Neurosci. 2021; 24:930–940. doi:10.1038/s41593-021-00827-3

35. Ruggiu M, McGovern VL, Lotti F, Saieva L, Li DK, Kariya S, et al. A role for SMN exon 7 splicing in the selective vulnerability of motor neurons in spinal muscular atrophy. Mol Cell Biol. 2012; 32:126–138. doi:10.1128/MCB.06077-11

36. Ling KK, Lin MY, Zingg B, Feng Z, Ko CP. Synaptic defects in the spinal and neuromuscular circuitry in a mouse model of spinal muscular atrophy. PLoS One. 2010; 5:e15457. doi:10.1371/journal.pone.0015457

37. Buettner JM, Sime Longang JK, Gerstner F, Apel KS, Blanco-Redondo B, Sowoindich L, et al. Central synaptopathy is the most conserved feature of motor circuit pathology across spinal muscular atrophy mouse models. iScience. 2021; 24. doi:10.1016/j.isci.2021.103376

38. Osman EY, Van Alstyne M, Yen P-F, Lotti F, Feng Z, Ling KKY, et al. Minor snRNA gene delivery improves the loss of proprioceptive synapses on SMA motor neurons. JCI Insight. 2020; 5. doi:10.1172/jci.insight.130574

39. Dubowitz V. Ramblings in the history of spinal muscular atrophy. Neuromuscul Disord. 2009; 19:69–73. doi:10.1016/j.nmd.2008.10.004

40. Ling KK, Gibbs RM, Feng Z, Ko CP. Severe neuromuscular denervation of clinically relevant muscles in a mouse model of spinal muscular atrophy. Hum Mol Genet. 2012; 21:185–195. doi:10.1093/hmg/ddr453

41. Kong L, Valdivia DO, Simon CM, Hassinan CW, Delestrée N, Ramos DM, et al. Impaired prenatal motor axon development necessitates early therapeutic intervention in severe SMA. Sci Transl Med. 2021; 13:eabb6871. doi:10.1126/scitranslmed.abb6871

42. Iascone DM, Henderson CE, Lee JC. Spinal muscular atrophy: from tissue specificity to therapeutic strategies. F1000Prime Rep. 2015; 7:4. doi:10.12703/P7-04

43. Vinay L, Ben-Mabrouk F, Brocard F, Clarac F, Jean-Xavier C, Pearlstein E, et al. Perinatal development of the motor systems involved in postural control. Neural Plast; 2005; 131–139. doi:10.1155/NP.2005.131

44. Clarac F, Brocard F, Vinay L. The maturation of locomotor networks. Prog Brain Res. 2004; 143:57–66. doi:10.1016/S0079-6123(03)43006-9

45. Cervero C, Blasco A, Tarabal O, Casanovas A, Piedrafita L, Navarro X, et al. Glial activation and central synapse loss, but not motoneuron degeneration, are prevented by the sigma-1 receptor agonist pre-084 in the SMN2B/- mouse model of spinal muscular atrophy. J Neuropathol Exp Neurol. 2018; 77:577–597. doi:10.1093/jnen/nly033
46. Ackermann B, Kröber S, Torres-Benito L, Borgmann A, Peters M, Hosseini Barkooie SM, et al. Plastin 3 ameliorates spinal muscular atrophy via delayed axon pruning and improves neuromuscular junction functionality. Hum Mol Genet. 2013; 22:1328–1347. doi:10.1093/hmg/dds540

47. Shorrock HK, van der Hoorn D, Boyd PJ, Llavero Hurtado M, Lamont DJ, Wirth B, et al. UBA1/GARS-dependent pathways drive sensory-motor connectivity defects in spinal muscular atrophy. Brain. 2018; 141:2878–2894. doi:10.1093/brain/awy237

48. Vukojicic A, Delestrée N, Fletcher E V., Pagiazitis JG, Sankaranarayanan S, Yednock TA, et al. The Classical Complement Pathway Mediates Microglia-Dependent Remodeling of Spinal Motor Circuits during Development and in SMA. Cell Rep. 2019; 29:3087-3100.e7. doi:10.1016/j.celrep.2019.11.013

49. Murray LM, Beauvais A, Bhanot K, Kothary R. Defects in neuromuscular junction remodelling in the Snn2B/- mouse model of spinal muscular atrophy. Neurobiol Dis. 2013; 49:57–67. doi:10.1016/j.nbd.2012.08.019

50. Lin T-L, Chen T-H, Hsu Y-Y, Cheng Y-H, Juang B-T, Jong Y-J. Selective Neuromuscular Denervation in Taiwanese Severe SMA Mouse Can Be Reversed by Morpholino Antisense Oligonucleotides. PLoS One. 2016; 11:e0154723–e0154723. doi:10.1371/journal.pone.0154723

51. Murray LM, Comley LH, Thomson D, Parkinson N, Talbot K, Gillingwater TH. Selective vulnerability of motor neurons and dissociation of pre- and post-synaptic pathology at the neuromuscular junction in mouse models of spinal muscular atrophy. Hum Mol Genet. 2008; 17:949–962.

52. Kim J-K, Caine C, Awano T, Herbst R, Monani UR. Motor neuronal repletion of the NMJ organizer, Agrin, modulates the severity of the spinal muscular atrophy disease phenotype in model mice. Hum Mol Genet. 2017; 26:2377–2385. doi:10.1093/hmg/ddx124

53. Feng Z, Lam S, Tenn EMS, Ghosh AS, Cantor S, Zhang W, et al. Activation of muscle-specific kinase (MuSK) reduces neuromuscular defects in the Delta7 mouse model of spinal muscular atrophy (SMA). Int J Mol Sci. 2021; 22:8015. doi:10.3390/ijms22158015

54. Tisdale S, Van Alstyne M, Simon CM, Mentis GZ, Pellizzoni L. SMN controls neuromuscular junction integrity through U7 snRNP. bioRxiv, 2021. doi: https://doi.org/10.1101/2021.08.31.458410.

55. Courtney NL, Mole AJ, Thomson AK, Murray LM. Reduced P53 levels ameliorate neuromuscular junction loss without affecting motor neuron pathology in a mouse model of spinal muscular atrophy. Cell Death Dis. 2019; 10. doi:10.1038/s41419-019-1727-6

56. Reedich EJ, Kalski M, Armijo N, Cox GA, DiDonato CJ. Spinal motor neuron loss occurs through a p53-and-p21-independent mechanism in the Smn2B/- mouse model of spinal muscular atrophy. Exp Neurol. 2021; 337. doi:10.1016/j.expneurol.2020.113587

57. Murray LM, Beauvais A, Gibeault S, Courtney NL, Kothary R. Transcriptional profiling of differentially vulnerable motor neurons at pre-symptomatic stage in the Smn (2b/-) mouse model of spinal muscular atrophy. Acta Neuropathol Commun. 2015; 3:55. doi:10.1186/s40478-015-0231-1
Fig 1. Behavioral characterization of Smn<sup>2B/+</sup> SMA mice. (A) Body weight of control Smn<sup>2B/+</sup> (n=32) mice and Smn<sup>2B/-</sup> SMA mice (n=31). Data represent mean and SEM. Statistics were performed with two-way ANOVA and Bonferroni’s multiple comparison test. ** P < 0.01; **** P < 0.0001. (B) Kaplan-Meier survival curves from the same experimental groups as in (A). Statistics were performed with Log-rank (Mantel-Cox) test. **** P < 0.0001. (C) Righting time from the same experimental groups shown in (A). Data represent mean and SEM. Statistics were performed with two-way ANOVA and Bonferroni’s multiple comparison test. Not Significant. (D) Time to fall in the hindlimb suspension test from the same experimental groups shown in (A). Data represent mean and SEM. Statistics were performed with two-way ANOVA and Bonferroni’s multiple comparison test. *** P < 0.001; **** P < 0.0001.
Fig 2. *Smn*^2B^−^−^ SMA mice display severe loss of proprioceptive synapses on motor neurons. (A) Immunostaining of VGlut1^+^ synapses (grayscale) and ChAT^+^ motor neurons (blue) in the L2 spinal cord from control (*Smn*^2B^+/+) and SMA (*Smn*^2B^-^−^−^) mice at P16. Scale bars: 25 μm. (B) Number of VGlut1^+^ synapses on the soma of L2 motor neurons from the same groups as in (A) at P16. The box-and-whiskers graph shows the individual values, median, interquartile range, minimum and maximum from 5 mice per group. Statistics were performed with two-tailed unpaired Student’s t-test. **** P < 0.0001.
Figure 3

(A) Quadratus lumborum

(B) Percentage of fully innervated NMJs from the same groups as in (A) and (C). The box-and-whiskers graph shows the individual values, median, interquartile range, minimum and maximum from Smn<sup>2B/+</sup> (n=4) and Smn<sup>2B−</sup> (n=5) mice. Statistics were performed with two-tailed unpaired Student’s t-test. **** P < 0.0001, ns=not significant.

Figure 3. Selective loss of NMJ innervation in axial but not distal muscles in Smn<sup>2B−</sup> SMA mice. (A and C) NMJ staining with bungarotoxin (BTX), Synaptophysin (SYP), and Neurofilament-M (NF-M) of the axial muscle quadratus lumborum (A) and the distal muscle tibialis anterior (C) from control (Smn<sup>2B/+</sup>) and SMA (Smn<sup>2B−</sup>) mice at P16. Arrowheads indicate denervated NMJs. Scale bars: 25 μm. (B and D) Percentage of fully innervated NMJs from the same groups as in (A) and (C). The box-and-whiskers graph shows the individual values, median, interquartile range, minimum and maximum from Smn<sup>2B/+</sup> (n=4) and Smn<sup>2B−</sup> (n=5) mice. Statistics were performed with two-tailed unpaired Student’s t-test. **** P < 0.0001, ns=not significant.
Figure 4

(A) ChAT immunostaining of motor neurons in the L1 spinal segment from control (Smn<sup>2B/+</sup>) and SMA (Smn<sup>2B/-</sup>) mice at P16. Scale bars: 50μm. (B) Total number of L1 motor neurons from the same groups as in (A). The box-and-whiskers graph shows the individual values, median, interquartile range, minimum and maximum from 5 mice per group. Statistics were performed with two-tailed unpaired Student’s t-test. ns=not significant. (C) ChAT immunostaining of motor neurons in the L2 spinal segment from control (Smn<sup>2B/+</sup>) and SMA (Smn<sup>2B/-</sup>) mice at P16. Scale bars: 50μm. (D) Total number of L2 motor neurons from the same groups as in (C). The box-and-whiskers graph shows the individual values, median, interquartile range, minimum and maximum from 5 mice per group. Statistics were performed with two-tailed unpaired Student’s t-test.

Fig 4. Death of lumbar motor neurons is not a disease feature of Smn<sup>2B/-</sup> SMA mice. (A) ChAT immunostaining of motor neurons in the L1 spinal segment from control (Smn<sup>2B/+</sup>) and SMA (Smn<sup>2B/-</sup>) mice at P16. Scale bars: 50μm. (B) Total number of L1 motor neurons from the same groups as in (A). The box-and-whiskers graph shows the individual values, median, interquartile range, minimum and maximum from 5 mice per group. Statistics were performed with two-tailed unpaired Student’s t-test. ns=not significant. (C) ChAT immunostaining of motor neurons in the L2 spinal segment from control (Smn<sup>2B/+</sup>) and SMA (Smn<sup>2B/-</sup>) mice at P16. Scale bars: 50μm. (D) Total number of L2 motor neurons from the same groups as in (C). The box-and-whiskers graph shows the individual values, median, interquartile range, minimum and maximum from 5 mice per group. Statistics were performed with two-tailed unpaired Student’s t-test.
ns=not significant. (E) ChAT immunostaining of motor neurons in the L5 spinal segment from control (Smn<sup>2B/+</sup>) and SMA (Smn<sup>2B/−</sup>) mice at P16. Scale bars: 50μm. (F and G) Total number of L5 LMC (F) and L5 MMC (G) motor neurons from the same groups as in (E). The box-and-whiskers graph shows the individual values, median, interquartile range, minimum and maximum from Smn<sup>2B/+</sup> (n=4) and Smn<sup>2B/−</sup> (n=5) mice. Statistics were performed with two-tailed unpaired Student’s t-test. ns=not significant.
Figure 5

A

L1 spinal cord

| Smn2B\(^{+}\) | Smn2B\(^{-}\) |
|----------------|----------------|

B

CHAT p53

C

CHAT p-p53(S18)

D

![Bar chart showing p53\(^{+}\) L1 MNs (%)]

E

![Bar chart showing p-p53\(^{+}\) L1 MNs (%)]
Fig 5. Smn deficiency induces p53 accumulation but not serine 18 phosphorylation in L1 motor neurons of Smn

2B- SMA mice. (A) ChAT and p53 immunostaining of the L1 spinal cord from control (Smn

2B/+ ) and SMA (Smn

2B/- ) mice at P16. Scale bars: 100 µm. (B) ChAT and p53 immunostaining of L1 motor neurons from the same groups as in (A). Scale bars: 50 µm. (C) ChAT and phospho-p53S18

immunostaining of L1 motor neurons from the same groups as in (A). Scale bars: 50 µm. (D) Percentage of p53

+ L1 motor neurons from the same groups as in (A). (E) Percentage of phospho-p53S18+ L1 motor neurons from the same groups as in (A). Data represents individual values, mean and SEM from 3 mice per group. Statistics were performed with two-tailed unpaired Student’s t-test. *** P < 0.001.
**Figure S1**

**A**

| Smn^{2B/2B} | Smn^{+/+} |
|-------------|-----------|
| Smn         |           |
| Tubulin     |           |

**B**

| Smn^{2B/+} | Smn^{2B/-} |
|------------|------------|
| Smn        |            |
| Tubulin    |            |

**Fig S1. Analysis of Smn expression from the Smn^{2B} allele in the mouse spinal cord.** (A) Western blot analysis of Smn levels in the spinal cord from Smn^{+/+} (wild type) and Smn^{2B/2B} mice at P16. (B) Western blot analysis of Smn levels in the spinal cord from Smn^{2B/+} an Smn^{2B/-} mice at P16. Two-fold serial dilutions of equal amounts of extracts are shown. Tubulin was probed as a loading control.
Fig S2. Smn deficiency induces p53 accumulation but not serine 18 phosphorylation in L2 motor neurons of Smn2B−/− SMA mice. (A) ChAT and p53 immunostaining of the L2 spinal cord from control (Smn2B+/+) and SMA (Smn2B−/−) mice at P16. Scale bars: 100 µm. (B) ChAT and p53 immunostaining of L2 motor neurons from the same groups as in (A). Scale bars: 50 µm. (C) ChAT and phospho-p53S18 immunostaining of L2 motor neurons from the same groups as in (A). Scale bars: 50 µm. (D) Percentage of p53+ L2 motor neurons from the same groups as in (A). (E) Percentage of phospho-p53S18+ L2 motor neurons from the same groups as in (A). Data represents individual values, mean and SEM from 3 mice per group. Statistics were performed with two-tailed unpaired Student’s t-test. ** P < 0.01.
Fig S3. Smn deficiency induces p53 accumulation but not serine 18 phosphorylation in L5 motor neurons of Smn<sup>2B−</sup>/SMA mice. (A) ChAT and p53 immunostaining of the L5 spinal cord from control (Smn<sup>2B+</sup>) and SMA (Smn<sup>2B−</sup>) mice at P16. Scale bars: 100 µm. (B) ChAT and p53 immunostaining of L5 motor neurons from the same groups as in (A). Scale bars: 50 µm. (C) ChAT and phospho-p53<sup>S18</sup> immunostaining of the L5 spinal cord from the same groups as in (A). Scale bars: 100 µm. (D) ChAT and phospho-p53<sup>S18</sup> immunostaining of L5 motor neurons from the same groups as in (A). Scale bars: 50 µm. (E and G) Percentage of p53<sup>+</sup> L5 LMC (E) and L5 MMC (G) motor neurons from the same groups as in (A). (F and H) Percentage of phospho-p53<sup>S18+</sup> L5 LMC (F) and L5 MMC (H) motor neurons from the same groups as in (A). Data represents individual values, mean and SEM from 3 mice per group. Statistics were performed with two-tailed unpaired Student’s t-test. ** P < 0.01; * P < 0.05.