Astrocytes influence the severity of spinal muscular atrophy

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Abstract
Systemically low levels of survival motor neuron-1 (SMN1) protein cause spinal muscular atrophy (SMA). α-Motor neurons of the spinal cord are considered particularly vulnerable in this genetic disorder and their dysfunction and loss cause progressive muscle weakness, paralysis and eventually premature death of afflicted individuals. Historically, SMA was therefore considered a motor neuron-autonomous disease. However, depletion of SMN in motor neurons of normal mice elicited only a very mild phenotype. Conversely, restoration of SMN to motor neurons in an SMA mouse model had only modest effects on the SMA phenotype and survival. Collectively, these results suggested that additional cell types contribute to the pathogenesis of SMA, and understanding the non-autonomous requirements is crucial for developing effective therapies. Astrocytes are critical for regulating synapse formation and function as well as metabolic support for neurons. We hypothesized that astrocyte functions are disrupted in SMA, exacerbating disease progression. Using viral-based restoration of SMN specifically to astrocytes, survival in severe and intermediate SMA mice was observed. In addition, neuromuscular circuitry was improved. Astrogliosis was prominent in end-stage SMA mice and in post-mortem patient spinal cords. Increased expression of proinflammatory cytokines was partially normalized in treated mice, suggesting that astrocytes contribute to the pathogenesis of SMA.

Introduction
Spinal muscular atrophy (SMA) occurs with a frequency of 1 in 11,000 newborns and is the most common inherited cause of infantile death (1). It is caused by the loss or functional inactivation of the gene survival motor neuron 1 (SMN1), leading to dysfunction of α-motor neurons in the spinal cord. SMN is expressed in all cell types, however it is unclear why motor neurons are particularly susceptible to reduced SMN levels.

SMA is widely considered a disorder caused by motor neuron dysfunction (2). However, recent findings have raised the possibility...
that the disease phenotype is not motor neuron-autonomous. In particular, transgenic ablution of SMN in motor neurons using an olig2-cre driver while maintaining normal expression levels in the rest of the animal induced only minor phenotypic abnormalities and did not shorten lifespan (3). Conversely, restoration of SMN specifically to motor neurons has met with unexpectedly minor phenotypic improvements even with seemingly robust SMN expression within neuronal populations. Using an Hb9-cre transgenic driver resulted in only a marginal extension of lifespan in two different mouse models of SMA (4,5). Similarly, restoring SMN to motor neurons using a ChAT-cre driver bred to the conditional inversion model (6) had only small effects on lifespan and gross motor function (7,8). Using a pan-neuronal driver, the prion protein (PrP) promoter, severe SMA mice were largely rescued to wild-type levels, however the PrP promoter is also leaky in a variety of tissues, including astrocytes (9). Although it is clear that motor neuron integrity is an essential component of SMA, collectively, these results suggest the importance of additional tissues (10).

Non-neuronal cells play an important role in the homeostasis of the CNS. In particular, astrocytes regulate blood flow and availability of oxygen, supply nutrients and survival factors to neurons, participate in the maintenance of synapses and modulate synaptic transmission. We hypothesized that low levels of SMN compromise astrocyte function and contribute to the pathology of SMA. To address this point, SMN expression was restored specifically to astrocytes in SMA mouse models in which SMN is at pathologically low levels throughout the rest of the organism. We observed increased lifespans and improvements in neuromuscular junctions (NMJs) as well as the numbers of vGLUT1+ motor neurons (11,12). To confirm the specificity in our experimental system, we generated a reporter virus, scAAV-GFP<sup>ΔP</sup>. Reporter-derived expression was appropriately restricted, as gfap-driven green fluorescent protein (GFP) was not detected in cervical or lumbar motor neurons (Fig.1B). Consistent with prior reports, low GFP expression was detected within peripheral tissues including the heart, liver and kidney compared with untreated tissues (Supplementary Material, Fig. S1). Having established expression specificity, 1.3 × 10<sup>11</sup> viral genomes of scAAV-SMN<sup>ΔP</sup> were delivered systemically to the severe SMNΔ7 mice via facial vein injection at postnatal day (PND) 2. This was the same route and dose for the scAAV-GFP<sup>ΔP</sup> vector. The SMNΔ7 mouse is a widely used model and recapitulates features of SMA, including loss of lower motor neurons, paralysis and premature death before weaning age (14). At PND 12, a time point at which the untreated SMA mice are fully symptomatic, an increase in SMN protein expression was detected in spinal cord lysates (Fig. 1C). This is not surprising given that ~65% of the cells in the mouse CNS are astrocytes (15). Importantly, survival of treated mice was nearly doubled as median survival of untreated SMA mice was 16 days versus 33 days for scAAV-SMN<sup>ΔP</sup>-treated SMA mice (Fig. 2A). Weight gain was significantly improved in parallel (Fig. 2B). Likewise, gross motor function as measured by the righting reflex, an indicator of overall muscle function in pre-weanlings (16), was improved (Fig. 2C).

Results

Restoring SMN in astrocytes improves lifespan and gross motor function

The initial questions we sought to address focused on SMN’s role in disease pathogenesis in astrocytes and whether specific restoration of SMN within astrocytes in an otherwise SMA-like background would decrease disease severity. To direct SMN expression within astrocytes in transgenic SMA model mice, we generated an expression cassette in which full-length SMN was driven by the astrocyte-specific glial fibrillary acidic protein (gfap) promoter (11,12). The expression cassette was cloned into the self-complementary adeno-associated virus (AAV) vector to generate scAAV-SMN<sup>ΔP</sup> (Fig. 1A) which was pseudotyped with the AAV serotype 9 capsid. AAV9 crosses the incompletely formed blood-brain barrier in newborn mice and effectively transduces cells of the central nervous system (CNS), including astrocytes (13). Although AAV9 also enters many cell types within the CNS, our central question is predicated on maintaining pathologically low levels of SMN in motor neurons. Therefore, to further ensure vector-derived SMN protein expression was restricted from neurons, the 3′ untranslated region (UTR) of the SMN transcript was engineered to include recognition sites for neuronally expressed miRNAs (miR-128 and -124). The gfap promoter used in this study has been characterized previously and expressed highly in astrocyte populations and at undetectable levels within motor neurons (11,12). To confirm the specificity in our experimental system, we generated a reporter virus, scAAV-GFP<sup>ΔP</sup>. Reporter-derived expression was appropriately restricted, as gfap-driven green fluorescent protein (GFP) was not detected in cervical or lumbar motor neurons (Fig. 1B). Consistent with prior reports, low GFP expression was detected within peripheral tissues including the heart, liver and kidney compared with untreated tissues (Supplementary Material, Fig. S1). Having established expression specificity, 1.3 × 10<sup>11</sup> viral genomes of scAAV-SMN<sup>ΔP</sup> were delivered systemically to the severe SMNΔ7 mice via facial vein injection at postnatal day (PND) 2. This was the same route and dose for the scAAV-GFP<sup>ΔP</sup> vector. The SMNΔ7 mouse is a widely used model and recapitulates features of SMA, including loss of lower motor neurons, paralysis and premature death before weaning age (14). At PND 12, a time point at which the untreated SMA mice are fully symptomatic, an increase in SMN protein expression was detected in spinal cord lysates (Fig. 1C). This is not surprising given that ~65% of the cells in the mouse CNS are astrocytes (15). Importantly, survival of treated mice was nearly doubled as median survival of untreated SMA mice was 16 days versus 33 days for scAAV-SMN<sup>ΔP</sup>-treated SMA mice (Fig. 2A). Weight gain was significantly improved in parallel (Fig. 2B). Likewise, gross motor function as measured by the righting reflex, an indicator of overall muscle function in pre-weanlings (16), was improved (Fig. 2C).

**Figure 1.** Construction and expression of the scAAV-SMN<sup>ΔP</sup> viral delivery module. (A) In the scAAV expression construct, the full-length human SMN1 cDNA was driven by the gfapABC<sub>1D</sub> promoter which is highly specific for astrocytes (11). Tandem binding sites for miR-128 and miR-124, which are expressed intrinsically in neurons, but not in astrocytes, were added to the 3′ UTR of the construct to further enhance the specificity of expression. The miR binding sites were inserted after the SMN coding region and before the polyadenylation site (pA). ITR, inverted terminal repeat. (B) Specificity of the gfap promoter. Unaffected mice were injected with scAAV-gfp<sup>ΔP</sup>. Spinal cords of PND 12 mice were fixed, dissected and processed for immunofluorescence. Motor neurons were highlighted with an antibody against choline acetyltransferase (ChAT). No gfp expression was detected in motor neurons in the lumbar or the cervical spinal cord. (C) Protein extracts from unaffected, untreated SMNΔ7 mice and SMNΔ7 mice treated with scAAV-SMN<sup>ΔP</sup> were detected by western blotting for SMN and a loading control (calnexin/IP90).
 SMA presents in an exceptionally broad clinical spectrum and the SMN7 mice, while valuable, likely represent only very severe cases of SMA. To determine whether the scAAV-SMN7 vector would result in a more profound phenotypic rescue in mild SMA mice, an equivalent dose was administered to a less severe SMA model: SMN7/− mice which have a lifespan of ∼30–35 days (17). Complementary to the results obtained using the severe model, scAAV-SMN7 vector conferred a dramatically extended lifespan to this milder model (Fig. 2D). In fact, all treated animals were alive 100 days of age and appeared to function normally and as anticipated based upon the lifespan data, treated mice weighed significantly more than untreated SMA mice and approached average weights similar to unaffected mice (Fig. 2E).

Defects in NMJs and proprioceptive synapses are partially rescued

Defects in NMJs and denervation of endplates in a set of susceptible muscles are a hallmark of SMA pathogenesis (18). The observed increase in motor function in scAAV-SMN7 vector-treated mice suggested that neuromuscular circuitry was improved. To determine the impact of astrocytic SMN restoration, we analyzed two muscles affected in SMA: splenius capitis and longissimus capitis. SMN7 mice receiving scAAV-SMN7 vector were compared with untreated littermates and unaffected controls at PND 12. Administration of scAAV-SMN7 vector corrected NMJ abnormalities in these muscles, as evidenced by the increased maintenance of fully innervated NMJs when compared with untreated cohorts (Fig. 3A and B). Conversely, the number of denervated NMJs was also reduced (Supplementary Material, Fig. S2). To assess the impact of the treatment regimen on overall muscle growth, we examined the extensor digitorum longus (EDL) which is atrophic in SMA mice. Administration of scAAV-SMN7 vector partially rescued the EDL size, likely reflecting the increased overall growth and body weight of the treated animals (Fig. 3C). Interestingly, in treated spinal cords, the number of lumbar motor neurons was unchanged compared with untreated SMA mice (Fig. 3D and E). Furthermore, the number of ventral root axons was increased only marginally (Fig. 3F). These results are in contrast to motor neuron numbers in SMA animals treated with a ubiquitously expressing vector in which motor neuron numbers are restored to near wild-type levels (19), suggesting an alternative mechanism in which SMN expression within astrocytes creates the appropriate cellular context to maintain functionality of SMA motor neurons.

We next investigated the presence of sensory synapses on lower motor neuron somata. Previously, it was reported that the reduction of vGLUT1+ synapses is a pathological feature of SMA in the SMN7 mice (20,21). Interestingly, NMJ morphology improvements were accompanied by an increase in the number of vGLUT1+ synapses, suggesting that scAAV-SMN7 vector improved existing motor neurons and enhanced maintenance of sensory synapses on motor neurons (Fig. 3G and H). Thus, astrocyte-mediated maintenance of higher vGLUT1+ bouton densities may contribute to the observed improvements of scAAV-SMN7 vector-treated mice.

Human SMA spinal cords present with astrogliosis

Glia-mediated neuroinflammation is a component of many neurodegenerative disorders (22). Activation of astrocytes and microglia may result in the production of proinflammatory cytokines that are thought to influence the severity or progression of CNS disorders, such as amyotrophic lateral sclerosis and Rett’s syndrome. Whether low levels of SMN in SMA astrocytes contribute to inflammatory responses or the modulation of neurotoxic mediators is not known. In order to assess whether neuroinflammation exists in SMA, thin sections of spinal cords from SMA patients and non-SMA control individuals (for patient sample characteristics, see Supplementary Material, Table S1) were stained with an antibody against GFAP, a marker for astrocyte activation. Then, GFAP+ cells were quantified manually in both grey
and white matter of dorsal and ventral horns of each sample (Fig. 4). A cell density in 0.1 mm² was calculated and compared. The numbers of GFAP+ cells were increased in human SMA spinal cords, although regional differences were observed. Although GFAP staining was unchanged in the white matter, a significant increase was found in the grey matter of the ventral horn where α-motor neurons are located (Supplementary Material, Fig. S3). This is in agreement with earlier case studies showing overall increased GFAP staining intensities in SMA patient spinal cords although regional variations were not investigated in these studies (23,24). These histological findings were paralleled by an increase in proinflammatory markers which are produced by activated glia, including astrocytes. Using reverse transcriptase-polymerase chain reaction (RT-PCR) to determine the mRNA levels, we found a trend to increased IL-6 and a significant elevation of IL-1β, which are markers for glial activation (Supplementary Material, Fig. S3). These proinflammatory cytokines can be generated by astrocytes as well as microglia, the CNS-resident immune cells, and we cannot rule out the possibility that both cell types contribute to the elevation of these markers. Nevertheless, our results suggest that neuroinflammation plays a role in SMA and that astrocytes in patient spinal cords are activated.

**Astrocyte activation is partially normalized in scAAV-SMNgfap-treated mice**

After confirming the presence of astrogliosis in patient spinal cords, we were interested in determining whether the widely used SMNΔ7 mouse recapitulates this facet of SMA. To this end, thin sections of lumbar spinal cords were prepared from mice at PND 4 which is considered a pre-symptomatic time point, to PND 14 at which time SMA-related deficits are apparent in this model. GFAP staining became progressively more widespread during this period in SMNΔ7 mice, but not in unaffected cohorts (Fig. 5A). This is in accordance with findings by Dachs et al. (25) who reported an increase in astroglia in a different, very severe mouse model of SMA. Using RT-PCR to determine mRNA abundance in lumbar spinal cords, we found progressively increasing expression levels of IL-1β, IL-6 and TNFα in SMNΔ7 mice when compared with unaffected controls (Supplementary Material, Fig. S4). Interestingly, SMNΔ7 mice receiving scAAV-SMNgfap
involved in the pathogenesis of SMA and provide evidence that a successful therapeutic must address SMN deficiency in a multitude of tissues to elicit the most robust response.

**Discussion**

The development of effective therapies for SMA depends on the identification and characterization of pathological changes across tissues and cell types. Although SMA has been widely regarded as a motor neuron-autonomous disorder in the past, a number of recent studies have challenged this view. SMN is a ubiquitously expressed protein, and homozygous deletion results in embryonic lethality (27). Motor neurons appear particularly susceptible to low levels of SMN, but tissues outside the CNS also present pathological changes in SMA mice, suggesting that SMA is rather a multi-system disorder (for review, see (10)). Cell-type-specific manipulation of SMN levels has been employed to explore the cell autonomy of SMA. Interestingly, motor neuron-specific ablation or restoration of SMN has not generated the phenotypes expected from a motor neuron-autonomous disease. Decreasing SMN levels in motor neurons using an inducible SMN allele and an olig2-cre driver to knockout SMN in motor neuron precursors resulted in a very mild, late-onset SMA-like phenotype (3). Conversely, inducing SMN expression using the motor neuron-specific Hb9-cre driver in an SMA mouse had only marginal effects on survival and motor function, but prevented the loss of vGLUT1+ synapses (4). Using a different inducible model in conjunction with the Hb9-cre driver did not result in significant improvements in survival and motor behavior either, although NMJ denervation was partially alleviated (5). Furthermore, restoring SMN to motor neurons with a ChAT-cre driver extended the lifespan of the SMNΔ7 mouse by about 50%, similar to muscle-specific restoration with a MyoD-cre driver (7). These reports suggest that motor neurons are not the only cell type contributing to the disease phenotype.

The phenotypic rescue obtained by the scAAV-SMNΔ7 treatment is predictably less than that observed with ubiquitous SMN expression constructs as delivery of scAAV-SMNΔ7 extends the lifespan of the SMNΔ7 mouse to ~1 year (28–32). This robust rescue with the ubiquitous expression cassette illustrates that SMA is a complex disease and multiple tissues are clearly involved, some are primary and others are secondary defects. In this report, we also observed gfap-driven transgene expression in peripheral tissues, including cardiac tissue which may also contribute to improvements in the phenotype. The purpose of this study, however, was not to fully rescue the SMA animals; rather, the hypothesis was: SMN restoration in astrocytes will improve the SMA phenotype. Our results indicate that SMA is a non-cell-autonomous disease and that astrocytes, and perhaps other tissues, contribute to the complex SMA pathology.

Constructing the appropriate cellular milieu for motor neurons is an important function of astrocytes and it is therefore not surprising that astrocytes contribute to the pathology of other neurological disorders (33,34). To begin to address the role of astrocytes in SMA, we sought to restore SMN expression specifically in astrocytes. Using the SMNΔ7 mouse and the less severe SmnΔmice model, we employed an AAV virus-based delivery to increase SMN expression specifically in astrocytes. Both resulted in a significant increase in lifespan, and further analysis of the AAV model also showed improved motor function and NMJ maturation (Figs 2 and 3). Collectively, our results support the hypothesis that SMA astrocytes exacerbate the SMA phenotype. Interestingly, recent work using ipscells shows that morphological and cellular changes in astrocytes precede overt motor

demonstrated a partial normalization of glial activation markers. Although TNFα was unchanged, the levels of IL-1β and IL-6 remained similar to those in unaffected controls (Supplementary Material, Fig. S4). These results suggest that restoration of SMN to astrocytes could exert its beneficial effect by preventing or reducing glial activation. Our data suggest that low levels of SMN in astrocytes lead to progressive astrocyte activation which in turn impacts the phenotype in SMA. This notion is supported by recent findings that SMA astrocytes derived from induced pluripotent stem (iPS) cells demonstrate morphologic and cellular changes and that these defects can be detected prior to the onset of motor neuron loss (26). Consistent with these results, human iPS cell-derived SMA astrocytes express unexpectedly low levels of SMN protein, manifesting in significantly shorter cellular processes (Fig. 5B). Lenti vector-mediated delivery of full-length SMN cDNA increases SMN expression and largely restores astrocyte process length to wild-type levels (Fig. 5B). Together, these results identify astrocytes as an important cell type.
neuron loss, suggesting that early astrocytic dysfunction affects the development of SMA (26). Astrocyte defects were largely corrected by restoring SMN levels (Fig. 5D).

Previous work demonstrated that SMNΔ7 mice showed a progressive loss of central synapses. In particular, glutamatergic excitatory synapses characterized by the expression of vGLUT1 and vGLUT2 were affected, whereas the number of GABAergic inhibitory synapses expressing vGAT remained unchanged (20). The majority of vGLUT+ synapses found on motor neurons transmit proprioceptive returns from sensory neurons (35). We confirmed the loss of vGLUT1+ synapses on motor neuron somata and showed that this deficit can be partially prevented by restoring SMN in astrocytes (Fig. 3). Remarkably, SMN restoration in astrocytes did not increase motor neuron populations. The observed phenotypical improvements suggest that the treatment rather protected and functionally enhanced the remaining motor neurons. Given the importance of the proprioceptive circuitry and the contribution of astrocytes to synaptic integrity, it is tempting to speculate that ‘rescued’ astrocytes enhance the functionality of the remaining motor neurons by preserving their sensory input from the periphery. This is in contrast to global induction of SMN via antisense oligonucleotides or viral vectors in which motor neuron populations are restored to wild-type levels (19,36).

The functional improvements and lifespan extension following astrocytic SMN restoration prompted us to investigate the possibility of glial activation in SMA. Neuroinflammation is a recognized component of many neurodegenerative disorders (22), but evidence in SMA is scant. We therefore assessed GFAP immunoreactivity as a marker for astrocyte activation in patient spinal cords and found increased GFAP staining compared with non-SMA controls, together with increased mRNA levels of proinflammatory cytokines. Astrocyte activation was also readily detectable in the SMNΔ7 mouse. Importantly, astrocyte rescue led to a partial normalization of cytokine expression (Fig. 5), suggesting that SMA astrocytes actively contribute to the pathogenesis of SMA. As mRNAs were measured in extracts from whole spinal cords, we cannot exclude the possibility that the cytokines were produced by microglia and that rescued astrocytes attenuated microglial activation through cross-talk between the two cell types. Indeed, increased immunoreactivity to Iba-1, a microglia marker, was observed in the SMNΔ7 mouse (20,37). To address this question, the potential interaction between these two cell types in SMA needs to be investigated in the future.

Our results indicate that non-neuronal cells, specifically astrocytes, play a role in SMA. Restoration of SMN specifically in astrocytes improved survival, motor function, NMJ morphology and vGLUT1+ synaptic bouton numbers. This suggests that SMA astrocytes contribute to the pathology of SMA and that glial activation plays an active role in this neurodegenerative disorder.

**Materials and Methods**

**Animals**

All mice were housed and handled in accordance with the Animal Care and Use Committees of the University of Missouri and the University of Southern California. SMNΔ7 mice were purchased from Jackson Labs (stock numbers 5025 and 7951), SMNΔ7Δ7 mice, which are a mixed genetic background of C57BL/6 and CD1, were a kind gift from Dr R. Kothary, Ottawa, Canada. Mice were housed under a 12 h light/dark cycle. SMNΔ7 mice used in this study had a homozygous deletion of the murine Smn gene and carried homozygous transgenes for human SMN2 and an SMN cDNA lacking exon 7. Animals live to ∼14 days of age and show signs of progressive muscle paralysis, NMJ abnormalities and a reduction in lower motor neurons. SmmΔ7Δ7 mice contain a targeted mutation in exon 7 of the Smn gene, show signs of SMA and have an average lifespan of ∼30 days (17). To assess gross motor function, the righting reflex was measured in pre-weanling mice. This is a sensitive and commonly performed test for SMA mice (16). Mice were genotyped using tail biopsies on the day of birth (PND 1) and assigned to treatment groups in random order as they became available. Litters were culled to five or fewer animals to minimize competition with unaffected littersmates. Animals of both sexes were used for all studies. Virus was administered systemically by injection of the facial vein on PND 2 mice as described (38). Mice that did not live at least 24 h following injection were excluded. At PND 2, the blood–brain barrier is not completely formed, and AAV delivered systemically will effectively penetrate into the CNS. Serotype 9 was chosen because it efficiently transduces astrocytes.

**RNA isolation and RT-PCR**

Total RNA from animal tissue was isolated with TriReagent (Sigma) and reverse-transcribed using Superscript III (Invitrogen). Real-time PCR was performed using Quant AR SYBR Green reagent. Data analysis was performed with the ABI System 7500 software. Test values were normalized to an internal housekeeping control (rpl7), and ΔACt values were calculated to determine the relative fold difference between unaffected and untreated SMA mice, and unaffected and scAAV-SMNΔ7Δ7-treated mice, respectively. Total RNA from human control and SMA thoracic tissue was isolated using Trizol reagent (Invitrogen) and RNeasy Kit (Qiagen). Isolated RNA was converted into cDNA via High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Reactions were run in triplicate using the ABI Prism 7900 Sequence Detector System. Relative levels of target gene expression (IL-1β, IL-6 and GFAP) were analyzed in reference to housekeeping gene 18s and normalized to a calibrator spinal cord sample from a control patient.

**Virus generation and purification**

HEK293T cells were cultured in 80 150 mm plates or 2 10-floor cell factories until ∼85% confluent. The cells were then transfected with a 1:1:1 molar ratio of the scAAV-SMN expression plasmid, the helper plasmid and the rep2cap9 plasmid, and 3 μl of a 1 mg/ml solution of polyethyleneimine in phosphate-buffered saline (PBS) per μg of DNA as precipitating agent (39). Media was changed 24 h after transfection. Cells were harvested 48 h after transfection and lysed by 3–4 freeze–thaw cycles in liquid nitrogen and the lysate treated with DNAse and protease. CsCl crystals were added to the lysate (0.631 g of CsCl per ml of the lysate) to generate a solution with a density of ∼1.4 mg/ml. After incubation at 37°C for 45 min, the solution was centrifuged at 4000 rpm in an Eppendorf 5810 R at 4°C. Following centrifugation, the debris at the top of the tubes was discarded, and the lysate was then ultracentrifuged for 48 h at 18°C at an average RCF of 158 000. Fractions of 500 μl were collected, diluted 1500-fold with water and the number of viral genomes was determined by real-time PCR using primers located in the intron of the expression construct. Fractions with the highest titer were combined and subjected to two additional ultracentrifugations. The functional improvements and lifespan extension follow-
were labeled with anti-choline acetyltransferase (ChAT, Chemicon) and anti-vesicular glutamate transporter 1 (vGLUT1) antibodies, respectively. Spinal cord sections were imaged using 20× (for motor neurons number) or 63× oil immersion (for vGLUT1 synapses) objectives on the Zeiss LSM confocal microscope with 5 and 1 μm serial intervals, respectively. All confocal images were taken using the same imaging parameters (e.g. laser intensities, amplification gains and offsets). Immuno-labeled axosomatic synapses were identified as boutons apposed to membrane of motor neuron somata and proximal dendrites (<50 μm from soma) with no visible intervening space. The number of synaptic boutons per motor neuron was manually counted.

**Immunofluorescence**

To visualize NMJs, experimental mice were euthanized at PND 12, cardiac perfused with PBS followed by buffered formalin and postfixed in buffered formalin for 2 h. Splenius capitis and longissimus capitis muscles, which are susceptible to denervation in SMA (18), were dissected and fibers were teased to facilitate penetration of antibodies. Whole mount preparations were used to quantify NMJ innervation. Muscle fibers were stained with anti-synaptophysin (Syn, Chemicon) marking the pre-synaptic terminal, anti-neurofilament to visualize axons and α-bungarotoxin (α-BTX, Invitrogen) staining the post-synaptic terminal. Fluorescently labeled NMJs were observed with epifluorescence or confocal microscopes and classified as fully innervated, partially innervated and denervated based on the co-localization between Syn and α-BTX as described (20). Further information is provided in the Supplementary Materials and methods.

**Motor axons**

To count motor axons, L4 ventral roots were fixed with glutaraldehyde, followed by osmium oxide incubation and embedding in Epon resin. One-micron cross-sections were stained with Toluidine blue and examined with light microscopy to count the number of myelinated axons.

**Muscle morphology**

To examine muscle morphology, the EDL muscle was fixed with glutaraldehyde, followed by osmium oxide incubation and embedding in Epon resin. One-micrometer-thick cross-sections were stained with Toluidine blue and examined by light microscopy. The size of the muscle cross-sectional area was measured using NIH Image software.

**iPS cells**

iPS cells derived from two independent Type I SMA patients and two independent unaffected individuals were used for this study. Each SMA iPS cell line was infected at the colony stage with a lentiviral vector containing both the CMV promoter driving SMN expression and EF1α promoter driving GFP expression. GFP-positive colonies were clonally selected for expansion. All iPS cells were maintained as floating spheres of neural progenitor cells until undergoing terminal differentiation (40).

**Astrocyte differentiation**

iPS cells were driven toward an astrocyte lineage as previously described (26). Briefly, progenitor cell spheres were dissociated and plated onto matrigel-coated glass coverslips (50,000 cells/coverslip) or cell culture plates (500,000 cells per well) and cultured in DMEM/F12 plus 2% B27 supplement (Life Technologies) for up to 6 weeks.

**iPS cell immunohistochemistry and western blotting**

Coverslips were collected 4 and 6 weeks after platedown and fixed using 4% paraformaldehyde for 20 min. Cells were blocked and permeabilized in 0.2% Triton X-100 and 5% normal goat serum for 30 min at room temperature, then incubated in mouse anti-GFAP primary antibody (Cell Signaling 3670) for 1 h at room temperature and goat anti-mouse RhoRed secondary antibody (Life Technologies R6393) for 1 h at room temperature. Coverslips were counterstained with Hoechst nuclear stain and mounted onto slides using FluoroMount media (SouthernBiotech 0100-01). Images were taken after coverslips from at least three independent differentiations per genotype. Five random fields per coverslip were taken at 40× magnification using a Nikon inverted microscope and Spot imaging software (Nikon Instruments, Inc., Melville, NY). Images were blinded for analysis. Cell process lengths were measured using MetaMorph Software (Molecular Devices, Inc., Sunnyvale, CA) and compared by unpaired Student’s t-test between groups with GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA; α = 0.05). Whole-cell lysates were prepared from iPSCs at neural progenitor cell stage and after 6 weeks of differentiation using Chaps buffer (Cell Signaling 9852S). Protein concentration was determined by Bradford Assay (Sigma-Aldrich B6916). Ten micrograms of protein/lane was separated in a 12% polyacrylamide gel (Bio-Rad) and transferred to polyvinylidene fluoride membrane. Membrane was blocked using 5% milk in tris-buffered saline and tween 20, incubated in primary antibody overnight at 4°C and secondary antibody for 1 h at room temperature before exposure. Primary antibodies used were mouse SMN (BD Biosciences 610646) or rabbit GAPDH (Sigma Aldrich G9545). Secondary antibodies used were anti-mouse HRP (Promega W402B) or anti-Rabbit HRP (Promega W4011).

**Human spinal cords**

SMA and control human tissue was collected at autopsy following parental informed consent as approved by the Johns Hopkins School of Medicine Institutional Review Board and the University of Utah Institutional Review Board. Some control human tissue was obtained from the Human Development Brain and Tissue Bank for Developmental Disorders at the University of Maryland, Baltimore, MD (Supplementary Material, Table S1). Thoracic spinal cord sections were processed for immunofluorescence with antibodies against SM132 to view neurons and against GFAP which serves as a cell-specific marker for differentiated and activated astrocytes. For purposes of quantitation, the total areas of the dorsal and ventral horns were distinguished by drawing a line from the center of the central canal to the lateral edge of the spinal cord white matter. Using SM132 staining, a region of interest was delineated for both the grey and white matter for both the ventral and dorsal horns. Both SM132+ and GFAP+ cells were counted using Zen Zeiss software from images taken on a Zeiss Imager fluorescent microscope. Analyses were performed on three independent samples for each group. Due to varying total areas between different cases, cell density in 0.1 mm² was calculated for all samples.

**Statistical analyses**

Comparisons between two groups were performed by two-tailed Student’s t-tests. Kaplan-Meier survival curves were compared...
using log-rank (Mantel–Cox) tests. A $P$-value of <0.05 was considered significant. Calculations were performed using GraphPad Prism software. Unless indicated otherwise, the following notation were used to indicate statistical significance: ns, not significant; *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$. Unless stated otherwise, the sample size number ($n$) is reported in the figures where appropriate, shown inside the bars of bar graphs or in parentheses behind the experimental groups in line graphs.

**Supplementary Material**

Supplementary Material is available at HMG online.

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**Conflict of Interest statement. None declared.**

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