SMN deficiency does not induce oxidative stress in SMA iPSC-derived astrocytes or motor neurons

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Abstract

Spinal muscular atrophy (SMA) is a genetic disorder characterized by loss of motor neurons in the spinal cord leading to muscle atrophy and death. Although motor neurons (MNs) are the most obviously affected cells in SMA, recent evidence suggests dysfunction in multiple cell types. Astrocytes are a crucial component of the motor circuit and are intimately involved with MN health and maintenance. We have previously shown that SMA astrocytes are altered both morphologically and functionally early in disease progression, though it is unclear what causes astrocytes to become reactive. Oxidative stress is a common feature among neurodegenerative diseases. Oxidative stress can both induce apoptosis in neurons and can cause astrocytes to become reactive, which are features observed in the SMA induced pluripotent stem cell (iPSC) cultures. Therefore, we asked if oxidative stress contributes to SMA astrocyte pathology. We examined mitochondrial bioenergetics, transcript and protein levels of oxidative and anti-oxidant factors, and reactive oxygen species (ROS) production and found little evidence of oxidative stress. We did observe a significant increase in endogenous catalase expression in SMA iPSCs. While catalase knockdown in SMA iPSCs increased ROS production above basal levels, levels of ROS remained lower than in controls, further arguing against robust oxidative stress in this system. Viral delivery of survival motor neuron (SMN) reversed astrocyte activation and restored catalase levels to normal, without changing mitochondrial respiration or expression of oxidative stress markers. Taken together, these data indicate that SMN deficiency induces astrocyte reactivity, but does not do so through an oxidative stress-mediated process.

Introduction

Spinal muscular atrophy (SMA) is a genetic disorder with a frequency of 1:11 000 live births (1). Though considered a rare disorder, SMA is the leading genetic cause of infant death. SMA is caused by the homozygous deletion or disruption of the SMN1 gene, which leads to motor neuron (MN) loss, muscle atrophy, respiratory distress and death (2). Despite disruption of SMN1, patients express low levels of survival motor neuron (SMN) protein from an almost identical gene, SMN2. Due to a nucleotide conversion in Exon 7, the gene is spliced incorrectly 90% of the time, yielding ~10% functional protein from each copy (3,4). However, because there is high variability in SMN2 copy number, SMN2 is a disease modifier such that the number of SMN2 copies inversely correlates with severity of the disease. Current clinical trials take advantage of the remaining SMN2 gene, using antisense oligonucleotides to correct the aberrant splice site and generate full-length protein (5–7). An alternate therapy in clinical trial uses viral delivery of scAAV9-SMN gene to increase SMN protein levels in the CNS (8–13). These therapies still require further optimization given that various vectors, modes and timing of delivery are optimal for targeting different cells in the body. For example, while it is well established that AAV9 is effective at crossing the blood–brain barrier (9,14,15), evidence suggests treatments lose efficacy with disease progression leaving only a narrow therapeutic window (13,16,17).

MN death via apoptosis is a hallmark of SMA, though what specifically triggers this apoptosis is still unknown (18,19). It seems unlikely that the process would be wholly MN autonomous given the ubiquitous expression of SMN protein. Indeed, SMN replacement in MNs using ChAT-Cre or HB9-Cre has shown only minimal benefit in two different SMA mouse models.
In the reciprocal experiment, SMN knockout in MN progenitors of mice using Olig2-Cre resulted in a surprisingly mild disease phenotype (23). In contrast, SMN replacement throughout CNS tissues using the prion promoter, which promotes expression in glia and a wide range of neuronal subtypes, showed substantial survival benefit (24), and global SMN replacement using scAAV9 SMN in SMA/Δ7 mice increased median survival from 13 days to 199 days (8). Interestingly, astrocyte-specific SMN replacement using viral vectors carrying glial-fibillary acidic protein (GFAP)-SMN extended survival in SMA/Δ7 mice from 16 to 33 days and provided an even more dramatic survival extension in SMN2B/Δ7 mice (25). These data demonstrate that while SMN replacement in SMA MNs is required, it is not sufficient to promote long-term survival of the whole animal. Multiple cell types likely work in concert during disease progression and therefore all must be considered during treatment.

Astrocytes serve crucial roles in the CNS, functioning to regulate the neural environment in the brain and spinal cord. Under resting conditions they maintain the blood-brain barrier, recycle neurotransmitters and secrete trophic factors necessary for neuronal growth and health. However, astrocytes can become reactive in response to stress, injury or disease. In this case, cells retract their processes, upregulate intermediate filament proteins such as GFAP, and alter their signaling processes. Additionally, they may stop secreting survival factors or start secreting destructive signals, which can directly affect cells in their immediate neural environment. For example, healthy astrocytes secrete glial cell line-derived neurotrophic factor (GDNF), which is especially important for MN health and survival (26) as growth factor deprivation can lead to MN apoptosis (27,28). Reactive gliosis has been observed in post-mortem tissue from SMA patients (25,29–31) as well as in mouse and stem cell models of the disease (25,32,33). Astrocyte abnormalities include shortened cell processes, increased GFAP protein levels, altered calcium homeostasis and decreased GDNF production and secretion (33). Reactive astrocytes are known to contribute to pathogenesis in a number of other neurodegenerative diseases including amyotrophic lateral sclerosis (ALS), Huntington’s disease (HD) and spinocerebellar ataxia suggesting that astrocytes can non-cell autonomously impact neuronal loss (34). However, it is unclear how reactive SMA astrocytes influence other cells in their direct environment, including other astrocytes and neighboring MNs.

Oxidative stress is a common feature among neurodegenerative diseases and has been well described as a contributing factor in the pathogenesis of Alzheimer’s disease (AD), Parkinson’s disease (PD), HD and ALS (35). Oxidative stress occurs when a cell’s ability to deal with reactive oxygen species (ROS) becomes overwhelmed, which can lead to toxic effects mediated by peroxides and free radicals. ROS can be produced by NAD(P)H oxidase and uncoupled nitric oxide synthase (36,37). Antioxidant enzymes are key for eliminating ROS before they become a problem. The best described of these enzymes are superoxide dismutase (SOD), glutathione peroxidase (GPX) and catalase. SODs convert superoxides (O\(^2−\)) to water and hydrogen peroxide. However, the resulting hydrogen peroxide must be rapidly converted to water so it does not negatively affect other cellular processes. This task can be handled by either GPX or catalase. Catalase is produced in most organisms as a crucial enzyme for maintaining homeostasis in an oxidative stress environment. Chronic oxidative stress can cause DNA damage, triggering cell death through apoptosis or necrosis. The role of oxidative stress in SMA pathogenesis is still poorly understood. Elevated levels of NAD(P)H subunits have been observed in cardiac tissue from SMN/Δ7 mice, but NAD(P)H levels were unchanged in the spinal cords from these mice (38). Conversely, SMN knockdown in SMA human embryonic stem cell (ESC)-derived MN progenitors caused severe mitochondrial oxidative stress, and treatment with the antioxidant, N-acetylcysteine, prevented apoptotic MN death (39).

In this study, we show that reactive SMA induced pluripotent stem cell (iPSC)-derived astrocytes can impact neighboring cells, and that viral mediated re-expression of SMN prevents astrocyte reactivity and restores astrocyte function. While mitochondrial bioenergetics and oxidative stress marker expression were normal, there were decreased levels of ROS and increased levels of catalase in iPSC-derived astrocyte and MN cultures, indicating possible oxidative stress in these cells. However, catalase knockdown did not increase ROS levels above the level of healthy control cells, indicating oxidative stress is not highly active, and likely does not contribute to astrocyte reactivity. In contrast, viral delivery of SMN restored astrocyte function and returned catalase levels to normal without altering mitochondrial bioenergetics and oxidative stress markers. Taken together, our data indicate SMN deficiency induces astrocyte reactivity, but does not readily activate oxidative stress.

Results
Reactive astrocytes induce neighboring astrocytes to change morphology

Evidence from models of ALS indicates that reactive astrocytes can have profound influences on neighboring cells (40–43). We first investigated how reactive SMA astrocytes would affect control astrocytes in their direct environment. Astrocytes derived from GFP-positive ESCs were cultured alone or in equal proportion with either SMA or control iPSC-derived astrocytes. Cells were immunostained for GFAP to mark astrocytes and GFP to mark the ESC population (Fig. 1A). As expected, 50% of cells in coculture conditions and 100% of cells when ESCs were cultured alone expressed GFP (Fig. 1B). Additionally, astrocyte differentiation was equivalent across all of the experimental conditions (Fig. 1C). The ESCs alone trended toward reduced astrocyte numbers at the 6-week time point, but this did not reach significance by one-way analysis of variance (ANOVA).

The morphology of SMA and control iPSC-derived astrocytes is consistent with previous observations with SMA astrocytes having significantly shorter cell process lengths than controls at both 4- and 6-week time points (33, Fig. 1D). Interestingly, the ESC-derived astrocytes mimicked the morphology of the cells they were contacting; ESCs cultured with SMA astrocytes had shorter process lengths than when cultured either with control astrocytes or by themselves (Fig. 1E). This effect was not observed when control astrocytes were not in direct contact with SMA astrocytes (data not shown) suggesting that the propagation of morphological changes was contact dependent. We then measured the calcium response to 10 \(\mu\)M ATP stimulation using live-cell imaging analyzing the response of the GFP-positive astrocytes. There was no significant difference in calcium response in ESC-derived astrocytes regardless of culture conditions. These data show that reactive SMA astrocytes can induce healthy astrocytes to exhibit a reactive morphology without substantially affecting their function.

MN survival and functional defects in SMA astrocytes are SMN-dependent

We have previously shown that SMA iPSC-derived astrocytes exhibit altered function (33) and now identify non-cell autonomous...
effects on neighboring otherwise healthy astrocytes. We, therefore, wanted to test if SMN replacement would reverse reactivity and improve functionality in SMA astrocytes. iPSCs from four independent SMA patients were infected with a lentiviral construct expressing SMN under control of the CMV promoter and GFP under control of the EF1α promoter (25). This allowed us to determine the effect of SMN replacement in cells with the same genetic background. GFP-positive colonies were clonally selected and expanded (Supplementary Material, Fig. S1A). Infected cells expressed significantly more SMN protein at pluripotent (Supplementary Material, Fig. S1B) and neural progenitor cell (NPC) stages (Supplementary Material, Fig. S1C), and have been shown to maintain that expression through 6 weeks of differentiation toward astrocytes (25).

Others have shown that SMN replacement in SMN deficient stem cell-derived MNs improves MN survival (44), and we re-capitulate the increased Tuj1+/SMI-32+ MN survival in SMA + SMN iPSC-derived MNs (Supplementary Material, Fig S2A). Next, control, SMA and SMA + SMN iPSCs were driven toward an astrocyte lineage and immunostained for GFAP (Supplementary Material, Fig. S2B). GFP is expressed at low levels in resting astrocytes but is upregulated in reactive astrocytes. Roughly 15% of total cells expressed detectable levels of GFAP by 4 weeks of differentiation with no significant difference in astrocyte (Supplementary Material, Fig. S2C). We have previously shown that SMN replacement in SMA iPSC-derived astrocytes significantly increased cell process lengths to control levels (25). To extend the analysis, we show here that SMN replacement also exhibited a trend toward increased GDNF production (Supplementary Material, Fig. S2D), significantly improved calcium response to ATP stimulation (Supplementary Material, Fig. S2E), and decreased GFAP expression (data not shown) in SMA iPSC-derived astrocytes. Therefore, these data demonstrate that astrocyte malfunction is due to SMN deficiency.

Mitochondrial bioenergetics and oxidative stress markers are unchanged in SMA iPSC-derived astrocyte and MN cultures

We next asked if astrocyte reactivity has any effect on mitochondrial health and dynamics. SMA, SMA + SMN and control iPSCs were seeded onto Seahorse Bioscience microplates and patterned toward astrocytes for 2 or 8 weeks prior to analysis. The plates were analyzed using a standard method for measuring oxygen consumption rates (OCRs) (45,46). There was no significant difference in ATP-linked respiration (Fig. 2A and B) or maximum respiration (Fig. 2B and D) between any of the experimental groups at either the 2-week (Fig. 2A and B) or 8-week time point (Fig. 2C and D). These data show that mitochondrial bioenergetics are not altered in reactive SMA astrocyte cultures.

We then asked if SMA iPSC-derived astrocyte or MN cultures had altered expression of oxidative stress markers or antioxidant genes. There was no difference in mRNA expression for any of the antioxidant genes examined between SMA, SMA + SMN or control astrocyte cultures: peroxisome proliferator-activated receptor c co-activator 1α (PGC1α), manganese superoxide dismutase (MnSOD), nuclear factor erythroid 2-related factor 2 (Nrf2), NAD(P)H quinone oxidoreductase 1 (NQO1), GPX1 or glutamate-cysteine ligase catalytic subunit (GCLC) (Supplementary Material, Fig. S3A). Likewise, there was also no difference in expression of these markers in MN cultures (Supplementary Material, Fig. S3B). NQO1 transcript levels appeared to be lower in SMA cells than controls in 6-week astrocyte cultures or 8-week MN cultures; however, this difference was not consistent across multiple differentiations. Protein levels of Nrf2 were also unchanged between the three groups for both astrocyte (Supplementary Material, Fig. S4A, C) and MN (Supplementary Material, Fig. S5A, C) cultures, in agreement with the results of Nrf2 transcript levels (Supplementary Material, Fig. S3). Levels of phosphorylated Nrf2 were also unaltered (data not shown). Additionally, protein levels of calpastatin, a calpain inhibitor involved in membrane

Figure 1. Reactive SMA astrocytes alter morphology in neighboring astrocytes. (A) Representative images of co-cultured cells immunostained for GFAP (red) to mark astrocytes, GFP (green) to mark ESCs and Hoechst nuclear stain (blue). (B–C) Percent of total cells expressing GFP (B) or GFAP (C). (D) Comparison of cell process lengths between control and SMA iPSC-derived astrocytes. *P < 0.01, **P < 0.001 by Student’s t-test (E–F) Cell process lengths (E) and average calcium response to stimulation with 10 μM ATP (F) of GFP-labeled ESC-derived astrocytes when cultured alone or co-cultured with SMA or control astrocytes. *P < 0.05, **P < 0.01, ***P < 0.001 by one-way ANOVA with Tukey’s post hoc test. Scale bar = 100 μm.
fusion events, and succinate dehydrogenase (complex II) and cytochrome c oxidase (complex IV) of the electron transport chain involved in oxidative phosphorylation (OXPHOS) were also unaltered in both astrocyte (Supplementary Material, Fig. S4B, D–F) and MN cultures (Supplementary Material, Fig. S5B, D–F) derived from SMA iPSCs compared with control and SMA + SMN iPSC cultures. There appeared to be a trend toward increased Calpastatin levels in the SMA and SMA + SMN MN cultures, but this again did not reach statistical significance as protein levels were highly variable. Together these data imply that oxidative stress genes and antioxidant defenses are not aberrantly expressed in SMA iPC-derived astrocyte or MN cultures.

**Endogenous catalase moderates ROS production in SMA iPSCs**

ROS overproduction or insufficient clearance can lead to oxidative stress. Therefore, we tested if SMA iPSC-derived astrocytes or MNs had increased ROS production using dihydroethidium (DHE) staining. Control and SMA NPCs were briefly patterned toward an astrocyte or MN lineage and stained for DHE and either GFAP (Fig. 3A) or SMI-32 (Fig. 3C). In contrast to our prediction, SMA cells had lower levels of DHE staining than control cells, indicating reduced levels of ROS in both the astrocyte (Fig. 3B) and MN (Fig 3D) progenitor cell cultures. In an effort to further explore the reduced ROS levels in SMA iPSC-derived astrocyte and MN cultures, we found that catalase was significantly upregulated in SMA iPSC-derived NPCs, astrocyte cultures (Fig. 4A and B) and MN cultures (Fig. 4C and D) at all time points tested, and levels were returned to normal upon SMN re-expression (Fig. 4).

We then knocked down catalase in SMA iPSC-derived NPCs using siRNA to test whether ROS levels would increase. Knockdown was confirmed by examining transcript (Fig. 5A) and protein (Fig. 5B) levels of catalase, which showed a 62.8% ± 5% knockdown of catalase at the protein level. Although transcript levels of oxidative stress markers were unaltered between the SMA sham treated and knockdown conditions (Fig. 5C), DHE levels in nestin-positive SMA iPSC-derived NPCs were significantly increased in the catalase knockdown SMA-derived cells compared with the sham treated cells (Fig. 5D); however, levels of DHE in knockdown NPCs were still lower than in controls. Therefore, we conclude that endogenous catalase mitigates ROS production in SMA iPSCs, but that other indicators of oxidative stress are not readily activated in the SMA iPSC system.

We next evaluated oxidative stress and catalase expression in SMNΔ7 mouse spinal cord. Others have shown no indication of increased oxidative stress in SMNΔ7 mouse spinal cord using NADPH (38). We confirmed a lack of oxidative stress here by staining spinal cord sections from pre- and post-symptomatic SMNΔ7 mice for the oxidative stress marker 8-hydroxydeoxyguanosine (8HOdG). 8HOdG is formed in response to oxidative stress (47) and can be used for analysis in fixed tissues, in contrast to DHE which is used in live cells. Minimal cytoplasmic and no nuclear 8OHdG immunoreactivity was observed in the ventral horn.
with no difference between control and SMNΔ7mice at post-natal day (PND) 4, 6 or 8 (data not shown). Next, we examined whether catalase expression was upregulated in SMNΔ7 mouse spinal cord as it was in the SMA iPSC system. Catalase expression was undetectable by western blot analysis of the lumbar spinal cord at PND 2, 6 and 8 (data not shown). Immunofluorescence staining intensity in the ventral spinal cord was minimal in both control and SMNΔ7 mice (data not shown). We did observe a change in the catalase distribution over time, with more diffuse staining throughout the cell body at PND 4 becoming more
punctuate at the later PND 6 and 8 time points; however, the staining distribution was not different between control and SMNΔ7 mice. Although an upregulation of catalase expression may be induced because of stressors unique to the SMA iPSC system, data from the in vitro and in vivo model systems both suggest a minimal role for oxidative stress in SMA.

**Discussion**

Like many neurodegenerative disorders, SMA is characterized by the loss of a target population of neurons with the origin of the neuron loss unclear. SMN1 was identified as the causative gene for SMA nearly 20 years ago, yet the scope of cellular consequences of that loss is just starting to be appreciated. For example, within the last 5 years nearly every cell type in the motor circuit has been shown to be affected in SMA including MNs, sensory neurons, Schwann cells, skeletal muscle and astrocytes (20,21,24,25,32,33,48,49). However, the order in which these cells become affected and how each cell type influences the greater circuit is still under investigation.

We have shown that reactive SMA astrocytes affect neighboring astrocytes by inducing them to acquire a reactive morphology. Although these otherwise healthy astrocytes did not show a defective response to ATP stimulation, it is possible that calcium response would be affected at later time points or that astrocytes were altered in ways we did not investigate such as in growth factor secretion or glutamate uptake. Alternatively, as these were healthy ESC-derived astrocytes, it is possible that they are more resistant to SMA astrocyte-induced insults. Nevertheless, the data indicate that astrocyte reactivity can propagate to previously unaffected cells and support the importance of SMA astrocytes in disease processes, highlighting their role in modulating the neural environment.

We and others have shown that SMN replacement in stem cell-derived SMN-deficient MNs prevents MN loss (39,44) and Fig. 2]. Additionally, the reactive SMA astrocyte morphology and diminished functionality can be ameliorated with SMN replacement. These findings may help explain why MN selective restoration has been ineffective in mouse models of SMA, whereas more broad neural SMN delivery has been highly effective (20,21,24). Converting SMA MNs to a ‘healthy’ state with targeted SMN replacement may not be sufficient if the astrocytes remain SMN deficient. As such, astrocytes will be an important additional target for future therapies as a way of improving the overall neural environment in the spinal cord to confer maximum benefit to the patient.

Although oxidative stress is a contributing factor to multiple age-related neurodegenerative diseases such as PD, HD, AD and ALS (35), its involvement in SMA pathogenesis is still unclear. One group has shown levels of NADPH-oxidase and Nox2 are unaltered in the spinal cords of post-symptomatic SMNΔ7 mice (38), in agreement with our studies here showing unaltered staining for 8OHdG in lumbar spinal cord sections from this same mouse model (data not shown). Additionally, transcript and protein levels of numerous oxidative stress markers and antioxidant genes were equivalent between control and SMA iPSC-derived astrocytes and MNs (Supplementary Material, Figs S3–S5). Mitochondria are important mediators of oxidative stress as ROS occur as a byproduct of OXPHOS, and they contain numerous redox enzymes (35). Mitochondrial oxidative stress has been shown in both ESC-derived MNs (39) and NSC-34 cells (50) following RNAi-mediated SMN knockdown. For example, Wang et al. (39) found increased levels of MitoSOX in ESC-derived MNs following shRNA-mediated knockdown of full-length SMN. Similarly, Acsadi et al. (50) observed reduced ATP levels and OXPHOS complex IV activity following siRNA knockdown of SMN in NSC-34 cells. However, we found no evidence of altered mitochondrial bioenergetics in SMA iPSC-derived astrocytes or MNs. This difference may be due to differences in the assays used or differences between RNAi-mediated SMN knockdown versus utilizing iPSCs derived from human SMA patients. Additionally, we were measuring complex IV protein levels while the other group measured activity. Nevertheless, our additional measurements of oxidative stress mediators and antioxidant proteins support our conclusions.

We observed no changes in levels of OXPHOS complex II, which is in direct contrast to what is seen in HD where complex II is reduced in HD brains (51). One possible explanation for these differences is age of onset. PD, HD, AD and ALS are all diseases of aging, such that oxidative stress and ROS could build up over time to affect neurons in a given area; Type 1 SMA is a childhood disorder and, therefore, neurons would have a much shorter exposure to stressful conditions. It is also possible that SMN deficient cells may have adopted endogenous mechanisms to attenuate stress and cell death, such as increasing catalase. Others have reported that overexpression of catalase eliminates toxic effects of oxidative stress in SOD1 mutant models of ALS (52). Therefore, the endogenous increase in catalase expression could be in response to an oxidative stress signal within SMA iPSC cultures. Since SMN re-expression returns catalase to normal levels, it is possible that reduced SMN creates a stressful environment that...
could contribute to the observed apoptotic cell death in iPSC models of SMA (19). An alternative explanation for the increased catalase in SMN deficient iPSCs could be that SMN participates in a cascade that regulates catalase expression. However, this seems unlikely given there was no difference in catalase immunostaining between control and SMNΔ7 lumbar spinal cord sections. Regardless, considering that ROS levels in SMA iPSCs were below control levels even with catalase knockdown (Fig. 5), our data argue against oxidative stress as a major player in SMA disease pathogenesis, which is consistent with the SMNΔ7 mouse spinal cord data described here and elsewhere (38). It remains possible that SMA cells have a reduced capacity to respond to additional stressors, but increased oxidative stress is not likely inducing the cellular demise. Taken together, our data demonstrate that low levels of SMN contribute to astrocyte reactivity and MN loss in SMA iPSCs but without inducing extreme oxidative stress. It is, therefore, unlikely that oxidative stress is a major contributor to SMA etiology.

Materials and Methods

Cell culture

Cell lines
Human iPSCs from four independent unaffected control and four SMA patient lines were grown as pluripotent colonies on Matrigel substrate (Corning) in the NutriStem medium (Stemgent). A subset of SMA iPSCs was infected with a lentivirus expressing CMV promoter driving SMN and EF1α driving GFP (multiplicity of infection = 10). Colonies were clonally selected and expanded based on GFP expression to indicate stable integration of the construct.

Neural progenitor cells
Colonies were detached from plates using 1 mg/ml Dispase (Gibco) and maintained as floating spheres of NPCs in the neural progenitor growth medium Stemline (Sigma) supplemented with 100 ng/ml human basic fibroblast growth factor-2 (Milenyi), 100 ng/ml epidermal growth factor (Milenyi) and 5 μg/ml heparin (Sigma-Aldrich) in ultra-low attachment flasks. Aggregates were passaged using a manual chopping technique as previously described (53,54).

Astrocyte differentiation
To induce astrocyte differentiation, spheres were dissociated with TrypLE Express (Gibco) and plated onto Matrigel-coated 12 mm coverslips or six-well plates in 1:1 Dulbecco’s modified Eagle’s medium (DMEM)/F12 (Gibco) medium supplemented with 1x B27 Supplement (Gibco) and 1x Antibiotic-Antimycotic (Gibco). Media was changed every 3–4 days for 4–8 weeks.

MN differentiation
All stages of MN differentiation were carried out in neural induction medium (NIM) composed of 1:1 DMEM/F12 (Gibco), 1x N2 Supplement (Gibco), 5 μg/ml Heparin (Sigma), 1x non-essential amino acids (Gibco) and 1x antibiotic-antimycotic (Gibco). To induce MN differentiation, spheres were floated in NIM plus 0.1 μM all-trans-retinoic acid (RA) for 2 weeks. One-micromolar purmorphamine (PMN, Stemgent) was added during the second week to help pattern the cells. Spheres were then dissociated with TrypLE Express (Gibco) and plated onto Matrigel-coated 12 mm coverslips or six-well plates in NIM + Goodies [NIM plus 1 μM RA, 1 μM PMN, 1x B27 supplement (Gibco), 200 ng/ml ascorbic acid (Sigma), 1 μM cAMP (Sigma), 10 ng/ml brain derived neurotrophic factor (Peprotech), 10 ng/ml GDNF (Peprotech)] and allowed to mature for an additional 2–4 weeks.

Immunocytochemistry
Coverslips were fixed in 4% paraformaldehyde (Electron Microscopy Sciences) for 20 min at room temperature and rinsed with phosphate-buffered saline (PBS). Cells were blocked with 5% normal donkey serum (Millipore) and/or 5% normal goat serum (Millipore) and permeabilized in 0.2% Triton X-100 (Sigma) for 30 min at room temperature. Cells were then incubated in primary antibody solution for 1 h, rinsed with PBS, and incubated in secondary antibody solution for 1 h at room temperature. Finally, nuclei were labeled with Hoechst nuclear stain (Sigma) to label DNA and mounted on glass slides using FluoroMount medium (SouthernBiotech). Primary antibodies used were rabbit anti-GFAP (Dako 20393, 1:1000), rabbit anti-GFAP (Life Technologies A11122, 1:1000), mouse anti-GFAP (Life Technologies A11120, 1:500), mouse anti-SMI-32 (Covance SMI-32R, 1:1000), chicken anti-Tuj1 (GeneTex GTX85469, 1:500), rabbit anti-Tuj1 (ECM Biosciences TP1721, 1:1000), mouse anti-Tuj1 (Sigma T8660, 1:1000), rabbit anti-Nestin (Millipore AB922, 1:1000). Secondary antibodies used were donkey anti-rabbit AF488 (Invitrogen A21206), goat anti-rabbit Rhod (Invitrogen R6394), donkey anti-mouse AF488 (Invitrogen A21202), goat anti-mouse Rhod (R6393), donkey anti-mouse AF647 (Invitrogen A31571) and donkey anti-chicken AF647 (Jackson Immunoresearch 703-604-155).

Immunohistochemistry
Fifty-micrometer sections through the lumbar spinal cord were analyzed in control and SMNΔ7 mice at PND 4, 6 and 8. Sections were permeabilized in 0.2% Triton X-100 and blocked in Normal Donkey Serum (Millipore). Sections were incubated in primary antibody overnight at 4°C and incubated with secondary antibody for 1 h at room temperature. Primary antibodies used were rabbit anti-catalase (Cell Signaling 12980, 1:1000), mouse anti-8HODG (Abcam ab62623, 1:1000) and goat anti-ChAT (Millipore AB144F, 1:1000). Secondary antibodies used were donkey anti-Rabbit (Invitrogen A21206), donkey anti-Mouse (Invitrogen A31571) and donkey anti-Goat (Invitrogen A11056).

Immunocytochemical analysis
Images were acquired from five random fields per coverslip or section using an inverted fluorescent microscope (Nikon) and either SPOT or NIS Elements software. Images were blinded and manually analyzed for antigen specificity or cell process length using the MetaMorph or NIS Elements software.

Mitochondrial bioenergetics
SMA, SMA + SMN and control astrocytes were seeded onto Seahorse Bioscience microplates and patterned toward astrocytes for 2 or 8 weeks prior to analysis. The plates were analyzed using a standard method for measuring OCRs using the XF96 Extracellular Flux Analyzer. Calculations were pooled from at least 10 replicates and compared using one-way ANOVA with Tukey’s post hoc test.

DHE staining
SMA or control iPSCs were patterned toward MNs or maintained as NPCs and then seeded onto matrigel-coated glass coverslips. Cells were incubated in 1 μM DHE (Sigma-Aldrich, Cat. D7008) diluted in
Catalase knockdown
NPCs were seeded onto matrigel-coated culture dishes for 1 week and then transfected with siRNA targeting human catalase (Santa Cruz, sc-45330) using Lipofectamine® RNAiMAX Transfection Reagent (Life Technologies, 13778-075) and collected 3 days after transfection for analysis. Knockdown was confirmed by reverse transcriptase–polymerase chain reaction (RT–PCR) using commercially available PCR primers (Santa Cruz, sc-45330-PR) and by immunoblot using rabbit anti-catalase primary antibody (Cell Signaling, 12980, 1:1000).

Reverse transcriptase–polymerase chain reaction
RNA was isolated from cell pellets using the RNeasy Mini Kit following manufacturer’s instructions (Qiagen, 74104). RNA was treated with RNase-free Dnase (Promega, M6101) and converted to cDNA using the Promega Reverse Transcription System (Promega, A3500). DNA was amplified using the ExTaq DNA Polymerase (TaKaRa, RR001A) following the manufacturer’s instructions. Catalase PCR was performed using commercial primer sets following manufacturer’s instructions (Santa Cruz, sc-45330-PR). Other primer sequences and cycling conditions are as indicated in Supplementary Material, Table S1.

Western blot
Immunoblots were performed on whole cell lysates from iPSC colonies, NPCs, 4- or 6-week MN cultures, 4- or 6-week astrocyte cultures, and mouse lumbar spinal cord. Whole cell lysates were prepared using 1x Chaps buffer with phenylmethylsulfonyl fluoride and 1x dithiothreitol (Cell Signaling). Protein concentration was determined using Bradford Assay (Sigma). Equal amounts of protein were loaded onto 10 or 12% pre-cast Tris-HCl Mini-PROTEAN gels and run at 105 V for 90 min (Bio-Rad), then transferred to polyvinylidene fluoride membrane (Bio-Rad). Membranes were blocked with 5% milk in tris-buffered saline with Tween-20 prior to overnight incubation in primary antibody and subsequent hour-long incubation in secondary antibody. Detection was performed using Western Bright ECL Substrate (Advansta). Primary antibodies used were mouse anti-SMN (BD Biosciences, 610646), mouse anti-GFAP (Cell Signaling, 3670), goat anti-GDNF (R&D Systems, AF-212-MA), rabbit anti-GAPDH (Sigma-Aldrich, G9545), rabbit anti-catalase (Cell Signaling, 12980), rabbit anti-Calpastatin (Cell Signaling, 4146S), rabbit anti-Nrf2 (Cell Signaling, 12721), anti-phospho Nrf2 (Abcam ab76026). Secondary antibodies used were anti-goat IgG HRP (Promega, V805A), anti-rabbit IgG HRP (Promega, W4011) and anti-mouse IgG HRP (Promega, W4021). Densitometry was performed using the ImageJ software.

Calcium imaging
Seven-week-old iPSC-derived astrocyte cultures were subjected to ratiometric live-cell calcium imaging to detect intracellular calcium levels, which were detected using ratiometric live-cell imaging with the dual-fluorescent calcium indicator FURA-2AM. Extracellular normal HEPE (ENH) buffer used throughout the experiment was composed of MilliQ water with 150 mM NaCl, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 8 mM glucose, 5.6 mM KCl, 1 mM MgCl2 and 2 mM CaCl2; pH 7.4; osmolality 320. iPS-derived astrocytes were cultured on 12 mm glass coverslips for 7 weeks prior to imaging, then loaded with 2.5 μM/l FURA-2AM for 1 h at room temperature, washed in ENH for 20 min, and mounted on a perfusion chamber. Solutions were superfused over cells at constant rate of 6 ml/min: 2 min ENH to establish baseline measurements, 1 min ENH + 10 μM ATP, 2 min ENH, 30 s ENH + 50 mM KCl and 1 min ENH. Videos were taken using an Ando Zyla sCMOS camera and detected and analyzed using the NIH Elements software (Nikon). Positive response was defined as >20% increase in fluorescence over baseline during the time of ATP stimulation. Cells that also responded to potassium-mediated depolarization were excluded from analysis.

Statistical analysis
Statistical analysis was performed with the GraphPad Prism software using one-way ANOVA with Tukey’s post hoc test or Student’s t-test as indicated in the figure legend. All comparisons were made within one time point, though multiple time points may be shown on the same graph. Results were considered statistically significant if P < 0.05. ns = no significance, *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, n = minimum of three biological replicates for each experiment.

Supplementary Material
Supplementary Material is available at HMG online.

Acknowledgements
The authors thank Steve Komas and Kelsey Kalous for assistance with the Seahorse Bioanalyzer experiments.

Conflict of Interest statement
None declared.

Funding
This work was supported by research grants from CureSMA (A.D.E.) and Advancing a Healthier Wisconsin (A.D.E.) and an equipment grant from The Gwendolyn Strong Foundation. T.N.P. was partially funded by the Quadracci Memorial Fellowship.

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