Published in final edited form as:

Glia. 2013 September ; 61(9): 1418–1428. doi:10.1002/glia.22522.

Spinal muscular atrophy astrocytes exhibit abnormal calcium regulation and reduced growth factor production

Jered V. McGivern[#], Teresa N. Patitucci[#], Joshua A. Nord, Marie-Elizabeth A. Barabas, Cheryl L. Stucky, and Allison D. Ebert

Department of Cell Biology, Neurobiology and Anatomy, Medical College of Wisconsin. 8701 Watertown Plank Rd, Milwaukee, WI 53226

[#] These authors contributed equally to this work.

Abstract

Spinal muscular atrophy is a genetic disorder caused by deletion of the survival motor neuron 1 (SMN1) gene that leads to loss of motor neurons in the spinal cord. Though motor neurons are selectively lost during SMA pathology, selective replacement of SMN in motor neurons does not lead to full rescue in mouse models. Due to the ubiquitous expression of SMN, it is likely that other cell types besides motor neurons are affected by its disruption and therefore may contribute to disease pathology. Here we show that astrocytes in SMA Δ 7 mouse spinal cord and from SMA induced pluripotent stem cells (iPSCs) exhibit morphological and cellular changes indicative of activation prior to overt motor neuron loss. Furthermore, our *in vitro* studies show misregulation of basal calcium and decreased response to ATP stimulation indicating abnormal astrocyte function. Together, these data show for the first time early disruptions in astrocytes that may contribute to SMA disease pathology.

Keywords

Motor neurons; stem cells; cell autonomous; astrocyte activation

Introduction

Spinal muscular atrophy (SMA) is an autosomal recessive disorder selectively affecting motor neurons in the spinal cord causing muscle weakness, respiratory difficulty, paralysis, and death (Crawford and Pardo, 1996; Lefebvre et al., 1997). SMA is most frequently caused by deletion of the survival motor neuron (SMN) gene and subsequent reduction of the SMN protein involved in biogenesis of RNA proteins, RNA transcription, and pre-mRNA splicing (Burghes et al., 1994; Lefebvre et al., 1995; Jablonka et al., 2000; Gabanella et al., 2007; Bebee et al., 2010). Recent evidence suggests that SMN is particularly important in U12-dependent splicing events that are important for motor neuron function (Lotti et al., 2012). Motor neurons appear to be the most vulnerable cell type, but evidence suggests that other cell types may be affected, including sensory neurons, skeletal muscle, and cardiomyocytes (Jablonka et al., 2006; Bevan et al., 2010; Heier et al., 2010; Ling et al., 2010; Shababi et al., 2010; Mentis et al., 2011; Gogliotti et al., 2012; Imlach et al., 2012; Martinez et al., 2012).

To whom correspondence should be addressed: Allison D. Ebert, 8701 Watertown Plank Rd, Milwaukee, WI53226, aeber@mcw.edu, 414-955-2979.

The authors have no conflicts of interest to declare.

Currently there is no clinically approved treatment for SMA, but recent studies using SMA transgenic mice have shown dramatic survival benefits following global SMN gene replacement and antisense oligonucleotide delivery (Azzouz et al., 2004; Foust et al., 2010; Hua et al., 2010; Passini et al., 2010; Porensky et al., 2012; Rigo et al., 2012). However, while SMN expression in motor neurons is necessary, it is not sufficient to elicit extended survival benefit (Park et al., 2010; Gogliotti et al., 2012; Lee et al., 2012; Martinez et al., 2012). For example, re-expression of SMN in SMA mice using either the HB9 motor neuron promoter or the choline acetyltransferase (ChAT) promoter provided only modest survival benefit (Gogliotti et al., 2012; Lee et al., 2012; Martinez et al., 2012). In contrast, SMN expression by the prion promoter, which expresses highly in both neurons and astrocytes, significantly extended survival of SMA mice (Gavrilina et al., 2008). Together these data suggest that other cell types likely contribute to motor neuron dysfunction and loss in SMA through paracrine signaling. A well studied example of this phenomenon has been shown for both *in vivo* and *in vitro* models of amyotrophic lateral sclerosis (ALS) in which astrocytes expressing mutated SOD1 cause increased motor neuron death demonstrating a non-cell autonomous nature of disease pathology (Clement et al., 2003; Di Giorgio et al., 2007; Marchetto et al., 2008; Benkler et al., 2013). Furthermore, astrocytes are known to be key mediators in disease processes in a variety of other neurodegenerative and neuropathic conditions, including Parkinson's disease, spinal cord injury, and chronic pain. Therefore, it is possible that astrocytes also contribute to disease processes in SMA.

Astrocytes are the most abundant cell type in the central nervous system and serve essential roles in both normal and pathological conditions. Under healthy conditions, astrocytes are critical for maintenance of the blood brain barrier, regulation of synapse formation and function, ion uptake, growth factor secretion, and neuronal metabolism and homeostasis (Wang and Bordey, 2008). They provide neurons with metabolic substrates through activation of the Na⁺/K⁺ ATPase and processing of glucose into lactate to fuel the variety of energy demanding activities within neurons (Giaume et al., 2010). Astrocytes also take up extrasynaptic glutamate, a portion of which is converted to glutamine and shuttled into the presynaptic terminals of glutamatergic neurons for later neurotransmission (Volterra and Steinhauser, 2004). Mammalian spinal cord motor neurons have been shown to release acetylcholine and glutamate (Nishimaru et al., 2005), suggesting glutamate uptake by astrocytes is critical for maintaining normal motor neuron function. In diseased and/or injured environments, these functions are disrupted, such that astrocytes become reactive and undergo distinct morphological restructuring: glial fibrillary acidic protein (GFAP) and other neurofilament proteins are upregulated, cell processes are retracted and thickened, and branching is reduced (Frisen et al., 1995; Pekny and Nilsson, 2005; Sun et al., 2010). Additionally, reactive astrocytes secrete pro-inflammatory cytokines following activation of the MAP kinase ERK, alter their ion channel and buffering properties, and induce a glial scar, all of which can promote neuron loss and impede repair (Ridet et al., 1997; Rousseau et al., 2008). Functional neural environments rely on proper signaling and interactions between the many cell types within the microenvironment. Astrocytes effectively communicate with other astrocytes and neurons through gap junctions, ATP release, and calcium-dependent gliotransmission (Parpura and Zorec, 2010). ATP also serves as one of the most robust signaling molecules for calcium-dependent communication in astrocytes by binding to purinergic receptors and facilitating communication between neurons, astrocytes, and vascular cells (Zhu and Kimelberg, 2001; Fields and Burnstock, 2006; Verkhratsky et al., 2012).

A number of mouse models of SMA have been generated with varying levels of severity by manipulating SMN expression levels. For example, the commonly used "severe" SMA mouse has 1-2 copies of human SMN2 on an *Smn*-null background (Hsieh-Li et al., 2000; Monani et al., 2000). SMN2 rescues embryonic lethality, but these mice exhibit extensive

motor neuron loss at 2-3 days of age and die between 5-7 days of age (Hsieh-Li et al., 2000; Monani et al., 2000). The inclusion of SMN Δ 7 cDNA on this severe background (“SMA Δ 7” mice) extends life span by ~7 days, but these mice still display extensive motor neuron loss and severe muscle weakness (Lee et al., 2005). An intermediate SMA model, Smn2B $^{-/-}$, which has a mutation in the exon 7 splicing enhancer, develops motor neuron loss and motor deficits after 10 days of age and survive ~30 days (Hammond et al., 2010; Bowerman et al., 2012). Importantly, these mice show loss of motor neurons in the spinal cord with corresponding behavioral pathologies characteristic of human SMA (Schmid and DiDonato, 2007; Sleight et al., 2011; Bebee et al., 2012), but no studies have thoroughly assessed what, if any, role astrocytes play in this pathology. We have previously shown selective apoptosis of motor neurons over time in a human induced pluripotent stem cell (iPSC) model of SMA (Sareen et al., 2012). Here, we aimed to investigate whether astrocytes also show a disease phenotype in SMA. Using both human iPSCs derived from two independent SMA patients and spinal cord sections of pre- and post-symptomatic SMA Δ 7 mice, we found significant changes in astrocyte morphology and activation states *in vitro* and *in vivo*. Further, we found an increase in basal intracellular calcium levels and a reduced calcium response to ATP in SMA iPSC-derived astrocytes, all of which may contribute to motor neuron loss.

Materials and Methods

Mice

Mouse tissue sections were taken from SMA Δ 7 mice (Jackson Labs, 005025, see (Le et al., 2005) for full description) where heterozygous mice were crossed to obtain homozygous mutant (SMA) and wild-type (WT) mice. Briefly, symptomatic mice carry the targeted mutation for *mSmn* and overexpress human SMN2 and SMA Δ 7.

Immunohistochemistry

SMA and WT littermates were sacrificed at postnatal day 3 (PND3) and PND9. Spinal cords were removed and fixed in 4% PFA for 3 days followed by 3 days in 30% sucrose. Spinal cords were cut into 30 μ m cryosections for immunofluorescence. Briefly, sections were washed in PBS. Nonspecific labeling was blocked and the tissue permeabilized with 5% normal goat serum and/or 5% normal donkey serum and 0.2% Triton X-100 in PBS for one hour at room temperature. Tissue was rinsed with PBS and then incubated with primary antibodies overnight at room temperature. Tissue was then labeled with the appropriate fluorescently-tagged secondary antibodies. Primary antibodies used were rabbit anti-GFAP (Dako, Z0334), rabbit anti-Nestin (Millipore AB5922), goat anti-Choline Acetyltransferase (ChAT) (Millipore AB144P), rabbit anti-S100 (abcam ab868). Secondary antibodies used were donkey anti-rabbit AF488 (Invitrogen A21206) and donkey anti-goat AF488 (Invitrogen A11055).

Cell culture

Human iPSCs were generated from commercially available fibroblast samples (unaffected 4.2 line, GM003814 Coriell Institute; unaffected 21.8 line, GM002183 Coriell; SMA 3.6 line, GM003813 Coriell Institute; SMA 7.12 line, GM09677 Coriell Institute) as previously described (Ebert et al., 2009; HD iPSC Consortium, 2012; Sareen et al., 2012; Ebert et al., 2013). iPSCs were grown in feeder free conditions on Matrigel substrate in Nutristem medium (Stemgent). Neural stem cells were generated by lifting intact colonies following dispase treatment (1mg/ml, Gibco) and placing them directly into a human neural progenitor growth medium (Stemline, Sigma-Aldrich) supplemented with 100ng/ml basic fibroblast growth factor (FGF-2, Chemicon), 100ng/ml epidermal growth factor (EGF, Chemicon), and 5 μ g/ml heparin (Sigma-Aldrich) in ultra-low attachment flasks and were passaged weekly using a chopping technique (Svendsen et al., 1998). To induce astrocyte

differentiation, spheres were dissociated with TrypLE (Invitrogen) and plated onto poly-ornithine/laminin (Sigma) coated coverslips in DMEM/F12 (Invitrogen) supplemented with 2% B27 (Invitrogen) with or without CNTF (Emdad et al., 2012) for 2-8 weeks.

Immunocytochemistry

Plated cells were fixed in 4% paraformaldehyde (PFA) for 20 minutes at room temperature and rinsed with PBS. Nonspecific labeling was blocked and the cells permeabilized with 5% normal goat serum and/or 5% normal donkey serum and 0.2% Triton X-100 in PBS for 30 minutes at room temperature. Cells were rinsed with PBS and then incubated with primary antibodies for one hour at room temperature or overnight at 4°C for transcription factors. Cells were then labeled with the appropriate fluorescently-tagged secondary antibodies. Hoechst nuclear dye was used to label nuclei. Primary antibodies used were rabbit anti-GFAP (Dako, Z0334), mouse anti-GFAP (Cell Signaling 3670), rabbit anti-S100 (abcam ab868), mouse anti-Vimentin (Developmental Studies Hybridoma Bank, 40E-C), rabbit anti-pERK1/2 (Cell Signaling 4370), mouse anti-ALDH1L1 (NeuroMab 73-140), and rabbit anti-P2Y2 (abcam ab10270). Secondary antibodies used were donkey anti-rabbit AF488 (Invitrogen A21206), goat anti-rabbit RhoRed (Invitrogen R6394), donkey anti-mouse AF488 (Invitrogen A21202), goat anti-mouse RhoRed (R6393).

Calcium imaging

iPSC-derived astrocyte cultures were cultured for 2, 4, 6, and 8 weeks and subjected to ratiometric live-cell calcium imaging using dual-wavelength fluorescent calcium indicator FURA-2AM (Invitrogen) to detect intracellular calcium levels (Barabas et al., 2012). Cells were loaded with 2.5 μ L/ml FURA-2AM in 2% BSA in extracellular buffer for 1 hour at room temperature, washed in extracellular buffer for 20 minutes, mounted onto a perfusion chamber, and superfused with extracellular buffer at 6mL/min. Coverslips were superfused with extracellular buffer containing 10 μ M ATP for one minute. Fluorescence images were taken with a cooled CCD camera (CoolSNAP FX; Photometrics, Tucson, AZ) before and after stimulation. Metafluor imaging software was used to detect and analyze intracellular calcium changes throughout the experiment (Molecular Devices, Sunnyvale, CA) where a 20% increase in intracellular calcium from baseline constituted a response. Extracellular buffer contained 150mM NaCl, 10mM HEPES, 8mM glucose, 5.6mM KCl, 1mM MgCl₂, and 2mM CaCl₂ (experiments with extracellular Ca²⁺) or 1mM EGTA (experiments without extracellular Ca²⁺, Sigma-Aldrich). To deplete intracellular Ca²⁺ stores, 1 μ M thapsigargin (Sigma-Aldrich) was added for 90 min prior to imaging.

Western Blot

Whole cell lysates were isolated from plated astrocyte cultures using Chaps buffer with protease inhibitors (Cell Signaling) or from conditioned medium collected from astrocyte cultures. Protein concentration determined by Bradford Assay (Sigma-Aldrich). 10 μ g/mL lysate or 45 μ g/mL conditioned medium protein was run on 10% Tris-HCl polyacrylamide gels (Bio-Rad), transferred to PVDF membrane (Millipore), and blocked in 5% milk solution for one hour at room temperature. The membrane incubated in primary antibody in 5% milk overnight at 4°C, washed in TBST, incubated in secondary antibody in 5% milk for one hour at room temperature and washed with TBST before developing using SuperSignal West Pico Detection Kit or SuperSignal West Femto Detection Kit (Thermo Scientific). Primary antibodies used were mouse anti-GFAP (Cell Signaling 3670), rabbit anti-GLT1 (ADI GLT11-A), rabbit anti-pERK1/2 (Cell Signaling 4370), rabbit anti-P2Y2 (abcam ab10270) goat anti-GDNF (R&D Systems AF-212-MA), rabbit anti-GAPDH (Sigma-Aldrich G9545), rabbit anti-Albumin (Cell Signaling 4929). Secondary antibodies used were anti-goat IgG HRP (Promega V805A), anti-rabbit IgG HRP (Promega W4011) and anti-mouse IgG HRP (Promega W4021).

GDNF ELISA

Conditioned medium was collected from iPSC-derived astrocyte cultures and assayed using DuoSet ELISA Development System for human GDNF according to the manufacturer protocol (R&D Systems).

Imaging and data analysis

Five images were taken on each of at least three different fluorescently labeled coverslips per condition or from at least 5 different spinal cord sections using a Nikon inverted microscope using Spot imaging software and then blinded for analysis. The images were analyzed for antigen specificity using MetaMorph Software (Molecular Devices Inc., Sunnyvale, CA). Fluorescence intensity and process lengths were measured using ImageJ (National Institute of Health). Data were statistically analyzed via one way ANOVA or Student's *t* test as appropriate with Prism software, $\alpha=0.05$ (GraphPad, La Jolla, CA).

Results

Well-characterized transgenic mouse models of SMA show consistent and significant loss of motor neurons in the ventral horn of the spinal cord (Schmid and DiDonato, 2007); however, there are little data examining whether astrocytes also exhibit a disease phenotype. We used the SMA $\Delta 7$ model, which show motor deficits as early as postnatal day (PND) 6 and survive approximately 14 days (Le et al., 2005). We first confirmed motor neuron loss in the ventral horn of WT and SMA mice using immunofluorescence for choline acetyltransferase (ChAT) at PND 3, a pre symptomatic time point, and at PND 9, a post-symptomatic time point. No significant difference was detected between WT and SMA mice at PND3, but SMA mice at PND9 had significantly fewer ChAT positive motor neurons compared to WT (Fig. 1A, $p<0.05$).

We next used immunofluorescence at PND3 and PND9 to assess whether SMA astrocytes undergo morphological and histological changes characteristic of reactive astrocytes in the ventral horn. S100 is a calcium binding protein highly enriched in astrocyte cell bodies and processes and labels typical resting astrocytes. Intermediate filament proteins Nestin and GFAP are generally expressed at low levels in resting astrocytes, but are highly expressed in reactive astrocytes (Frisen et al., 1995; Ridet et al., 1997). We found no statistical difference between WT and SMA spinal cord in the number of S100 positive astrocytes (Fig. 1B,C) or in their staining intensity (Fig. 1D) at either time point in the ventral spinal cord. In contrast, WT astrocytes displayed minimal cytoplasmic GFAP labeling and lightly stained thin, fine processes (Fig. 1E), whereas SMA astrocytes displayed enlarged cell bodies, intense cytoplasmic GFAP staining, and thick, short processes (Fig. 1E) with a corresponding significant up-regulation in GFAP fluorescence intensity in SMA spinal cord at both PND3 and PND9 (Fig. 1F). Furthermore, we found dramatic morphological differences in SMA GFAP positive astrocytes at both PND3 and PND9, where the cell process length was significantly reduced compared to WT astrocytes (Fig. 1G). Although Nestin is not exclusively expressed in astrocytes (data not shown), we did observe a significant up-regulation in Nestin expression in SMA spinal cord at PND9 (Fig. 2). Taken together, these characteristics are consistent with activated astrocytes (Frisen et al., 1995; Kanaan et al., 2010; Sun et al., 2010) and indicate these early changes may be a contributing factor to SMA pathology.

In order to further dissect functional properties of SMA astrocytes, we utilized human induced pluripotent stem cells (iPSCs) derived from two independent SMA patients and two independent healthy individuals. We have previously characterized this *in vitro* system to show a significant loss of motor neurons over time in the SMA iPSCs compared to control,

although the number of post-mitotic Tuj1 positive neurons were unaffected (Ebert et al., 2009; Sareen et al., 2012). Therefore, we next aimed to determine if SMA iPSC-derived astrocytes exhibit altered phenotypes compared to control iPSC-derived astrocytes over time. We differentiated astrocytes from SMA and control iPSCs using standard protocols (Emdad et al., 2012) and examined them after 2, 4, 6, and 8 weeks of differentiation. We found no detectable Iba-1 staining indicating that microglia were not present in the differentiated cultures (data not shown). We labeled astrocytes with S100 (Fig. 3A) to assess differentiation capability between lines and found that there was a trend toward fewer astrocytes in the SMA iPSC cultures, they were not statistically different from control iPSC lines (Fig. 3C). In order to further characterize the iPSC-derived astrocytes, we found ALDH1L1 positive astrocytes by immunocytochemistry (Fig. 3B) and GLT1 expression by western blot consistent with maturing astrocytes (Fig. 3D). Of note, we did not observe a decrease in GLT1 expression over time in SMA iPSC-derived astrocytes, which is in contrast to what is observed in ALS (Bruijn et al., 1997). These data collectively show that control and SMA iPSCs are capable of producing astrocytes that can be used as a robust model for astrocyte activity in SMA.

We aimed to see if SMA iPSC-derived astrocytes displayed an activated phenotype similar to that seen in the SMA Δ 7 mice. Control and SMA iPSCs were driven toward astrocyte fate and stained for GFAP (Fig. 4A). As GFAP is highly expressed in cultured astrocytes, we used both GFAP intensity measurements and morphological characteristics that would discriminate normal resting GFAP positive astrocytes from reactive GFAP positive astrocytes (Kanaan et al., 2010). We found that SMA iPSC astrocyte cultures have increased GFAP protein levels at 4 and 6 weeks of differentiation as detected by western blot (Fig. 4B), and also have significantly higher GFAP fluorescence intensity compared to control iPSC astrocyte cultures at all time points tested (Fig. 4C). Furthermore, we found that GFAP positive SMA iPSC-derived astrocytes have significantly shorter processes than control iPSC-derived astrocytes at all time points tested (Fig. 4D), indicative of a reactive state. Similar experiments staining for Vimentin, another intermediate filament protein up-regulated in reactive astrocytes, showed a trend toward increased fluorescence intensity at all time points, though results did not reach significance (data not shown). Since we do not observe motor neuron loss in SMA iPSCs until after 4 weeks of differentiation (Ebert et al., 2009; Sareen et al., 2012), these data are consistent with our *in vivo* results (Fig. 1) such that astrocyte activation is observed prior to overt motor neuron loss.

Although SMA iPSC astrocytes exhibit an altered phenotype, we next aimed to determine if they also displayed functional impairments. We used ratiometric live-cell calcium imaging to assess levels of free, intracellular calcium at baseline (Fig. 5A) and following stimulation with ATP (Fig. 5B). We selected astrocytes in culture based on morphological characteristics and a lack of KCl-induced depolarization (data not shown). SMA iPSC-derived astrocytes showed increased basal calcium levels at all time points compared to control iPSC-derived astrocytes (Fig. 5A). Additionally, stimulation with 10 μ M ATP produced only a minimal calcium response at all time points (Fig. 5B). In contrast, control iPSC-derived astrocytes showed a developmental increase in calcium response to ATP suggesting a maturation of purinergic receptors and the intracellular signaling processes necessary for effective cellular communication (Fig. 5B). In particular, the purinergic receptor P2Y2 is known to be involved in internal calcium release due to ATP signaling (Zhu and Kimelberg, 2001; Verkhratsky et al., 2012). In order to verify that the reduced activity in SMA iPSC astrocytes was not due to a loss of this receptor, we analyzed six week old astrocyte cultures and observed co-labeling of ALDH1L1 and P2Y2 by immunofluorescence (Fig. 5C) and P2Y2 protein level by western blot (Fig. 5C). Ion gradients are crucial for signal induction, so we next asked if reducing basal calcium levels in SMA astrocytes would re establish proper gradients and rescue the ATP response. By

eliminating extracellular calcium, basal calcium levels in SMA iPSC-derived astrocytes returned to control levels (Fig. 5D); however, the ATP response was unchanged (Fig. 5E) indicating SMA iPSC astrocytes have deficits in internal calcium signaling mechanisms affecting proper cellular communication. As expected, treatment with thapsigargin depleted internal calcium stores and eliminated the response to ATP in both control and SMA iPSC astrocytes (Fig. 5F). Together these results suggest that deficits in SMA iPSC-derived astrocytes may negatively impact astrocyte exocytosis of gliotransmitters and influence motor neuron health.

Previous data have shown that abnormal calcium signaling can activate ERK1/2 signaling in reactive astrocytes (Zhuang and Schnellmann, 2006; Rousseau et al., 2008). Using immunofluorescence, we found increased pERK1/2 and GFAP co-expression in SMA iPSC astrocyte cultures (Fig. 6A). Similarly, western blot analysis showed increased levels of pERK1/2 by 6 weeks in SMA iPSC-derived astrocyte cultures compared to controls (Fig. 6B). Since ERK activation can occur in response to growth factor deprivation (Zhuang and Schnellmann, 2006; Rousseau et al., 2008), we used whole cell lysates and conditioned medium from control and SMA iPSC astrocyte cultures to examine GDNF production and secretion by western blot (Fig. 6D) and ELISA (Fig. 6C), respectively. We found that SMA iPSC astrocyte cultures produced and secreted less GDNF than control iPSC astrocyte cultures (Fig. 6C,D). GDNF is known to promote survival of spinal motor neurons and its absence leads to apoptosis (Henderson et al., 1994; Oppenheim et al., 1995). Several studies suggest that motor neurons die through an apoptotic process in SMA, suggesting reduced GDNF production from SMA astrocytes may contribute to disease pathogenesis.

Discussion

Motor neuron degeneration has been well documented in SMA, but much less is known about the role of astrocytes in this disease. Here we demonstrate the first evidence that there are distinct differences between SMA and wild type glia in form and function that may contribute to motor neuron loss. Considering that selective re-expression of SMN in motor neurons is not sufficient to effectively impede SMA disease pathology (Lee et al., 2012), our data indicate that astrocytes may be an additional target for therapeutic intervention.

We have shown that SMA astrocytes show signs of activation by characteristic morphological changes and up-regulation of GFAP and Nestin (Figs. 1, 2, 4). These changes are observed prior to motor neuron loss *in vitro* and *in vivo*, suggesting that astrocyte activation may be an early contributor to motor neuron loss. In this regard, others have reported increased GFAP immunoreactivity in the spinal cord of severe SMA mice as early as post-natal day 0-1 (Dachs et al., 2011). Western blot data (Fig. 4C) are from whole cultures which contain mixed populations of neurons, glia, and non-neuronal cells. Although impure iPSC culture conditions can dilute astrocyte-specific signals, mixed cultures may be more representative of the native environment. For example, previous data have shown that isolated motor neurons from SMA mouse spinal cord do not die in culture (Rossoll et al., 2003) despite the fact that motor neurons die *in vivo*. In contrast, the SMA iPSC cultures exhibit selective motor neuron loss over time (Ebert et al., 2009; Sareen et al., 2012), indicating interactions with non-motor neuron cell types is critical to disease processes, and in the SMA iPSC system, astrocytes appear to be a key mediator.

ERK1 and ERK2 are members of the MAPK signaling pathway and are activated by dual phosphorylation by MEK1 and MEK2. ERK1/2 had generally been known as a survival pathway, but recent studies have shown it to serve an apoptotic role as well (reviewed by (Zhuang and Schnellmann, 2006)). Studies have shown that ERK1/2 can be activated by calcium leading to apoptosis (Vossler et al., 1997; Li et al., 2005). The up regulation of

pERK1/2 observed in the SMA iPSC-derived astrocytes may be a consequence of the increased basal calcium levels, but as we are not observing significant astrocyte loss (Fig. 3), pERK1/2 must have an additional role in this system. Previous studies have shown that ERK1/2 activation can increase expression of pro-inflammatory cytokines such as TNF α , IL-1 and IL-6 (Ridet et al., 1997; Wang et al., 2004; Jo et al., 2005), which can subsequently induce apoptosis in surrounding cells. Up-regulation of pro-inflammatory cytokines trigger apoptotic cascades in motor neurons. Although we failed to detect significant amounts of TNF α secretion in SMA iPSC astrocyte cultures (unpublished observations), it is possible that our detection methods were not sensitive enough to identify secreted cytokines that may be binding to death receptors on neighboring motor neurons and inducing apoptosis. However, ERK can also be activated by growth factor deficiency (Sinha et al., 2004), which may correspond to the decreased GDNF secretion observed in the SMA iPSC astrocyte cultures (Fig 6) and suggest pERK is a consequence rather than a cause of reduced GDNF expression. Motor neuron apoptotic cell death is well established to be induced by trophic factor deprivation through a fas-mediated mechanism (Oppenheim et al., 1991; Raoul et al., 1999). Reduced GDNF expression from SMA iPSC astrocytes may be a critical induction signal to activate apoptotic pathways contributing to motor neuron loss in SMA. Fas ligand binds to the cell surface fas receptor to initiate caspase-8 activation and down-stream induction of apoptotic cascades. We have previously found that SMA iPSC motor neuron cultures have increased membrane bound fas ligand and undergo caspase-8 mediated apoptosis (Sareen et al., 2012). Interestingly, this is selective for motor neurons in the culture, as other post-mitotic neurons are not affected (Ebert et al., 2009; Sareen et al., 2012). Therefore, increasing astrocyte-derived growth factor support may be effective at reducing motor neuron death in SMA.

In addition to apoptosis, other functional consequences arise due to increased basal calcium levels. For example, another study identified increased basal calcium levels in astrocytes in a transgenic mouse model of Alzheimer's disease (AD) (Kuchibhotla et al., 2009). Kuchibhotla and colleagues found that astrocytes in AD mice with plaques had higher resting calcium concentrations than wild type mice, which the authors suggested allowed astrocytes to amplify the effects of focal amyloid deposits across large neural networks thereby altering cortical and cognitive functions (Kuchibhotla et al., 2009). Regarding SMA, previous research has shown that there is defective calcium channel clustering in motor neuron axon terminals in SMA that may affect excitability (Jablonka et al., 2007). Taken together, these data suggest that calcium homeostasis is important for the maintenance of healthy neural circuits. Increased basal calcium levels may activate cell stress pathways and alter the signaling properties of the cell leading to motor neuron loss. For example, secretion of gliotransmitters by astrocytes is a calcium-dependent process by which synaptotagmin proteins on vesicles and their target membrane merge to release signaling molecules stored in the vesicular lumen (Parpura et al., 2011). These vesicles can carry luminal cargo and membrane associated molecules, and activated glia may have altered secretion efficiency, thereby changing their local environments within the cellular milieu of the brain and culture dish (Parpura et al., 2010). Interestingly, iPSC-derived SMA astrocytes never develop a robust response to ATP stimulation despite presence of purinergic receptors (Fig. 5C), which may lead to defects in ATP-regulated calcium events. The specific cause of the minimal ATP response has yet to be fully elucidated. As ion gradients are necessary for proper cell signaling, it is possible that the increased basal levels disrupted the ion gradients enough to prohibit SMA iPSC-derived astrocytes from responding to ATP. However, this is unlikely as SMA iPSC-derived astrocytes still showed minimal response to ATP in the absence of external calcium when baseline levels were normalized (Fig. 5D,E). Therefore, a more likely explanation is that SMA iPSC astrocytes have disrupted internal signaling processes, which is a path of further investigation.

In conclusion, these data provide novel insight into early dysfunction in SMA astrocytes that may contribute to motor neuron dysfunction. It is unclear at this time whether the observed astrocyte activation and functional deficits are an SMN-dependent process. Nevertheless, these data contribute to our understanding of SMA disease pathogenesis and suggest that therapies targeting both motor neurons and astrocytes may be more beneficial for treating SMA.

Acknowledgments

Funding support was provided by the National Institutes of Health R21HD060899 (ADE), R01NS40538 (CLS), and Advancing a Healthier Wisconsin (ADE). TNP was supported by the Quadracci Memorial Fellowship. The vimentin antibody generated by Dr. Alvarez-Buylla was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The Department of Biology at the University of Iowa.

References

- Azzouz M, Le T, Ralph GS, Walmsley L, Monani UR, Lee DCP, Wilkes F, Mitrophanous KA, Kingsman SM, Burghes AHM, Mazarakis ND. Lentivector-mediated SMN replacement in a mouse model of spinal muscular atrophy. *J Clin Invest*. 2004; 114:1726–1731. [PubMed: 15599397]
- Barabas ME, Kossyreva EA, Stucky CL. TRPA1 is functionally expressed primarily by IB4-binding, non peptidergic mouse and rat sensory neurons. *PLoS One*. 2012; 7:e47988. [PubMed: 23133534]
- Bebee TW, Dominguez CE, Chandler DS. Mouse models of SMA: tools for disease characterization and therapeutic development. *Hum Genet*. 2012; 131:1277–1293. [PubMed: 22543872]
- Bebee TW, Gladman JT, Chandler DS. Splicing regulation of the survival motor neuron genes and implications for treatment of spinal muscular atrophy. *Frontiers Biosci*. 2010; 15:1191–1204.
- Benkler C, Ben-Zur T, Barhum Y, Offen D. Altered astrocytic response to activation in SOD1(G93A) mice and its implications on amyotrophic lateral sclerosis pathogenesis. *Glia*. 2013; 61:312–326. [PubMed: 23280929]
- Bevan AK, Hutchinson KR, Foust KD, Braun L, McGovern VL, Schmelzer L, Ward JG, Petruska JC, Lucchesi PA, Burghes AH, Kaspar BK. Early heart failure in the SMN Δ 7 model of spinal muscular atrophy and correction by postnatal scAAV9-SMN delivery. *Hum Mol Genet*. 2010; 19:3895–3905. [PubMed: 20639395]
- 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. *Neuromuscular Disord*. 2012; 22:263–276.
- Brujin LI, Becher MW, Lee MK, Anderson KL, Jenkins NA, Copeland NG, Sisodia SS, Rothstein JD, Borchelt DR, Price DL, Cleveland DW. ALS-linked SOD1 mutant G85R mediates damage to astrocytes and promotes rapidly progressive disease with SOD1-containing inclusions. *Neuron*. 1997; 18:327–338. [PubMed: 9052802]
- Burghes AH, Ingraham SE, Kote-Jarai Z, Rosenfeld S, Herta N, Nadkarni N, Didonato CJ, Carpten J, Hurko O, Florence J, Et Al. Linkage mapping of the spinal muscular atrophy gene. *Hum Genet*. 1994; 93:305–312. [PubMed: 8125483]
- Clement AM, Nguyen MD, Roberts EA, Garcia ML, Boillee S, Rule M, McMahon AP, Doucette W, Siwek D, Ferrante RJ, Brown RH Jr, Julien JP, Goldstein LSB, Cleveland DW. Wild-type nonneuronal cells extend survival of SOD1 mutant motor neurons in ALS mice. *Science*. 2003; 302:113–117. [PubMed: 14526083]
- Crawford TO, Pardo CA. The neurobiology of childhood spinal muscular atrophy. *Neurobiol Dis*. 1996; 3:97–110. [PubMed: 9173917]
- Dachs E, Hereu M, Piedrafita L, Casanovas A, Caldero J, Esquerda JE. Defective neuromuscular junction organization and postnatal myogenesis in mice with severe spinal muscular atrophy. *J Neuropathol Exp Neurol*. 2011; 70:444–461. [PubMed: 21572339]
- Di Giorgio FP, Carrasco MA, Siao MC, Maniatis T, Eggan K. Non-cell autonomous effect of glia on motor neurons in an embryonic stem cell-based ALS model. *Nat Neurosci*. 2007; 10:608–614. [PubMed: 17435754]

- Ebert AD, Shelley BC, Hurley AM, Onorati M, Castiglioni V, Patitucci TN, Svendsen SP, Mattis VB, McGivern JV, Schwab AJ, Sareen D, Kim HW, Cattaneo E, Svendsen CN. EZ spheres: A stable and expandable culture system for the generation of pre-rosette multipotent stem cells from human ESCs and iPSCs. *Stem Cell Res.* 2013; 10:417–427. [PubMed: 23474892]
- Ebert AD, Yu J, Rose FF Jr, Mattis VB, Lorson CL, Thomson JA, Svendsen CN. Induced pluripotent stem cells from a spinal muscular atrophy patient. *Nature.* 2009; 457:277–280. [PubMed: 19098894]
- Emdad L, D'souza SL, Kothari HP, Qadeer ZA, Germano IM. Efficient differentiation of human embryonic and induced pluripotent stem cells into functional astrocytes. *Stem Cells Dev.* 2012; 21:404–410. [PubMed: 21631388]
- Fields RD, Burnstock G. Purinergic signalling in neuron glia interactions. *Nat Rev Neurosci.* 2006; 7:423–436. [PubMed: 16715052]
- Foust KD, Wang X, McGovern VL, Braun L, Bevan AK, Haidet AM, Le TT, Morales PR, Rich MM, Burghes AH, Kaspar BK. Rescue of the spinal muscular atrophy phenotype in a mouse model by early postnatal delivery of SMN. *Nat Biotechnol.* 2010; 28:271–274. [PubMed: 20190738]
- Frisen J, Johansson CB, Torok C, Risling M, Lendahl U. Rapid, widespread, and longlasting induction of nestin contributes to the generation of glial scar tissue after CNS injury. *J Cell Biol.* 1995; 131:453–464. [PubMed: 7593171]
- Gabanella F, Butchbach ME, Saieva L, Carissimi C, Burghes AH, Pellizzoni L. Ribonucleoprotein assembly defects correlate with spinal muscular atrophy severity and preferentially affect a subset of spliceosomal snRNPs. *PLoS One.* 2007; 2:e921. [PubMed: 17895963]
- Gavrilina TO, McGovern VL, Workman E, Crawford TO, Gogliotti RG, Didonato CJ, Monani UR, Morris GE, Burghes AH. Neuronal SMN expression corrects spinal muscular atrophy in severe SMA mice while muscle-specific SMN expression has no phenotypic effect. *Hum Mol Genet.* 2008; 17:1063–1075. [PubMed: 18178576]
- Giaume C, Koulakoff A, Roux L, Holcman D, Rouach N. Astroglial networks: a step further in neuroglial and gliovascular interactions. *Nat Rev Neurosci.* 2010; 11:87–99. [PubMed: 20087359]
- Gogliotti RG, Quinlan KA, Barlow CB, Heier CR, Heckman CJ, Didonato CJ. Motor neuron rescue in spinal muscular atrophy mice demonstrates that sensory-motor defects are a consequence, not a cause, of motor neuron dysfunction. *J Neurosci.* 2012; 32:3818–3829. [PubMed: 22423102]
- Hammond SM, Gogliotti RG, Rao V, Beauvais A, Kothary R, Didonato CJ. Mouse survival motor neuron alleles that mimic SMN2 splicing and are inducible rescue embryonic lethality early in development but not late. *PLoS One.* 2010; 5:e15887. [PubMed: 21249120]
- HD iPSC Consortium. Induced Pluripotent Stem Cells from Patients with Huntington's Disease Show CAG Repeat Expansion-Associated Phenotypes. *Cell Stem Cell.* 2012; 11:264–278. [PubMed: 22748968]
- Heier CR, Satta R, Lutz C, Didonato CJ. Arrhythmia and cardiac defects are a feature of spinal muscular atrophy model mice. *Hum Mol Genet.* 2010; 19:3906–3918. [PubMed: 20693262]
- Henderson CE, Phillips HS, Pollock RA, Davies AM, Lemeulle C, Armanini M, Simmons L, Moffet B, Vanden RA, Simpson LC. GDNF: a potent survival factor for motoneurons present in peripheral nerve and muscle. *Science.* 1994; 266:1062–1064. [PubMed: 7973664]
- Hua Y, Sahashi K, Hung G, Rigo F, Passini MA, Bennett CF, Krainer AR. Antisense correction of SMN2 splicing in the CNS rescues necrosis in a type III SMA mouse model. *Genes Dev.* 2010; 24:1634–1644. [PubMed: 20624852]
- Imlach WL, Beck ES, Choi BJ, Lotti F, Pellizzoni L, McCabe BD. SMN is required for sensory-motor circuit function in *Drosophila*. *Cell.* 2012; 151:427–439. [PubMed: 23063130]
- Jablonka S, Beck M, Lechner BD, Mayer C, Sendtner M. Defective Ca²⁺ channel clustering in axon terminals disturbs excitability in motoneurons in spinal muscular atrophy. *J Cell Biol.* 2007; 179:139–149. [PubMed: 17923533]
- Jablonka S, Karle K, Sandner B, Andreassi C, Von Au K, Sendtner M. Distinct and overlapping alterations in motor and sensory neurons in a mouse model of spinal muscular atrophy. *Hum Mol Genet.* 2006; 15:511–518. [PubMed: 16396995]
- Jablonka S, Rossoll W, Schrank B, Sendtner M. The role of SMN in spinal muscular atrophy. *J Neurol.* 2000; 247(Suppl 1):I37–42. [PubMed: 10795885]

- Jo SK, Cho WY, Sung SA, Kim HK, Won NH. MEK inhibitor, U0126, attenuates cisplatin-induced renal injury by decreasing inflammation and apoptosis. *Kidney Int.* 2005; 67:458–466. [PubMed: 15673293]
- Kanaan NM, Kordower JH, Collier TJ. Age-related changes in glial cells of dopamine midbrain subregions in rhesus monkeys. *Neurobiol Aging.* 2010; 31:937–952. [PubMed: 18715678]
- Kuchibhotla KV, Lattarulo CR, Hyman BT, Bacskaï BJ. Synchronous hyperactivity and intercellular calcium waves in astrocytes in Alzheimer mice. *Science.* 2009; 323:1211–1215. [PubMed: 19251629]
- Le TT, Pham LT, Butchbach ME, Zhang HL, Monani UR, Coover DD, Gavrilina TO, Xing L, Bassell GJ, Burghes AH. 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. [PubMed: 15703193]
- Lee AJ, Awano T, Park GH, Monani UR. Limited Phenotypic Effects of Selectively Augmenting the SMN Protein in the Neurons of a Mouse Model of Severe Spinal Muscular Atrophy. *PLoS One.* 2012; 7:e46353. [PubMed: 23029491]
- Lefebvre S, Burglen L, Reboullet S, Clermont O, Burlet P, Viollet L, Benichou B, Cruaud C, Millasseau P, Zeviani M. Identification and characterization of a spinal muscular atrophy-determining gene. *Cell.* 1995; 80:155–165. [PubMed: 7813012]
- Lefebvre S, Burlet P, Liu Q, Bertrand S, Clermont O, Munnich A, Dreyfuss G, Melki J. Correlation between severity and SMN protein level in spinal muscular atrophy. *Nat Genet.* 1997; 16:265–269. [PubMed: 9207792]
- Li DW, Liu JP, Mao YW, Xiang H, Wang J, Ma WY, Dong Z, Pike HM, Brown RE, Reed JC. Calcium-activated RAF/MEK/ERK signaling pathway mediates p53-dependent apoptosis and is abrogated by alpha B-crystallin through inhibition of RAS activation. *Mol Biol Cell.* 2005; 16:4437–4453. [PubMed: 16000378]
- 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. [PubMed: 21085654]
- Lotti F, Imlach WL, Saieva L, Beck ES, Hao Le T, Li DK, Jiao W, Mentis GZ, Beattie CE, McCabe BD, Pellizzoni L. An SMN-dependent U12 splicing event essential for motor circuit function. *Cell.* 2012; 151:440–454. [PubMed: 23063131]
- Marchetto MC, Muotri AR, Mu Y, Smith AM, Cezar GG, Gage FH. Non-cell-autonomous effect of human SOD1 G37R astrocytes on motor neurons derived from human embryonic stem cells. *Cell Stem Cell.* 2008; 3:649–657. [PubMed: 19041781]
- Martinez TL, Kong L, Wang X, Osborne MA, Crowder ME, Van Meerbeke JP, Xu X, Davis C, Wooley J, Goldhamer DJ, Lutz CM, Rich MM, Sumner CJ. Survival motor neuron protein in motor neurons determines synaptic integrity in spinal muscular atrophy. *J Neurosci.* 2012; 32:8703–8715. [PubMed: 22723710]
- Mentis GZ, Blivis D, Liu W, Drobac E, Crowder ME, Kong L, Alvarez FJ, Sumner CJ, O'donovan MJ. Early functional impairment of sensory-motor connectivity in a mouse model of spinal muscular atrophy. *Neuron.* 2011; 69:453–467. [PubMed: 21315257]
- Nishimaru H, Restrepo CE, Ryge J, Yanagawa Y, Kiehn O. Mammalian motor neurons corelease glutamate and acetylcholine at central synapses. *Proc Natl Acad Sci USA.* 2005; 102:5245–5249. [PubMed: 15781854]
- Oppenheim RW, Housenou LJ, Johnson JE, Lin LFH, Li L, Lo AC, Newsome AL, Prevette DM, Wang S. Developing motor neurons rescued from programmed and axotomy-induced cell death by GDNF. *Nature.* 1995; 373:344–346. [PubMed: 7830769]
- Oppenheim RW, Prevette D, Yin QW, Collins F, Macdonald J. Control of embryonic motoneuron survival in vitro by ciliary neurotrophic factor. *Science.* 1991; 251:1616–1618. [PubMed: 2011743]
- Park GH, Maeno-Hikichi Y, Awano T, Landmesser LT, Monani UR. Reduced survival of motor neuron (SMN) protein in motor neuronal progenitors functions cell autonomously to cause spinal muscular atrophy in model mice expressing the human centromeric (SMN2) gene. *J Neurosci.* 2010; 30:12005–12019. [PubMed: 20826664]

- Parpura V, Baker BJ, Jeras M, Zorec R. Regulated exocytosis in astrocytic signal integration. *Neurochem Int.* 2010; 57:451–459. [PubMed: 20156504]
- Parpura V, Grubisic V, Verkhratsky A. Ca(2+) sources for the exocytotic release of glutamate from astrocytes. *Biochimica et Biophysica Acta.* 2011; 1813:984–991. [PubMed: 21118669]
- Parpura V, Zorec R. Gliotransmission: Exocytotic release from astrocytes. *Brain Res Rev.* 2010; 63:83–92. [PubMed: 19948188]
- Passini MA, Bu J, Roskelley EM, Richards AM, Sardi SP, O’riordan CR, Klinger KW, Shihabuddin LS, Cheng SH. CNS-targeted gene therapy improves survival and motor function in a mouse model of spinal muscular atrophy. *J Clin Invest.* 2010; 120:1253–1264. [PubMed: 20234094]
- Pekny M, Nilsson M. Astrocyte activation and reactive gliosis. *Glia.* 2005; 50:427–434. [PubMed: 15846805]
- Porensky PN, Mitrpant C, McGovern VL, Bevan AK, Foust KD, Kaspar BK, Wilton SD, Burghes AH. A single administration of morpholino antisense oligomer rescues spinal muscular atrophy in mouse. *Hum Mol Genet.* 2012; 21:1625–1638. [PubMed: 22186025]
- Raoul C, Henderson CE, Pettmann B. Programmed cell death of embryonic motoneurons triggered through the Fas death receptor. *J Cell Biol.* 1999; 147:1049–1062. [PubMed: 10579724]
- Ridet JL, Malhotra SK, Privat A, Gage FH. Reactive astrocytes: cellular and molecular cues to biological function. *Trends Neurosci.* 1997; 20:570–577. [PubMed: 9416670]
- Rigo F, Hua Y, Krainer AR, Bennett CF. Antisense-based therapy for the treatment of spinal muscular atrophy. *J Cell Biol.* 2012; 199:21–25. [PubMed: 23027901]
- Rossoll W, Jablonka S, Andreassi C, Kroning AK, Karle K, Monani UR, Sendtner M. Smn, the spinal muscular atrophy-determining gene product, modulates axon growth and localization of beta-actin mRNA in growth cones of motoneurons. *J Cell Biol.* 2003; 163:801–812. [PubMed: 14623865]
- Rousseau S, Papoutsopoulou M, Symons A, Cook D, Lucocq JM, Prescott AR, O’garra A, Ley SC, Cohen P. TPL2-mediated activation of ERK1 and ERK2 regulates the processing of pre-TNF alpha in LPS-stimulated macrophages. *J Cell Sci.* 2008; 121:149–154. [PubMed: 18187448]
- Sareen D, Ebert AD, Heins BM, McGivern JV, Ornelas L, Svendsen CN. Inhibition of apoptosis blocks human motor neuron cell death in a stem cell model of spinal muscular atrophy. *PLoS One.* 2012; 7:e39113. [PubMed: 22723941]
- Schmid A, Didonato CJ. Animal models of spinal muscular atrophy. *J Child Neurol.* 2007; 22:1004–1012. [PubMed: 17761656]
- Shababi M, Habibi J, Yang HT, Vale SM, Sewell WA, Lorson CL. Cardiac defects contribute to the pathology of spinal muscular atrophy models. *Hum Mol Genet.* 2010; 19:4059–4071. [PubMed: 20696672]
- Sinha D, Bannerjee S, Schwartz JH, Lieberthal W, Levine JS. Inhibition of ligand-independent ERK1/2 activity in kidney proximal tubular cells deprived of soluble survival factors up-regulates Akt and prevents apoptosis. *J Biol Chem.* 2004; 279:10962–10972. [PubMed: 14701865]
- Sleigh JN, Gillingwater TH, Talbot K. The contribution of mouse models to understanding the pathogenesis of spinal muscular atrophy. *Disease Models Mechanisms.* 2011; 4:457–467. [PubMed: 21708901]
- Sun D, Lye-Barthel M, Masland RH, Jakobs TC. Structural remodeling of fibrous astrocytes after axonal injury. *J Neurosci.* 2010; 30:14008–14019. [PubMed: 20962222]
- Svendsen CN, Ter-Borg MG, Armstrong RJ, Rosser AE, Chandran S, Ostenfeld T, Caldwell MA. A new method for the rapid and long term growth of human neural precursor cells. *J Neurosci Methods.* 1998; 85:141–152. [PubMed: 9874150]
- Verkhratsky A, Rodriguez JJ, Parpura V. Calcium signalling in astroglia. *Mol Cell Endocrinology.* 2012; 353:45–56.
- Volterra A, Steinhauser C. Glial modulation of synaptic transmission in the hippocampus. *Glia.* 2004; 47:249–257. [PubMed: 15252814]
- Vossler MR, Yao H, York RD, Pan MG, Rim CS, Stork PJ. cAMP activates MAP kinase and Elk-1 through a B-Raf- and Rap1-dependent pathway. *Cell.* 1997; 89:73–82. [PubMed: 9094716]
- Wang DD, Bordey A. The astrocyte odyssey. *Prog Neurobiol.* 2008; 86:342–367. [PubMed: 18948166]

- Wang W, Shi L, Xie Y, Ma C, Li W, Su X, Huang S, Chen R, Zhu Z, Mao Z, Han Y, Li M. SP600125, a new JNK inhibitor, protects dopaminergic neurons in the MPTP model of Parkinson's disease. *Neurosci Res.* 2004; 48:195–202. [PubMed: 14741394]
- Zhu Y, Kimelberg HK. Developmental expression of metabotropic P2Y(1) and P2Y(2) receptors in freshly isolated astrocytes from rat hippocampus. *J Neurochem.* 2001; 77:530–541. [PubMed: 11299315]
- Zhuang S, Schnellmann RG. A death promoting role for extracellular signal-regulated kinase. *J Pharmacol Exp Ther.* 2006; 319:991–997. [PubMed: 16801453]

Fig. 1.

SMA astrocytes show morphological signs of activation in SMA Δ 7 spinal cord before motor neuron loss. **(A)** Choline Acetyltransferase (ChAT) positive motor neurons in the ventral horn show significant reduction of motor neurons by PND 9 in SMA mice. ANOVA $*=p<0.05$ ($n=3$). **(B-D)** S100+ astrocytes in the ventral horn show no difference in number **(C)** or intensity **(D)** in WT compared to SMA. **(E)** GFAP immunofluorescence in WT and SMA spinal cord at PND3. White line in upper panels indicates ventral horn. **(F)** SMA astrocytes have significantly increased immunoreactivity and **(G)** shorter processes compared to WT at PND3 and PND9. ANOVA $*=p<0.05$ ($n=4-5$). Scale bar = 50 μ m.

Fig. 2. SMA spinal cord shows increased Nestin expression by PND9. (A) Immunohistochemistry shows no significant difference in Nestin expression between WT and SMA spinal cord at PND3. (B) Nestin expression is up-regulated in SMA spinal cord compared to WT by PND9. Scale bar = 50 μ m.

Fig. 3.

Both control and SMA iPSCs differentiate into astrocytes in culture. (**A-B**) Immunostaining for S100 (**A**) and ALDH1L1 (**B**) shows astrocytes from control and SMA iPSCs at 4 weeks of differentiation. (**C**) Staining for S100 shows a non-significant trend toward fewer astrocytes in SMA iPSC cultures compared to control iPSCs (ANOVA, $p=0.31$). (**D**) Western blot analysis confirmed control and SMA iPSC astrocytes express the mature astrocyte glutamate transporter GLT1. Scale bar = 50 μ m.

Fig. 4.

SMA iPSC astrocytes show morphological signs of activation. (A) GFAP positive astrocytes in SMA iPSCs display an activated phenotype compared to control iPSCs. (B-D) SMA iPSC-derived astrocytes have increased GFAP protein (B), intense GFAP immunoreactivity (C), and truncated processes (D) consistent with an activated morphology. ANOVA $*=p<0.05$ (n=4-5). Scale bars = 50 μ m.

Fig. 5.

SMA iPSC-derived astrocyte cultures have disrupted calcium signaling. **(A)** SMA iPSC-derived astrocytes have significantly increased basal calcium compared to control iPSC-derived astrocytes. **(B)** Control, but not SMA, iPSC-derived astrocytes show a significant increase in calcium response to ATP administration over time. Example calcium transients are shown for 8wk control and SMA iPSC astrocytes. **(C)** P2Y2 receptor expression was observed in 6 week SMA iPSC-derived astrocytes cultures by immunocytochemistry (ALDH1L1, green; P2Y2, red) and western blot. Nuclei are labeled with Hoechst dye in blue; GAPDH serves as the protein loading control. **(D-E)** Removal of extracellular calcium from medium stabilizes basal calcium levels in SMA iPSC astrocytes to control levels **(D)** but does not rescue response to ATP **(E)**. **(F)** Thapsigargin treatment reduces calcium response to ATP in both control and SMA iPSC-derived astrocytes. Student's t test * $p < 0.05$ (n=4-8)

Fig. 6. SMA iPSC-derived astrocytes have increased ERK1/2 activation and decreased GDNF production. **(A)** Western blot and **(B)** immunocytochemistry indicate a significant increase in pERK1/2 in SMA compared to control iPSC astrocytes. pERK1/2 (green); GFAP (red). SMA iPSC astrocyte cultures show reduced GDNF production by **(C)** ELISA and **(D)** western blot. Student's t-test * $p < 0.05$ ($n = 9$). Scale bar = $50\mu\text{m}$.