Neurobiology of Disease

Defects in Motoneuron–Astrocyte Interactions in Spinal Muscular Atrophy

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Spinal muscular atrophy (SMA) is a motoneuron disease caused by loss or mutation in Survival of Motor Neuron 1 (*SMN1*) gene. Recent studies have shown that selective restoration of SMN protein in astrocytes partially alleviates pathology in an SMA mouse model, suggesting important roles for astrocytes in SMA. Addressing these underlying mechanisms may provide new therapeutic avenues to fight SMA. Using primary cultures of pure motoneurons or astrocytes from *SMNΔ7* (SMA) and wild-type (WT) mice, as well as their mixed and matched cocultures, we characterized the contributions of motoneurons, astrocytes, and their interactions to synapse loss in SMA. In pure motoneuron cultures, SMA motoneurons exhibited normal survival but intrinsic defects in synapse formation and synaptic transmission. In pure astrocyte cultures, SMA astrocytes exhibited defects in calcium homeostasis. In motoneuron–astrocyte contact cocultures, synapse formation and synaptic transmission were significantly reduced when either motoneurons, astrocytes or both were from SMA mice compared with those in WT motoneurons cocultured with WT astrocytes. The reduced synaptic activity is unlikely due to changes in motoneuron excitability. This disruption in synapse formation and synaptic transmission by SMN deficiency was not detected in motoneuron–astrocyte noncontact cocultures. Additionally, we observed a downregulation of Ephrin B2 in SMA astrocytes. These findings suggest that there are both cell autonomous and non–cell-autonomous defects in SMA motoneurons and astrocytes. Defects in contact interactions between SMA motoneurons and astrocytes impair synaptogenesis seen in SMA pathology, possibly due to the disruption of the Ephrin B2 pathway.

Key words: astrocytes; ephrin B2; motoneurons; spinal muscular atrophy; synapses; synaptic transmission

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Significance Statement

Astrocytes have been implicated in various neurological diseases, including spinal muscular atrophy (SMA), a childhood motoneuron disease. However, how bidirectional motoneuron–astrocyte communications may contribute to SMA disease mechanisms is unclear. Using *in vitro* culture systems, we demonstrate that SMN deficiency affects both astrocytes and motoneurons, as well as their contact interactions, leading to defects in synapse formation and synaptic transmission onto motoneurons, probably through downregulation of Ephrin B2 expression in astrocytes. Our work suggests that defects in motoneuron–astrocyte interactions exacerbate SMA synaptic pathology and that therapeutic restoration of SMN should target multiple cell types, including astrocytes.

Introduction

Spinal muscular atrophy (SMA) is an autosomal recessive motoneuron disease characterized by loss of synapses and motoneurons in the spinal cord, in addition to neuromuscular junction

defects and skeletal muscle atrophy (Crawford and Pardo, 1996; Tisdale and Pellizzoni, 2015). It has been well established that SMA is caused by the loss or mutation of the telomeric *Survival of Motor Neuron* (*SMN1*) gene with the retention of the centromeric *SMN2* gene, which results in a low level of full-length SMN protein (Lefebvre et al., 1995; Crawford and Pardo, 1996). Currently, there are no effective therapies available for this leading genetic cause of infant mortality.

Although SMN deficiency mainly affects motoneurons, motoneuron-specific SMN restoration only modestly extends the survival of SMA mice (Gogliotti et al., 2012; Martinez et al., 2012; Paez-Colasante et al., 2013). Conversely, neuronal depletion of SMN protein results in synaptic transmission defects but does not significantly affect animal survival (Park et al., 2010). These findings suggest that reduced SMN may impair not only motoneurons but also other components in the motor circuitry. Indeed,

recent studies have implicated that non-neuronal cells in the neuromuscular circuit, such as glial cells (McGivern et al., 2013; Hunter et al., 2014; Rindt et al., 2015), muscles (Nicole et al., 2003; Ruiz et al., 2010; Boyer et al., 2014), and sensory afferent connections (Ling et al., 2010; Mentis et al., 2011; Gogliotti et al., 2012) contribute to SMA pathogenesis. Furthermore, emerging evidence suggests that SMA is a multisystem disorder, including defects in non-neuronal organs, such as the heart, lung, liver, pancreas, intestines, vasculature, and bone (Hamilton and Gillingwater, 2013; Shababi et al., 2014; Iascone et al., 2015).

Astrocytes play critical roles in neuron survival, synapse formation, and maturation through diffusible factors and contact-mediated mechanisms (Barres, 2008; Allen and Barres, 2009; Clarke and Barres, 2013). Thus, dysfunction of astrocytes likely leads to synapse and/or neuronal loss. This has been implicated in various neurological diseases, such as ALS, Rett syndrome, fragile X syndrome, and Down's syndrome (Ballas et al., 2009; Ilieva et al., 2009; Garcia et al., 2010; Jacobs and Doering, 2010). A recent study showed that selective increase of SMN in astrocytes significantly extends the life span of severe SMA mice (*SMN*Δ7 mice) and partially rescues synapse loss onto spinal motoneurons and neuromuscular junction defects (Rindt et al., 2015). However, the mechanisms underlying astrocyte-mediated rescue in SMA remain unknown. Given that the loss of synapses on motoneurons is one of the key pathological features seen in SMA mice (Ling et al., 2010; Mentis et al., 2011), the role and interactions of astrocytes with motoneurons in this context need to be addressed.

To study intrinsic defects of motoneurons and astrocytes in SMA, we used primary cultures of pure motoneurons or astrocytes from *SMN*Δ7 mice, a severe SMA mouse model (Le et al., 2005). To study the interactions between motoneurons and astrocytes, we used mixed cocultures with wild-type (WT) or mutant motoneurons and astrocytes. Neuron and astrocyte cocultures provide a simplified model to understand the complex cross talk between them (Ullian et al., 2001; Di Giorgio et al., 2007; Nagai et al., 2007; Ballas et al., 2009; Haidet-Phillips et al., 2011). Cocultures between motoneurons and astrocytes can allow us to study and differentiate whether cross talk is mainly mediated via direct interaction (contact cocultures) or by diffusible factors from astrocytes. Here, we have found that SMN deficiency results in cell-autonomous defects of both motoneurons and astrocytes, as well as non–cell-autonomous defects observed in contact interactions between motoneurons and astrocytes. These interactions are likely mediated by a downregulation of Ephrin B2. The results suggest that, beyond defects in spinal motoneurons, SMA synaptic pathology is also characterized by astrocytes that are defective in promoting synaptogenesis on motoneurons. Thus, astrocytes, like motoneurons, could be a therapeutic target for SMA.

Materials and Methods

Animals. The Institutional Animal Care and Use Committee of the University of Southern California approved the care and use of animals and the experimental protocol of this study. *SMN*Δ7 mice were purchased from The Jackson Laboratory (#005025, FVB.Cg-Tg(SMN2*δ7)4299Ahmb Tg(SMN2)89Ahmb *Smn1*$^{tm1Msd/J}$) and were bred in the university animal facility. Mouse genotypes were determined as described previously (Le et al., 2005). Food and water were provided *ad libitum*; daylight cycle was set to 6:00 A.M. to 6:00 P.M.

Primary astrocyte culture. Isolated spinal cord astrocytes were prepared as described previously with some modifications (Ullian et al., 2004). WT and SMA mice of either sex were killed at postnatal day 5 with Euthasol (Virbac). Whole spinal cords were removed and immersed in ice-cold HBSS (Invitrogen) with 1% glucose. After meninges were removed, spinal cords were minced and trypsinized (0.25%) for 30 min at 37°C, triturated with glass pipettes, and centrifuged 1000 rpm at room temper-

ature for 15 min (Hettich, Rotofix 32A). The pellets were resuspended and transferred to DMEM (Invitrogen) supplemented with 10% FBS (Invitrogen) in tissue culture flasks (BD Biosciences). When the cells reached confluence, the flasks were shaken at 250 rpm for 6–8 h to remove contaminating cells. The astrocytes were harvested and reseeded onto 24 well culture plates, 35 mm dishes, or 12 mm glass coverslips precoated with 0.01% poly-L-lysine. The astrocytes were fed twice a week.

Primary motoneuron culture. Spinal cord motoneurons were prepared as described previously (Ullian et al., 2004). Briefly, spinal cords were dissected from embryonic day 13–14 WT or SMA mice and incubated into ice-cold EBSS (Invitrogen). Spinal cords from 4 or 5 embryos with the same genotype were pooled together, minced, and digested in 20 U/ml papain (Sigma)/EBSS at 37°C for 30 min, then were triturated and centrifuged (1000 rpm for 10 min). After the pellets were suspended with EBSS, the cell suspensions were layered >8.5% metrizamide and centrifuged 1100 rpm for 15 min. The supernatant and the cell interface layers were diluted into the equal volume of EBSS. Cells were centrifuged for another 10 min at 1000 rpm at room temperature. The resulting motoneuron pellets were resuspended and cultured on poly-L-lysine, and laminin-precoated 12 mm coverslips with a density of 6000–8000 cells per coverslip. Motoneurons were maintained in neurobasal medium supplemented with B27 (Invitrogen), BDNF (10 ng/ml), GDNF (10 ng/ml), and CNTF (10 ng/ml) (Invitrogen). Only half of media was replaced with fresh media every other day. Motoneuron astrocyte contact or noncontact cocultures were performed as previously described (Ullian et al., 2004; Környei et al., 2005). For motoneuron astrocyte direct contact cocultures, motoneurons were seeded directly onto astrocyte monolayers on 12 mm glass coverslips. For motoneuron astrocyte noncontact cocultures, motoneuron inserts were placed into confluent astrocytes for cocultures. Motoneuron growth media were the same for both contact and noncontact cocultures.

Electrophysiological recording. Neurons were visualized with phase-contrast illumination under an Olympus microscope equipped with 10× and 40× air objectives. The electrophysiological signals of neurons were recorded with whole-cell patch-clamp techniques. The setup has an Axopatch 200B amplifier (Molecular Devices), a Digidata 1200 analog-to-digital converter (Molecular Devices), and is controlled by Clampex 8.0 (Molecular Devices). The patch electrode had a resistance of 5–7 MΩ, when filled with the intrapipette solution as follows (in mM): 135 potassium gluconate, 5 EGTA, 0.5 CaCl$_2$, 10 HEPES, 2 Mg-ATP, and 0.1 GTP. The extracellular solution contained the following (in mM): 140 NaCl, 5 KCl, 10 HEPES, 2 MgCl$_2$, 2 CaCl$_2$, 10 glucose, pH 7.3. Drugs were added in external solution and applied with bath perfusion. Action potentials were recorded in current-clamp mode. Miniature EPSCs (mEPSCs) were recorded from voltage-clamped neurons (at −50 mV) in the presence of TTX (1 μM). Evoked action potential was recorded with injection of 800 ms current pulse in current-clamp mode. Change of membrane potential was evoked by current steps from −20 pA to 100 pA with a 10 pA increment. All recordings were performed at room temperature.

The mEPSCs and action potential were screened, counted, and analyzed using the Event Detection feature in Clampfit 10.3 after the traces were low-pass filtered at 2 kHz. The frequency and amplitude of mEPSCs and action potentials recorded from motoneurons were compared among different culture conditions with one-way ANOVA. Rheobase current for generation of action potential was defined as minimal current required to produce an action potential. Voltage threshold of action potential was measured at the beginning of upward rise of action potential. The input membrane resistance of the neuron was calculated by change of membrane potential in response to injected current, according to Ohm's law.

Fluorescent immunocytochemistry. Cultures were fixed with 4% PFA for 10 min, permeabilized with 0.01% Triton X-100 in PBS for 5 min, and blocked with 10% serum in PBS for 30 min. The cultures were incubated 1 h at room temperature with following primary antibodies: rabbit anti-Hb9 antibody (1:200, Millipore), rabbit anti-synaptophysin (1:200, Synaptic Systems), mouse anti-PSD95 (1:1000, NeuroMab), and rabbit anti-S100 antibody (1:200, Sigma). The samples then were incubated with fluorescence-conjugated secondary antibodies at room temperature

Table 1. Primers for the target genes amplification

Genes	Forward primer	Reverse primer	Tm	Product (bp)
Ephrin-A3 (Carmona et al., 2009)	GCAGCACGCCTCGCACAGC	AAGGATGTAGGCCCCACAGCA	65	171
Ephrin-A4 (Du et al., 2012)	CAGCGCTACACACCCTTCCC	GTGATGACCCGCTCTCCTTG	65	142
Ephrin-B2 (Pickles, 2003)	TTGAAAAGCCAAAGGTCAAAC	CTCTAAGAGGTGGGCCATGTG	55	227

for 1 h. The samples were immersed in Vectorshield mounting medium (Vector Laboratories) and were covered by coverslips.

Samples processed with fluorescent immunocytochemistry were imaged with a confocal microscope (Zeiss). ImageJ (National Institutes of Health) was used for image processing. Synaptic puncta were counted manually as described previously (Ullian et al., 2004). Neuron survival at 12 DIV was normalized as a percentage of initial number counted at day 0. All data were obtained from three coverslips at each experiment and at least three different cell preparations.

RT-PCR of individual genes. Total RNA was extracted from cultured astrocytes using TRIzol reagent (Invitrogen) and treated with DNase using the DNA-free kit (Thermo Fisher Scientific); 2 μg of total RNA was reverse-transcribed using the SuperScript III (Invitrogen) to synthesize cDNA. Quantitative PCR was performed using a CFX96 real-time PCR detection system (Bio-Rad). The relative expression level for each gene was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001), and all PCR values were normalized with the housekeeping genes, such as GAPDH or actin (Table 1) (Pickles, 2003; Carmona et al., 2009; Du et al., 2012).

Calcium imaging. Astrocytes or motoneurons (WT and SMA) on 12 mm glass coverslips were washed with extracellular solution the same as one used for electrophysiological recordings, and then loaded for 30 min at room temperature with 5 μM fura-2 (Invitrogen) and 1 mg/ml BSA in extracellular solution. Pseudo-color fluorescence ratio at excitation of 340 and 380 nm was obtained with Olympus IX70 fluorescent microscope and Metafluor software (Molecular Devices). All data presented were obtained from three coverslips of each experiment and at least three different cell preparations.

Statistical analysis. Unpaired t test or one-way ANOVA as needed was applied with Prism 5.0 software. The p values <0.05 are considered significant. Results are expressed as mean ± SEM.

Results

SMN deficiency in motoneurons impairs synapse formation and function

To determine whether the synapse loss onto spinal motoneurons seen in *SMNΔ7* mice (Ling et al., 2010; Mentis et al., 2011) is a cell-autonomous defect of SMA motoneurons, motoneurons were isolated from spinal cords of WT and SMA mice at embryonic day (E) 13–14 and cultured for 12 DIV. First, we quantified the number of synapses formed onto motoneurons. Synapses were immunostained with antibodies against presynaptic and postsynaptic markers, synaptophysin (Fig. 1A, green fluorescence) and PSD95 (Fig. 1A, red fluorescence), respectively. Synapse numbers were counted within the area twice the diameter of the cell body. The vast majority of presynaptic synaptophysin puncta overlapped with postsynaptic PSD95 staining on the soma and along the neurite of motoneurons (Fig. 1A), suggesting that synaptophysin puncta correspond to structural synapses. As shown in Figure 1B, a significant reduction (~20%) in synapse number was observed on SMA motoneurons compared with WT motoneurons ($p < 0.001$). Next, the viability of WT and SMA motoneurons was measured to rule out the possibility of motoneuron loss, which in turn could cause a reduction of synapse number on SMA motoneurons. There was no difference in the survival of WT and SMA motoneurons at 12 DIV ($p = 0.75$) (Fig. 1C). Additionally, the

difference in synapse numbers was not due to the variation in the purity of motoneurons because >90% of Nissl-positive neurons were also stained by Hb9, a motoneuron marker (data not shown).

To investigate whether the reduction of synapses, formed onto SMA motoneurons, leads to the decrease in synaptic activity, we recorded synaptic currents from WT and SMA motoneurons at 12–14 DIV using the whole-cell patch-clamp technique. The recorded mEPSCs from motoneuron cultures were completely blocked by 20 μM CNQX, a specific AMPA receptor blocker, suggesting that they were glutamatergic (Fig. 1D). This result was consistent with the previous study (Ullian et al., 2004) showing that primary cultured motoneurons form glutamatergic synapses with each other. Although primary motoneurons do secrete acetylcholine (Camu and Henderson, 1992; Martinou et al., 1992), it would be improbable to record acetycholine-mediated mEPSCs given that motoneurons express none or few nicotinic acetylcholine receptors. Frequency and amplitude of mEPSC were measured. The mean frequency of mEPSC represents the amount of glutamate release from presynaptic sites, whereas the mean amplitude of mEPSCs represents the property of postsynaptic glutamate receptors. We observed an ~58% reduction in mEPSC frequency ($p < 0.001$) but no change in mEPSC amplitude ($p = 0.26$) from SMA motoneurons, compared with that from WT motoneurons (Fig. 1E,F), indicating that SMN deficiency adversely affects presynaptic glutamate release without changing postsynaptic glutamate receptor function. To further confirm that glutamate receptors on motoneurons are not affected by SMN deficiency, we used Ca^{2+} imaging approach with fura-2 AM to record activation of glutamate receptors on WT and SMA motoneurons elicited by 100 μM glutamate. The kinetics of intracellular calcium concentration $[Ca^{2+}]_i$ were quantified by the A340/A380 ratio (Fig. 1G). WT and SMA motoneurons displayed no difference in resting $[Ca^{2+}]_i$ level ($p = 0.24$) (Fig. 1H). In addition, no difference in the $[Ca^{2+}]_i$ response elicited by 100 μM glutamate was detected between WT and SMA motoneurons ($p = 0.35$) (Fig. 1I). These results suggest that the function of glutamate receptors on motoneurons is not altered by SMN deficiency, which is also consistent with our patch-clamp data showing no change in mEPSC amplitude. Together, our results suggest that SMN deficiency in motoneurons, although not affecting motoneuron survival, results in reduced synapse formation and synapse transmission, demonstrating an intrinsic deficit of SMA motoneurons in synapse formation *in vitro*.

SMN deficiency in astrocytes alters calcium concentrations

Having demonstrated the intrinsic defects in SMA motoneurons, we then asked whether there were also intrinsic defects in SMA astrocytes. To address this question, we used pure astrocytes in culture and examined astrocyte Ca^{2+} signaling, which is regulated by cellular intrinsic properties and essential to neuron-astrocyte communication in the brain (Perea and Araque, 2005; Araque, 2008).

In WT and SMA spinal astrocyte cultures, >95% cells were positively stained by S100 antibody, an astrocyte marker, confirming the purity of the population (Fig. 2A). SMA astrocytes displayed similar cell density and size after 6 d of culture as WT astrocytes (Fig. 2B,C). At resting condition, SMA astrocytes had modest but significant lower basal $[Ca^{2+}]_i$ compared with WT astrocytes, as illustrated in Figure 2D, E ($p < 0.0001$). Applying 50 mM KCl evoked an increase in $[Ca^{2+}]_i$ in >80% of WT or SMA astrocytes tested (WT: $n = 94$ of 112, SMA: $n = 156$ of 191), but the $[Ca^{2+}]_i$ increase (peak-baseline) was ~25% higher

in SMA astrocytes than that in WT astrocytes ($p < 0.001$; Fig. 2 F, G). Similarly to KCl, application of 10 μM ATP evoked ~25% greater increase of $[Ca^{2+}]_i$ in SMA astrocytes compared with WT astrocytes ($p < 0.0001$; Fig. 2 H, I). These results indicate that SMN deficiency alters intracellular calcium signaling in astrocytes, which in turn may impair the communication between motoneurons and astrocytes. In contrast to our results shown here, a recent study has shown that astrocytes from SMA-induced pluripotent stem cells (iPSCs) have higher basal $[Ca^{2+}]_i$ and decreased response to ATP stimulation than control iPSC astrocytes (McGivern et al., 2013). The inconsistency may be due to the difference in species (human vs mouse) and preparations (human iPSC-derived astrocytes with the influence of other cell types in the same culture vs pure primary mouse spinal astrocytes). In addition, many astrocyte-specific genes (e.g., GFAP, S100 β, NFIA/B, GLAST, Sox9) are differentially regulated during astrocyte development (Cahoy et al., 2008; Molofsky et al., 2012); thus, it is likely that astrocytes at different maturation stages (adult vs neonatal) are molecularly and functionally different (Molofsky et al., 2012).

SMN deficiency alters motoneuron–astrocyte interactions in synapse formation and function in contact cocultures

Given that SMA astrocytes have abnormal calcium signaling (Fig. 2) and SMN restoration in astrocytes mitigates SMA disease pathologies (Rindt et al., 2015), SMN deficiency in astrocytes might alter normal motoneuron–astrocyte interactions. To address whether SMA astrocytes are involved in motoneuron dysfunction and loss in SMA, we used a coculture system in which motoneurons were directly plated onto a feeder layer of astrocytes (illustrated in Fig. 3A).

First, to examine whether SMN deficiency in astrocytes affect neuronal survival, WT or SMA astrocytes were cultured with WT or SMA motoneurons for 12–14 DIV and neuronal survival was quantified using Hb9 antibody staining (Fig. 3B). We observed that astrocytes, regardless of their genotypes, significantly enhanced the survival of WT and SMA motoneurons by ~8-fold ($p < 0.01$, 3 experiments per condition; Fig. 3C, bars 1–4) compared with pure motoneuron cultures (Fig. 3C, bars 5, 6), which is consistent with previous studies that astrocytes promote motoneuron survival in vitro (Ullian et al., 2004). Interestingly, the survival of WT and SMA motoneurons did not differ when cocultured with WT or SMA astrocytes (Fig. 3C, bars 1–4; $p > 0.05$, 3 experiments per condition, one-way ANOVA). These results demonstrate that SMA astrocytes are capable of promoting motoneuron survival to the same degree as WT astrocytes in vitro.
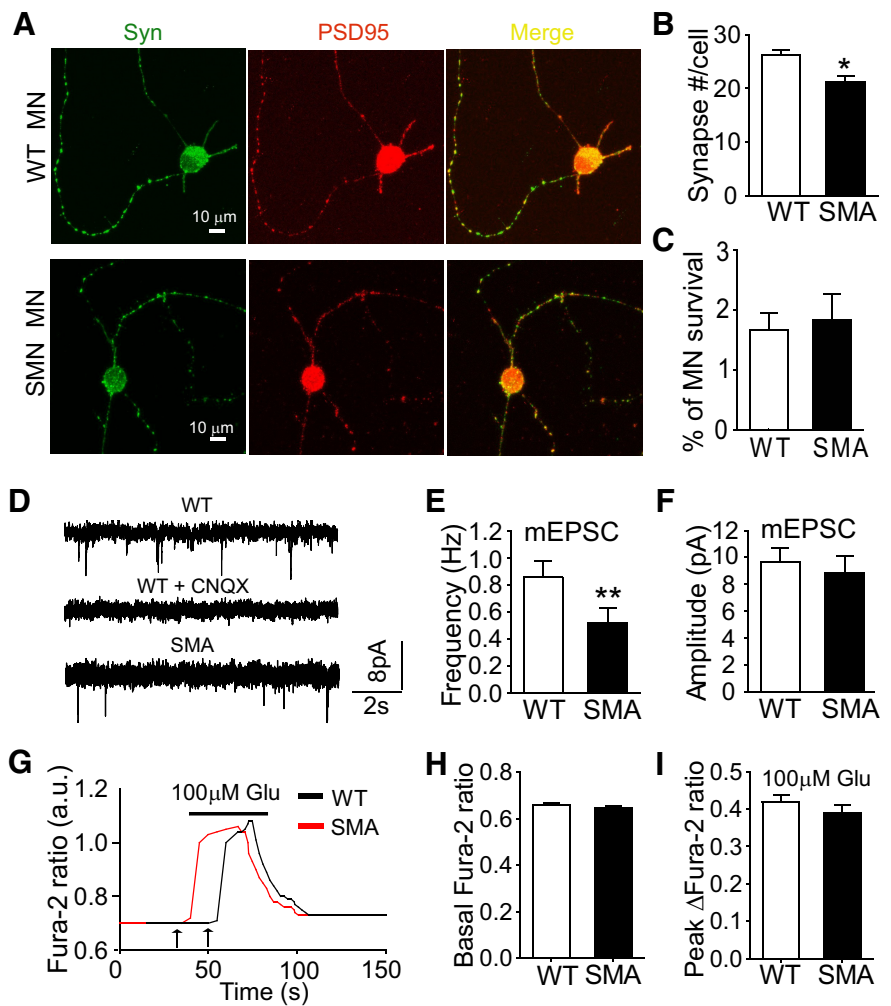

Figure 1. SMN deficiency in motoneurons impairs synapse formation and synaptic transmission. *A*, Representative images of a WT and SMA motoneuron (MN) immunostaining with specific antibodies against presynaptic and postsynaptic markers, synaptophysin (Syn) and PSD95, respectively. More than 95% synaptophysin-positive puncta are colocalized with postsynaptic PSD95 staining. *B*, Synapse number per cell is significantly decreased in SMA motoneurons than that in WT motoneurons (WT: 26.2 ± 0.9, $n = 71$; SMA: 21.2 ± 1.1, $n = 71$). $p < 0.001$ (unpaired t test). *C*, SMA motoneurons display normal survival as WT motoneurons at 12 DIV (WT: 1.7 ± 0.3%; SMA: 1.8 ± 0.4%; $p = 0.75$). *D*, Glutamatergic mEPSCs are recorded from a WT and SMA motoneuron, which can be blocked by CNQX. *E*, *F*, The frequency (*E*) but not amplitude (*F*) of mEPSC is decreased on SMA motoneurons, compared with that on WT motoneurons (frequency: WT, 0.76 ± 0.19 Hz, $n = 35$; SMA, 0.34 ± 0.18 Hz, $n = 35$; $p < 0.001$, unpaired t test; amplitude: WT, 9.7 ± 1.1 pA; SMA, 8.8 ± 1.3 pA; $p = 0.24$, unpaired t test). *G*, $[Ca^{2+}]_i$ increase elicited by 100 μM glutamate. Arrow indicates application of glutamate. *H*, *I*, $[Ca^{2+}]_i$ basal level (*H*) and $[Ca^{2+}]_i$ increase with 100 μM glutamate application (*I*) in WT and SMA motoneurons (calcium basal level: WT, 0.66 ± 0.008, $n = 75$; SMA, 0.64 ± 0.009 $n = 63$; $p = 0.24$, unpaired t test). *$p < 0.05$. **$p < 0.01$. Error bars indicate SE.

Next, we tested whether the formation of synapses onto motoneurons is impaired by SMN deficiency in astrocytes. We found that more synapses were formed in WT motoneurons cocultured with WT astrocytes (Fig. 4 A, B, bar 1) than synapses formed in WT motoneurons cultured alone (Fig. 4 A, B, bar 5), which is consistent with a previous study (Ullian et al., 2004). The addition of SMA astrocytes also increased synapse number in WT motoneurons (Fig. 4 A, B, bars 2, 5). However, a significant ~40% reduction in synapse number was observed in WT motoneurons cocultured with SMA astrocytes (Fig. 4 A, B, bar 2) compared with cocultured with WT astrocytes (Fig. 4 A, B, bar 1). These results suggest that SMA astrocytes have defects in promoting synapse formation in vitro. In addition, we observed that WT astrocytes promoted synapse formation in SMA motoneurons by ~2-fold compared with SMA motoneurons alone (Fig. 4B, bars
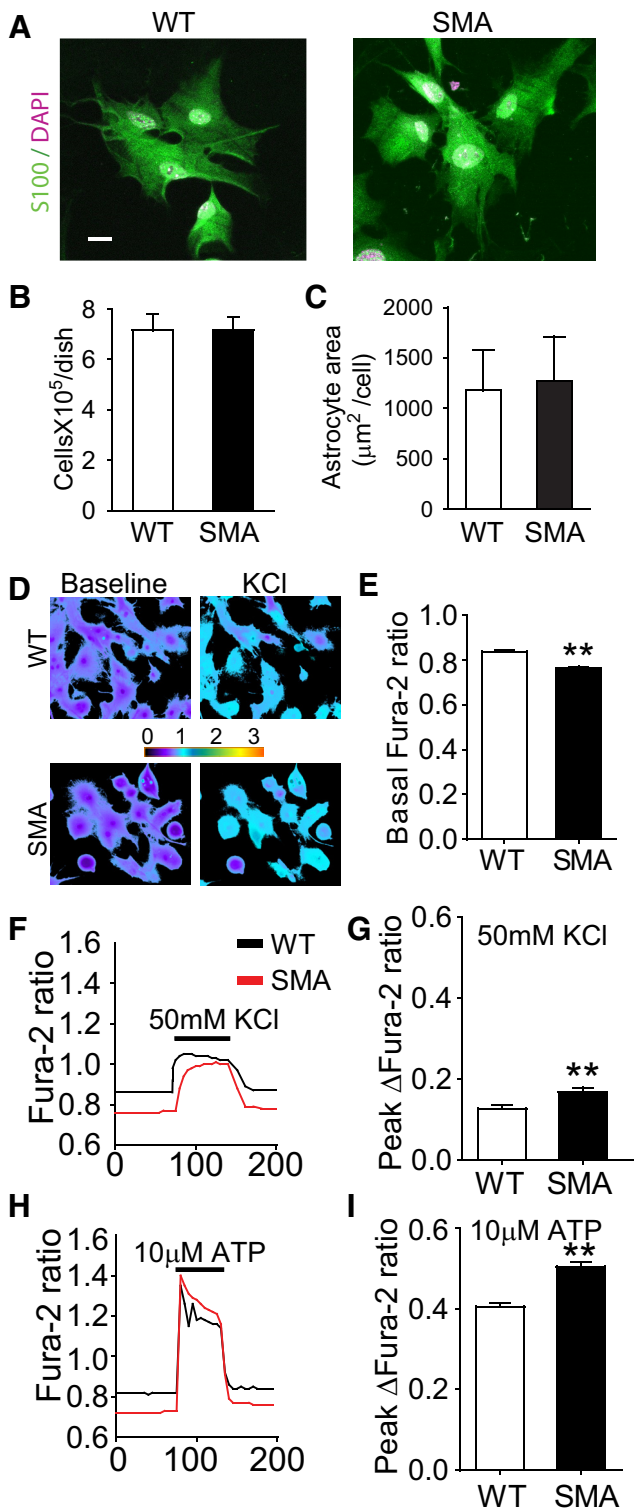
Figure 2. SMN deficiency in astrocytes alters calcium signals. **A**, WT and SMA spinal astrocytes immunostaining with S100 antibody (green) and DAPI (magenta). Scale bar, 25 μm. **B**, WT astrocytes and SMA astrocytes show a similar cell density 6 d after culture. Astrocytes are plated in 35 mm dishes. Average cell number is counted on day 6 from 4 dishes in each group. **C**, WT and SMA astrocytes show similar cell size. Area of individual astrocyte is analyzed from >200 astrocytes in each group. **D**, Pseudocolored Fura2 fluorescence imaging in WT and SMA astrocytes shows rise of $[Ca^{2+}]_i$ concentration with 50 mM KCl application. **E**, $[Ca^{2+}]_i$ basal level is significantly lower in SMA astrocytes than that in WT astrocytes (WT: 0.84 ± 0.007 a.u., $n = 102$; SMA, 0.76 ± 0.006 a.u., $n = 162$; $p < 0.001$, unpaired t test). **F**, $[Ca^{2+}]_i$ increase elicited by 50 mM KCl. **G**, SMA astrocytes have stronger increase of $[Ca^{2+}]_i$ to KCl application than WT astrocytes (WT, 0.13 ± 0.0 a.u., $n = 110$; SMA, 0.17 ± 0.01 a.u., $n = 174$; $p < 0.001$,

3, 6), but the synapse number did not reach the level of WT motoneurons cocultured with WT astrocytes (Fig. 4B, bars 1, 3). Thus, these data indicate that, in addition to the cell-autonomous defect, SMA motoneurons also display a defective response to astrocyte-derived signals. Furthermore, synapse numbers were at the same level between cocultures of SMA motoneurons with WT astrocytes and SMA motoneurons with SMA astrocytes (Fig. 4B, bars 3, 4). These data suggest that the synapse formation deficit in SMA may be due to a defect in the interactions between motoneurons and astrocytes. That is, defects of either component causes deficit in motoneuron synapse formation.

To investigate whether the reduction of synapse numbers on motoneurons upon SMN deficiency could lead to impaired synaptic transmission, we performed whole-cell patch-clamp recordings of mEPSCs from motoneurons (WT or SMA) in contact cocultures at 12–14 DIV (Fig. 4C). In WT motoneurons cocultured with WT astrocytes, we recorded a ~3.0-fold increase in mEPSC frequency compared with that in WT motoneurons alone (Fig. 4D, bars 1, 5). The frequency of mEPSCs on WT motoneurons was also increased when cocultured with SMA astrocytes compared with that in WT motoneurons alone (Fig. 4D, bars 2, 5). However, there was an ~46% reduction of mEPSC frequency between WT motoneurons cocultured with SMA astrocytes and WT motoneurons cocultured with WT astrocytes (Fig. 4D, bars 1, 2). This result suggests that SMA astrocytes exhibit a defect in enhancing synaptic function in motoneurons. In SMA motoneurons cocultured with either WT or SMA astrocytes, there was also a significant increase in mEPSC frequency compared with that of SMA motoneurons alone (Fig. 4D, bars 3, 4, 6). These data suggest that astrocytes from both WT and SMA are capable of enhancing synaptic function even in SMA motoneurons. However, there was still a significant reduction in mEPSC frequency compared with that in WT motoneurons cocultured with WT astrocytes (Fig. 4D, bars 1, 3, 4), indicating a defective response of SMA motoneurons to astrocyte signaling. Analysis of mEPSC amplitude revealed that the mean amplitude of mEPSCs on WT motoneurons was increased by ~60% in the presence of WT astrocytes ($p < 0.05$; Fig. 4E, bars 1, 5), consistent with a previous study showing that astrocytes enhance postsynaptic AMPA receptor responsiveness (Ullian et al., 2004). We also noticed that, regardless of their genotypes, astrocytes increased the mean amplitude of mEPSCs on either WT or SMA motoneurons ($p < 0.05$; Fig. 4E, bars 1–4 vs bars 5, 6) to a similar level, implicating that SMN deficiency on either astrocytes or motoneurons has little effect on postsynaptic AMPA receptors. Our data show that SMN deficiency impairs motoneuron–astrocyte cross talk and leads to defects in both synapse formation and function.

SMN deficiency does not alter motoneuron excitability
It is possible that the reduced synaptic activity of motoneurons driven by SMN deficiency (Fig. 4D) may be associated with the change in motoneuron excitability. To test this hypothesis, we recorded evoked action potential upon increasing hyperpolarizing or depolarizing current injection. Action potential threshold and intrinsic membrane properties were analyzed and compared between WT and SMA motoneurons when cultured with or with-

←

unpaired t test). **H**, $[Ca^{2+}]_i$ increase elicited by 10 μM ATP. **I**, SMA astrocytes have stronger increase of $[Ca^{2+}]_i$ to ATP application than WT astrocytes (WT, 0.4 ± 0.009 a.u., $n = 240$; SMA, 0.5 ± 0.01 a.u., $n = 236$; $p < 0.001$, unpaired t test). **p < 0.01. Error bars indicate SE.
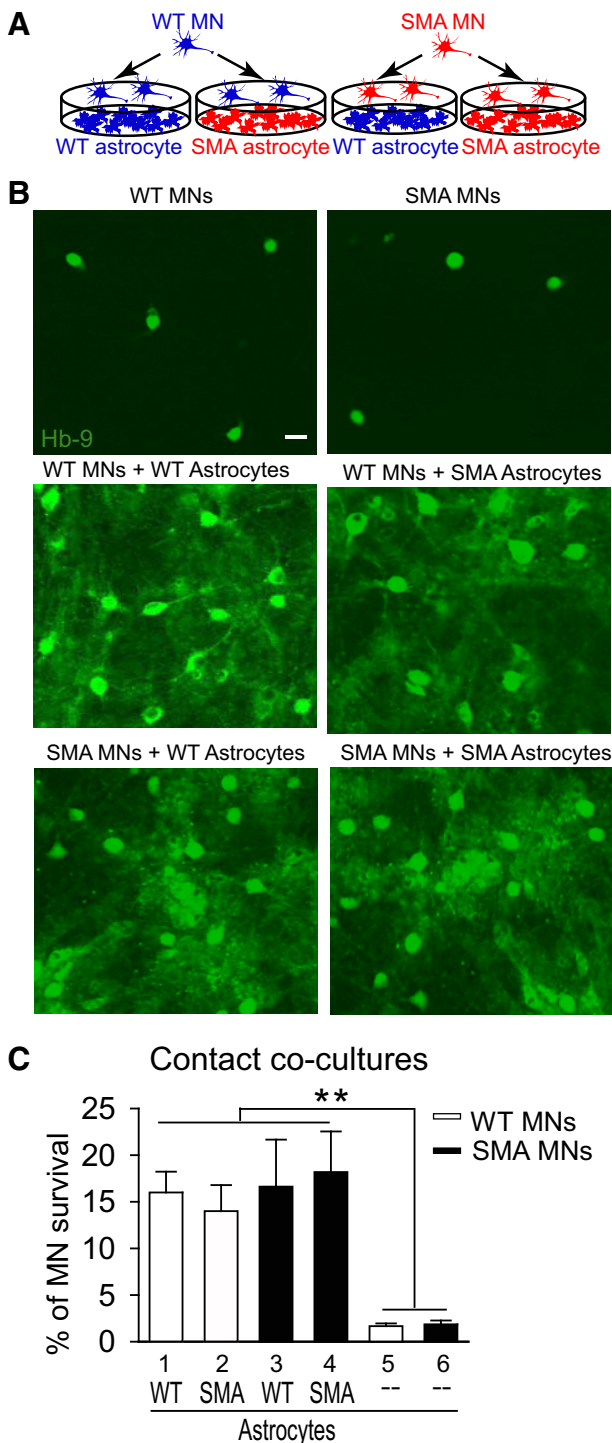
Figure 3. Motoneuron survival is not changed in motoneuron–astrocyte cocultures by SMN deficiency. ***A***, Schematic diagram of motoneuron astrocyte contact cocultures. Motoneurons (WT or SMA) are grown on a feeder layer of astrocytes (WT or SMA) allowing physical contacts between motoneurons and astrocytes. ***B***, Representative images of WT or SMA motoneurons grown alone or in astrocyte contact cocultures. The motoneurons are stained with Hb9 antibody (green). Scale bar, 20 μm. ***C***, The survival of motoneurons in contact cocultures. The survival of SMA motoneurons does not differ from WT motoneurons in culture alone (WT, bar 5: 1.7 ± 0.3%; SMA, bar 6: 1.8 ± 0.4%; $p = 0.75$). The survival of motoneurons (WT or SMA, bars 5, 6) is significantly increased in the presence of either WT or SMA astrocytes (bars 1–4; $p = 0.005$, one-way ANOVA). In contrast, the survival of WT or SMA motoneurons shows no difference when cocultured with either WT or SMA astrocytes (WT MNs with WT astrocytes, bar 1: 16 ± 2.2%; WT MNs with SMA astrocytes, bar 2: 14 ± 2.8%; SMA MNs with WT astrocytes, bar 3: 16.6 ± 5%; SMA MNs with SMA astrocytes, bar 4: 18.2 ± 4.4%). **$p < 0.01$, n is equal to 4 different cell preparations. Error bars indicate SE.

out WT or SMA astrocytes. We observed that both SMA and WT motoneurons were able to readily fire action potential upon depolarization current injection (Fig. 5A). Both resting membrane potential and rheobase current for evoked action potential did not differ between WT and SMA motoneurons (Table 2). Furthermore, there was no significant difference regarding resting membrane potential, input resistance, rheobase current, and voltage threshold on motoneurons (WT and SMA) in contact cocultures (Table 2). However, pure motoneuron cultures from both WT and SMA displayed similarly higher input resistance and rheobase current compared with those in motoneuron–astrocyte contact cocultures (one-way ANOVA, $p < 0.05$; Table 2). Because cells with less surface area tend to have a greater input resistance (Mentis et al., 2011), we measured the size of motoneurons. The size of motoneuron when cultured alone was significantly smaller than that when cocultured with astrocytes (Fig. 5B). Therefore, the change in input resistance and rheobase current of WT or SMA motoneurons cultured alone is likely due to the change in motoneuron size rather than SMN deficiency in motoneurons. These results indicate that motoneuron excitability is less likely affected by SMN deficiency in either motoneurons or astrocytes.

Reduction of synapse formation and function on motoneuron by SMN deficiency is not mediated by astrocyte-diffusible factors *in vitro*
It is known that astrocytes modulate synapse formation and maintenance through both direct contact machinery and astrocyte-diffusible factors, such as neurotrophic factors, tumor necrosis factor-α, or synaptogenic molecules (Christopherson et al., 2005; Barker et al., 2008; Clarke and Barres, 2013). To examine whether the reduction of synapse formation is due to a defect of SMA astrocytes in contact support or a defect in the release of diffusible factors, we seeded motoneurons on coverslips and set coverslips on top of a feeder layer of astrocytes for coculture (Fig. 6A). Such an approach using noncontact coculture allows astrocytes to interact with motoneurons through diffusible factors without physical contact.

In noncontact cocultures, astrocytes from WT or SMA significantly increased the survival of both WT and SMA motoneurons ($p < 0.05$; Fig. 6B, bars 1–4 vs bars 5, 6). The result is similar to that in contact cocultures shown above (Fig. 3C). Additionally, the survival of WT motoneurons (grown above either WT or SMA astrocytes) was not different from SMA motoneurons (grown above either WT or SMA astrocytes) ($p > 0.05$, 3 experiments per condition, one-way ANOVA; Fig. 6B, bars 1–4). The number of synapses formed on motoneurons in noncontact cocultures were at the same level in all four different combinations of motoneurons and astrocytes ($p = 0.54$, one-way ANOVA, 3 experiments in each condition; Fig. 6C). Consistent with these data, there was no significant difference in either mEPSC frequency ($p = 0.63$, one-way ANOVA; Fig. 6D) or amplitude ($p = 0.43$, one-way ANOVA; Fig. 6E) in these noncontact cocultures. These data suggest that SMA astrocytes are capable of secreting diffusible factors for promoting synapse formation and function to the same level as WT astrocytes. Thus, synaptogenic deficits in SMA are less likely to be mediated by astrocyte-diffusible factors. The defect in contact support might be a major pathophysiological feature of motoneuron–astrocyte interactions in SMA.

SMN deficiency alters Ephrin B2 expression in astrocytes
Our findings (Figs. 4, 6) suggested that the defects in contact support may disrupt motoneuron–astrocyte interactions in
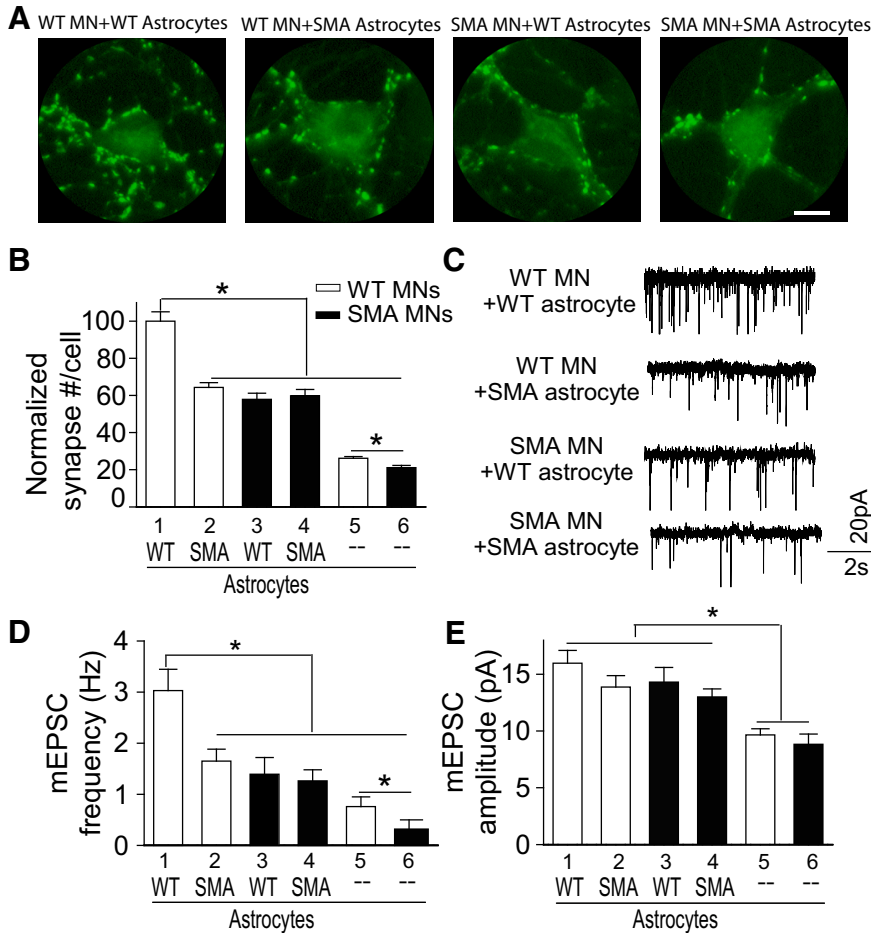
Figure 4. SMN deficiency alters motoneuron–astrocyte interactions in synapse formation and activation in contact cocultures. **A**, Images of a motoneuron labeled with synaptophysin antibody for synapses (green) in contact cocultures. Scale bar, 10 μm. **B**, Summary of normalized synapse number on motoneurons. SMA MNs (black bar) have less synapse number than WT MNs (white bar). Astrocytes from both WT and SMA significantly increase synapse number on either WT or SMA motoneurons. However, cocultures where deficiency of SMN in either MNs or astrocytes or both have less synapse number than WT MNs cocultured with WT astrocytes: WT MNs with WT astrocytes, bar 1: $100 \pm 5.1\%$; WT MNs with SMA astrocytes, bar 2: $64.3 \pm 2.6\%$; SMA MNs with WT astrocytes, bar 3: $57.9 \pm 3.3\%$; SMA MNs with SMA astrocytes, bar 4: $59.8 \pm 3.4\%$; WT MN, bar 5: $26.2 \pm 0.9\%$; SMA MN, bar 6: $21.1 \pm 1.1\%$; $n > 30$ for each group. $*p < 0.05$ (one-way ANOVA). **C**, Representative mEPSC traces recorded from either a WT or an SMA motoneuron in contact coculture. **D, E**, Summary of mEPSC frequency (**D**) and amplitude (**E**) on motoneurons. mEPSC frequency recorded on MNs shows similar trend as synapse number in **B**. Astrocytes from both WT and SMA increase mEPSC amplitude on either WT or SMA MNs to a similar level (WT MNs with WT astrocytes, bar 1: 3.0 ± 0.4 Hz, 16 ± 1.1 pA; WT MNs with SMA astrocytes, bar 2: 1.7 ± 0.2 Hz, 13.9 ± 1.0 pA; SMA MNs with WT astrocytes, bar 3: 1.4 ± 0.3 Hz, 14.3 ± 1.3 pA; SMA MNs with SMA astrocytes, bar 4: 1.3 ± 0.2 Hz, 13.4 ± 0.7 pA; WT MNs, bar 5: 0.76 ± 0.19 Hz, 9.7 ± 1.1 pA; SMA MNs, bar 6: 0.34 ± 0.18 Hz, 8.8 ± 1.3 pA). $n > 25$ for each group. $*p < 0.05$ (one-way ANOVA). Error bars indicate SE.

SMA. We then explored the contact molecules that might be involved in these processes. It has been widely recognized that membrane binding ligands, Ephrins, originating from astrocytes, play positive roles in axon guidance and synapse development through binding to their neuron-bond receptors (Murai et al., 2003; O'Leary and McLaughlin, 2005; Lai and Ip, 2009). Ephrins are categorized into two types (Ephrin A and B) based on the properties of a glycosylphosphatidylinositol anchor or a transmembrane domain (Lai and Ip, 2009). The receptors for Ephrin A and Ephrin B are receptor tyrosine kinases, which are able to initiate intracellular signaling cascades. As astrocytes in murine express Ephrin A3, A4, and B2 (Ota et al., 2013), we used qRT-PCR to detect mRNA levels of these subtypes of Ephrins in both WT and SMA primary cultured astrocytes (Fig. 7A–C). We found that, whereas the expression of Ephrin A3 and A4 was not changed, Ephrin B2 mRNA was reduced in SMA astrocytes by

~73%, compared with that in WT astrocytes ($p < 0.05$, Fig. 7C). It is possible that disruption of Ephrin B2 signaling may mediate impaired synapse formation on motoneurons in SMA.

Discussion

The present study used primary cultures of WT and SMA spinal motoneurons and astrocytes, to investigate their intrinsic deficits, as well as their interactions in SMA. First, SMA motoneurons alone exhibited intrinsic deficits in synapse formation and glutamatergic synaptic transmission. Second, SMA astrocytes had defects in calcium homeostasis, demonstrated by a lower basal $[Ca^{2+}]_i$ level but a stronger $[Ca^{2+}]_i$ increase to ATP application, in comparison with WT astrocytes. Third, in motoneuron and astrocyte cocultures, SMN deficiency in motoneurons, astrocytes, or both impaired the formation and function of synapse onto motoneurons, probably through a contact-dependent mechanism. Fourth, the transcript of contact molecule Ephrin B2 in SMA astrocytes was markedly reduced compared with WT astrocytes. Together, we demonstrate that, in SMA, there is a deficit in motoneuron–astrocyte contact interactions probably associated with downregulation of Ephrin B2, which could impair synapse formation.

Intrinsic defects of SMA motoneurons in synapse formation and function

Previous studies from our and Mentis' groups have found decreases in synapse number and function on motoneurons in the SMA mouse model (Ling et al., 2010; Mentis et al., 2011). Here we used pure motoneuron cultures to specifically isolate and characterize dysfunction of motoneurons in SMA. We provide evidence that SMN deficiency causes intrinsic defect in synaptogenesis in motoneurons (Fig. 1). First, SMA motoneurons had ~20% reduction in synapse number in comparison with WT motoneurons. Second, mEPSC frequency, but not amplitude, in SMA motoneurons was decreased compared with that in WT motoneurons. Other studies showed that SMN reduction causes axon development defect of motoneurons (McWhorter et al., 2003; Rossoll et al., 2003) and that SMN-selective restoration in motoneurons is capable of restoring synapse numbers onto motoneurons (Gogliotti et al., 2012; Martinez et al., 2012). Our results confirm previous evidence in addition to supporting the hypothesis that SMN in motoneurons serves an important function in synapse formation and function (Rossoll et al., 2003; Park et al., 2010; Martinez et al., 2012). We also observed that SMA motoneurons displayed similar survival rate as WT motoneurons. The result is consistent with previous studies (Rossoll et al., 2003; Zhang et al., 2009), suggesting that synapse loss on motoneurons preceding motoneuron death in
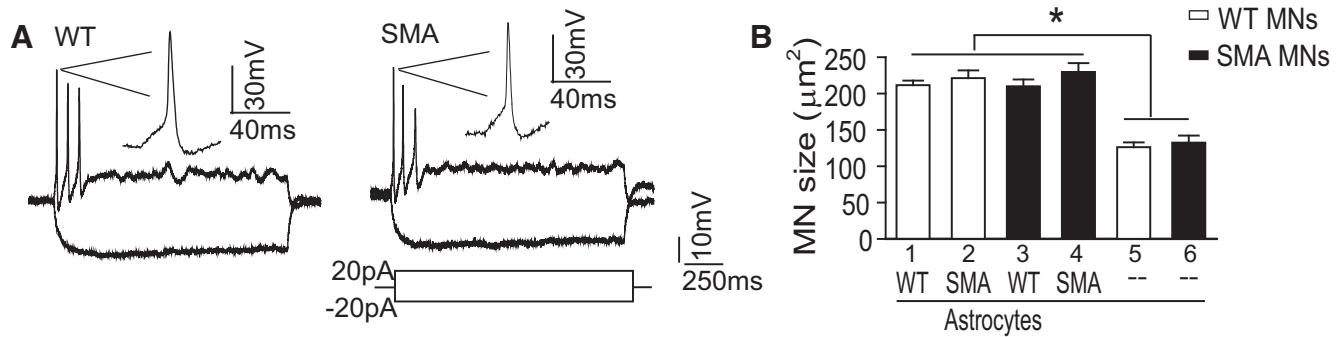
Figure 5. Electrophysiological properties and size of motoneuron are not changed by SMN deficiency. ***A***, Representative membrane potential changes in response to current injections in a WT and SMA motoneuron in culture alone. ***B***, Summary of cross-sectional area of motoneurons in astrocyte contact cocultures. The size of MNs (WT or SMA) is significantly enlarged in the presence of astrocytes (WT or SMA). However, motoneuron sizes are not changed by SMN deficiency (compare bars 1– 4). WT MNs with WT astrocytes, bar 1: $211.5 \pm 6.1 \ \mu m^2$; WT MNs with SMA astrocytes, bar 2: $221.2 \pm 10.4 \ \mu m^2$; SMA MNs with WT astrocytes, bar 3: $209.7 \pm 9.5 \ \mu m^2$; SMA MNs with SMA astrocytes, bar 4: $229.5 \pm 12.3 \ \mu m^2$; WT MNs cultured alone, bar 5: $126.1 \pm 6.6 \ \mu m^2$; SMA MNs cultured alone, bar 6: $132.4 \pm 9.5 \ \mu m^2$. $n > 50$ for each group. $*p < 0.05$ (unpaired t test). Error bars indicate SE.

Table 2. Electrophysiological properties of motoneurons in astrocyte contact cocultures[a]

Motoneurons	WT	WT	SMA	SMA	WT	SMA
Astrocytes	WT	SMA	WT	SMA	—	—
Resting membrane potential (mV)	-56.6 ± 1.8 (12)	-55.3 ± 2.2 (10)	-63.5 ± 3.0 (8)	-56.3 ± 3.7 (10)	$-53.60.9 \pm 2.5$ (14)	-55.7 ± 3.2 (9)
Input resistance (MΩ)	545.5 ± 51.2 (12)	525.0 ± 126.1 (10)	469.2 ± 73.1 (8)	425.0 ± 72.7 (10)	1579 ± 128 (14)*	1128 ± 84.4 (9)*
C threshold (pA)	33.8 ± 8.3 (12)	41.1 ± 3.2 (10)	41.2 ± 12.9 (8)	33 ± 8.6 (10)	14 ± 1.9 (14)*	16.7 ± 2.9 (9)*
V threshold (mV)	-44.6 ± 1.3 (12)	-41.9 ± 2.1 (9)	-40.9 ± 1.9 (8)	-44.2 ± 2.2 (10)	-42.2 ± 1.7 (14)	-42.6 ± 2.4 (9)

[a]The numbers of neurons are shown in parentheses.

*$p < 0.05$, compared with WT motoneurons plus WT astrocytes contact coculture (one-way ANOVA).

addition to disturbances in functional synaptic formation on motoneurons, may ultimately lead to motoneuron loss and behavioral abnormalities in SMA. The fact that significant motoneuron death occurs near the end stage (postnatal day 9) of *SMNΔ7* mice (Le et al., 2005) supports a possibility that motoneurons might show neurodegenerative properties only after they have been maintained in culture for a prolonged period of time.

Abnormal Ca²⁺ signal in SMA astrocytes

Intracellular calcium signal in astrocytes plays important roles in their communication with neurons as it can trigger the release of glia transmitters, including glutamate, ATP, and prostaglandin E2, which in turn modulate synaptic transmission and plasticity (Verkhratsky and Steinhäuser, 2000; Haydon, 2001). The dysfunction of astrocyte calcium signaling has been reported in various pathological states, including epilepsy, Alzheimer's disease, and amyotrophic lateral sclerosis (Bezprozvanny, 2009; Kawamata et al., 2014). In pure astrocyte cultures, we found a dysfunction in calcium signaling in SMA astrocytes compared with WT astrocytes as demonstrated by lower resting Ca^{2+} concentration, and a stronger Ca^{2+} concentration increase in responses to ATP stimulation. The reduction in resting Ca^{2+} in SMA astrocytes suggests that SMN deficiency hampers the availability of calcium in astrocytes. Given that the increase of calcium in astrocytes cytoplasm by ATP stimulation is primarily mediated by calcium release from the endoplasmic reticulum after P2Y receptor activation (Verkhratsky, 2006), the increase of Ca^{2+} flux in SMA astrocytes in response to ATP application (Fig. 2G) suggests that either purinergic receptors on membrane or calcium storage/release from the endoplasmic reticulum were modified by SMN deficiency. Alterations of Ca^{2+} homeostasis in SMA astrocytes may impair physiological roles, which include regulation of neuronal synaptogenesis, proliferation, glutamate uptake, and release. Interestingly, we have found that SMN deficiency

does not affect $[Ca^{2+}]_i$ signals in motoneurons (Fig. 1G–I), suggesting that the defects in calcium homeostasis following SMN deficiency are restricted to astrocytes.

SMN deficiency disrupts motoneuron–astrocyte interactions in synapse formation and synaptic transmission

In motoneuron–astrocyte contact cocultures (Fig. 4), we examined the role of motoneuron–astrocyte interactions in synapse formation and synaptic transmission onto motoneurons. We observed that SMA astrocytes were less supportive than WT astrocytes for synaptic formation and excitatory synaptic transmission onto WT motoneurons. However, WT astrocytes did not further improve synapse formation and synaptic transmission on SMA motoneurons in comparison with SMA astrocytes in our coculture system. Our data suggest that interactions between motoneurons and astrocytes might be disrupted due to SMN deficiency, and either motoneurons or astrocytes from SMA might contribute to the defects in synapse formation and synaptic transmission. Different from *in vitro* results here, our recent *in vivo* study has shown that selective SMN restoration in astrocytes actually increases synapse number onto motoneurons in SMNΔ7 mice (Rindt et al., 2015). One possible explanation is that, in our culture system, there are only motoneurons and astrocytes, as well as primary motoneurons forming synapses with each other. However, motoneurons in the spinal cord receive synaptic inputs from other neuron types, such as interneurons and sensory neurons. Selective SMN restoration in astrocytes may be beneficial not only to motoneurons but also to interneurons or sensory neurons, which may further improve maintenance of synapses on motoneurons in SMA mice.

One potential concern is that the reduced synapse numbers in WT motoneurons cocultured with SMA astrocytes could be secondary to a toxic effect of SMA astrocytes, as suggested in other
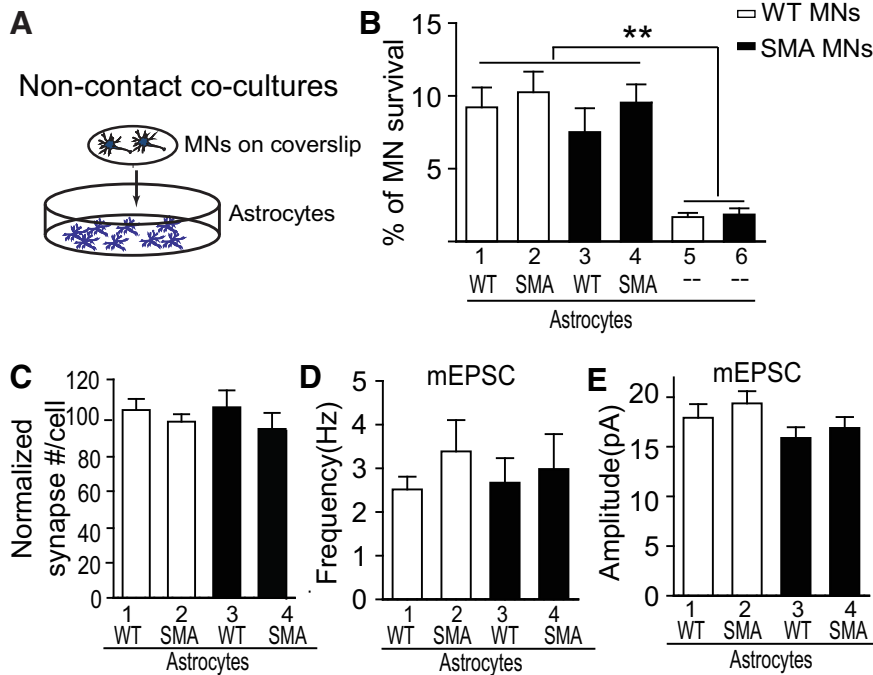
Figure 6. Synapse formation and function are not changed in motoneuron astrocyte noncontact cocultures. **A**, Schematic diagram of noncontact cocultures. Motoneurons (WT or SMA) are seeded on coverslip and inserted in astrocytes (WT or SMA) cultures at day 0 for cocultures 12–14 DIV. **B**, The survival of motoneurons in noncontact cocultures. Survival of MNs (WT or SMA) is significantly improved in the presence of astrocytes (WT or SMA) (WT MNs with WT astrocytes, bar 1: 9.2 ± 1.4%; WT MNs with SMA astrocytes, bar 2: 10.3 ± 1.4%; SMA MNs with WT astrocytes, bar 3: 7.5 ± 1.6%; SMA MNs with SMA astrocytes, bar 4: 9.5 ± 1.2%; WT MNs cultured alone, bar 5: 1.7 ± 0.3%; SMA MNs cultured alone, bar 6: 1.8 ± 0.4%). **$p < 0.01$ (one-way ANOVA). $n = 3$ different cell preparations. **C**, Summary of normalized synapse number on motoneurons in noncontact cocultures (WT MNs with WT astrocytes, bar 1: 100 ± 5.9%; WT MNs with SMA astrocytes, bar 2: 93.7 ± 4.0%; SMA MNs with WT astrocytes, bar 3: 101.4 ± 8.9%; SMA MNs with SMA astrocytes, bar 4: 89.9 ± 6.2%). $n > 30$ for each group, 3 experiments in each condition. $p = 0.54$ (one-way ANOVA). **D**, **E**, Summary of mEPSC frequency (**D**) and amplitude (**E**) on motoneurons (WT MNs with WT astrocytes, bar 1: 2.5 ± 0.3 Hz, 17.9 ± 1.4 pA; WT MNs with SMA astrocytes, bar 2: 3.4 ± 0.7 Hz, 19.4 ± 1.2 pA; SMA MNs with WT astrocytes, bar 3: 2.7 ± 0.7 Hz, 19.4 ± 1.2 pA; SMA MNs with SMA astrocytes, bar 4: 3.0 ± 0.8 Hz, 15.9 ± 1.0 pA). $n > 25$ for each group. $p = 0.63$ for frequency comparison. $p = 0.43$ for amplitude comparison (one-way ANOVA). Error bars indicate SE.
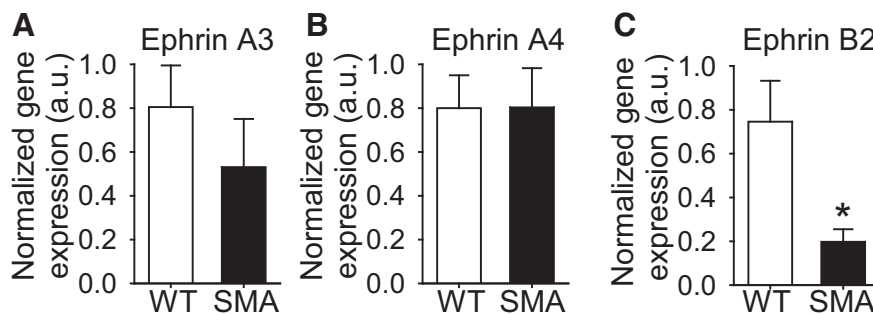

Figure 7. SMN deficiency alters Ephrin B2 expression in astrocytes. **A–C**, Quantification of mRNA expression of Ephrin A3, Ephrin A4, and Ephrin B2 in astrocytes using quantitative RT-PCR. Ephrin A3: WT, 0.8 ± 0.19; SMA, 0.53 ± 0.22; $p = 0.37$; Ephrin A4: WT, 0.8 ± 0.15; SMA, 0.80 ± 0.18, $p = 0.99$; Ephrin B2: WT, 0.75 ± 0.19; SMA, 0.20 ± 0.06, $p = 0.02$; $n = 6$ for each group. *$p < 0.05$ (unpaired t test). Error bars indicate SE.

neurological diseases, such as ALS and Rett syndrome (Di Giorgio et al., 2007; Nagai et al., 2007; Ballas et al., 2009). We ruled out such possibility because the survival of motoneurons in contact or noncontact cocultures was not affected when either motoneurons, astrocytes, or both were from SMA (Figs. 3, 6). Therefore, it is likely that SMA astrocytes exhibit a defect in providing synaptogenic support for motoneurons.

During neuronal communication, synaptic inputs drive dramatic membrane potential changes and alter firing patterns. One

might anticipate that reduced excitatory synaptic inputs would lead to abnormal excitability in SMA motoneurons. However, we did not find changes in the excitability of SMA motoneurons (Fig. 5; Table 2). The result is contradictory with the hyperexcitability of SMA motoneurons observed in *in vivo* studies (Mentis et al., 2011; Gogliotti et al., 2012). The difference between *in vitro* and *in vivo* systems might account for the discrepancy. The hyperexcitability of motoneurons in *in vivo* studies may originate from motoneuron homeostatic reaction in response to reduced excitatory synaptic inputs, perhaps from the proprioceptive dorsal root ganglion neurons or spinal interneurons (Mentis et al., 2011).

Our observations from noncontact cocultures of motoneurons and astrocytes (Fig. 6) demonstrated that contact support from astrocytes contributes to the impaired interactions between motoneurons and astrocytes in SMA. In our experimental condition, all motoneuron cultures (contact and noncontact cocultures) were supplemented with neurotrophic factors (BDNF, GDNF, CNTF) (Ullian et al., 2004; Graber and Harris, 2013). Therefore, our data cannot rule out the possibility that SMA astrocytes might have defects in releasing diffusible factors *in vivo*.

It has been reported recently that SMA Schwann cells also exhibit intrinsic defects, and their interactions with motor axons are disrupted by SMN reduction, likely mediated by extracellular matrix defects, but not Schwann cell secreted factors (Hunter et al., 2014). Thus, it is likely that both astrocytes and Schwann cells contribute to the overall development of SMA pathology.

Downregulation of Ephrin B2 in SMA astrocytes
Among several contact molecules that contribute to contact interactions between motoneurons and astrocytes, we found, for the first time, that transcription of Ephrin B2 is reduced in SMA astrocytes (Fig. 7C). Considering that (1) Ephrin B2 coordinates development of both presynaptic and postsynaptic compartment and regulation of glutamatergic synaptogenesis (Kayser et al., 2006; McClelland et al., 2009) and (2) loss of Ephrin B2 results in specific defects in synapse formation in cortex neurons (Henkemeyer et al., 2003), it is therefore possible that EphrinB2 reduction in astrocytes could be a potential mechanism underlying the impaired synapse formation and glutamatergic synaptic transmission on SMA motoneurons. However, further investigation needs to be followed to understand the involvement of Ephrin B2 in SMA.

In conclusion, the present study reveals critical pathophysiological signs of early-stage SMA: impaired contact-dependent interactions between motoneurons and astrocytes result in reduced synaptogenesis. SMA astrocytes display impairments *in vitro* in intracellular Ca^{2+} signaling, synaptic promotion function, and Ephrin B2 expression. It will be interesting to determine whether these impairments contribute to disease phenotypes in SMA animal models or patients as well. Together, our results indicate that astrocytes play a role in the pathogenesis of SMA and that targeting astrocytes, especially improving their contact communication with motoneurons, could be a potential therapy for SMA.

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