Rho-kinase inactivation prolongs survival of an intermediate SMA mouse model

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ABSTRACT

Spinal muscular atrophy (SMA) is an inherited disease resulting in the highest mortality of children under the age of two. SMA is caused by mutations or deletions in the survival motor neuron 1 (SMN1) gene, leading to aberrant neuromuscular junction development and the loss of spinal cord α-motor neurons. Here, we show that Smn depletion leads to increased activation of RhoA, a major regulator of actin dynamics, in the spinal cord of an intermediate SMA mouse model. Treating these mice with Y-27632, which inhibits ROCK, a direct downstream effector of RhoA, dramatically improves their survival. This lifespan rescue is independent of Smn expression and is accompanied by an improvement in the maturation of the neuromuscular junctions and an increase in muscle fiber size in the SMA mice. Our study presents evidence linking disruption of actin cytoskeletal dynamics to SMA pathogenesis and for the first time, identifies RhoA effectors as viable targets for therapeutic intervention in the disease.
INTRODUCTION

Spinal muscular atrophy (SMA) is an autosomal recessive disease caused by homozygous mutations and or deletions of the survival of motor neuron 1 (SMN1) gene (1, 2). Affecting 1:10 000 live births, it is the number one inherited killer of children below the age of two (2, 3). The depletion of the SMN protein results in the loss of α-motor neurons in the spinal cord, muscular atrophy of the limbs and trunk, paralysis and, in the most severe cases, fatality (3). Recent studies in SMA mouse models suggest that the loss of motor neurons and muscular atrophy of SMA patients is preceded by and may be due to defects in neuromuscular junction (NMJ) maturation and function (4-6). Indeed, analysis of myotubes from SMA type I (severe) patients shows an absence of nicotinic acetylcholine receptor aggregation, an essential initial step for proper NMJ formation (7).

While the SMN gene is evolutionarily conserved and all eukaryotic organisms have only one copy of the gene, humans have two copies (1, 8). SMN1 is the telomeric copy while SMN2 is a duplicated centromeric copy on chromosome 5q (1). The critical difference within the SMN2 gene is a C to T substitution at position 6 of exon 7 (9). This substitution results in the loss of an exon splicing enhancer and/or the gain of an exon splicing silencer, which promotes exon 7 skipping and production of an unstable truncated protein, termed Δ7SMN (1, 10, 11). Although, the Δ7SMN protein is the major product of the SMN2 gene, a small amount of full-length SMN protein (~ 10%) is still produced (1). Thus, the number of SMN2 copies present in SMA patients can modulate the severity of the disease (1).
Therapeutic approaches to SMA have included neuronal cell replacement and increasing SMN expression via gene therapy (12, 13). Alternatively, others have attempted to alter the splicing pattern and/or the transcription of the SMN2 gene to produce more full-length SMN mRNA and ultimately protein (14-18). While these approaches have resulted in improvement in weight, motor function and motor neuron loss in SMA mouse models, the increase in survival have to date been modest (12-14, 16). More importantly, although encouraging, most of these approaches are still a long way from clinical translation.

To date no SMA therapeutic strategy has addressed the cellular and molecular pathways that are dysregulated as a result of the infraphysiologic levels of SMN observed in the disorder. We have previously shown that Smm depletion in PC12 cells is characterized by an increase in profilin IIa (small actin binding protein) (19) availability, and an increase in total and active RhoA (20, 21). The defects in these cells include perturbation of neuronal differentiation and integrity (20). Furthermore, we have also identified a cell-autonomous increase in profilin IIa and a decrease in plastin 3 (actin-bundling protein) (22) levels in neuronal cells of an intermediate SMA mouse model (23). While knocking out profilin II restored plastin 3 levels, it neither improved survival and weight, nor did it reduce motor neuron degeneration in these SMA mice (23). One explanation for the failure of profilin IIa and plastin 3 reconstitution to ameliorate the SMA phenotype may be because they are end-point effectors of the actin cytoskeletal dynamics signaling pathways. It is indeed possible that more upstream regulators of actin dynamics are misregulated due to Smm depletion, and other targets in addition to profilin IIa and plastin 3 would therefore also be affected.
In the present study, we investigate the effect of Smn depletion on upstream regulators of actin dynamics in the Smn\(^{2B/-}\) intermediate SMA mouse model (20, 23). We show an increased activation of RhoA, a major regulator of actin dynamics, in the spinal cord of this SMA mouse model. More importantly, we demonstrate that the pharmacological inhibition of Rho-kinase, a direct downstream effector of active RhoA, dramatically increases the survival and neuromuscular junction maturation in these mice. This is the greatest increase in lifespan of an SMA mouse model reported to date; the fact that it was achieved through the use of a synthetic compound that modulates the RhoA pathway highlights the importance of the regulation of actin cytoskeletal dynamics in SMA pathogenesis. Our study thus holds significant promise for a novel therapeutic approach for this incurable disorder.

RESULTS

Abnormal RhoA activation in the Smn\(^{2B/-}\) SMA mouse model

We have previously reported that Smn-depleted PC12 cells have increased total and active RhoA, a critical regulator of actin cytoskeletal dynamics (20, 21). To determine if a similar effect was observed in vivo, we took advantage of an intermediate SMA mouse model that we have recently described (23). Briefly, this line has a three nucleotide substitution mutation within exon 7 of the endogenous mouse Smn gene. This change leads to the alternative splicing of Smn, resulting in a marked shift from full length SMN mRNA to \(\Delta 7\)Smn mRNA (24). This new allele is termed Smn\(^{2B}\). Smn\(^{2B/-}\) mice (which have \(~15\%\) Smn protein levels) present features of SMA after the second week of life. Upon disease onset, they become significantly smaller than their wild type
littermates and exhibit motor neuron loss in the ventral horn region of the spinal cord. The majority of $Smn^{2B/-}$ mice die by one month of age.

Here, we analyzed spinal cord extracts of wild type and $Smn^{2B/-}$ mice at three stages. Postnatal days (P) 0 and 10 were considered to be pre-phenotype stage as $Smn^{2B/-}$ mice were indistinguishable from their wild type littermates, while P21 was considered to be phenotype stage. Total RhoA levels in the spinal cords of the $Smn^{2B/-}$ mice did not show significant changes when compared to wild type, however there was sample to sample variability, particularly at P21 (Fig. 1A and B). In contrast, while at P0, the active RhoA levels are unchanged (1.68% +/- 15%), we observe a consistent increase of RhoA-GTP in the spinal cords of $Smn^{2B/-}$ mice at P10 (82% +/- 10%) and P21 (84% +/- 3%) (Fig. 1A and B). This is of importance since it is the GTP-bound RhoA that dictates its effects and the activated signaling pathways within the cell. These results suggest that RhoA activity in the spinal cord is altered when Smn is depleted. Indeed, whereas RhoA-GTP levels in the spinal cord of $Smn^{2B/-}$ mice are unchanged at birth, they consistently increase prior to and at onset of disease.

**Y-27632 administration improves survival of $Smn^{2B/-}$ mice**

As a therapeutic strategy, we decided to target the signaling pathway of active RhoA in the spinal cord of the $Smn^{2B/-}$ mice. Y-27632 is a synthetic compound that inhibits Rho-kinase (ROCK) (25), a direct downstream effector of RhoA-GTP (26), by competing with its ATP binding site (27). We have previously demonstrated that Y-27632 partially rescues the neuronal differentiation defects in Smn-depleted PC12 cells (20). We thus set out to assess if the increased activation of RhoA in the spinal cord of
the Smn2B−/− mice could contribute to the observed SMA phenotype and reduced lifespan of this mouse model. The Y-27632 compound was administered to wild type, Smn2B+/+ (normal littermates) and Smn2B−/− mice at a concentration of 1 mg/kg twice daily or 30 mg/kg once daily starting at P3 and ending at P21. Control groups were given the peptide vehicle (water). Neither treatment regime prevented the weight loss of the Smn2B−/− mice that occurs at about P10 and beyond (Fig. 2A). Concordantly, treatment of Smn2B−/− mice with the lower dose did not improve their lifespan although the trend was towards improvement (Fig. 2B, \( P = 0.2981 \)). Remarkably, Smn2B−/− mice treated with the higher dose exhibited a dramatic rescue in survival (Fig. 2B, \( P = 0.018 \)). Indeed, the mice survived far beyond their normal lifespan and expired between 14 and 33 weeks of age. These mice move about the cage, albeit with a slight neurological phenotype (see movie in Supplemental Fig. 1A). In contrast, vehicle treated Smn2B−/− mice display a much more severe neurological phenotype and refrain from moving about the cage (see movie in Supplemental Fig. 1B). Finally, Y-27632 administration did not negatively impact weight or survival of Smn2B+/+ littermates (Fig. 2A and B). Overall, these results show that treatment of Smn2B−/− mice with the higher dose of Y-27632 significantly increases their lifespan.

To determine if the Y-27632 compound had a similar effect on a severe SMA mouse model, we administered the compound to the Smn−/−;SMN2 mice that die within the first postnatal week (28). Pregnant females were administered either 30 mg/kg of Y-27632 or vehicle starting at gestational day E14. Once born, pups were administered Y-27632 by gavage daily starting at P3. We observed no significant improvement in the lifespan of Smn−/−;SMN2 mice treated with the Y-27632 compound (Fig. 3, \( P = 0.42 \),
perhaps because the severity of the disease in this mouse model was too extreme to benefit from the treatment.

_Y-27632 inhibits ROCK activity but does not alter Smn expression in the spinal cord of Smn^{2B/-} mice_

To ensure that Y-27632 was present and active following gavage, we analyzed downstream effectors of ROCK within spinal cord protein extracts of treated animals. Activated ROCK phosphorylates LIM kinase (LIMK), which subsequently leads to the phosphorylation of cofillin (29, 30). The spinal cords of P10 Smn^{2B/-} mice treated with 30 mg/kg of Y-27632 for one week (P3 to P10) show a decrease in both phosphorylated LIMK and phosphorylated cofillin compared to untreated Smn^{2B/-} mice (Fig. 4A). Total LIMK and cofillin protein, however, were unchanged (Fig. 4A). We can thus conclude that the Y-27632 compound reaches the spinal cord and appropriately inhibits ROCK activity.

One possible explanation for the observed improvement in survival of the Smn^{2B/-} mice is that Y-27632 increases Smn protein or mRNA levels. However, our analysis shows that Smn protein levels did not change in the spinal cords of Smn^{2B/-} mice treated with 30 mg/kg of Y-27632 compared to untreated Smn^{2B/-} mice (Fig. 4B). Additionally, administration of Y-27632 (30 mg/kg) did not alter the ratio of full-length Smn to Δ7Smn mRNAs (P = 0.56; Fig. 4C and D). These results suggest that the increased lifespan of Smn^{2B/-} mice treated with 30 mg/kg of Y-27632 is due to its direct effect on ROCK inhibition and not because of an unanticipated increase in Smn expression.
**Y-27632 improves neuromuscular junction maturation in Smn\(^{2B/-}\) mice**

Recent studies have identified a role for Smn in the maturation of neuromuscular junctions (NMJs) with poor terminal arborization, denervation, impaired synaptic vesicle release and decreased NMJ size all observed in SMA mouse models. We thus elected to examine the impact of Y-27632 treatment on this structure, comparing NMJs in the tibialis anterior (TA) muscle of P21 wild type (untreated) and Smn\(^{2B/-}\) mice (untreated and treated). Alpha-bungarotoxin was used to identify NMJs, and SV2 and SMI312 antibodies were used to identify the synaptic vesicles and neurons, respectively. Although the NMJs of Smn\(^{2B/-}\) mice treated with 30 mg/kg of Y-27632 were smaller than those of wild type mice (Fig. 5A and B, \(P = 0.027\)), they were significantly larger than those observed in untreated Smn\(^{2B/-}\) mice (Fig. 5A and B, \(P = 0.015\)). The percentage of NMJs with mature terminals was next quantified using sophisticated folds and perforations as criteria for NMJ maturity (31). Specifically, only the terminals in which SV2 and SMI312 staining showed full endplate occupancy as well as multiple perforations and folds were considered as being mature NMJs. Once again, although not equivalent to that observed in wild type (Fig. 5A and C, \(P = 0.0054\)), NMJ maturation is markedly improved in treated Smn\(^{2B/-}\) mice when compared to untreated mice (Fig. 5A and C, \(P = 0.018\)). The improvement in NMJ maturation is in accordance with other studies implicating Y-27632 in axonal sprouting and regeneration (32-34). Our results suggest that the beneficial effect of Y-27632 on the lifespan of Smn\(^{2B/-}\) mice is due to a direct improvement in NMJ maturation.

**Y-27632 increases muscle fiber size**
To complement the weight and NMJ analyses, we investigated the effect of Y-27632 on the muscle fiber size of the tibialis anterior (TA) of P21 mice. The muscle fibers of Smn2B/- mice were significantly smaller than their WT littermates (Fig. 6A and B, $P = 0.0021$). Surprisingly, while the vehicle- and Y-27632-treated Smn2B/- mice did not differ in weight (Fig. 2A), mice that were administered the Y-27632 compound displayed a significant increase in muscle fiber size (Fig. 6A and B, $P = 0.0007$). The percentage of centrally located nuclei of vehicle-treated Smn2B/- mice was not significantly different from WT or Y-27632-treated Smn2B/- mice (Fig. 6C, $P = 0.37$ and 0.8, respectively).

_Y-27632 does not increase the number of motor neurons in the ventral horn region of the spinal cord_

Since the Smn2B/- mice are characterized by a loss of spinal cord motor neurons (23), we wanted to assess if an improvement in NMJ maturation translated to a protection of these neurons from degeneration. Wild type mice had significantly more motor neurons than vehicle-treated Smn2B/- mice (Fig. 7A and B, $P = 0.002$). Treatment with the Y-27632 compound did not prevent the loss of motor neurons in the Smn2B/- mice (Fig. 7A and B, $P = 0.12$). This inability of the Y-27632 treatment to rescue such a pathological feature of SMA is most likely due to the fact that the Y-27632 is acting via an Smn-independent pathway. Thus, neuronal degeneration and/or denervation may still occur through other intrinsic pathways directly affected by the loss of the Smn protein. Indeed, administration of the Y-27632 may only strengthen the remaining NMJs and thus, enable the treated mice to survive longer.
DISCUSSION

In this study, we show that the level of active RhoA (RhoA-GTP) is increased in the spinal cords of the $Smn^{2B/-}$ intermediate SMA mouse model. Inactivation of ROCK, a direct downstream effector of RhoA-GTP (26), via administration of Y-27632 (30 mg/kg), results in the most significant increase in the lifespan of SMA mice reported to date. Significantly, treatment with Y-27632 did not increase Smn RNA or protein levels suggesting the modulation of an event or events downstream of SMN depletion. Importantly, $Smn^{2B/-}$ mice treated with Y-27632 displayed larger NMJs and a greater percentage of mature NMJ terminals when compared to untreated $Smn^{2B/-}$ mice. Recent work in mouse models has suggested that SMA could be a motor neuron synaptopathy (4-6). As well, perturbed NMJ formation has been reported in myotubes from severe SMA patients (7). Consistent with these observations, we propose that disrupted RhoA regulation in an SMN depleted neuron contributes to a failure of the NMJs to fully mature. The correcting of RhoA/ROCK signaling with Y-27632 represents one promising means of ameliorating NMJ formation and function, with a consequent improvement in lifespan.

RhoA is a small GTPase that plays an important role in the regulation of actin cytoskeletal dynamics (21). By signaling through various pathways, RhoA mediates neuronal growth, formation, polarization, regeneration, branching, pathfinding, guidance and retraction (reviewed in (35, 36)). Furthermore, dysregulation of RhoA has been implicated in mental retardation, amyotrophic lateral sclerosis and lissencephaly (37-39). The depletion of Smn in motor neurons leads to defects in β-actin mRNA and protein localization (40). This defect may then result in anomalous signaling between the actin
cytoskeletal signaling pathways and RhoA. Furthermore, an abnormal upregulation of RhoA-GTP also results in defects in actin localization (41). Previous studies have shown that the Y-27632 compound leads to actin rearrangements through the decreased phosphorylation of cofilin (42), promoting the advancement and enlargement of the neuronal growth cone and increasing (43, 44) the activity of voltage-gated calcium channels (45). The effects of the RhoA/ROCK inhibitor on the actin dynamics in neurons have thus been well characterized.

RhoA-GTP activates ROCK through phosphorylation (26), which in turn leads to the activation and phosphorylation of LIMK (29). Interestingly, in Drosophila, LIMK normally functions as a suppressor of synaptic sprouting and growth at the NMJ (46). Thus, increased RhoA activation in the spinal cord motor neurons of the Smn<sup>2B/−</sup> mice and a subsequent increase in LIMK activity may explain the smaller NMJs and the fewer mature terminals observed in these mice (Fig. 8). The inactivation of ROCK through the administration of Y-27632 decreased the levels of phosphorylated LIMK. This reduction in phosphorylated LIMK would therefore suppress its inhibitory effect on growth and sprouting at the NMJ. A critical improvement in NMJ maturation and function, would thus lead to a subsequent enhancement in the lifespan of SMA mice. Studies attempting to alleviate the phenotype and lethality of rodent models of SMA and amyotrophic lateral sclerosis, another fatal neurodegenerative disease, also show that improvement of NMJ innervation is associated with increased survival (47-49).

Interestingly, while Y-27632 administration dramatically ameliorates the survival of the Smn<sup>2B/−</sup> mice, it does not fully rescue the neurological phenotype. Indeed, we observed no improvement in the weight loss phenotype or age of onset of the disease in
these mice. Further, the motor neuron loss in the ventral horn region of the spinal cord was not prevented. Given that the Y-27632 compound acts via an Smn-independent pathway, loss of the Smn protein itself might have residual pathogenic effects. Indeed, Smn has been shown to have roles in mRNA splicing and axonal transport (40, 50). These functions might still be perturbed due to the persistent Smn depletion in the Y-27632-treated SMA mice. An alternative explanation for the inability of the Y-27632 to completely rescue the phenotype of the Smn²B/⁻ mice is its simple incapacity to fully restore the maturity of NMJs. Indeed, although the NMJs of treated Smn²B/⁻ mice are significantly larger and more mature than those of untreated Smn²B/⁻ mice, they still remain significantly smaller and less mature than wild type NMJs. The improvement in NMJ maturation without a concomitant rescue in motor neuron and muscle fiber pathology therefore suggests that the Y-27632 treatment improves the functionality of the remaining NMJs without preventing motor neuron denervation and/or degeneration.

Surprisingly, Smn²B/⁻ mice that received the Y-27632 compound displayed larger TA muscle fibers than their vehicle-treated counterparts. An increase in muscle fiber cross-sectional area without being accompanied by an increase in strength can occur, such as in instances of sarcoplasmic hypertrophy (51). Why this occurs in the TA muscles of treated Smn²B/⁻ mice is most likely due to the secondary effects of the systemically delivered Y-27632 compound. Indeed, activated ROCK has been shown to negatively impact the differentiation and fusion of skeletal myoblasts (52) (53, 54). More importantly, the ROCK inactivation in skeletal myoblasts through Y-27632 administration results in enhanced differentiation, myoblast fusion, and an increase in myotube size (52, 54). Thus, in addition to the effects observed in the spinal cord and
NMJs of the Smn$^{2B/-}$ mice, the Y-27632 compound may also act on inactivating ROCK in skeletal muscle thereby enhancing differentiation and fusion, resulting in an increase in muscle fiber size. Whether this is consequential in the improved survival observed in the Y-27632-treated mice remains uncertain due to the absence of a concomitant increase in weight.

While administration of the Y-27632 compound significantly improved the survival of the Smn$^{2B/-}$ mice, an intermediate SMA mouse model, it had no beneficial effect on the most severe SMA mouse model, Smn$^{-/-};SMN2$ (28). We have shown that the Y-27632 compound acts via an Smn-independent pathway, most likely by improving NMJ maturity. The rodent NMJ undergoes dramatic physical and molecular changes during the first two postnatal weeks (reviewed in (55)). As the Smn$^{-/-};SMN2$ mice expire within the first week of life, the beneficial effect of the Y-27632 compound on NMJ maturation and size likely does not have time to occur in this severe SMA mouse model. Severe and intermediate mouse models of SMA, akin to human SMA types I, II and III, are very different in phenotype and severity of disease. In the present case in particular, the beneficial effect of Y-27632 in our intermediate mouse model is not due to “Smn” rescue but rather due to a “biological” rescue, bypassing the Smn defect altogether. These findings highlight the fact that treatment modalities for the different severities of SMA will have to be different. Indeed, it is most likely that for SMA, the “one size fits all” approach will not be tenable.

Although we have identified RhoA as a major player in the disruption of actin cytoskeletal dynamics in the Smn$^{2B/-}$ mouse model, we cannot exclude the involvement of other actin regulators. Indeed, our work in Smn-depleted PC12 cells shows a decrease in
total and active Cdc42, another small Rho GTPase (20, 21). Investigation of the \textit{in vivo} effects of Smn depletion on Cdc42 expression would help elucidate if SMA pathogenesis is due to a more general disruption of actin cytoskeletal dynamics. Nevertheless, we show that abnormal activation of the RhoA pathway in the spinal cord of an intermediate SMA mouse model leads to some of the hallmarks of SMA pathogenesis. We are the first to provide evidence that the modulation of disrupted actin cytoskeletal dynamics dramatically improves the survival of SMA mice. The present work provides novel avenues for therapeuetic approaches to treat this devastating disease.

\section*{MATERIAL AND METHODS}

\subsection*{Antibodies}

The mouse monoclonal primary antibodies used were as follows: anti-actin (1:800; Fitzgerald), anti-SMI312 (1:1000; Covance), anti-SV2 (1:100; Developmental Studies Hybridoma Bank), anti-RhoA (1:200 (IB), 1:5 (IF); Santa Cruz), and anti-Smn (1:5000; Transduction Laboratories). The rabbit polyclonal primary antibodies used were as follows: anti-LIMK (1:500; Cell Signaling), anti-phospho-LIMK (1:250; Cell Signaling), anti-cofilin (1:500; Chemicon); anti-phospho-cofilin (1:250; Chemicon) and anti NF (N4142) (1:200; Sigma).

The secondary antibodies used were as follows: horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (1:5000; Bio-Rad), HRP-conjugated goat anti-rabbit IgG (1:5000), Alexa Fluor 555 goat anti-mouse (1:100; Molecular Probes) and Alexa Fluor 488 goat anti-rabbit IgG (1:100; Molecular Probes).

The alpha-bungarotoxin Alexa Fluor 555 conjugate (1:10) was from Invitrogen.
Animal models

The $\text{Smn}^{2B/-}$ mice were established in our laboratory and maintained in our animal facility on a C57BL/6 x CD1 hybrid background (23). The 2B mutation consists of a substitution of 3 nucleotides in the exon splicing enhancer of exon 7 (24). The $\text{Smn}$ knock-out allele was previously described by Schrank et al. (56) and $\text{Smn}^{+/}$ mice were obtained from The Jackson Laboratory. The $\text{Smn}^{-/-};\text{SMN2}$ mice (28) are maintained on a FVB/N background and were obtained from The Jackson Laboratory. All animal procedures were performed in accordance with institutional guidelines (Animal Care and Veterinary Services and Ethics, University of Ottawa).

Y-27632 experimental procedure

The Y-27632 compound (Calbiochem) was diluted in water and administered by a modified oral gavage procedure (57) to $\text{Smn}^{2B/-}$ and $\text{Smn}^{2B/+}$ mice from P3 to P21. The $\text{Smn}^{-/-};\text{SMN2}$ mice were given the compound by administering it by gavage to pregnant females at gestational day E14 and by reinstating gavage to the pups at P3. $\text{Smn}^{2B/-}$ and $\text{Smn}^{2B/+}$ animals received low (1 mg/kg, twice daily) or high (30 mg/kg, once daily) doses of Y-27632 while vehicle-treated animals received water. $\text{Smn}^{-/-};\text{SMN2}$ pups and their mothers received only the higher dose (30 mg/kg). Survival and weight were monitored daily.

Immunoblot analysis

Equal amounts of spinal cord samples of P0, P10 and P21 mice were separated by electrophoresis on 10% SDS-polyacrylamide gels and blotted onto a PVDF membrane.
The membranes were blocked in 5% nonfat milk in TBST (10 mM Tris-HCl pH 8.0, 150 mM NaCl, and 0.1% Tween 20 (Sigma)), incubated overnight at 4°C with the first primary antibody, rinsed in TBST and incubated again overnight at 4°C with the second primary antibody. Then, membranes were incubated at room temperature with the secondary antibodies, 1 hour for each one, with TBST washes in between. Signals were visualized using the ECL or the ECL plus detection kit (Amersham).

**RhoA activity assay**

Rhotekin-Rho-GTP binding domain GST (GST-RBD) beads were from Cytoskeleton. Thirty µg of GST-RBD beads was added to one hundred µg of spinal cord lysates. After a 1 hour incubation at 4°C, beads were washed three times with lysis buffer B (50 mM Tris pH 7.6, 150 mM NaCl, 1% Triton X-100, and 0.5 mM MgCl₂) supplemented with 1 mM PMSF, 0.01 mg/ml leupeptin and 0.01 mg/ml aprotinin. The bound RhoA-GTP was detected by immunoblot analysis using an anti-RhoA antibody. The average increase or decrease of total and active RhoA was quantified by densitometry comparisons of the RhoA-GTP/actin and total RhoA/actin ratios of 3 mice per genotype using the Image J software.

**RT-PCR**

RNA from spinal cords was extracted with the TRIzol Reagent (Gibco) according to the manufacturer’s instructions. Reverse transcription was performed using 0.5 µg of total RNA. The following primers were used for RT-PCR analysis: Smn forward (5’ GCACAGCCAGAAGAAAACCT 3’), Smn reverse (5’ CGACACGCACACTCCACT
3’), actin forward (5’ CCGTCAGGCAGCTCATAGCTCTTC 3’) and actin reverse (5’ CTGAACCCTAAGGCCAACCGT 3’). FL Smn mRNA/Δ7 Smn mRNA ratios were determined by densitometry using the Image J software.

**Immunohistochemistry of neuromuscular junctions**

Dissected whole tibialis anterior (TA) muscles of P21 mice were dissected and fixed in 4% paraformaldehyde (PFA). Following the removal of connective tissue, the TA muscles were incubated with alpha-bungarotoxin Alexa Fluor 555 conjugate for 20 min at room temperature (RT). The TA muscles were then incubated in methanol at -20°C for 5 min, followed by an overnight incubation at 4°C with the SV2 and SMI312 antibodies. Incubation with the secondary antibodies was performed the following day at RT for 1 hour. Finally, two to three thin files per stained TA were cut and mounted in Fluorescent Mounting Medium (Dako). All files were single sections of the surface of the TA muscle. Images were taken with a Zeiss confocal microscope, with a 20X objective, equipped with filters suitable for FITC/Cy3/fluorescence. For the blind quantification analysis, a minimum of 90 NMJs were analyzed for each genotype/treatment group. The Zeiss AxioVision software was used to calculate the area of NMJs. Maturity of the NMJs was determined by the sophisticated complexity of folds displayed by the terminals.

**Hematoxylin and Eosin staining**

Spinal cord sections and TA muscle sections were first deparaffinized in xylene and then fixed in 100% ethanol. Following a rinse in water, samples were stained in
hematoxylin (Fisher) for 3 minutes, rinsed in water, dipped 40 times in a solution of 0.02% HCl in 70% ethanol and rinsed in water again. The sections were next stained in a 1% eosin solution (BDH) for 1 minute, dehydrated in ethanol, cleared in xylene and mounted with Permount (Fisher). Images were taken with a Zeiss Axioplan2 microscope, with a 20X objective.

Quantitative assays were performed on 3 mice for each genotype and 5 sections per mouse. Analyzed sections were at least 10 µm apart. Motor neurons were identified by their shape and size in the same designated area of the ventral horn region of the spinal cord sections. The area of muscle fiber within designated regions of the TA muscle sections was measured using the Zeiss AxioVision software.

**Statistical methods**

For the Kaplan-Meier survival analysis, the log-rank test was used and survival curves were considered significantly different at p<0.05.

For the remaining statistical analyses, the Student’s two-tail t test for paired variables was used to test for differences between samples and data were considered significantly different at p< 0.05.
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REFERENCES


**FIGURE LEGENDS**

**Figure 1.** Total and active RhoA in spinal cords of wild type (WT) and *Smn<sup>2B/c</sup>* mice. A) The spinal cords of *Smn<sup>2B/c</sup>* mice at pre-phenotype (P10) and phenotype (P21) stages have an increase in RhoA-GTP (active) levels compared to WT mice. B) RhoA-GTP/actin ratios of *Smn<sup>2B/c</sup>* mice (n = 3) were compared to WT (n = 3). RhoA-GTP levels are increased in the spinal cords of *Smn<sup>2B/c</sup>* mice at P10 and P21 while total RhoA levels are more variable and not significant (data are mean +/- S.E).

**Figure 2.** Weight and survival analysis of wild type (WT), *Smn<sup>2B/+</sup>* and *Smn<sup>2B/c</sup>* mice treated with low and high doses of Y-27632 or vehicle (water). The different groups analyzed were: untreated WT mice (n = 9), vehicle-treated *Smn<sup>2B/+</sup>* mice (n = 24), 1 mg/kg Y-27632-treated *Smn<sup>2B/+</sup>* mice (n = 12), 30 mg/kg Y-27632-treated *Smn<sup>2B/+</sup>* mice (n = 10), vehicle-treated *Smn<sup>2B/c</sup>* mice (n = 12), 1 mg/kg Y-27632-treated *Smn<sup>2B/c</sup>* mice (n = 8) and 30 mg/kg Y-27632-treated *Smn<sup>2B/c</sup>* mice (n = 10). A) Administration of 1 mg/kg or 30 mg/kg of Y-27632 does not attenuate the weight loss observed in vehicle-treated *Smn<sup>2B/c</sup>* mice (data are mean +/- S.E.). B) Treatment of *Smn<sup>2B/c</sup>* mice with 30 mg/kg Y-
27632 dramatically improves lifespan compared to vehicle-treated $Smn^{2B/-}$ mice ($P = 0.018$).

**Figure 3.** Administration of Y-27632 (30 mg/kg) to a severe SMA mouse model ($Smn^{-/-};SMN2$) does not improve lifespan. The different age-matched groups analyzed were: vehicle-treated $Smn^{-/-};SMN2$ (n = 3) and Y-27632-treated $Smn^{-/-};SMN2$ (n = 3). There was no significant difference between the survival curves of both groups ($P = 0.42$).

**Figure 4.** Y-27632 inhibits ROCK activity but does not alter Smn expression in spinal cords of $Smn^{2B/-}$ mice. A) Spinal cords of $Smn^{2B/-}$ mice treated with 30 mg/kg of Y-27632 show decreased levels of phospho-LIMK and phospho-cofilin compared to vehicle-treated $Smn^{2B/-}$ mice. LIMK and cofilin are usually phosphorylated by ROCK, the Y-27632 target. Total levels of LIMK and cofilin were unchanged between both treated and untreated $Smn^{2B/-}$ mice. B) The spinal cords of $Smn^{2B/-}$ mice treated with 30 mg/kg of Y-27632 did not show increased expression of Smn protein when compared to vehicle-treated $Smn^{2B/-}$ mice. C) The spinal cords of $Smn^{2B/-}$ mice treated with 30 mg/kg of Y-27632 did not show an increase in full-length (FL) Smn mRNA when compared to vehicle-treated $Smn^{2B/-}$ mice. D) Quantification of FL Smn mRNA/Δ7 Smn mRNA ratios shows no significant differences between vehicle- and Y-27632-treated $Smn^{2B/-}$ mice ($P = 0.56$) (data are mean +/- S.E)

**Figure 5.** Analysis of neuromuscular junction (NMJ) maturity in the tibialis anterior (TA) muscle of postnatal day 21 (P21) wild type and $Smn^{2B/-}$ mice. The different age-
matched groups analyzed were: untreated WT (n = 4), vehicle-treated Smn$^{2B/-}$ mice (n = 3) and 30 mg/kg Y-27632-treated Smn$^{2B/-}$ mice (n = 5). A) Representative images showing the NMJs of WT and untreated and treated Smn$^{2B/-}$ mice. NMJs were stained with alpha-bungarotoxin (red), the axons with SMI312 (green) and the synaptic vesicles with SV2 (green) (scale bar, 50 µm). B) Quantification of NMJ size. The NMJs of Smn$^{2B/-}$ mice treated with 30 mg/kg of Y-27632 are significantly larger compared to vehicle-treated Smn$^{2B/-}$ mice ($P = 0.015$). However, the NMJs of treated Smn$^{2B/-}$ mice are still significantly smaller than WT NMJs ($P = 0.027$) (data are mean +/- S.E.). C) The percentage of NMJs with mature terminals is significantly greater in Smn$^{2B/-}$ mice treated with 30 mg/kg Y-27632 than with the vehicle ($P = 0.018$). However, NMJs of treated Smn$^{2B/-}$ mice still remain more immature than WT NMJs ($P = 0.0054$) (data are mean +/- S.E.).

**Figure 6.** Treatment of Smn$^{2B/-}$ mice with Y-27632 (30 mg/kg) increases fiber diameter in tibialis anterior (TA) muscle. The different age-matched groups analyzed were: untreated wild type (WT) (n = 3), vehicle-treated Smn$^{2B/-}$ mice (n = 3) and Y-27632-treated Smn$^{2B/-}$ mice (n = 3). A) Representative images showing hematoxylin and eosin staining of muscle fibers within a designated area of the TA muscle. (scale bar, 10 µm). B) Quantification of the cross-sectional area of TA muscle fibers. Vehicle-treated Smn$^{2B/-}$ mice have significantly smaller muscle fibers than their WT littermates ($P = 0.0021$). Administration of the Y-27632 compound significantly increased the muscle fiber size of Smn$^{2B/-}$ mice ($P = 0.0007$). C) Quantification of the percentage of muscle fibers displaying a centrally located nuclei. There was no significant difference (NS).
between WT and vehicle-treated $Smn^{2B/-}$ mice ($P = 0.37$) or between vehicle- and Y-27632-treated $Smn^{2B/-}$ mice ($P = 0.8$).

**Figure 7.** Y-27632 (30 mg/kg) administration does not protect from motor neuron loss in $Smn^{2B/-}$ mice. The different groups analyzed were: untreated wild type (n = 3), vehicle-treated $Smn^{2B/-}$ (n = 3) and Y-27632-treated $Smn^{2B/-}$ mice (n = 3). A) Representative images showing hematoxylin and eosin staining of the motor neurons in the ventral horn region of the spinal cord for each group (scale bar, 50 $\mu$m). B) Quantification of the number of motor neurons within the ventral horn region of the spinal cord. While untreated $Smn^{2B/-}$ mice show significantly fewer motor neurons than their WT littermates ($P = 0.002$), treatment of the SMA mice with Y-27632 did not have a positive impact on the number of motor neurons ($P = 0.12$) (data are mean +/- S.E.).

**Figure 8.** A model of how SMN depletion may affect RhoA activity in spinal cords and neuromuscular junction (NMJ) maturation. In SMA patients, a depletion in SMN results in abnormal localization and distribution of actin which alters regulation of the actin cytoskeletal signaling pathways. A major upstream regulator of actin dynamics, RhoA, is thus misregulated, and its activity increases in the spinal cord. RhoA-GTP phosphorylates and activates ROCK, which in turn phosphorylates and activates cofilin and LIMK. An abnormal increase in p-LIMK suppresses synaptic sprouting and growth at the NMJ. This would eventually lead to the denervation and/or degeneration of the motor neurons within the ventral horn region of the spinal cord.
Figure 1

A

pre-phenotype

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<th></th>
<th>Actin</th>
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<th>RhoA-GTP</th>
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</table>

B

Change in RhoA expression compared to wild-type (%)

- Total RhoA
- Active RhoA

(PO) (P10) (P21)
Figure 2

A

B
Figure 3

![Graph showing percent survival over age (day) for different treatments. The legend indicates two groups: Smn−/−;SMN2 (vehicle) and Smn−/−;SMN2 (Y-27632 30 mg/kg).]
Figure 4

A

p-cofilin
Total coflin
Smn2B/- (vehicle) Smn2B/- (Y-27632)
Smn2B/- (vehicle) Smn2B/- (Y-27632)
p-LIMK
Total LIMK

B

Actin
Smn
WT Smn2B/- (vehicle) Smn2B/- (Y-27632)

C

FL-Smn Δ7 Smn
Actin
Smn2B/- (vehicle) Smn2B/- (Y-27632)

D

FL-Smn Rln/Δ7 Smn mRNA

Smn2B/- (vehicle) Smn2B/- (Y-27632)
0.00 0.50 1.00
NS

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Figure 5

A

\[ \alpha \text{-bungarotoxin} \quad \text{SV2 + SMI312} \quad \text{Merge} \]

WT

Smn2B/- (vehicle)

Smn2B/- (Y-27632)

B

\[
\begin{array}{c}
\text{NMU size (um)}^2
\end{array}
\]

WT  Smn2B/- (vehicle)  Smn2B/- (Y-27632)

C

\[
\begin{array}{c}
\text{NMU with mature terminals (%)}
\end{array}
\]

WT  Smn2B/- (vehicle)  Smn2B/- (Y-27632)
Figure 6

A

WT    Smn2B/- (vehicle)    Smn2B/- (Y-27632)

B

C

TA muscle fiber area (µm²)

Percentage of centrally located nuclei per designated area

NS

*
Figure 7

A

WT  Smn2B/- (vehicle)  Smn2B/- (Y-27632)

B

Number of motor neurons in the ventral horn region of the spinal cord

WT  Smn2B-/-  Smn2B/- (Y-27632)

*  NS
### Figure 8

<table>
<thead>
<tr>
<th><strong>SMA patients</strong></th>
<th><strong>Normal individuals</strong></th>
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<tr>
<td>Smn depleted spinal cord</td>
<td>Normal levels of Smn in the spinal cord</td>
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<td>Defects in actin localization</td>
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<td>Increase in RhoA activity</td>
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<tr>
<td>Increase in ROCK activity, p-LIMK and p-cofilin</td>
<td>Normal regulation ROCK, p-LIMK and p-cofilin</td>
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<tr>
<td>Defects in NMJ development, maturity and function</td>
<td>Normal NMJ development, maturity and function</td>
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