The *Smn*-Independent Beneficial Effects of Trichostatin A on an Intermediate Mouse Model of Spinal Muscular Atrophy

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Abstract

Spinal muscular atrophy is an autosomal recessive neuromuscular disease characterized by the progressive loss of alpha motor neurons in the spinal cord. Trichostatin A (TSA) is a histone deacetylase inhibitor with beneficial effects in spinal muscular atrophy mouse models that carry the human SMN2 transgene. It is currently unclear whether TSA specifically targets the SMN2 gene or whether other genes respond to TSA and in turn provide neuroprotection in SMA mice. We have taken advantage of the SMN2 mouse model that does not harbor the human SMN2 transgene, to test the hypothesis that TSA has its beneficial effects through a non-SMN mediated pathway. TSA increased the median lifespan of SMN2 mice from twenty days to eight weeks. As well, there was a significant attenuation of weight loss and improved motor behavior. Pen test and righting reflex both showed significant improvement, and motor neurons in the spinal cord of SMN2 mice were protected from degeneration. Both the size and maturity of neuromuscular junctions were significantly improved in TSA treated SMN2 mice. Of interest, TSA treatment did not increase the levels of Smn protein in mouse embryonic fibroblasts or myoblasts obtained from the SMN2 mice. In addition, no change in the level of Smn transcripts or protein in the brain or spinal cord of TSA-treated SMA model mice was observed. Furthermore, TSA did not increase Smn protein levels in the hind limb muscle, heart, or liver of SMN2 mice. We therefore conclude that TSA likely exerts its effects independent of the endogenous mouse Smn gene. As such, identification of the pathways regulated by TSA in the SMN2 mouse model could lead to the development of novel therapeutics for treating SMA.

Introduction

Spinal Muscular Atrophy (SMA) is an autosomal recessive neuromuscular disorder characterized by the progressive loss of alpha motor neurons in the anterior horn region of the spinal cord. SMA is caused by homozygous deletions or rare missense mutations in the survival motor neuron 1 (SMN1) gene [1]. Although SMA is caused by deletions in SMN1, all patients retain at least one copy of the near identical SMN2 gene. SMN1 produces full-length SMN transcript and protein while SMN2 produces only 10% full-length SMN transcript and protein, and therefore 90% of SMN2 transcript lacks exon 7 and encodes an unstable truncated protein [2], [3]. The severity of the disease in SMA is inversely correlated with SMN2 gene copy number [4], [5]. Unlike humans, rodent species only carry a single survival of motor neuron gene (*Smn*) and its inactivation leads to early embryonic lethality [6]. A number of strategies have been employed to generate viable mouse models of SMA. Some of the most commonly used models rely on the addition of a human SMN2 transgene onto an Smn<sup>-/-</sup> background [7], [8]. For example, the Smn<sup>-/-</sup>;SMN2;Smn<sup>47</sup> mouse model of SMA (herein referred to as the delta 7 mouse model) contains 2 copies of the human SMN2 onto a Smn<sup>-/-</sup> background and also carries 2 copies of the delta 7 SMN cDNA. An alternative approach has been to introduce a three-nucleotide substitution in the exon 7 splicing enhancer of the murine Smn gene, generating a model known as the Smn<sup>2B/-</sup> mouse model [9], [10].

Numerous strategies have been proposed for treating SMA, including the use of histone deacetylase (HDAC) inhibitors. HDAC inhibitors can modify the chromatin architecture by relaxing the chromatin conformation through a net increase in the acetylation state of histones. HDAC inhibitors have been shown to increase SMN2 expression through direct modification of histone acetylation state at the SMN2 promoter [11]. Indeed, a number of different HDAC inhibitors have had promising results in mouse models of SMA that carry the SMN2 transgene [12], [13], [14],

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[15], [16]. Specifically, trichostatin A (TSA) has produced some of the most dramatic results, increasing life span of the delta 7 mouse model by 19% [13]. However the exact mechanism for the beneficial effects of HDAC inhibitors in SMA are currently unclear. These beneficial effects may be mediated via the small but significant increase in SMN levels observed. Numerous studies have now demonstrated that very small increases in SMN protein level can have a dramatic effect upon the phenotype of mouse models. They further demonstrated that TSA can act directly upon the SMN2 transgene to increase its expression [11]. However, it should also be noted that HDAC inhibitors can act globally across the genome and orchestrate the activity of many genes to provide neuroprotection. For this reason, a number of HDAC inhibitors have been used in clinical trials for a variety of other neurodegenerative conditions. Therefore the identification of novel pathways that TSA may regulate for neuroprotection could provide new therapeutic avenues in treating SMA.

In this study, we assessed whether TSA can have beneficial effects on an SMA mouse model in the absence of the SMA2 transgene. We have taken advantage of the Smn2B/- intermediate mouse model of SMA, which does not carry human SMN2. Here we show that TSA treatment significantly improved survival, reduced weight loss and improved motor behavior in Smn2B/- mice. Daily administration of TSA reduced motor neuron loss in the spinal cord, improved the maturity and size of the neuromuscular junctions (NMJs), and improved myofiber histology in the tibialis anterior (TA) muscle. Importantly, administration of TSA led to no increase in Smn transcript or protein levels in brain, spinal cord, muscle or liver of Smn2B/- mice. We also observed no increase in Smn protein levels in TSA treated embryonic fibroblasts and myoblasts cultured from Smn2B/- mice. We therefore suggest that the beneficial effects observed in this study are likely mediated independent of Smn.

**Materials and Methods**

**Animals, intraperitoneal injection, injection, growth and motor function test**

The intermediate SMA mouse model, Smn2B/-, were established in our laboratory and maintained on a C57BL/6 x CD1 hybrid background [9], [10]. The Smn2B+/m heterozygous littersmate do not display any detectable phenotype and were used as littermate controls. TSA was dissolved in DMSO at a concentration of 4 μg/μl as described previously [13]. Mice were administered with TSA (10 mg/kg of body weight) or vehicle DMSO through intraperitoneal injection daily from postnatal day (P)12 to P25. Soft bedding, water-soaked food cubes and transgel were provided to the pups in TSA- and vehicle-treated groups after weaning at P21. Warm pads were also provided to Smn2B/- pups under the cages from P21 to P25. In order to eliminate the synergistic effects of diet and TSA [16], mice received a regular diet without any caloric or dietary enhancement. During TSA or vehicle treatment at P12-P25, mice were kept in the surgical surveillance area. Mice were monitored daily by animal facility professionals, and additionally monitored by the researchers once per day. Animal facility reported to researchers for any identified animal deaths and recommended to sacrifice animals in need. After the treatment period, mice were kept in the standard area. Animal facility professionals provided feeding and general monitoring daily and cage changing weekly. In addition, from P26 to P41, mice were monitored daily by the researchers. From P42 to P119, mice were monitored weekly by the researchers. The University of Ottawa Animal Care Committee approved all experimental protocols. The protocols conformed to or exceeded those defined in the Canadian Council on Animal Care’s Guide to the Care and Use of Experimental Animals, and the Animals for Research Act.

The mice were weighed daily from P12 to P25. During the survival study, humane endpoints were applied as per veterinary recommendation (severe dehydration, low body temperature, and desetration by feeding mother). At endpoints, mice were anesthetized via intraperitoneal injection of tribroromethanol (Avertin) followed by instant cervical dislocation. Righting reflex test and pen test examined the mice for motor function. For righting reflex test, the mice were placed on their backs, and the time the mice took to right themselves was recorded. Thirty seconds was also defined as the maximum time. TSA and vehicle treated Smn2B/- mice were assessed daily from P12-P18. For pen test, the mice were placed on a pen and the time that a mouse took before it fell off was recorded. Average of three consistent records for each mouse was used for statistical analysis. Thirty seconds was set as the maximum testing time. TSA and vehicle treated Smn2B/- mice were assessed daily from P18 to P25.

**Histology, NMJ analysis and immunohistochemistry**

To harvest tissues for histological examination, mice were transcardially perfused with 4% paraformaldehyde. Spinal cords from the L1 level downwards and tibialis anterior (TA) muscles at the thickest point were dissected and processed for paraffin embedding. Transverse sections of L1 spinal cord spanning 500 μm with intervals of 40 μm were processed for hematoxylin/eosin staining and motor neuron analysis. Three to five animals were analyzed for each treatment group. For each animal, six sections were examined. Images were taken on a Zeiss/Axioplan2 microscope with a Plan-Neofluar 20x/0.5 lens and processed using Adobe Photoshop CS software. Analyses were performed using the Axiovision Rel 4.6 program. Motor neuron cell bodies were determined by their morphology and a diameter of 18 μm or larger. Number of motor neuron cell bodies located at each side of the ventral horn below the horizontal line at the level of the central canal was recorded. The transversus abdominis (TVA) and rectus abdominis (RA) muscles were harvested and NMJs were labeled by immunohistochemistry as described previously [17], [18]. Briefly, muscles were immediately dissected from recently sacrificed mice and fixed in 4% paraformaldehyde (Electron Microscopy Science) in PBS for 15 min. Post-synaptic AChRs were labeled with alpha-bungarotoxin (αBTX) for 30 min. Muscles were permeabilized in 2% Triton X-100 in PBS for 30 min, then blocked in 4% bovine serum albumin (BSA)/1% Triton X-100 in PBS for 30 min before incubation overnight in primary antibodies [neurofilament (NF; 2H3) - Developmental Studies Hybridoma Bank; synaptic vesicle protein 2 (SV2) - Developmental Studies Hybridoma Bank] and visualized with Cy3-conjugated secondary antibodies [Cy3 goat anti-mouse; all 1:250, Jackson]. Muscles were then whole-mounted in Dako Fluorescent mounting media. Images were taken with a Zeiss LSM-510 meta confocal microscope. Histological analysis of the TA muscle was performed for assessing myofiber morphology.

**Cell culture and drug treatment**

Smn2B/- mouse primary myoblasts were isolated from hind limb muscles of 3-week-old mice as originally described [19]. The primary myoblasts were cultured on collagen-coated plates (Gibco) and maintained under growth conditions using Hams F10 (Wisent) media supplemented with 20% fetal bovine serum (FBS), 2.5 ng/mL human recombinant basic fibroblast factor (Invitrogen), and 2% penicillin/streptomycin (Gibco). In all experiments, cells were maintained at 37°C with 5% CO2 and were provided fresh media every second day. The cells were grown and treated with 100 nM...
of TSA or DMSO immediately added to the media prior to each use for a 24 hr period and were maintained at 37°C with 5% CO2.

For nuclear extraction, the cells were harvested after 2 washes with PBS and trypsinized and pelleted followed by an additional wash in PBS. The cells were pelleted and resuspended (2-10^10 cells/mL) in hypotonic buffer (10 mM Hepes pH 7.9, 1.5 mM MgCl2, 10 mM KCl, 0.5 μL 1 mM DTT/mL, 1 μL 200 mM PMSF/mL). After resuspension, the cells were placed on ice for 5 minutes and then centrifuged at 13,000 rpm for 15 sec at 4°C. Hypotonic buffer was added at half the volume with 0.15% NP-40 and the cells were placed on ice for 5 minutes. The cells were then centrifuged at 13,000 rpm for 15 sec at 4°C and the nuclei were suspended in 0.2 N HCl and placed on a rotator at 4°C overnight. The cells were then centrifuged at 13,000 rpm for 15 minutes at 4°C and the supernatant that contains the histones was collected.

For the total protein isolation, the cells were treated with a single dose of TSA (100 nM) for a 24 hr period and were maintained at 37°C with 5% CO2. The cells were then washed twice with ice cold PBS and lysed using RIPA buffer. After the lysis on ice for 10 minutes, the cells were centrifuged at 13,000 rpm for 15 minutes and the supernatant collected.

Smn^2f-/- primary mouse embryonic fibroblasts (MEFs) were established from E13 embryos obtained from pregnant female mice. The uterine horns from the mice were dissected and rinsed with 70% ethanol and placed in PBS. Each embryo was carefully separated from the placenta and surrounding tissue and the brain and dark red organs were dissected out. The dissected head was used for genotyping performed by PCR. The embryo carcass was then washed with fresh PBS and blood removed. The embryo was then finely minced in PBS using razor blades. After this step, the obtained tissue was suspended in 2 mL of ice cold trypsin-EDTA and using gentle shaking was incubated at 37°C for 15 minutes. After sedimentation, the suspension was transferred to a 50 mL Falcon tube and mixed with 2 volumes of fresh MEF media which contained high glucose DMEM supplemented with 10% FBS, 1% L-glutamine, 1% penicillin/streptomycin. After incubation, the supernatant was removed and was subjected to low speed centrifugation for 5 minutes and the resulting pellet was resuspended in fresh warm MEF media and cells plated in a 10 cm dish. Each embryo was plated into a separate 10 cm dish and was maintained at 37°C with 5% CO2 until cells reached confluency (1-2 days). The experiments were performed at the 4th passage and confluent plates were treated with TSA (100 nM) or DMSO for a 24 hr period. After treatment, the cells were lysed and total and/or nuclear protein was obtained.

For nuclear protein isolation, the cells were harvested after 2 washes with PBS and trypsinized and pelleted. The pellet was then washed with 10 mL of PBS and centrifuged at 300 g for 10 minutes. The pellet was then resuspended in 1 mL of hypotonic lysis buffer containing 10 mM Tris-HCl pH 0.9, 1 mM KCl, 1.5 mM MgCl2 and 1 mM DTT and incubated for 30 minutes on a rotator at 4°C. The intact nuclei preparation was centrifuged at 10,000 g for 10 minutes at 4°C. The pellet was then resuspended in 0.5 mL of 0.2 N HCl and incubated overnight on a rotator. The samples were then centrifuged at 16,000 g for 10 minutes and the supernatant was obtained for quantification.

Reverse transcriptase-polymerase chain reaction (RT-PCR) and quantitative RT-PCR (qRT-PCR)

To harvest tissues for mRNA analysis, mice were sacrificed by cervical dislocation four hours after the last injection. Brains and spinal cords were dissected, frozen in liquid nitrogen, and stored at -80°C. RNA was extracted and RT-PCR performed as described previously [20] with the following primers: Smn long (194-1117): Forward 5’ GCA CAG CCA GAA GAA AAC CT 3’, Reverse 5’ CGA CAC GCA CAC TTG ACT 3’; Pollastinin: Forward 5’ CCT TTC AAG TGG ATG ATT TTC 3’, Reverse 5’ ACA GTA GGC ATT ATG GGT CTG 3’; β-actin: Forward 5’ CCG TGA GGC AGC TAG TTC TTG 3’, Reverse 5’ CTG AAC CCT AAG GCC AAC CTG 3’. For qRT-PCR, 1 μL of cDNA was used in a total reaction volume of 20 μL Superscript (BIORAD) according to the manufacturer’s instructions. The PCR reaction was performed on a CFX connect (BIORAD) qPCR system with 40 amplification cycles. Changes in gene expression after TSA treatment were quantified based on the 2ΔΔCtDMsO-ΔCt/TSA value. Ct was normalized to Gapdh (ΔCt = Ct target gene - Ct Gapdh). Triplicate samples were used for each PCR run. Primers for qPCR include: Smn (final concentration at 50 nM): Forward 5’ GCC AGC AAT CCC TGG AAA CAG 3’, Reverse 5’ TGG GGG AAA GTA GGT CATA AG 3’; Gapdh (50 nM): Forward 5’ TGA AGG GGT CGT TGA TGG 3’, Reverse 5’ AAA ATG GTG AAG GTC GTT GT 3’.

Histone isolation and western blot analysis

Histone isolation was performed using the protocol adapted from Mishra et al. [21]. One hundred mg of brain or liver tissue was homogenized using PowerGen tissue homogenizer (Fisher Scientific) in 500 μL of ice-cold histone lysis buffer (10 mM Tris-HCl, pH 6.5, 50 mM sodium bisulfitie, 1% Triton X-100, 10 mM MgCl2, and 8.6% sucrose). For nuclear fraction isolation, the samples were centrifuged at low speed (1,000 g) for 10 minutes. This was followed by two additional washes in cold lysis buffer and once with cold Tris-EDTA (10 mM Tris-HCl, pH 7.4, and 13 mM EDTA). The nuclei were then resuspended in 100 μL of cold water followed by acidification with HCl at a final concentration of 0.2 N. The samples were vortexed and placed on a rotator for 1 hour. Acid soluble proteins were obtained using centrifugation at 15,000 g for 10 minutes and were precipitated in 1 mL acetone overnight at 25°C. Following this step, acetone precipitated proteins were collected by centrifugation at 15,000 g for 10 minutes and the protein pellets were air dried and suspended in 50 μL water. The Bradford assay was used for determination of protein concentration. SDS-PAGE and western blot were performed and membranes probed with a rabbit anti-acetylated H3 histone antibody (Millipore; 1:5000), rabbit anti-acetylated H4 histone antibody (Millipore; 1:5000), and anti-pan H3 histone antibody (Millipore; 1:2000). The bands were visualized with a peroxidase-linked goat anti-rabbit IgG secondary antibody (Chemicon; diluted 1:10,000) by chemiluminescence detection (Thermo Scientific Pierce ECL Western Blotting Substrate). Densitometry was performed using Image J software and acetylated histone H3 were normalized to pan-histone H3 values. To harvest tissues for total protein analysis, mice were sacrificed by cervical dislocation four hours after the last injection. Brains, spinal cords and livers were dissected, frozen in liquid nitrogen and stored at -80°C. Protein extracts were prepared, and SDS-PAGE and western blot performed as previously described [20]. The primary antibodies consisted of mouse monoclonal anti-Smn (BD Transduction Laboratory; 1:5000), mouse monoclonal anti-GAPDH (Abcam; 1:5000), and anti-β-tubulin (Developmental Studies Hybridoma Bank; clone E7; 1:5000). AlphaEaseFC software was used for quantification analysis.

Statistical analysis

Statistical analyses were performed with Microsoft Office Excel and GraphPad Prism software (San Diego, CA). Analysis for statistical significance between groups was performed in Microsoft Office Excel and GraphPad Prism software (San Diego, CA).
Excel. The dataset for each genotype and treatment group was compared to achieve the P value by Student’s t-test (two-sample assuming equal variances and two-tailed). Y-axis values in the graphs were expressed as mean +/- standard deviation (SD) or standard error of the mean (SEM). P<0.05 was considered to indicate statistical significance.

Results
TSA treatment improves survival and motor function in Smn2B/- mice

Previous work has shown that TSA leads to improvements in survival and weight in the delta 7 mouse model of SMA [13]. Here, we tested the efficacy of TSA in the Smn2B/- mouse model, which does not carry the human SMN2 transgene. TSA was delivered using daily intraperitoneal injections at a dose of 10 mg/kg from P12 to P25. Vehicle (DMSO) was used as a control. Daily administration of TSA led to a significant improvement in survival of Smn2B/- mice. Median lifespan of the Smn2B/- mice was increased from twenty days to eight weeks after TSA treatment (Fig. 1A). We also observed a trend towards improvements in growth of TSA treated Smn2B/- mice, however this did not reach statistical significance (Fig. 1B).

To investigate motor function, Smn2B/- mice were monitored daily to record righting reflex time from P12 to P18, and latency to fall using the pen test from P18 to P25. The TSA treated animals displayed faster righting times at P15 and P18 (Fig. 1C), and increased grip strength measured using the pen test from P21 to P25 (Fig. 1D).

Therefore, TSA treatment leads to significant improvement in survival and motor function. It is important to note that even after TSA treatment was discontinued at P25, the animals continued to survive and show signs of improvement past the normal lifespan of non-treated Smn2B/- mice. This provides further evidence that there is a critical time window in SMA pathogenesis.

TSA improves the neuromuscular pathology that is observed in Smn2B/- mice

To investigate the cellular mechanisms underlying the phenotypic improvements observed in TSA treated mice, we investigated the effects of TSA on the neuromuscular pathology of Smn2B/- mice. Motor neuron loss in the anterior horn region of spinal cords is a key hallmark of SMA in both humans and Smn2B/- mice [9], [22]. Quantification of motor neuron cell body number revealed a statistically significant increase in TSA treated Smn2B/- mice compared with the DMSO treated controls (Fig. 2A and C).

Figure 1. TSA treatment promotes survival, attenuates weight loss, and enhances motor function in Smn2B/- mice. (A) Kaplan-Meier Survival curves of TSA or vehicle (DMSO) treated Smn2B/- mice and littermate controls. Median lifespan is 8 weeks in TSA treated Smn2B/- mice (black arrow) in comparison to 23 days in DMSO-treated (black arrow). n = 9 (Smn2B/- DMSO), 7 (Smn2B/- TSA), 5 (Smn2B/+ DMSO), 4 (Smn2B/+ TSA). (B) Growth curves of TSA or vehicle (DMSO) treated Smn2B/- mice and littermate controls from P13 to P25. DMSO-treated Smn2B/- mice gradually lost weight during this time period. n = 16 (Smn2B/- DMSO), 15 (Smn2B/- TSA), 11 (Smn2B/+ DMSO), 14 (Smn2B/+ TSA). (C) Righting reflex test. Mild improvement observed in the righting ability of TSA-treated Smn2B/- mice from P15 onwards. n = 16 (Smn2B/- DMSO) and 15 (Smn2B/- TSA). (D) Pen test assay on Smn2B/- mice treated with TSA or DMSO. Thirty seconds was set as the maximum time period. Improvement in latency to fall in TSA treated SMA mice (red line) became more apparent after P21 in comparison to DMSO treated SMA mice (black line). n = 16 (Smn2B/- DMSO) and 15 (Smn2B/- TSA). doi:10.1371/journal.pone.0101225.g001
Interestingly, this data contrasts with previous work reporting that TSA was unable to reduce the motor neuron loss that is normally observed in the delta 7 model of SMA [13]. This is perhaps reflective of the relative difference in severity of these two SMA mouse models, with the more prolonged pathology being observed in the Smn2B+/− mice.

We went on further to investigate whether the neuroprotective properties of TSA extends to the NMJ. Smn2B+/− mice display significant levels of denervation, which is accompanied by a decrease in NMJ size and complexity [9]. This finding was recapitulated in our DMSO treated Smn2B+/− mice, where we observed widespread NMJ pathology as evidenced by denervation, neurofilament accumulation and immature post-synaptic endplates (Fig. 3A, top panels). TSA administration significantly improved the overall appearance of Smn2B+/− NMJs, with a decrease in neurofilament accumulation and an increase in post-synaptic size and maturity (Fig. 3A, bottom panels). Importantly, quantification of the percentage of fully occupied endplates also revealed a significant increase in TSA treated Smn2B+/− mice compared to vehicle treated controls (Fig. 3B and C).

The data above demonstrate that TSA has neuroprotective properties at the level of motor neurons and NMJs. Since the neuromuscular pathology is significantly improved in Smn2B+/− mice and this is accompanied by an improvement in motor behaviour, we investigated whether the beneficial effects could reach the muscle. Smn2B+/− mice have smaller and more immature myofibers and display a delay in muscle development [23], [24], [25]. TSA treatment led to improvements in myofiber histology (Fig. 2B). We therefore conclude that TSA significantly improves the pervasive neuromuscular pathology that is evident in Smn2B+/− mice; this consequently leads to positive functional outcomes such as improved survival, growth, and motor behavior.

TSA administration does not result in an increase in Smn in Smn2B+/− mice or cells

In previous clinical trials and studies of HDAC inhibitors in SMA patients and animal models, the beneficial effects have been attributed to the increase in SMN transcript and protein levels [13], [26], [15], [27]. We therefore investigated the effects of TSA on Smn expression in various tissues and primary cells obtained from the Smn2B+/− mice. RT-PCR analysis of RNA from the spinal cord of Smn2B+/− mice revealed that there is no change in the ratio of full-length and delta7 Smn splice isoforms following TSA treatment (Fig. 4A). Furthermore, TSA administration did not increase Smn transcript levels in the spinal cord or the brain of Smn2B+/− mice (Fig. 4B). However, it is noteworthy that follistatin from the same animals was upregulated after treatment as a proof of TSA efficacy (Fig. 4A).

Although there was no change in Smn transcript levels, we measured Smn protein levels in various tissues obtained from Smn2B+/− mice. TSA administration resulted in no change in Smn protein levels in the brain and spinal cord (Fig. 5A and B). Furthermore, TSA treatment did not increase Smn protein levels in muscle, heart or liver of Smn2B+/− mice (Fig. 5A and B).

The data above suggest that TSA treatment did not increase Smn transcript or protein levels in various tissues obtained from Smn2B+/− mice. To confirm this result, we obtained MEFs and myoblasts from Smn2B+/− mice to investigate whether TSA can increase Smn protein levels in vitro. MEFs and myoblasts obtained from Smn2B+/− mice were treated with 100 nM of TSA for 24 hours. At this dose, TSA increased the acetylation of histone H3 in MEFs and myoblasts obtained from Smn2B+/− mice (Fig. 6A and C). However, TSA did not increase Smn transcript levels or protein levels obtained from Smn2B+/− mice (Fig. 6A and C).

We therefore conclude that Smn is not a specific or direct target of TSA at the time points tested, and other genes are responsible for the phenotypic and neuroprotective effects observed in Smn2B+/− mice. Since HDAC inhibitors and TSA can cause global transcriptional changes, and there are no changes in Smn transcript or protein levels in various tissues and cells obtained from Smn2B+/− mice, we suggest that the beneficial effects of TSA on the Smn2B+/− phenotype are Smn-independent.
In this study, we have shown that TSA administration have beneficial effects in the Smn2B/- mouse model. Repeated administration of TSA leads to a significant improvement in survival and growth, motor behavior, motor neuron survival, NMJ size and maturity, and muscle growth. It is important to note that this was not accompanied by an increase in Smn protein or RNA levels in either tissues from TSA treated mice or in primary cell cultures treated with TSA.

The Smn2B allele is a knock-in mutation that disrupts splicing of the endogenous Smn gene, resulting in an increase in the production of delta7 Smn mRNA with a concomitant reduction of full-length Smn transcripts. Presenting about 15% of full-length Smn protein levels compared to the wild type allele, the Smn2B/- mice display a neurological phenotype beginning at two weeks of age [9], [10]. The data presented here suggest that the beneficial effects of TSA in the Smn2B/- mice are likely independent of changes in the endogenous Smn gene. Previous reports of HDAC inhibitors in mouse models of SMA have shown similar beneficial effects. Daily administration of TSA to the delta 7 mouse model from P5 to P13 lead to improved survival, attenuated weight loss and enhanced motor behavior, increased myofiber size and number, and increased anterior horn cell size [13]. Full-length

Discussion

In this study, we have shown that TSA administration have beneficial effects in the Smn2B/- mouse model. Repeated administration of TSA leads to a significant improvement in survival and growth, motor behavior, motor neuron survival, NMJ size and maturity, and muscle growth. It is important to note that this was not accompanied by an increase in Smn protein or RNA levels in either tissues from TSA treated mice or in primary cell cultures treated with TSA.

The Smn2B allele is a knock-in mutation that disrupts splicing of the endogenous Smn gene, resulting in an increase in the
Smn transcript levels were not increased in the brain, liver, and spinal cord of TSA treated delta 7 mice, however, there was a 1.5 fold increase in Smn protein levels in these tissues. It should be emphasized that the delta 7 model carries the human SMN2 transgene, which has been reported to be a direct target of TSA [11]. It still remains unclear whether the small increase in SMN protein levels is sufficiently capable to cause dramatic changes in phenotype. Regardless, our results demonstrate that TSA exerts beneficial effects on a mouse model of SMA, which lacks the human SMN2 transgene. Therefore, the current available studies do not indicate a direct nor specific detectable increase in Smn transcript levels in response to TSA, further supporting the hypothesis that the observed beneficial effects of TSA are independent of Smn.

TSA treatment enhanced motor behaviour, gene expression of choline acetyltransferase (ChAT), but not ventral horn neuron number in the delta 7 SMA model [13]. In contrast, we observed a rescue of the motor neuron loss along with motor function improvement in the TSA-treated Smn2B/- mice. While severity and time of onset differ between these two models, there is no systematic comparison of SMN/Smn protein levels, correlation of SMN/Smn protein levels to motor neuron morphology and global gene expression, and extent of perturbation in motor function. Differences in these aspects can certainly influence the overall outcome of TSA treatment. Therefore, one speculation to the discrepancy in motor neuron rescue in the two studies is that there may be a certain developmental window, after which motor neuron loss is less likely reversible. TSA has general neuroprotective properties in various other cellular conditions and animal models of neurodegenerative disease. TSA increases alpha-synuclein levels and protects against glutamate induced excitotoxicity in the cerebellum and frontal cortex as well as in cortical neurons [30]. Oxidative stress and disturbed glutamate transport has been reported in type I SMA patients [31]. TSA also increases cytoprotective HSP70 levels and concurrently suppresses P53 upregulation, consequently inhibiting the effects of this proapop-

**Figure 4. No alteration in the splicing pattern or levels of Smn mRNA in the brains of TSA-treated mice.** (A) RT-PCR for Smn and a known target of TSA, follistatin, was conducted in total RNA extracted from brains of Smn2B/- mice or littermate controls treated with TSA or DMSO vehicle. Ratio of full length Smn versus ∆7Smn transcripts remained similar after TSA treatment in Smn2B/- or in control mice. Transcript level of follistatin is elevated in TSA-treated mice. Actin serves as a loading control. (B) Real-time quantitative RT-PCR analyses of RNA from spinal cord and brain. No significant change in the level of Smn transcripts (full length Smn plus ∆7Smn) was revealed after TSA treatment (n = 3). Fold increase normalized to reference gene Gapdh.

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totic transcription factor [32]. Other than playing a key role in neuroprotection, HSP70 also plays a role in the muscle and protects against the atrophic features also present in SMA [33]. Aberrant apoptosis through the downregulation of Bcl-2 proteins in motor neurons of type I SMA patients is well documented [34] and TSA can increase the levels of Bcl-2 [32]. TSA also increases neurotrophic support to existing neurons by robustly increasing the transcription of BDNF and GDNF [35]. Therefore, TSA utilizes multiple neuroprotective pathways to improve neuronal function.

In summary, we have shown that TSA, a potent pan-HDAC inhibitor, significantly improves the phenotype caused by Smn deficiency in the Smn2B/- intermediate mouse model of SMA. TSA treated Smn2B/- mice were significantly better on outcome measures such as survival, growth, motor behavior, motor neuron survival, NMJ maturity, and muscle histology. These beneficial changes were not accompanied by an increase in Smn levels in various tissues and cells obtained from the Smn2B/- mice. Therefore, the beneficial effects that we observed are likely mediated through Smn-independent pathways. Identification of these pathways will be of therapeutic value in SMA.

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Author Contributions

Conceived and designed the experiments: HL, AY LMM AB RK. Performed the experiments: HL, AY LMM AB. Analyzed the data: HL, AY LMM AB. Contributed reagents/materials/analysis tools: HL, AY LMM AB RK. Contributed to the writing of the manuscript: HL, AY LMM RK.
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