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SAHA ameliorates the SMA phenotype in two mouse models for spinal muscular atrophy

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Proximal spinal muscular atrophy (SMA) is a common autosomal recessively inherited neuromuscular disorder determined by functional impairment of α-motor neurons within the spinal cord. SMA is caused by functional loss of the *survival motor neuron gene 1* (*SMN1*), whereas disease severity is mainly influenced by the number of *SMN2* copies. *SMN2*, which produces only low levels of full-length mRNA/protein, can be modulated by small molecules and drugs, thus offering a unique possibility for SMA therapy. Here, we analysed suberoylanilide hydroxamic acid (SAHA), a FDA-approved histone deacetylase inhibitor, as potential drug in two severe SMA mouse models each carrying two *SMN2* transgenes: US-SMA mice with one *SMN2* per allele (*Smn*$^{-/-}$;*SMN2*$^{tg/tg}$) and Taiwanese-SMA mice with two *SMN2* per allele (*Smn*$^{-/-}$;*SMN2*$^{tg/wt}$), both on pure FVB/N background. The US-SMA mice were embryonically lethal with heterozygous males showing significantly reduced fertility. SAHA treatment of pregnant mothers rescued the embryonic lethality giving rise to SMA offspring. By using a novel breeding strategy for the Taiwanese model (*Smn*$^{-/-}$;*SMN2*$^{tg/tg}$ × *Smn*$^{-/+}$ mice), we obtained 50% SMA offspring that survive ∼10 days and 50% control carriers in each litter. Treatment with 25 mg/kg twice daily SAHA increased lifespan of SMA mice by 30%, significantly improved motor function abilities, reduced degeneration of motor neurons within the spinal cord and increased the size of neuromuscular junctions and muscle fibers compared with vehicle-treated SMA mice. *SMN* RNA and protein levels were significantly elevated in various tissues including spinal cord and muscle. Hence, SAHA, which lessens the progression of SMA, might be suitable for SMA therapy.

INTRODUCTION

Proximal spinal muscular atrophy (SMA) is a neuromuscular disorder characterized by the loss of motor neurons in the spinal cord that results in muscle atrophy and weakness of voluntary muscles (1). With an incidence of 1 in 6000 live-births and a carrier frequency of 1:35 in the Caucasian population, SMA is the second most frequent autosomal recessive disorder in humans and the leading genetic cause of infant death today, for which no therapy is available (2–4).

SMA is caused by functional loss of the *survival motor neuron gene 1* (*SMN1)* due to homozygous deletion, gene conversion or subtle mutations (5–7). The severity of SMA, which varies from severe type I to very mild type IV is mainly influenced by the number of *SMN2* copy genes (2,8,9). *SMN2* carries a translationally silent mutation that affects proper pre-mRNA splicing of exon 7 and produces only ∼10% full-length (FL) *SMN2* transcript and protein that is identical to the protein encoded by *SMN1* (5,10). The majority of *SMN2* transcripts lack exon 7 (*SMN2Δ7*) and encode a truncated and unstable protein (11,12).

SMN2, which varies between one and six copies per genome in SMA patients, is considered as the main target for a successful therapy. Different therapeutic interventions have been reported so far including: (i) elevation of *SMN*2 RNA and protein levels by transcription activation, correction of splicing or stabilization of protein, (ii) gene replacement by use of gene therapy approaches, (iii) neuroprotective therapy

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and (iv) stem cell therapy (13). Despite these efforts, there is still no therapy and cure for SMA patients available.

Currently, *SMN2* targeted therapeutics seem to be most promising. *SMN2* can be epigenetically modulated using histone deacetylase inhibitors (HDACi) and DNA methyltransferase inhibitors (14–19). Most importantly, all HDACi used so far [sodium butyrate, valproic acid, phenylbutyrate, Trichostatin A, suberoylanilide hydroxamic acid (SAHA), M344 and LBH589] proved to increase SMN levels *in vitro* and some also *in vivo* in SMA mice and SMA patients, delivering the proof of principle for the ability to activate *SMN2* via HDACi (14,15,17–24). Two of these drugs, valproic acid (VPA) and phenylbutyrate, have already been enrolled in clinical trials in SMA patients. Both drugs seem to activate *SMN2* expression only in a subset of patients, the so-called responders, which account for ~35% (20,25). Placebo-controlled clinical trials with VPA or phenylbutyrate failed, however, to show significant improvements in SMA patients (26,27). Nonetheless, since in neither study, a stratification of patients in responders and non-responders has been undertaken, these results are still questionable and need further investigation. Nevertheless, there is still an urgent need for new SMA therapeutics.

In the present work, we focused on the FDA-approved drug SAHA or vorinostat (marketing name *Zolinza*), which is a HDACi shown to inhibit virtually all classical HDACs (28). SAHA has been demonstrated to reduce glioma progression *in vivo* (29). Moreover, it has been proven that the oral therapy with SAHA ameliorates motor deficits in a mouse model of Huntington's disease; additionally, it has been verified that SAHA crosses the blood-brain barrier, which is essential for prospective SMA therapy (30). We have previously shown that SAHA is able to up-regulate SMN on protein and RNA levels *in vitro* and *ex vivo* using organotypic hippocampal brain slices derived from rat and human (31). To further explore the therapeutic potential *in vivo*, different SMA mouse strains were analyzed in the present study.

Since the homozygous ubiquitous knock-out of the endogenous *Smn* gene leads to early embryonic lethality, several groups generated SMA mice by either specifically knocking out *Smn* in spinal cord using the Cre-loxP system or more frequently by introducing the human *SMN2* as a transgene on a murine *Smn* null background (32–34). Comparable to SMA patients, the severity of the disease inversely correlates with the *SMN2* copy number in transgenic SMA models (33,34). While $Smn^{-/-}$;$SMN2^{tg/tg}$ mice, carrying one *SMN2* copy per integrate, die between 1 and 8 days, $Smn^{-/-}$;$SMN2^{tg/tg}$ mice with 8–16 *SMN2* copies show a complete rescue of the phenotype (34). Hsieh-Li *et al*. (33) integrated a BAC clone containing the human *SMN2* gene into the murine DNA. Depending on the number of *SMN2* copies, mice have developed type I–III like SMA phenotypes.

In the present study, we investigated two SMA mouse models (USA and Taiwanese), both on pure FVB/N background, for the *in vivo* effect of SAHA to lessen the progression of SMA. Most importantly, since the US-SMA model turned out to be embryonic lethal, we developed a novel breeding strategy for the Taiwanese model, which then produced 50% control carriers and 50% SMA in each litter, the latter living in average 10 days and thus representing an excellent model for pre-clinical trials. In both models, SAHA significantly ameliorated the SMA phenotype. Treated animals showed an increase in mean lifespan, improvement in motor function, increased number of motor neurons and increased size and occupancy of post-synaptic sites of neuromuscular junctions (NMJ) and muscle fibers. SAHA elevated expression of *SMN2* in the spinal cord and muscle. Since SAHA is FDA-approved, currently being tested in a large number of clinical trials, and able to lessen the progression of SMA in mice, we propose SAHA as an appropriate candidate for SMA therapy.

RESULTS

Identification of an appropriate SMA mouse model for drug screening

In our study, we used two severely affected SMA mouse models published in the year 2000 by the group of Burghes and Hsieh-Li, respectively (33,34). The first model, here termed US-SMA mouse model, carries one *SMN2* copy per allele. Homozygous mice ($Smn^{-/-}$;$SMN2^{tg/tg}$) on C57BL/6J background have been described to develop very severe SMA with a mean age of survival of 5 days (34). The mice used in the frame work were kindly provided by Dr Sendtner (Würzburg) and were backcrossed on pure FVB/N background. After exact quantification of the number of *SMN2* transgene copies in the breeder pairs by a newly developed real-time quantitative PCR assay (Supplementary methods), mice carrying the genotype $Smn^{-/+}$;$SMN2^{tg/tg}$ were bred with each other. Following a Mendelian inheritance, 25% of the offspring was expected to carry a $Smn^{-/-}$;$SMN2^{tg/tg}$ genotype, which would resemble the expected SMA phenotype. However, SMA animals with this respective genotype were never observed to be born. Five breeder pairs produced 35 offspring with an average litter size of 7.2 ± 1.2, but none of the theoretically expected 9 SMA animals were observed (Fig. 1). Thus, we concluded that the US-SMA model on FVB/N background is embryonic lethal. Furthermore, we observed a severe reduction in fertility. Most heterozygous mice ($Smn^{-/+}SMN2^{tg/tg}$) were infertile at an age of 6 months when compared with a normal fertility until usually at least 10 months in wild-type mice (35) (Supplementary Material, Fig. S1). However, we did not further analyse the reason for this observation. We can definitively exclude infertility due to inbreeding.

The second model, here termed Taiwanese-SMA mouse model, was initially generated by the Hsieh-Li group (33). In the initial publication, several founders on a mixed background have been reported for these mice that developed type I–III SMA depending on the copy number (33). We purchased a pair of mice from the Jackson's Laboratory, each mouse carrying four *SMN2* copies (two copies per integrate) on *Smn* null background ($Smn^{-/-}SMN^{tg/tg}$) and pure FVB/N background. The homozygous mice did not develop a SMA phenotype; however, they presented a shortened tail and necrotic ears. In order to generate SMA mice, we crossbred homozygous *SMN2* transgenic *Smn* knockout mice ($Smn^{-/-}$;$SMN2^{tg/tg}$) with heterozygous *Smn* knockout mice ($Smn^{+/-}$). This led to 50% SMA mice ($Smn^{-/-}$;$SMN2^{tg/+}$) and 50%

A

Substance	# SMA mice expected	# SMA mice observed	p-value (χ^2-test)
water control	9	0	0.012
HP-β-CD	8	1	0.042
SAHA	12	5	0.119

B

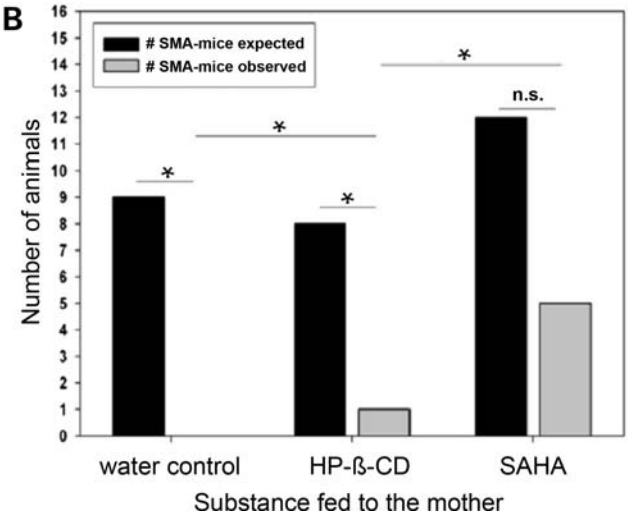

Figure 1. US-SMA mice show embryonic lethality that can be rescued by SAHA. (**A**) Overview table. *P*-values of SAHA-treated mice suggest that the treatment of pregnant mice leads to a higher *in utero* survival rate of SMA mice. (**B**) Diagrammatic representation of significant increase in number of born SMA mice after SAHA treatment of the pregnant mothers, $^{*}P < 0.05$.

control carriers ($Smn^{-/+}$;$SMN2^{tg/+}$) with an identical *Smn/SMN2* genotype. The $Smn^{-/-}$;$SMN2^{tg/+}$ mice developed a severe SMA phenotype with a mean age of survival of 9.9 (± 0.45) days with a median of 10 days ($n = 42$). This breading strategy of the Taiwanese-SMA model turned out to be very successful: first, the number of SMA mice that can be generated is double when compared with all other SMA mouse models and second, 10 days of survival are appropriate for drug screening. In comparison, the delta7SMA mouse model ($SMN^{-/-}$;$SMN2^{tg/tg}$;$SMN\Delta7^{tg/tg}$) widely used for drug screenings by the SMA community (Table 1) has a mean age of survival of 11.4 ± 0.7 days (19,36–42).

SAHA rescues embryonic lethality in the US-SMA model and prolongs survival by 30% in the Taiwanese-SMA model

In order to prove whether SAHA could rescue the embryonic lethality in the US-SMA model, pregnant mother mice were treated from gestational day 15 on either with SAHA or with its respective solvent. Since SAHA is a hydrophobic substance and cannot be solved in water alone, hydroxypropyl-β-cyclodextran (HP-β-CD) was used as a carrier substance. Given the fact that a 20 g mouse drinks 3 ml water per day, this dosage equals 200 mg SAHA per kg body weight per day, which was previously described to be well tolerated in wild-type adult mice and shown to increase H3/H4 acetylation levels in the CNS (30).

Strikingly, prenatal SAHA treatment resulted in a first observation of live born SMA animals (Fig. 1). In contrast, treatment with the solvent alone failed to rescue the embryonic lethality, except for one mouse, which died at postnatal day (PND) 1. The five observed SMA mice, which were born under SAHA treatment, survived for 2 (two mice), 8, 11 and 14 days, respectively. This suggests a beneficial effect of SAHA on the *in utero* progression of SMA. This preliminary positive effect of SAHA prompted us to (i) identify a second more suitable mouse model for drug testing and (ii) to continue the pre-clinical testing of SAHA. Since SMA is a neuromuscular disorder leading to impairment in motor abilities, it was necessary to use a SMA mouse model, which reaches an age at which motor abilities can be assessed. All further analyses were performed in the Taiwanese-SMA model.

Since SAHA was used here for the first time in newborn mice, the right application method and adequate treatment concentration had to be determined. The use of 200 mg per kg per day as described for adult mice (30) turned out to be highly toxic in newborn SMA mice as well as heterozygous mice (Supplementary Material, Fig. S2). Therefore, diverse concentrations and application methods (oral or subcutaneous) were tested (Supplementary Material, Fig. S2). Of all the tested methods, oral application using a feeding needle of 25 mg/kg twice daily SAHA (solved in DMSO) was the adequate regimen. This treatment led to a statistically significant improvement in the mean survival of the severely affected SMA mice when compared with vehicle-treated SMA mice ($n = 38$). SAHA-treated mice ($n = 42$) showed a 30.3% ($P < 0.001$) increased survival rate, from 9.9 ± 0.32 to 12.9 ± 0.76 days (Fig. 2). At this concentration, SAHA had no influence on development and survival of heterozygous littermates ($n = 38$). Noteworthy, the SMA mouse representing the longest survivor in the vehicle-treated group lived for 16 days, while ~10% of the SAHA-treated animals lived 16 days or longer: one mouse died at the age of 16 days, two mice lived for 20 days, one mouse each survived 23, 24 and 32 days, respectively (Fig. 2B). The 32-day-old SMA mouse developed severe necrosis of the tail and the hind limbs and was therefore sacrificed. Necrosis of hind limbs and tail was also observed in the other long-time SMA survivors but never in heterozygous littermates treated with SAHA. Therefore, we concluded that necrosis is not a side-effect of SAHA, but rather a feature of progressing SMA as previously described (43). The statistically highly significant difference in survival of SAHA versus vehicle-treated SMA mice ($P < 0.001$) suggests that SAHA is reducing the progression of SMA development.

SAHA counteracts weight loss in SMA mice

To characterize the progression of SMA and the development of the SMA animals under SAHA when compared with vehicle-treated animals, the weight of each mouse was determined every morning before drug treatment.

Up to PND3 all mice showed the same weight progression regardless of the respective genotypes. From PND3 on, SAHA-treated heterozygous littermates increased almost linearly in weight. In contrast, the mean weight of both SAHA- and vehicle-treated SMA mice showed a slow elevation, which was similar until PND8 (Fig. 3A). At

Table 1. Overview of *in vivo* approaches to ameliorate the SMA phenotype of diverse SMA mouse models in comparison to the results discussed in the present work (marked in grey)

Mechanism of action	Compound	SMA mouse model	Increase in mean survival	Effect on SMA progression	SMN protein increase	Original publication
Histone deacetylase inhibition (activation of *SMN2* transcription and/or splicing)	Suberoylanilide hydroxamic acid (SAHA)	US model ($Smn^{-/-}$;$SMN2^{tg/tg}$) (on FVB/N) Taiwanese model ($Smn^{-/-}$;$SMN2^{tg/wt}$) (on FVB/N)	Treatment of mothers overcomes embryonic lethality 30%	Treatment of mothers overcomes embryonic lethality Increased number of motor neurons, motor function improvement, bigger neuromuscular junction size and muscle fibers size	2.7-fold (spinal cord), 5.5-fold (brain), 17.6-fold (muscle)	This paper
Histone deacetylase inhibition (activation of *SMN2* transcription and/or splicing)	Sodium butyrate (NaBu)	Taiwanese model ($Smn^{-/-}$;$SMN2^{tg/w}$t) (on mixed background)	39%	Treatment of mothers overcomes embryonic lethality, muscular atrophy of tails is ameliorated	Slightly increased (not quantified)	(21)
	Valproic acid (VPA)	Taiwanese model ($Smn^{-/-}$;$SMN2^{tg/wt}$) (on C57BL/6)	Not determined	Better motor function, larger motor-evoked potentials, less degeneration of spinal motor neurons, less muscle atrophy, and better neuromuscular junction innervation	~1.4-fold	(24)
	Trichostatin A (TSA)	$Smn^{-/-}$;$SMN2^{tg/tg}$;$SMN\Delta7^{tg/tg}$ (on FVB/N)	19%	Increased myofiber size and number, better motor behavior, attenuated weight loss, increased spinal motor neuron size	1.5-2-fold	(19)
	Trichostatin A (TSA) plus nutrition	$Smn^{-/-}$;$SMN2^{tg/tg}$;$SMN\Delta7^{tg/tg}$ (on FVB/N)	170%	Increased myofiber size, weight gain, maintained stable motor function, retained intact neuromuscular junctions, long-lived mice show necrosis	Not determined	(56)
Read-through of stop codon	TC007	$Smn^{-/-}$;$SMN2^{tg/tg}$;$SMN\Delta7^{tg/tg}$ (on FVB/N)	~30%	Increase in number of spinal motor neurons, motor function improvement	~2-4-fold	(38)
	Geneticin (G418)	$Smn^{-/-}$;$SMN2^{tg/tg}$;$SMN\Delta7^{tg/tg}$ (on FVB/N)	No increase (due to toxicity)	Motor function improvement	~3-fold	(39)
Non-steroidal anti-inflammatory drug (transcriptional activation)	Indoprofen	US model ($Smn-/-$;*SMN2*tg/wt) (on C57BL/6/FVB/N)	Treatment of mothers prolongs development of SMA embryos but failed to overcome embryonic lethality		Not determined	(57)
Decapping enzyme, scavenger (DcpS) inhibitor (transcriptional activation)	2, 4 Diaminoquinazoline (D156844)	$Smn^{-/-}$;$SMN2^{tg/tg}$;$SMN\Delta7^{tg/tg}$ (on FVB/N)	~21-30% (dependent on treatment start)	Motor function improvement, less degeneration of spinal motor neurons, activation of β-galactosidase expression (in *Smn* KO)	~1.7-fold	(58)
Oligonucleotides (splicing correction)	Bifunctional RNA (high affinity to SFRS10)	$Smn^{-/-}$;$SMN2^{tg/tg}$;$SMN\Delta7^{tg/tg}$ (on FVB/N)	'trend towards increased life expectancy'	Increase in weight gain	~2-fold	(37)
	Steric block antisense oligonucleotide	$Smn^{-/-}$;$SMN2^{tg/tg}$;$SMN\Delta7^{tg/tg}$ (on FVB/N)	Not determined	Increase in weight gain, partial correction of motor deficits (righting reflex)	~2-3-fold	(59)
Induction of muscle growth	Recombinant follistatin	$Smn^{-/-}$;$SMN2^{tg/tg}$;$SMN\Delta7^{tg/tg}$ (on FVB/N)	30%	Increased muscle mass, increase in number of spinal motor neurons, motor function improvement	Unchanged	(60)
Lentiviral gene transfer	Lentivector expressing human SMN	$Smn^{-/-}$;$SMN2^{tg/tg}$;$SMN\Delta7^{tg/tg}$ (on FVB/N)	20–38%	Increase in weight gain, increased motor neuron survival	Restoration in motor neurons	(61)
Stem cell therapy	Neuronal stem cells	$Smn^{-/-}$;$SMN2^{tg/tg}$;$SMN\Delta7^{tg/tg}$ (on FVB/N)	~40%	Increased motor neuron survival, improvement in muscular phenotype, neuroprotection, motor function improvement, increase in weight gain	~2-fold	(62)
Transplantation	Bone marrow transplantation (wt)	*HSA-Cre;* $Smn^{fl7/fl7}$ (muscle-specific *Smn* KO)	Not determined	Improvement of myopathic phenotype (muscular regeneration), improvement of motor performance (+85%)	Not determined	(63)
Physical training	Exercises (running)	Taiwanese model ($Smn^{-/-}$;*SMN2*) different types on mixed background	57.3%	Increased motor neuron survival, splicing correction, improvement in muscular phenotype, neuroprotection	Not determined	(64)

Results are summarized from the given publications.

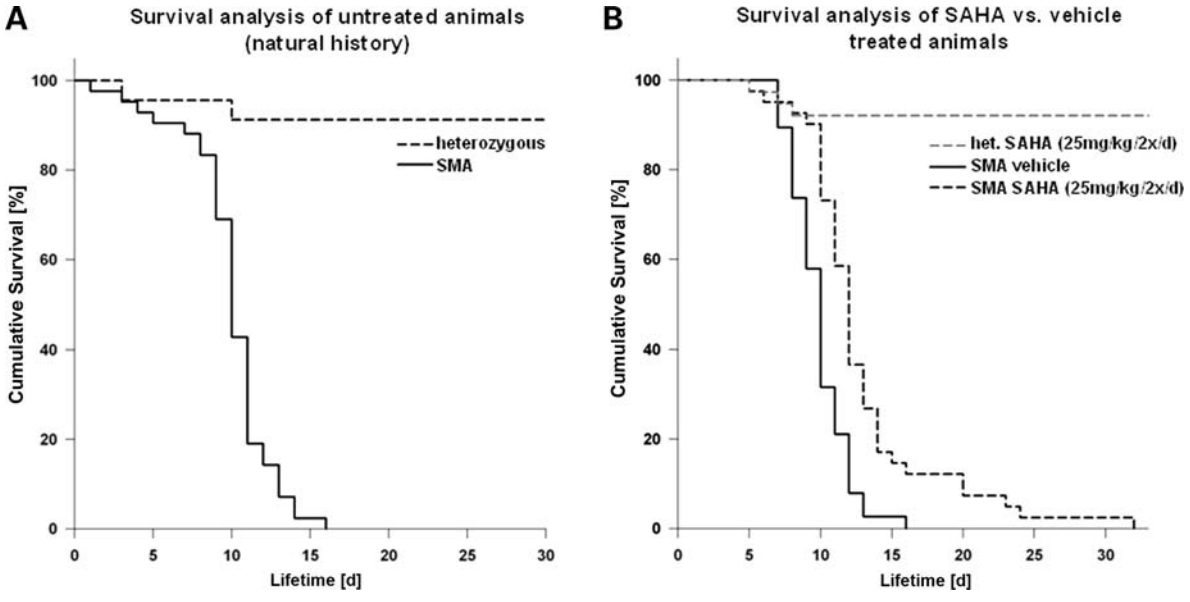

Figure 2. Comparison of the mean survival of SAHA-treated versus vehicle-treated SMA mice. (**A**) Kaplan–Meier curve representing the natural history of untreated mice. Black line, SMA mice ($n = 42$); dashed black line, heterozygous littermates ($n = 36$); (**B**) Kaplan–Meier curve comparing SAHA-treated heterozygous mice ($n = 38$, grey dashed line), vehicle-treated SMA mice ($n = 38$, black line) and SAHA-treated SMA mice ($n = 42$, black dashed line).

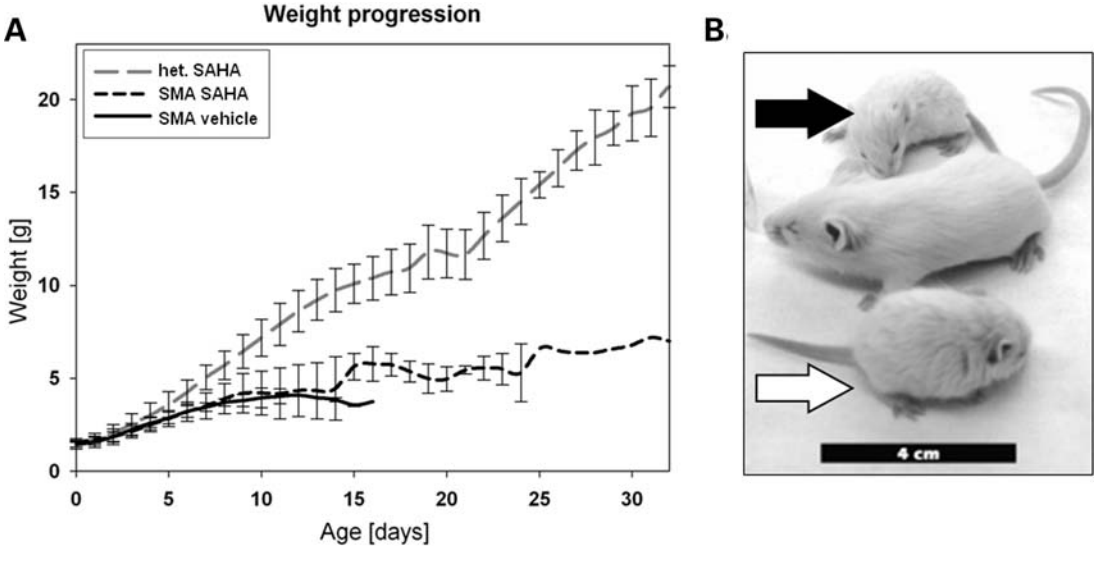

Figure 3. Weight development under SAHA regimen. (**A**) Diagrammatic representation of the mean weight progression of SAHA-treated heterozygous mice ($n = 38$, grey dashed line), vehicle-treated SMA mice ($n = 38$, black line) and SAHA-treated SMA mice ($n = 42$, black dashed line). (**B**) Photograph of vehicle-treated SMA mice (black arrow), SAHA-treated heterozygous littermate and SAHA-treated SMA mouse (white arrow) at PND14.

PND9, first differences between SAHA- and vehicle-treated mice were recorded: SAHA-treated mice were ~10% heavier than vehicle-treated SMA mice (4 versus 3.6 g; Fig. 3A), which further increased to ~5.5 g at PND16. In contrast, SAHA-treated heterozygous littermates revealed a mean weight of 10.4 ± 1.2 g at PND16.

These data indicate that SAHA only slightly improves weight progression in the SMA mice (Fig. 3B). Longer SAHA-treated SMA survivors developed a dwarf-like phenotype. These animals were only half the size as their heterozygous littermates at an age of 14 days. This discrepancy was even higher at later ages.

SAHA improves motor abilities of SMA mice

Since SMA is a motor neuron disease severely affecting the motor abilities of patients, a potential prospective therapy should ameliorate motor impairments. Therefore, the effect of SAHA on motor abilities was chosen as a major outcome measure of this pre-clinical trial.

As a motor test, the so-called tube test, which has been especially developed for neonates (40), was chosen. In brief, to perform a tube test, pups were placed headfirst into a vertical 50 ml reaction tube. Depending on their motor abilities, mice were rated from 4 (best tube score) to 1 (bad tube

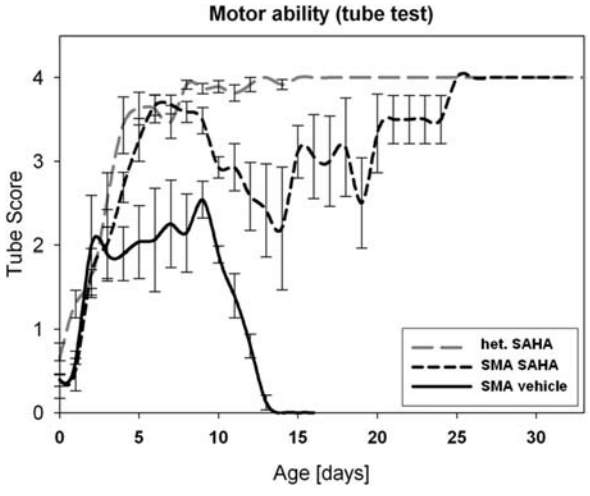

Figure 4. Analysis of the impact of SAHA on motor function. Diagrammatic representation of the mean motor ability progression of SAHA-treated heterozygous mice ($n = 38$, grey dashed line), vehicle-treated SMA mice ($n = 38$, black line) and SAHA-treated SMA mice ($n = 42$, black dashed line).

score, hind limbs in a clasped position). The worst tube score of 0 was recorded when mice were not able to hold onto the tube.

Motor performance of all neonates, regardless of their genotype and treatment situation, showed a similar development for the first 2 days after birth (Fig. 4). As expected, the motor abilities of SAHA-treated heterozygous mice developed normally and reached the maximum tube score of 4 at PND8 and remained stable thereafter (Fig. 4). SAHA-treated SMA mice performed similarly to heterozygous littermates and reached a maximum tube score of 3.7 ± 0.12 at PND6 when compared with vehicle-treated SMA mice, which reached a maximum tube score of only 2.5 ± 0.22 at PND9. Vehicle-treated SMA animals showed a clear stagnation in their motor performance, with values around 2 to 2.5 from PND2 to PND9, followed by a steep decline with scores decreasing from 2.5 to 0 until death (Fig. 4). Although SAHA-treated SMA mice showed a decline in motor performance starting at PND9, their achieved tube score was always significantly higher ($P = 0.0048$) than the tube score of the vehicle-treated SMA mice (Fig. 4).

These data show that SAHA treatment significantly ameliorates the motor impairment of SMA mice, demonstrating that SAHA improves the major outcome of SMA symptoms.

SAHA elevates SMN expression in spinal cord and muscle

In order to evaluate the impact of SAHA treatment on SMN expression, SMA mice and heterozygous littermates were compared with vehicle-treated mice at PND5 and PND10, respectively ($n = 3$ per group). Mice were sacrificed and liver, whole brain, the *gastrocnemius* muscle and spinal cord were quickly harvested, snap frozen in liquid nitrogen and used for RNA and protein isolation.

SMN protein expression was determined by semi-quantitative western blot analysis using β-actin as internal control. Since the SMN antibody recognizes both the murine and human SMN protein, in heterozygous mice the SMN band represents the sum of both orthologs. Most importantly, in all tissues derived from SAHA-treated SMA mice, an up-regulation of SMN protein levels was observed, with a significant elevation in spinal cord and muscle at PND5 (2.7- and 17.6-fold, respectively) and in brain and muscle at PND10 (5.5- and 3.5-fold, respectively) (Figs 5 and 6). Furthermore, we determined the *FL-SMN2* and *SMN2Δ7* transcript levels to verify whether SAHA is transcriptionally activating *SMN2* in the same four tissues. While at PND5, only SAHA-treated SMA mice showed elevated *FL-SMN2* levels, at PND10 both transcripts were significantly elevated in spinal cord and particularly in muscle, the latter one obviously correlating well with the high increase also observed on protein level (Supplementary Material, Fig. S3).

These data confirm the previous observation that SAHA crosses the blood-brain barrier (30) and provides first evidence that SAHA is able to augment the *SMN*2 RNA and SMN protein levels in spinal cord and muscle, the two main tissues being involved in SMA pathology.

SAHA counteracts motor neuron loss in SMA mice

The primary feature of SMA is the deterioration of the α-motor neurons in the anterior horns of the spinal cord, which causes subsequent muscular atrophy. To investigate whether SAHA is capable to rescue α-motor neuron loss, the number of motor neurons was quantified at PND5 and PND10 in SMA mice and heterozygous littermates, which were either SAHA or vehicle treated ($n = 3$ per group). Twenty cross-sections of the lumbar region of each mouse were generated and every third or fourth (depending on quality) section was counted. Cell bodies of motor neurons, which were visualized by Nissl staining, can easily be distinguished from the surrounding cells based on their size and particular shape (Fig. 7). The mean number of motor neurons per anterior horn was counted. At PND5, no significant difference between SMA animals and heterozygous mice of both groups, SAHA or vehicle-treated was observed (Fig. 7). The mean number of α-motor neurons in each anterior horn region ranged between 12 and 13 (Fig. 7).

In contrast, at PND10, SAHA-treated SMA mice as well as the vehicle-treated heterozygous mice showed a mean number of ∼14 and ∼18 motor neurons, respectively, and thus significantly more motor neurons than vehicle-treated SMA mice, which have only ∼8 motor neurons (Fig. 7). This data clearly demonstrate that SAHA treatment stabilizes the mean number of motor neurons at a level only slightly lower than in heterozygous mice at PND10, and is therefore delaying the neuronal impairment in SMA mice.

SAHA increases the size of motor neuron junctions in SMA mice

It has been shown that in SMA mice, in which SMN protein levels are severely reduced, the maturation of the NMJs is seriously impaired and their size is markedly reduced (44,45). To investigate the effect of SAHA on the integrity of the NMJs in the *gastrocnemius* muscle, the surface size area of 100 NMJs per animal ($n = 3$ per group) was measured (in μm^2) using Axio Vision computer software (Zeiss).

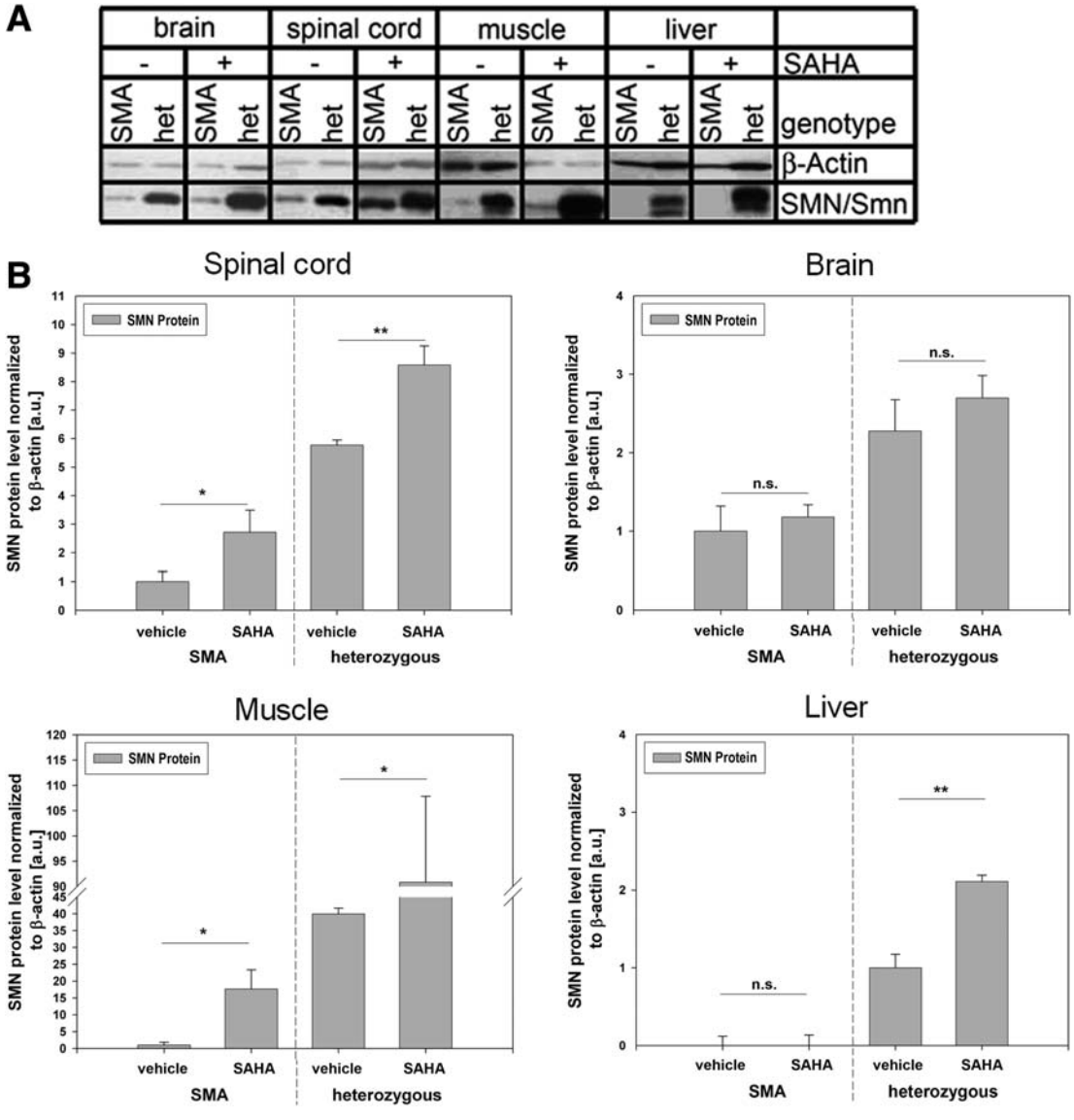

Figure 5. Impact of SAHA treatment on SMN expression at PND5. (**A**) Representative pictures of western blot analyses. Equal protein amounts were simultaneously stained with anti-SMN and anti-β-actin as loading control. SMA, SMA mouse; het, heterozygous mouse. (**B**) Diagrammatic representation of the semi-quantitative protein quantification in spinal cord, brain, muscle and liver relative to β-actin, $^{*}P < 0.05$; $^{**}P < 0.01$.

As expected, the mean NMJ size of both SAHA and vehicle-treated heterozygous mice was similar (229.8 ± 3.4 and 240.3 ± 4.3 μm^2, respectively) (Fig. 8). In contrast, vehicle-treated SMA mice showed a severely reduced area size of 100.1 ± 2.5 μm^2, indicating the progression of SMA. Notably, the SAHA-treated SMA mice displayed a significantly higher mean of the NMJ size of 161.6 ± 3.2 μm^2 when compared with vehicle-treated mice ($P < 0.001$) (Fig. 8A). Furthermore, we analysed the occupancy of the post-synaptic sites at the NMJs and identified a significant improvement of the occupancy level and the arborisation complexity of the NMJs in SAHA-treated SMA mice compared with vehicle-treated mice (Fig. 8C). These results suggest that SAHA treatment ameliorates the NMJ phenotype of SMA mice.

SAHA increases the size of muscle fibers in SMA mice

One major hallmark of SMA is the atrophy of the proximal muscles. It has been described that SMA mouse models show a reduction of the muscle fiber size in the hind limb muscles (46). Therefore, cross-sections of the *rectus femoris* muscle were stained with an H&E staining in the four groups of mice at PND10. Similar to the NMJ measurement, the surface area of 100 muscle fibers was measured using Axio Vision computer software (Zeiss).

Vehicle- or SAHA-treated heterozygous mice showed virtually no difference in muscle fiber size (280.3 ± 8.9 and 277.8 ± 12 μm^2) (Fig. 9). In contrast, the vehicle-treated SMA mice showed with 125.8 ± 4 μm^2 severe reduction in the surface area size (Fig. 9), which reflects the profound pro-

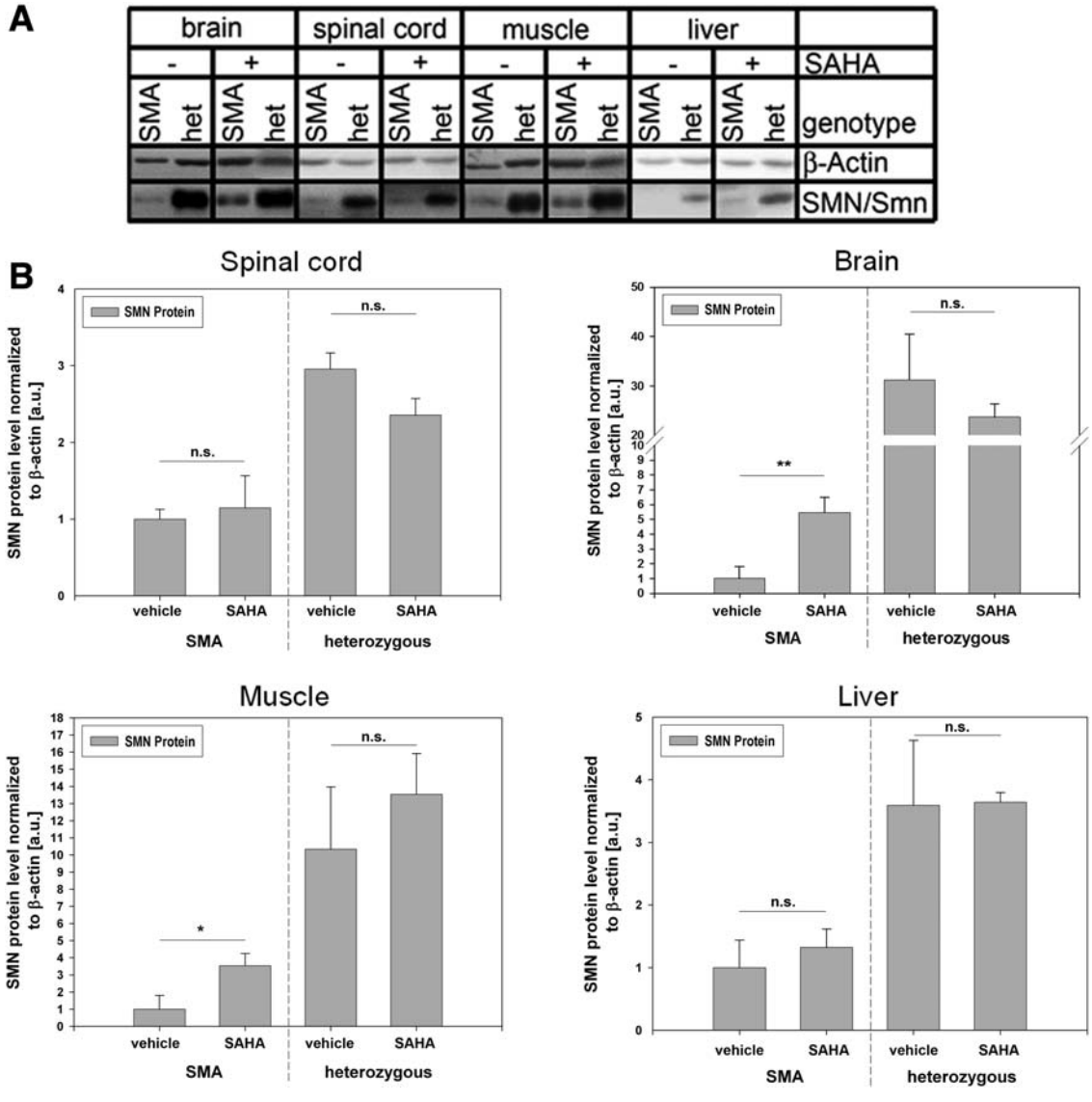

Figure 6. Impact of SAHA treatment on SMN expression at PND10. (**A**) Representative pictures of western blot analyses. Equal protein amounts were simultaneously stained with anti-SMN and anti-β-actin as loading control. SMA, SMA mouse; het, heterozygous mouse. (**B**) Diagrammatic representation of the semi-quantitative protein quantification in spinal cord, brain, muscle and liver relative to β-actin, $^{*}P < 0.05$; $^{**}P < 0.01$.

gression of SMA. Strikingly, the SAHA-treated SMA mice showed a significantly augmented mean muscle fiber size of $187.7 \pm 5.6\ \mu m^2$ when compared with vehicle-treated SMA mice ($P < 0.001$) (Fig. 9).

To summarize these results, SAHA treatment ameliorates the muscle fiber size phenotype in SMA mice, most likely as a secondary effect, by protecting the α-motor neurons from deterioration. However, the highly elevated SMN levels in muscle upon SAHA treatment may also contribute to the improvement of the muscle functionality.

DISCUSSION

We have previously been able to show that SAHA, a pan-HDACi (28), is able to elevate SMN levels *in vitro* in fibroblast cell lines derived from SMA patients and *ex vivo* using cultured hippocampal brain slices derived from rat and human (31). Since SAHA was approved in 2006 by the US Food and Drug Administration (FDA) for the treatment of cutaneous T-cell lymphoma (CTCL) and has been shown to cross the blood-brain barrier (30), its direct application to SMA patients would be feasible.

Identification of an appropriate SMA mouse model for drug screenings

Two transgenic SMA mouse models have been generated and published in 2000 (33,34). The two models (US and Taiwanese) differ in the size of the *SMN2* transgene: the US model contains the genomic region of *SMN2* only, the Taiwanese model, a BAC including also some of the adjacent region to *SMN2*. Furthermore, the US model contains one *SMN2* per allele whereas the Taiwanese model contains two *SMN2* copies per allele. Consequently, to produce SMA mice carry-

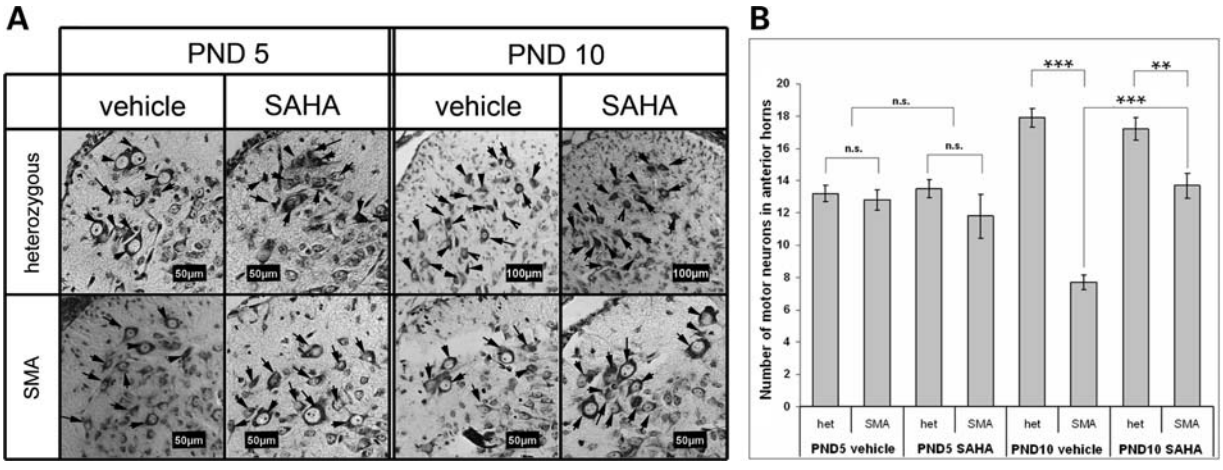

Figure 7. Influence of SAHA regimen on the number of motor neurons. (**A**) Depiction of the characteristic shape and size of α-motor neurons in the anterior horns of the spinal cord. Representative cross-sections of either vehicle-treated or SAHA-treated SMA and heterozygous mice at an age of 5 and 10 days. Black arrows indicate counted α-motor neurons. (**B**) Diagrammatic representation of the α-motor neuron counts of cross-sections derived from SAHA-treated and vehicle-treated SMA and heterozygous mice at PND5 and PND10. $n = 3$ per group. The mean values of motor neurons from 20 cross-sections per animal were determined, $^{**}P < 0.01$; $^{***}P < 0.001$.

ing two *SMN2* copies, a homozygous state is needed for the US model and a heterozygous state for the Taiwanese model.

Although the initial publication showed that the US-SMA mice have a mean lifespan of 5 days on C57BL/6J background (34), a pure congenic FVB/N background (this work) led to embryonic lethality. This finding suggests an obvious impact of the congenic background on the severity of the SMA phenotype. This may be due to modifier genes as shown to be the case also in humans (47) or due to epigenetic influences (17). A protective effect of the C57BL/6J background has also been published for the Taiwanese-SMA mouse model, which on C57BL/6J background lives 13 months (24), whereas on FVB/N background only 10 days (this work). The differences in the two SMA models carrying the same *SMN2* copy number and the same background could also be due to epistatic effects at the integration site or the size of the transgene.

An appropriate SMA mouse model for drug screening should have several features: (i) reduced life expectancy (at best between 10 and 30 days) so that survival can be taken as primary outcome measurement, (ii) they should live long enough to enable motor function tests, (iii) SMA mice should resemble clinical features of SMA, such as reduced SMN levels, motor neuron deterioration, NMJ and muscular phenotype and (iv) they should produce a high percentage of SMA mice in each litter.

Since the Taiwanese-SMA mice on FVB/N background fulfill all of the above-mentioned criteria (mean survival of 10 days, capable to perform tube test, clear SMA phenotype, 50% SMA mice in each litter), we propose these mice as an excellent SMA mouse model for the *in vivo* characterization of potential SMA drugs. In comparison, the SMA mouse model most widely used for pre-clinical tests (Table 1) is the delta7SMN2 SMA mouse ($Smn^{-/-}$;$SMN2^{tg/tg}$;$\Delta 7SMN2^{tg/tg}$) that lives on average only 1.5 days longer, but gives only 25% SMA mice, usually only 1–2 per litter (36). Furthermore, the healthy offspring has different genotypes, i.e. either *Smn* knockout carrier (50%) or *Smn* wild-type (25%), when compared with the Taiwanese model (using our breading scheme) where all healthy offspring are *Smn* carriers, having the same genotype ($Smn^{+/-}$;$SMN2^{tg/+}$). Thus, potential influences based on different Smn levels can be excluded in control littermates.

SAHA rescues embryonic lethality in the US-SMA model and prolongs survival by 30% in the Taiwanese-SMA mice

Oral SAHA treatment of pregnant mothers of severely affected US-SMA mice rescued embryonic lethality, which reflects its *in utero* capacity to ameliorate the severe SMA phenotype. However, with regard to a potential SMA therapy, treatment of pregnant mothers with HDAC inhibitors (as shown for valproic acid) has to be performed very carefully, due to their teratogeneity during the first trimester of pregnancy (48–50). Based on these preliminary encouraging results on an otherwise inappropriate model for pre-clinical trials, we were prompted to identify a second model, in which SMA mice were born and could be properly analysed avoiding maternal treatment.

SAHA (25 mg/kg twice daily) regimen in the Taiwanese-SMA model statistically significantly prolonged the mean survival time by more than 30%. The fact that ∼10% of the treated SMA mice lived longer than the longest surviving vehicle-treated SMA mouse indicates that some mice respond beneficially to SAHA, whereas others may be non-responders. Similar observations have been described for VPA treatment of SMA patients (20) and might explain the heterogeneity of the life-prolonging effect of SAHA. However, even in the SAHA responders, SAHA treatment was not capable to restore the lifespan to normal.

SAHA increases body weight in SMA mice

It is obvious that SAHA treatment was not able to restore normal development, since the weight progression in SMA mice lies inbetween heterozygous and vehicle-treated SMA

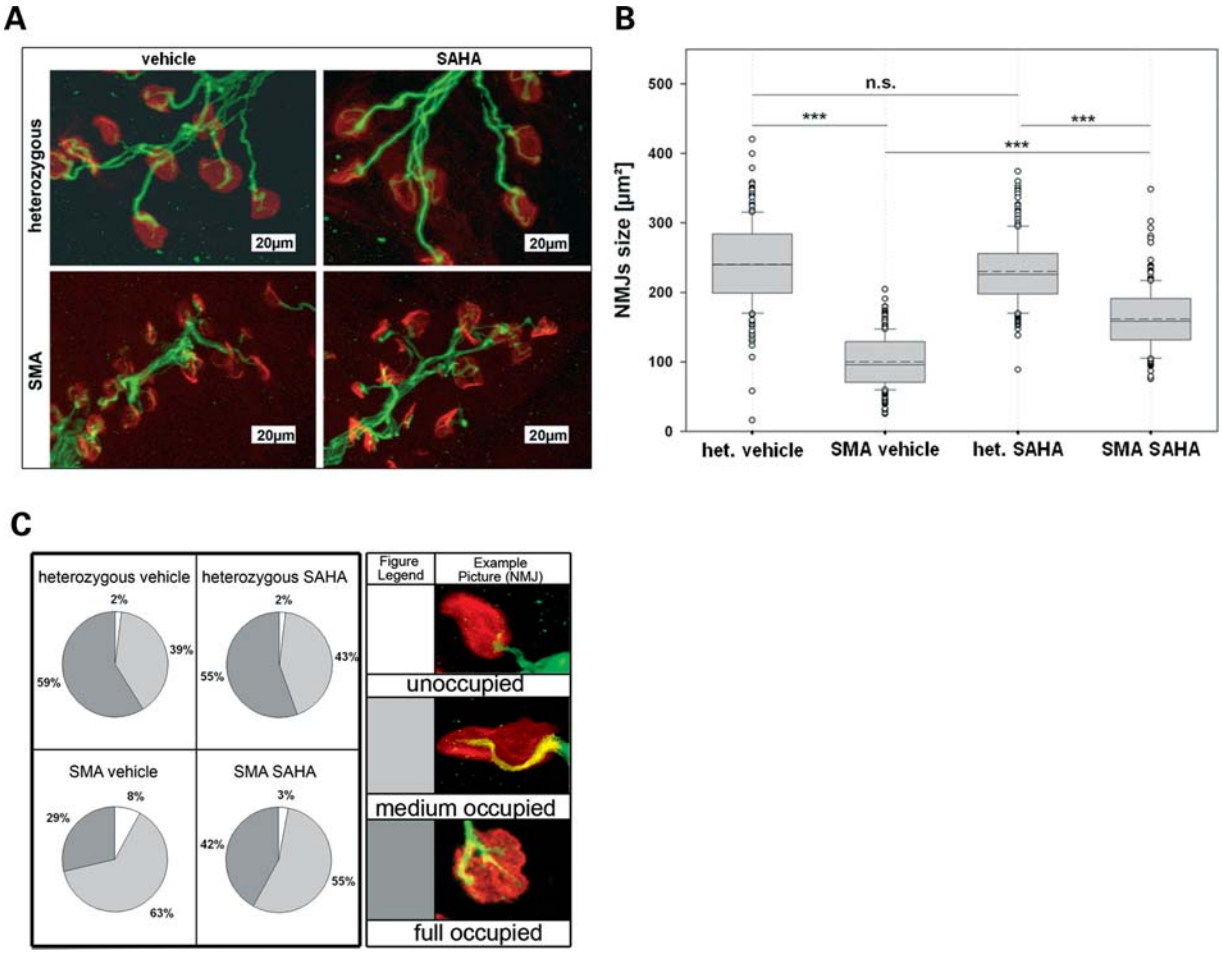

Figure 8. Influence of SAHA regimen on the size and occupancy of neuromuscular junctions. (**A**) Representative microscopic illustration of the immunofluorescence staining of the NMJs derived from longitudinal sections of the *gastrocnemius* muscle of either a vehicle- or SAHA-treated heterozygous mouse or a SMA mouse. Red staining: α-BTX-rhodamine, stains acetylcholine receptors; green staining: anti-neurofilament M (labeled with AlexaFlour 488), stains neurofilament M-proteins. (**B**) Boxplot of surface area sizes of NMJs measured in longitudinal sections of vehicle or SAHA-treated heterozygous or SMA mice at PND10. $n = 3$ per group. 100 NMJs per mouse were measured. Mean value, dashed black line; median value, black line, $^{***}P < 0.001$. (**C**) Quantification of occupancy levels of NMJs. 100 NMJs per mouse were classified as unoccupied (no nerve terminal at the NMJ), medium occupied (one or two nerve terminals in the NMJ) or fully occupied (three or more nerve terminals in the NMJ) depending on the complexity of the arborisation of the nerve terminals in the NMJs.

mice. SAHA-treated SMA mice showed a stagnation of weight improvement starting around PND10 and resulting in a dwarf-like appearance in the long-surviving SMA mice. Moreover, some of these showed a severe necrosis of the hind limbs, which was only observed for the SAHA-treated SMA mice surviving for 20 days or longer. Necrosis is not an effect of SAHA, but rather a characteristic of progressive SMA as previously reported (43), which was not seen in vehicle-treated SMA mice due to the severely reduced life expectancy.

SAHA improves motor abilities of SMA mice

Initially, the well-described righting reflex test was performed to determine motor abilities of mice (36). Since we could not observe any statistical difference between SMA and heterozygous mice in righting time (Supplementary Material, Fig. S4), the more sophisticated tube test was applied. This motor function test (40) revealed a significant improvement in motor abilities under the SAHA regimen. Motor function of SAHA-treated SMA mice developed similarly to the healthy littermates, but declined at later stages, indicating that motor function deficits of the SMA mice were ameliorated but not restored by the SAHA regimen.

SAHA elevates SMN expression in spinal cord and muscle

It was detected that SAHA stimulated the expression of both *SMN2* transcripts, FL-*SMN2* and Δ7-*SMN2* in virtually all tested organs, such as brain, liver, spinal cord and muscle (Supplementary data). This indicates that Sfrs10, the main splicing factor known to be able to correct the *SMN2* splicing (51), although up-regulated in some tissues (data not shown), does not significantly influence *SMN2* splicing in these mice. The SMN protein level was also significantly augmented in virtually all tissues of treated SMA mice, but never reached levels seen in heterozygous mice. Interestingly, some of the tissues showed a much higher elevation on protein than on RNA level. This has been observed also for other HDACi and may be due to a stabilization of the protein and reduction of the SMN ubiquitinylation and protein degradation (22,52)

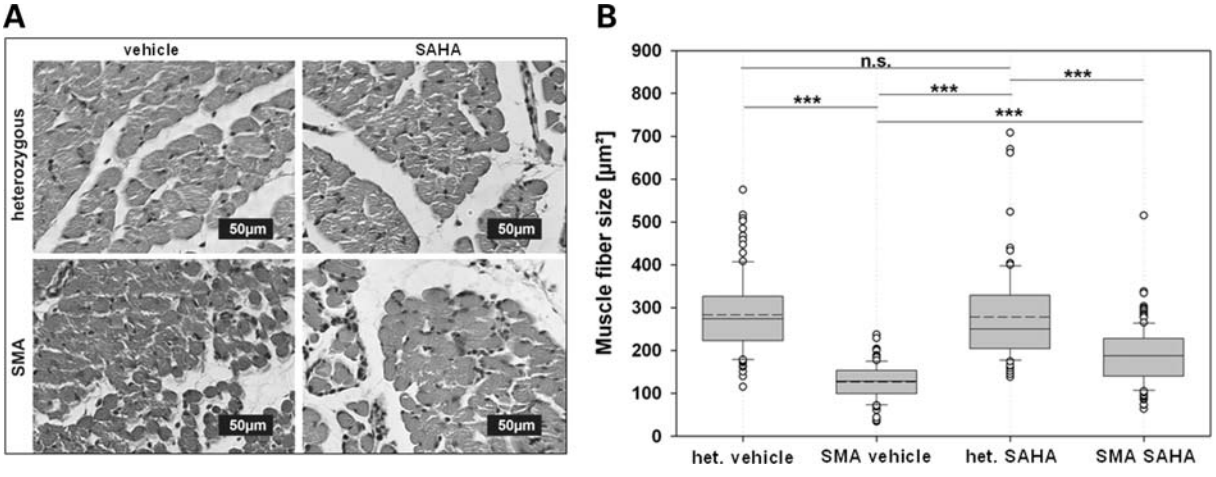

Figure 9. Effect of SAHA regimen on the surface area size of muscle fibers. (**A**) Representative microscopic illustration of the H&E staining of the muscle fibers derived from cross-sections of the *rectus femoris* muscle of a vehicle or a SAHA-treated heterozygous mouse or a SMA mouse. The surface area size of the muscle fibers of the vehicle-treated SMA mouse is obviously smaller than the surface area sizes of a SAHA-treated SMA-mouse. (**B**) Boxplot of surface area sizes of muscle fiber measured in cross-sections derived from vehicle or SAHA-treated heterozygous and SMA mice at PND10. $n = 3$ per group. One hundred muscle fibers per mouse were measured. Mean value, dashed black line; median value, black line, $^{***}P < 0.001$.

Of particular importance was the finding that SAHA increased the SMN level also in neuronal tissues, underlining its ability to cross the blood-brain barrier. This would be essential for a prospective SMA drug.

SAHA stabilizes the number of motor neurons and increases the size of neuromuscular junctions and muscle fibers in SMA mice

Histological examinations revealed that the SAHA regimen reduces the loss of spinal motor neurons, improves the size of NMJs and the arborisation complexity of the nerve terminals at the NMJs (occupancy level), and most likely as a secondary effect, is able to increase the surface area size of cross-sections of muscle fibers. All histological improvements of the phenotype resembled an amelioration of the SMA progression. The lack of potency of SAHA to fully rescue the SMA phenotype might be explainable by its relatively short half life of only ∼45 min in mice (53,54), which could not be overcome by higher dosages, since they were not tolerated by the newborn mice. Nevertheless, the obviously favorable function of SAHA might be based not only on the activation of *SMN2* but also on an additional neuroprotective property of SAHA, which has previously been described (30).

Results in research context

So far, several *in vivo* approaches have been performed in order to ameliorate the SMA phenotype in a mouse model for SMA (summarized in Table 1).

In comparison with previous findings, it is noteworthy that SAHA treatment resulted in one of the highest increases of survival ever observed under treatment with a chemical compound. SAHA treatment of pregnant mice (from E15 on) overcame embryonic lethality in a very severe SMA mouse model and prolonged survival by 30% in a neonatal severely affected SMA mouse. However, HDACi should not be used in humans during early pregnancy due to potential teratogenic effects. Although 30% increase in lifespan is still not enough to cure a patient with severe SMA, it may significantly ameliorate the progression in an intermediate or mildly affected SMA patient. Moreover, the SAHA-treated SMA mice exhibited increased muscle mass, decreased motor neuron loss, larger NMJ size and higher NMJ occupancy at post-synaptic sites as well as motor function improvement. SAHA regimen resulted in a profound increase of SMN levels in all tested tissues except the liver. The ∼17-fold increase of the SMN amount in the muscle was the highest ever observed, demonstrating that SAHA may ameliorate the SMA phenotype in two ways on the neuronal and the muscle level. Altogether these data provide a strong proof of principle for the specific effect of SAHA on *SMN2* and the SMA pathogenesis.

To summarize these results, SAHA treatment was not capable to protect against the onset of SMA, but turned out to efficiently ameliorate the SMA phenotype in severely affected SMA mouse models. Since SAHA is a FDA-approved drug, it may be considered a useful drug for clinical trials in SMA patients. These findings are of particular interest, since recent studies revealed only a modest beneficial improvement in SMA patients, which were treated with valproic acid (27). SAHA may be a good alternative second generation HDACi which significantly increase the mean lifespan and ameliorate the SMA phenotype in SMA mice so far.

MATERIALS AND METHODS

Mice

Two different SMA mouse models were used in this project. (i) The US-SMA model, FVB.Cg-Tg(SMN2)89Ahmb *Smn1*^{tm1Msd}/J, was kindly provided by the group of Michael Sendtner (University of Würzburg). It is also available at Jackson's Laboratory (Stock Number: 005024) and carries 1 *SMN2* copy per integrate/allele. (ii) The Taiwanese-SMA mouse model, FVB.Cg-Tg(SMN2)2Hung Smn1^{tm1Hung}/J, was purchased from Jacksoń s Laboratory (Stock Number: 005058).

Since the Taiwanese-SMA mice are produced in our laboratory by a novel breeding scheme, a more detailed description is given next. The Taiwanese mouse model (FVB.Cg-Tg(SMN2)2Hung Smn1^{tm1Hung}/J) carries homozygously the *SMN2* transgene on null murine *Smn* background (33) and were defined in our paper as *Smn*$^{-/-}$;*SMN2*$^{tg/tg}$. These mice fail to develop any SMA phenotype, are fertile and live for >1 year, but develop a short and thick tail and necrotic ears. In order to generate a potential SMA mouse, the *Smn*$^{-/-}$;*SMN2*$^{tg/tg}$ mice were crossbred to heterozygous *Smn* knockout mice (*Smn*$^{+/-}$). The later strain was obtained by crossing back the original strain purchased from Jackson's Laboratories (No. 005058) to wild-type FVB/N mice for two generations. By real-time quantitative PCR (see Supplementary material), we determined four *SMN2* copies in the homozygous mice (*Smn*$^{-/-}$;*SMN2*$^{tg/+}$) and two *SMN2* copies in the heterozygous mice (*Smn*$^{-/-}$;*SMN2*$^{tg/+}$) indicating that there must be two *SMN2* copies per integrate. The mice being heterozygous for the *SMN2* transgene on *Smn* null background (*Smn*$^{-/-}$;*SMN2*$^{tg/+}$) developed a severe SMA phenotype with a mean age of survival of ~10 days. They turned out to be an excellent SMA mouse model. In each litter, 50% of offspring was SMA mice (*Smn*$^{-/-}$;*SMN2*$^{tg/+}$) and 50% was control carriers (*Smn*$^{-/+}$;*SMN2*$^{tg/+}$). As a wild-type strain, the inbred strain FVB/NJ (Jacksoṅs Laboratory, Stock Number: 001800) was used.

The Taiwanese-SMA mice were housed in micro-isolation chambers in the mouse facility of the Institute of Genetics, Cologne. All mice were humanely euthanized according to protocols set forth by the 'Landesamt für Natur, Umwelt und Verbraucherschutz NRW'.

The animal breeding and all mouse experiments were approved by the local animal protection committee. The animal experiment application form was confirmed. All *in vivo* experiments were performed under the reference number 9.93.2.10.31.07.292.

The US-SMA mice were kept under the same conditions, but in the 'Franz-Penzoldt-Zentrum' in Erlangen. The reference number for the animal experiments performed in Erlangen was 621-2531.31-18/05.

Genotyping

For each animal used in this work, the genotype was checked by previously described genotyping PCR reactions on respective tail-tip DNA. Primer sequences and PCR conditions were used as previously described: US-SMA mice in reference (36) and Taiwanese-SMA mice in reference (33).

Determination of disease progression in SMA mice

For the evaluation of SMA progression, mice were weighted daily in the morning. To characterize motor abilities of the animals, the tube test was applied as described elsewhere (40). In brief, to perform a tube test, first the whole litter was taken together with some bedding into an adequate vessel to separate it from the mother. Next, each pup was placed headfirst into a vertical 50 ml reaction tube. The pups were placed into the tube in such a way that they could hold themselves by their muscular strength of their hind limbs. If a mouse was able to hold itself for a while (>20 s) and if the hind limbs were spread, it was scored to the highest tube score of 4. Depending on the spreading of the hind limbs, each mouse was rated from 4 (best score wide spreading) to 1 (bad score, hind limbs in a clasped position). A mouse was rated with the worst score of 0 when it was not able to hold onto the tube. The tube test was performed daily starting from PND0 in the morning between 9:00 and 10:30 a.m.

Preparation of proteins and RNA from mouse organs

For the preparation of mouse organs, the respective mouse was sacrificed by decapitation either at day PND5 or day PND10. Subsequently, the mouse was fixed onto an operating table and its body was opened by abdominal incision.

For the subsequent RNA or protein preparation of organs, whole liver, brain, the *gastrocnemius* muscle and spinal cord were removed. While liver, muscle and brain were simply withdrawn, spinal cord was rinsed out of the spinal canal with PBS. All organs were immediately snap-frozen and stored in liquid nitrogen.

For subsequent histological experiments, biopsies from the *gastrocnemius* muscle and the *rectus femoris* muscle were taken as well as the whole spine including the spinal cord. All organs for histological stains were immediately stored and fixed in 4% paraformaldehyde at 4°C.

Western blot analysis

After sacrificing the mice at the respective time-points, tissues were freshly harvested and analysis was performed as follows: for the quantification of protein amounts, tissue lysates were homogenized by the use of an Ultra Turrax homogenizer (*IKA*) in RIPA buffer (Sigma-Aldrich, Germany) and western blot analysis was performed as previously described (14). The following antibodies were used: mouse monoclonal anti-β-actin (1:10,000, Sigma-Aldrich, Germany), mouse monoclonal anti-SMN (1:2,000, BD Transduction laboratories, USA) and rabbit polyclonal SFRS10 [1:3000 (55)].

Determination of transcript levels by qRT–PCR

To isolate total RNA from mouse organ biopsies, the RNeasy Kit (Qiagen) and the QIAshredder (Qiagen) were used according to the manufacturer's protocols. For the isolation of total RNA out of fatty organs like brain or spinal cord, some preparative steps with Qiazol® according to the manufacturer's protocol were included before starting with the RNeasy protocol. DNase I digest was carried out using the RNase-Free DNase kit (Qiagen) according to the protocol included in the RNeasy kit.

Subsequent analysis of *FL-SMN2* and *SMN2Δ7* transcripts by quantitative real-time RT–PCR on a LightCycler 1.5 (Roche, Basel, Switzerland) were performed as described in detail earlier (23).

Histology

Before whole mouse organs or biopsies were embedded in paraffin, they were fixed in 4% paraformaldehyde (PFA) over

night and afterwards dehydrated with the help of an infiltration machine (Leica). Embedded samples were cut with the help of microtome (Leica) into 8 μm thick sections. For the quantification of the α-motor neurons, these neurons were quantified in SMA mice and heterozygous littermates at PND5 and PND10 ($n = 3$ per group). To do so, motor neurons of 20 cross-sections (using every third or fourth section) of the lumbar region were generated and *Nissl* stained. The specific localization of the motor neurons in the anterior horns of the spinal cord and the specific size and shape allowed a clear discrimination between α-motor neurons and the surrounding remaining neurons. For each mouse, the mean number of motor neurons per anterior horn evaluated (Microsoft Excel).

Although one should not expect an increase in motoneurons from P5 to P10, we observed a slight increase in number which may be attributed to the size of the spinal cord and the better recognizable motor neurons in P10 compared with P5. In P10, they showed already a more relaxed distribution are larger and easier to be counted. Three independent persons in the lab counted each section and similar results were obtained.

To measure the surface size of the NMJs, the area of the NMJs was measured (in μm^2). Therefore, 200 μm thick longitudinal sections of the *gastrocnemius* muscle were stained with anti-Neurofilament M (labeled with AlexaFlour 488, Millipore), which labels the neurons, and α-Bungarotoxin (labeled with rhodamine, Invitrogen), which binds to the muscular acetycholine-receptors (AChR). The surface area of 100 NMJ per animal was measured with the help of *Axio Vision* computer software (Zeiss).

To quantify the size of the muscle fibers, cross-sections of the *rectus femoris* muscle were stained with an H&E staining. The surface area size of 100 muscle fibers was measured per animal with the help of *Axio Vision* computer software (Zeiss).

Quantification of the occupancy of post-synaptic AChR clusters was carried out as previously described (44,46). Depending on arborisation level of the nerve terminals, the occupancy at the AChR clusters was classified as unoccupied (no innervation), medium occupancy (one to two nerve terminals in the NMJ) and fully occupied (three or more nerve terminals). For each mouse, the classified NMJs were evaluated (Microsoft Excel).

Statistical analysis

Whenever the significance of the RNA expression levels after SAHA treatment as well as the changed protein levels were tested, it was done by the use of a directional student's *t*-test for uncorrelated samples. In all cases, three levels of statistical significance were distinguished: $P < 0.05$, $P < 0.01$ and $P < 0.001$.

To evaluate the significant variance in the number of born US-SMA mice compared with the expected number of SMA mice, a chi-square (χ^2) test was performed.

A Wilcoxon's rank-sum test was performed in order to determine the significance in the increase of survival (the shift of the Kaplan–Meier curves) of the SMA mice after SAHA treatment. Each value is given as a mean of three experiments ± SEM if not indicated otherwise.

The significance in the differences of NMJ and muscle fiber surface area size as well as motor neuron numbers was determined by the use of a directional student's *t*-test for uncorrelated samples. NMJ and muscle fiber surface area size are presented in boxplot diagram, reflecting mean and median values.

All statistical analyses were performed by use of the software programs Excel 2003 (Microsoft) and Sigma Plot 9.0 (Systat Software).

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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Conflict of Interest statement. None declared.

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REFERENCES

1. Montes, J., Gordon, A.M., Pandya, S., De Vivo, D.C. and Kaufmann, P. (2009) Clinical outcome measures in spinal muscular atrophy. *J. Child. Neurol.*, **24**, 968–978.
2. Feldkotter, M., Schwarzer, V., Wirth, R., Wienker, T.F. and Wirth, B. (2002) Quantitative analyses of SMN1 and SMN2 based on real-time lightCycler PCR: fast and highly reliable carrier testing and prediction of severity of spinal muscular atrophy. *Am. J. Hum. Genet.*, **70**, 358–368.
3. Pearn, J. (1978) Incidence, prevalence, and gene frequency studies of chronic childhood spinal muscular atrophy. *J. Med. Genet.*, **15**, 409–413.
4. Czeizel, A. and Hamula, J. (1989) A Hungarian study on Werdnig–Hoffmann disease. *J. Med. Genet.*, **26**, 761–763.
5. Lefebvre, S., Burglen, L., Reboullet, S., Clermont, O., Burlet, P., Viollet, L., Benichou, B., Cruaud, C., Millasseau, P., Zeviani, M. *et al.* (1995) Identification and characterization of a spinal muscular atrophy-determining gene. *Cell*, **80**, 155–165.
6. Wirth, B. (2000) An update of the mutation spectrum of the survival motor neuron gene (SMN1) in autosomal recessive spinal muscular atrophy (SMA). *Hum. Mutat.*, **15**, 228–237.
7. Alias, L., Bernal, S., Fuentes-Prior, P., Barcelo, M.J., Also, E., Martinez-Hernandez, R., Rodriguez-Alvarez, F.J., Martin, Y., Aller, E., Grau, E. *et al.* (2009) Mutation update of spinal muscular atrophy in Spain: molecular characterization of 745 unrelated patients and identification of four novel mutations in the SMN1 gene. *Hum. Genet.*, **125**, 29–39.
8. McAndrew, P.E., Parsons, D.W., Simard, L.R., Rochette, C., Ray, P.N., Mendell, J.R., Prior, T.W. and Burghes, A.H. (1997) Identification of proximal spinal muscular atrophy carriers and patients by analysis of SMNT and SMNC gene copy number. *Am. J. Hum. Genet.*, **60**, 1411–1422.
9. Wirth, B., Brichta, L., Schrank, B., Lochmuller, H., Blick, S., Baasner, A. and Heller, R. (2006) Mildly affected patients with spinal muscular atrophy are partially protected by an increased SMN2 copy number. *Hum. Genet.*, **119**, 422–428.

10. Helmken, C., Hofmann, Y., Schoenen, F., Oprea, G., Raschke, H., Rudnik-Schoneborn, S., Zerres, K. and Wirth, B. (2003) Evidence for a modifying pathway in SMA discordant families: reduced SMN level decreases the amount of its interacting partners and Htra2-beta1. *Hum. Genet.*, **114**, 11–21.
11. Lorson, C.L. and Androphy, E.J. (2000) An exonic enhancer is required for inclusion of an essential exon in the SMA-determining gene SMN. *Hum. Mol. Genet.*, **9**, 259–265.
12. Lorson, C.L., Hahnen, E., Androphy, E.J. and Wirth, B. (1999) A single nucleotide in the SMN gene regulates splicing and is responsible for spinal muscular atrophy. *Proc. Natl Acad. Sci. USA*, **96**, 6307–6311.
13. Wirth, B., Riessland, M. and Hahnen, E. (2007) Drug discovery for spinal muscular atrophy. *Expert Opin. Drug. Discov.*, **2**, 437–451.
14. Brichta, L., Hofmann, Y., Hahnen, E., Siebzehnrubl, F.A., Raschke, H., Blumcke, I., Eyupoglu, I.Y. and Wirth, B. (2003) Valproic acid increases the SMN2 protein level: a well-known drug as a potential therapy for spinal muscular atrophy. *Hum. Mol. Genet.*, **12**, 2481–2489.
15. Sumner, C.J., Huynh, T.N., Markowitz, J.A., Perhac, J.S., Hill, B., Coovert, D.D., Schussler, K., Chen, X., Jarecki, J., Burghes, A.H. *et al.* (2003) Valproic acid increases SMN levels in spinal muscular atrophy patient cells. *Ann. Neurol.*, **54**, 647–654.
16. Kernochan, L.E., Russo, M.L., Woodling, N.S., Huynh, T.N., Avila, A.M., Fischbeck, K.H. and Sumner, C.J. (2005) The role of histone acetylation in SMN gene expression. *Hum. Mol. Genet.*, **14**, 1171–1182.
17. Hauke, J., Riessland, M., Lunke, S., Eyupoglu, I.Y., Blumcke, I., El-Osta, A., Wirth, B. and Hahnen, E. (2009) Survival motor neuron gene 2 silencing by DNA methylation correlates with spinal muscular atrophy disease severity and can be bypassed by histone deacetylase inhibition. *Hum. Mol. Genet.*, **18**, 304–317.
18. Andreassi, C., Angelozzi, C., Tiziano, F.D., Vitali, T., De Vincenzi, E., Boninsegna, A., Villanova, M., Bertini, E., Pini, A., Neri, G. *et al.* (2004) Phenylbutyrate increases SMN expression in vitro: relevance for treatment of spinal muscular atrophy. *Eur. J. Hum. Genet.*, **12**, 59–65.
19. Avila, A.M., Burnett, B.G., Taye, A.A., Gabanella, F., Knight, M.A., Hartenstein, P., Cizman, Z., Di Prospero, N.A., Pellizzoni, L., Fischbeck, K.H. *et al.* (2007) Trichostatin A increases SMN expression and survival in a mouse model of spinal muscular atrophy. *J. Clin. Invest.*, **117**, 659–671.
20. Brichta, L., Holker, I., Haug, K., Klockgether, T. and Wirth, B. (2006) In vivo activation of SMN in spinal muscular atrophy carriers and patients treated with valproate. *Ann. Neurol.*, **59**, 970–975.
21. Chang, J.G., Hsieh-Li, H.M., Jong, Y.J., Wang, N.M., Tsai, C.H. and Li, H. (2001) Treatment of spinal muscular atrophy by sodium butyrate. *Proc. Natl Acad. Sci. USA*, **98**, 9808–9813.
22. Garbes, L., Riessland, M., Holker, I., Heller, R., Hauke, J., Trankle, C., Coras, R., Blumcke, I., Hahnen, E. and Wirth, B. (2009) LBH589 induces up to 10-fold SMN protein levels by several independent mechanisms and is effective even in cells from SMA patients non-responsive to valproate. *Hum. Mol. Genet.*, **18**, 3645–3658.
23. Riessland, M., Brichta, L., Hahnen, E. and Wirth, B. (2006) The benzamide M344, a novel histone deacetylase inhibitor, significantly increases SMN2 RNA/protein levels in spinal muscular atrophy cells. *Hum. Genet.*, **120**, 101–110.
24. Tsai, L.K., Tsai, M.S., Ting, C.H. and Li, H. (2008) Multiple therapeutic effects of valproic acid in spinal muscular atrophy model mice. *J. Mol. Med.*, **86**, 1243–1254.
25. Brahe, C., Vitali, T., Tiziano, F.D., Angelozzi, C., Pinto, A.M., Borgo, F., Moscato, U., Bertini, E., Mercuri, E. and Neri, G. (2005) Phenylbutyrate increases SMN gene expression in spinal muscular atrophy patients. *Eur. J. Hum. Genet.*, **13**, 256–259.
26. Mercuri, E., Bertini, E., Messina, S., Solari, A., D'Amico, A., Angelozzi, C., Battini, R., Berardinelli, A., Boffi, P., Bruno, C. *et al.* (2007) Randomized, double-blind, placebo-controlled trial of phenylbutyrate in spinal muscular atrophy. *Neurology*, **68**, 51–55.
27. Swoboda, K.J., Scott, C.B., Reyna, S.P., Prior, T.W., LaSalle, B., Sorensen, S.L., Wood, J., Ascsadi, A., Crawford, T.O., Kissel, J.T. *et al.* (2009) Phase II open label study of valproic acid in spinal muscular atrophy. *PLoS ONE*, **4**, e5268.
28. Hahnen, E., Hauke, J., Trankle, C., Eyupoglu, I.Y., Wirth, B. and Blumcke, I. (2008) Histone deacetylase inhibitors: possible implications for neurodegenerative disorders. *Expert Opin. Investig. Drugs*, **17**, 169–184.
29. Eyupoglu, I.Y., Hahnen, E., Buslei, R., Siebzehnrubl, F.A., Savaskan, N.E., Luders, M., Trankle, C., Wick, W., Weller, M., Fahlbusch, R. *et al.* (2005) Suberoylanilide hydroxamic acid (SAHA) has potent anti-glioma properties in vitro, ex vivo and in vivo. *J. Neurochem.*, **93**, 992–999.
30. Hockly, E., Richon, V.M., Woodman, B., Smith, D.L., Zhou, X., Rosa, E., Sathasivam, K., Ghazi-Noori, S., Mahal, A., Lowden, P.A. *et al.* (2003) Suberoylanilide hydroxamic acid, a histone deacetylase inhibitor, ameliorates motor deficits in a mouse model of Huntington's disease. *Proc. Natl Acad. Sci. USA*, **100**, 2041–2046.
31. Hahnen, E., Eyupoglu, I.Y., Brichta, L., Haastert, K., Trankle, C., Siebzehnrubl, F.A., Riessland, M., Holker, I., Claus, P., Romstock, J. *et al.* (2006) In vitro and ex vivo evaluation of second-generation histone deacetylase inhibitors for the treatment of spinal muscular atrophy. *J. Neurochem.*, **98**, 193–202.
32. Frugier, T., Tiziano, F.D., Cifuentes-Diaz, C., Miniou, P., Roblot, N., Dierich, A., Le Meur, M. and Melki, J. (2000) Nuclear targeting defect of SMN lacking the C-terminus in a mouse model of spinal muscular atrophy. *Hum. Mol. Genet.*, **9**, 849–858.
33. Hsieh-Li, H.M., Chang, J.G., Jong, Y.J., Wu, M.H., Wang, N.M., Tsai, C.H. and Li, H. (2000) A mouse model for spinal muscular atrophy. *Nat. Genet.*, **24**, 66–70.
34. Monani, U.R., Sendtner, M., Coovert, D.D., Parsons, D.W., Andreassi, C., Le, T.T., Jablonka, S., Schrank, B., Rossol, W., Prior, T.W. *et al.* (2000) The human centromeric survival motor neuron gene (SMN2) rescues embryonic lethality in Smn(−/−) mice and results in a mouse with spinal muscular atrophy. *Hum. Mol. Genet.*, **9**, 333–339.
35. Silver, L.M. (1995) *Mouse Genetics*, Oxford University Press.
36. Le, T.T., Pham, L.T., Butchbach, M.E., Zhang, H.L., Monani, U.R., Coovert, D.D., Gavrilina, T.O., Xing, L., Bassell, G.J. and Burghes, A.H. (2005) SMNDelta7, the major product of the centromeric survival motor neuron (SMN2) gene, extends survival in mice with spinal muscular atrophy and associates with full-length SMN. *Hum. Mol. Genet.*, **14**, 845–857.
37. Baughan, T.D., Dickson, A., Osman, E.Y. and Lorson, C.L. (2009) Delivery of bifunctional RNAs that target an intronic repressor and increase SMN levels in an animal model of spinal muscular atrophy. *Hum. Mol. Genet.*, **18**, 1600–1611.
38. Mattis, V.B., Ebert, A.D., Fosso, M.Y., Chang, C.W. and Lorson, C.L. (2009) Delivery of a read-through inducing compound, TC007, lessens the severity of a spinal muscular atrophy animal model. *Hum. Mol. Genet.*, **18**, 3906–3913.
39. Heier, C.R. and DiDonato, C.J. (2009) Translational read through by the aminoglycoside geneticin (G418) modulates SMN stability in vitro and improves motor function in SMA mice in vivo. *Hum. Mol. Genet.*, **18**, 1310–1322.
40. El-Khodor, B.F., Edgar, N., Chen, A., Winberg, M.L., Joyce, C., Brunner, D., Suarez-Farinas, M. and Heyes, M.P. (2008) Identification of a battery of tests for drug candidate evaluation in the SMNDelta7 neonate model of spinal muscular atrophy. *Exp. Neurol.*, **212**, 29–43.
41. Butchbach, M.E., Edwards, J.D. and Burghes, A.H. (2007) Abnormal motor phenotype in the SMNDelta7 mouse model of spinal muscular atrophy. *Neurobiol. Dis.*, **27**, 207–219.
42. Thurmond, J., Butchbach, M.E., Palomo, M., Pease, B., Rao, M., Bedell, L., Keyvan, M., Pai, G., Mishra, R., Haraldsson, M. *et al.* (2008) Synthesis and biological evaluation of novel 2,4-diaminoquinazoline derivatives as SMN2 promoter activators for the potential treatment of spinal muscular atrophy. *J. Med. Chem.*, **51**, 449–469.
43. Araujo Ade, Q., Araujo, M. and Swoboda, K.J. (2009) Vascular perfusion abnormalities in infants with spinal muscular atrophy. *J. Pediatr.*, **155**, 292–294.
44. Kariya, S., Park, G.H., Maeno-Hikichi, Y., Leykekhman, O., Lutz, C., Arkovitz, M.S., Landmesser, L.T. and Monani, U.R. (2008) Reduced SMN protein impairs maturation of the neuromuscular junctions in mouse models of spinal muscular atrophy. *Hum. Mol. Genet.*, **17**, 2552–2569.
45. Murray, L.M., Comley, L.H., Thomson, D., Parkinson, N., Talbot, K. and Gillingwater, T.H. (2008) Selective vulnerability of motor neurons and dissociation of pre- and post-synaptic pathology at the neuromuscular junction in mouse models of spinal muscular atrophy. *Hum. Mol. Genet.*, **17**, 949–962.
46. Gavrilina, T.O., McGovern, V.L., Workman, E., Crawford, T.O., Gogliotti, R.G., DiDonato, C.J., Monani, U.R., Morris, G.E. and Burghes, A.H. (2008) Neuronal SMN expression corrects spinal muscular atrophy

in severe SMA mice while muscle-specific SMN expression has no phenotypic effect. *Hum. Mol. Genet.*, **17**, 1063–1075.
47. Oprea, G.E., Krober, S., McWhorter, M.L., Rossoll, W., Muller, S., Krawczak, M., Bassell, G.J., Beattie, C.E. and Wirth, B. (2008) Plastin 3 is a protective modifier of autosomal recessive spinal muscular atrophy. *Science*, **320**, 524–527.
48. Kultima, K., Nystrom, A.M., Scholz, B., Gustafson, A.L., Dencker, L. and Stigson, M. (2004) Valproic acid teratogenicity: a toxicogenomics approach. *Environ. Health Perspect.*, **112**, 1225–1235.
49. Alsdorf, R. and Wyszynski, D.F. (2005) Teratogenicity of sodium valproate. *Expert Opin. Drug Saf.*, **4**, 345–353.
50. Wyszynski, D.F., Nambisan, M., Surve, T., Alsdorf, R.M., Smith, C.R. and Holmes, L.B. (2005) Increased rate of major malformations in offspring exposed to valproate during pregnancy. *Neurology*, **64**, 961–965.
51. Hofmann, Y., Lorson, C.L., Stamm, S., Androphy, E.J. and Wirth, B. (2000) Htra2-beta 1 stimulates an exonic splicing enhancer and can restore full-length SMN expression to survival motor neuron 2 (SMN2). *Proc. Natl Acad. Sci. USA*, **97**, 9618–9623.
52. Burnett, B.G., Munoz, E., Tandon, A., Kwon, D.Y., Sumner, C.J. and Fischbeck, K.H. (2009) Regulation of SMN protein stability. *Mol. Cell Biol.*, **29**, 1107–1115.
53. Duvic, M., Talpur, R., Ni, X., Zhang, C., Hazarika, P., Kelly, C., Chiao, J.H., Reilly, J.F., Ricker, J.L., Richon, V.M. *et al.* (2007) Phase 2 trial of oral vorinostat (suberoylanilide hydroxamic acid, SAHA) for refractory cutaneous T-cell lymphoma (CTCL). *Blood*, **109**, 31–39.
54. Venkatesh, P.R., Goh, E., Zeng, P., New, L.S., Xin, L., Pasha, M.K., Sangthongpitag, K., Yeo, P. and Kantharaj, E. (2007) In vitro phase I cytochrome P450 metabolism, permeability and pharmacokinetics of SB639, a novel histone deacetylase inhibitor in preclinical species. *Biol. Pharm. Bull.*, **30**, 1021–1024.
55. Hofmann, Y. and Wirth, B. (2002) hnRNP-G promotes exon 7 inclusion of survival motor neuron (SMN) via direct interaction with Htra2-beta1. *Hum. Mol. Genet.*, **11**, 2037–2049.
56. Narver, H.L., Kong, L., Burnett, B.G., Choe, D.W., Bosch-Marce, M., Taye, A.A., Eckhaus, M.A. and Sumner, C.J. (2008) Sustained improvement of spinal muscular atrophy mice treated with trichostatin A plus nutrition. *Ann. Neurol.*, **64**, 465–470.
57. Lunn, M.R., Root, D.E., Martino, A.M., Flaherty, S.P., Kelley, B.P., Coovert, D.D., Burghes, A.H., Man, N.T., Morris, G.E., Zhou, J. *et al.* (2004) Indoprofen upregulates the survival motor neuron protein through a cyclooxygenase-independent mechanism. *Chem. Biol.*, **11**, 1489–1493.
58. Butchbach, M.E., Singh, J., Thornorsteinsdottir, M., Saieva, L., Slominski, E., Thurmond, J., Andresson, T., Zhang, J., Edwards, J.D., Simard, L.R. *et al.* (2009) Effects of 2,4-diaminoquinazoline derivatives on SMN expression and phenotype in a mouse model for spinal muscular atrophy. *Hum. Mol. Genet.*, [Epub ahead of print November 25].
59. Williams, J.H., Schray, R.C., Patterson, C.A., Ayitey, S.O., Tallent, M.K. and Lutz, G.J. (2009) Oligonucleotide-mediated survival of motor neuron protein expression in CNS improves phenotype in a mouse model of spinal muscular atrophy. *J. Neurosci.*, **29**, 7633–7638.
60. Rose, F.F. Jr, Mattis, V.B., Rindt, H. and Lorson, C.L. (2009) Delivery of recombinant follistatin lessens disease severity in a mouse model of spinal muscular atrophy. *Hum. Mol. Genet.*, **18**, 997–1005.
61. Azzouz, M., Le, T., Ralph, G.S., Walmsley, L., Monani, U.R., Lee, D.C., Wilkes, F., Mitrophanous, K.A., Kingsman, S.M., Burghes, A.H. *et al.* (2004) Lentivector-mediated SMN replacement in a mouse model of spinal muscular atrophy. *J. Clin. Invest.*, **114**, 1726–1731.
62. Corti, S., Nizzardo, M., Nardini, M., Donadoni, C., Salani, S., Ronchi, D., Saladino, F., Bordoni, A., Fortunato, F., Del Bo, R. *et al.* (2008) Neural stem cell transplantation can ameliorate the phenotype of a mouse model of spinal muscular atrophy. *J. Clin. Invest.*, **118**, 3316–3330.
63. Salah-Mohellibi, N., Millet, G., Andre-Schmutz, I., Desforges, B., Olaso, R., Roblot, N., Courageot, S., Bensimon, G., Cavazzana-Calvo, M. and Melki, J. (2006) Bone marrow transplantation attenuates the myopathic phenotype of a muscular mouse model of spinal muscular atrophy. *Stem Cells*, **24**, 2723–2732.
64. Grondard, C., Biondi, O., Armand, A.S., Lecolle, S., Della Gaspera, B., Pariset, C., Li, H., Gallien, C.L., Vidal, P.P., Chanoine, C. *et al.* (2005) Regular exercise prolongs survival in a type 2 spinal muscular atrophy model mouse. *J. Neurosci.*, **25**, 7615–7622.