Pharmacologically induced mouse model of adult spinal muscular atrophy to evaluate effectiveness of therapeutics after disease onset

Zhihua Feng1,†, Karen K.Y. Ling1,†, Xin Zhao2,†, Chunyi Zhou1, Gary Karp2, Ellen M. Welch2, Nikolai Naryshkin2, Hasane Ratni3, Karen S. Chen4, Friedrich Metzger3, Sergey Paushkin4, Marla Weetall2,‡ and Chien-Ping Ko1,*,‡

1Section of Neurobiology, Department of Biological Sciences, University of Southern California, Los Angeles, CA 90089-2520, USA, 2PTC Therapeutics, Inc., South Plainfield, NJ 07080, USA, 3F. Hoffmann-La Roche Ltd, Pharmaceutical Research and Early Development, Roche Innovation Center Basel, Grenzacherstrasse 124, 4070 Basel, Switzerland and 4SMA Foundation, 888 Seventh Avenue, Suite 400, New York, NY 10019, USA

*To whom correspondence should be addressed. Tel: +1 9089129111; Fax: +1 9082227231; Email: mweetall@ptcbio.com (M.W.); Tel: +1 2137409182; Fax: +1 2137405687; Email: cko@usc.edu (C.-P.K.)

Abstract

Spinal muscular atrophy (SMA) is a genetic disease characterized by atrophy of muscle and loss of spinal motor neurons. SMA is caused by deletion or mutation of the survival motor neuron 1 (SMN1) gene, and the nearly identical SMN2 gene fails to generate adequate levels of functional SMN protein due to a splicing defect. Currently, several therapeutics targeted to increase SMN protein are in clinical trials. An outstanding issue in the field is whether initiating treatment in symptomatic older patients would confer a therapeutic benefit, an important consideration as the majority of patients with milder forms of SMA are diagnosed at an older age. An SMA mouse model that recapitulates the disease phenotype observed in adolescent and adult SMA patients is needed to address this important question. We demonstrate here that Δ7 mice, a model of severe SMA, treated with a suboptimal dose of an SMN2 splicing modifier show increased SMN protein, survive into adulthood and display SMA disease-relevant pathologies. Increasing the dose of the splicing modifier after the disease symptoms are apparent further mitigates SMA histopathological features in suboptimally dosed adult Δ7 mice. In addition, inhibiting myostatin using intramuscular injection of AAV1-follistatin ameliorates muscle atrophy in suboptimally dosed Δ7 mice. Taken together, we have developed a new murine model of symptomatic SMA in adolescents and adult mice that is induced pharmacologically from a more severe model and demonstrated efficacy of both SMN2 splicing modifiers and a myostatin inhibitor in mice at later disease stages.

Introduction

Spinal muscular atrophy (SMA), the leading genetic cause of infant mortality, is a human autosomal recessive neuromuscular disease characterized by the loss of spinal motor neurons and the atrophy of proximal muscles (1,2). There are four clinical types of SMA based on the age of disease onset and achievement of motor milestones. SMA results from the loss of or mutations in the Survival Motor Neuron 1 (SMN1) gene (3,4). The nearly identical SMN2 gene, which differs from SMN1 in a single translationally synonymous C-to-T transition at position 6 of exon 7, results in
exclusion of exon 7 due to alternative splicing (5,6). As a result, the SMN2 gene fails to generate adequate levels of SMN protein. The copy number of the SMN2 gene modifies disease severity (7,8). Currently, there are no approved treatments for SMA, except for supportive care. Given that SMA is caused by insufficient levels of SMN protein, several therapeutic strategies have been developed aiming to restore the level of SMN protein (9–11). A number of therapeutics approaches to increase SMN protein are being tested in clinical trials (12).

One critical question to the success of a therapy is to determine the efficacy of therapeutics when treatment is initiated at varying times in the disease progression. Existing evidence from a severe mouse model of SMA (Δ7 mice) indicates that the therapeutic window of opportunity for severe SMA is limited to varying times in the disease progression. Existing evidence mine the efficacy of potential therapies that increase SMN will be effective if initiated at symptomatic stages in adolescence/adulthood.

In addition to SMN-restoring therapies, efforts have also been devoted to explore strategies that improve motor circuit function independent of SMN in SMA. For example, to help individuals whose disease has progressed and muscle atrophy has developed, therapeutic interventions targeted to muscle growth could be especially beneficial. One such approach is to inhibit myostatin, a negative regulator of muscle growth. Myostatin inhibition induces a drastic increase in muscle mass due to hypertrophy and/or hyperplasia (17,18). In the context of SMA, however, myostatin inhibition results in little-to-no increase in the survival or increase in muscle mass in Δ7 mice (19–22). Given that Δ7 mice have a median lifespan of only ~14 days, the limited effect observed could be attributed to the possibility that early postnatal muscle development is not effectively regulated by myostatin (20). Whether enhancing muscle mass by inhibiting myostatin could be effective in milder forms of SMA in adulthood still remains to be answered.

Recently, we have described small molecules that penetrate the blood–brain barrier and selectively correct SMN2 alternative splicing that results in elevated levels of full-length SMN protein both in SMA patient fibroblasts and in SMA mouse models (23). These compounds correct pathology of Δ7 mice in a dose-dependent manner, with a suboptimal dose achieving partial rescue and prevention of disease progression. This observation prompted us to treat Δ7 mice with a suboptimal dose of SMN-C3, an SMN2 splicing modifier, to allow survival into adulthood. These mice presented with an intermediate SMA phenotype that provides an important tool to evaluate postsymptomatically the efficacy of potential therapies for treating SMA. We show that increasing the dose of SMN-C3 after symptoms have presented provides measurable benefit to young adult SMA mice. Additionally, we show that myostatin inhibition by treating mice with follistatin, an endogenous myostatin inhibitor (24,25), also mitigates muscle atrophy in SMA mice suboptimally dosed with SMN-C1, a second SMN2 splicing modifier. Our findings illustrate that these pharmacologically induced young adult SMA mice provide a useful, convenient and relevant testing platform to evaluate the efficacy of SMN-enhancing treatments, as well as SMN-independent regenerative therapies, for treating symptomatic SMA patients. Importantly, we have provided evidence that both of these approaches could be treatment options for older SMA patients.

**Results**

**Symptomatic adult Δ7 mice are generated using a suboptimal dose of the splicing modulator SMN-C3**

We have recently shown that an SMN2 splicing modifier, SMN-C3, increases SMN protein expression, as well as mitigates spinal motor neuron loss, neuromuscular junction (NMJ) denervation and muscle atrophy, in a dose-dependent manner in the Δ7 model of severe SMA at disease end-stage (PND14) (23). To characterize further the dose-dependent effects of SMN-C3 on disease pathologies of Δ7 mice, Δ7 mice were treated from PND3 at two different doses (Fig. 1A). Both doses extended survival beyond that of the vehicle-dosed mice, so no vehicle-dose Δ7 mice survived at PND28. At PND28, relative to the high-dose-treated mice (3 mg/kg), Δ7 animals treated with a suboptimal dose (0.1 mg/kg, denoted as ‘low dose’) of SMN-C3 expressed a significantly lower level of SMN protein both in the brain and in the muscle, reported here as arbitrary units (AU): (i) brain Δ7 low dose: 66.5 ± 9.5; Δ7 high dose: 160.8 ± 10.6; non-SMA: 669.1 ± 7.8, Figure 1B and (ii) muscle Δ7 low dose: 41.8 ± 6.0; Δ7 high dose: 80.8 ± 4.9; non-SMA: 121.2 ± 4.0, Figure 1B. Treatment of Δ7 mice with high dose of SMN-C3 is fully effective in preventing disease progression (23). Low-dose-treated Δ7 mice, although surviving into adulthood with a median survival time of 28 days (23, Fig. 1C), displayed SMA disease phenotypes as detailed subsequently. Compared with non-SMA mice, low-dose-treated Δ7 mice displayed reduced body weight growth (Fig. 1D) and strength as measured by inverted-hang test (Δ7 low dose: 12.4 ± 2.1 s; Δ7 high dose: 34.4 ± 9.5 s; non-SMA: 107.9 ± 15.2 s, Fig. 1E), as well as progressive peripheral necrosis at the tail (Δ7 low dose: 33.0 ± 0.9 mm; Δ7 high dose: 54.8 ± 1.7 mm; non-SMA: 63.3 ± 0.7 mm, Fig. 1F). Taken together, treatment with a suboptimal dose of SMN-C3 extends the survival of Δ7 mice into adulthood with reduced body weight and associated pathological changes.

We next tested whether these suboptimally dosed adult Δ7 mice display any SMA relevant disease phenotype, especially within the neuromuscular circuitry. As one of the characteristic symptoms in SMA is profound muscle atrophy, we first examined whether low-dose-treated Δ7 mice exhibit any muscle pathology. We found that at PND32, the muscle cross-sectional area of the extensor digitorum longus (EDL) muscle in low-dose-treated Δ7 mice was only ~67% of that in vehicle-treated non-SMA mice, whereas the EDL muscle in high-dose-treated Δ7 mice attained a size similar to that of non-SMA controls (Δ7 low dose: 0.34 ± 0.03 mm²; Δ7 high dose: 0.51 ± 0.02 mm²; non-SMA: 0.52 ± 0.04 mm²; Fig. 2A and C). The reduced size of the EDL muscle was not attributed to loss of myofibers (Δ7 low dose: 1185 ± 88; Δ7 high dose: 1373 ± 30; non-SMA: 1360 ± 42; Fig. 2B). In addition, we have recently demonstrated that unlike the EDL muscle, which remains fully innervated in Δ7 mice even at disease end-stage at PND14 (i.e., are more resistant to disease), the longissimus capitis muscle of Δ7 mice undergoes severe NMJ denervation and is more vulnerable to disease (26). When we examined the size of the longissimus muscle at PND32, we found that over 80% of muscle fibers degenerated in the longissimus muscle of low-dose-treated Δ7 mice relative to non-SMA controls (Δ7 low dose: 75 ± 7; Δ7 high dose: 162 ± 25; non-SMA: 666 ± 16; Fig. 2E), which could be attributed to NMJ denervation (see Discussion). As a result, a striking reduction in the size of longissimus muscle was observed in low-dose-treated Δ7 mice (Δ7 low dose: 0.08 ±
To assess whether low-dose-treated Δ7 mice exhibit any synaptic defects in muscles that are susceptible (i.e., vulnerable) to NMJ denervation in the Δ7 mouse model (26), we examined NMJ innervation in the longissimus muscle from Δ7 mice. Muscles were immunostained for nerves, presynaptic terminals and postsynaptic acetylcholine receptors (AChRs) with anti-neurofilament, anti-synaptophysin and α-bungarotoxin, respectively. Consistent with our previous findings in another vulnerable muscle (the splenius capitis muscle) (23), only high- but not low-dose treatment with SMN-C3 improved the percentage of fully innervated NMJs in the longissimus muscle (Δ7: 23.4 ± 4.4%; Δ7 low dose: 32.8 ± 5.3%; Δ7 high dose: 53.2 ± 6.8% at PND14 in Fig. 3A and B). At PND32, as demonstrated earlier (Fig. 2), over 80% of longissimus muscle fibers were lost in Δ7 mice with low-dose treatment, and only ∼20% of NMJs on the remaining fibers were fully innervated (Δ7 low dose: 21.0 ± 5.7%; Fig. 3C). In contrast, fewer fibers were degenerated and more NMJs were fully innervated at PND32 in high-dose-treated Δ7 mice (Δ7 high dose: 76.2 ± 7.3%; Fig. 3C). Thus, similar to mice with severe SMA, low-dose-treated Δ7 mice also display NMJ denervation in vulnerable muscles in adulthood.

In addition to synaptic defects in the muscle, we and others have previously demonstrated a loss of synaptic inputs onto SMA motor neurons that innervate hindlimb muscles, which might also contribute to motor impairment in SMA (27,28). To assess whether such central synapse defects are also present...
in adult Δ7 mice treated with low-dose SMN-C3, we immunolabeled motor neurons and proprioceptive primary afferents in the lumbar (L3–L5) segments of the spinal cord with choline acetyltransferase (ChAT) and vesicular glutamate transporters (vGLUT) 1, respectively. At PND32, motor neurons in low-dose-treated Δ7 mice had fewer vGLUT1-positive synapses when compared with non-SMA controls (Δ7 low dose: 14 ± 1.2; Δ7 high dose: 21.6 ± 0.8; non-SMA: 22.0 ± 0.6; Fig. 3E), suggesting that synaptic inputs onto motor neurons are reduced in low-dose-treated adult Δ7 mice. 

Taken together, our findings illustrate that Δ7 mice treated with SMN-C3 at a suboptimal dose survive into adulthood and exhibit disease-relevant defects in the motor circuit, including muscle atrophy, and the loss of synapses at the NMJ (in vulnerable muscles) and in the spinal cord. These adult Δ7 mice are a model for symptomatic patients with SMA.

**SMN-C3 at a fully efficacious dose mitigates SMA-related phenotype in symptomatic adult Δ7 mice**

We sought to take advantage of our pharmacologically induced mouse model of milder SMA to determine whether increasing SMN protein levels at later stages provides therapeutic benefit in adolescence/adulthood after symptoms have already developed. To address this question, one group of Δ7 mice treated with low-dose SMN-C3 was switched to the high dose starting at PND32, whereas a second group of Δ7 mice remained on low-dose SMN-C3 (Fig. 4A). At PND60, Δ7 mice treated from PND32 with the higher dose of SMN-C3 had increased body weight gain compared with animals that were continuously treated with the low dose over the entire period (purple versus green line in Fig. 4B). Of the 18 mice in the low-dose group at PND32, 10 survived through PND60 (56%, Fig. 4C); whereas of the nine mice in the low-to-high-dose group at PND32, eight of the mice survived through PND60 (89%, P = 0.21 comparing survival in low dose to low-to-high dose, log rank test, Fig. 4C). In addition, tail necrosis (Δ7 low dose: 14.6 ± 2.1 mm; Δ7 high dose: 32.1 ± 2.1 mm; Δ7 high dose: 64.2 ± 1.1 mm; non-SMA: 68.2 ± 0.8 mm; Fig. 4D) and eye necrosis (Δ7 low dose: 2.9 ± 0.1 AU; Δ7 low-high: 1.7 ± 0.4 AU; Δ7 high dose: 0.7 ± 0.2 AU; Fig. 4E) were reduced when the dose was switched to the optimal dose at PND32. These results demonstrate that increasing the dose of SMN-C3...
promotes body weight gain and prevents necrosis in symptomatic adult Δ7 mice, even when the enhanced treatment regimen is initiated postsymptomatically.

We next tested whether the observed muscle atrophy in low-dose-treated Δ7 mice could be mitigated by increasing the dose at PND32. At PND60, switching the treatment from the low to the
high dose at PND32 increased the muscle size as demonstrated by a significant ~20% increase in the cross-sectional area of the EDL muscle when compared with A7 mice that continued to receive the low dose of SMN-C3 (Δ7 low dose: 0.51 ± 0.03 mm²; Δ7 low-high: 0.61 ± 0.04 mm²; Δ7 high dose: 0.67 ± 0.03 mm²; non-SMA: 0.78 ± 0.04 mm²; Fig. 5A and B). As we found no change in the number of muscle fibers among all groups (Δ7 low dose: 1240 ± 17; Δ7 low-high: 1193 ± 17; Δ7 high dose: 1284 ± 74; non-SMA: 1242 ± 35; Fig. 5C), the changes in muscle size are likely driven by changes in individual myofiber size (Δ7 low dose: 330.7 ± 40.0 µm²; Δ7 low-high: 442.7 ± 21.0 µm²; Δ7 high dose: 504.9 ± 36.8 µm²; non-SMA: 593.2 ± 28.8 µm²; Fig. 5D). In the vulnerable longissimus muscle, a 30% increase was observed in the size of the muscle when the treatment was switched from the low to the high dose (Δ7 low dose: 0.11 ± 0.01 mm²; Δ7 low-high: 0.15 ± 0.02 mm²; Fig. 5F), although this difference did not reach statistical significance. Switching to the high dose did not affect the longissimus muscle growth in adulthood.

To explore the effect of increasing SMN within adult NMJs, we examined NMJ innervation in the vulnerable longissimus muscle at PND60. Although the fiber number remained the same, all NMJs were fully innervated in high-dose-treated A7 mice (Δ7 high dose: 100 ± 0.001%; non-SMA: 100 ± 0.001%; Fig. 6A). In low-dose-treated A7 mice, although ~85% of NMJs were fully innervated in the remaining muscle fibers following severe muscle fiber loss (Fig. 5G), there was a significant decrease in the percentage of fully innervated NMJs compared with non-SMA controls or with high-dose treatment (Fig. 6A). Switching from the low to the high dose slightly increased the percentage of fully innervated NMJs, although the difference did not reach statistical significance (Δ7 low-high: 92.3 ± 4.1%), indicating that switching to high-dose treatment in adulthood may still promote NMJ innervation even though denervation is occurring.

We next examined whether increasing the dose from a low to a high dose at PND32 can ameliorate the central synaptic defects observed in low-dosed symptomatic adult A7 mice. At PND60, we found that increasing to a high dose at PND32 significantly increased the number of vGLUT1 synapses to a level similar to that of non-SMA controls, as well as similar to the level measured in A7 mice that had been treated continuously with the high dose (Δ7 low dose: 16.5 ± 1.0; Δ7 low-high: 22.4 ± 1.3; Δ7 high dose: 23.4 ± 1.0; non-SMA: 24.2 ± 0.6; Fig. 6B). The beneficial effect of increasing to a higher dose of SMN-C3 was not limited to vGLUT1 synapses. Spinal motor neurons receive vGLUT2 synapses as well, and these synapses are also reduced in A7 spinal cords (27). As shown in Figure 6C, motor neurons in low-dose-treated A7 mice had fewer vGLUT2-positive synapses at PND60. Δ7 mice treated with the low dose and switched to the higher dose from PND32 to PND60 had a level of vGLUT2-positive synapses similar to that of non-SMA mice and high-dose-treated A7 mice (Δ7 low dose: 14.0 ± 0.2; Δ7 low-high: 22.4 ± 1.3; Δ7 high dose: 25.9 ± 0.2; non-SMA: 23.6 ± 0.2; Fig. 6D). The beneficial effect of increasing to a higher dose of SMN-C3 was not limited to vGLUT1 synapses. Spinal motor neurons receive vGLUT2 synapses as well, and these synapses are also reduced in A7 spinal cords (27). As shown in Figure 6C, motor neurons in low-dose-treated A7 mice had fewer vGLUT2-positive synapses at PND60. Δ7 mice treated with the low dose and switched to the higher dose from PND32 to PND60 had a level of vGLUT2-positive synapses similar to that of non-SMA mice and high-dose-treated A7 mice (Δ7 low dose: 14.0 ± 0.2; Δ7 low-high: 19.3 ± 1.0; Δ7 high dose: 19.3 ± 1.0; non-SMA: 21.3 ± 0.9 per 100 µm motor neuron perimeter; Fig. 6C). In addition, Δ7 mice also display other hallmark phenotypes associated with severe SMA, including loss of spinal motor neurons. Previously, we showed that SMN-C3 treatment alleviated motor neuron loss when measured at PND14 in Δ7 mice in a dose-dependent manner (23). Consistently, we have observed an increase in the number of L4 ventral root axons in A7 mice treated with either dose compared with vehicle-treated Δ7 mice at PND14 (data not shown). At PND60, however, we did not detect any change in the number of L4 ventral root axons when the treatment was switched from low to high dose at PND32 when compared with continuous low-dose treatment (Δ7 low dose: 753.6 ± 19.2; Δ7 low-high: 741 ± 18; Δ7 high dose: 803 ± 25; non-SMA: 910 ± 13; Fig. 6D). Taken together, an increased dose of SMN-C3 in the low-dose-treated adult Δ7 mice resulted in the restoration of the central synapses.
Myostatin inhibition promotes muscle growth in symptomatic adult Δ7 mice

As one of the characteristic symptoms in SMA is profound muscle atrophy and weakness, strategies to promote muscle growth and muscle maintenance could be effective in treating SMA. It has been shown that myostatin inhibition using recombinant follistatin, an endogenous myostatin inhibitor protein, had limited effect in Δ7 mice (21,22), which could be attributed to the short lifespan of Δ7 mice. To further take advantage of the pharmacologically generated adult Δ7 mice, we asked whether myostatin inhibition mitigates SMA muscle atrophy in our suboptimally dosed Δ7 mice. Adeno-associated virus serotype 1 (AAV1) encoding follistatin (FS344) has been shown to induce an increase in endogenous follistatin (29,30) as well as in muscle size and strength (29,31). The same AAV-follistatin was administered at PND14 via intramuscular injection (31) (5 × 10¹¹ viral particles per animal) to Δ7 mice treated with a low dose of SMN-C1 (23), a compound similar to SMN-C3. At approximately PND60, the addition of AAV-follistatin did not affect the survival of low-dose-treated Δ7 mice (Fig. 7A). However, AAV-follistatin did result in an increase in the body weight of low-dose-treated Δ7 mice (Fig. 7B), as well as non-SMA mice (data not shown). Consistent with previous findings (31), AAV-follistatin treatment resulted in an increase in the weight of several hindlimb muscles in non-SMA mice (Fig. 7C–E), including the gastrocnemius muscle (non-SMA: 149.4 ± 6.6 mg; non-SMA + AAV-follistatin: 353.4 ± 23.8 mg), the anterior tibialis muscle (non-SMA: 46.3 ± 3.3 mg; non-SMA + AAV-follistatin: 59.2 ± 3.1 mg) and the EDL muscle (non-SMA: 8.6 ± 0.3 mg; non-SMA + AAV-follistatin: 11.6 ± 1.3 mg). In addition, we found a similar increase in the weight of these muscles in low-dose-treated Δ7 mice that received AAV-follistatin (Fig. 7C–E), including the gastrocnemius muscle (low dose: 60.4 ± 4.1 mg; low dose + AAV-follistatin: 130.9 ± 13.0 mg), the anterior tibialis muscle (low dose: 17.9 ± 1.3 mg; low dose + AAV-follistatin: 30.4 ± 2.8 mg) and the EDL muscle (low dose: 4.0 ± 0.3 mg; low dose + AAV-follistatin: 5.9 ± 0.7 mg). As follistatin does not affect SMN expression in Δ7 mice (22), the increase in the muscle size of low-dose-treated Δ7 mice with AAV-follistatin is likely due to the effect of follistatin on muscle growth. These
data suggest that enhancing muscle growth in SMA in later stages of development may be an additional therapeutic approach for the treatment of intermediate/mild SMA patients.

Discussion

Our findings show that Δ7 mice treated with a suboptimal dose (i.e., low dose) of an SMN2 splicing modifier (SMN-C3 or SMN-C1) have increased survival when compared with untreated Δ7 mice, but display a range of disease phenotypes that are reminiscent of the milder forms of SMA. These adult Δ7 mice represent a unique tool to evaluate efficacy of potential therapies to treat SMA in adolescence/adulthood when symptoms have already appeared. Here, we show that increasing SMN protein levels using SMN2 splicing modifier compounds in adolescent/adulthood mice displaying disease symptoms is beneficial. Furthermore, the enhanced muscle growth induced by recombinant follistatin delivery in adult Δ7 mice demonstrates that myostatin inhibition, an SMN-independent approach, may also provide significant benefit in alleviating muscle atrophy in SMA patients.

The Δ7 model, which has a median survival time of 14 days and displays severe muscle weakness (32), has provided tremendous insights into SMA biology and is widely used for translation studies. As human SMA patients exhibit diverse clinical severities, Δ7 mice only mimic a subset of SMA patients (severe or type I). A model with a less severe phenotype may provide a more relevant system to evaluate drug efficacy for patients with milder forms of SMA (reviewed in 33–35). Our findings have demonstrated that Δ7 mice treated with a suboptimal dose of SMN-C3 have a longer lifespan when compared with untreated Δ7 mice and still display SMA-like pathology including synaptic defects in the spinal cord and the NMJ as well as muscle atrophy. In these suboptimally dosed Δ7 mice, the SMN protein level remains deficient prenatally and is pharmacologically increased postnatally. Nevertheless, our suboptimally dosed Δ7 mouse model provides a novel approach to evaluate drug efficacy for SMA patients in late childhood and young adulthood when symptoms are already apparent.

SMA is a disease caused by reduced levels of the SMN protein. Restoring SMN protein has been the primary focus of treatment strategies in the field, and several therapeutics aimed to increase SMN levels are being tested in clinical trials. Recent studies have demonstrated that restoring SMN protein during early postnatal development is critical to mitigate disease pathophysiology, and the beneficial effect of restoring SMN wanes as the disease progresses in severe SMA (13–15,36). Noteworthy, the recent study performed in Taiwanese mice showed that repetitive administration from birth of low-dose SMN upregulating morpholino oligomer confers cumulative benefits, whereas a single low dose at birth and subsequent single high dose of morpholino oligomer are protective only when administered in the first 2 weeks of life (37). In addition, it has been reported recently that antisense
SMN deficiency in the muscle might exacerbate myofiber loss in the longissimus muscle. However, even high-dose SMN-C3 at PND32 cannot rescue muscle fibers from degeneration in suboptimally dosed Δ7 mice, suggesting that earlier administration of the SMN-upregulating compounds might be required for the maintenance of myofibers in these vulnerable muscles. Furthermore, the reduction of myofiber number in the longissimus muscle at PND32 emphasizes that at least in Δ7 mice, denervated myofibers would degenerate over time. In patients, SMA pathology is characterized by the presence of small myofibers that are deficient in innervation and developmentally arrested (1). It is unknown whether the increase of SMN protein itself would be sufficient to promote the growth of those small myofibers in human SMA patients. It is possible that SMN protein-enhancing treatments might provide incomplete restoration of muscle function. Thus, strategies that promote muscle regeneration should be considered in combination therapy with strategies that promote SMN protein expression.

Central synapse loss is another prominent pathology in SMA mouse models. SMA motor neurons receive fewer excitatory inputs from proprioceptive sensory neurons and local spinal interneurons, which might contribute to severe motor dysfunction in SMA (27,28). Here, we demonstrate that this central synaptic defect also presents in low-dose-treated adult Δ7 mice. Increasing the dose of SMN-C3 restores synaptic inputs onto motor neurons, indicating a possible synaptogenesis effect of SMN-C3 in adult spinal cord. The increased synaptic inputs onto motor neurons would promote the restoration of normal motor function. Furthermore, we did not observe any increase in the number of motor axons in lumbar 4 ventral roots, which often correlates with the number of motor neurons. Once motor neuron death
occurs, interventions aimed at neuroprotection might be too late to be clinically relevant. Thus, in adult SMA patients, a therapeutic strategy that targets blocking the progression of motor neuron loss and/or promoting axonal regeneration or sprouting should be validated.

Currently, multiple clinical trials in SMA that are focussed on increasing SMN protein are ongoing (9–11). Given the possibility that some molecular or cellular components might be irreversibly damaged due to insufficient SMN protein during development, SMN-independent therapeutics, complementary to SMN-restoring approaches, are also being explored. These include strategies that target biological processing that are downstream of SMN, neuroprotective agents that might prevent disease progression, as well as therapies that promote motor circuit function (9,41). In this study, a low dose of SMN-C3 slightly increases the SMN level in Δ7 mice, which results in an SMA model that survives into adulthood and manifests pathological phenotypes that recapitulate symptomatic adult SMA patients. Our proof-of-concept study with myostatin inhibition in low-drug dose-induced symptomatic adolescent Δ7 mice indicates that promoting muscle growth in SMA may provide an additional therapeutic approach. This SMA mouse model allows for the validation of an SMN-independent therapy that might otherwise fail in the severe Δ7 mice. It will be of interest to revisit approaches that failed to increase survival in Δ7 mice, such as insulin-like growth factor I and plastin (42–45), using these low-dose-treated Δ7 mice. In the future, a combination of SMN-restoring compounds and SMN-independent approaches may exert a synergistic effect and provide the most effective treatment option for SMA patients.

Materials and Methods

Animals

All animal studies were performed under Institutional Animal Care and Use Committee-approved protocols at AAALAC-certified animal facilities. Δ7 mice were generated from breeder pairs of heterozygous Δ7 mice purchased from the Jackson Laboratory [no. 009025, FVB.Cg-Tg(SMN2*delta7)4299Ahmb Tg(SMN2)89Ahmb Smn1Δ7m1Mad/J]. Genotyping was performed as described previously (27). For each study, litters were randomized across groups, with 15–16 mice per group. Body weight and survival were assessed daily.

Drug treatment

From PND3 through PND23, homozygous Δ7 mice were dosed with SMN-C3 [low dose: 0.1 mg/kg and high dose: 3 mg/kg, all as solutions in 100% dimethyl sulfoxide (DMSO)] or vehicle (100% DMSO) via intraperitoneal (i.p.) injection once daily (23). At PND24, animals were treated by oral gavage at a dose 3-fold higher in 0.5% hydroxypropyl methylcellulose and 0.1% Tween 80. In the study of the effect of myostatin inhibition on muscle physiology, Δ7 mice were treated with SMN-C1 (0.01 mg/kg) using a dosing regimen, as described for SMN-C3 earlier. To inhibit myostatin, AAV1-encoding follistatin (5 × 1011 viral particles per animal, hFST344, Vector BioLabs) was given at PND14 via intramuscular injections in forelimbs and hindlimbs (31).

Inverted-hang screen test (four-limb hanging wire test)

Mice were placed on a wire grid (1 cm × 1 cm grid), and the grid was turned over so that the mice were ~35 cm above the ground. Soft bedding was placed under the grid so that the mice were not harmed on falling. Mice were monitored for up to 5 min. If mice remained on the grid for 5 min, a time of 300 s was used for subsequent calculations. Each mouse was tested three times with 5 min between each trial.

Quantification of SMN protein in animal tissues

SMN protein in animal tissues was quantified using homogeneous Time-Resolved Fluorescence (HTRF, Cisbio Bioassays) assay, as described previously (23). Brain and quadriceps muscles were collected, homogenized in water and the tissue lysates were transferred to a 384-well plate containing antibody solution of anti-SMN d2 and anti-SMN cryptate (Cisbio). Fluorescence was measured at 665 and 620 nm using EnVision multilabel plate reader (Perkin Elmer). Total protein of each homogenate was measured using the BCA assay, according to the manufacturer’s protocol. The HTRF signal for SMN protein was normalized to the total protein concentration for each sample. Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by the Dunnett’s multiple comparison test. A P < 0.05 was considered as significant.

Mouse histology

Mice were euthanized and perfused with Ringer’s solution followed by 4% paraformaldehyde (Sigma). Muscles and spinal cords were removed and fixed overnight. To examine muscle size, transverse sections of the EDL and longissimus muscles were processed by standard hematoxylin and eosin staining, and the size of muscle cross-sectional area was measured using NIH Image software. To examine NMJ innervation, either whole mount preparations (PND14) or teased muscle fibers (PND32 and 60) of the longissimus capitis muscles were used. Standard immunofluorescent staining was used to label motor nerve terminals with anti-neurofilament (Millipore), anti-synaptophysin (Invitrogen) and AChRs with α-bungarotoxin (Invitrogen) (26). The percentage of endplates (identified by AChR clusters) that are fully, partially or not occupied by nerve terminals was calculated. To assess synaptic inputs to motor neurons, lumbar spinal cords were sectioned at 80 μm and stained with anti-ChAT (Millipore) and anti-vGLUT1 (Synaptic System) or anti-vGLUT2 (Synaptic System) antibodies. The number of synaptic boutons onto motor neuron soma and proximal dendrites was counted with a stereological method using serial confocal imaging (27,28). To examine motor axons, 14 ventral roots were fixed with glutaraldehyde, postfixed with osmium tetroxide and embedded in Epon. One-micron cross-sections were stained with toluidine blue and examined with light microscopy. The number of myelinated axons was measured using NIH Image software.

Statistical analysis

Values for each mouse were averaged, and then these values were averaged across the group. Values are expressed as mean ± SEM, and unequal variance Student’s t-tests were used to determine statistical differences and significance (**P < 0.001, *P < 0.01 and *P < 0.05; ns, P > 0.05). Survival analysis was performed using GraphPad Prism (log-rank test), and a P < 0.05 was considered as significant.

Acknowledgement

The authors thank A. Mollin for the SMN protein analysis and genotyping, J. Sheedy for support with the in-life work, W. Lennox and O. Vaze for formulation support and C. Mazzasette, S. Sunshine, V. Le Verche, and P.-F. Yen for providing comments on the manuscript.
Conflict of Interest statement. X.Z., G.K., E.M.W., N.N. and M.W. are employees of PTC Therapeutics. H.R. and F.M. are employees of F. Hoffmann-La Roche Ltd.

Funding
This work was supported by the Spinal Muscular Atrophy Foundation.

References


