Biochemical and Biophysical Research Communications 417 (2012) 376–381

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Direct central nervous system delivery provides enhanced protection following vector mediated gene replacement in a severe model of Spinal Muscular Atrophy

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ARTICLE INFO

Article history:
Received 21 November 2011
Available online 8 December 2011

Keywords:
Survival Motor Neuron (SMN)
Spinal Muscular Atrophy (SMA)
scAAV
Gene therapy
Neurodegeneration
Therapeutics

ABSTRACT

Spinal Muscular Atrophy (SMA), an autosomal recessive neuromuscular disorder, is the leading genetic cause of infant mortality. SMA is caused by the homozygous loss of Survival Motor Neuron-1 (SMN1). SMA, however, is not due to complete absence of SMN, rather a low level of functional full-length SMN is produced by a nearly identical copy gene called SMN2. Despite SMN's ubiquitous expression, motor neurons are preferentially affected by low SMN levels. Recently gene replacement strategies have shown tremendous promise in animal models of SMA. In this study, we used self-complementary Adeno Associated Virus (scAAV) expressing full-length SMN cDNA to compare two different routes of viral delivery in a severe SMA mouse model. This was accomplished by injecting scAAV9-SMN vector intravenously (IV) or intracerebroventricularly (ICV) into SMA mice. Both routes of delivery resulted in a significant increase in lifespan and weight compared to untreated mice with a subpopulation of mice surviving more than 200 days. However, the ICV injected mice gained significantly more weight than their IV treated counterparts. Likewise, survival analysis showed that ICV treated mice displayed fewer early deaths than IV treated animals. Collectively, this report demonstrates that route of delivery is a crucial component of gene therapy treatment for SMA.

1. Introduction

Spinal Muscular Atrophy (SMA) is caused by the homozygous loss of Survival Motor Neuron-1, SMN1 [1,2]. The human genome contains two nearly identical SMN genes, SMN1 and SMN2, however, only SMN1 functions as the disease-determining gene [3,4]. SMN1 and SMN2 differ by a silent C to T transition at the 5′ end of exon 7 [5,6]. This difference alters the alternative pre-mRNA splicing ratios from the two genes, resulting in high levels of full-length product from SMN1, whereas SMN2 produces low levels of full-length SMN and an abundant alternatively spliced isoform, SMNΔ7. The truncated isoform is unstable and cannot compensate for the loss of SMN1 [3]. Despite the ubiquitous expression of SMN, preferential loss of motor neurons occurs in SMA. Because SMA is monogenic, vector-based gene replacement of SMN1 is an attractive option for the treatment of SMA. Encouraging reports have been published using a relatively severe model of SMA called SMNΔ7. These mice lack endogenous mouse *Smn*, but express the human SMN2 gene and the cDNA encoding the alternatively spliced isoform produced by SMN2, SMNΔ7 ($Smn^{-/-}$; $SMN2^{+/+}$; $SMN\Delta7^{+/+}$) [7]. Untreated SMNΔ7 animals live approximately 14 days with disease symptoms becoming overtly apparent around day 7 [7]. Delivery of full-length SMN cDNA to SMNΔ7 neonates using scAAV8 or scAAV9 vectors resulted in significant extensions in survival ranging from an average of 60–200+ days [8–11], with some treated mice displaying a full rescue in terms of lifespan and motor function. However, it remains unclear whether the different injection paradigms or the vector serotype was the primary cause for the differences in the degree of phenotypic rescue.

In this report we utilized a scAAV9-SMN vector and examined two routes of injection in neonatal SMNΔ7 mice [12]. Pups received injections of 2×10^{10} viral genomes via the facial vein (IV) or directly into the brain ventricles (ICV) on postnatal day 2 (PND2). We demonstrate that at this relatively low viral titer, animals receiving ICV injections gained significantly more weight and lived longer than animals receiving IV injections. As expected, animals receiving ICV injections also had higher SMN protein levels in the brain and lumbar spinal cord as compared to IV injected animals. From these results, we conclude that the route of injection for scAAV9-SMN has a significant impact upon the degree of

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doi:10.1016/j.bbrc.2011.11.121

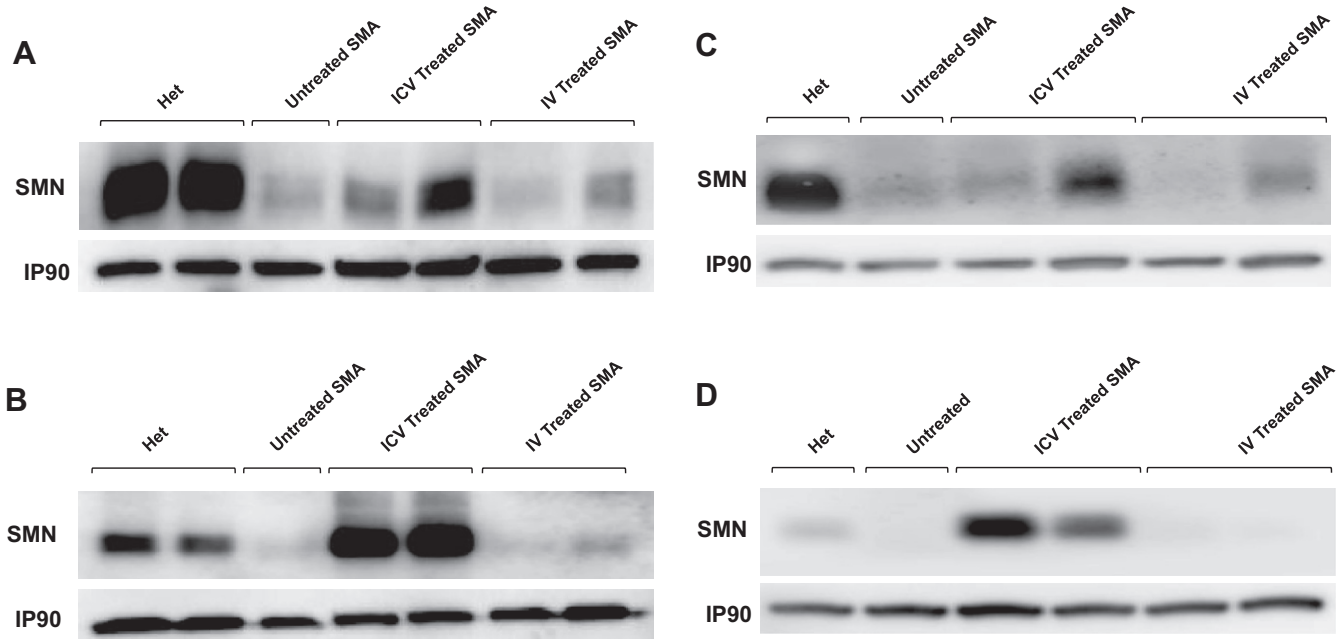

Fig. 1. Western blot showing protein expression is increased to near normal levels following ICV treatment with scAAV9-SMN, while IV treatment results in a more modest increase. Western blots of (A) PND 7 and (B) PND14 brain tissue. (C) PND7 and (D) PND14 spinal cord. All tissues were collected on the respective days from animals injected on PND2 with 2×10^{10} viral particles. Controls were untreated SMA ($Smn^{-/-}$; $SMN2^{+/+}$; $SMN\Delta7^{+/+}$) and unaffected, heterozygous (het) animals ($Smn^{+/-}$; $SMN2^{+/+}$; $SMN\Delta7^{+/+}$).

phenotypic rescue and sheds light upon the development of disease and potential therapeutic implications.

2. Materials and methods

2.1. Genotyping and mouse handling

Animals were handled according to the University of Missouri Animal Care and Use Committee approved Protocols. Mice heterozygous for *mSmn* ($Smn^{+/-}$; $SMN2^{+/+}$; $SMN\Delta7^{+/+}$) were interbred to generate experimental SMA cohorts ($Smn^{-/-}$; $SMN2^{+/+}$; $SMN\Delta7^{+/+}$). The day of birth was counted as PND1 and the neonates were genotyped within 24 h. Animals were genotyped using PCR conditions as previously described [13]. SMA mice were raised with two heterozygous siblings. Additional heterozygous and wild-type animals were culled at the time of injection in experimental cages to control for litter size.

2.2. Tissue collection

Dissections were done as follows: the vertebral column was separated from the torso, then the spinal cord was removed and divided into the cervical, thoracic, and lumbar (C-T-L) regions. The C-T-L sections were immediately frozen. The brain was removed from the skull and divided into four equal sections and each section was immediately frozen. The hindlimbs of each animal were removed at the highest point possible. The foot was bent at a 90° to control for differences in muscle stretching and the tissue was fixed in 4% paraformaldehyde overnight. After fixation, the gastrocnemius and tibialis anterior were removed from the bone, embedded in paraffin, and cross sectioned. The sections were stained with hematoxylin and eosin stain for muscle fiber size analysis. Quantification of fiber size was done as previously described [14].

2.3. Western blotting

Tissues were harvested at indicated times and analysis was performed as previously described [15,16]. Mouse monoclonal anti-SMN (BDBiolabs), 1:2000, and anti-IP90 polyclonal rabbit antibody, 1:2000, were used for SMN and calnexin detection, respectively.

2.4. Production of scAAV-SMN viral vector

scAAV9-SMN was produced via triple transfection in HEK293T cells using polyethyleneimine as previously described [17]. The scAAV plasmid expresses the SMN full-length cDNA (NCBI accession number NM_000344) under the control of the chicken beta actin promoter. Forty eight hours post transfection, the cells were collected and the vector was purified by two cesium chloride density gradient ultracentrifugation steps and dialyzed against HEPES buffer (20 mM HEPES, 100 mM NaCl). Viral particles were tittered by quantitative realtime PCR using SYBR green.

2.5. In vivo injections

Beginning on PND2 $mSmn^{-/-}$; $hSMN2^{+/+}$; $SMN\Delta7^{+/+}$ mice were injected with 2×10^{10} viral particles of scAAV9-SMN. Due to the volume restrictions of the ICV technique, a series of three injections were given to obtain a titer of 2×10^{10}. Animals chosen for ICV injections were injected twice on PND2 (AM and PM) and once on PND3 (AM). Animals chosen for IV injection received a single injection on PND2. Injections were visualized for accuracy by the additional of filter sterilized food dye.

2.6. Motor function analysis

Time to right was measured from p10 to p18. Mice were placed on their backs and given a maximum of 30 s to right themselves onto their paws. Failure to right within 30 s was considered failure. Grip strength and rotarod tests were performed with mice older than 80 days. The rotarod and grip strength assessments were done for 20 consecutive days. The first 10 days served as an initiation and learning period for the animals and the last 10 days were used for analysis. Each individual mouse was given three trials and the best trial was graphed.

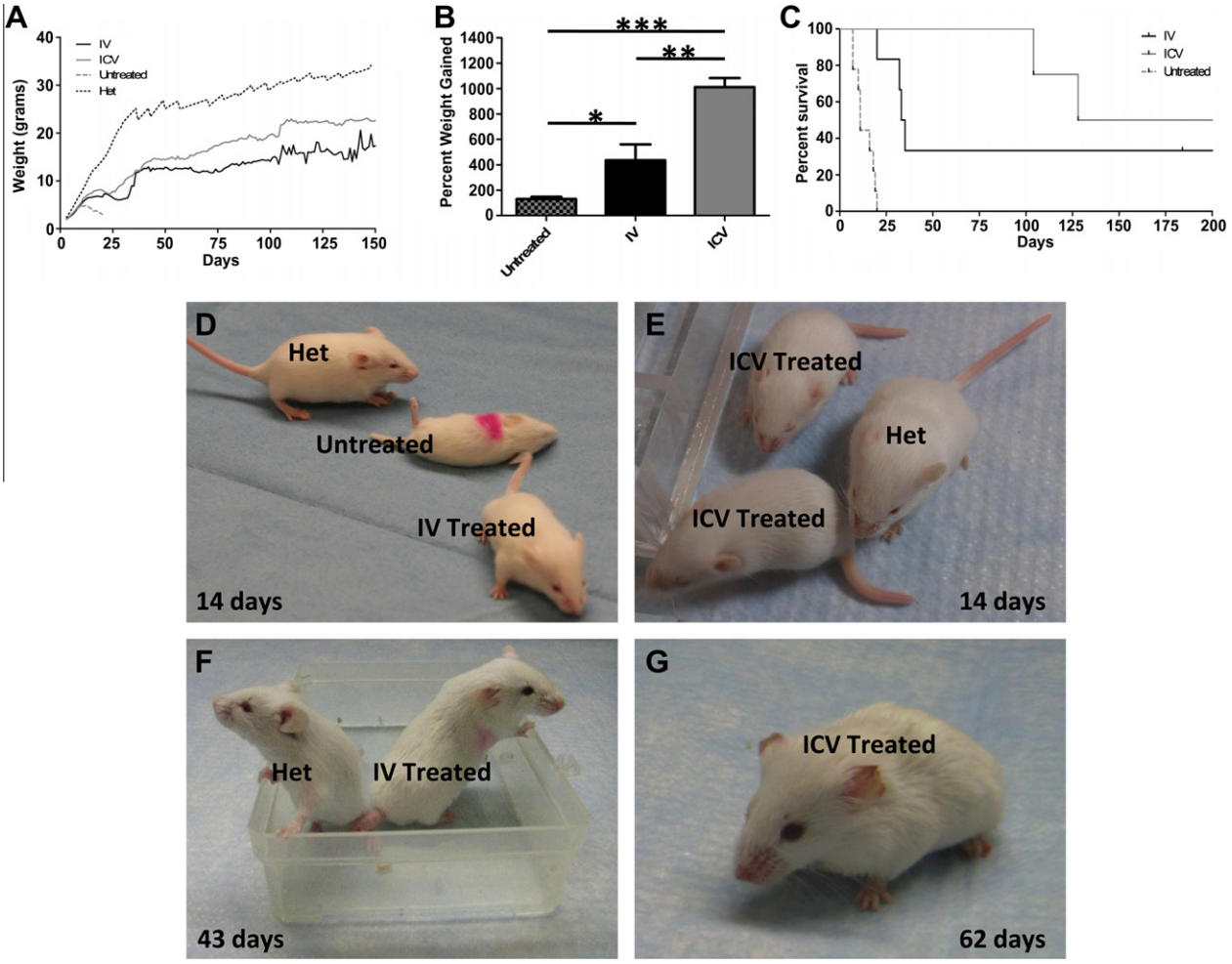

Fig. 2. scAAV-SMN ICV treated animals gain significantly more weight and experience fewer early deaths than IV treated SMA animals while both groups gain significantly more weight and live longer than untreated SMA controls ($Smn^{-/-}$; $SMN2^{+/+}$; $SMN\Delta7^{+/+}$). (A) Average weight, in grams, of animals at each day of life: untreated ($n = 9$), IV treated ($n = 6$), ICV ($n = 4$), and Het ($n = 24$). Each treatment group is significantly different from the others by one way ANOVA ($p < 0.0001$). Tukey's multiple comparison test: Untreated vs. ICV $p < 0.001$, Untreated vs. IV $p < 0.001$ and IV vs. ICV $p < 0.001$. (B) Percent body weight gained from PND3 to peak weight of untreated, IV treated, and ICV treated SMA animals: untreated = 130.6%, IV treated = 435.9%, and ICV treated = 1011.7%. Unpaired two-tailed student's t test: untreated vs. ICV $p < 0.0001$, untreated vs. IV $p = 0.0157$, and IV vs. ICV $p = 0.0085$. (C) Kaplan–Meier survival curve of untreated, IV treated, and ICV treated SMA animals. Log-rank (Mantel–Cox) test: untreated vs. IV $p = 0.0004$, untreated vs. ICV $p = 0.0019$, and IV vs. ICV $p = 0.3251$. (D–G) Representative images of treated SMA animals with both untreated SMA ($Smn^{-/-}$; $SMN2^{+/+}$; $SMN\Delta7^{+/+}$) and unaffected controls ($Smn^{+/-}$; $SMN2^{+/+}$; $SMN\Delta7^{+/+}$). (D) PND14 IV treated with controls, (E) PND14 IV treated with controls, (F) PND43 IV treated with unaffected littermate, and (G) PND63 ICV treated.

3. Results and discussion

To determine if the route of injection significantly impacts the degree to which the SMA phenotype is corrected in SMA mice following treatment with scAAV9-SMN, we performed a titration experiment to identify a relatively low concentration of vector that still resulted in significant survival (data not shown). This concentration (2×10^{10}) as opposed to a maximal dose was used in subsequent experiments since a lower concentration was more likely to reveal differences regarding the ability to rescue the SMA phenotype. SMNΔ7 neonatal pups received IV or ICV injections with identical concentrations of the self-complementary AAV9 vector expressing full-length SMN cDNA. SMN protein levels were examined at PND7 and PND14 in disease relevant tissues, including brain and spinal cord, following scAAV9-SMN delivery (Fig. 1). As anticipated, following the ICV injection directly into the brain, SMN expression was dramatically increased in the brain and spinal cord to levels comparable to unaffected heterozygous animals (Fig. 1). IV injected animals expressed higher levels of SMN as well, although not to the same extent as ICV injected tissues. To monitor the gross phenotypic changes in treated mice, weight and survival were measured in each of the treatment groups (Fig. 2). The ICV and IV treated animals gained significantly more weight than untreated controls (Fig. 2A and B), gaining nearly 2-fold more weight from PND3 to their peak weight. We also observed that IV treated animals experienced more early deaths compared to ICV treated mice (Fig. 2C).

Not all phenotypic parameters, however, were different between ICV and IV treatment groups. At early time points both treatment groups were visually indistinguishable from unaffected controls (Fig. 2D–F). Later on despite the differences in body weight and survival, animals in both treatment groups developed mild ear and tail necrosis around 50–60 days of age (Fig. 2G), similar to previous reports [9,14,18]. However, the distal necrosis resolved shortly thereafter and did not progress further.

Time-to-right is an established means to monitor gross mobility for SMA mice [19]. Therefore, SMA mice were subjected to a time-to-right assessment beginning on PND10. Interestingly, IV treated animals begin righting themselves at an earlier age than

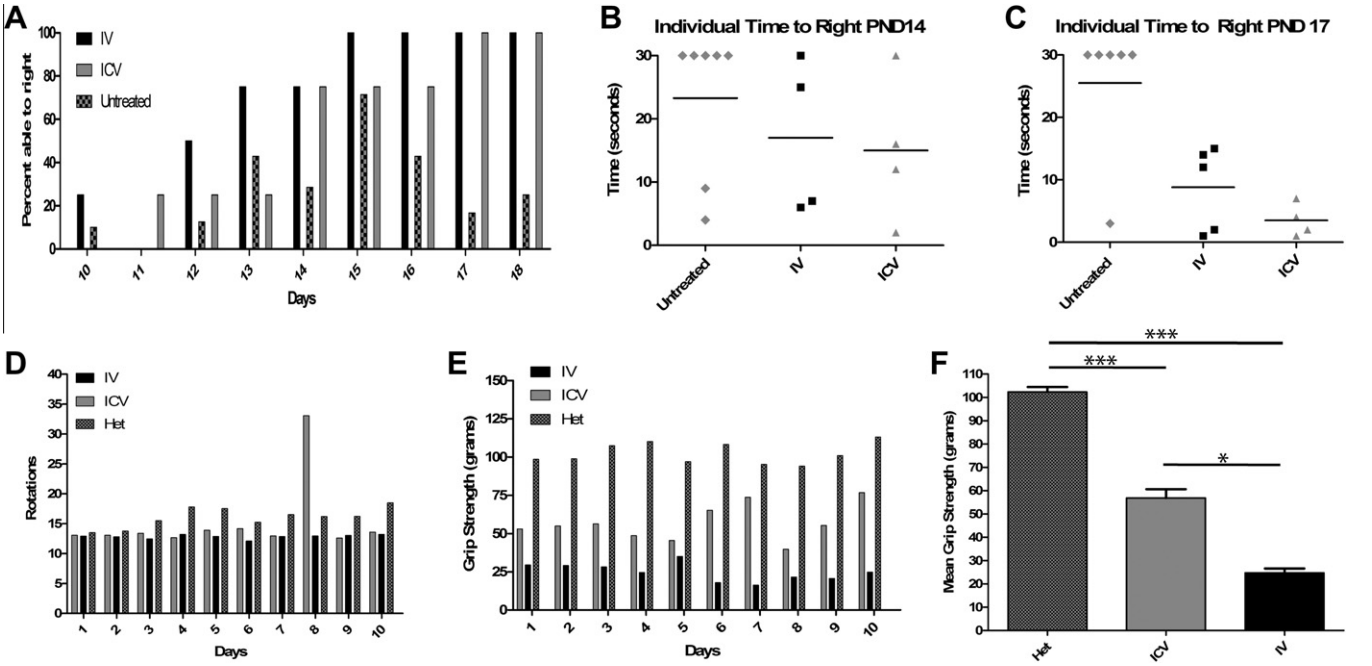

Fig. 3. ICV and IV scAAV9-SMN treatment improves motor function in SMA animals ($Smn^{-/-}$; $SMN2^{+/+}$; $SMN\Delta7^{+/+}$). (A) Percent of pups able to right themselves on PND10–PND18. Time it took for each individual pup assessed to right on (B) PND14 and (C) PND17. (D) Rotorod performance and (E) Grip strength assessed for 10 consecutive days. (F) Mean grip strength quantified from the 10 day course of assessment. (D–F) Unaffected het controls ($Smn^{+/-}$; $SMN2^{+/+}$; $SMN\Delta7^{+/+}$) $n = 10$, ICV treated $n = 2$, and IV treated $n = 2$. All tested mice >80 days of age.

ICV treated animals and a greater percentage of the IV treated animals as compared to the ICV treated animals were able to right throughout PND10 to PND18 (Fig. 3A). However, by PND17 ICV animals that did right, righted faster than IV treated animals (Fig. 3B and C). Later in life, motor function and coordination in surviving animals was measured by rotarod and grip strength tests. Both ICV and IV treated animals performed as well as normal animals in the rotarod test (Fig. 3D) indicating that motor coordination, as measured by this assay, is fully rescued regardless of the route of injection. Grip strength analysis revealed that ICV treated animals have significantly better forearm strength when compared to IV treated animals (Fig. 3E and F). Likewise, ICV treated animals also have slightly larger muscle fibers (Fig. 4), perhaps accounting for the difference in strength observed in the grip strength assays. As expected based on their dramatic improvement in weight and survival, both IV and ICV treated animals have significantly improved tibalis anterior fiber size when compared to untreated SMA controls (Fig. 4A–D).

Recently, four reports have demonstrated that viral-mediated gene replacement in SMA can significantly rescue the SMNΔ7 mouse model [8–11]. These reports differ in the usage of a variety of parameters in their therapeutic approach including: viral serotype, viral promoter, route of injection, and time of injection [9]. Together these studies represent the most profound improvement in phenotype and survival seen in the SMNΔ7 mouse model to date. However, without a direct comparison, it is difficult to compare these studies in order to determine the most efficient and effective course of treatment and to assess serotype and promoter activity that can profoundly impact gene expression in AAV vectors [20,21].

This study was performed to directly compare the influence of the injection route on the SMA phenotype. Here we chose to compare the two injection techniques that have been used thus far in viral gene therapy treatment of SMA: ICV and IV injections [8–11]. In doing so, we have found that route of injection makes a dramatic difference in survival and mouse phenotype. ICV injections have proven to be more effective in rescuing the SMNΔ7 mouse. We propose that this is due to the direct introduction of virus into the central nervous system (CNS) [12]. Because SMA is primarily a disease of the CNS, using ICV injections to physically overcome the blood–brain barrier is advantageous. Conversely, systemically injected viral particles must cross the blood–brain barrier before they are able to transduce motor neurons, reducing their efficiency and requiring more viral particles to achieve results similar to those of the ICV injection. However, it is known that there is a need for SMN protein in the peripheral organ systems, including, but not limited, to the heart [18,22,23]. While IV injections are able to meet this need directly, it is likely that some of the ICV injected vector is able to escape the blood–brain barrier in the same fashion that the IV delivered vector is able to penetrate it, especially within the context of SMA compromised neonatal animals. Thus, ICV injections are able to meet the need for SMN protein in peripheral tissues, while first meeting the primary need for SMN protein in the CNS, resulting in a more complete phenotypic rescue.

When considering the clinical translation of these approaches, ICV injections, while easily executed in mice, are difficult to perform in humans. However, intrathecal injections, while still not as straightforward as IV administration, directly overcome the blood–brain barrier and can be performed in safely in humans [24]. Additionally, intrathecal injections have recently been shown to result in efficient motor neuron transduction [20] when using AAV9. Thus, intrathecal delivery may prove to be a desirable route of administration should AAV gene therapy for SMA reach clinical trials. While the results of this study emphasize the importance of route of delivery when examining gene therapy in the context of SMA, route of delivery is an important component of any therapeutic.

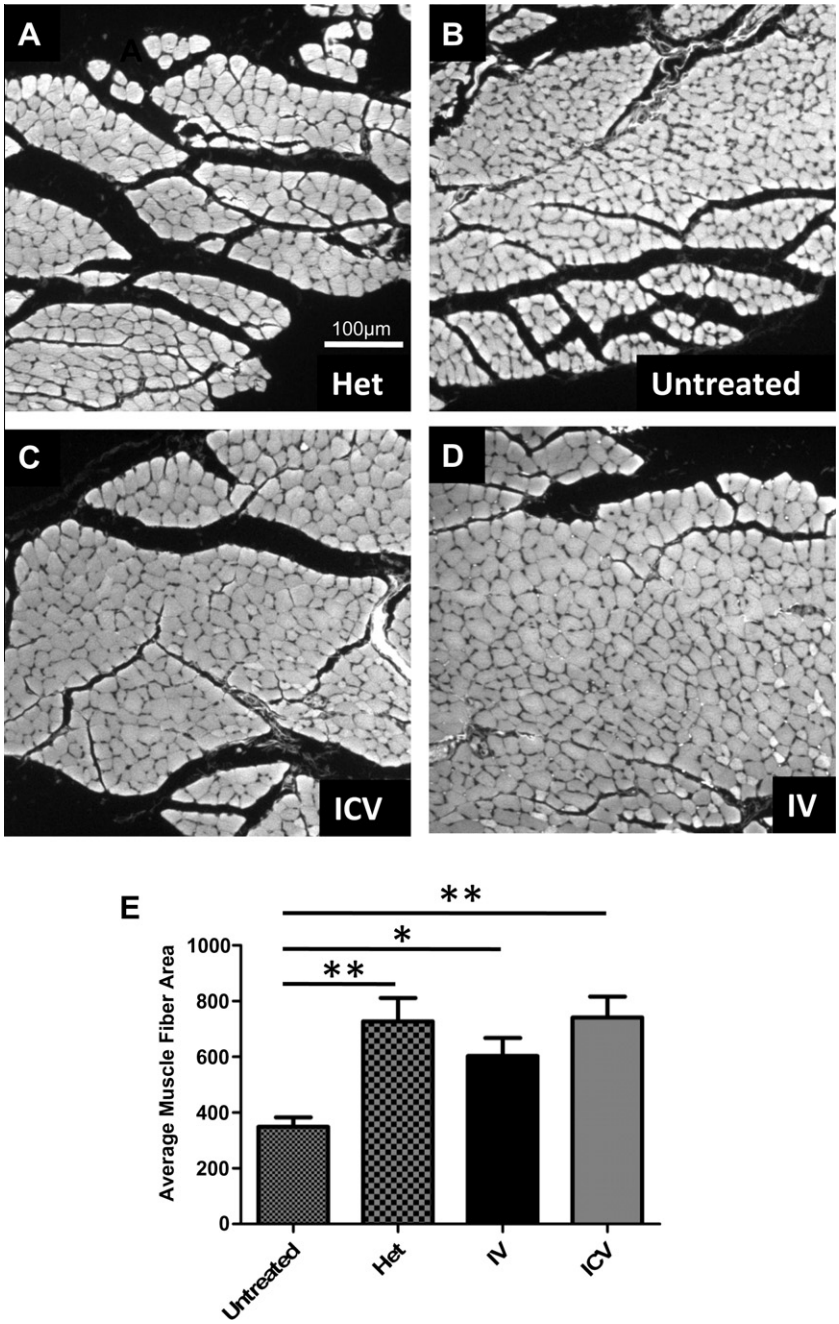

Fig. 4. Treatment with scAAV9-SMN results in increased muscle fiber size. (A–D) Representative muscle cross sections taken at 10× of an unaffected het control ($Smn^{+/-}$; $SMN2^{+/+}$; $SMN\Delta7^{+/+}$), (B) untreated SMA ($Smn^{-/-}$; $SMN2^{+/+}$; $SMN\Delta7^{+/+}$), (C) ICV treated SMA animal, and (D) IV treated SMA animal. (E) Average tibialis anterior muscle fiber size (in um^2) at P14 following treatment with 2×10^{10} viral particles. Untreated SMA $n = 5$, Unaffected het $n = 8$, IV treated $n = 8$, and ICV treated $n = 5$.

Acknowledgments

We thank John R. Marston for expert technical assistance and Hansjörg Rindt, PhD for his assistance in statistical analysis. This work was supported by Grants from the National Institutes of Health [R01 HD054413; R01 HS41584], a training fellowship for JJG [NIGMS T32] and a Grant from SMA Europe (MS).

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