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ARTICLE

Efficacy and biodistribution analysis of intracerebroventricular administration of an optimized scAAV9-*SMN1* vector in a mouse model of spinal muscular atrophy

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Spinal muscular atrophy (SMA) is an autosomal recessive disease of variable severity caused by mutations in the *SMN1* gene. Deficiency of the ubiquitous SMN function results in spinal cord α-motor neuron degeneration and proximal muscle weakness. Gene replacement therapy with recombinant adeno-associated viral (AAV) vectors showed therapeutic efficacy in several animal models of SMA. Here, we report a study aimed at analyzing the efficacy and biodistribution of a serotype-9, self-complementary AAV vector expressing a codon-optimized human *SMN1* coding sequence (co*SMN1*) under the control of the constitutive phosphoglycerate kinase (PGK) promoter in neonatal SMNΔ7 mice, a severe animal model of the disease. We administered the scAAV9-co*SMN1* vector in the intracerebroventricular (ICV) space in a dose-escalating mode, and analyzed survival, vector biodistribution and SMN protein expression in the spinal cord and peripheral tissues. All treated mice showed a significant, dose-dependent rescue of lifespan and growth with a median survival of 346 days. Additional administration of vector by an intravenous route (ICV+IV) did not improve survival, and vector biodistribution analysis 90 days postinjection indicated that diffusion from the cerebrospinal fluid to the periphery was sufficient to rescue the SMA phenotype. These results support the preclinical development of *SMN1* gene therapy by CSF vector delivery.

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INTRODUCTION

Spinal muscular atrophy (SMA) is a severe, autosomal recessive neuromuscular disease that represents the most common genetic cause of infant death, with an incidence of approximately 1 in 10,000 live births and a carrier frequency of 1 in 40–60.[1–3] SMA is caused by homozygous loss of the *SMN1* telomeric gene function by deletion, conversion or mutation, leading to reduced levels of the full-length SMN protein.[4–6] SMN is ubiquitously expressed and involved in multiple aspects of RNA metabolism, including splicing.[7–9] SMN deficiency affects multiple tissues and organs at variable extent, although the neuronal tissue is invariably affected resulting in α-motor neuron degeneration in the spinal cord with subsequent neuromuscular junction dysfunction and proximal muscle weakness.[10,11] The human genome contains a centromeric *SMN2* gene, a highly homologous version of *SMN1* which differs in a translationally silent C to T transition in exon 7 (ref. 5). The mutation disrupts an exonic splicing enhancer and results in enhanced skipping of exon 7 and synthesis of only 10% of full-length transcripts.[12] The truncated SMNΔ7 protein is highly unstable and rapidly degraded. In general, the *SMN2* copy number—and thus the total amount of full-length SMN—is inversely correlated with the severity of the disease.[13–15]

SMA is generally classified into five clinical variants (type 0 to 4) according to age of onset and severity of symptoms.[16] Type-1 SMA accounts for ~50% of all patients, affects infants under 6 months of age and is lethal within the first 2 years of life.[17] A fundamental strategy for treating SMA is to increase SMN levels in the affected tissues: this has been attempted by modulating *SMN2* exon 7 splicing, by increasing *SMN2* transcriptional levels, or by *SMN1* gene replacement with recombinant adeno-associated viral (AAV) vectors.[18–22] We and others previously reported that intravenous (IV) administration of a self-complementary, serotype-9 (scAAV9) vector expressing a human *SMN1* cDNA gene rescues the phenotype of SMNΔ7 mice, a severe animal model of the disease.[23–27] AAV9 vectors are able to cross the blood–brain barrier (BBB) and mediate transgene expression in the central nervous system (CNS) in rodents and larger animals.[28–32] However, since high doses of vector are required to deliver efficaciously a transgene to the CNS by IV injections and a transient hepatitis that is controlled by a short course of

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glucocorticoid therapy has been associated to this route of administration,[33,34] other delivery modes have been investigated in preclinical models, such as the intramuscular, intracerebroventricular (ICV) and combined ICV and intrathecal delivery.[27,35,36] In particular, one study showed that administration of an AAV9 vector directly in the cerebrospinal fluid (CSF) leads to rapid and long lasting correction of SMN levels and phenotypic rescue of SMNΔ7 mice at lower vector doses compared to a systemic administration.[37]

In this study, we investigated the therapeutic efficacy of administering a scAAV9 vector expressing a codon-optimized (co) version of the human *SMN1* cDNA under the control of the phosphoglycerokinase (PGK) promoter (scAAV9.PGKco*SMN1*) in the ICV space in neonatal SMNΔ7 mice. We show prolonged and robust correction of the SMA phenotype at a dose of 4×10^{10} vg/mouse (3×10^{13} vg/kg), with median and maximum survival of 346 and 406 days respectively, the longest reported so far in this animal model. Vector and protein biodistribution analysis showed robust transduction of the CNS and expression of *SMN1* at substantial levels in liver, skeletal muscles, and heart. IV administration of different doses of the same vector in combination with ICV administration led to a significant increase of genome and protein levels in the peripheral organs but not in the CNS, did not prolong survival and provided no obvious additional benefit. This study therefore supports the concept that CSF delivery of an AAV vector could be sufficient to treat SMA patients.

Table 1 Study design and overview of the different animal cohorts included in the study

N (mice)	ICV (vg/mouse)	IV (vg/mouse)	Ratio IV/ICV	Total dose in (vg/kg)
ICV delivery:				
16	2.5E10			1.9E13
16	4E10			3.0E13
16	1E11			7.5E+13
ICV+IV delivery:				
16	2E10	5E9	0.25	1.9E13
16	2E10	2E10	1	3.0E13
16	2E10	8E10	4	7.5E+13

The doses of AAV9-SMN1 administered in the various experimental groups are reported and expressed as total vg/mouse, and for better comparison as vg/kg on the right side of the table. The ICV+IV delivery groups received beside the constant ICV dose, a systemic injection through the facial vein; the Ratio indicates the proportion between vector doses administered through the two delivery routes. Mice (n=8 per group) were euthanized at postnatal day 90 (PND90) and organs were harvested for the biodistribution study. The other half number of mice per group was used for the survival study (n=8, with equally males and females). The same number of uninjected WT and KO sex-matched littermates was used as controls respectively.
ICV, intracerebroventricular.

RESULTS

ICV administration of AAV9-*SMN1* increases survival and rescues the SMA phenotype of SMNΔ7 mice

The scAAV9-co*SMN1* vector used in this study contains a codon-optimized human *SMN1* coding sequence and a chimeric intron under the control of the constitutive PGK promoter, as previously described.[24] Systemic, IV delivery of this vector (referred hereafter as AAV9-*SMN1*) at a dose of 4.5×10^{10} vg/mouse showed therapeutic efficacy in the SMNΔ7 mouse model of severe SMA.[24] In the present study, we aimed at testing the efficacy of ICV administration of the vector in the same animal model, by injecting escalating doses of AAV9-*SMN1* (2.5×10^{10}, 4×10^{10}, and 10^{11} vg/mouse) into the ICV space at birth (PND0) in 16 mice per group (Table 1). As shown in Figure 1a, the lowest dose of AAV9-*SMN1* was already sufficient to prolong the survival of SMNΔ7 mice from <14 days to up to 1 year (median survival: 201 days). The intermediate dose, corresponding to 3×10^{13} vg/kg (weight of SMNΔ7 mice at PND0: 1.34 ± 0.18 g, $n = 16$) provided the longest, statistically significant median survival of 346 days (Mantel-Cox log-rank test, P-value = 0.0032). The first death within this group occurred at day 208 and the longest survival was 406 days. The group of mice treated at the highest dose, corresponding to 7.5×10^{13} vg/kg, showed a higher degree of early mortality compared to the other groups, with half of the mice dying before 3 months of age and an overall median survival of 154 days. However, mice surviving over 3 months exhibited a median survival of 284 days and the highest observed survival, with a mouse euthanized at the end of the study at the age of 510 days (Figure 1a).

A second cohort of mice received at birth a constant ICV dose of 2×10^{10} vg/mouse combined with three different doses (5×10^{9}, 2×10^{10}, and 8×10^{10} vg/mouse) delivered IV, corresponding to an ICV:IV ratio of 0.25, 1, and 4. The median survival observed in the three groups was 283, 188, and 262 days, a statistically nonsignificant difference with respect to the 201 median survival of mice receiving an ICV only dose of 2.5×10^{10} vg/mouse (Mantel-Cox log-rank test, P-value = 0.346) (Figure 1b). Of note, we observed a statistically significant higher median survival in mice receiving an ICV dose of 4×10^{10} vg/mouse (346 days, Figure 1a) compared to mice receiving the same dose split 1:1 by ICV+IV administration (188 days, ratio 1 in Figure 1b) (Mantel-Cox log-rank test, $P = 0.023$).

We then assessed the effect of AAV9-*SMN1* administration on the growth of SMNΔ7 mice, which undergo a significant loss of weight starting around day 9 when untreated (3.3 ± 0.9 g at PND9 versus 6.1 ± 1 g in WT controls), and die before day 14 (Figure 1c). All treated mice showed a progressive increase in body weight reaching a plateau around 110 days, with no significant differences between groups, with the exception of mice treated at 1×10^{11} vg/mouse that lost weight between day 28 and 50 compared to the other treated animals ($P < 0.05$) due to the mice that died early. Mice surviving beyond day 50 showed no significant difference in growth compared to the other mice (Figure 1c). Overall, all treated mice remained smaller than WT control animals.

Biodistribution of AAV9-*SMN1* in SMNΔ7 mice after ICV and ICV+IV administration

We analyzed the biodistribution of AAV9-*SMN1* by quantifying the vector copy number (VCN) in the spinal cord and peripheral tissues in separate cohorts of SMNΔ7 mice injected at birth ICV or ICV+IV and sacrificed 90 days postinjection (Figure 2). Primers and probes were specifically designed to distinguish the codon-optimized human *SMN1* transgene from the h*SMN2* sequences introduced in the SMNΔ7 mouse genome. Mice that received ICV injections at a dose of 4×10^{10} vg/mouse showed a high VCN in the spinal cord (6.9 ± 1.3 vector copies per diploid genome, vg/dg) and a comparable amount of vector in the liver (Figure 2a,b). The liver tropism of AAV9 in mice is known and was in line with previous reports.[38,39] The group of mice

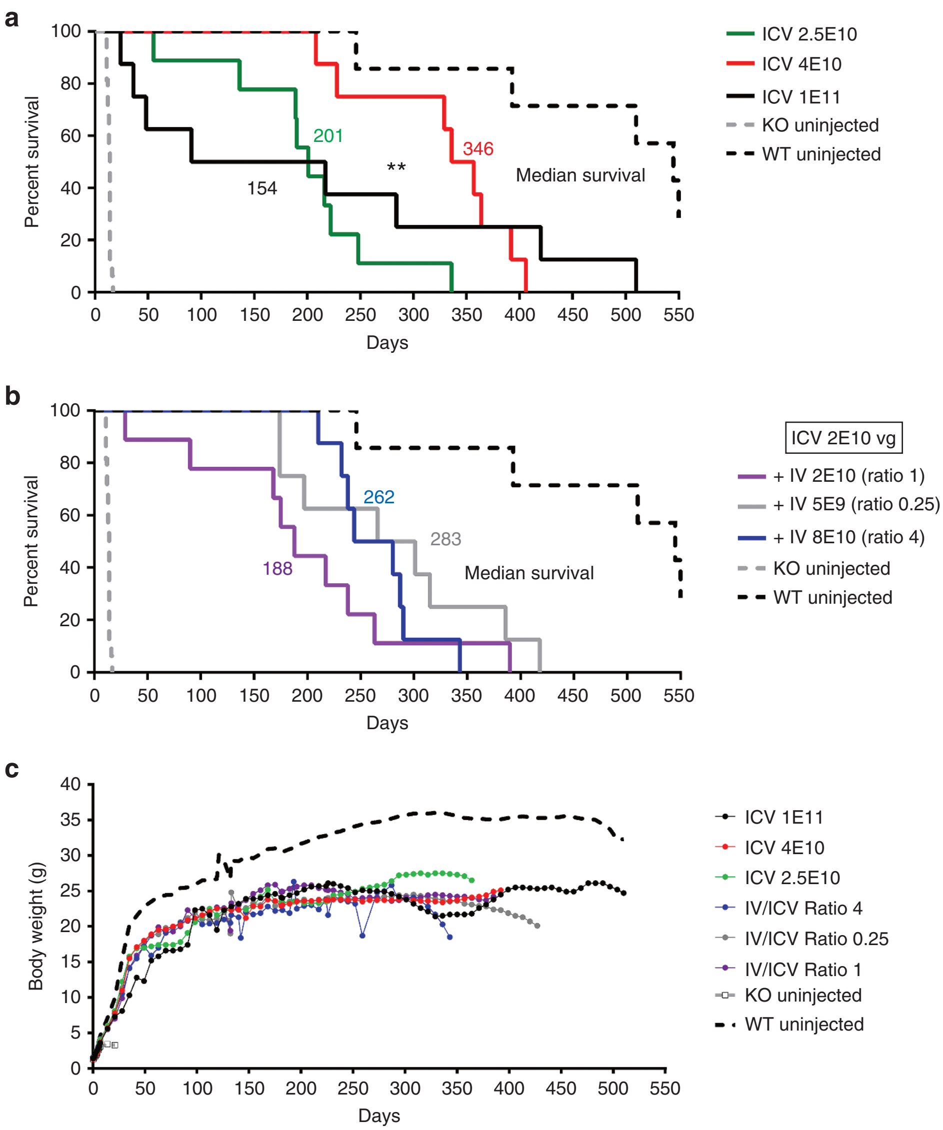

Figure 1 Effect of single ICV and ICV+IV combined injections of AAV9-SMN1 in SMNΔ7 mice. Kaplan-Meier survival curves (a, b): all AAV9-SMN1 doses prolonged lifespan. (**a**) Intracerebroventricular (ICV) injection of AAV9-SMN1 at various doses (2.5E10 vg, 4E10 vg, and 1E11 vg/mouse) in newborn SMNΔ7 mice. The median survival is given as number next to each curve ($n = 8$ per group). (**b**) Combination of ICV (2E10 vg/mouse) and intravenous (IV) vector administration at various ratios (ratio 0.25: 5E9 vg IV + 2E10 vg ICV/mouse, ratio 1: 2E10 vg IV + 2E10 vg ICV/mouse, and ratio 4: 8E10 vg IV + 2E10 vg ICV/mouse). The median survival is given as a number next to each curve ($n = 8$ per group). Untreated WT ($n = 8$) and KO mice ($n = 8$) were used as controls, and groups were compared using the log-rank test in **a** and **b**. (**c**) Body weight of untreated and treated SMNΔ7 mice with AAV9-SMN1 (ICV and ICV+IV at various doses) ($n = 16$ mice per group until day 90, which includes $n = 8$ mice/group from the biodistribution study, and thereafter "n" depending on the number of surviving mice).

that received a dose of 2.5×10^{10} vg/mouse showed a lower VCN in the spinal cord (2.4 ± 1 vg/dg). Both groups showed a low VCN in heart (1.8 ± 0.5, Figure 2c) and in the *Tibialis anterior* (TA) and *Gastrocnemius* (GA) skeletal muscles (TA: 0.2 ± 0.1; GA: 0.1 ± 0.02, Figure 2d,e). In mice treated at the highest dose of 10^{11} vg/mouse that survived until 90 days postinjection, we found a VCN in spinal cord and liver comparable to the intermediate dose group (Figure 2a,b) but a significantly higher vector accumulation in skeletal muscles and particularly in the heart (17.3 ± 9.4 vg/dg) (Figure 2c–e), likely due to a significant passage of vector toward the peripheral organs.

We then assessed vector distribution in the ICV+IV cohorts: VCN in the spinal cord remained constant at increasing IV doses from 5×10^9 to 8×10^{10} vg/mouse (1.4 ± 0.2, 1.3 ± 0.2, and 1.6 ± 0.3 vg/dg, respectively), at levels comparable to those observed in mice injected ICV only at a dose of 2.5×10^{10} vg/mouse (2.3 ± 1 vg/dg), while VCN in liver and heart increased with the IV vector dose, up to >15 and >9 vg/dg respectively (Figure 2a–c). These data suggest that CNS transduction is achieved essentially by the vector delivered ICV, while vector delivered IV transduces mainly the peripheral organs, and particularly liver and heart. Only the highest IV dose (8×10^{10} vg/mouse, ratio 4) resulted in a significant increase in vector copies in skeletal muscles (TA: 1.6 ± 0.5 vg/dg; GA: 0.7 ± 0.2), indicating a lower tropism of AAV9 for this tissue in newborn mice.

Synthesis of SMN protein in the spinal cord and peripheral organs of treated SMNΔ7 mice

We analyzed the level of SMN protein in various tissues of SMNΔ7 mice 3 months after AAV9-*SMN1* administration by western blotting. Densitometry data were normalized against the expression

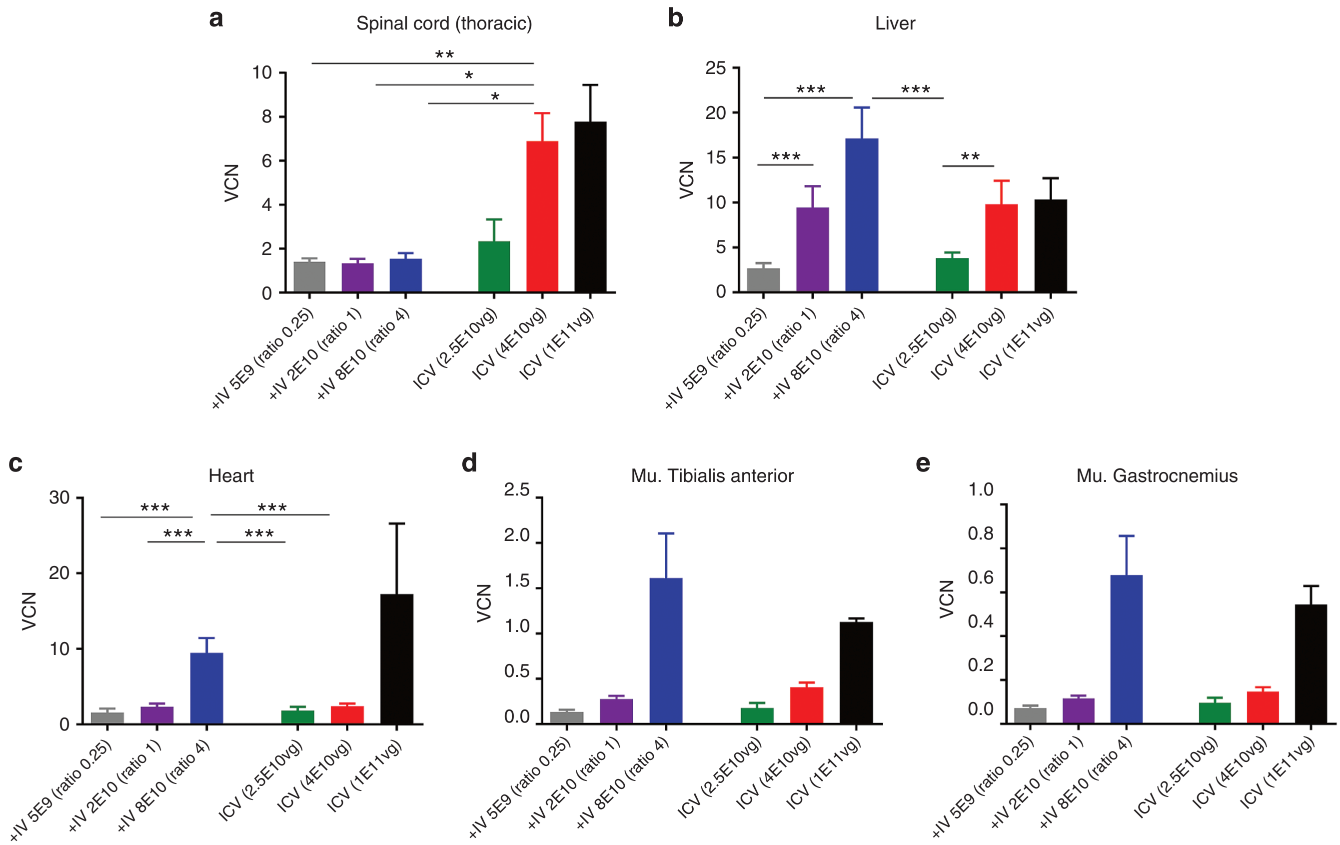

Figure 2 AAV9-SMN1 vector biodistribution 3 months after injection. (**a–e**) Vector copy number (VCN), which corresponds to viral genomes/diploid genome (vg/dg), was quantified in various tissues (thoracic part of the spinal cord, liver, heart, tibialis anterior, and gastrocnemius muscles) of mutant SMNΔ7 mice 90 days post-injection. Mice received the vector at different doses by intracerebroventricular injection alone (ICV, 2.5E10 vg, 4E10 vg and 1E11 vg /mouse) and in combination with intravenous (IV) delivery at various ratios (ratio 0.25: 5E9 vg IV + 2E10 vg ICV/mouse, ratio 1: 2E10 vg IV + 2E10 vg ICV/mouse, and ratio 4: 8E10 vg IV + 2E10 vg ICV/mouse) ($n = 8$ for each group, with exception of the group treated by ICV at 1E11 vg/mouse, $n = 3$). Tissues from untreated WT ($n = 8$) and KO mice ($n = 3$) were used as negative controls (undetectable, data not shown). VCN with values <0.0005 were considered as undetectable. Spinal cord: one-way ANOVA; liver, heart, tibialis anterior, and gastrocnemius muscles: two-way analysis of variance and Bonferroni post-test; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

of a constitutive protein (tubulin) and expressed as SMN/Tubulin ratios. Mice in the group with the highest median survival (ICV administration, 4×10^{10} vg/mouse) and two ICV+IV groups (ratio 1 and ratio 4) were compared to aged-matched WT mice and to untreated SMNΔ7 littermates at the age of 14 days. SMN expression data were in line with vector biodistribution in the same groups of mice. Mice in all groups had similar levels of SMN expression in the spinal cord, reaching >50% of the WT values, indicating significant restoration of overall protein levels and confirming that IV delivery has no major impact in transducing the spinal cord (Figure 3a). Mice in the ICV+IV ratio 4 group showed the highest amount of SMN protein in peripheral organs (Figure 3b–e), reaching endogenous SMN levels in the heart (Figure 3c).

We further investigated SMN protein expression by immunohistochemistry in a separate group of mice treated ICV with the dose that allowed for the longest survival in the long-term study (4×10^{10} vg/mouse). Mice were euthanized 3 months post-injection together with age-matched WT controls and untreated SMNΔ7 mice. The lumbar SC was cryostat-cut and processed for immunohistochemistry. The staining revealed robust SMN expression in the lumbar spinal cord of WT mice with an intense staining in α-motor neurons (Figure 3g). On the contrary, the SMN signal was very low and scattered in the same region of untreated SMNΔ7 mice. We detected a significant recovery of SMN expression, with the typical staining in α-motor neurons, in the lumbar spinal cord of mice treated with the AAV9-*SMN1* vector (Figure 3g).

DISCUSSION

SMA is caused by partial or complete loss of the *SMN1* gene function and is classified in five clinical variants of increasing severity depending on the level of residual, ubiquitous SMN protein. Past attempts to treat murine models of SMA by gene replacement therapy have shown very significant results in terms of efficacy. Administration of AAV vectors expressing the human SMN protein caused partial rescue of the SMA phenotype and significant prolongation of survival in the SMNΔ7 severe mouse model.[24–26,36,37] However, because of differences in the delivery route, dosage, age at injection, viral vector serotype, and transgene expression cassette, these studies are difficult to compare and defining the best strategy to treat this disease remains challenging. Nevertheless, on the basis of the promising results obtained by systemic IV delivery, a phase 1/2 clinical trial started in 2014 at Nationwide Children's Hospital (Ohio State University, Columbus USA; trial ID: NCT02122952), with the aim of proving safety and efficacy of gene therapy by intravascular administration of a scAAV9 vector expressing the human *SMN1* transgene.

The SMA disease is considered to be primarily related to lower motor neuron loss in the spinal cord. However, whether targeting motor neurons only would be sufficient to treat the disease,

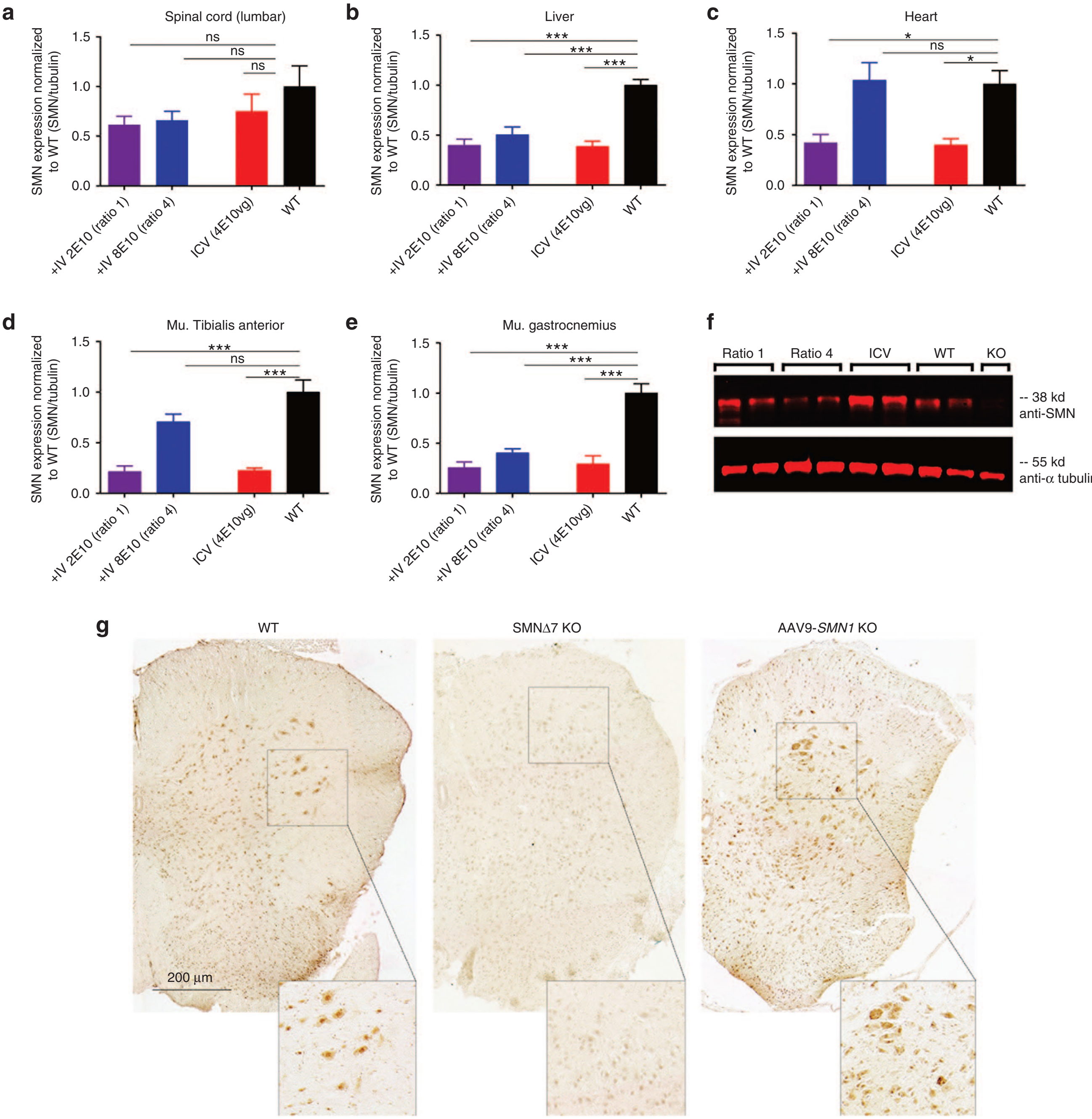

Figure 3 SMN protein localization in the spinal cord three months after vector administration in SMNΔ7 mice. (**a–e**) Intracerebroventricular-only delivery provided a higher amount of SMN protein in the lumbar part of the SC (shown in red, 4E10 vg/mouse). With the highest given IV dose (ratio 4) more SMN protein was detected in the heart and TA muscle. SMN levels were normalized to the loading control (α-tubulin) and the WT controls (set as 1). (**f**) Exemplary western blot analysis of spinal cord samples. 30 μg protein lysates were loaded (except for the muscles: 60 μg), $n = 8$ for each group. Spinal cord: one-way analysis of variance (ANOVA), liver, heart, tibialis anterior, and gastrocnemius muscles: two-way ANOVA and Bonferroni post-test; $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. (**g**) Immunohistochemistry for SMN protein in the anterior horn of the lumbar spinal cord. Pictures from untreated SMNΔ7 WT (left), KO (middle), and AAV9-SMN1 treated KO (right) mice are shown (AxioSCAN microscope). Scale bar, 200 μm. Insets show α-motor neurons at higher magnification.

particularly in type-I patients presenting associated pathologies unrelated to the nervous system,[40–42] is a highly controversial issue. In particular, the question of whether transduction of peripheral tissues, such as skeletal muscles and heart, would be necessary for all patients and all clinical variants is of high importance.[40,42–47] Several studies have suggested that SMN expression in muscles might be advantageous,[48–52] although SMN restoration in skeletal muscles alone did not rescue the SMA phenotype in transgenic mice.[53] A recent report showed that SMN reduction solely in skeletal muscles had no phenotypic effect in SMA mice carrying two *SMN2* copies, strongly suggesting that low SMN levels might be sufficient for a normal muscle function in moderately severe SMA patients.[46] In this study, we show that ICV delivery of scAAV9-*SMN1* is sufficient in rescuing the phenotype of severely affected SMNΔ7 mice and provide substantial levels of SMN also to peripheral organs, while no significant additional benefit was observed by combining an IV coadministration that further increased SMN expression in muscle and heart. ICV delivery of the scAAV9-*SMN1* vector at an optimal dose of 3×10^{13} vg/kg in newborn SMNΔ7 mice led to a complete rescue of the disease-associated perinatal mortality and a significant recovery of the clinical symptoms, with the longest median and maximal survival reported so far in this animal model, *i.e.*, 346 and 406 days. These results were obtained with an average of 7 vector copies per diploid genome in the spinal cord measured 90 days postinjection, which led to synthesis of SMN protein in the CNS at levels comparable to those observed in WT animals. Previous studies using the same vector delivered IV or IM resulted in a median lifespan of 163 and 199 days, respectively,[24,35] while ICV delivery of a similar vector, scAAV9.CBA.SMN, achieved a maximal survival of 282 days.[37] Delivery of a higher vector dose, up to 7.5×10^{13} vg/kg allowed to achieve a longer survival in 30% of the mice, up to more than 500 days. Some degree of early mortality was observed in some groups of treated mice, and was more pronounced in the group of animals given the highest vector dose. We could not determine the cause of death in these animals, which may be related to variabilities in vector response, or other factors associated to the procedure.

It is well established that serotype-9 AAV vectors can cross the BBB when administrated either intravenously or into the CSF.[28,30,54] Accordingly, we found significant VCNs of AAV9-*SMN1* in liver, heart, and two types of skeletal muscles 90 days after ICV delivery, with SMN protein levels reaching 25 to 50% of the normal levels in these organs. These levels were apparently sufficient to correct the disease phenotype in the whole body. In fact, splitting the efficacious dose of 4×10^{10} vg/mouse in a combined ICV+IV delivery in a 1:1 ratio reduced the median survival to 188 days, compared to the 346 days obtained by the full dose administrated ICV only. Raising the IV dose up to 8×10^{10} vg/mouse (IV:ICV ratio of 4:1) increased the survival to a median of 262 days but was still less efficacious than a unique dose of 4×10^{10} vg administrated ICV. Increasing the IV dose caused no augmentation of vector and protein levels in the CNS, while it increased significantly the vector load in the liver and heart, two organs targeted at high efficiency in the mouse by an AAV9 vector. These results clearly support the CSF administration as a delivery route for gene therapy of SMA by a scAAV9 vector. This is particularly plausible for type 2 to 4 SMA patients in whom peripheral abnormalities are uncommon,[55,56] and given the results on vector biodistribution, it could be beneficial even for type 1 patients that manifest additional cardiac and/or vascular defects.[44,57,58] A recent study in a large animal model of SMA supports these conclusions and confirms the therapeutic benefit of administering an AAV9 vector directly in the CSF. Knock-down of SMN obtained by intrathecal delivery of a scAAV9-shRNA vector targeting the *SMN1* gene in newborn pigs resulted in a SMA phenotype, with loss of spinal motor neurons and proximal muscle weakness.[59] Restoration of SMN levels and a striking phenotype correction were observed after pre- and postsymptomatic delivery of a scAAV9-*SMN* vector in the *cisterna magna* of these animals.[59] The impressive phenotype amelioration after disease onset is highly encouraging, as it widens the therapeutic window of AAV-mediated gene therapy beyond the evidence obtained in the mouse model.[32,60]

Additional advantages of administering an AAV vector directly into the CSF are related to potential immune responses. In humans, anti-AAV antibody titers are lower in CSF than in serum, and it has been reported that high serum levels of anti-AAV9 antibodies only partially blocks CSF-mediated gene transfer to the brain in dogs.[54] This and previous studies predict that a lower vector dose may be required to achieve a therapeutic effect when an AAV vector is administrated in the CSF as compared to an IV route, with a consequent reduction of the predicted T-cell-mediated liver toxicity and a reduced need for steroid treatment or immune suppression.[34,37,61]

MATERIALS AND METHODS

Generation of recombinant scAAV9-co*SMN1* vectors

The human *SMN1* AAV2 plasmid has been described previously.[24] Briefly, it contains the inverted terminal repeat of AAV2, a codon-optimized version of the human *SMN1* coding sequence under the control of the human PGK promoter, a chimeric intron and a SV40 polyadenylation signal. Adenovirus-free pseudotyped rAAV2/9 vector preparations were generated by triple transfection of HEK293 cells with the plasmids pSMNopti, pXX6 encoding adenovirus helper functions and p5E18-VD2/9 that contains the AAV2 rep and AAV9 cap genes. Recombinant vectors were purified by double cesium chloride ultracentrifugation gradients from cell lysates, followed by dialysis against sterile formulation buffer PBS-MK (1 mmol/l $MgCl_2$- 2.5 mmol/l KCl). The viral preparations were desalted and concentrated using Amicon Ultra columns (Ultra cell 100 K; Milipore, France). Physical particles were quantified by real-time polymerase chain reaction (PCR) and vector titers are expressed as viral genomes per ml (vg/ml). The pseudotyped packaged vector was designated scAAV9-co*SMN1* (lots: T12079.VEC; T12080.VEC).

Animals

SMNΔ7 founder mice were purchased from Jackson (stock number: 5025). Beside the disruption of exon2 of the endogenous mouse *Smn* gene, these mice harbor two transgenic alleles, the entire human *SMN2* gene and a *SMN1* cDNA lacking exon 7 (*SMNΔ7*). WT mice corresponded to *Smn*+/+, *SMN2*+/+, *SMNΔ7*+/+, heterozygous to *Smn*+/-, *SMN2*+/+, *SMNΔ7*+/+ and knockout mice to *Smn*-/-, *SMN2*+/+, *SMNΔ7*+/+. Heterozygous breeding pairs were mated and litters were genotyped at birth. Mice were kept under a 12-hour light 12-hour dark cycle and fed with a standard diet, with food and water *ad libitum*. Care and manipulation of mice were performed in accordance with national and European legislations on animal experimentation and approved by the institutional ethical committee.

Vector administration

The study was performed with two scAAV9-co*SMN1* production batches with a titer of 1.8×10^{13} and 1.6×10^{13} viral genomes per ml (vg/ml) respectively. The vectors were diluted accordingly to the treatment group with saline and injections were performed at the day of birth after genotyping. A mounted Hamilton glass micropipette (connected to a 32 G needle; both: NH BIO, France) was used to slowly inject (2–3 seconds) the vector into the lateral ventricle of the right cerebral hemisphere (ICV injection volume: 7 µl). For the systemic delivery, a volume of 30 µl was injected into the facial vein. Noninjected WT or SMNΔ7 littermates were used as controls.

Tissue sampling

Tissues were collected 90 days postinjection for VCN and SMN protein analysis. Mice were deeply anesthetized by intraperitoneal injection of 100 mg/kg ketamine 500 (50 mg/ml, Virbac, France) and 10 mg/kg xylazine (2 %, Rompun, Bayer, France) and intracardially perfused with phosphate buffer solution (PBS) via the left ventricle. The spinal cord (cervical, thoracic, and lumbar parts), heart, gastrocnemius (GA) and tibialis anterior (TA) muscles (the left and right muscles were used for protein and gDNA extraction, respectively) and liver were harvested and immediately flash-frozen in liquid nitrogen.

Immunohistochemistry

Mice dedicated to immunohistochemistry received subcutaneously 0.1 mg/kg buprenorphine (Buprecare, 0.3 mg/ml, Axience, France) and were deeply anesthetized by intraperitoneal injection of 100 mg/kg ketamine 500 (50 mg/ml, Virbac, France) and 10 mg/kg xylazine (2%, Rompun, Bayer, France) and intracardially perfused via the left ventricle with PBS followed by 4% paraformaldehyde. Neural tissues (brain and spinal cord) were postfixated for 24 hours in 4% paraformaldehyde (PFA), equilibrated for 24 hours in 30% sucrose in PBS and quickly frozen in 7.5% gelatine or OCT (CML, France) by using isopentane cooled in dry ice. Serial coronal cryostat (12–14 µm-thick) sections were collected and processed for anti-SMN staining (anti-SMN primary antibody, goat polyclonal Santa Cruz, sc-7804). After a heat-induced epitope retrieval step, with an incubation of the sections for 5 minutes (microwave, 700W) in citrate buffer (DAKO S1699, unmasking solution pH6, 1/10 diluted in distilled water), the sections were subsequently incubated with blocking solution (10% Normal Rabbit Serum (DAKO X0902) + 0.3% Triton X-100 in PBS) for 1 hour at RT and then incubated overnight at 4 °C with primary antibody diluted 1/600 in blocking solution (1/100 Normal Rabbit Serum, Dako X0902, France). After thorough washing (PBS, 3×5 minutes each), antibody staining was revealed using species-specific horseradish peroxidase (HRP)-conjugated secondary antibodies diluted in PBS (rabbit anti-goat HRP, DAKO P0449, Dilution: 1/200, 1 hour, RT). Sections were washed in PBS (3×5 minutes each), and the substrate, DAB+ (3,3′ diaminobenzidine, DAKO K3468, France), was applied for 5 minutes to reveal the staining, followed by a washing step in PBS. Tissue sections were dehydrated and mounted with permanent mounting media (Coverquick 4000, VWR, France) and images were taken using a digital slide scanner microscope (Axio Scan Z1, Zeiss).

Western blot analysis

Tissues were weighed and homogenized in lysis buffer (10 mmol/l Tris-HCl pH7.4, 1 mmol/l EDTA, 1 mmol/l EGTA, 150 mmol/l NaCl, 4 mmol/l sodium pyrophosphate, 100 mmol/l NaF, 2 mmol/l Na3VO4, 0.5% IGEPAL, 1% Triton-X 100 and protease inhibitors (Complete Mini; Roche Diagnostics, France) using MP FastPrep-24 tubes and a Polytron homogenizer (MP Biomedicals, France). After centrifugation (2,600 g, 4 °C, 10 minutes) the supernatant was recovered and kept on ice for 30 minutes with occasionally vortexing. Lysates were cleared (12,000 g, 4 °C, 20 minutes) and proteins were quantified with the Biorad Protein Assay detection kit (Bio-Rad, France). Equal amounts of protein (30 µg of total protein for lumbar part of the SC, liver and heart samples and 60 µg for the GA and TA muscle samples) were loaded on 10 % Mini-Protean TGX precast gels (Biorad, France) and transferred to nitrocellulose membranes (0.2 µm; Life Technologies-Invitrogen, France). Membranes were incubated with Ponceau S staining solution (0.1% (w/v) Ponceau S in 5% (v/v) acetic acid) and cut for separate probing with the anti-SMN and loading control antibody. After blocking for 1 hour at room temperature with the Odyssey blocking buffer (Li-Cor), the membranes were probed overnight with a specific monoclonal anti-SMN antibody (BD #610646, dilution: 1/4,000) and anti-αTubulin antibody respectively (Santa Cruz sc-5286, Dilution: 1/1,000). Secondary antibodies were incubated for 90 minutes at room temperature (IR Dye 680 Goat anti-mouse, A21058 Invitrogen, dilution: 1/10,000). All antibodies were diluted in Dulbecco's phosphate-buffered saline (DPBS)/Odyssey blocking buffer (1/1 dilution). The immunoreaction was visualized and quantified using the Odyssey Infrared Imager (LI-COR Biotechnology).

Genomic DNA extraction and vector copy number analysis

Tissues were weighed (~20–100 mg) and homogenized in 500–700 µl lysis buffer from the Puregene Blood Core kit (Qiagen, France) using MP FastPrep-24 tubes and a Polytron homogenizer (MP Biomedicals, France). After a centrifugation step (2,600 g, 4 °C, 10 min) the supernatant was recovered, 30 µl Puregene Proteinase K (10 mg/ml) was added following overnight incubation under rotation at 55 °C. The genomic DNA (gDNA) was isolated according to the manufacturer's protocol (Puregene Blood Core kit, Qiagen, France), quantified using a Nanodrop ND-8000 spectrophotometer (Thermo Scientific, France) and stored at 4 °C.

Vector copies per diploid genome were quantified by TaqMan analysis, starting from 100 ng mouse gDNA by amplifying the codon-optimized human *SMN1* transgene and *Ttn* as a mouse housekeeping gene. The absolute amount of each gene was obtained by referring to a standard curve consisting of a 10-fold serial dilution of a plasmid containing one copy of the codon-optimized human *SMN1* and *Ttn* gene (six concentrations, ranging from 10–10^6 copies). The absolute number of vector copies is given as VCN (co*SMN1*) per diploid genome and was obtained by dividing the co*SMN1* amount by half of the *Ttn* amount. The real-time PCR reactions were run in simplex with an ABI Prism 7900 HT SDS v2.3 system (Applied Biosystems). Serial dilutions of the plasmid or 100 ng/10 µl mouse genomic DNA were included in a mix containing the absolute QPCR Rox mix (Thermo Scientific, France), 0.3 µmol/l of specific primers, 0.2 µmol/l of the corresponding TaqMan probe and water to a final volume of 25 µl. The run conditions were as follows: 10 minutes 95°C polymerase activation step, followed by 40 cycles of a two-step qPCR (15 seconds of 95 °C denaturation, 1 minute of 60 °C combined annealing/extension). Primers and probes (5′-3′) were used as follows: op*SMN1*-F: TGCTCCTCCCGGTTTCC, co*SMN1*-R: CGCGAGCATCGACTTCAAG, co*SMN1* probe: AGCCGGTGTAAACCACCACACAAGTCTC [5′] VIC [3′] TAMRA. *Ttn*-F: AAAACGAGCAGTGACGTGAGC, *Ttn*-R: TTCAGTCATGCTGCTAGCGC, *Ttn* probe: TGCACGGAAGCGTCTCGTCTCAGTC [5′] FAM [3′] TAMRA.

Statistical analysis

Data were analyzed with Graphpad Prism version 5 (Graphpad Software) and expressed as mean ± standard error of the mean (with $n = 8$, if not otherwise stated). Statistical differences between mean values were tested using one-way analysis of variance and two-way analysis of variance followed by a Bonferroni correction for false discovery rate where appropriate. From the survival data Kaplan–Meier curves were generated and tested using the Mantel–Cox log-rank test. Differences between values were considered to be significant with: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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