Intravenous scAAV9 delivery of a codon-optimized SMN1 sequence rescues SMA mice

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Spinal muscular atrophy (SMA) is the most common genetic disease leading to infant mortality. This neuromuscular disorder is caused by the loss or mutation of the telomeric copy of the ‘survival of motor neuron’ (Smn) gene, termed SMN1. Loss of SMN1 leads to reduced SMN protein levels, inducing degeneration of motor neurons (MN) and progressive muscle weakness and atrophy. To date, SMA remains incurable due to the lack of a method to deliver therapeutically active molecules to the spinal cord. Gene therapy, consisting of reintroducing SMN1 in MNs, is an attractive approach for SMA. Here we used postnatal day 1 systemic injection of self-complementary adeno-associated virus (scAAV9) vectors carrying a codon-optimized SMN1 sequence and a chimeric intron placed downstream of the strong phosphoglycerate kinase (PGK) promoter (SMNopt) to overexpress the human SMN protein in a mouse model of severe SMA. Survival analysis showed that this treatment rescued 100% of the mice, increasing life expectancy from 27 to over 340 days (median survival of 199 days) in mice that normally survive about 13 days. The systemic scAAV9 therapy mediated complete correction of motor function, prevented MN death and rescued the weight loss phenotype close to normal. This study reports the most efficient rescue of SMA mice to date after a single intravenous injection of an optimized SMN-encoding scAAV9, highlighting the considerable potential of this method for the treatment of human SMA.

INTRODUCTION

Spinal muscular atrophy (SMA) is an autosomal recessive neuromuscular disorder characterized by a selective loss of α motor neurons (MNs) in the ventral horn of the spinal cord leading to a progressive muscle atrophy and paralysis. With no effective treatment currently available, SMA remains the leading genetic cause of infant mortality (1). The gene responsible for the most common form of SMA was identified in 1995 and named ‘survival motor neuron’ (Smn) (2). Two inverted copies of Smn are present on chromosome 5. Homozygous disruption (by deletion, conversion or mutation) of the telomeric copy of Smn, termed SMN1, leads to decreased levels of full-length SMN protein (FL-SMN) in MNs (3). The centromeric copy, SMN2, critically differs from SMN1 by a C–T nucleotide transition in exon 7 thereby promoting exon skipping during alternative splicing (3). As a result, SMN2 expression leads primarily to a truncated SMNd7 protein, which cannot fully compensate for the decrease in FL-SMN due to SMN1 disruption. However, SMN2 (whose number of copies differs between patients) can control disease severity by expression of low levels of functional FL-SMN (4). Due to the monogenic and recessive nature of this disease, gene therapy, consisting of the SMN1 gene...
transfer into affected MNs, is an attractive approach. However, a first SMN gene therapy study, consisting of multiple intramuscular injections of a SMN-expressing lentiviral vector, only allowed a minimal extension of the survival (3–5 days) in severe SMA mice (5), possibly due to a restricted temporal window for full SMN expression in this severe mouse model.

Interestingly, adeno-associated virus (AAV) vectors based on a double-stranded genome were demonstrated to have the general ability to bypass the step of second-strand DNA synthesis mediated by the host cells, achieving a greater and faster transgene expression compared with single-stranded AAV vectors, offering new promising perspectives for gene therapy (6). We recently identified self-complementary (sc) AAV9 as a vector of choice for central nervous system (CNS) gene therapy (7,8). We found that this vector had a remarkable capacity to transduce both neurons and astrocytes in adult mice and cats following intravenous delivery, suggesting efficient crossing of the mature blood-brain barrier (7,8). An independent study similarly demonstrated the high potential of self-complementary AAV of serotype 9 (scAAV9) for neuronal transduction, albeit only in neonatal mice (9). In this study, we investigated whether systemic delivery of SMN-expressing scAAV9 into post-natal MNs could prevent or delay disease onset in a transgenic mouse model of severe SMA.

RESULTS

We constructed a scAAV9 vector expressing the human SMN1 gene (hSMN1) under control of the phosphoglycerate kinase (PGK) promoter. Since codon optimization strategies have proven useful to increase translation efficiency (10–12), codon optimization of hSMN1 (pSMNopt) was carried out using a gene optimization algorithm to achieve the highest possible levels of protein in our expression system. To further improve scAAV9-SMN expression, we also inserted a chimeric intron immediately downstream from the PGK promoter (SMNpopt) (Fig. 1A). Heterologous introns have been indeed reported to improve gene expression, by influencing transcripational initiation or elongation (via enhancers or other cis-acting elements), by enhancing mRNA stability in the nucleus (via the process of mRNA splicing), or by facilitating opening of chromosomal domains (13,14). The PGK promoter was chosen for its ability to direct a high, and constitutive expression of foreign genes (15). In particular, the stability of PGK-driven expression of transgenes confers to this promoter a great advantage over the cytomegalovirus promoter whose transcriptional activity has been reported to significantly decrease with time (16). In the CNS, a lentiviral vector expressing lacZ under control of the PGK promoter was shown to drive in vivo expression preferentially in neurons (17).

The efficacy of this optimized cassette (pSMNopt) for SMN expression was first evaluated on primary astrocytes that were derived from SMA severe mice (SMN2+/+, Smn−/−) (18). Cells were transduced with different plasmids carrying either the optimized SMN cassette or the human SMN1 cDNA with or without the chimeric intron, and were compared for transgene expression after 24 h in culture. Western-blot analysis of SMN levels revealed that both optimization of the sequence and insertion of the chimeric intron (pSMNopt) dramatically increased SMN expression (~37-fold versus pSMN, P < 0.0001, n = 3) (Fig. 1B–D). A superiority of pSMNopt (71.7 ± 3.6) versus pSMN (14 ± 0.7) was similarly observed for SMN expression in transfected wild-type (WT) astrocytes (P < 0.0001, n = 3). Transfected WT astrocytes, KO astrocytes (SMN2+/+, Smn−/−) and NSC-34 cells displayed numerous SMN-positive aggregates located mainly in the cytoplasm, but also in the nucleus where they formed complex structures resembling to gems (Fig. 1E). This expression pattern was consistent with that previously described (5,18). The human SMNpopt sequence was thus engineered into the scAAV backbone and a scAAV9-SMNpopt vector was produced and purified as previously described (7).

To verify the potential of this vector for inducing SMN expression after in vivo administration, mild SMA mice (SMN A2G+/−, SMN2+/+, Smn−/−; n = 3) (19) were injected at 6 weeks of age into the cervical and the lumbar segment of the spinal cord with 4.3 × 1011 vector genome (vg) of scAAV9-SMNpopt. SMN expression was investigated by immunofluorescence analysis of transversal spinal cord sections 16 weeks later (Fig. 2A–C). Overexpression of SMN triggered formation of cytoplasmic aggregates and punctuated nuclear granules in cells of the ventral spinal cord, confirming the in vivo functionality of scAAV9-SMNpopt. To further investigate the ability of scAAV9 to target the spinal cord following intravenous injection, 1.5 × 1010 vg of green fluorescent protein (GFP)-expressing scAAV9 were injected into the temporal vein of neonatal (PI) C57Bl6 mice (n = 3). As expected, strong GFP expression was detected in cells and nerve fibers in the ventral spinal cord, including in NeuN-co-labelled neurons (Fig. 2D–F). SMNpopt-expressing scAAV9 (n = 3) were thus administered to mild SMA mice under similar conditions (4.5 × 1010 vg). Four weeks after injection, SMN expression was found in the ventral spinal cord, including in large cells with a typical MN morphology (>20 μm) (Fig. 2G–I). Examination of the transduced cells revealed the presence of SMN in numerous aggregates throughout the cytoplasm, in gems-like punctuated nuclear granules, and along the neurites.

We then determined the effect of systemic scAAV9-mediated SMN gene transfer on the phenotype of SMNdelta7 mice (SMN2+/+, SMNdelta7+/+, Smn−/−) (20). These mice present a particularly severe phenotype, showing weight loss by 11.7 ± 0.2 days of age (P < 0.01) and a mean life expectancy of 13.7 ± 0.6 days. SMNdelta7 mice were injected into the temporal vein at P1 with scAAV9-SMNpopt (4.5 × 1010 vg) (n = 13) and were compared by survival analysis and weight monitoring to non-injected SMNdelta7 (n = 13), heterozygous (SMN2+/+, SMNdelta7+/+, Smn−/−) (n = 20) or WT littermate mice (n = 19). Additional WT (n = 4) and heterozygous (n = 7) mice were injected with the same vector to verify the absence of deleterious side effects of the treatment.

Intravenous scAAV9-SMNpopt delivery in SMA mice allowed restoration of survival in 100% of the treated mice, increasing mean life expectancy to 160 ± 39 days (median = 199 days) compared with 13.7 ± 0.6 days for untreated SMA mice (P < 0.001) (at time of re-submission) (Fig. 3A). No untreated SMA littermate passed the age of 16 days, whereas all treated SMA mice survived from 27 to
over 340 days. One injected SMA mouse was excluded from the analysis at 34 days of age because of inability to feed due to abnormal teeth, and was euthanized at 46 days old for ethical purposes.

The survival curve revealed two groups of mice, a first group that had a median lifespan of 40 days and a second one whose median survival surpassed 242 days (two mice are still alive at 260 and 340 days, at the time of re-submission). In the first group of treated mice that died prematurely, two mice died suddenly at 27 and 32 days of age without presenting signs of SMA such as weakness or muscular paralysis. In one of these mice, gross examination revealed heart enlargement. This pathological feature was recently reported in SMNdelta7 mice (21) and suggests that, at least in this mouse, premature death was due to heart disease that was not offset by adequate SMN supplying in cardiac tissue (possibly due to failure of intravenous scAAV9 delivery).

Remarkably, systemic administration of scAAV9-SMNopti dramatically improved the weight loss phenotype of most treated SMNdelta7 mice (Fig. 3B and C). The scAAV9 treated mice gained weight throughout the study period and their body weight was significantly higher when compared with non-treated SMNdelta7 mice from the age of 11 days (\( P < 0.0001 \)). Only a slight decrease in weight gain was observed at 24 days of age in treated male and female SMA mice compared with sex-matched WT mice (\( P = 0.0005 \) and \( P = 0.01 \) for males and females, respectively). Unexpectedly, the weight loss appeared less pronounced in females than in males. Male SMA mice displayed a lower body weight than age-matched control mice from 4 weeks of age (\( P < 0.05 \) to \( P < 0.001 \)) (Fig. 3C), whereas the body weight of females...
was not statistically different from that of heterozygous or WT controls at 11–12 weeks of age, and from 15 to 27 weeks of age (Fig. 3B). We noted that the overall general appearance of treated SMNdelta7 and WT mice was the same (Fig. 4A, C, F, G, I). However, both the tails and ears of treated mice showed overt edema and displayed degenerative necrotic changes at \( \approx 25 \) and \( \approx 40 \) days of age, respectively (Fig. 4G and I, upper panel). These pathological features were not present in WT or heterozygous mice injected with the same doses of vector (Fig. 4H). Moreover, bilateral cataracts were also detected from the age of 210 days in the two oldest treated SMA mice, in contrast to age-matched scAAV9-injected controls (Fig. 4I, lower panel).

Unlike non-treated SMNdelta7 mice, which displayed a severe neurological phenotype and showed impaired movement by 10 days of age, AAV-injected SMNdelta7 mice moved without any difficulty as demonstrated by monitoring spontaneous motor activity (Fig. 4J–M and Supplementary Material, Movies). The cumulative measurements over a 1 h period did not reveal any significant differences in overall spontaneous activity between scAAV9-SMNopti treated, WT or heterozygous littermates (maximal speed, \( P = 0.99 \); distance, \( P = 0.52 \); resting time, \( P = 0.71 \); rearing, \( P = 0.34 \)). It is noteworthy that from \( \approx 40 \) days of age, treated mice were hyperactive (in particular when the experimenter tried to manipulate them), an effect that could be due to SMN deficiency in specific brain areas.

We analyzed SMN expression levels using western blotting with protein extracts from different tissues of scAAV9-SMNopti-injected SMA mice (\( n = 2 \)), untreated SMA mice (\( n = 2 \)) and WT mice (\( n = 2 \)). Very low SMN levels were found in the untreated SMA mice (below the threshold of detection at the selected exposure time) (Fig. 5) In contrast, the SMN protein levels were dramatically increased in both the spinal cord and the muscles (tibialis anterior) of

![Figure 2](https://academic.oup.com/hmg/article-abstract/20/4/681/587045)
scAAV9-SMNopt-injected SMA mice, 14 days after vector injection into the temporal vein. In one mouse, the SMN levels in both the spinal cord and muscles were even higher than the WT control (≏140% of the WT control). Unexpectedly, the increase of SMN levels in the brain tissues was lower than in the other tissues (≏40% of the WT control). This could explain the behavioral disturbances (notably the hyperactivity) observed in some treated SMNdelta7 mice after ~40 days of age, possibly due to regional changes in brain morphology as recently reported in SMA mice (22).

To identify the cell types expressing SMNopt in the spinal cord, immunofluorescence analysis using anti-NeuN or anti-GFAP antibodies and in situ hybridization (ISH) analysis using mRNA antisense probes specific for the SMNopt sequence were performed in the cervical spinal cord of scAAV9-opti-injected mice (n = 3). Fourteen days after vector injection, SMNopt mRNAs were clearly detected in a
large number of spinal cord cells including MNs (29 ± 2% of the transduced cells) and astrocytes (14 ± 2% of the transduced cells) (Fig. 6). We found that 49 ± 16% of MNs expressed SMN in the cervical spinal cord (with up to 78% of transduction in one mouse).

We next examined the effect of the systemic scAAV9-SMNopti treatment on the number of calcitonin gene-related peptide (CGRP)-positive MNs in the cervical spinal cord of SMA mice. Immunohistochemical analysis of spinal cord tissue sections at 14 days revealed a reduced number of MNs in SMNdelta7 mice (6.3 ± 0.6) compared with that observed in WT mice (15.2 ± 0.7) (P < 0.01) (Fig. 7). In contrast, the number of CGRP-positive MNs was significantly increased in the treated SMNdelta7 mice (13.8 ± 0.2) compared with non-treated SMNdelta7 mice (P < 0.01) and did not differ from that of WT mice, demonstrating a strong protective effect of scAAV9-SMNopti gene therapy on the survival of MNs.

DISCUSSION
In this study, we describe a gene therapy strategy for SMA based on systemic delivery of a SMN-expressing scAAV9 vector in a severe mouse model of SMA. In contrast to previous studies using the endogenous hSMN1 sequence, we used a codon-optimized version of the human sequence to enhance SMN expression in mouse tissues. Moreover, an intronic sequence was placed downstream of the strong PGK

Figure 4. Systemic scAAV9-SMNopti rescues the SMNdelta7 phenotype. (A–I) Photographs of WT mice, SMNdelta7 mice and AAV9-SMN treated SMNdelta7 mice (SMNdelta7 AAV9-SMN) at (A, F) 2–3 weeks, (G, H) 27 weeks, (I) 6 and 30 weeks. (A, C) and (E–H) illustrate the near complete rescue of the treated SMNdelta7 mice. In (G), the arrow indicates tail necrosis in a 108-day-old mouse. (B) and (D) illustrate the severe SMNdelta7 phenotype. (I) Ear necrosis (upper panel) and cataract (lower panel) are also observed in the AAV9-SMN treated SMNdelta7 mice from the age of 40 and 210 days, respectively. (J–M) Evaluation of the spontaneous activity of wild-type (WT, white), heterozygous (HT, gray) and scAAV9-SMNopti treated SMNdelta7 mice (KO AAV9-SMN, black) using an actimeter system. Monitoring of (J) maximal speed, (K) distance, (L) resting time and (M) rearing demonstrates the normalization of motor activity in SMNdelta7 mice intravenously injected with scAAV9-SMNopti. Circles represent individual data, and squares are means (+ SEM). None of the parameters showed any statistical difference between groups (ANOVA, P > 0.05).
promoter to further enhance expression of the therapeutic protein.

A single intravenous injection of SMA mice with the SMNopti-expressing scAAV9 vector resulted in a substantial extension in mouse survival, increasing life expectancy by 14 to more than 310 days in mice that normally survive until ~13 days. This treatment restored body weight and rescued motor activity to near normal levels. In addition, MN death was completely prevented in the spinal cord of the treated-SMA mice at 14 days of age.

These results far exceeded the therapeutic improvement described in previous studies based on pharmacological or RNA transsplicing approaches (23–25), as well as that of a previous gene therapy study using multiple intramuscular lentivirus injections (5). In the latter study, it was noted that the SMNdelta7 mice develop a pathological phenotype by 5 days after birth, suggesting that the therapeutic window may be too short for gene therapy protocols; therefore, the success of our approach may be due to the use of a scAAV vector that is as soon as 48 h after injection (6). It is possible that there is also a requirement for SMN expression in cells other than MNs. After intramuscular injection, the lentiviruses are transported specifically to the spinal cord MNs, whereas systemic scAAV9-SMNopti injections result in SMN expression also in non-nervous tissues. Although CNS-directed SMN expression has been reported to be highly efficient in ameliorating SMA-associated manifestations in transgenic mice (26,27), post-natal expression of SMN in non-nervous tissues (such as muscle or liver) may also play an important role in SMA pathogenesis (28–31). Post-natal systemic delivery of vectors expressing SMN under the control of tissue-specific promoters should help finally determine the role of non-neuronal tissues in SMA pathology.

Recently, the high potential of scAAV9 vectors for systemic gene therapy of SMA has been similarly reported in two independent studies (32,33). However, a set of differences was observed in comparison with our study, such as the number of injected vector particles, the SMN expression levels and the level of phenotypic correction. Importantly, we used ~2.5- to 10-fold less virus particles than in the previous reports [4×10^{10} vg per mouse versus 10^{11} vg per mouse (33) and 5×10^{11} vg per mouse (32)], with a higher phenotypic rescue. As an example, the median survival was increased to 199 days versus 69 days in the previous report of Valori et al. (33) using 2.5-fold more vector particles, and was similar to that reported in the study of Foust et al. (32) using ~10 times more vector particles. Moreover, the body weight phenotype of the SMA mice was almost fully rescued by intravenous injection of our optimized vector, in contrast to the only partial rescue (50 and 65% of controls) that was previously reported (32,33). It is noteworthy that only the ‘carriers’ (Smm+) were considered as controls in these two previous studies, and that no comparison with WT mice was available (as shown in the present study, the ‘carriers’ weight body was significantly lower than that of ‘WT’ from ~19–23 weeks of age).

Just as the level of therapeutic rescue, the SMN expression levels were higher than those previously reported. In vitro, up to 35-fold higher SMN expression was found in SMNopti expressing cells compared with hSMN1 expressing cells, and in vivo, the SMN expression levels in AAV9-SMNopti injected SMA mice was similar to or even surpassed the SMN concentrations found in WT mice in the spinal cord (80–140% of WT) or in the muscles (120–140% of WT).

Importantly, intravenous injection of scAAV9-SMNopti induced the complete survival of the CGRP-positive MNs in the spinal cord of SMNdelta7 mice. Such an effect on MN survival has never been observed in previous SMN gene therapy studies, either after intravenous scAAV9 injections (32,33) or injection of scAAV8-SMN into the CNS of SMA mice (27). However, as demonstrated after AAV8-SMN injections into the CNS (27), and as suggested in several previous studies (34–36), the therapeutic effect of SMN overexpression in SMA mice could also result from axonal sprouting and remodeling of the neuromuscular junction (NMJ).

Unexpectedly, our results further suggest sex differences in the responsiveness to the AAV9-SMN treatment. Influence of sex on the course of SMA has been previously suggested in a chronic form of childhood SMA (37). This phenomenon could logically result from a hormonal interaction with the course of SMA and/or the treatment response. Interestingly, it could be related to differences in the expression levels of plastin, which has been recently described as a sex-specific modifier of SMA. Indeed plastin 3 expression has been associated with disease severity only in postpubertal females (38). However, additional studies will be necessary to confirm and explain these sex-related differences.

The treated SMA mice also displayed degenerative necrotic changes affecting both the ears and the tail at about 25 and 40 days of age, respectively. These abnormalities were not due to any undesired effect of scAAV9 delivery, as no necrotic change was observed in WT or heterozygous mice injected with similar amounts of vector. Rather, necrosis was probably due to the lack of SMN protein in these specific tissues. Similar ear and tail abnormalities have been described previously in mild and partially rescued severe SMA mice.
Although tail length of the scAAV9-SMNopti treated mice was not quantitatively evaluated, tail necrosis was not complete (in all mice except one), even at advanced ages. Moreover, this pathological feature was observed from 3 to 4 weeks of age in the scAAV9-SMNopti treated severe SMA mice, similarly to what was recently described after antisense correction of SMN2 splicing in a mild SMA model (42). These results highlight the potential of systemic scAAV9-SMNopti delivery for correction of peripheral distal necrosis linked to SMA. Strikingly similar abnormalities affecting the tips of the feet and the fingers digits have, indeed, been described in infants with SMA (43,44). Although the clear cause of these symptoms has not yet been identified, digital necrosis was reported to be due to insufficient vascular perfusion (43,44). Interestingly, a relationship between innervation and vascularization has been previously suggested in several diseases such as ALS and SMA with respiratory distress, associated to a dysregulation of the VEGF angiogenic factor (44–46).

We also noted bilateral cataracts in the oldest treated SMA mice, a pathology which, to our knowledge, has never been described in mouse models of SMA. Interestingly, one clinical study has, however, reported the diagnosis of a bilateral anterior polar cataract in two SMA patients at the age of 9–11 months (47). Although additional work is necessary to determine the cause of this pathological feature, a role of vascular perfusion abnormalities could also be considered (48).

In summary, our results demonstrate that it is possible to significantly increase life expectancy and alleviate the clinical symptoms of mice with a severe form of SMA by reintroducing the SMN protein after birth using a single systemic injection of a scAAV9 vector. Modification of the human endogenous SMN1 sequence by codon-optimization and introduction of a chimeric intron downstream of the promoter significantly improved the therapeutic impact of the systemic scAAV9-mediated SMA gene therapy. This work constitutes an important step towards the development of a gene therapy protocol for the treatment of this devastating MN disease.

MATERIALS AND METHODS

Animals

Pregnant and adult (6–8 week old, female) C57Bl6 mice were purchased from Charles River Laboratories (Les Oncins, France). Severe SMNdelta7 mice were purchased from Jackson Laboratory (SMN2+/-, SMNdelta7+/-, Smn-/-, JACKSON no. SN 5025). These mice are triple mutant...

Figure 6. ISH analysis of SMN mRNA in scAAV9-SMNopti injected SMNdelta7 mice. Cervical spinal cord sections of (B–I) AAV9 treated and (A) untreated SMNdelta7 mice were hybridized with digoxigenin-UTP labeled riboprobes corresponding either to (A, B; D–I) SMNopt antisense or (C) sense sequences. No specific staining was detected in a section from untreated SMNdelta7 mice hybridized with the SMNopt antisense sequence (A) or in a section from AAV9-treated SMNdelta7 mice hybridized with SMNopt sense sequence (C) in contrast to specific SMNopt mRNA staining observed in sections from AAV9-treated SMNdelta7 mice hybridized with SMNopt antisense sequence (B). (D–I) ISH was followed by (D–F) anti-GFAP immunofluorescence (green) or (G–I) anti-NeuN immunofluorescence (red) (F–I) merge showing double-labeled SMN-expressing astrocytes (GFAP positive cells, arrows in F) or MN (large NeuN-positive cells, arrows in I). Scale bar = 100 μm.
harboring two transgenic alleles (a SMN cDNA lacking exon 7 and the entire SMN2 gene) and a single targeted mutant (disruption of exon 2 in the endogenous mouse Smn gene). WT corresponded to \((\text{SMN}2^+/-, \text{SMN}\text{delta7}^+/+, \text{Smn}^+/-)\) mice, and heterozygous (carriers) to \((\text{SMN}2^+/-, \text{SMN}\text{delta7}^+/-, \text{Smn}^+/-)\). The clinical signs are apparent at 5 days, and mean survival is approximately 13 days. Mild ‘A2G’ mice were purchased from Jackson Laboratory \((\text{SMN} A2G^+/-, \text{SMN}2^+/-, \text{Smn}^2/-; \text{JACKSON no. SN 5026})\). These mice are homozygous for the targeted mutant Smn allele, homozygous for the SMN2 transgene and hemizygous for the SMN1∗A2G transgene. These mice were reported to show first clinical signs at 3 weeks of age and have a shortened lifespan (less than a year) (19). The SMA mice were bred to generate self-sustaining colonies and maintained under controlled conditions \((22 \pm 1 ^\circ \text{C}, 60 \pm 10% \text{ relative humidity}, 12 \text{ h}/12 \text{ h} \text{ light/dark cycle}, \text{ food and water ad libitum})\). All animal experiments were carried out according to the European guidelines for the care and use of experimental animals.

**Optimized SMN sequence**

The hSMN1 sequence (gift from Dr J. Melki, Evry, France) was inserted into the AAV2 plasmid downstream of the PGK promoter (pSMN). A chimeric intron (Promega) was added between the promoter and the transgene using polymerase chain reaction (PCR) (pSMNi). This intron is composed of the 5′-donor splice site of the human \(\beta\)-globin intron 1 and the branch point, and the 3′-acceptor splice site from an intron derived from the immunoglobulin gene heavy chain variable region. Codon optimization of hSMN1 (pSMNopt) was carried out using GENEART’s gene optimization system. This technology uses a specific algorithm which not only accounts for the codon bias of the expression system (expression of human SMN in the mouse), but also accounts for different factors that can compromise the mRNA stability (such as extreme GC content, ribosomal binding sites, consensuses and cryptic splice sites, repeats and secondary structures).

The pSMNopt \(i\) plasmid carried the SMNopt sequence placed downstream from the PGK promoter and the chimeric intron.

**Transfection of cultured cells**

Primary astrocytes were prepared from cerebral cortices of 1-day-old \((\text{SMN}2^+/-, \text{Smn}^2/-)\) mice. After removal of the meninges, the tissues were mechanically dissociated after incubation with trypsin-EDTA \((10 \text{ min}, 37^\circ \text{C})\). After addition of complete culture medium \([\text{Dulbecco’s modified Eagle’s medium (DMEM) supplemented with Glutamax}^\text{w}, 10% \text{ heat-inactivated fetal calf serum and 1% (w/v) penicillin/streptomycin}]\), the cell suspension was centrifuged \((900 \text{ g; 3 min})\) and the pellet was re-suspended in 10 ml of complete culture medium and seeded in 25 cm\(^2\) culture flask for cells derived from one pup. Primary astrocytes were harvested at 37°C in 5% \(\text{CO}_2\) and half of the culture medium was changed twice a week. The NSC34 cells (neuroblastoma \(\times\) spinal cord), kindly provided by Dr Neil Cashman (49), were harvested at 37°C in 5% \(\text{CO}_2\) in the same culture medium than primary
astrocytes. Primary astrocytes were seeded in six-well plates at the density of $5 \times 10^5$ cells/well for western blotting experiments, and in four-well plates at the density of $10^5$ cells/well for immunofluorescence analyses. For the latter, cells were cultured on glass cover slides coated with polyornithine (Sigma-Aldrich).

Twenty-four hours after seeding, astrocytes were transfected in the presence of lipofectamine 2000 (Invitrogen) with each of the four AAV2 plasmids according to the manufacturer’s protocol. Cells were collected 24 h after transfection.

NSC34 cells were seeded in four-well plates at the density of $2 \times 10^5$ cells/well. Twenty-four hours later, cells were transfected in the presence of lipofectamine with Reagent Plus (Invitrogen) with pSMNopt. Cells were collected 2 days after transfection.

**Production of scAAV vectors**

Pseudotyped AAV9 vectors were generated as previously described in ref. (7) with slight modifications. Briefly, AAV9-SMN was produced by helper virus-free three-plasmid transfection in HEK293 cells, using (i) the adenovirus helper plasmid, (ii) the AAV packaging plasmid encoding the rep2 and cap9 genes (pSE18-VD2/9) and the AAV2 plasmid expressing the SMNopt transgene under control of the PGK promoter. The vector genome containing plasmid was constructed by deleting the D sequence and the terminal resolution site (trs) from one of the inverted terminal repeats. The recombinant vector was purified by ultracentrifugation on an iodixanol density gradient. Viral preparation was desalted and concentrated using Amicon Ultra—Ultra cell 100 K filters units (Millipore). Aliquots were stored at $-80^\circ$C until use. Vector titers were determined by real-time PCR and expressed as viral genomes per milliliter (vg/ml).

**In vivo AAV injections**

Six weeks old A2G$^{+/−}$ mice ($n = 3$) were injected with scAAV9-SMNopti into the cervical ($3.4 \times 10^{11}$ vg, $20 \mu$l) and lumbar ($9 \times 10^{10}$ vg, $2.5 \mu$l) spinal cord. Intravenous injections of scAAV9-GFP ($1.5 \times 10^{10}$ vg, $28 \mu$l) ($n = 3$) or scAAV9-SMNopti ($4.5 \times 10^{10}$ vg, $70 \mu$l) ($n = 3$) were performed in the temporal vein at post-natal day 1 (P1). The GFP expression analysis was performed 1 month later.

**Spontaneous activity**

scAAV9-SMNopti-injected mice ($n = 4$) were compared with age-matched WT ($n = 3$) and HT mice ($n = 3$) for spontaneous activity (40–60 days of age). Spontaneous activity was assessed for 60 min by numbering infrared crossed beams in an actimeter (Harvard Apparatus/Panlab, Bioseb) placed in a quiet room. Data were acquired by the Track software (Harvard Apparatus/Panlab, Bioseb).

**Tissue preparation**

Fourteen days after injection, mice were lethally anesthetized with Dolethal (Vetoquinol, 120 mg/kg) and perfused transcardially with 0.1 mol/l PBS followed by 4% paraformaldehyde in PBS. Tissues were removed, post-fixed for 4 h in the same solution and cryoprotected overnight at 4°C in a PBS-sucrose solution (30% sucrose for the spinal cord, 15% sucrose for the other organs). The tissue samples were then frozen in cold isopentane ($−50^\circ$C) and serial 14 μm thick sections were cut on a cryostat and stored at $−80^\circ$C.

**Western blotting**

_In vitro._ Cell protein extracts were prepared using lysis buffer from CAT ELISA kit (Roche Diagnostics) supplemented with proteases cocktail inhibitors (Complete Mini, Roche Diagnostics). Protein concentration was measured using Biorad DC Protein Assay (Biorad). Equal amounts of proteins were loaded on a 15% acrylamide gel and transferred on a nitrocellulose membrane (Hybond ECL, Amersham Biosciences, GE Healthcare). After overnight incubation with a blocking buffer (Odyssey, Li-Cor), membranes were probed with primary antibodies (mouse anti-SMN, 1/5000, BD Biosciences; rabbit anti-GFAP, 1/1000, DakoCytomation) in the same buffer followed by goat anti-mouse Irdye-680 or goat anti-rabbit Irdye-800 (1/10 000, Li-Cor). Immunoreaction was visualized using Odyssey Infrared Imager (Li-Cor). Quantifications were performed on scanned images using the Odyssey software (Li-Cor).

_In vivo._ Protein extracts were prepared from brains, muscles and spinal cords from 14 days old untreated SMNdelta7 mice ($n = 2$), scAAV9-SMNopht treated SMNdelta7 mice ($n = 2$) and WT mice ($n = 2$). Brains and spinal cords extracts were prepared using the Qproteome FFPE tissue kit according to the manufacturer’s protocol (Qiagen). Muscles were lysed in RIPAE buffer (150 mM NaCl, 50 mM Tris–HCl, 0.5% sodium deoxycholate, 1% NP40, 1% SDS) supplied with a protease inhibitors cocktail (Complete Mini, Roche Diagnostics). Fifty micrograms of total protein extracts were run on SDS 10% polyacrylamide gels and transferred to Immobilon-p membrane (Millipore). The membranes were probed with primary antibodies (mouse anti-SMN, rabbit anti-GFAP, 1/1000, DakoCytomation) and secondary antibodies (goat anti-mouse Irdye-680, goat anti-rabbit Irdye-800 (1/10 000, Li-Cor) in the Odyssey software (Li-Cor). After four washes in TBST buffer, membranes were incubated with a peroxidase-conjugated swine anti-mouse antibody (Amersham Pharmacia Biotech, 1:10 000) in blocking buffer. Membranes were further processed using the chemiluminescence Super Signal Ultra reagent (Pierce).

**In situ hybridization**

Complete SMNopt cDNA was introduced in a pGEM-T Easy vector (Promega). Antisense and sense riboprobe were synthesized from 1 μg of linearized plasmid in the presence of 3.5 nmol of digoxigenin-11-UTP (Roche Diagnostics) using the Riboprobe system kit (Promega). ISHs were performed as previously described (50,51) on 14 μm thick serial sections of cervical spinal cord. Probes were detected with alkaline phosphatase-conjugated anti-Dig antibodies (Roche). Colorimetric detection was achieved with 5-bromo-4-chloro-3-indolylphosphate (BCIP) and nitroblue tetrazolium (NBT;...
Promega) to obtain a blue staining. Sections were then processed for further NeuN or GFAP immunostaining.

Immunofluorescence
For in vitro SMN immunofluorescence, cells were washed with PBS and fixed with 3.7% paraformaldehyde. After washing in PBS, cells were permeabilized (PBS, 0.4% Triton X-100) for 1 h and incubated in a blocking solution for preventing nonspecific binding due to endogenous mouse immunoglobulin (Mouse On Mouse Immunodetection kit, Vector Laboratories) according to the manufacturer’s protocol. Primary antibody (mouse anti-SMN, BD Biosciences, 1/750) was diluted in the MOM diluent and incubated overnight. Cells were washed with PBS and incubated with Alexa 488-conjugated goat anti-mouse (1/1000, Molecular Probes). After final washing, cells were mounted with fluoromount-G (Calbiochem) and were observed by confocal microscopy [Leica, laser emission: 488 nm (green) and 594 nm (red)].

For in vivo SMN immunofluorescence, the sections were pre-incubated (1 h, room temperature) in a PBS buffer containing 3% goat serum (Interchim) and 0.3% Triton X-100, and then incubated in the same buffer supplemented with primary antibody rabbit anti-SMN H-195 (1/300, Santa Cruz Biotechnology) overnight at 4°C. After washing in PBS, sections were incubated for 1 h at room temperature with Alexa 488-conjugated goat anti-rabbit (1/1000, Molecular Probes) antibody. The sections were then washed in PBS and mounted with Dapi-Fluoromount-G solution (Calbiochem). GFP/NeuN immunostaining was performed in two steps. Sections were first pre-incubated in a blocking solution containing 3% donkey serum (Millipore), 5% Bovine Serum Albumin (Sigma) and 0.4% Triton X-100 diluted in PBS (1 h, room temperature), and was then incubated in the same solution supplemented with rabbit anti-GFP ab6556 (1/2000, Abcam, overnight, 4°C). After PBS washing, sections were incubated for 1 h at room temperature with Alexa 488-conjugated Donkey anti-rabbit (1/500, Molecular Probes) antibody. The sections were then washed in PBS and incubated in a specific blocking solution containing the MOM reagent according to the manufacturer’s protocol. Primary antibody mouse anti-NeuN (1/300, Chemicon International) was diluted in the MOM diluent and incubated 2 h at room temperature. After washing in PBS, sections were incubated for 1 h at room temperature with Alexa 594-conjugated Goat anti-mouse (1/500, Molecular Probes) antibody. The sections were then washed in PBS and mounted with Dapi-Fluoromount-G solution (Calbiochem). For CGRP immunohistochemistry, the sections were pre-incubated (1 h, room temperature) in a PBS buffer containing 3% donkey serum (Millipore) and 0.4% Triton X-100, and then incubated in the same buffer supplemented with primary rabbit anti-CGRP (1/1000, Calbiochem) overnight at 4°C. After washing in PBS, sections were incubated for 1 h at room temperature with a donkey Alexa 488-conjugated anti-rabbit antibody (1/1000, Molecular Probes). Sections were observed by confocal microscopy (Axio Imager Z1, Zeiss) and AxioVision 4.7 software (Zeiss). For NeuN or GFAP immunostaining following SMN ISH, the sections were washed with PBS after ISH, and pre-incubated in the MOM Kit (as described for in vitro immunostaining). The sections were then incubated with the primary mouse anti-NeuN (1/100) and rabbit anti-GFAP (1/500, DakoCytomation) antibodies. Alexa 594-conjugated goat anti-mouse (1/500) and Alexa 488-conjugated donkey anti-rabbit (1/1000, Molecular Probes) were used as secondary antibodies.

SMN + astrocyte and MN counts
The total number of SMN0pt positive cells was counted on sections treated for ISH. SMN0pt positive cells were scored on 20 serial sections of 14 μm thick per mouse (n = 3) spanning the cervical spinal cord. Astrocytes were identified using GFAP immunostaining. The number of SMN0pt-positive astrocytes and SMN0pt-positive MNs was estimated using NeuN and GFAP immunostaining, respectively. Large NeuN-immunopositive cells (diameter > 20 μm) with a visible nucleus and located in the ventral horn were considered as MNs. The percentage of SMN0pt expressing MNs was estimated relative to the total number of MNs.

MN survival
The CGRP-immunopositive MNs were counted on 30–60 s (14 μm thick) per mouse spanning the cervical spinal cord from four WT, four SMNdelta7 and three scAAV9-treated SMNdelta7 mice using confocal microscopy (laser emission 488 nm). Serial sections were 70 μm apart and only MNs with clearly visible nuclei were counted.

Statistical analysis
Differences of SMN expression in transfected (SMN2+/+, Smn+/−) and WT astrocytes were analyzed using a one-way variance analysis (ANOVA) followed by post-hoc Scheffe’s test (two-tailed tests). Differences in mean life expectancy between SMNdelta7 and WT mice were analyzed using unpaired Student’s t-test. Differences in body weight, in MN number and in spontaneous activity were analyzed using an ANOVA followed by post-hoc Scheffe’s tests (two-tailed tests). P < 0.05 was considered significant.

SUPPLEMENTARY MATERIAL
Supplementary Material is available at HMG online.

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REFERENCES