A New AAV10-U7-Mediated Gene Therapy Prolongs Survival and Restores Function in an ALS Mouse Model

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One of the most promising therapeutic approaches for familial amyotrophic lateral sclerosis linked to superoxide dismutase 1 (SOD1) is the suppression of toxic mutant SOD1 in the affected tissues. Here, we report an innovative molecular strategy for inducing substantial, widespread, and sustained reduction of mutant human SOD1 (hSOD1) levels throughout the body of SOD1G93A mice, leading to therapeutic effects in animals. Adeno-associated virus serotype rh10 vectors (AAV10) were used to mediate exon skipping of the hSOD1 pre-mRNA by expression of exon-2-targeted antisense sequences embedded in a modified U7 small-nuclear RNA (AAV10-U7-hSOD). Skipping of hSOD1 exon 2 led to the generation of a premature termination codon, inducing production of a deleted transcript that was subsequently degraded by the activation of nonsense-mediated decay. Combined intravenous and intracerebroventricular delivery of AAV10-U7-hSOD increased the survival of SOD1G93A mice injected either at birth or at 50 days of age (by 92% and 58%, respectively) and prevented weight loss and the decline of neuromuscular function. This study reports the effectiveness of an exon-skipping approach in SOD1-ALS mice, supporting the translation of this technology to the treatment of this as yet incurable disease.

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is the most common adult-onset motor neuron (MN) disorder, characterized by MN degeneration, severe paralysis, and death within 3–5 years after diagnosis.1 No treatment is currently available, except the drug Riluzole, which offers only a modest survival benefit.2 ALS is epidemiologically classified into sporadic (90%–95%) and familial (5%–10%) forms (fALS);3 approximately 12% of fALS cases are caused by over 180 mutations in the superoxide dismutase 1 (SOD1) gene, conferring a toxic gain of function to the mutant protein.4,5 Continuous infusion of antisense oligonucleotides (ASOs) into the brain ventricles has been reported as a promising approach to induce significant SOD1 silencing in SOD1G93A rats, but only 10 days of survival extension was achieved with infusion prior to disease onset.6 A phase I clinical study using intrathecally delivered ASOs to patients revealed the absence of serious adverse effects, highlighting the feasibility of this strategy in humans.7 Significant SOD1 reduction has also been provided by injection of lentiviral vectors encoding short hairpin RNA targeting human SOD1 (SOD1-shRNA) into the spinal cord parenchyma or multiple muscle groups of SOD1G93A mice.8,9 These approaches resulted in a substantial increase in ALS mouse survival, but their clinical applicability remains questionable, due notably to the difficult translation of the vector administration routes to patients.

More recently, the self-complementary adeno-associated virus (AAV) serotype 9 vector was used to deliver SOD1-shRNA in ALS mice after a single intravascular injection.10 This systemic AAV9 approach was successful in extending the lifespan of SOD1G93A mice injected at birth or later.10 Another study using AAV serotype rh10 to express artificial microRNA (miRNA) also showed slowing of disease progression and extended survival in SOD1G93A mice intrathecally injected with the vector at 65 days of age.11 These and other2,12,13 recent AAV studies have provided encouraging therapeutic results and the potential of feasible translation to the clinic. However, these approaches have only led to incomplete rescue and better SOD1-silencing approaches in mice are still needed to provide promising treatment options to patients.

Transcriptional SOD1 silencing can be achieved by skipping of a constitutive SOD1 exon (exon skipping) using ASOs complementary to splicing regulatory elements on the primary transcript. The resulting deleted mRNA, containing a premature termination codon, is then degraded by the endogenous cellular surveillance nonsense-mediated decay pathway.14,15

Here, we report the high therapeutic potential of this exon-skipping strategy in both newborn (P1) and adult (P50) SOD1G93A mice using...
the administration of ASOs against mutant human SOD1 (hSOD1) inserted in an AAV10-U7 vector (AAV10-U7-hSOD1). The AAV vector was co-injected into the central nervous system and the peripheral organs, combining intravenous (i.v.) and intracerebroventricular (i.c.v.) injections. This gene therapy approach induced efficient hSOD1 exon skipping in the spinal cord, resulting in a large reduction of hSOD1 mRNA and protein levels. SOD1G93A mice survival was prolonged, with a mean increase in life expectancy of 92% and 58% for mice injected either at birth or 50 days of age, respectively. Disease onset was also delayed by 95 and 63 days, respectively, relative to untreated mice. Finally, AAV10-U7-hSOD1 delivery prevented weight loss and preserved motor function and skeletal muscle force.

RESULTS
Specific Antisense Sequences Promote hSOD1 Exon Skipping In Vitro
We rationally designed five steric, blocking RNA-based ASOs that masked the splicing acceptor site (SA) in intron 1 or exonic splicing enhancer sequences (ESEs)\(^{16}\) in exon 2 (E2) to promote efficient E2 skipping (Figures 1A and S1A). Skipping of hSOD1 E2 generates a
frameshift in the mRNA transcript, with production of a premature stop codon in exon 4 (E4), 92 nt upstream of the E4-E5 junction (Figure 1A). The deleted transcript (∆E2 hSOD1) is a likely substrate for the endogenous cellular surveillance nonsense-mediated decay pathway and is subsequently degraded because the premature stop codon is located greater than 55 nt upstream of the last exon-exon junction.17

The ASOs were chemically modified (2′-O-methyl phosphorothioate [2′OMePS]) to increase their stability, resist nuclease and RNase H degradation,18 and their ability to promote E2 skipping was evaluated (hSOD1-ASO). A scrambled fluorescently (FAM)-labeled ASO was used as control (ASO-CTR). After ASO transfection into HEK293T cells, RT-PCR analysis revealed a 258-nt band corresponding to the ∆E2 hSOD1 mRNA variant in the samples transfected with hSOD1-ASO and a 355-nt product corresponding to the complete hSOD1 mRNA in all samples (Figures S1B and S1C). Although all ASOs resulted in a substantial reduction of hSOD1 mRNA levels relative to non-transfected cells, ASO1 and ASO4 showed the highest efficiency (Figure 1B). These two ASOs, targeting different ESE regions in E2, were both used in the following studies to expand the masked sequence and maximize steric hindrance to splicing factors.

**AAV10-U7-Mediated Delivery of Antisense Sequences in SOD1G93A Mice Reduces hSOD1 Protein and mRNA Levels**

We sub-cloned the two AS sequences corresponding to ASO1 and ASO4 into the optimized U7-sRNA cassette (U7-AS-1/AS-4), previously described by Schumperli et al.19, to protect them from degradation. The embedding of ASOs into the U7snRNP particle has been reported to improve their nuclear entry and incorporation into the spliceosome and increase their stability in vivo.18,20 We then engineered the U7-AS-1/AS-4 cassette or a control AS, previously tested in vivo,21 into the AAV backbone, and the corresponding AAV10 vectors were produced (AAV10-U7-hSOD1 or AAV10-U7-CTR) (Figure 2A).

We locally injected the AAV10-U7-hSOD1 or the AAV10-U7-CTR vectors (4.7 × 1012 viral genome [vg]/kg, two sites, 4.8 × 1010 per site in 5 μl) into the lumbar spinal cord of 50-day-old SOD1G93A mice to analyze the efficiency of AAV10-U7-hSOD1 in reducing hSOD1 mRNA and protein levels in vivo. As expected, we found the ∆E2 hSOD1 mRNA variant in the spinal cords of AAV10-U7-hSOD1-injected animals 1 month after injection, with a more than 80% reduction of full-length hSOD1 mRNA for each injected mouse (Figure 2B) and a 70% average reduction in hSOD1 protein relative to AAV10-U7-CTR-injected animals (Figure 2C). Due to the high degree of homology between hSOD1 and murine SOD1 sequences, ASOs were designed to be human specific, with AS-1/AS-4 presenting a total of ten mismatches to the mouse SOD1 mRNA (Figure S2A). Accordingly, the endogenous SOD1 protein levels were unchanged in all injected animals, confirming the specificity of AAV10-U7-hSOD1 for the hSOD1 form (Figures S2B and S2C).

**Co-i.v./i.c.v. Administration of AAV10-U7-hSOD1 in Newborn SOD1G93A Mice Prolongs Survival and Delays Disease Onset**

To test the therapeutic efficacy of hSOD1 exon skipping, we injected SOD1G93A mice on postnatal day 1 (PND1) with 4.5 × 1014 vg/kg of AAV10-U7-hSOD1 (n = 15) or AAV10-U7-CTR vectors (n = 17) co-injected into the temporal vein (i.v., 4 × 1014 vg/kg) and lateral ventricles (i.c.v., 5 × 1013 vg/kg) (co-i.v./i.c.v.) (Figure 3A). All AAV10-U7-hSOD1-injected SOD1G93A mice survived substantially longer than did non-injected (NI) (n = 12) or AAV10-U7-CTR-injected mice (239 days versus 118 days or 125 days, respectively; p < 0.0001), for an extension of the median lifespan of 121 and 114 days, respectively. No statistically significant difference was found between the lifespans of AAV10-U7-CTR-injected and NI mice (Figure 3B).

The mean lifespan of AAV10-U7-hSOD1-injected mice was 95% and 92% longer than that of NI or AAV10-U7-CTR-injected mice, respectively (Figure 3C).

AAV10-U7-hSOD1 delivery also slowed the weight loss observed in NI SOD1G93A mice at the end stage (23.9 ± 1.1 g versus 20.5 ± 0.9 g at 17 weeks, p < 0.05) (Figure 3D), and, importantly, AAV10-U7-hSOD1-injected mice gained weight until ~25 weeks of age, at which point weight reached a plateau. The weights of AAV10-treated SOD1G93A mice and wild-type (WT) mice were statistically significantly different at 25 weeks of age (25.6 ± 1.4 g versus 30.7 ± 1.4 g, respectively; p < 0.05) (Figure 3D).

AAV10-U7-hSOD1 delivery significantly delayed disease onset, based on the age of peak body weight, by ~102 and 95 days relative to NI and AAV10-U7-CTR-injected mice, respectively (199.5 days versus 97.5 days and 104.5 days; p < 0.0001) (Figure 3E). No statistically significant difference was observed between disease onset of AAV10-U7-CTR-injected and NI mice (Figure 3E). The mean disease progression, defined as the time from disease onset to death, was significantly delayed by 14 days in AAV10-U7-hSOD1-injected mice (36.6 ± 4.9 days) relative to NI mice (23.1 ± 1.7 days, p < 0.05) and AAV10-U7-CTR-injected mice (22.8 ± 3.7 days, p < 0.05) (Figure 3F).

**Co-i.v./i.c.v. Administration of AAV10-U7-hSOD1 in Newborn SOD1G93A Mice Reduces hSOD1 Levels and Preserves Pathological Signs**

We analyzed the efficacy of E2 skipping for the silencing of hSOD1 in the spinal cords of SOD1G93A mice, co-i.v./i.c.v. injected at birth, both at 112 days of age (n = 3) and at the end stage (n = 3) (Figure S3). We observed a significant reduction of full-length hSOD1 mRNA at both ages relative to those of NI mice (76% and 65% reduction at 112 days of age and end stage, respectively; p < 0.0001) and AAV10-U7-CTR-injected mice (77% and 66% reduction at 112 days of age and end stage, respectively; p < 0.0001) (Figure S3A). The levels of hSOD1 protein were also reduced by 71% and 66% in the spinal cord of AAV10-U7-hSOD1-injected SOD1G93A mice at 112 days of age relative to those of NI mice and AAV10-U7-CTR-injected mice,
respectively (p < 0.05). However, we detected no significant difference in hSOD1 protein levels at the end stage among AAV10-U7-hSOD1-injected, AAV10-U7-CTR-injected, or NI animals, despite the significant decrease of mRNA, suggesting progressive accumulation of residual amounts of mutant hSOD1 protein in the treated mice (Figure S3B).

We investigated the possible production of a protein from the truncated SOD1 mRNA after the prolonged expression of AAV10-U7-hSOD1 in the spinal cords of SOD1G93A mice injected at birth (n = 4) (Figure S4). If the ΔE2 hSOD1 mRNA is translated into protein, the latter will have a different amino acid sequence downstream of the end of exon 1 and will be made of 55 amino acids. Using the hSOD1-specific antibody, recognizing the N-terminal portion of the hSOD1 protein, we detected no protein signal at the presumed size of the truncated protein (6.05 kDa) (Figure S4A). As expected, with the same antibody, we confirmed that the levels of the hSOD1 protein were reduced by 76% in AAV10-U7-hSOD1-injected SOD1G93A mice relative to those of NI mice at 112 days of age (p < 0.0001) (Figure S4B).

Double-immunofluorescence analysis of spinal cord sections using antibodies against hSOD1 and either choline acetyl transferase (ChAT) (a marker of MNs) or glial fibrillary acid protein (GFAP) (a marker of astrocytes) confirmed the reduction of hSOD1 protein levels in individual MNs from AAV10-U7-hSOD1-injected mice at 112 days of age, but also in astrocytes, another cell type involved in ALS pathogenesis22 (Figure S5).

Figure 2. AAV-U7-Mediated hSOD1 Exon Skipping Reduces mRNA and Protein Levels In Vivo
(A) Design of the AAV vectors used to target hSOD1 (AAV10-U7-hSOD1) or control (AAV10-U7-CTR) sequences. The antisense sequences (ASs) corresponding to ASO1 and ASO4 were embedded into the optimized human U7 small nuclear RNA (snRNA)19,20 and cloned between two AAV inverted terminal repeats (ITRs). (B) The upper panel corresponds to a semiquantitative RT-PCR analysis of mRNA extracted from the spinal cord (SC) of SOD1G93A mice, in which the lumbar SC was injected with 4.7 × 10^{12} vg/kg of AAV10-U7-hSOD1 (n = 3) or AAV10-U7-CTR (n = 2). The lower panel shows results of the corresponding qRT-PCR analysis, showing lower levels of full-length hSOD1 mRNA in SC from AAV10-U7-hSOD1-injected mice than AAV10-U7-CTR-injected controls. Data are expressed as the mean ± SEM. (C) Western blot (WB) analysis of hSOD1 protein expression in SOD1G93A mice, in which the SC was injected with AAV10-U7-hSOD1 (n = 3) or AAV10-U7-CTR (n = 3) (4.7 × 10^{12} vg/kg). Antibody specificity for the human form of SOD1 was demonstrated by the absence of signal in protein extracts from WT mice. α-Tubulin was used as a loading control. The lower panel corresponds to the densitometric analysis of WB results, showing the significant reduction of hSOD1 levels in the AAV10-U7-hSOD1-injected SCs. Values are expressed as the mean ± SEM, and differences between groups were analyzed by the Student’s t test (**p < 0.01).
Skeletal muscles have also been shown to be involved in the pathogenesis of ALS; thus, we further quantified the level of hSOD1 silencing in the triceps muscle. We found 40% lower hSOD1 mRNA levels in the triceps muscles of AAV10-U7-hSOD-injected SOD1G93A mice than in those of NI or AAV10-U7-CTR-injected mice (n = 3; p < 0.01) (Figure S6A).

We then quantified the main neuropathological features of ALS observed in SOD1G93A mice, such as MN loss, astrogliosis, and microglia activation. AAV10-U7-hSOD1 delivery significantly prevented ChAT+ MN degeneration at 112 days of age (11.8 ± 0.4 versus 9.7 ± 0.2 and 8.7 ± 0.2, for NI and AAV10-U7-CTR delivery, respectively; p < 0.0001) (Figures 4A and 4B, upper panels), reduced the intensity of GFAP fluorescence (astrocytosis) (27.2 ± 1.8 versus 54.0 ± 1.9 and 49.5 ± 2.3 for NI and AAV10-U7-CTR, respectively; p < 0.001) (Figures 4A and 4B, middle panels), and decreased the number of ionized calcium-binding adaptor molecule 1 positive (Iba1+) microglial cells (38.3 ± 2.2 versus 151.1 ± 6.0 and 138.5 ± 4.7 for NI and AAV10-U7-CTR, respectively; p < 0.0001) (Figures 4A and 4B, lower panels).

We assessed skeletal muscle denervation by analyzing the occupancy of neuromuscular junctions (NMJs) in AAV10-U7-hSOD1-injected SOD1G93A mice, NI SOD1G93A mice, and WT mice at 112 days by double staining of the extensor digitorum longus (EDL) muscles for bungarotoxin (BTX) (binding to the nicotinic acetylcholine receptor of NMJ) and neurofilament (NF) (fibrillar component of the axons) (Figure 4C). After AAV10-U7-hSOD1 injection, 64.0 ± 3.2% of endplates were innervated in SOD1G93A mice, with no significant difference to WT mice (80.3 ± 7.0%) (Figure 4D, left panel). Conversely, NI SOD1G93A mice presented 31.5 ± 8.5% of innervated endplates and were significantly different from both AAV-treated and WT mice (p < 0.05 and p < 0.01; respectively) (Figure 4D, left panel). Endplates’
surface was preserved in AAV10-U7-hSOD1-injected SOD1 G93A mice (480.5 ± 50.52 m²) compared with NI and WT animals (324.3 ± 12.48 m², p < 0.05 and 580.3 ± 17.13 m²) (Figure 4D, right panel).

Furthermore, measurement of tibialis anterior (TA) muscle sections showed a 53% greater mean section area in AAV10-U7-hSOD1-treated mice (3.0 × 10⁶ ± 0.2 × 10⁵ m²) than in either AAV10-U7-CTR-injected (3.0 × 10⁶ ± 0.2 × 10⁵ m²; p < 0.05) or NI...
AAV10-U7-hSOD1-Mediated Gene Therapy Rescues 50-Day-Old ALS Mice Survival

We further investigated the potential therapeutic effects of AAV10-U7-hSOD1-mediated SOD1 silencing in adult mice, when muscle denervation is already present, by co-i.v./i.c.v. injecting 50-day-old SOD1G93A mice (n = 25) with the vector (i.v., 4.2 × 10^{14} vg/kg and 3.0 × 10^{13} vg/kg, respectively). The median survival of AAV10-U7-hSOD1-treated mice was extended by 63 days relative to NI controls (n = 24) (186 days versus 123 days; ****p < 0.0001, log rank Mantel-Cox test). The mean life expectancy was extended by 58% (195.0 ± 6.7 days versus 122.8 ± 0.9 days; ****p < 0.0001, Student’s t test). AAV10-U7-hSOD1 delivery also significantly prevented weight loss starting at 15 weeks of age in NI SOD1G93A mice (24.2 ± 0.6 g versus 21.0 ± 0.6 g, p < 0.01) (Figure 5C). Disease onset was delayed by 71.5 days in the AAV10-U7-hSOD1-injected mice relative to the NI mice (165.0 days versus 93.5 days; p < 0.0001) (Figure 5D). There was no significant increase in the duration of disease progression between AAV10-U7-hSOD1-injected mice and NI mice (31.7 ± 1.8 days and 28.2 ± 3 days, respectively; p = 0.33).

Widespread AAV10-U7-hSOD1-Mediated hSOD1 Suppression Delays Disease Signs in Adult SOD1G93A Mice

We observed a significant reduction of hSOD1 mRNA and protein levels in both the CNS and peripheral organs of mice injected with...
AAV10-U7-hSOD1 at the age of 50 days (n = 3) relative to NI mice (n = 3) (Figure S7), similar to the results of SOD1G93A mice injected at birth and confirming the consistent targeting of multiple tissues. Histopathological analysis of the spinal cord and TA muscle in SOD1G93A mice injected at the age of 50 days also showed 35% more MNs relative to age-matched NI mice (Figure S8A) and a total area and frequency distribution of the myofiber area in the TA muscle that was similar to that of WT mice (Figure S8C).

The overall phenotype of the treated SOD1G93A mice was considerably improved relative to that of NI mice, as illustrated in Figure 6A, with 36% of SOD1G93A-treated mice never showing signs of hindlimb paralysis. When present, the duration of paralysis, calculated as the time from muscle rigidity onset to death, was significantly extended by the treatment (16.7 ± 2.7 days versus 10.0 ± 1.4 days in NI; p < 0.05; Student’s t test), although there was no increase in disease duration in the AAV10-U7-hSOD1-injected mice, suggesting a potential beneficial effect of the treatment on muscle innervation. This was confirmed by the observation of NMJ protection in the gastrocnemius muscle of SOD1G93A mice injected with AAV10-U7-hSOD1 at 50 days relative to NI SOD1G93A mice (48.0 ± 2.5% versus 16.0 ± 2.0% of innervated endplates; p < 0.001; Student’s t test) (Figure S8B).

A further comparison of the contractile properties of the TA muscle from AAV10-U7-hSOD1-injected and NI mice confirmed the impact of hSOD1 silencing on neuromuscular function. The absolute maximal force generated during isometric TA muscle contraction after direct stimulation of the TA muscle of 112-day-old AAV10-U7-hSOD1-injected mice was double that of NI mice (79.2 ± 3.1 g versus 35.6 ± 5.1 g; respectively, p < 0.01), but still lower than that of WT mice (130.5 ± 10.0 g, p < 0.001) (Figure 6B, left panel). However, when the absolute force was normalized to muscle mass, the specific maximal force was 69% greater in treated than in NI mice (1.8 ± 0.1 g versus 1.1 ± 0.1 g; p < 0.01) with no statistically significant difference from that of WT mice (2.1 ± 0.1 g) (Figure 6B, middle panel). Moreover, the maximal activation capacity of the TA muscle (defined as the percentage of force generated in response to sciatic nerve stimulation relative to muscle stimulation) was more than 100% higher in treated mice than in NI controls (101.6 ± 3.1 versus 41.2 ± 10.0; p < 0.0001), and was not different from that of WT mice (90.3 ± 11.0) (Figure 6B, right panel), demonstrating the functional maintenance of neuromuscular transmission and functional force generation.
We confirmed the beneficial effect of AAV10-U7-hSOD1 on neuromuscular function by analyzing muscle strength in a grip test, showing the maintenance of muscle force over the lifespan of treated mice that had a significantly greater grip force than did NI mice (5.6 ± 0.2 g versus 4.5 ± 0.3 g, p < 0.05) and no statistically significant difference from that of WT mice (6.5 ± 0.3, NS), at 15 weeks of age (Figure 6C). The analysis of spontaneous motor activity in an actimeter also showed the complete rescue of the overall activity of the treated mice, which was significantly greater than that of NI mice and similar to that of WT mice (Figure 6D; Movie S1). In contrast, motor coordination of the SOD1G93A mice measured by the rotarod test was only partially restored by AAV10-U7-hSOD1 delivery because the latency to fall of the injected mice was still significantly higher than that of NI mice at 15 weeks of age (31.0 ± 3.3 s versus 21.4 ± 2.2 s, p < 0.05), but remained lower than that of WT mice (59.2 ± 2.6 s; p < 0.0001) (Figure S9).

DISCUSSION

In summary, we showed the efficacy of a new gene therapy approach for silencing mutant hSOD1 expression throughout the body of SOD1G93A mice by AAV10 delivery of a U7-AS construct that functionally skips exon 2 of hSOD1 out of frame. Survival and the results of functional tests exceeded any reported results from previous hSOD1-silencing approaches. This increased effect is likely due to the combination of a more efficient gene-silencing method and the targeting of both central and peripheral tissues.

The reduction in mutant SOD1 levels induced by AAV10-mediated exon skipping was much greater than those reported in previous studies using AAV9- or AAV10-mediated RNA interference10,11,13 with a nearly 70% reduction of hSOD1 protein levels in the spinal cord.

This effect is most likely due to the use of U7-delivered antisense molecules, acting at the pre-mRNA level and hampering the formation of a full-length hSOD1 mRNA and its toxic product. To establish whether the described exon-skipping approach outperforms RNA-interference techniques (artificial miRNA or short hairpin RNA), a direct comparison of the different molecular strategies tested under the same conditions (e.g., same amount of vectors and delivery route) is needed. These studies will be helpful to identify the best method for hSOD1 reduction in vivo.

Moreover, co-delivery of the therapeutic vector into the cerebrospinal fluid and bloodstream resulted in efficient SOD1 silencing in tissues known to be involved in SOD1G93A-mediated toxicity, including MNs and glial cells in the spinal cord24 and skeletal muscle fibers.25 Appropriate biodistribution studies, determining the histotypes targeted by the co-i.v./i.c.v. delivery of AAV10, will enable the identification of the specific cell populations responsible for the therapeutic effect. This will be possible in the immediate future, taking advantage of a tagged AAV10-U7-hSOD1 co-expressing the AS molecules and a reporter gene.

Unlike chemically modified ASOs, for which the short lifetime requires their regular re-injection and which can cause toxicity,7,29 our AAV-U7 strategy to deliver AS molecules allows sustained therapeutic effects in injected animals and reduction of neuroinflammatory signs. The loco-regional delivery of an AAV-U7-mediated exon skipping approach is currently in clinical development for the restoration of truncated dystrophin in patients with Duchenne muscular dystrophy, and the first preclinical results showed a good safety profile in large animals.30,31 The therapeutic effect observed in our study could also be due to the high AAV dose used (4.5 × 1014 vg/kg), initially tested in proof of concept studies in newborn mice and then scaled up in adult mice. Indeed, thanks to the combination of the two delivery routes (i.c.v. and i.v.), it was possible to inject larger AAV quantities compared to studies in which the two delivery routes were used independently. Dose-finding studies will be necessary to determine the optimal AAV dosage for the translation of this therapy in large animals before translation to humans. Results from both preclinical and clinical studies showed that higher doses allow better therapeutic effects in neuromuscular disorders (C. Le Guiner, personal communication; J.R. Mendell et al., 2016, Am. Soc. Gene Cell Ther., conference),35 but difficulties to manufacture high vector doses and potential immunotoxicity remain obstacles to overcome the development of whole-body rescue strategies.

The efficacy and the clinical feasibility of our innovative SOD1-silencing approach highlights its considerable and realistic potential for ALS treatment, although some concerns regarding safety and the regulatory process of the combined central and peripheral mode of AAV delivery should be considered. The therapeutic translation of this strategy could thus benefit from a direct comparison of the three administration routes, namely, i.v., i.c.v., and co.i.v./i.c.v., using equal AAV doses. This will imply the injection of highly concentrated AAV productions to respect the volume limitations in brain ventricles compared to those of the bloodstream.

The use of AAV in clinical and preclinical studies has demonstrated transgene expression for several years.33,34 Furthermore, to prolong the AAV-mediated therapeutic effect, different strategies are currently developed to allow vector re-administration and escape to the immune response, such as exosome-embedded AAVs35 or the use of tolerogenic nanoparticles.36 In our study, we appreciated the prolonged AAV-mediated effect on hSOD1 mRNA at the end stage of treated animals (more than 250 days), although the major part of treated animals died developing progressive signs of limb paralysis. This finite therapeutic effect observed in treated SOD1G93A mice is likely due to the accumulation of a residual amount of protein produced by the unskipped mRNA, attributable to the described prion-like properties of mutant hSOD1.37,38 Complete SOD1 suppression could be achieved by combining several U7-AS cassettes, as recently described by Schümperli and colleagues39 for SMA or by coupling other methods to suppress SOD1, such as RNA interference or single chain antibodies40 counteracting protein aggregation.
One-third of treated mice died from atypical ALS, without any sign of classical limb paralysis, rather showing a weight loss phenotype without ambulatory alterations, similar to the phenotype described by Stoica et al.13 This variability within the treated group could be due to stochastic distribution of the vector, which could have preferentially preserved limb motor units for yet unknown reasons.

The antisense sequences injected in SOD1G93A mice were able to specifically silence hSOD1, without affecting endogenous SOD1 levels, thanks to the presence of ten mismatches between the human and mouse sequences. Such discrimination will not be possible in man because the described exon-skipping method will not distinguish between the product of the mutant or WT allele. Consequently, hSOD1 exon skipping could be virtually applied to all patients with SOD1 mutations, demonstrating SOD1 accumulation. Whether the partial knockdown of functional SOD1 is detrimental to normal cellular physiology is still a controversial matter.41 SOD1 knockout mice do not develop evident pathological phenotypes,12 and the first clinical trial using infused ASO against total hSOD1 has proven safe.7 The use of in vitro models, such as induced pluripotent stem-cell-derived motor neurons,13 could help in the identification of potential toxic manifestations after such treatment. Moreover, a possible therapeutic solution to solve this problem would be to co-administer with the AAV10-U7-hSOD1, an antisense-resistant SOD1 coding sequence.

Interestingly, AAV10-U7-mediated exon 2 skipping mimicked a natural event observed in a Canadian SOD1-linked ALS family, carrying a heterozygous or homozygous deletion in a specific ESE sequence of SOD1 E2.26 In this family, natural skipping of E2 reduced transcription of the mutant SOD1 allele, explaining the low penetrance of the mutation. This mutation was found in ALS patients or carriers who had low levels of toxic hSOD1 protein and were either weakly affected or unaffected.44 In these patients, an exon 3 skipped form, which was never detected in our experiments, was observed.

When we tested the ASO in vitro, we observed some variability in exon-skipping efficacy. In some experiments, such as in the one shown in Figure S1B, the ΔE hSOD1 mRNA was much more evident in ASO2-treated cells relative to ASO1-treated cells. The thorough qPCR analysis on total mRNA levels (Figure 1B) showed a major efficacy in reducing the full-length RNA by the ASO1 sequence that was chosen for in vivo testing. One possible explanation for the lack of correspondence between the presence of the skipped form and the full-length hSOD1 mRNA reduction could involve other mechanisms than nonsense-mediated decay, such as nuclear RNA surveillance pathways for decay, as described by Ward et al.14 in the example of STAT3 exon skipping.

To evaluate SOD1 knockdown, we used an antibody recognizing the N-terminal region of the human SOD1 protein. The specific epitope is unknown and could detect some amino acids within exon 2. In this case, the effect on the protein would be attributed to the lack of a specific portion of the protein rather than to total suppression of hSOD1 protein. To corroborate the observed effect on protein suppression, we performed western blot analysis with an antibody raised against the full recombinant protein and confirmed the SOD1 suppression of the total protein in AAV10-U7-hSOD1-injected mice (Figure S10).

Different studies have suggested the involvement of WT SOD1 misfolding in sporadic ALS,23,45,46 but this involvement was not confirmed by one recently published study.47 Thus, the therapeutic application of AAV10-U7-mediated exon skipping may not be limited to the 12% of fALS patients who present with SOD1 mutations, but may also be applicable to some sporadic patients when hSOD1 accumulation is confirmed. This gene-silencing strategy could further be applied to a number of neurological disorders caused by “gain-of-function” gene mutations. In particular, because an U7-AS therapy has been demonstrated to be effective in the context of a trinucleotide repeat expansion pathology, namely Myotonic dystrophy type 1,48 we can speculate that it could be further applied to other diseases caused by repeated nucleotide expansion and in particular to another form of ALS not linked to SOD1, the most common form of dominantly inherited ALS linked to C9Orf72 mutations49 caused by hexanucleotide repeat expansion.

MATERIALS AND METHODS

Animals

Animals were maintained following the European guidelines for the care and use of experimental animals and approved by the Charles Darwin N.5 Ethics Committee on Animal Experiments (agreement number 04830.02). High copy SOD1G93A mice, B6SJLTg (SOD1*G93A)1Gur/J (JACKSON SN 272640), were purchased from Jackson Laboratory. The Colony Management Considerations published in “Working with ALS Mice” were taken into account for the maintenance of the strain. SOD1G93A mice were genotyped by PCR and assessed for hSOD1 genome copy number. To take into account the effect of gender on disease progression,51 sex-balanced groups of animals were injected.

AON Design

ESE finder 3.0 software22 was used to determine the ESEs in E2 of hSOD1. This software can predict binding sites for the most abundant SR proteins (SF2/ASF, IgM-BRCA1, SC35, SRp40, and SRp55). The consensus sequence for the SA was identified in the first hSOD1 intron. Five RNA-based ASOs masking these sequences on hSOD1 pre-mRNA were rationally designed. The ASOs were designed following the specific rules published by Aartsma-Rus et al.5 and using the RNAstructure 5.3 software. Each ASO was designed to be 20 nt long and to have the highest melting temperature (Tm) and the highest binding energy between the ASO and the target E2 sequence. ASO 1: 5′-CCACACCUUCAGUGCCA-3′; ASO 2: 5′-GGCCCUUCAGUCGUCCCUU-3′; ASO 3: 5′-UGGCUUCAUACUUUCCU-3′; ASO 4: 5′-CAAUGCAGGCCUCAGUCUCAG-3′; and ASO 5: 5′-UCUCUGCUAUACUUCCAAAC-3′. A scrambled ASO sequence was also selected as a negative control (ASO-CTR: 5′-GCUUUCCUUUCACUUCUU-3′).

Cell Transfections

HEK293T cells were cultured in DMEM containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin at 37°C in 5% CO₂ and
2'-O-methyl phosphorothioate (2’OMePS) ASOs were purchased from Eurogentec and re-suspended in H2O RNase-free water at a final concentration of 1 µg/µL. Cells were transfected with 5 µg of each ASO with Oligofectamine (Invitrogen) following the manufacturer’s instructions. The cells were harvested, 48 hr after transfection, for RNA extraction.

**AAV Productions**

The DNA sequences corresponding to the two best-performing ASOs were juxtaposed and cloned into pAAVsc_U7DTex23 (kindly provided by GENETHON) using PCR-mediated mutagenesis, as previously described. The scAAV serotype 10 vectors were produced using the tri-transfection method, as described in Dominguez et al. Vector titers were determined by qPCR on ITRs and expressed as vg/mL.

**AAV Injections**

SOD1G93A mice of 50 days of age were used for direct injection into the lumbar spinal cord. Mice were anesthetized by an intraperitoneal injection of a ketamine/xylazine mixture (100 mg/kg ketamine and 10 mg/kg xylazine; 0.1 mL per 20 g of body weight). Injections were performed as reported by Raoul et al., and a total volume of 10 µL (two sites, 5 µL per site) containing 9.5 x 10^10 vg (4.7 x 10^12 vg/kg) of each vector was injected per mouse.

Combined i.c.v. and i.v. injections were performed in newborn mice with a total of 7.8 x 10^15 vg (4.5 x 10^14 vg/kg) per animal. Each mouse was injected with 70 µL of vector solution in the temporal vein and 10 µL in the lateral ventricles (unilateral injection, coordinates: −1 mm anterior-posterior, ±1 mm medio-lateral, and −1 mm dorso-ventral from bregma) using a Hamilton syringe (32G and 30-mm length needle).

A total of 7.8 x 10^15 vg (4.5 x 10^14 vg/kg) was administered to 50-day-old adult mice using the combined i.c.v. and i.v. delivery route. A roughly 20 µL viral suspension was stereotactically injected into the lateral ventricles (−0.2 mm anterior-posterior, ±1 mm medio-lateral, and −1.8 mm dorso-ventral from bregma). An average of 320 µL of viral solution was injected into the tail vein or the retrobulbar sinus using an insulin syringe (29G, Terumo).

**RT-PCR and qPCR Analyses**

For in vivo analyses of mRNA levels, animals were anesthetized by intraperitoneal injection of ketamine (200 mg/kg, Imalgene, Merial) and xylazine (20 mg/kg, Rompun 2%, Bayer) at 112 days of age or at the end stage and transcardially perfused with PBS. Tissues (spinal cord, skeletal muscle, forebrain, brain stem, cerebellum, liver, and heart) were removed, snap frozen in liquid nitrogen, and stored at −80°C.

Total RNA was extracted either from frozen tissues or transfected HEK293 cells using the NucleoSpin RNA II RNA extraction kit (Macherey-Nagel) and quantified with a DeNovix DS-11 spectrophotometer.

For RT-PCR analyses, cDNA was synthesized from 1 µg of total RNA using oligo (dT) and random hexamer primers, according to the iScript cDNA Synthesis kit protocol (Bio-Rad). E2 skipping in the hSOD1 mRNA was revealed by RT-PCR analysis of 200 ng of cDNA using the following primers (Eurogentec):

- Primer Fw1, matching the hSOD1 exon 1: 5’-CTAGCGAGT TATGGCGAC-3’
- Primer Rev 4/5, matching the hSOD1 exon 4-exon 5 boundary: 5’-GCCAATGATGCAATGGTCTC-3’.

For qRT-PCR (qPCR) analyses, cDNA was synthesized from 200 ng of RNA using the High Capacity cDNA RT Kit (Life Technologies) following the manufacturer’s instructions. Amplified cDNA (30 ng) was mixed with 10 µL of Taqman Universal PCR Master Mix II - 2X (Life Technologies) and 1 µL of FAM probe for hSOD1 (TaqMan Gene expression assay Hs00533490_m1, Life Technologies). For in vitro studies, 1 µL of VIC probe for human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (TaqMan Gene expression assay Hs03929097_g1, Life Technologies) was used as endogenous control, and for in vivo analysis, 1 µL of VIC probe for mouse hypoxanthine-guanine phosphoribosyltransferase (HPRT) (Taqman gene expression assay Mm00446968_m1, Life Technologies) was used as endogenous control. Each sample was deposited in triplicate in a 96-well plate (Applied Biosystems). The thermal cycling conditions were: 1 min at 60°C and 10 min at 95°C, followed by 39 cycles of 15 s at 95°C and 1 min at 60°C in the StepOne Plus Real Time PCR System (Applied Biosystems).

The relative quantity of hSOD1 mRNA was calculated using the delta Ct/delta Ct method, taking into account the PCR signal of the target gene transcript of each sample (normalized to the endogenous control) relative to that of the control sample. The qPCR analyses were performed with the StepOne software v2.3 (Life Technologies).

**Western Blot Analyses**

Protein extracts from cells or freshly frozen tissues (see above) were prepared using a lysis buffer (150 mM NaCl, 50 mM Tris-HCl, 0.5% sodium deoxycholate, 1% NP40, and 0.1% SDS) supplied with a protease inhibitor cocktail (Complete Mini, Roche Diagnostics). Protein lysates were quantified using the DC protein assay kit (BioRad). 15-µg proteins (except for western blot in Figure S4) were separated on a 12% polyacrylamide gel (Criterion XT 12% bis-Tris, Bio-Rad) and analyzed by western blot using anti-α-tubulin (T5168, Sigma-Aldrich), anti-actin (A2066, Sigma-Aldrich), anti-human SOD1 (sc-8636, Santa Cruz Biotechnology), and anti-human SOD1 (556360, BD Pharmingen) antibodies. Peroxidase-conjugated mouse (VWR), rabbit (VWR), or goat (Life Technologies) immunoglobulin (Ig) antisera were used as secondary antibodies. Western blots were developed using the SuperSignal West Dura kit (Life Technologies), and densitometric analyses were performed using ImageJ software.

**Histological Analyses and Microscopy**

Animals were anesthetized by intraperitoneal injection of ketamine (100 mg/kg, Imalgene, Merial) and xylazine (10 mg/kg, Rompun in 2% saline) i.p. Animals were used for histological analyses 6 days post-injection. The spinal cords were dissected and fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 24 h before being transferred to 30% sucrose in PBS. Serial 30-µm coronal sections were cut on a freezing microtome and processed for cryostat permeabilization and adenosine triphosphate (ATP) detection as described previously.

Histological analyses and confocal imaging were performed as described previously.
2%, Bayer) at 112 days of age or at the end stage and transcardially perfused with PBS. The TA and *gastrocnemius* muscles were removed and perpendicularly or horizontally, respectively, placed on a cork support with tragacanth, frozen in cold isopentane, and stored at −80°C. Mice were then perfused with 4% paraformaldehyde (PFA) (Sigma-Aldrich) in PBS. The spines were explanted and stored in 4% PFA at +4°C for at least 24 hr, then transferred into a PBS-sucrose solution (30%) and stored for at least 24 hr at +4°C. Spinal cords were extracted, embedded in Tissue-Tek (OCT; Sakura Finetek), and frozen in cold isopentane (between −45°C and −50°C). 14-µm-thick sections were serially cut from the whole spinal cord at −22°C.

8-µm-thick cryosections were serially cut from TA muscles using a cryostat (Leica Microsystems) at −24°C, starting from the proximal origin, and stored at −80°C. Cryosections were fixed with 4% PFA and permeabilized in 0.1% Triton X-100 in PBS. Only spinal cord sections were also subjected to antigen retrieval with citrate buffer (10 mM citric acid, pH 6) at 85°C. Non-specific epitopes were blocked in a solution containing 5% BSA (IgG free, protease free, Jackson Laboratory), 0.1% Triton X-100 and 10% normal donkey serum (Millipore) (for ChAT), 10% normal goat serum (Life Technologies) (for GFAP, Iba1, hSOD1, and Laminin), or 1% FBS (Life Technologies) (for IIa-MHC) in PBS. The following primary antibodies were used in the corresponding blocking solution overnight at +4°C: goat anti-ChAT (1:50; AB144P); rabbit anti GFAP (1:250; Dako #Z033401); rabbit anti-Iba1 (1:400, Wako #019-19741); mouse anti-hSOD1 (1:100, BD Pharmigen #556360), rabbit anti-Laminin (1:400; Sigma-Aldrich #L9393), and mouse anti-MHC-IIa (undiluted SC-71, DSHB #AB2147165). After washing, tissues were incubated with the appropriate fluorescent-conjugated secondary antibodies: donkey anti-goat 488 (1:750, Life Technologies # A-11055, for ChAT), goat anti-rabbit 488 (1:750, Life Technologies # A-11008, for GFAP, Iba1, and Laminin), goat anti-mouse 594 (1:200, Life Technologies # A-11005 for hSOD1), or goat anti-mouse Cy3 (1:400, Jackson #115165206 for MHC-IIa), combined with DAPI staining (1:5,000, Sigma-Aldrich). Slides were mounted with FluoroMount-G Mounting Medium (Interchim).

Skeletal muscle images were obtained using an epifluorescence microscope (Leica Camera AG), digitized with a Nikon camera, and acquired using MetaMorph software. Spinal cord images were obtained using a Leica SPE confocal microscope (Leica Camera AG). Photographs were contrast enhanced by applying the brightness/contrast regulation of the Photoshop CS 8.0 software (Macintosh version, Adobe). Double immunofluorescence images were obtained by superimposing two or three single-color images of the same field.

ChAT-positive motor neurons (with a diameter >20 µm) were manually counted in the ventral horn of the cervical, thoracic, and lumbar spinal cord segments using an epifluorescence microscope (Leica Camera AG). Astroglial activation was evaluated by measuring the fluorescence intensity of the GFAP staining in the gray matter of the spinal cords using ImageJ software. Microglial activation was evaluated by counting the Iba-1-positive cells in the gray matter of spinal cord sections. The myofiber area and number were quantified using ImageJ software. Fiber areas were calculated in square pixels and then converted into µm². Cell counting and morphological analyses were performed in a blinded manner.

**NMJ Analysis**

For whole-mount staining of the EDL muscles, the protocol described in the SOP SMA.M_1.2.003 of “Treat-NMD neuromuscular network” was used with slight modifications. EDL muscles were freshly explanted, washed in PBS, and incubated on a rotating shaker (Stuart rotator SB3) for 10 min in a solution containing 500 µg/mL of 594-conjugated BTX (Life Technologies # B-13423) in PBS. After PBS washings, muscles were fixed with 4% PFA in PBS for 15 min and dissected under a stereomicroscope (Zeiss). Non-specific epitopes were blocked in a solution containing 5% BSA, 0.1% Triton X-100, and 10% normal goat serum for 30 min. The monoclonal antibody anti-NF (1:200, MAB5254) was incubated overnight at +4°C. After washings, EDL muscles were incubated with goat anti-mouse 488 (1:200, Life Technologies A-21121) and mounted on slides with FluoroMount-G.

20-µm-thick cryosections were serially cut from *gastrocnemius* muscles using a cryostat at −24°C and stored at −80°C. Sections were fixed with cold acetone, incubated with 594-conjugated BTX, and then processed for anti-NF immunofluorescence, as described above.

Fluorescence images along the z axis were taken by a Leica SPE confocal microscope. Z stacks were done using ImageJ software, and images were processed as described above. Endplates were scored as innervated when BTX and NF signals co-localized and were scored as denervated if they showed only BTX staining. Endplate areas were measured using ImageJ software and were calculated in square pixels and then converted into µm².

**Analyses of Muscle Contractile Properties**

Absolute maximal force, specific maximal force, and maximal activation capacity were evaluated by measuring the in situ isometric muscle contraction of the TA in response to nerve stimulation (as described by Ferry et al.33). Mice were anesthetized by intraperitoneal administration of a pentobarbital solution (60 mg/kg). The knee and foot were fixed with clamps and stainless steel pins, and the distal tendon of the TA muscle was attached to an isometric transducer, dual-mode lever (Aurora Scientific) using a silk ligature, under constant tension. Force responses to muscle and nerve electrical stimulation (square wave pulses of 0.1-ms duration, pulse frequency of 75–150 Hz, and stimulation train of 500 ms) were successively recorded. Absolute maximal isometric force was determined at optimal length (length at which maximal tension was obtained during the tetanus) and then normalized to the muscle mass to estimate specific maximal force. We also compared the maximal tetanic force produced following stimulation of either the sciatic nerve or the muscle directly. If neuromuscular transmission is impaired, indirect muscle stimulation would be expected to produce lower maximal tetanic.
force values than when the muscle is stimulated via the nerve. In this study, we used this comparison as a functional index of the capacity of the neuromuscular transmission to maximally activate the muscle (maximal activation capacity).

**Behavioral Analyses**

Body weight, strength, motor coordination, and spontaneous activity were assessed twice a week. The grip strength of the four limbs was evaluated using a grip strength meter (Bioseb) to determine the peak force (in grams) generated when the mouse is pulled back from a metal gird. For each test, five measures per animal were recorded. Motor coordination was assessed using an accelerating rotarod instrument (Bioseb), starting at 4 rpm/min. The time spent on the rotarod before falling was recorded in two runs per animal. At 17 weeks of age, the spontaneous activity of mice was assessed overnight (12 hr) using an actimeter to record the number of crossed infrared light beams, and data were analyzed using Track software (Bioseb).

Survival was determined as the time when the mouse was unable to right itself in 30 s when placed on its side (defined as the end stage).

**Statistical Analyses**

Statistical significance was assessed using the Student’s paired t test, one-way ANOVA, or two-way ANOVA, depending on the experimental protocols, as stated in the figure legends. Survival and disease-onset curves were compared using the log-rank Mantel-Cox test. Results were considered to be significant for p values under 0.05. All statistical tests were performed using Prism software (version 4.0, GraphPad). The therapeutic study of AAV-U7-hSOD1 delivery in adult SOD1(G93A) mice was designed following the Prize4Life criteria and the recommendations published by Scott et al.50

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes ten figures and one movie and can be found with this article online at http://dx.doi.org/10.1016/j.molther.2017.05.017.

**AUTHOR CONTRIBUTIONS**

M.G.B. and M.B. planned, designed, interpreted the experiments, and wrote the manuscript. T.V. intellectually contributed to the study and supervised the revision process. M.G.B., M.C.-T., C.B., and A.C. carried out experiments. M.G.B., M.R., B.G., and T.M. maintained the animals and performed behavioral analyses and injections. M.C.-T. and S.A. produced the AAV vectors. A.F. performed the electrophysiological analyses. Aurore Besse for her technical assistance, and Ilaria Di Emidio for the illustrations. We also thank G. Haase and M. Crescenzi for critical reading of the manuscript. This work was funded by the Association Française contre les Myopathies (AFM), the University Pierre et Marie Curie (UPMC), the Institut National de la Santé et de la Recherche Médicale (INSERM), the Centre National de la Recherche Scientifique (CNRS), the Association Institut de Myologie (AIM), the Otto per Mille Waldensian Church, and the Association pour la Recherche sur la Sclérose Latérale Amyotrophique (ARSLA). T.V. is supported by the NIHR GOSH Biomedical Research Centre.

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