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Lorelei I. Stoica

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GENE THERAPY FOR AMYOTROPHIC LATERAL SCLEROSIS:

AN AAV DELIVERED ARTIFICIAL MICRORNA AGAINST HUMAN SOD1 INCREASES SURVIVAL AND DELAYS DISEASE PROGRESSION OF THE SOD1^{G93A} MOUSE MODEL

A Dissertation Presented

By

LORELEI IOANA STOICA

Submitted to the Faculty of the

University of Massachusetts Graduate School of Biomedical Sciences, Worcester

in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

DECEMBER 7, 2015

GENE THERAPY FOR AMYOTROPHIC LATERAL SCLEROSIS:

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Interdisciplinary Graduate Program

December 7th, 2015

Dedication

To the patients.

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Abstract

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease characterized by loss of motor neurons, resulting in progressive muscle weakness, atrophy, paralysis and death within five years of diagnosis. About ten percent of cases are inherited, of which twenty percent are due to mutations in the superoxide dismutase 1 (SOD1) gene. Since the only FDA approved ALS drug prolongs survival by just a few months, new therapies for this disease are needed. Experiments in transgenic ALS mouse models have shown that decreasing levels of mutant SOD1 protein alters and in some cases entirely prevents disease progression. We explored this potential therapeutic approach by using a single stranded AAV9 vector encoding an artificial microRNA against human SOD1 injected bilaterally into the cerebral lateral ventricles of neonatal $SOD1^{G93A}$ mice. This therapy extended median survival from 135 to 206 days (a 50% increase) and delayed hind limb paralysis. Animals remained ambulatory until endpoint, as defined by a sharp drop in body weight. Treated animals had a reduction of mutant human SOD1 mRNA levels in upper and lower motor neurons. As compared to untreated $SOD1^{G93A}$ mice, the AAV9 treated mice also had significant improvements in multiple parameters including the number of motor neurons, diameter of ventral root axons, and degree of neuroinflammation in the spinal cord. These studies clearly show that an AAV9-delivered artificial microRNA is a translatable therapeutic approach for ALS.

Table of Contents

List of Figures

List of Multimedia Files

Movie 2.1A. An untreated SOD1^{G93A} mouse at 135 days shows signs of hind limb paralysis.

Movie 2.1B. An AAV9-amiRNA treated SOD1^{G93A} mouse at its humane endpoint (>200 days) is thin and has signs of kyphosis.

Movie 2.1C. An AAV9-amiRNA treated SOD1^{G93A} mouse at 198 days does not show signs of paralysis, or any movement impairment.

Movie 2.1D. An AAV9-amiRNA treated SOD1^{G93A} mouse at its humane endpoint (>200 days) is still able to climb and hang from a metal food hopper.

Preface

All of the work presented here was completed in Dr. Miguel Sena-Esteves' Laboratory at the University of Massachusetts Medical School.

Sections of Chapter II have been submitted to Annals of Neurology and are currently under review. I am the primary author on that article, and completed the first stage of manuscript composition. Dr. Sena-Esteves and Dr. Robert Brown edited the manuscript into its final form. Dr. Sena-Esteves is the corresponding author.

Dr. Christian Mueller designed the artificial microRNA to human SOD1, and provided a plasmid containing the artificial microRNA. Gabriela Toro Cabrera performed the initial *in vitro* screening of the artificial microRNA constructs, and the digital PCR.

Stacy Maitland packaged the original ssAAV9 containing the therapeutic cassette. Sophia Todeasa provided partial technical assistance with animal care, tissue sectioning and staining.

Dr. Johnny Salameh performed all electromyography recordings and analysis.

Dr. Mai ElMallah performed pulmonary function testing and analysis, and contributed to the study design. Kaitlyn Wetmore performed pulmonary function testing.

The UMMS electron microscopy core performed nerve embedding, cutting and staining. The UMMS small animal imaging core performed all CT imaging.

Dr. Robert Brown was involved throughout with concept formation.

I performed all other experiments, data analysis and manuscript composition.

CHAPTER I – INTRODUCTION

AMYOTROPHIC LATERAL SCLEROSIS

Amyotrophic Lateral Sclerosis (ALS) is a fatal neurodegenerative disease of upper and lower motor neurons [1]. Patients develop focal weakness, usually in the limbs or bulbar muscles. Disease progresses to the diaphragm and intercostal muscles, leading to breathing impairment and restrictive lung disease. The average age of onset is 55, and death occurs within three to five years of diagnosis, usually due to respiratory failure [2,3]. There is currently no effective cure, with Riluzole being the only approved treatment, extending life by only a few months [4].

ALS is a heterogeneous disease, with over 40 causal genes identified to date. Ninety percent of cases are sporadic in nature, while ten percent are familial, with a Mendelian inheritance pattern. A genetic cause has been identified for 68% of familial ALS (fALS) cases, and only 11% of sporadic ALS (sALS) cases. Some of the most commonly mutated genes are superoxide dismutase 1 (*SOD1*), fused in sarcoma/translocatd in liposarcoma (*FUS/TLS*), trans-active response (TAR) DNA binding protein (*TARDBP*), and *C9orf72*. *SOD1* was the first gene discovered to cause ALS [5], and is responsible for approximately 20% of familial cases and 1% of sporadic cases. Over a hundred mutations of *SOD1* have been identified to date, and most are inherited in an autosomal dominant manner [6,7].

Mechanism of SOD1 toxicity in ALS

SOD1 is an ubiquitously expressed soluble antioxidant enzyme that protects against reactive oxygen species through its conversion of superoxide radicals (O_2^-) to the less oxidizing H_2O_2 and molecular oxygen. In its native conformation, it is a beta barrel homodimer, stabilized by disulfide bonds, and contains a catalytic copper ion and structural zinc ion. The copper chaperone for SOD1 (CCS) facilitates copper insertion and disulfide bond formation, required for SOD1 maturation and enzymatic function. SOD1 is primarily localized in the cytosol, but it can also be found in the mitochondria [8].

Most cases of SOD1 fALS exhibit an autosomal dominant inheritance pattern. The exact molecular pathways by which SOD1 mutations lead to neuronal degeneration are unknown. Multiple mechanisms have been proposed, including 1) toxic protein aggregation, 2) loss of proteostasis, 3) mitochondrial dysfunction, 4) oxidative stress, 5) axonal structure and transport deficits, 6) excitotoxicity and 7) non-cell autonomous effects. It is likely that disease results from a complex interplay between these multiple, non-mutually exclusive cellular events, but the exact relationships still need to be elucidated.

It is widely accepted that SOD1 misfolding and aggregation plays a key role in disease. SOD1 positive protein aggregates are observed in spinal cord post-mortem fALS and sALS patient tissue, as well as in SOD1 mouse and cell models [9,10]. Most SOD1 mutations do not cause a loss of enzymatic activity, but instead destabilize the native conformation of the homodimer [10-12]. This leads to dimer dissociation and the formation of monomeric SOD1. Monomeric SOD1 is susceptible to losing the stabilizing zinc ion

and disulfide bridges, and subsequently refolding into non-native oligomers that accumulate in the cell [13]. These small, misfolded species are selectively enriched in motor neurons, and become insoluble aggregates as disease symptoms develop. The aggregates associate with soluble oligomers at disease endpoint, as observed in a $SOD1^{G85R}$ mouse model [14]. It has been postulated that misfolded SOD1 acts in a prion like manner – capable of cell-to-cell transmission, and initiation of aggregation of normal cellular counterparts. *In vitro*, misfolded SOD1 proteins are able to propagate from cell to cell, and induce aggregation of native SOD1. *In vivo,* transgenic $hSOD1^{G93A/WT}$ mice develop hSOD1 aggregates containing both the mutant and wild type forms of the protein [14-17]. Additionally, non-genetic perturbations to the structure of wild type SOD1, such as oxidation and metal depletion, lead to aberrant conformations and misfolding, and can induce aggregation in a manner similar to mutant SOD1. Indeed, wild type SOD1 aggregates have been found in a subset of SALS patients [9,18]. Since specific mutations in SOD1 have distinct survival times ($SOD1^{A4V}$ 1 year average; $SOD1^{H46R}$ 18 years average), it was postulated that aggregation propensity could be correlated with disease duration. However more recent studies suggest this not to be true [19,20]. It has also been postulated that while the unfolded SOD1 monomers are the toxic species, the aggregates have a protective role - sequestering free unfolded SOD1 monomers and reducing their concentration in the cell [15]. Thus, while protein aggregates are a pathological hallmark of SOD1 ALS, it is still unclear if they are disease causing, harmless byproducts, or a protective response.

Accumulation of SOD1 aggregates in ALS suggests a potential saturation of cellular mechanisms responsible for clearing misfolded proteins. Two such responses that have been implicated in ALS are proteasomal degradation through the ubiquitin-proteasome system (UPS) and endoplasmic reticulum (ER) stress activation of the unfolded protein response (UPR). Excess misfolded mutant SOD1 overloads the proteasome and inhibits its function [21]. Similarly, the UPR is normally protective, suppressing translation while promoting misfolded ER protein degradation, but its prolonged activation leads to apoptotic signaling [22]. Transgenic SOD1 mice have increased levels of ER stress sensors and chaperones confirming activation of the protein degradation mechanisms [23-27]. Additionally, mutant SOD1 binds proteins involved in both proteasomal [28,29] and ER associated degradation [30], directly impeding their function. In an attempt to reduce the protein burden in the cell, the levels of autophagy were increase in transgenic SOD1 mice. This led to clearance of the mutant protein and an extension in lifespan [31]. Interestingly, mutations in proteosome degradation protein UBQLN2 and UPR protein VAPB (vesicle-associated membrane protein-associated protein B) have also been linked to ALS [32,33], suggesting protein degradation dysfunction as a common mechanism of cell death in multiple types of ALS.

Axonal structure and transport are important for proper motor neuron function. Neurofilament is a component of the axonal cytoskeletal, and maintains axon structural integrity. Large caliber motor axons in ALS are selectively vulnerable in ALS, potentially due to changes in cytoskeletal organization and neurofilament assembly. Studies using induced pluripotent cells (iPSCs) from SOD1 patients show that motor neurons

selectively exhibit neurofilament aggregation. This is due to mutant SOD1 binding of the 3'UTR of NF-L mRNA, leading to a decrease in its stability, and thus altered proportions of NF subunits [34]. A re-establishment of proper neurofilament levels was shown to extend the lifespan of ALS mice [35]. Mutant SOD1 also binds motor complexes required for axonal transport. Subsequently, SOD1 mice have slowed axonal anterograde and retrograde transport, and impaired trafficking of mitochondria and cytoskeletal components [36,37]. This can potentially lead to cell death through energy deficiencies due to a lack of mitochondria at the distal synapses, or impaired neuromuscular communication. However, ALS mice with intact axonal transport still display motor neuron degeneration [38]. Mutation in genes leading to neurofilament disruption and transport have also been linked to ALS, further supporting this mechanism as a common pathway for disease progression[39]. Axonal transport and cytoskeletal pathology contribute to disease progression, but are unlikely primary causes of disease for SOD1-ALS.

Mitochondrial swelling and vacuolization has been found in the spinal cord of ALS patients and SOD1 mouse models, although it is unclear if changes in this organelle are a cause or consequence of neurodegeneration [40,41]. Wild type SOD1 is predominantly localized to the cytoplasm, but can be imported into the mitochondria [42,43]. Due to its misfolded shape, mutant SOD1 can become trapped in the mitochondria, and accumulate in the intermembrane space and on the outer membrane, altering mitochondrial shape and distribution [44,45], impairing its function, and leading to cell death [46]. Additionally, accumulation in the intermembrane space blocks mitochondrial protein import [47].

Mitochondria are responsible for ATP production and calcium homeostasis, and dysregulation of both of these processes has been implicated in ALS [47]. Specifically, $SOD1^{G93A}$ mice display defects in ATP synthesis [48] and lower levels of ATP [49] as well as lowered calcium buffering capacity [50]. Additionally, ALS patients have excess intracellular calcium levels at motor nerve terminals [51]. Motor neurons might be particularly sensitive to changes in calcium levels due to their need for calcium in action potential generation. An increase in mitochondrial calcium buffering in $SOD1^{G93A}$ mice led to maintenance of motor neurons, although disease progression was not affected [52]. Lastly, misfolded SOD1 oligomers could trigger apoptosis through their binding of mitochondrial anti-apoptotic proteins such as Bcl-2, causing conformational changes and subsequent conversion to a toxic species [53]. Given the selective damage and accumulation of mutant SOD1 in mitochondria of cells specifically affected in ALS, as well as the functional impairments seen in this organelle in both ALS models and patients, it is likely that mitochondria play a crucial role in disease pathogenesis.

Blood and CSF samples, as well as biochemical analysis of post mortem tissue samples from SOD1 fALS and sALS patients has revealed signs of oxidative damage [54,55]. It is unlikely that the reactive oxygen species are produced by a lack of dismutase activity or an aberrant enzymatic function of mutant SOD1 since a complete lack of SOD1 does not cause motor neuron degeneration, and an enzymatically inactive mutant SOD1 still causes motor neuron disease [56]. However, mutant SOD1 has been shown to disrupt regulation of NADPH Oxidase (Nox), and cause its over-activation, leading to a persistent production of extracellular superoxide [57]. The reactive oxygen species are toxic

to cells, and cause microglia activation, which leads to further neuroinflammation [58]. In fact, activated microglia are found in ALS rodent models before symptom onset [59,60]. Additionally, mRNA oxidation is commonly seen in ALS patients. SOD1 mice show mRNA oxidation primarily in motor neurons and spinal cord oligodendrocytes, and decreased translation of these molecules. Interestingly, certain mRNA species are more vulnerable to oxidation, including those involves in mitochondrial electron transport chain, folding and degradation pathways, and cytoskeleton structure [61]. Additionally, oxidized wild type SOD1 found in sALS patients displays axonal transport deficits similar to those observed in SOD1 ALS [62]. Thus, aberrant oxidative damage could be affecting other cellular processes, and accumulation of reactive oxygen species could be directly contributing to cellular stress, causing the premature neuronal degeneration.

Another possible mechanism by which SOD1 causes disease pathology is thorough glutamate excitotoxicity, a process of neuronal cell death caused by excess cellular calcium influx, due to excessive stimulation of glutamate receptors. Some ALS patients have raised levels of CSF glutamate, and concentrations correlate with faster disease progression [63]. Additionally, astrocytes of both ALS patients and mouse models show a decrease in the expression and activity of glutamate transporter EAAT2, especially in areas affected by neurodegeneration [64-66]. The strongest evidence for this hypothesis comes from Riluzole, the only approved therapeutic for ALS, which acts by inhibiting glutamate release and increasing its uptake [67-69]. However, patient survival is only increased by a few months, thus deficient glutamate reuptake is likely only one of the mechanisms involved in disease progression.

Loss of motor neurons is the major driver of disease phenotype although there is significant involvement of non-neuronal cells, including astrocytes, oligodendrocytes, and microglia [70]. Thus, it has been questioned whether motor neuron death is cell autonomous – caused by the intrinsic toxicity in neurons themselves; or if it is non-cell autonomous – triggered by disease progression in other cell types. Several mouse models of SOD1 ALS, as well as multiple *in vitro* studies have contributed to the understanding of mechanism of disease and contribution of different cell types [71-74]. Restricting expression of mutant SOD1 to specific cell populations has shown that non-cell autonomous mechanisms are required for disease progression. Motor neuron restricted expression of mutant SOD1 does not cause paralysis [75,76], although there is some neurodegeneration when expressed at supraphysiological levels [77]. Expression of mutant SOD1 in astrocytes (murine $SOD1^{G86R}$) [78], microglia ($hSOD1^{G93A}$) [79], or Schwann cells ($hSOD1^{G93A}$) [80] alone does not cause an ALS phenotype. However, delivery of $hSOD1^{G93A}$-derived astrocytes into the cervical spinal cord induces death in neighboring motor neurons [81]. Skeletal muscle restricted expression of mutant $hSOD1^{G93A}$ also leads to motor deficits [82]. Thus accumulation of human SOD1 in certain non-neuronal cells, such as astrocytes and skeletal muscle, can initiate non-autonomous degeneration of motor neurons.

Studies have also been done to determine the effect of cell specific SOD1 reduction. Decreasing mutant SOD1 in astrocytes [83], microglia [84], and oligodendrocytes [85] increases survival, while decreasing mutant SOD1 in Schwann cells decreases survival [86] of the SOD1 mouse. Decreasing hSOD1 in the hindlimb muscle does not affect disease

progression [87]. Furthermore, survival is increased when hSOD1^{G93A} motor neurons are surrounded by wild types cells, as seen in a chimeric mouse, or after spinal cord transplantation of wild type glial cells [88,89]. Taken together, these experiments use transgenic mice to demonstrate the interdependence of different cell types in disease progression.

The mechanisms of non-cell autonomous motor neuron degeneration are still being investigated, and it remains unclear if the primary site of dysfunction is the motor neuron itself. Motor neuron death promotes neuroinflammation, causing the recruitment and activation of astrocytes and microglia. While this is initially a protective mechanism, continuous microglia activation in ALS causes a switch to an inflammatory neurotoxic phenotype, potentially through NF-kB activation [90-92]. Oligodendrocytes degenerate and die early on in ALS progression, and new ones are generated by differentiation of oligodendrocyte precursors. However, these newly differentiated cells have compromised myelination abilities and are unable to provide metabolic support to motor neurons [93,94]. Lastly, mutant SOD1 astrocytes have also been shown to be toxic to motor neurons, either through the secretion of a soluble toxic factor [95], or through changes in glutamate handling, leading to glutamate excitotoxicity [96]. *In vitro*, astrocytes from the SOD1 mouse or sALS patients are toxic to co-cultured motor neurons [97,98], while iPSC SOD1^{G93A} astrocytes induce motor neuron death in wild type rats [81]. However, reducing mutant SOD1 levels in these cells abrogates the toxicity. Additionally, co-culture studies have shown motor neuron death is triggered by fALS or sALS astrocytes through

necroptosis [99]. Taken together, these studies prove that neighboring cells have a crucial role in motor neuron degeneration.

Although the exact mechanism by which SOD1 causes motor neuron degeneration remains uncertain, both cell autonomous and non-cell autonomous mechanisms are implicated, and accumulation of misfolded SOD1 has a crucial role.

Mouse model of SOD1 ALS

Since its discovery in 1993 as the first ALS causative gene [5], SOD1 has been studied in multiple *in vitro* and *in vivo* models. The $SOD1^{G93A}$ mouse is one of the most commonly used models of ALS, due to its reproduction of ALS pathophysiology and phenotype. Specifically, the $SOD1^{G93A}$ mouse develops progressive hind limb weakness, leading to paralysis and death [100]. Histologically, the mouse has ubiquinated aggregations of SOD1, inflammation in the spinal cord, denervation of neuromuscular junctions, and loss of both upper and lower motor neurons, of the layer V of the motor cortex and spinal cord, respectively [100-102]. This phenotype is due to the expression of multiple copies of the full human SOD1 (hSOD1) gene, containing the G93A mutation [100]. The level of $hSOD1^{G93A}$ gene expression is directly correlated with disease severity - mice with fewer $hSOD1^{G93A}$ transgene copies have a milder phenotype and delayed disease development [103,104]. The observed phenotype is thought to be caused by a gain of function mechanism and accumulation of mutated hSOD1 protein, consistent with the autosomal dominance inheritance patterns seen in patients. Transgenic mice expressing wild type hSOD1 have mitochondrial dysfunction but no paralysis [41]. However, transgenic mice expressing

higher levels of wild type hSOD1 do develop late onset paralysis and have aggregates of the wild type protein [105]. This further supports the notion that wild type SOD1 may indeed play a role in disease progression, since some sALS patients have SOD1 aggregates but no SOD1 mutation [9]. Furthermore, SOD1-fALS is through to be a gain of function mechanism, since $hSOD1^{G93A}$ is enzymatically active, and endogenous mouse Sod1 levels are unchanged [106]. Additionally, *sod1* knock out mice do not have motor dysfunction [107]. Due to its extensive characterization and reproduction of multiple aspects of human disease the $SOD1^{G93A}$ mouse is still one of the best models for testing potential therapeutics, especially those aimed at treating SOD1 ALS.

REDUCTION OF GENE EXPRESSION FOR NEUROLOGICAL DISORDERS

Dominantly inherited diseases caused by a gain of toxic protein function can be treated by reducing the toxic protein levels. This can be done at the protein level itself, by using antibodies to bind and remove the protein, or at the mRNA level by utilizing antisense oligonucleotides (ASO) and RNA interference (RNAi) to silence gene expression [108-110]. While each of these methods is effective at reducing protein expression, they work through distinct mechanisms, with different advantages and disadvantages.

Monoclonal antibodies have been developed to recognize toxic species in neurological diseases, especially misfolded protein that have an aberrant conformation. This approach takes advantage of passive immunization, and uses the body's immune system to clear the misfolded proteins. An advantage is that antibodies can be specifically

developed against the misfolded conformation, leaving the wild type protein intact [109-111]. These engineered antibodies can be directly infused into the central nervous system (CNS) at multiple times points, or they can be expressed from a viral vector for sustained long-term expression[110]. ASOs are single stranded nucleic acids that bind to a target sequence of mRNA. The oligonucleotide-mRNA duplex is recognized and degraded by ribonuclease H (RNAse H) [112]. Neither monoclonal antibodies nor ASOs can cross the blood brain barrier (BBB) without the aid of chemical modification. However, direct delivery into the CSF, such as through intrathecal infusion, has been used successfully to treat neurological disease in pre-clinical studies [113,114]. RNAi uses the endogenous cell machinery of post-transcriptional gene silencing to reduce the expression of target mRNAs (Figure 1). All of these approaches are effective at silencing gene expression and choosing which to use depends on the specific needs of each therapeutic approach.

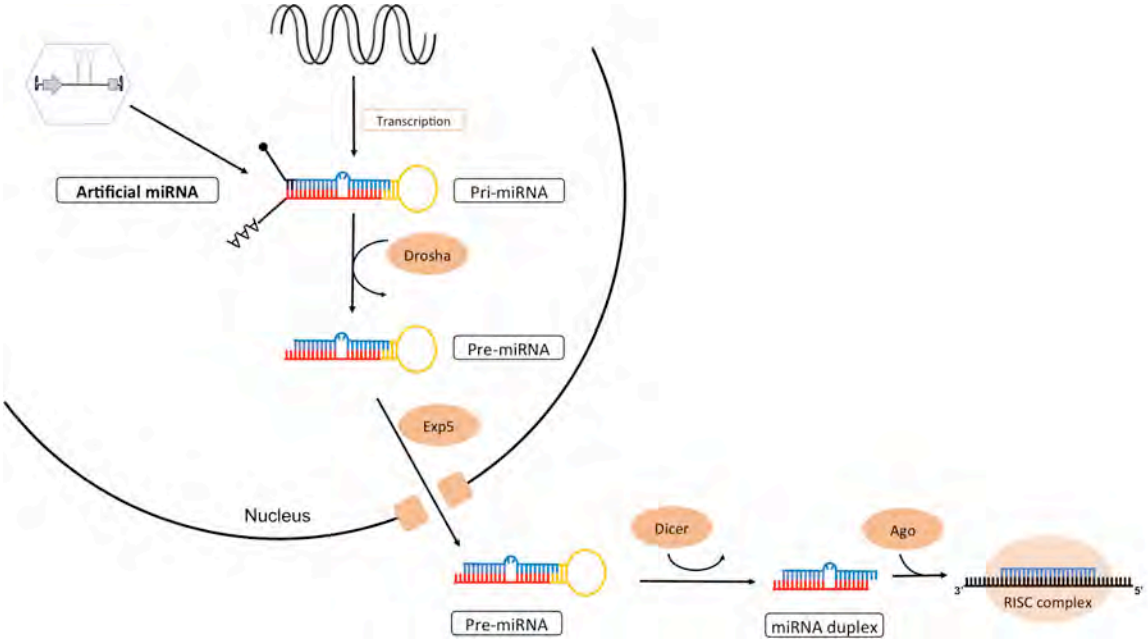

Figure 1. MicroRNA biogenesis and processing of RNAi molecules. The primary microRNA (pri-miRNA) is transcribed from the genome and forms a stem-loop structure. It next cleaved by Drosha into a pre-microRNA (pre-miRNA) hairpin structure. Exportin 5 shuttles the pre-miRNA out of the nucleus through the nuclear pore. The pre-miRNA is further cleaved by Dicer into the microRNA (miRNA) duplex. One strand of the miRNA duplex is preferentially loaded into RISC (guide strand, blue). The activated RISC binds complementary mRNAs. Catalytically active Ago2 degrades mRNAs that are perfectly complementary to the miRNA seed sequence. The endogenous microRNA pathway can be used for therapeutic gene silencing. The different RNAi species (amiRNANA, shRNA, and siRNA) mimic endogenous molecules (pri-miRNA, pre-miRNA, miRNA, respectively), and are processed in a similar manner at the respective steps of the pathway. AAVs can be used to deliver amiRNAs and shRNAs into the nucleus, while siRNAs directly enter the cell and are processed in the cytoplasm.

RNA interference is an inherent cellular mechanism used to regulate gene expression [115]. It uses short double stranded RNA strands processed from endogenously expressed transcripts to target complementary cellular mRNA for translational repression or degradation [116-119]. The double stranded RNA molecules can be transcribed by either polII or polIII promoters from independent genomic transcription units, or from the introns of protein-coding genes into long stem-loop primary-microRNA transcripts (pri-

miRNAs) [120-123]. These pri-miRNAs are cleaved in the nucleus by the microprocessor complex composed of Drosha, an RNAse III enzyme, and DGCR8, a double stranded RNA binding protein. This results in a ~60-80nt hairpin structured precursor-miRNA (pre-miRNAs), with a two nucleotide 3' overhang and stem loop [124-126]. The pre-miRNA is then transported to the cytoplasm though the nuclear pore complex by the nuclear export machinery, composed of Exportin-5 and Ran guanosine triphosphate (Ran-GTP) [123,127,128]. Once in the cytoplasm, pre-miRNAs are recognized by another RNAse III enzyme, Dicer. Dicer, in cooperation with its partners, transactivation-response RNA-binding protein (TRBP) and protein kinase R-activating protein (PACT), binds the base of the hairpin and cleaves the loop [129-131]. The resulting ~22nt RNA duplex consists of the antisense guide strand and the sense passenger strand. The guide strand is preferentially loaded into the RNA Induced Silencing Complex (RISC) based on 5' end base pairing thermostabilty [118,132,133].

RISC is a multiprotein complex; key components are the RNA guide strand and a catalytically active argonaute protein such as Ago2 [134]. The RNA guide strand has a "seed" sequence at nucleotides 2-8 of its 5' end, which binds to mRNAs by sequence complementarity [135,136]. This can be anywhere in the mRNA, but it is usually in the 3'UTR [137]. RISC cleaves perfectly complementary mRNA through the RNAse H like PIWI domain of Ago2, or represses translation of imperfectly complementary mRNAs primarily through inhibition of translation initiation. [116,138-140]. After binding and cleavage, the miRNAs are either degraded or recycled [141]. This mechanism is commonly

used for cellular gene regulation as well as for host defense by degradation of viral RNAs[117].

For therapeutic purposes, there are three different species of small RNA targeting molecules – short interfering RNAs (siRNAs), short hairpin RNAs (shRNAs) or artificial microRNAs (amiRNAs), and each enters the RNAi pathway at different steps [142] (Figure 1). siRNAs, usually double stranded duplexes or single stranded nucleotides, are delivered to the cytoplasm, and processed by Dicer or loaded directly into RISC, respectively [143,144]. shRNAs and artificial microRNAs can be delivered into the nucleus as vector encoded transgenes [145,146]. shRNAs have a hairpin structure that is similar to pre-miRNA. They do not undergo any processing in the nucleus and are exported out into the cytoplasm for cleavage by Dicer and loading into RISC [147]. amiRNAs, as the name implies, use the backbone of endogenous microRNAs and an artificially designed guide strand that is complementary to the gene of interest [148]. Thus, amiRNA are processed through all the steps of the miRNA pathway [126,149,150]. All RNAi species have been successfully used for gene silencing *in vitro* and *in vivo* [151].

One of the concerns with using RNAi for gene silencing is toxicity due to overexpression of the therapeutic molecule. High levels of expression have lead to reports of dose dependent toxicity with the use of U6 promoter expressed shRNAs; the toxicity is not specific to the targeting sequence or the tissue types. Both targeting and control shRNAs proved toxic when expressed at high levels, both in the liver and the brain, in multiple species [152-156]. It was hypothesized that high expression levels of RNAi molecules lead to saturation of the endogenous RNAi machinery due to competition with

endogenous miRNA, as well as subsequent dysregulation of endogenous miRNA expression. The rate-limiting factors could be Exportin 5 and Ago2, as their increased expression led to an attenuation of the toxicity [152,157]. Additionally, even when delivering the same amounts of shRNAs, toxicity was directly correlated with guide strand RNA levels [154]. Multiple studies have shown that reducing guide strand expression reduces toxicity. This has been achieved through the use of weaker promoters, such as Pol III H1 and Pol II cellular promoters, amiRNA instead of an shRNA, and less effective means of gene delivery [146,157-159]. In a direct comparison of U6 expressing shRNA and miRNAs, the shRNA proved to be toxic and to be disrupting endogenous miRNA biogenesis [146]. Additionally, even when optimized for identical strand processing, the guide strands of shRNA were expressed at higher quantities and were more effective at decreasing target mRNA levels [160]. Artificial miRNA, which tend to be expressed from cellular pol-II promoters, have been used with no reportable toxicity. Thus artificial miRNAs have proven to be safe and effective as gene silencing molecules.

Many Phase I and Phase II clinical trial are ongoing for RNAi therapeutics. siRNAs are the most advanced in the clinic, with two ongoing Phase III trials aimed at reducing TTR for the treatment of TTR mediated amyloidosis [161]. For neurological diseases, RNAi molecules are either delivered directly to the CNS by a direct infusion, or use a delivery vehicle that can cross the blood brain barrier [162]. Although ASOs are not RNAi molecules, they also act by targeting mRNA, and have similar delivery requirements as siRNAs. ASOs are currently being used in the clinic for neurological diseases such as ALS, to decrease expression of SOD1 [163]. However, both species are

transient in nature, and require re-administration for maintenance of therapeutic effect. Thus attention has been turned to viral vector delivered RNAi to achieve sustained expression of the therapeutic molecules.

AAV GENE THERAPY FOR GENETIC DISEASES

Gene therapy is a promising option for treatment of diseases driven by gene mutations, where the cause is a missing, dysfunctional, or toxic gene product. Viral vectors can be used to deliver therapeutic molecules aimed at restoring functional protein levels or decreasing the amount of toxic protein. Additionally, a single administration of the therapeutic molecule is often sufficient for sustained gene expression. Adeno associated virus (AAV) is a commonly used viral delivery vehicle for gene therapy.

AAV is a small (~22nm), nonpathogenic, nonenveloped, single stranded DNA virus. Wild type AAV has a single stranded 4.7kb genome flanked by two hairpin shaped inverted terminal repeats (ITRs). Its genome codes for proteins required for replication, viral packaging, and capsid assembly [164,165]. However, it is replication deficient and it depends on either adenovirus or herpes simplex virus for replication [166]. The capsid is responsible for the tissue tropism of the virus – the residues on the capsid surface bind cell surface receptors and co-receptors, leading to internalization into the cell. Thus capsid variants can display different tissue tropisms, and retrograde or anterograde transport abilities, depending on variations in capsid structure and affinity for specific cellular receptors [167-172]. Once inside the cell, AAV enters the nucleus, the capsid is uncoated and the genome is released [173]. Since AAV is a single stranded DNA virus,

second strand synthesis takes place, followed by transcription. This is one of the rate limiting steps for AAV transduction, and only a small percentage of AAV vector genomes become transcriptionally functional [174]. Wild type AAV integrates into the host genome at a high frequency, with a preference for the AAVS1 site on human chromosome 19, although it can also have an episomal persistence [175-177]. To date, over 12 AAV serotypes have been identified from human and nonhuman primate origins, as well as over 100 additional genomovars [178,179].

Due to the non-pathogenic nature and long terms gene expression of wild type AAV, recombinant AAV (referred to as AAV throught this manuscript) was developed as a gene transfer vector. The wild type AAV genome contains two ITRs flanking genes encoding regulatory (Rep) and capids (Cap) proteins, and needs adenoviral helper elements to replicate [180]. In the recombinant AAV genome, the viral genes are removed, and the two ITRs are kept, the only *cis* elements, required for replication and packaging into the capsid [181,182]. During packaging, the *rep* and *cap* genes, along with the adenovirus helper genes, are delivered in *trans* [183,184]. This ensures that no wild type viral proteins are packaged into the recombinant AAV. To be used as a gene transfer vector, a transgene cassette is inserted between the two ITRs. In single stranded AAVs, the size of the transgene cassette is limited to 4.5kb [185]. Self-complementary AAVs (scAAV) were also developed, to bypass the rate-limiting step of second strand synthesis after nuclear localization. This was accomplished by a mutation in the right ITR. Replication continues through the mutated ITR generating a dimeric DNA genome. This leads to scAAVs having transcriptionally competent dimeric DNA genomes that become active

immediately after decapsidation in the nucleus [186]. These vectors have more rapid transgene expression, but also half the packaging capacity, of 2.3-2.4kb. Unlike wild type AAV, recombinant AAV very rarely integrates, instead having a mostly episomal persistence in various forms, including circular, linear, and large concatemers [175]. Much work has been dedicated to vector development since the discovery of AAVs, and multiple capsid variants have been developed in the lab, leading to improved transduction efficiencies and modulation of tissue tropism profiles [168,187].

One concern with AAV therapy is the pre-existence or generation of neutralizing antibodies. Neutralizing antibodies against wild type AAV naturally exist in humans and other large mammals [188]. Additionally, recombinant AAVs cause a strong humoral immune response, leading to the development of neutralizing antibodies against the injected capsid [189]. Neutralizing antibodies against the capsid being infused remove the AAVs from circulation [190]. Studies have shown that administration of the same virus in a previously injected animal leads to significantly reduced gene transfer [191]. Thus, if re-administration is necessary, a different serotype must be used. Lastly, an immune response to the transgene itself, rather than just the AAV capsid protein, has also been observed. Neuronal loss of transduced cells was reported after expression of GFP and a therapeutic transgene, when both proteins were novel to the host immune system [192]. Recent clinical trials with scAAV8 for Hemophilia B have shown that a tapered course of prednisolone is sufficient to blunt the immune response to the capsid and allow for long-term stable gene expression [193]. Thus, when considering translation to larger animal

models and clinical trials, the effect of the immune response to both the capsid and the transgene must be monitored.

It has been postulated that AAV2 insertional mutagenesis could cause hepatocellular carcinoma (HCC). A recent publication discovered the presence of wild type AAV2 sequences in several tumor driver genes, in 6% of examined HCCs[194]. Although this study raised potential concerns regarding the pathogenicity of AAV, further studies need to be performed in order to definitively establish a causal link between AAV2 integration and development of HCC. As mentioned by Berns et al.[195], it would be informative to know the level of AAV2 antibody in control and case subjects. The potential for co-infection with adenovirus or HSV should also be determined, since these viruses are known provide helper virus functions for AAV replication[166]. Additionally, the analysis was done for the integration of wild type AAV2. However, recombinant AAV, the clinically used therapeutic molecule, has different properties than the wild type species. Recombinant AAV has a mostly episomal persistence, with much lower integration incidence than wild type AAV[177]. Additionally, previous animal studies using recombinant AAVs have shown the likelihood of integration to be correlated with vector dose, and promoter strength, and integration to be preferential to transcriptionally active genes[196]. For this reason, recombinant AAV associated HCCs have only been seen after vector delivery to neonatal mice, and has not been reported in adult rodents, dogs, or nonhuman primates [197-199]. Furthermore, none of the almost 100 clinical trials using recombinant AAV have reported development of HCCs [195,200]. Nevertheless, the long-term effects of recombinant AAV administration still need to be monitored.

AAV delivery to the CNS

Initial studies for neurological disorders were performed using intracranial injection of AAV into the brain region of interest. Most serotypes will transduce neurons at the site of injection, as well as microglia, astrocytes, and oligodendrocytes. However, the transduction profiles show differences in cell type preference and expression levels. Comparison studies using direct intracranial injections in adult mice have determined that AAV5, AAV7, AAV8, AAV9, AAVrh10 are particularly efficient in neurons, while AAV5, AAV8, and AAVrh8 are also efficient in astrocytes. Additionally, AAV4 and AAVrh8 transduce ependymal cells, while AAVrh8 also transduces oligodendrocytes. AAV9 and AAVrh10 also had the largest vector spread from the injection site. [201-205]. However, direct intracranial injections have limited vector spread beyond the injection site, and thus are most effective for diseases where benefit can be achieved when treating a specific region.

The first gene therapy trial for a neurological disease was done for Canavan's disease, a childhood leukodystophy, using AAV2. The missing enzyme, aspartoacylase (ASPA), was delivered by six direct intracranial injections, bilaterally in the frontal, parietal, and occipital regions of the brain. This trial proved AAV2 to be safe for use in humans as a therapeutic vector, but the patients showed only modest phenotypic improvement [206,207]. All subsequent trials confirmed AAV to be a safe vector, and several phase II trials are underway or have been completed with several different serotypes (AAV2, AAV5, AAVrh10), for diseases such as Parkinson's, Alzheimer's, AADC and lysosomal storage disorders [187]. However, only mild symptom improvements have been

reported in any of these trials, and a full Phase III efficacy evaluation has yet to be performed [208]. Since almost all of these trials for neurological diseases used direct intraparenchymal injections, it is likely necessary to target a larger brain area, rather than just the most affected regions for significant phenotypic improvement. Despite recent advanced infusion modalities, such as convection-enhanced delivery and microbubble facilitated focused ultrasound, widespread CNS distribution has not been achieved [209,210]. A better approach is needed, especially since most neurodegenerative diseases are multifocal, affecting multiple brain regions.

The discovery of AAV9's ability to cross the blood brain barrier raised the possibility that an intravenous infusion (IV) could achieve global CNS gene transfer [211]. However, AAV9 transduction patterns are dependent on the age of the animal model and the route of delivery. In the initial study by Foust et al. [211,212], intravenous injections of a AAV9 in neonate mice resulted in transduction of all CNS cell types, including neurons of the spinal cord, neocortex, hippocampus and cerebellum. The same vector injected in adult mice had a transduction pattern that was almost exclusively glia and endothelium. However, other studies using IV injections in adult mice have observed neuronal transduction in the brain and spinal cord [213,214]. Systemic administration of AAV9 to adult non-human primates resulted in mostly astrocytes transduction, with scattered neuronal transduction throughout the brain and spinal cord [214-216], while fetal or neonate injections had a neuronal transduction profile [217,218]. Thus, systemic AAV delivery is more clinically relevant for early childhood neuronal disorders, when a larger number of

neuronal populations can be targeted. Direct CSF infusion deliveries have been explored to target neuronal populations in adult animal models.

Intrathecal (IT) injections into the subarachnoid space of the spinal canal are used to deliver AAV vectors into the CSF. This method led to successful gene transfer to spinal cord motor neurons in both rodent and large animal models [215,216,219,220]. However widespread gene transfer to the brain was only seen with non-human primates [221]. Recently, analysis of gene expression from NHPs that had been placed in the Trendelenburg position after an IT AAV9 injection revealed transduction of more than 50% of spinal cord motor neurons throughout the entire spinal cord, as well upper motor neurons in layer V of the cortex [222]. IT injections are challenging in mice, and do not achieve widespread brain transduction. Gene transfer is mostly localized to the injection site, with limited vector spread to distal spinal cord regions and the brain [219,221,223] However, neonate injections into the lateral ventricles result in transduction of motor neurons in the spinal cord as well as neurons in the motor cortex [224]. Taken together, these studies prove the feasibility of widespread AAV transduction of the CNS.

The current status of AAV gene therapy is very encouraging, with one AAV product already approved for clinical use (Glybera) in Europe. The most advanced clinical trial to date in the United States is a phase III using AAV2 to deliver hRPE65 for Leber Congenital Amaurosis. However, many trials are underway using new generation of AAV capsids, such as the use of AAV9 in intramuscular injections for Pompe disease or AAVrh10 for Batten's and Sanfillipo disease [225]. Two ongoing trials have reported encouraging results. The first trial uses AAV2 to deliver AADC to children via direct

intracranial injections into the putamen. This group reported with marked improvements in motor function, with no vector associated side effect or loss of treatment effect. Impressively, without treatment, these children would be unable to stand, while after treatment one patient was learning to walk. The second trial uses a double stranded AAV9 to deliver SMA to the CNS of infants, via intravenous infusion. Preliminary data from this study has shown the approach to be safe and well tolerated, and a CSF delivery of the same vector is being planned.

RNAI-GENE THERAPY FOR ALS

Gene therapy for ALS is not a new concept, but the availability of new tools, such as AAV9, and artificial microRNAs has made AAV mediated RNAi a therapeutic possibility. These types of approaches have been tested in the $SOD1^{G93A}$ mouse and other ALS animal models, with various degrees of success.

Two transgenic mouse model studies showed reduction of hSOD1 as a possible therapeutic option. The first [226] crossed mice containing an anti-SOD1 siRNA transgene with the $SOD1^{G93A}$ mice, and the double transgenic mice did not develop an ALS phenotype. In this study, hSOD1 expression was inhibited in all cells, presumably from embryonic development, stopping the production and accumulation of the toxic protein. A parallel study [84] used transgenic mice with the mutant hSOD1 transgene flanked by loxP sites. These mice were crossed with other transgenic mice expressing Cre under a neuronal or microglial/macrophage promoter, leading to the excision of the hSOD1 in those cell types. While both mouse lines displayed an increase in survival, elimination of

hSOD1 in neurons delayed onset, while elimination of hSOD1 in glia slowed the disease progression. These studies showed early on that although motor neurons are selectively vulnerable in ALS, it is important to target multiple cell types to treat disease.

The largest increase in lifespan in a mouse model was observed with a lentivirus delivered shRNA against hSOD1. When delivered at postnatal day 7, into multiple muscle groups, survival was increased by 77%, due to a delay in onset only. The muscles injected included the hindlimb, to target lower motor neurons, and the facial, tongue, and intercostal, since ALS has not only mobility, but feeding and respiration components as well. Unfortunately, the multitude of injections in this study made clinical translatability unfeasible. It is interesting to note that this study chose injections into the muscle to take advantage of the retrograde transport capabilities of their (equine infectious anemia virus) EIAV psudotyped lentivirus, and target motor neurons. Thus, it is not surprising that while disease onset was delayed, disease progression was not changed, as motor neurons are mediators of disease onset not progression [227]. Another study used a lentivirus to deliver an shRNA, by two bilateral injections on either side of the lumbar spinal cord, in 40-day-old mice $SOD1^{G93A}$ mice. Although motor neuron survival was increased by 60%, lifespan was only mildly increased [228]. Together, these studies further highlight the importance of targeting multiple cell types, and the potential involvement of motor neurons at multiple levels of the spinal cord.

The most successful AAV mouse study used an intravenous infusion of a H1 driven shRNA against hSOD1, encoded in a self complementary AAV9 [229]. With this approach median survival was increased by 39% when delivered to SOD1 mice at

postnatal day 1, and by 30% when delivered at postnatal day 21. An intravenous infusion of AAV9 is neurotropic when delivered to neonates, but more glia-tropic when delivered to older mice [211]. Thus, P1 injections resulted in transduction of more motor neurons (64%) than glia (34%); and P21 injection resulted in transduction of fewer neurons (8%) than glia (54%). Consequently, there was a 60% reduction of hSOD1 protein after the P1 injections, but only a 45% reduction after the P21 injection. Thus, while an intravenous infusion is the easiest delivery method, current AAV vectors are not efficient enough at crossing the blood brain barrier in adult mice to transduce the majority of neurons in the CNS.

It has been hypothesized that ALS is caused by a "dying back" mechanism, where degeneration starts in the muscles, travels up the axons to the spinal cord motor neurons and then up to the neurons of the brain. AAV6 has been shown to efficiently transduce skeletal muscle as well as undergo retrograde transport to motor neurons after an intramuscular injection [230,231]. Thus, several studies used AAV6 to deliver an shRNA against hSOD1 in SOD1 mice. In the first study, Towne et al. [232] injected adult mice intravenously, and observed a systemic transduction in skeletal muscle, as well a 50% reduction in hSOD1 in transduced tissues. However, less than 5% of motor neurons were transduced, and there was a lack of significant hSOD1 reduction in the spinal cord. In the subsequent study Towne et al.[233] injected AAV6-shRNA into multiple muscle groups of neonate SOD1 mice, similar to the EIAV lentivirus study. However this treatment had no effect on survival, despite efficient retrograde transport to motor neurons as evidenced by the greater than 50% reduction of hSOD1 mRNA levels in those cells. This discrepancy

is likely due to the lower number of motor neurons transduced in the AAV6 study compared to the EIAV lentivirus study (40% versus >50% in the lumbar spinal cord, respectively). In a separate experiment, a shRNA against hSOD1 was delivered by an intramuscular injections of a lentivirus or a AAV. Only the AAV was capable of retrograde transport. Subsequently, while the same level of hSOD1 reduction was seen in the muscle with both treatments, hSOD1 reduction in motor neurons was only observed in AAV treated mice. This lead to an improvement in motor function solely in the AAV treated SOD1 mouse [87]. Thus, a therapeutic strategy aimed at treating just motor neurons is not sufficient, and it is possible that a threshold percentage of motor neuron transduction is necessary for therapeutic benefit.

Additionally, a recent study aimed to definitively answer the question as to whether neurons or astrocytes alone can be therapeutic targets. AAV vectors encoding SOD1-specific artificial microRNAs under cell specific promoters were infused into the lateral ventricles of neonate SOD1 mice [234]. The infusion with the neuronal promoter extended survival by 26% and with the glial promoter by 14%. The combined injection of both AAV vectors showed no additional benefit, and was not able to recreate the large increase in lifespan seen with the intravenously delivered, ubiquitously expressed shRNA. From these studies, it is clear that hSOD1 must be reduced in all cells of the CNS, and targeting of only specific subpopulations is not sufficient.

Recently, more attention has been given to the potential role of upper motor neuron degeneration on disease phenotype. Ozdinler et al. [101], showed them to be a cell population susceptible to degeneration in $SOD1^{G93A}$ mice. This degeneration is also seen

in ALS patients. Following up on this, Thomsen et al. [235] showed that multiple motor cortex injections of an AAV-H1-shRNA in a SOD1 rat model improved survival. This raised questions regarding the hypothesis that ALS is caused by a "dying back" mechanism, since treating the neurons of the CNS resulted in a therapeutic benefit. Thus, it is possible that motor neuron death starts concomitantly in at all levels of the CNS.

Widespread CNS transduction has been achieved in adult non-human primates with a AAV intrathecal infusion into the CSF of the lumbar spinal cord. However, this delivery method has variable reproducibility in the much smaller mouse model, and only limited vector spread is achieved in distal spinal cord regions and in the brain. Wang et al. [236] used a AAVrh10 vector encoding an amiRNA against hSOD1, and injected it intrathecally into the lumbar spinal cord of adult $SOD1^{G93A}$ mice. This resulted in only an 11% increase in survival that was directly correlated with the level of transduction of the spinal cord. Patel et al. used a similar injection method to deliver a AAV1 encoding a single chain antibody against misfolded hSOD1, leading to an increased in survival by up to 28%. However, they observed large variations in the survival time of treated mice, which directly correlated with antibody titers in the spinal cord [237]. Intrathecal injections are technically challenging in mice due to their small size, but easily overcome when using larger animals, and are feasible in the clinic. This highlights the still unsolved problem of widespread AAV delivery to motor neurons in adult mice.

Thus, this work sought to achieve widespread delivery of an AAV-amiRNA against hSOD1 in the CNS of a $SOD1^{G93A}$ mouse model. We chose an intraventricular delivery of AAV9 in neonate mice, since it has been shown to transduce the upper motor

neurons in the motor cortex, the motor neurons at all levels of the spinal cord, as well as glial cells [238]. Due to the current challenges in achieving widespread CNS transduction in adult mice, we believe this delivery route most closely reproduce what is feasible in adult non-human primates, and eventually in the clinic. We chose the ubiquitous CBA promoter to achieve expression of our artificial microRNA in all cell types, since SOD1-ALS disease progression involves both neuronal and non-neuronal cells. This proof of concept study proves the viability of AAV-amiRNA treatment for treating SOD1-ALS, and can be the basis for future non-human primate safety studies and subsequent clinical trials.

CHAPTER II – RESULTS

An amiRNA is effective at reducing expression of hSOD1 mRNA and protein in vitro and in vivo

We designed an artificial microRNA against hSOD1 (amiRNA) that is perfectly complementary to human SOD1 (Figure 2.1A). The amiRNA targets the coding sequence of exon 2 of the human SOD1 gene (Figure 2.1A), is based on the Invitrogen Block-iT PolII miR vector system originally developed in the laboratory of David Turner (US Patent No 2004/0053876), and uses the endogenous murine miR-155 flanking sequences. We used two tandem copies of the amiRNA in our constructs for increased efficiency. The full tandem sequence is as follows, with the guide strand underlined: CCTCTTGCTGAAGGCTGTATGCTGATGAACATGGAATCCATGCAGGTTTTGGC CACTGACTGACCTGCATGGTCCATGTTCATCAGGACACAAGGCCTGTTACTAG CACTCACATTGGCCCAGATCCTCTTGCTGAAGGCTGTATGCTGATGAACATGG AATCCATGCAGGTTTTGGCCACTGACTGACCTGCATGGTCCATGTTCATCAGG ACACAAGGCCTGTTACTAGCACTCACATTGGCC. In order to facilitate loading into Dicer, two nucleotides are missing from the passenger strand, creating a bulge. The folded pre-miRNA is shown in Figure 2.1B.

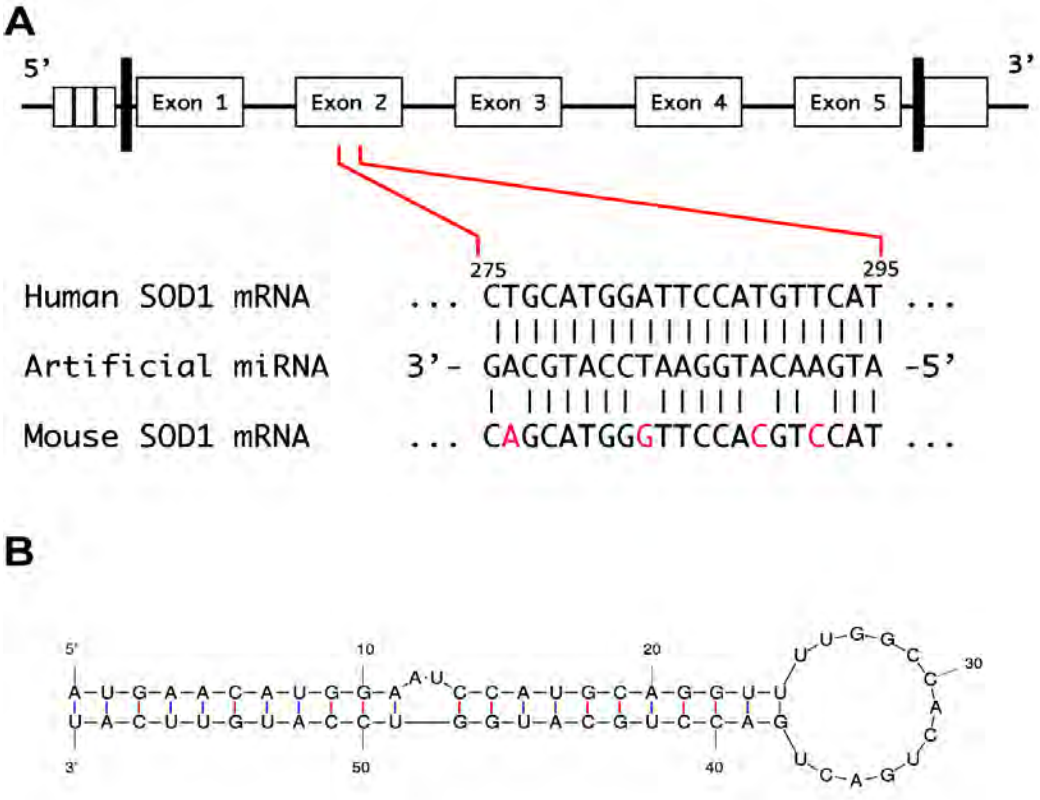

Figure 2.1. The amiRNA is perfectly complementary to human SOD1. A) The amiRNA is perfectly complementary to the coding region of human SOD1 in exon 2, but has four base pair mismatches to mouse SOD1, indicated in red. B) The stem-loop structure of the artificial microRNA used in this study, designed based on the murine miR-155, has two nucleotides missing in the passenger strand.

We first tested the amiRNA *in vitro* by transfecting human embryonic kidney (HEK293T) cells, and observed an 80% reduction in hSOD1 mRNA as measured by RT-qPCR and a 35% reduction in hSOD1 protein as measured by immunoblot (Figure 2.2A). Our therapeutic construct has a chicken beta acting (CBA) promoter driving a green fluorescent protein (GFP) expression cassette, with two tandem copies of the amiRNA

cloned in the 3'UTR, between the GFP stop codon and the Woodchuck Hepatitis Virus Posttranscriptional Regulatory Element (WPRE) and is packaged into a single stranded AAV9 (Figure 2.2D). We tested it *in vitro* by infecting HEK293T at two different doses, and observed a greater than 90% reduction in hSOD1 mRNA (Figure 2.2B). We then tested it *in vitro* in adult $SOD1^{G93A}$ mice by an intravenous infusion. AAV9 has high transduction of the liver after an intravenous infusion, thus we analyzed hSOD1 expression levels in this organ. Again, we observed very efficient reduction in both hSOD1 mRNA (>95%) and protein (80%) (Figure 2.2C). Lastly, we validated the efficiency of our vector in the CNS by directly injecting the striatum of adult $SOD1^{G93A}$ mice. We observed an almost 70% reduction in hSOD1 mRNA when measured in a tissue punch of the GFP positive brain region (Figure 2.2C).

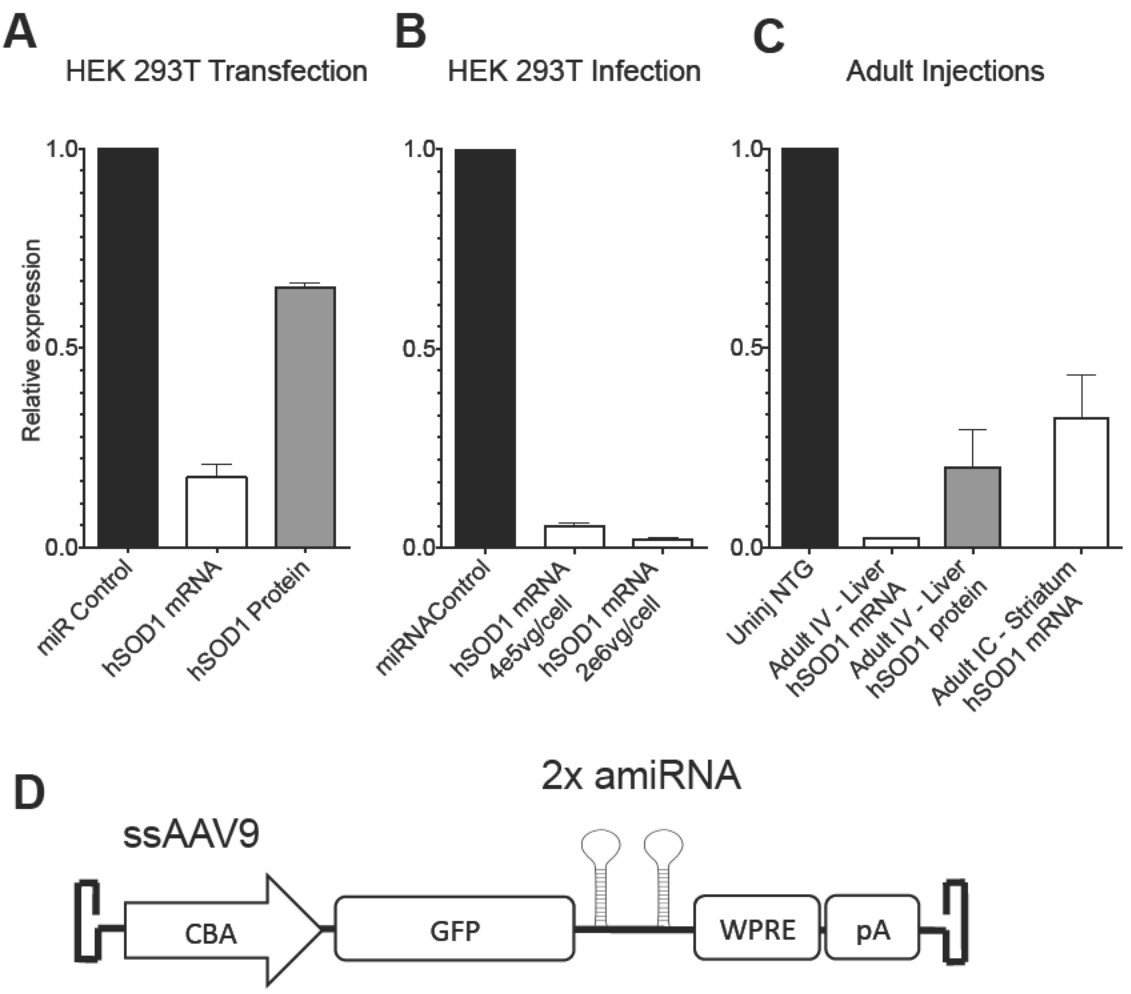

Figure 2.2. The amiRNA is efficient at reducing expression of human SOD1 *in vitro* and *in vivo*. A) Transfection of HEK293T cells with a plasmid containing two copies of the amiRNA results in reduced expression of hSOD1 mRNA and protein. B) Infection of HEK293T cells with a single stranded AAV9 containing two copies of the amiRNA results in reduced expression of hSOD1 mRNA. C) Delivery of the AAV encoded amiRNA either by intravenous infusion or direct intra-striatal injection results in reduction of human SOD1 mRNA and protein in the liver and reduction of hSOD1 mRNA in the striatum, respectively. D) The therapeutic construct used in this study is a single stranded AAV vector containing a CBA promoter driving a GFP expression cassette, followed by two tandem copies of the amiRNA cloned in the 3'UTR, a WPRE, and a polyadenylation signal.

An AAV9-GFP injection into the ventricles of neonate mice results in transduction of multiple cell types of the CNS

We first analyzed the CNS transduction profile of a ssAAV9-GFP vector injected into the lateral ventricles of neonatal $SOD1^{G93A}$ mice at a total dose of $1x10^{11}$ vector genomes, to determine if it is an appropriate delivery route for targeting upper and lower motor neurons. Analysis of GFP expression at four weeks post-injection revealed transduced neurons in the motor cortex and ventral horn of the spinal cord (Figure 2.3, arrows). Based on location, size, and morphology, many of these GFP+ neurons are layer V cortical (Figure 2.3A) and spinal cord motor neurons (Figure 2.3B). It also appears that astrocytes or microglia were transduced in the spinal cord (Figure 2.3B, arrowhead), as gauged by the morphology of the cells and the lack of GFP/NeuN co-localization. This pattern of targeting (cortical and spinal motor neurons, glial cells) corresponds to the cell types most affected by ALS.

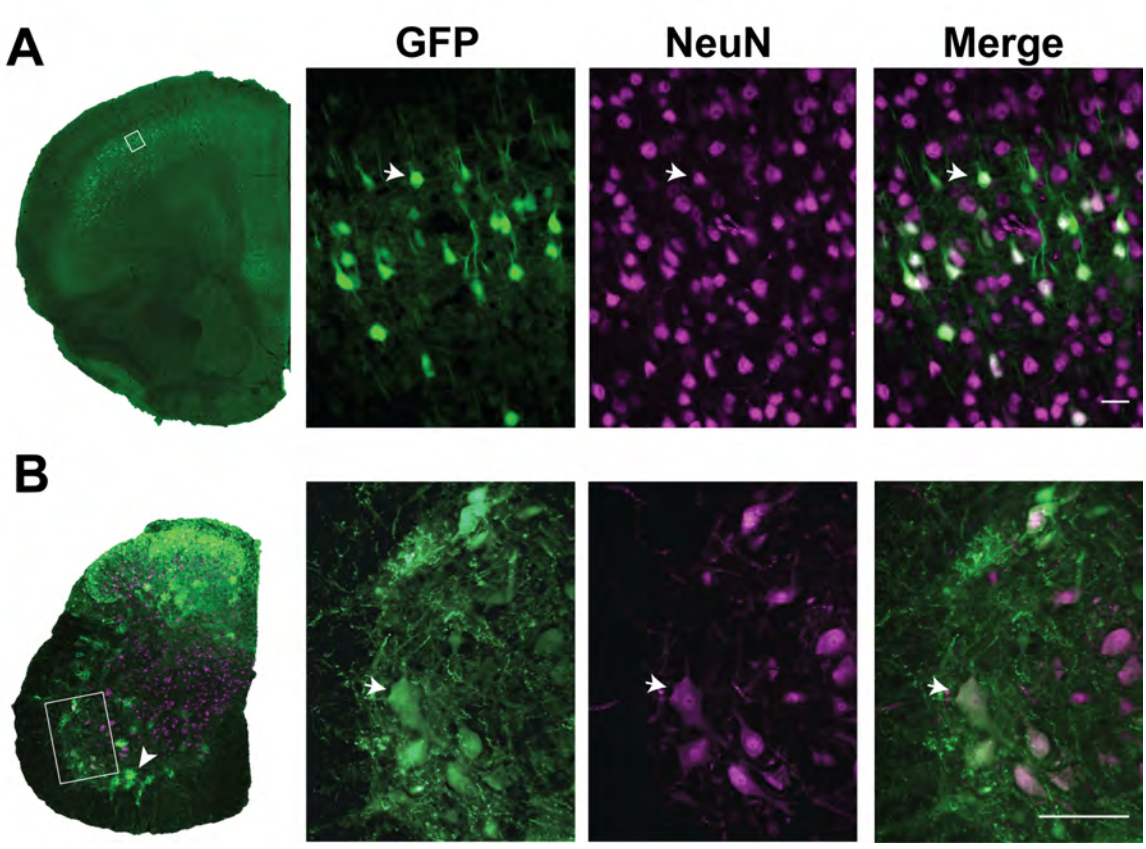

Figure 2.3. A single neonate ICV injection of AAV9-GFP transduces neurons in motor cortex and spinal cord. Immunofluorescence staining of brain (A) and spinal cord (B) sections with antibodies to GFP and NeuN revealed broad neuronal transduction. Boxes indicate locations of magnified regions. Arrows indicate double labeled cells. Arrowhead indicates non-neuronal transduced cells. Scale bars, 25µm brain, 50µm spinal cord

An AAV9-amiRNA injection into the ventricles of neonate mice results in a decrease of hSOD1 gene expression

We investigated the therapeutic effectiveness of our vector by injecting $SOD1^{G93A}$ mice at post-natal day 0-1 (P0-P1) and quantified hSOD1 mRNA levels at four weeks post injection (Figure 2.4). Human SOD1 mRNA levels in spinal cord were reduced up to 50% with no significant difference between cervical, thoracic, and lumbar regions. In peripheral tissues, hSOD1 mRNA levels were reduced by more than 80% in heart and gastrocnemius muscle, but were unchanged in liver or lung. We also analyzed the levels of mouse SOD1 mRNA and found no changes in the thoracic spinal cord. This was expected, since amiRNA was designed to be human specific with four mismatches to the mouse SOD1 mRNA (Figure 1.2A).

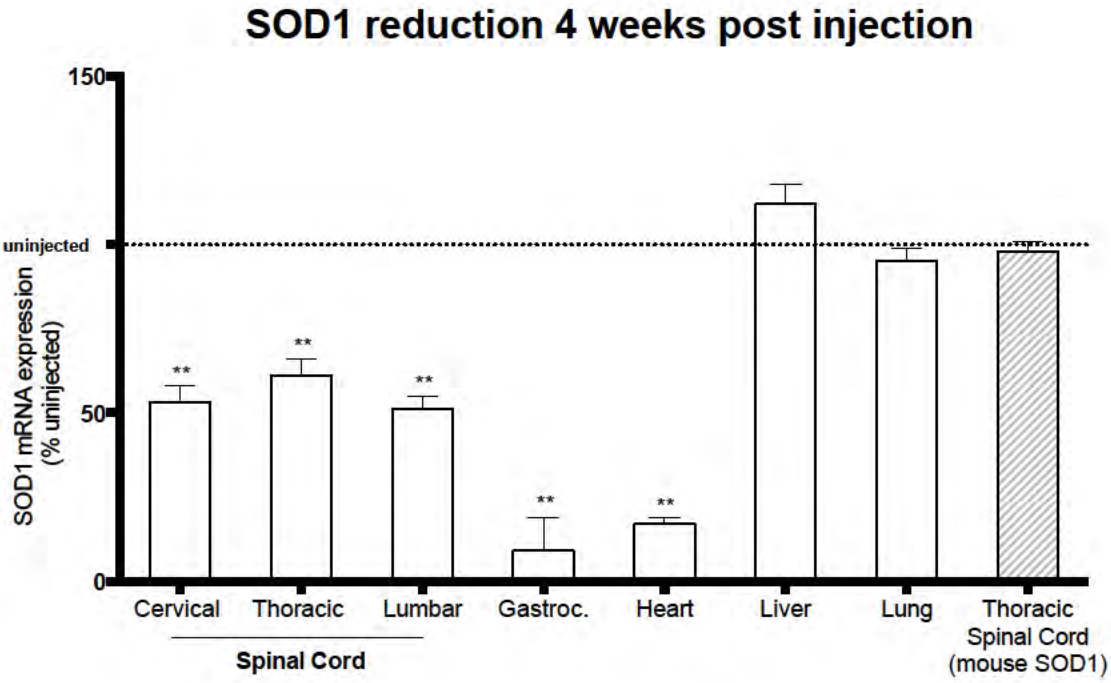

Figure 2.4. A single neonate ICV injection of AAV9-amiRNA reduces human SOD1 mRNA. RT-qPCR analysis was performed for human and mouse SOD1 mRNAs. Human SOD1 mRNA was reduced by almost 50% at all three levels of the spinal cord, and more than 70% in the gastrocnemius and heart. No change was apparent in liver or lung. Endogenous mouse SOD1 mRNA remained unchanged. Dotted line indicates uninjected $SOD1^{G93A}$ mice. **$p<0.005$. Unpaired two-tailed t-test was used for statistical comparison.

We measured the quantity of vector genomes in the thoracic spinal cord of injected $SOD1^{G93A}$ mice at four weeks post injection, and at 135 days post injection. There was no change in the number of vector genomes per diploid genome in injected SOD1 animals at any time points. Additionally, there was no difference in the number of vector genomes present between $SOD1^{G93A}$ and non-transgenic (NTG) mice (Figure 2.5A).

We also measured the number of copies of the mature guide strand of the amiRNA, in injected SOD1^{G93A} mice at four weeks post injection, and at endpoint. There was not a significant difference in guide strand presence at the two time points (Figure 2.5B).

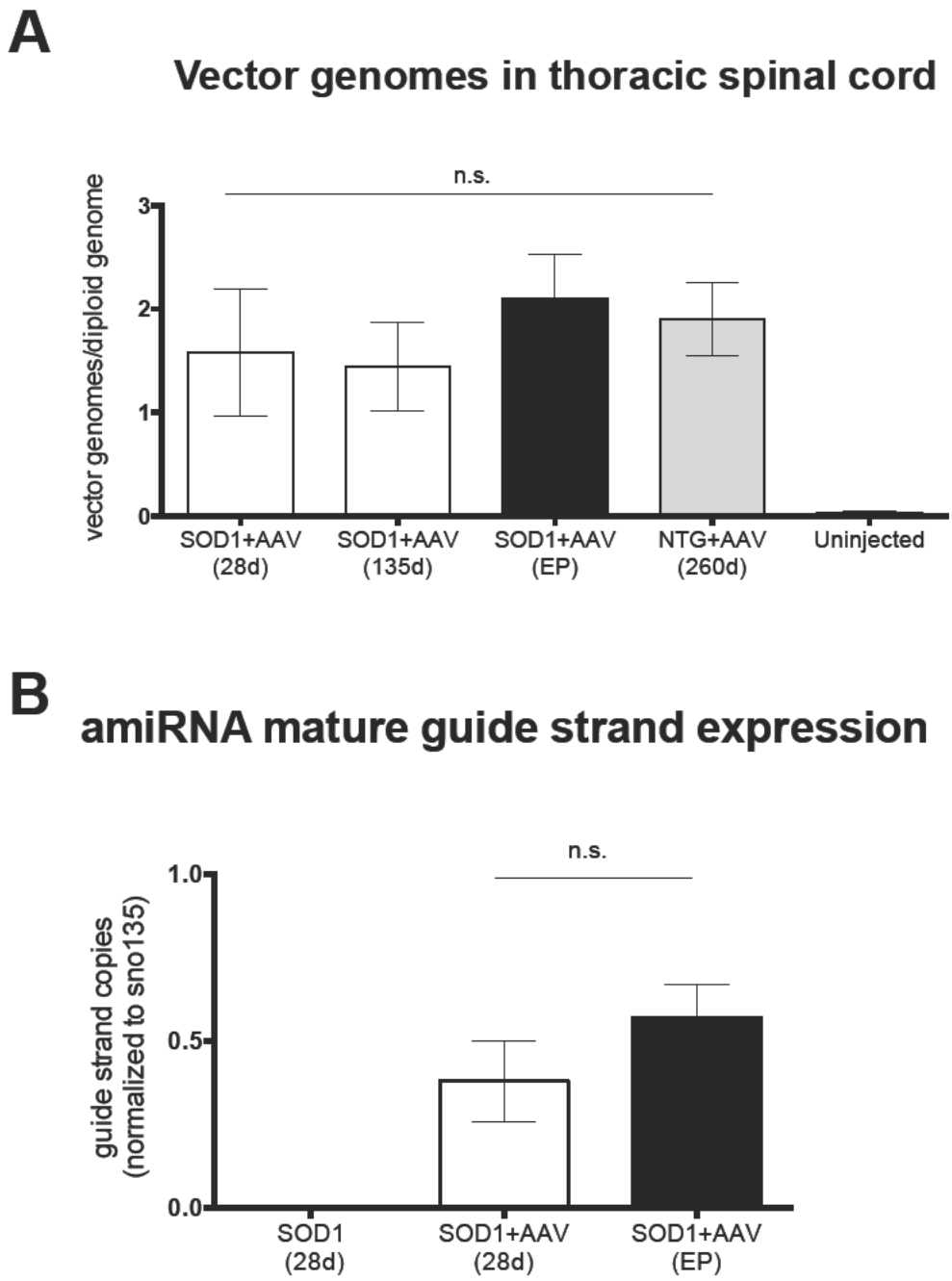

Figure 2.5. A single neonate ICV injection of AAV9 transduces the spinal cord. A) RT-qPCR analysis was performed for quantification of vector genomes in the thoracic spinal cord of AAV injected and uninjected mice. B) Digital droplet PCR was performed for expression of the mature guide strand of the amiRNA. Injected animals had comparable levels of vector genomes and mature miRNA guide strand expression at all time points. **p<0.005. Unpaired two-tailed t-test was used for statistical comparison.

We also analyzed changes in hSOD1 mRNA levels in AAV9-transduced upper and lower motor neurons using multiplex fluorescent *in situ* hybridization (RNAscope). This technique provides detailed spatial information on changes in hSOD1 mRNA in specific cell populations such as spinal cord motor neurons and layer V upper motor neurons, identified with probes specific for ChAT (choline acetyl transferase) or Etv1 (ets variant 1) [239], respectively (Figure 2.6). The intensity of hSOD1 mRNA signal was considerably reduced in cells expressing GFP mRNA both in motor cortex (Figure 2.6A) and spinal cord (Figure 2.6B). Furthermore, co-localization of GFP mRNA signal with probes specific for Etv1 and ChAT mRNAs confirms the transduction of both layer V cortical neurons (Figure 2.6A, arrows) and spinal cord motor neurons (Figure 2.6B, arrows) in AAV9-injected mice. Non-transduced ChAT-positive neurons (GFP-negative) show strong hSOD1 mRNA signal (Figure 2.6B, arrowhead).

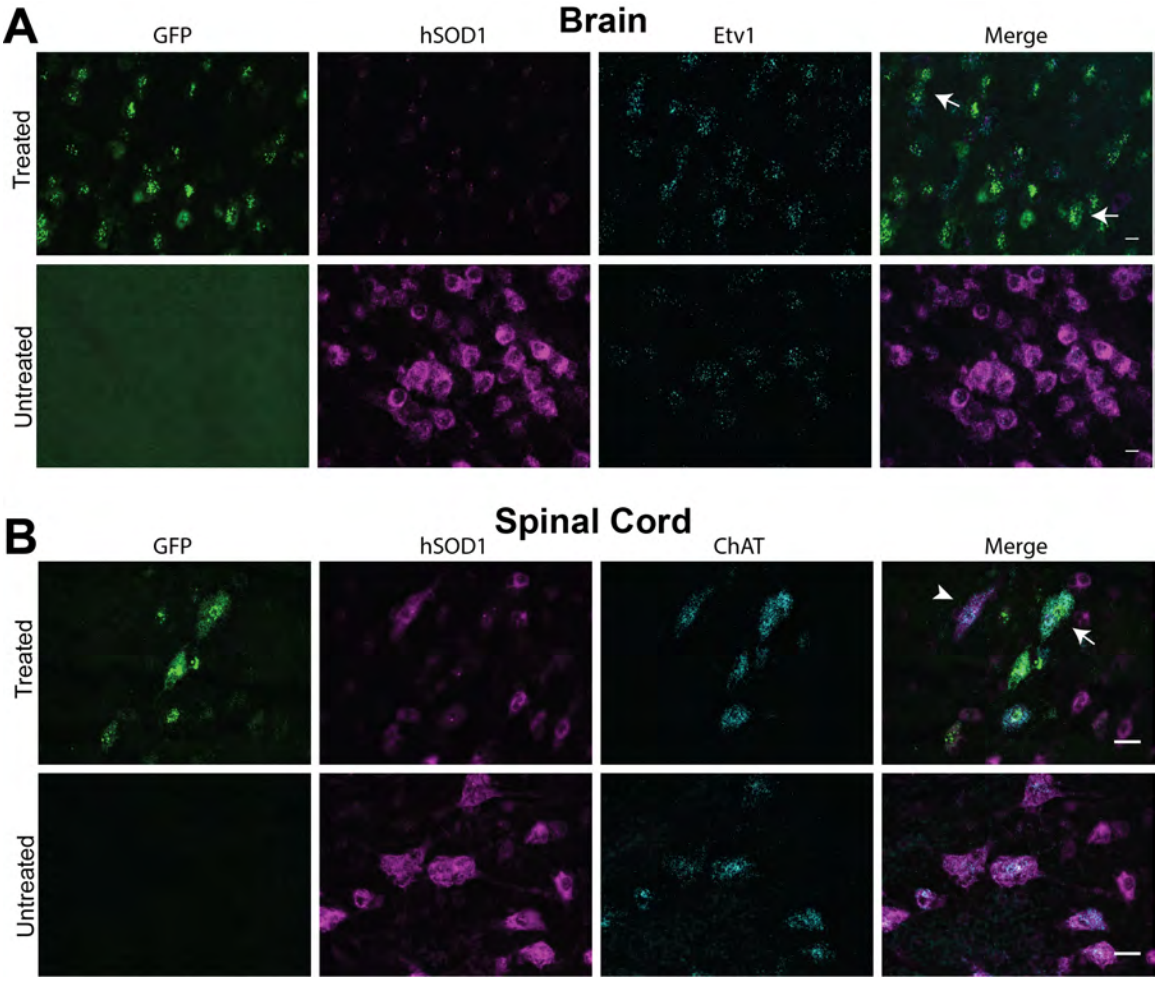

Figure 2.6. AAV9-amiRNA treatment reduces SOD1 mRNA expression in transduced cortical and spinal cord motor neurons. Multiplex fluorescent *in situ* hybridization was used to assess changes in human SOD1 mRNA at the cellular level in brain and spinal cord of AAV9 treated and untreated $SOD1^{G93A}$ mice at 4 weeks of age. Probes for GFP (green), and human SOD1 mRNA (magenta) were multiplexed with probes to genes in cortical (Etv1) or spinal cord (ChAT) motor neurons (cyan). Cells expressing GFP mRNA, indicating transduced cells, had reduced levels of hSOD1 mRNA, in both the brain (A) and spinal cord (B). Arrow indicates AAV9 transduced cells with reduced hSOD1 mRNA signal. Arrowhead indicates non-transduced cells, lacking GFP mRNA, and retaining high SOD1 mRNA signal in the spinal cord. Scale bars, 10 μm (A) and 25 μm (B).

AAV9-amiRNA treatment improves survival and delays the onset of paralysis in SOD1^{G93A} mice

Based on the initial indications of efficient reduction of hSOD1 mRNA in upper and lower motor neurons we next assessed the therapeutic benefit of neonatal ICV injection of AAV9-amiRNA vector in SOD1^{G93A} mice. We injected the therapeutic vector into the cerebral lateral ventricles of 22 neonatal ALS SOD1^{G93A} mice. As controls, we used 18 untreated SOD1^{G93A} mice, 12 treated NTG B6/SJL mice and 12 untreated NTG B6/SJL mice. Four mice from each group were sacrificed for histological and biochemical studies at 135 days, the median age of survival of untreated SOD1^{G93A} mice. The remaining eighteen AAV9 treated SOD1^{G93A} mice were followed until euthanized at endpoint. AAV9 treated and untreated NTG B6/SJL mice (8 per group) were euthanized at 260 days, well past the age of the longest surviving treated SOD1^{G93A} mouse. Histological and biochemical studies were performed in tissues from AAV9 treated SOD1^{G93A} mice at endstage and NTG controls at 260 days.

AAV9-amiR treatment extended median survival by 50%, from 137 days for untreated SOD1^{G93A} mice to 206 days ($p<0.0001$; Figure 2.7A). Untreated SOD1^{G93A} mice usually show clear signs of hind limb paralysis by 135 days (Movie 2.1A), and are considered at endpoint when they are unable to right themselves. Unexpectedly, AAV9 treated SOD1^{G93A} mice did not die from paralysis but instead were euthanized due to rapid weight loss and the development of kyphosis, or a hunched appearance (Figure 2.7B, Movie 2.1B, >200 days). Up to that point, mice were fully ambulatory (Movie 2.1C, 198 days); and even at endpoint, treated mice remained active and continued to

display behaviors that required considerable limb strength, such as rearing and hanging from the wire food hopper (Movies 2.1D, >200 days).

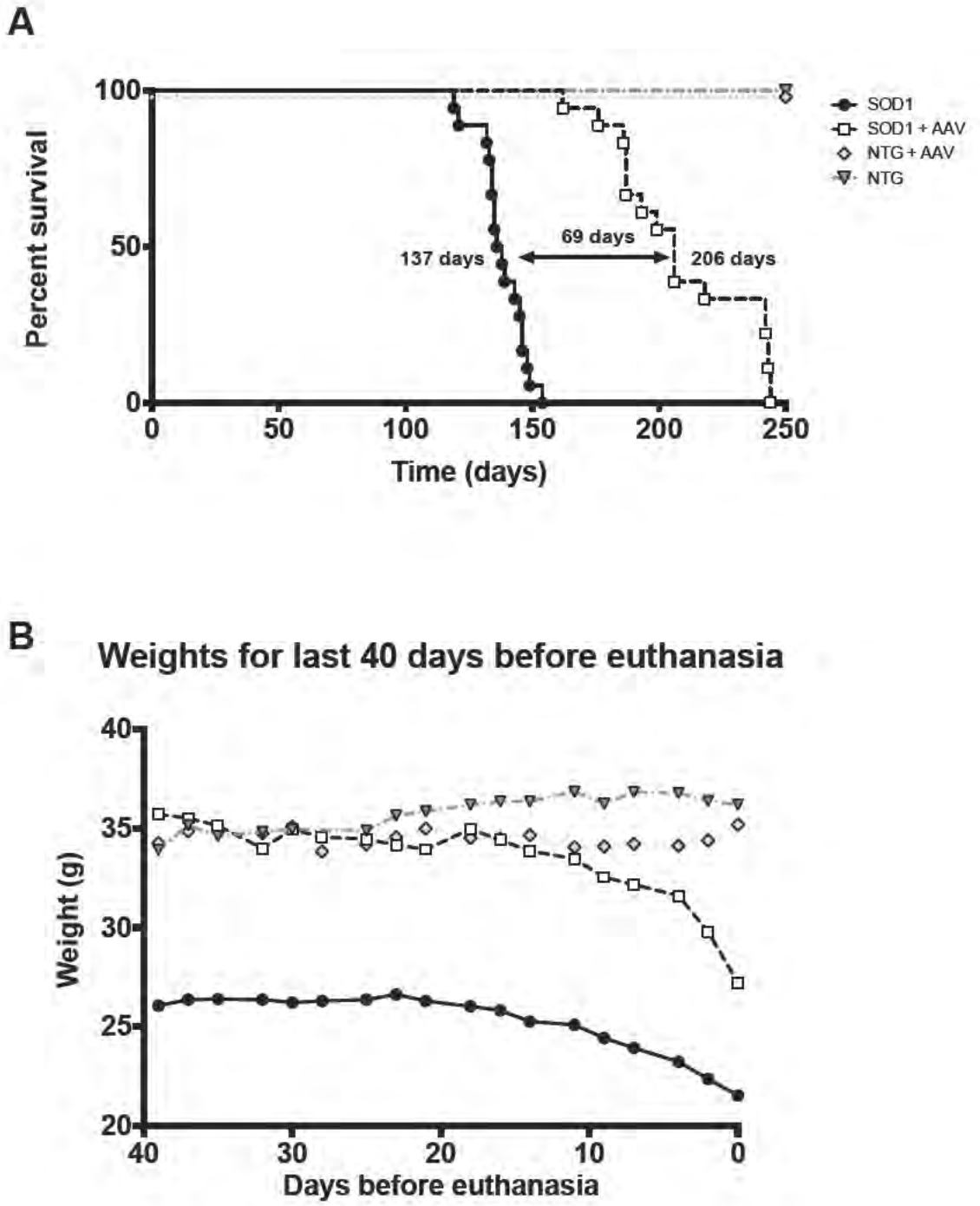

Figure 2.7. AAV9-amiRNA treatment increases lifespan of SOD1^{G93A} mice. A) Kaplan-Meier survival plot shows a 69 day increase in median survival of AAV9 treated mice compared to untreated SOD1^{G93A} littermates. Log-rank test, $p<0.001$. B) Treated SOD1^{G93A} mice have a sharp drop in body weigh; untreated SOD1^{G93A} mice have a more gradual decrease in weight, concurrent with progressing paralysis.

In addition to observing the mice for limb paralysis, we also assessed neuromuscular function in AAV9 treated SOD1^{G93A} mice and controls at different ages using quantitative electrophysiological measures [240,241]. Needle electromyography (EMG) assesses several critical muscle parameters including the presence of fibrillation potentials and the amplitude of the compound muscle action potential. The results were scored as 0 being normal to 5 being highly abnormal. The EMG scores of NTG control animals were zero while untreated SOD1^{G93A} animals scored in the 3 to 5 range, corresponding to extensive acute muscle denervation (Figure 2.8A). In contrast AAV9 treated SOD1^{G93A} mice scored 0 to 2 throughout the experiment. The scores of 2 were evident in only a subset of AAV9 treated SOD1^{G93A} mice and only at older ages. Even at the latest time point analyzed (ranging from 207 to 242 days), some AAV9 treated SOD1^{G93A} mice had normal EMGs (scored zero).

We further assessed neuromuscular function by estimating the number and sizes of motor units [242]. AAV9 treated SOD1^{G93A} mice maintained a normal number of motor units, and only a few mice, which had scored 2 on the EMG scale, showed a decrease at older ages (Figure 2.8B). We also quantified the motor unit size, which corresponds to the number of muscle fibers innervated; in ALS, this parameter can be a measure of axonal collateral re-innervation, which increases as motor neurons degenerate. Untreated SOD1^{G93A} mice showed an increase in motor unit size compared to NTG controls while AAV9 treated SOD1^{G93A} mice maintained a normal motor unit size (Figure 2.8C). These quantitative measures of neuromuscular function were consistent with the absence of overt signs of paralysis in AAV9 treated SOD1^{G93A} mice.

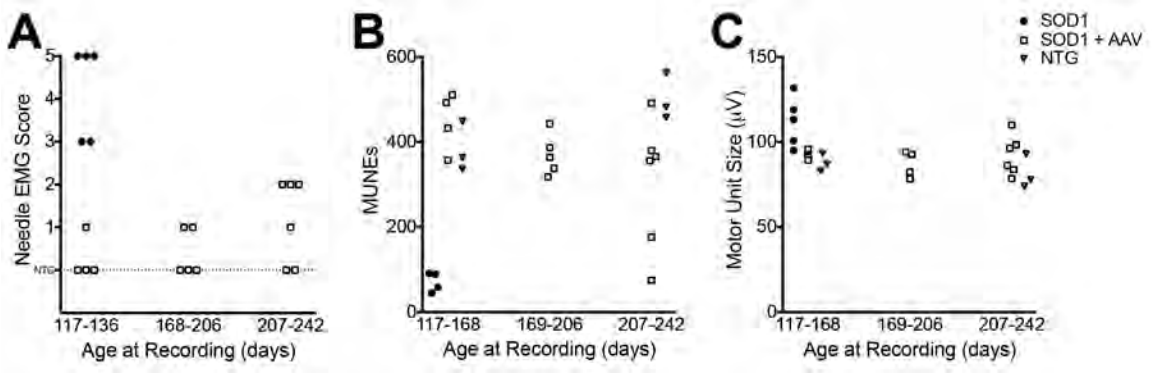

Figure 2.8. AAV9-amiRNA treatment improves neuromuscular function of SOD1^{G93A} mice. Electrophysiological recordings revealed preservation of motor neuron function in AAV9 treated SOD1^{G93A} mice as assessed by (A) needle electromyography scores, (B) number of motor units, and (C) motor unit size.

In ALS the most frequent cause of death is respiratory failure resulting from denervation of the diaphragm and the chest wall muscle. We therefore assessed pulmonary function on awake, spontaneously breathing animals. At 127 days, AAV9 treated and untreated SOD1^{G93A} mice had greater minute ventilation (MV) than NTG mice ($p<0.05$). However, when subjected to a respiratory challenge using hypercapnia AAV9 treated and untreated SOD1^{G93A} mice had a significantly attenuated MV response compared to NTG controls ($p<0.001$). By day 192, the AAV9 treated SOD1^{G93A} mice had a further decline in their MV response to the respiratory challenge compared to the day 127 recordings ($p<0.05$). Figure 2.9 illustrates the absolute change in MV (Figure 2.9A) and peak inspiratory flow (PIF) (Figure 2.9B) during hypercapnia relative to baseline in each group. PIF is a primary measurement of respiratory muscle strength, specifically of diaphragm strength; progressive diaphragm weakness seems likely in AAV9 treated SOD1^{G93A} mice since PIF values declined with age. Additionally, chest computerized

tomography (CT) scans of > 200 day old animals showed decreased chest volume in AAV9 treated SOD1^{G93A} mice compared to NTG controls (Figure 2.9C). Chest volume is a measure of the distortion of the chest wall due to kyphoscoliosis, and the decrease is indicative of physical lung restriction.

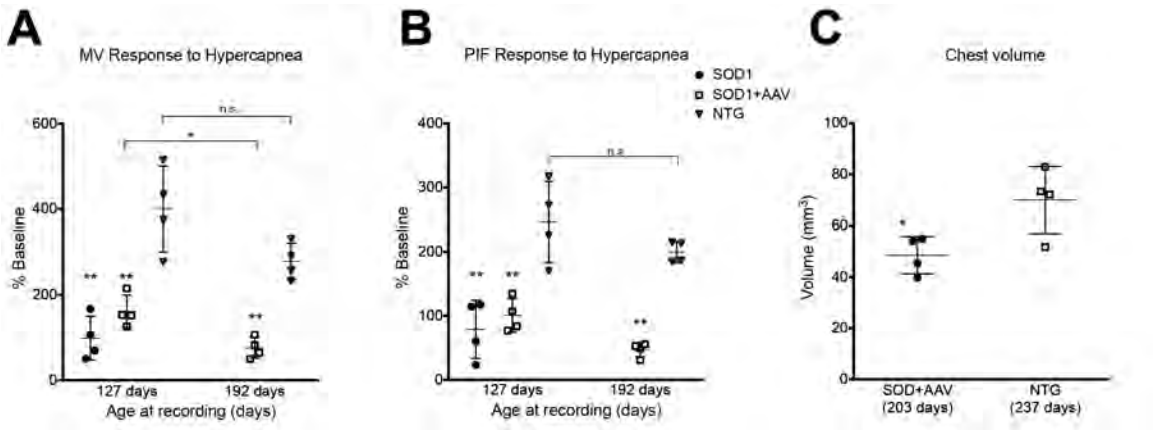

Figure 2.9. AAV9-amiRNA treated SOD1^{G93A} mice display evidence of pulmonary dysfunction at endpoint. Plethysmography recordings show a drop in response to hypercapnea in both (A) minute ventilation (MV) and (B) peak inspiratory flow (PIF) in both AAV9 treated and untreated SOD1^{G93A} mice when compared to age matched NTG mice, indicating breathing impairment due to diaphragm dysfunction. *$p<0.05$, **$p<0.001$. Near the humane endpoint there is a significant decrease in (C) chest volume of AAV9 treated SOD1^{G93A} compared to NTG mice. *$p<0.05$

AAV9-amiRNA treatment improves axonal integrity and surviving motor neuron numbers in SOD1^{G93A} mice

Next we evaluated axonal integrity in the sciatic nerve and ventral roots of the lumbar spinal cord as gauged by fiber morphology and distribution of fiber sizes. On comparing the sciatic nerves of end-point (EP) untreated SOD1^{G93A} mice, AAV9 treated and NTG mice at 135 days of age, we observed extensive axonal loss in untreated

SOD1^{G93A} mice, while the sciatic nerves of AAV9 treated SOD1^{G93A} and NTG mice were indistinguishable (Figure 2.10A). We also analyzed lumbar ventral roots, which are composed solely of motor axons; it is well documented that the large caliber alpha axons degenerate in the ventral roots of ALS patients and SOD1^{G93A} mice [243-246]. We observed this degeneration in untreated SOD1^{G93A} mice when compared to NTG mice as denoted by a shift in axonal size distribution towards small caliber axons (Figure 2.10B,C). The distribution of ventral root axon size in AAV9 treated SOD1^{G93A} mice at endpoint is intermediate between that in untreated SOD1^{G93A} and NTG mice. The numbers of large and small diameter fibers in the ventral roots of AAV9 treated SOD1^{G93A} mice differs statistically from untreated SOD1^{G93A} mice and NTG controls (Figure 2.10C). Thus, treated mice display remarkable preservation of axonal integrity.

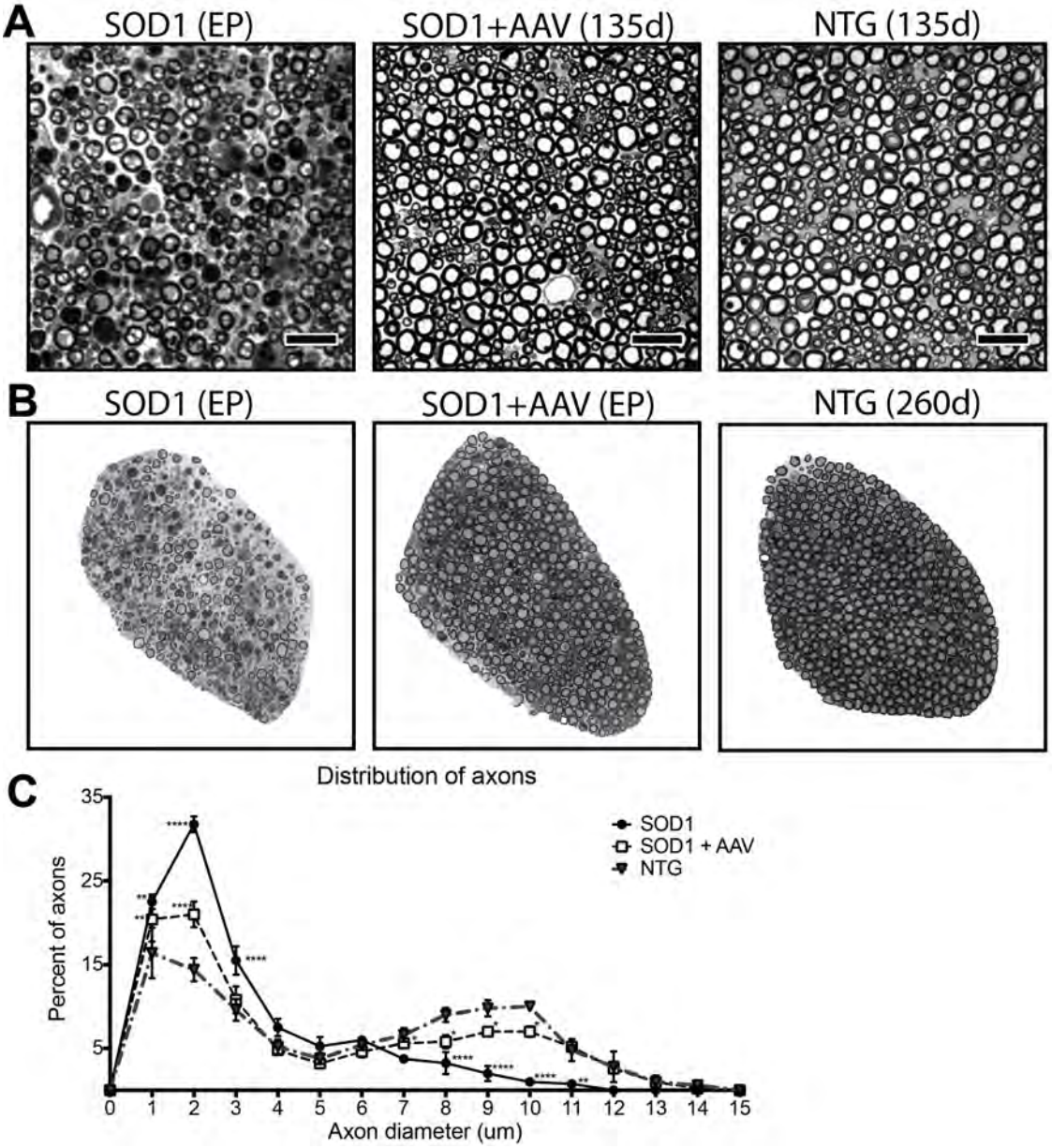

Figure 2.10. AAV9-amiRNA treatment preserves axons in sciatic nerve and lumbar ventral roots. A) Cross sections of sciatic nerves of untreated SOD1^{G93A} mice at endpoint were compared with AAV9 treated SOD1^{G93A} and NTG mice at 135 days. Representative toluidine blue stained sections show degeneration only in untreated SOD1^{G93A} mice. Scale bar, 25µm. B) Ventral roots of untreated SOD1^{G93A} mice were compared with treated SOD1^{G93A} mice at their respective endpoints, and NTG mice at 260 days. Representative toluidine blue stained sections are shown for all groups. C) Quantitative analysis of ventral root axon fiber distribution in SOD1^{G93A} mice shows loss of large diameter fibers with a shift toward small diameter fibers compared to NTG mice. Treated SOD1^{G93A} mice displayed a fiber distribution pattern between untreated NTG and SOD1^{G93A} mice. All mice were compared with NTGs. *p<0.05, **p<0.01, ****p<0.001

ALS has been described as a dying back neuropathy, a term that implies greater distal than proximal peripheral nerve degeneration. We therefore also assessed the integrity of neuromuscular junctions (NMJ) of the gastrocnemius in all cohorts. AAV9 treated SOD1^{G93A} mice at the humane endpoint revealed variable degrees of mild NMJ denervation, and overall distinctly less disorganization than detected in untreated SOD1^{G93A} mice (Figure 2.11).

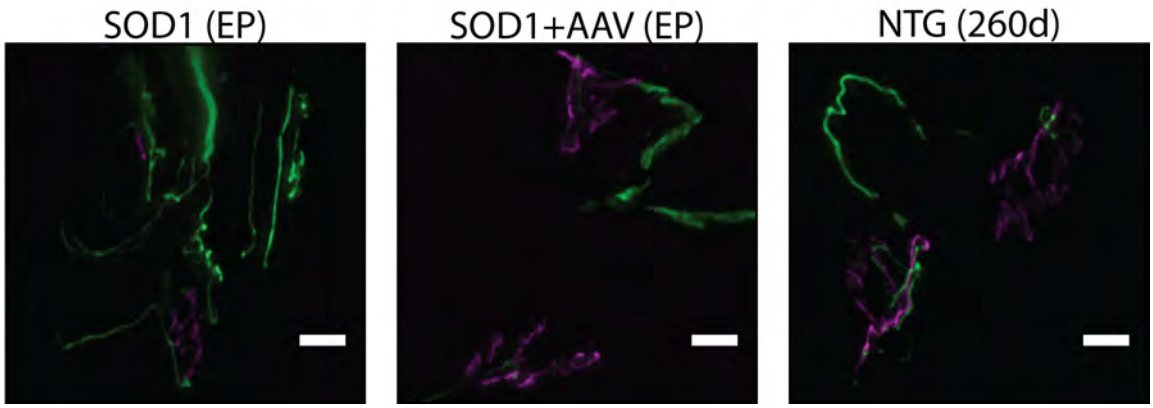

Figure 2.11. AAV9-amiRNA treatment preserves neuromuscular junctions. NMJs were analyzed in AAV9 treated and untreated SOD1^{G93A} mice at their respective endpoints and in NTG mice at 260 days. Immunofluorescence staining with antibodies against neurofilament-200 (green) and α-bungarotoxin (magenta) revealed disorganized neuromuscular junctions in untreated SOD1^{G93A} at endpoint. AAV9 treated SOD1^{G93A} mice had variable degrees of NMJ denervation, depending on the animal and NMJ analyzed. Scale bar, 10μm;

We also quantified the number of motor neurons in the ventral horn of the lumbar spinal cord at endpoint by counting ChAT+ neurons. The end stage spinal cords of untreated SOD1^{G93A} mice had a significant reduction in the number of ChAT+ neurons compared to control NTG mice ($p<0.005$; Figure 2.12A). In contrast, there was no statistical difference between end stage AAV9 treated SOD1^{G93A} and NTG mice (Figure

2.12C). To account for the possibility that we were including ChAT+ neurons compromised by the disease we also quantified numbers of motor neurons using a Nissl stain (Figure 2.12B). Stained cells in the ventral horn with a cell body area >250 μm^2 were considered motor neurons [247]. This method also showed a significantly higher number of motor neurons in AAV9 treated $SOD1^{G93A}$ mice compared to untreated $SOD1^{G93A}$ mice, albeit lower than in control NTG mice (Figure 2.12B,D). Thus, with the ChAT+ stain we counted all motor neurons, regardless of status of degeneration, while with the Nissl stain we counted only healthy motor neurons. This confirms that our AAV treatment dramatically preserved motor neurons, and slowed their degeneration.

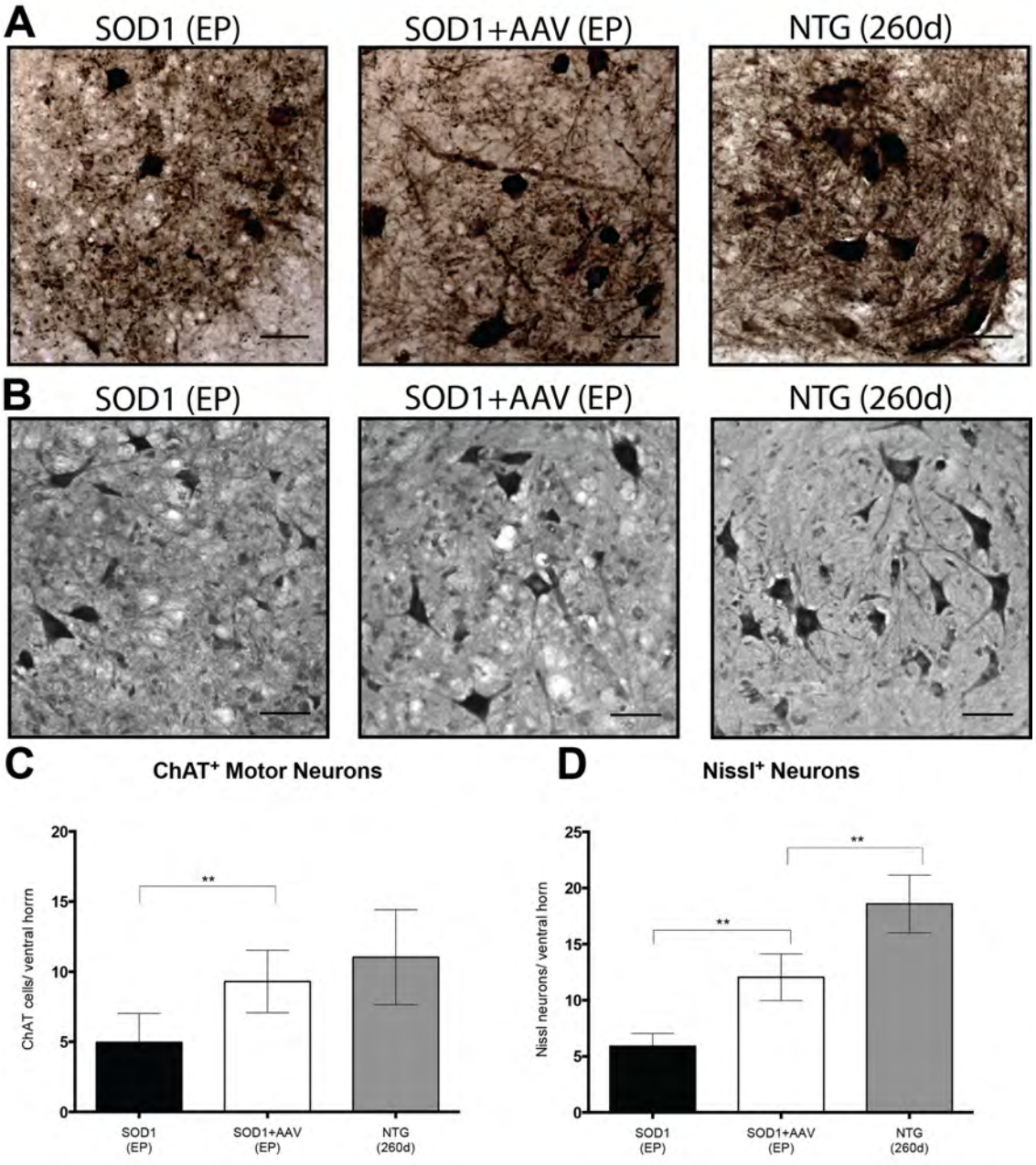

Figure 2.12 AAV9-amiRNA treatment improves survival of spinal cord motor neurons. Untreated $SOD1^{G93A}$ mice were compared with AAV9 treated $SOD1^{G93A}$ mice at their respective endpoints, and NTG mice at 260 days. Immunostaining for ChAT positive motor neurons (A) and Nissl-positive neurons (B) of the lumbar spinal cord showed significant preservation of motor neurons in treated mice when compared to untreated mice. Scale bar, 50 μm. Motor neurons were quantified, defined as either ChAT-positive cells (C) or Nissl-positive cells larger than 250 μm^2 (D), in the ventral horn of the lumbar spinal cord. **p<0.001, *p<0.005

Recently it has been shown that upper motor neurons undergo degeneration in SOD1^{G93A} mice [101] as seen in human ALS. Therefore we analyzed the motor cortex of mice in all three cohorts for the presence of layer V motor neurons identified by immunofluorescence staining with a Ctip2 (COUP-TF-Interacting Protein 2) specific antibody [101,248]. A qualitative assessment suggests that there are lower numbers of neurons in the untreated SOD1^{G93A} mice at endpoint compared to NTG mice, and that AAV9 treatment had a modest impact on the survival of cortical layer V motor neurons. Thus, although a large number of cortical neurons were transduced, it was not sufficient to fully protect the whole layer V neuronal population (Figure 2.13).

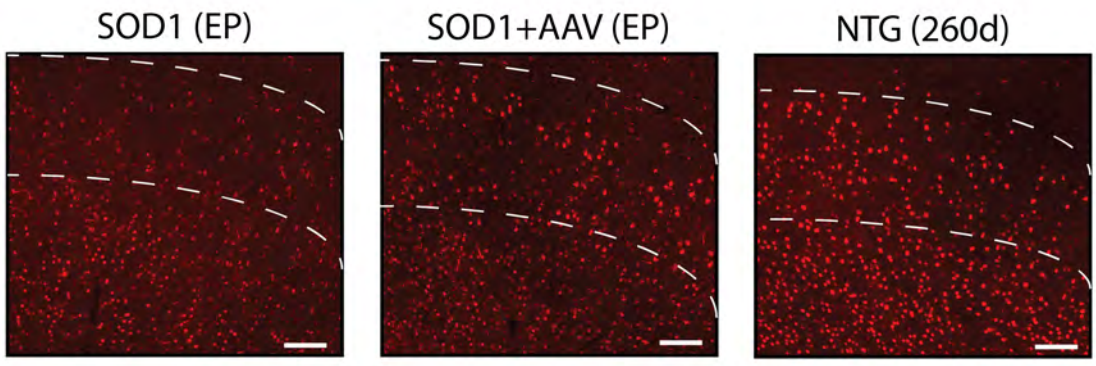

Figure 2.13 AAV9-amiRNA treatment improves survival of corticospinal motor neurons. Immunostaining for Ctip2 positive neurons of motor cortex showed preservation of neurons in treated mice when compared to untreated mice. Dotted lines represent region of layer V neurons. Scale bar, 100 μm.

AAV9-amiRNA treatments delayed onset of inflammation in the spinal cord of SOD1^{G93A} mice

Motor neuron death in both human and mouse ALS is accompanied by a neuroinflammatory process, characterized by activation of astrocytes and microglia. We assessed the levels of inflammatory markers in the lumbar spinal cord of our ALS mouse cohorts. The AAV9-mediated silencing of hSOD1 in the SOD1^{G93A} mice markedly delayed the onset of microgliosis and astrocytosis (Figure 2.14). The spinal cord of AAV9 treated SOD1^{G93A} mice at 135 days of age showed a marginal increase in activated Iba1^{+} microglia (Figure 2.14A) and GFAP^{+} reactive astrocytes (Figure 2.14B) compared to NTG control animals at 260 days. A considerable increase in these markers was apparent by the humane endpoint (median age 206 days) of AAV9 treated SOD1^{G93A} mice, but perhaps to a lower extent than observed in untreated SOD1 mice at their endpoint (median age 137 days). To validate these observations using a quantitative assay, we performed RT-qPCR for genes up-regulated in activated microglia (Tyrobp, Cybb) [249] and reactive astrocytes (GFAP) [250]. All three genes were significantly increased in the spinal cord of untreated SOD1^{G93A} mice at endpoint, but only mildly increased in AAV9 treated SOD1^{G93A} animals at 135 days of age. However, treated and untreated SOD1^{G93A} mice had comparable levels of inflammatory markers at their respective human endpoints. The expression levels of these three genes were unchanged by AAV treatment of NTG animals (Figure 2.14C).

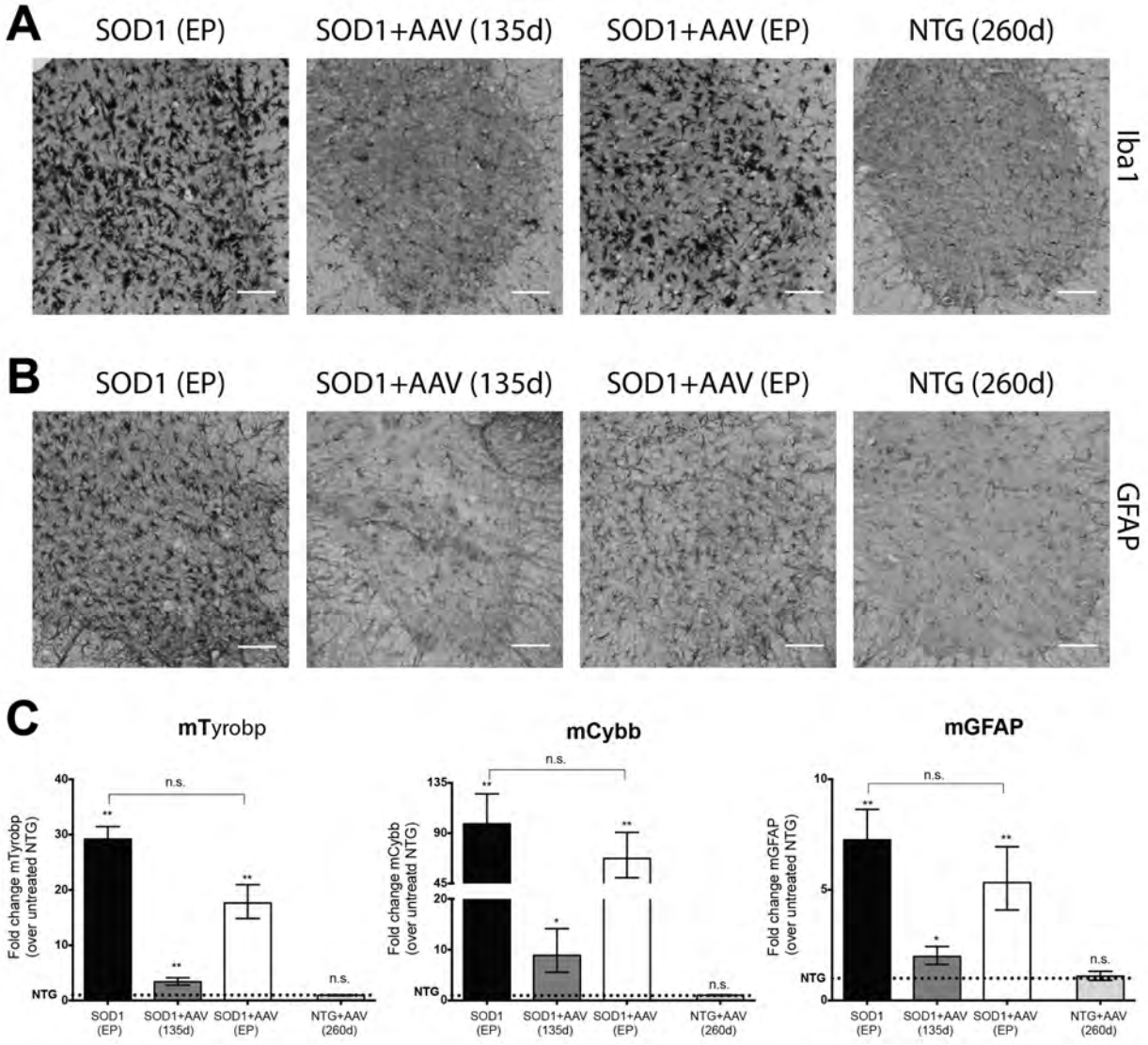

Figure 2.14. AAV9-amiRNA treatment delays the onset of spinal cord inflammation in SOD1^{G93A} mice. The presence of inflammatory markers in the spinal cord of AAV9 treated SOD1^{G93A} mice at 135 days and humane endpoint (EP) was compared to that in untreated SOD1^{G93A} also at their humane endpoint (EP), and NTG mice at 260 days of age. Immunohistochemistry for Iba1, a marker for activated microglia, (A) and for GFAP, a marker for astrogliosis (B), showed mild changes in AAV9 treated mice at 135 days but a strong increase in these histological markers at endpoint. (C) RT-qPCR analysis of genes up-regulated in activated microglia (Tyrobp, Cybb) and reactive astrocytes (GFAP) confirmed the histological findings. All mice were compared to SOD1^{G93A} untreated mice. Dotted line represents age matched untreated NTG mice at 260 days. Scale bar, 100 μm, **p<0.005

Treatment of neonate SOD1^{G93A} mice with an AAV9-amiRNA successfully delays all symptoms of disease

Our single stranded AAV9-CBA-amiRNA-WPRE therapeutic construct was successful at reducing hSOD1 mRNA and protein expression both *in vitro* and *in vivo*. This resulted in a 50% increase in lifespan of treated mice with our therapeutic approach. At the median age of euthanasia of untreated SOD1^{G93A} mice (~135 days), AAV9 treated mice were almost indistinguishable from NTG mice. Additionally, AAV9 treated mice showed a delay in paralysis, as determined by visual observations, and electromyography recordings. However, although at that age AAV9 treated mice had normal looking sciatic nerves, there were already signs of inflammation in the spinal cord. Untreated SOD1^{G93A} mice were euthanized due to hind limb paralysis, but treated mice were euthanized due to a sudden and rapid weight loss and development of kyphosis. This is likely due to an extrinsic restrictive pulmonary phenotype. Kyphoscoliosis, which results from axial muscle weakness, is known to disrupt the chest wall architecture and disturb the mechanics of respiratory muscle function. This phenotype is similar to that seen in ALS patients, but was previously unseen in SOD1^{G93A} mice due to the earlier death due to paralysis. In fact, AAV9 treated mice maintained good motor function, as indicated by visual observation, and supported by maintenance in the number of upper and lower motor neurons and integrity of neuromuscular junctions and ventral roots. Surprisingly, the levels of inflammatory markers in the spinal cord of treated and untreated mice were not significantly different at their respective endpoints. This indicates that our treatment was successful at slowing down, but not halting, disease progression.

CHAPTER III – DISCUSSION

In this work we show that using AAV9 delivered artificial microRNAs to silence hSOD1 is a viable gene therapy approach for SOD1-ALS, as validated in the $SOD1^{G93A}$ mouse model. In this mouse model ALS is caused by an accumulation of mutant hSOD1 protein. Thus, for our therapeutic approach, we wanted to decrease the amount of mutant hSOD1 protein being made. Artificial miRNAs have been shown to successfully decrease mRNA expression *in vivo*. Additionally, AAV viral vectors are good gene delivery vehicles for ALS due to their ability to transduce all cells types involved in the disease, including upper (corticospinal) and lower (spinal cord) motor neurons, as well as astroglia, oligodendrocytes, and skeletal muscle. Several studies have used lentivirus or AAV delivered RNAi molecule against hSOD1 for treating SOD1-ALS with various degrees of success [227-229]. However we were the first to use an AAV vector and delivery route that targeted both upper and lower motor neurons, astrocytes and skeletal muscle. Reducing hSOD1 mRNA expression in all these cell types led to a 50% increase in survival and almost complete delay of paralysis in the $SOD1^{G93A}$ mouse model. This is the largest survival achieved to date with AAV gene therapy.

Multiple studies have used viral vectors to deliver an RNAi species aimed at reducing hSOD1 mRNA expression in the SOD1 mouse model. Some of the most successful thus far used a lentivirus-shRNA delivered intramuscularly at P7 (77% extension in median lifespan), an AAV9-shRNA delivered intravenously at P1 (39% extension in median lifespan), and an AAV6-amiRNA delivered intravenously at P1

(26% extension in median lifespan) [227,229,234]. These studies successfully delivered a therapeutic RNAi species to different cell population in the CNS. The intramuscular lentivirus study used retrograde transport to target both skeletal muscle and their innervating motor neurons. The intravenous study used AAV9 to cross the blood brain barrier and transduce spinal cord motor neurons as well as other non-neuronal cells. The intraventricular AAV6 study used direct CSF delivery to target both upper and lower motor neurons. In our study we used a AAV9 vector containing an artificial microRNA against hSOD1, expressed from the CBA promoter, and delivered it into the lateral ventricles of neonate $SOD1^{G93A}$ mice. We used the direct CSF intraventricular infusion of AAV9 for widespread CNS delivery, AAV9 to for its superior transduction properties, and the ubiquitous CBA promoter for broad gene expression in neuronal and non-neuronal cell populations. With this approach, we achieved a 50% increase in median survival of our mouse model. Additionally, the AAV9 treated $SOD1^{G93A}$ mice in our study remained fully ambulatory up to end stage and were euthanized due to rapid weight loss and the development of progressively worsening kyphosis (hunched posture) rather than the usual limb paralysis. We believe the success of our approach is due to simultaneous reduction of hSOD1 in upper motor and lower motor neurons, as well as in non-neuronal cells of the spinal cord, and in skeletal muscle; as well as the specific design of our targeting cassette.

Recent work has shown progressive degeneration of upper motor neurons in $SOD1^{G93A}$ mice to be a major contributing factor to the paralysis phenotype. Ozdinler et al. [101] showed that upper motor neurons in layer V of the motor cortex degenerate in the

SOD1^{G93A} mouse. Thomsen et al. [235] injected a AAV9-shRNA directly into the motor cortex of adult SOD1 rats, to selectively silence hSOD1 in cortical but not spinal motor neurons. This led to an extension in survival of 20 days, demonstrating the importance of targeting this neuronal population. Our intraventricularly injected AAV9-amiRNA transduced the layer V motor neurons in the cortex and in situ hybridization revealed a considerable reduction of the hSOD1 mRNA in transduced cells. This led to increased preservation of these neurons in our treated mice.

The presence of hSOD1 in non-neuronal cells is important for disease progression and multiple studies have shown that motor neurons are selectively vulnerable to astrocyte-mediated toxicity [71]. Several studies have shown that reduction of hSOD1 specifically in astrocytes leads to an increase in survival, in multiple SOD1 mouse models [83,84]. Additionally, delivery of hSOD1 carrying astrocytes is toxic to motor neurons, both *in vivo* and *in vitro* [95,97,98]. An increase in astrocytosis is also associated with SOD1 astrocyte driven disease progression [81]. We observed some GFP positive astrocytes in the spinal cord after a neonate intraventricular injection of our AAV9-GFP vector. We also observed a delay in astrocytosis in the SOD1^{G93A} mice treated with our AAV9-amiRNA, although it is difficult to determine if that is specifically due to a decrease of hSOD1 in astrocytes.

Skeletal muscles have also been implicated in disease progression. Our intraventricular injection delivers vector into the CSF, but there is leakage into the periphery, and transduction of peripheral organs. AAV9 is known to transduce skeletal muscle at high efficiency, and accordingly we noted significant reduction of hSOD1

mRNA in the gastrocnemius. Reduction of mutant hSOD1 in the muscle has not been shown to have a significant effect on motor function [87], although expression of mutant SOD1 in muscle alone leads to motor impairment by causing neuromuscular junction dysfunction [77,82]. Thus, while this reduction is unlikely to be a principal component of increased survival in our study, it likely plays a role, especially since most of our mice displayed no obvious signs of paralysis.

While previous studies used AAVs to deliver therapeutic RNAi molecules, none of them achieved widespread distribution throughout the CNS and periphery. IV infusion of AAV9 into neonate mice, such as the Foust et al. [229] study, transduce peripheral organs and a large number of motor neurons in the spinal cord, but not in the cortex. However, delivery of the same vector into older mice leads to a loss of neuronal transduction and an increase in non-neuronal cell transduction. Similarly, intrathecal injections in adult mice can transduce the spinal cord, but not cortical motor neurons [219]. Additionally, studies using this type of delivery method have shown significant inter-animal transduction variations [236,237]. The study using intramuscular lentiviral injections used the vector's ability to undergo retrograde transport, and transduce the motor neurons, but only those neurons innervating the injected muscles [227]. Lastly, although Dirren et al. used a neonatal intraventricular delivery approach, they transduce very few neurons in the cortex with their AAV6 vector driving GFP expression from the CMV promoter [251]. Is apparent from these previous studies that treatment of either upper or lower motor population alone is beneficial, but not sufficient to substantially affect the course of disease progression. Thus, the significant increase in survival and delay in paralysis we achieved

with our AAV injection is primarily due to the widespread reduction of hSOD1 in both upper and lower motor neurons.

Additionally, several design features in our AAV9 vector may account for its efficacy, such as the use of the CBA promoter or the presence of a WPRE element in the vector-derived mRNA. We chose the strong ubiquitous CBA promoter to achieve expression of our amiRNA in all cell types. It is interesting to note that the two previous studies with the largest increase in survival both used the strong and ubiquitous H1 promoter to drive shRNA expression. These studies suggest that high levels of expression of the RNAi molecule are required in multiple cell types to achieve a strong therapeutic effect. While we do not use a strong polIII promoter, we have both an enhancer and a chimeric intron flanking the basic chicken beta actin promoter, as well as a WPRE element. These features have been shown to enhance transgene expression [252]. Additionally, we used the murine pre-miRNA155 backbone in the design of our artificial microRNA. The exact contribution of each element in our vector remains to be determined, and future modifications should maintain a high enough level of expression for efficacy, yet not cause toxicity.

Cell-specific promoters could be used to determine the exact contribution of each cell type in our therapeutic approach. However, since the CBA promoter is ubiquitously expressed, it would be challenging to assess every cell type that could be contributing, especially given the heterogeneous nature of astroglial cells. Cell specific promoters could also lead to different expression levels than with the CBA promoter. Furthermore, it is most likely that the therapeutic effect is a due to simultaneous expression in multiple

cell types. Testing this would require the use of combination of vectors with cell-specific promoters. This would also be challenging, since both volume and quantity of vector genomes have an effect on the spatial distribution of the vectors. Thus, such studies may end up raising more questions regarding the contribution of different cell types to the therapeutic effect.

While we achieved significant survival improvement with our AAV9-amiRNA vector, the question still remains as to why were not able to cure the $SOD1^{G93A}$ mice. The electrophysiological recordings for the AAV9 treated animals remained within normal range until end-stage, when only a few mice showed evidence of mild motor dysfunction. Additionally, an increase in spinal cord inflammatory markers and decreased motor neuron counts in AAV9 treated $SOD1^{G93A}$ mice at endpoint indicated that disease was still progressing although with delayed onset. This could be a result of 1) an insufficient decrease in the level of mutant hSOD1 expression in transduced cells, 2) an insufficient number of transduced cells to completely prevent disease onset 3) an insufficient transduction of specific cell types, such as astrocytes. The identity of transduced cells is particularly important for SOD1 ALS, where there is a strong non-cell autonomous effect. Transplantation of a small number of $SOD1^{G93A}$ astrocytes into the spinal cord of normal animals is sufficient to cause motor neuron death [81]. Additionally, recent work with co-culture models shows that SOD1 containing astrocytes can release toxic factors to induce motor neuron death [95,99]. A disadvantage of neonate injections is the loss of expression due to cell proliferation during post-natal development. Since AAV is a non-integrating vector, its presence in dividing cells is diluted over time. This is seen with the

liver and lung of our treated mice, where we found no evidence of hSOD1 mRNA silencing, despite preferential AAV9 transduction to these organs. Astrocytes continue to proliferate during post-natal brain development, so it is likely that expression of the amiRNA is lost in those cells as well. Since the accumulation of hSOD1 in astrocytes is implicated in disease progression, a loss of expression of amiRNA from these cell types during post-natal brain development could be diminishing the efficacy of our treatment. In future studies, valuable information could be gained about the levels of hSOD1 in both transduced and non-transduced cells at endpoint, through the use of immunostaining for the presence of hSOD1.

Our initial studies both *in vitro* and *in vivo* showed our artificial microRNA to be effective at reducing hSOD1 mRNA expression by more than 90% in HEK293T cells, and by more than 60% in the brain at the site of a direct injection. In situ hybridization data for hSOD1 mRNA in the cortex and spinal cord showed successful silencing in transduced cells, although residual hSOD1 mRNA expression remained. However, only a subset of motor neurons in the spinal cord was transduced. It is currently unknown what level of hSOD1 reduction is sufficient to stop disease progression. As a significant number of motor neurons in the spinal cord are preserved at endpoint, it would be interesting to know if there is a correlation between neuronal survival and extent of hSOD1 reduction. This could be inferred by a correlation between the size of motor neurons at endpoint and their GFP status. It would be expected that the smaller, degenerating neurons, would have less GFP expression as compared to normal size motor neurons, assuming that GFP expression correlates with hSOD1 reduction. There is no

difference in the number of AAV vector genomes and amiRNA guide strand expression in the spinal cord of injection SOD1^{G93A} mice at four weeks of age and endpoint, indicating that loss of amiRNA expression and subsequent loss of hSOD1 silencing is not a likely cause of disease progression.

Although effective therapeutics to cure ALS do not exist, patients have multiple options to help manage their symptoms. Thus, while limb weakness is usually a primary symptom of disease, the cause of death is respiratory failure, due to weakness of the diaphragm and intercostal muscles [1]. However, untreated SOD1 mice are euthanized due to the primary symptoms of hind limb paralysis, and do not display the respiratory phenotype seen in patients. Recent work has shown there are some signs of respiratory impairment in the SOD1^{G93A} mice, but only a few days before their paralysis endpoint [253]. Our AAV9-amiRNA treated SOD1 mice did not exhibit paralysis, but instead developed severe kyphosis (hunched posture) and rapid weight loss, requiring euthanasia. A kyphotic posture is consistent with axial muscle weakness, causing disruption of the chest wall architecture, and direct physical restriction of lung inflation. This would also cause a subsequent disturbance of the mechanics of respiratory muscle function. CT measurements confirmed a decrease in chest volume in the treated SOD1 mice, as compared to age matched non-transgenics. Additionally, loss of the motor neurons innervating the diaphragm and intercostal muscle would directly contribute to respiratory muscle weakness. This would affect the ability of the mice to maintain lung volume both during inspiration and at rest. Thus, respiratory impairment is a potential cause of death. To assess respiratory function we used a hypercapnic challenge and measured breathing

response to an increase in CO_2; a decreased response implies an increase in breathing effort at rest, and is commonly seen in restrictive pulmonary disease of neuromuscular origin [254]. Indeed, the treated SOD1 mice had a significant decrease in minute ventilation in the hypercapnic respiratory challenge, and their ability to respond to the challenge continued to decline with age. Thus, our treatment successfully delayed hind limb paralysis, and revealed a respiratory phenotype in the $SOD1^{G93A}$ mouse model, similar to the restrictive lung disease seen in ALS patients.

Further experiments need to be performed to determine the exact cause of death of the treated mice. While respiratory impairment due to intercostal and diaphragm muscle weakness seems to be a likely cause of death, this argument would be strengthened with histological proof. The status of the phrenic motor neurons in the cervical spinal cord and their innervation of the neuromuscular junctions on the diaphragm should be examined. As mentioned before, since our motor neuron transduction efficiency is not 100%, we likely rescued a sufficient number of phrenic motor neurons to alleviate the respiratory phenotype, but not halt the disease.

The degree of SOD1 mRNA reduction we achieved was sufficient to extend survival but not cure the disease. Mostly likely, there is a critical amount of mutant SOD1 accumulation that triggers motor neuron death, although this threshold and the cell type(s) responsible are not yet determined. However, it is clear that in the SOD1 mouse model disease is correlated with the level of mutant SOD1 transgene expression, and a “cure” was achieved only with 100% removal of the mutant SOD1 transgene in the double transgenic model [103,104,226]. Thus, a cure of the $SOD1^{G93A}$ mouse may only be

possible with 100% reduction of hSOD1 in all cells. However, our results suggest that with enough mutant SOD1 reduction, symptom free survival could be extended to the normal lifespan of the nontransgenic mice.

FUTURE DIRECTIONS

Future studies should test our therapeutic vector for efficacy in adult mice. However, the mode of delivery still remains a challenge. Foust et al. [229] has shown some therapeutic efficacy (23% extension in median lifespan) after an intravenous infusion at 85 days. Thus, an intravenous infusion is a good starting point. However, their shRNA is expressed from an H1 promoter, which is likely stronger than our CBA promoter. While the WPRE in our vector is known to enhance transgene expression, it is unknown if it has an effect for amiRNAs embedded in the 3'UTR. Additionally, intravenous infusions are ineffective at transducing upper motor neurons of cortical layer V. Thus, an approach coupling an intravenous infusion as well as several direct intracranial injections into the motor cortex should also be tested. Also, it is possible that we could achieve a greater therapeutic success if we reduced hSOD1 expression in more astrocytes. Thus, an adult IV injection coupled with the neonate ICV injection would deliver our amiRNA to non-proliferating astrocytes. Lastly, multiple groups have shown some success in targeting the spinal cord motor neurons and brain with a AAVrh10 intrathecal or AAV9 intra-cisterna magna injection [255,256]. Thus, more efficient capsid variants and delivery approaches should be considered. Nevertheless, a lack of significant therapeutic success in adult mice

due to inadequate vector distribution should not be discouraging, since effective delivery has been shown to be possible in larger animal models [222,257,258].

For clinical translation our vector will also need to be tested in non-human primates for safety, delivery, and SOD1 reduction efficacy, although it cannot be tested for therapeutic efficacy due to the lack of an NHP ALS model. Translation into larger animal models is also important for determination of doses for clinical trials. The level of SOD1 mRNA reduction should be the same as that demonstrated in the mouse model in order to expect similar therapeutic results. Large animal models for ALS do exist, including a newly developed $SOD1^{G93A}$ swine model [259], and a naturally occurring dog model [260]. However these models are long lived, have late symptom onset, and have yet to be fully characterized and assessed for faithful reproduction of human disease. As non-human primates are the closest mammals to humans in terms of assessing delivery methods and distribution, methods similar to those used in the clinic can be tested, and vector distribution and transduction efficacy can be evaluated. Our artificial microRNA is perfectly complimentary to both human and primate SOD1, allowing us to test the exact RNAi molecule that would be used in the clinic. Also, since they are not transgenic models, non-human primates will provide additional information regarding the potential negative impact of reducing endogenous SOD1 levels, or potential changes in endogenous microRNA expression profiles. This is particularly important as our therapeutic approach involves delivering a high dose of an amiRNA, which uses the endogenous RNAi pathway. As microRNA-155 is already overexpressed in ALS [261,262], an additional increase in microRNA processing due to expression of our amiRNA could

lead to a saturation of the RNAi pathway. These experiments will provide valuable information for translation to the clinic.

A potential concern for translating this therapy to the clinic is the effect of loss of normal SOD1 function. Given that our amiRNA does not distinguish between mutant and normal SOD1 mRNA, it is likely that it will lead to a reduction in both proteins. We are unable to address this in our current $SOD1^{G93A}$ mouse, where the human $SOD1^{G93A}$ gene is highly overexpressed, and the endogenous mouse Sod1 is unchanged. The transgenic knock out Sod1 mouse does not develop overt ALS like symptoms [107]. Thus, it is unlikely that when delivered to patients, a reduction in SOD1 will have negative effects. However a lack of phenotype in transgenic models could be due to backup or compensatory mechanisms. Nevertheless, we expect our therapeutic approach to be safe and well tolerated, since treated non-transgenic animals did not show any symptoms of toxicity. Another concern with the reduction of SOD1 is the overabundance of unused CCS (responsible for copper loading into SOD1), especially since mouse models overexpressing CCS display severe toxicity [263]. Thus, levels of ROS and CCS should be carefully monitored, and enzyme replacement therapy to restore normal SOD1 levels may become necessary. An alternative therapeutic strategy would be simultaneously delivering an amiRNA and a SOD1 cDNA that is resistant to the particular amiRNA being used.

The widespread gene transfer achieved with neonatal ICV infusions provides insight into therapeutic outcomes that may be attainable in adults. If a neuronal transduction profile similar to neonates can be replicated in adults, we anticipate the

therapeutic outcomes to be superior to those achieved by neonatal intervention. This is because in the adult brain there is minimal cell proliferation, and thus fewer vector genomes will be lost due to post-natal cell division. This would result in sustained silencing of hSOD1 expression in all CNS populations, and thus suppression, either partial or complete, of non-cell autonomous effects on disease progression.

Although the $SOD1^{G93A}$ mouse reproduces many aspects of the patient phenotype, it is not an accurate model of disease progression, as the phenotype develops much more rapidly in the mouse due to the supraphysiological levels of mutant hSOD1. This raises concerns when attempting to test therapeutics in the mouse model. Therapeutic intervention in adult mice has only achieved a small increase in survival of the mouse model. However, it is possible that the effect in patients will be greater, since patients have a much milder disease progression than the $SOD1^{G93A}$ mice. In our case, delivery to neonatal mice does not represent clinical administration of the therapeutic, which would occur after disease onset. However, a reduction in the misfolded protein burden should alleviate cellular stress, and lead to a deceleration of disease progression. As earlier interventions would yield the most benefit, development of biomarkers for ALS diagnosis is as important as development of therapies.

Large animal studies have already shown the ability of AAV9 to transduce the CNS using alternative delivery approaches, such as cisterna magna [257] and intrathecal infusions [222]. Additionally, the use of the Trendelenburg position after intrathecal infusion has shown transduction of cortical neurons in adult non-human primates. Both AAVs and siRNAs have been safely delivered to the CNS in multiple clinical trials. Our

proof of concept study proves that an AAV delivered amiRNA successfully decreases hSOD1 mRNA expression in multiple cell types, and significantly extends lifespan of the $SOD1^{G93A}$ mouse model. Thus, delivery of our AAV9-amiRNA through the use of a direct CSF infusion is directly translatable to the clinic.

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