Therapeutic suppression of mutant SOD1 by AAV9-mediated gene therapy approach in Amyotrophic Lateral Sclerosis

Dissertation

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By

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Abstract

Amyotrophic Lateral Sclerosis is one of the most common, adult-onset neurodegenerative disorder, characterized by progressive and fatal loss of motor neurons in spinal cord, motor cortex and brainstem which results into muscular paralysis and ultimate respiratory failure leading to death. Dominant mutations in Superoxide Dismutase 1 (SOD1) gene are one of the frequent causes of familial ALS. Mutant SOD1 confers cell autonomous as well as non cell autonomous toxicity towards motor neurons in ALS. Therefore, along with motor neurons, surrounding non-neuronal cells like astrocytes, microglia and oligodendrocytes play important role in the pathogenesis of familial ALS. Non cell autonomous toxicity towards the motor neurons is also evident in sporadic ALS cases. Thus, suggesting that reduction of motor neuron toxicity from multiple cell types within the CNS is required to achieve the maximum therapeutic benefits in ALS.

Transgenic removal of mutant SOD1 from motor neurons and astrocytes significantly delays the disease onset and progression of ALS mice with significant extension in survival. Here, we determined the feasibility and efficacy of post-natal downregulation of mutant SOD1 via AAV9-mediated shRNA delivery in two distinct mouse models of ALS. Our results show that AAV9-mediated delivery of SOD1 shRNA before or even after the disease onset results in significant extension in the lifespan of
SOD1\textsuperscript{G93A} and SOD1\textsuperscript{G37R} mice. We also show that AAV9 SOD1 shRNA administration is safe and well-tolerated in wild-type mice. Moreover, widespread spinal cord transduction and efficient SOD1 reduction in non-human primates provide strong basis for the translation of AAV9 mediated SOD1 shRNA delivery approach to the human settings. Furthermore, in accordance with the guidelines established by the Food & Drug Administration (FDA), USA, we show successful construction of clinical AAV SOD1 shRNA plasmid with non-coding stuffer sequence, replacing the GFP expression cassette. We show that in vitro administration of clinical AAV SOD1 shRNA vector into human cells results into efficient downregulation of SOD1 protein levels with lack of GFP expression as well as any unnatural transcript production from the stuffer. Here, we show successful generation of clinical AAV SOD1 shRNA vector that can be utilized directly in human clinical trials for ALS.

Microglia are one of the key players involved in non-cell autonomous toxicity in ALS pathogenesis. Transgenic removal of mutant SOD1 from microglia has confirmed their role in the disease progression. Transgenic inhibition of NF-κB pathway in ALS microglia has also confirmed their role in neuroinflammation induced motor neuron death in ALS. However, none of the currently available approaches are able to target the microglia post-natally. In Chapter 4, we show that conditioning of endstage microglia from SOD1\textsuperscript{G93A} mice with exogenous Galectin 1, a lectin binding carbohydrate, results in reduction of their neurotoxic potential towards the motor neurons. We also show that Galectin 1 treatment of ALS microglia reduces the secretion of proinflammatory cytokines
like TNF-α. Finally, our results show that Galectin 1 exerts its immunomodulatory effects on ALS microglia via inhibition of NF-κB pathway. As astrocytes act as a source of endogenous Galectin 1 and remain to be one of the cell types readily targeted by AAV9, our results suggest a plausible approach for post-natal immunomodulation of ALS microglia by AAV9-mediated overexpression of exogenous Galectin 1.
Dedication

This document is dedicated to my parents.
Acknowledgments

I am incredibly thankful to my mentor, Dr. Brian Kaspar for his constant support, guidance and confidence in me. His enthusiasm and passion for research and his untethered dedication and commitment to science have always inspired me to be persistent, perseverant and do better and meaningful science. I cannot thank him enough for spending his time and energy in training me to be an independent researcher. With the intense training under his supervision, I feel ready for any future challenges.

I would like to thank my committee members, Dr. Arthur Burghes, Dr. Stephen Kolb and Dr. Christine Beattie. Their invaluable critics and meticulous suggestions have certainly helped shape my scientific career. The strong basic and translational research background of Dr. Burghes and Dr. Beattie blended with Dr. Kolb’s clinical expertise helped me comprehend the big picture of science. I feel honored and extremely obliged to have these elite scientists looking over my shoulders.

Being an international student, I feel extremely lucky to be one of “the Kaspars”! It was never just a research lab for me but was my family outside India! Every member of Kaspar lab, past or current, holds my deepest respect and gratification for making it the best lab I could have ever asked for.

I would like to thank Dr. Kevin Foust for his constant advice and feedback throughout my graduate training. Being easily approachable and patient enough, he really helped me build confidence in myself and my work.

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I cannot imagine my graduate work here in the Kaspar laboratory without my colleagues Sungwon Song and Ashley Frakes. I would like to thank them for their constant feedback and constructive criticism which kept the work environment always innovative and thought-provoking. Having such friends definitely made all the stress and hardships of graduate life bearable.

I also want to thank our new members, Jillian Liu and Anton Blatnik for constant discussions and debates. Trying to answer the continuous string of questions coming from them has certainly helped me to be prompt, precise and up to date with the literature. Also, their never-ending interest in research made the lab environment innovative and exciting.

I would like to thank all the visiting scholars, Olivia Michels, David Baker and Dr. Alessandra Govoni. It was a great experience to learn and exchange both scientific and non-scientific knowledge with them. I am also thankful to have Dr. Nicolas Wein as a colleague and a friend whom I could turn to if there was any problem whatsoever.

I would also like to thank my friends outside the lab who have played a very important part in keeping me socially sane. I would like to extend special thanks to my friend Wenyan Jiang for always being there to support me in tough times. I would also like to thank my friends Balasubramani Hariharan and Krishna Patel for their support and patience with me.

Finally, I would like to thank my parents, Balkrishna Likhite and Surekha Likhite. I would not have been here if it was not for their constant support, encouragement and confidence in me. I feel extremely obliged with all the energy, efforts and time they’ve spent on me. Their lessons of hard work, perseverance and humbleness have helped me complete this intense task of graduate study. I hope I could keep them happy and make them proud with my future endeavors.
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Publications


Fields of Study

Major Field: Molecular, Cellular and Developmental Biology
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Chapter 1

Introduction

1.1 Amyotrophic Lateral Sclerosis

1.1.1 General description

Amyotrophic Lateral Sclerosis (ALS) is an adult onset, neurodegenerative disorder. It is characterized by progressive and fatal loss of motor neurons, primarily in spinal cord, brain stem and motor cortex. This leads to muscular paralysis and finally death due to respiratory failure. By definition, “Amyotrophic” stands for atrophy of muscle fibers caused due to degeneration of the innervated motor neurons from the ventral horn of spinal cord that leads to progressive muscle weakness and muscle death. “Lateral Sclerosis” refers to hardening of anterior and lateral corticospinal tracts due to death of corresponding motor neurons in the ventral horn and subsequent gliosis[1]
Rather than a single disease, Amyotrophic Lateral Sclerosis is considered as a syndrome of various disorders characterized by motor neuron degeneration. Hence, it is extremely important to clearly diagnose a patient with primary ALS and not any of the other mimic disorders. Primary ALS can be manifested as bulbar onset ALS or spinal onset ALS[2]. Bulbar ALS involves degeneration of motor neurons in the corticobulbar region of the brainstem. These motor neurons innervate the muscles of face, head and neck. Hence the initial symptoms of bulbar onset ALS include difficulty in speaking and swallowing. Bulbar ALS has faster progression than spinal ALS. Spinal onset ALS, also called as limb onset ALS, involves the degeneration of motor neurons in the corticospinal tract which innervate the limb muscles. Spinal-onset ALS presents with focal weakness in proximal and/or distal muscles. Minority of ALS patients also manifest respiratory onset ALS in which the disease starts with the degeneration of motor neurons innervating the intercostals muscles and thus affecting breathing.

1.1.2 Epidemiology

ALS is one of the most common neurodegenerative disorders worldwide. Almost 90% of ALS cases are sporadic (SALS), meaning without a known genetic cause while 10% of ALS cases are familial (FALS) where the disease is inherited in Mendelian pattern. Unlike sporadic ALS, familial ALS is caused due to autosomal dominant mutation in certain genes[3]. These include mutation in Superoxide dismutase 1 (SOD1), TAR DNA binding protein (TARDBP), Fused in sarcoma (FUS), Optineurin (OPTN),
Valocin containing protein (VCP), Ubiquillin 2 (UBQLN2), C9ORF72 and Profilin 1 (PFN1). Very recently, hexanucleotide repeat expansions found in C9ORF72 have also been shown to be associated with familial ALS cases[4-6]. The incidence of ALS is estimated to be 1.5 to 2.1 per 100,000 population per year in Europe and North America. The estimated life time risk of sporadic ALS is 1:400. It is also reported that males are more prone to sporadic ALS than females with M:F ration of about 1.5:1. ALS is an adult onset disorder with the mean age of onset between 55-65 years for ALS. ALS cases with the onset at the age of less than 25 years of age are considered as juvenile onset ALS. ALS is a highly progressive disorder with median survival of only 2-3 years for bulbar onset ALS while 3-5 years for spinal onset ALS. Respiratory onset ALS has the poorest prognosis. Familial ALS patients have disease onset almost 10 years earlier than sporadic cases. FALS patients also have much shorter survival rate than SALS patients[2].

1.1.3 Clinical presentation

Almost 65% of ALS cases have spinal onset, 30% cases manifest as bulbar onset while a minority (5%) of cases show respiratory onset. Spinal onset ALS patients present with asymmetric pattern of symptoms with weakness in proximal or distal muscles of upper or lower limbs. In these cases, degeneration of both upper and lower motor neurons is involved. All the spinal onset ALS cases progressively develop bulbar symptoms and finally the death occurs due to respiratory muscle impairment. The initial symptoms of
bulbar ALS include dysarthria of speech and dysphagia (difficulty in swallowing). Bulbar ALS later culminates into limb ALS. Patients with respiratory onset ALS present with respiratory weakness including respiratory failure or nocturnal hypoventilation. All types of primary ALS finally culminate into degeneration of both upper motor neurons as well as lower motor neurons in motor cortex, brainstem, bulbar, cervical, thoracic and lumbar region of the spinal cord with respiratory failure or pulmonary complications being the final cause of death[7].

1.1.4 Diagnosis

Previously established criteria like EL Escorial criteria (established in 1994), revised Airlie House Criteria (established in 1998) are used for the diagnosis of primary ALS in clinics[8,9]. The diagnosis of primary ALS requires the involvement of both upper motor neuron and lower motor neuron degeneration in the same region of brainstem, bulbar, cervical, thoracic or lumbar spinal cord[10,11]. The definitive diagnosis of ALS depends on the identification of lower motor neuron degeneration by clinical, electrophysiological and pathological assessment; identification of upper motor neuron degeneration by clinical examination and most importantly progression of the motor symptoms within the same region or to the other regions. Also, as primary ALS comes under the umbrella of various motor neuronopathies, it is extremely important to confirm the absence of any other disorders that could cause the observed clinical and electrophysiological signs[2,7]. For most patients, simultaneous manifestation of both
upper motor neuron and lower motor neuron abnormalities is absent which makes the
diagnosis of primary ALS more difficult. This time gap between the actual onset of
disease and the definitive diagnosis in the clinic can be as long as 9 to 15 months. This is
worth taking a note as it underlines the importance of developing the clinical therapeutics
that would act on prolonging the disease progression than just delaying the disease onset.

1.1.5 Disease management

Despite the advances in understanding the basic mechanisms of pathogenesis of
ALS, there is no cure for the disease till date. Riluzole is the only available drug that is
shown to alter the disease outcome[12-16]. Management of ALS is hence mainly focused
on the symptomatic treatment and palliative care.

1.2 Pathogenic mechanisms of ALS

Majority of the ALS cases are sporadic (~90%), that is, without any mutations in
specific genes while 10% of the cases are caused due to autosomal dominant mutations in
certain genes and hence are called familial ALS. More than 10 genes have been linked to
the familial cases of ALS including Superoxide dismutase 1 (SOD1), TAR DNA binding
protein (TARDBP), Fused in sarcoma (FUS), Optineurin (OPTN), Valocin containing
protein (VCP), Ubiquilllin 2 (UBQLN2), C9ORF72 and Profilin 1 (PFN1)[3]. Among
these, Superoxide Dismutase 1 (SOD1, ~20%)[17], Fused in sarcoma (FUS, 1-
5%)[18,19] and TAR DNA binding protein (TARDBP, 1-5%)[20-22] are the most
common genes carrying the FALS causing mutations. Recently found hexanucleotide repeat expansion (GGGGCC)n in C9ORF72 is identified as the most frequent cause of the familial ALS (~40%) as well as sporadic ALS (~6%). C9ORF72 mutation is also considered to be the common link between ALS and familial frontotemporal dementia (FTD), another adult-onset neurodegenerative disease[4-6].

Among the identified causative genes, Superoxide dismutase 1 (SOD1) remains the most studied gene in pathogenesis of familial ALS[17]. SOD1 gene encodes for superoxide dismutase 1 enzyme that catalyzes the reaction of converting free superoxide radicals into hydrogen peroxide as follows:

\[ 2 \text{O}_2^- + 2 \text{H}^+ \rightarrow \text{O}_2 + \text{H}_2\text{O}_2 \]

SOD1 is one of the three superoxide dismutase enzymes present in the body. It is a soluble, cytoplasmic enzyme which is also present in mitochondrial inter-membrane space. Over 150 mutations in the SOD1 gene have been discovered so far to be responsible for ALS[23]. In the United States, A4V mutation is the most prevalent mutation in familial cases[24] while H46R mutation is the most frequent mutation found in Japanese population[25,26]. Although most of the SOD1 mutations are inherited in an autosomal dominant manner where a single copy of the mutated gene is enough for manifestation of ALS, a few recessive mutations have also been reported, for example, an autosomal recessive D90A mutation. Surprisingly, not all the ALS-linked mutations in SOD1 gene result in the loss of its enzymatic activity, suggesting that ALS is not caused due to loss of functionally active SOD1. Mouse studies have also shown that complete
knockout of the endogenous SOD1 do not develop ALS in mice[27]. Thus, mutations in SOD1 are considered to be toxic gain-of-function mutations which render mutant SOD1 responsible for the pathogenesis of ALS, irrespective of its native enzymatic activity. This was proven for the first time in 1993 with the generation of SOD1\textsuperscript{G93A} mice by overexpressing the human SOD1 transgene carrying G93A mutation in the mouse genome. Since then, SOD1\textsuperscript{G93A} mice stand to be the most widely studied mouse model of ALS[28].

SOD1\textsuperscript{G93A} mice carry 20 copies of the human mutant SOD1 construct under endogenous mouse SOD1 promoter, producing 10-15 fold higher levels of mutant SOD1 protein all throughout the body[28]. This mouse model closely resembles the clinical as well as pathological features of human ALS[28,29]. There is a continuous loss of motor neurons in the spinal cord, brainstem and motor cortex as the disease progresses. SOD1\textsuperscript{G93A} mice show the clinical disease onset around 90 days of age which includes peak body weight, hindlimb tremor and muscle weakness. These mice have a fast progression phase which is evident from the muscle atrophy, weight loss and hindlimb paralysis. Clinical endstage of SOD1\textsuperscript{G93A} mice is marked as the time point when they fail to right themselves within 30 seconds. SOD1\textsuperscript{G93A} mice reach endstage at around 130 days of age. Due to much shorter lifespan, SOD1\textsuperscript{G93A} mouse model is considered as the fast progressing mouse model of ALS. Being an adult onset disorder, ALS takes almost 45 years for its manifestation in humans and around 5 years till the disease endstage. Mouse models resembling this late onset and slow progression of the disease
have also been generated. LoxSOD1\textsuperscript{G37R} is one of the most studied, slow progressing mouse models of ALS. These mice have the disease onset at around 200 days of age with the median age at endstage being 350 days[30].

Transgenic mice carrying several other SOD1 mutant constructs (G85R, G86R, G127X, H46R, D90A) have also been generated for better understanding of the pathogenic mechanisms of ALS[31,32]. However, after more than 2 decades of research, there is still no consensus about the exact pathogenic mechanisms by which mutations in SOD1 gene cause ALS.

1.2.1 Cell autonomous toxicity of mutant SOD1 in FALS pathogenesis

Excitotoxicity has been considered as one of the most direct consequences of mutant SOD1 expression within motor neurons[33]. Several \textit{in vitro} as well as \textit{in vivo} studies have shown that expression of mutant SOD1 renders the motor neurons vulnerable to Glutamate-induced excitotoxicity[34-40]. In a normal situation, glutamate is released from the pre-synaptic terminal which then binds to the glutamate receptors on the post-synaptic motor neurons and causes the influx of Ca\textsuperscript{2+} ions, thus generating the action potential. The residual glutamate is uptaken by surrounding glial cells, especially astrocytes with the help of Glutamate transporter EAAT2. This process controls the level of glutamate in the synapse[41]. In ALS, however, there is a buildup of extracellular glutamate which results in increased influx of Ca\textsuperscript{2+} ions inside the motor neurons.
Increased intracellular Ca2+ overloads the Ca2+ buffering capacity of the mitochondria, resulting into mitochondrial damage and further activating the apoptotic cascade and neuronal death. The accumulation of extracellular glutamate has been attributed to reduced uptake of glutamate due to reduced expression/activity of glutamate transporters on the astrocytes and Ca2+ permeability[42]. Increased levels of glutamate have been found in plasma as well as CSF of ALS patients[43-45]. Decreased expression of glutamate transporter EAAT2 on astrocytes as well as altered glutamate synthase expression has also been well-documented in both ALS mouse models and human ALS patients[41,46-50]. It was shown that the glutamate-induced excitotoxicity of ALS motor neurons can be rescued by using glutamate receptor antagonists. An antiglutametergic drug, Riluzole, has been shown to reduce the glutamate release in the synaptic terminal as well as enhance the activity of glial glutamate transporters and thus increase the glutamate uptake. Being able to improve the survival of ALS patients by almost 2 months in three different clinical trials, Riluzole remains to be the only available FDA approved drug for the treatment of ALS so far[12-16,43,51].

Mutant SOD1 has a high propensity to misfold[52]. Misfolded mutant SOD1 has been shown to accumulate inside the cells and cause detrimental effects on ER function[53]. Endoplasmic reticulum (ER) is an important cell organelle required for the synthesis and folding of cellular proteins. Misfolded mutant SOD1 has been shown to inhibit the activity of ER chaperons like ER-luminal polypeptide chain binding protein (BiP)[54]. This results in the activation of ER stress pathway. Mutant SOD1 can also
inhibit ER associated degradation by binding to Derlin-1, a transmembrane protein that translocates misfolded proteins from the lumen of ER to the cytosol where they are degraded by proteasome[55]. Thus, accumulation of mutant SOD1 protein leads to improper functioning of ER which eventually affects the synthesis and distribution of other essential proteins.

Misfolded mutant SOD1 has also been postulated to inhibit cytosolic proteasomal degradation[56-58]. This leads to an accumulation of mutant SOD1 aggregates within the motor neurons as well as non-neuronal cells in the spinal cords of ALS mice as well as patients. Reduced levels of proteasomal activity in the lumbar spinal cord have been shown in different SOD1 ALS mice and human patients[59-62]. Inhibition of proteasomal degradation not only results in [63] uncontrolled accumulation of the misfolded mutant SOD1 within the cell but also results in the sequestration of the essential cellular components within the aggregates, leading to further damage to the cell[64].

Mitochondria play a crucial role in cellular energy homeostasis. Mutant SOD1 has been postulated to affect the mitochondrial function by depositing on the cytoplasmic face of the outer mitochondrial membrane[63,65]. Changes in the mitochondrial morphology have been shown in certain mouse models of ALS. It has been proposed that mutant SOD1 accumulation affects the mitochondria by interfering with the electron transport chain, mitochondrial protein import or regulation of apoptosis[66].
Mutant SOD1 has been postulated to interfere with axonal transport in the motor neurons[66]. Antero and retrograde transport, carried out by kinesin and dynein motor proteins, are critical for the transport of essential components between the neuronal cell body and their synaptic termini. Studies have shown that mutant SOD1 can slow down both antero and retrograde transport in vitro as well as in vivo[67-70]. Disorganization of cytoskeletal components of the axon is also documented in human ALS patients. Moreover, mutant SOD1 has also been postulated to cause synaptic vesicle defects in susceptible motor neurons[71,72]

Mouse studies have demonstrated that toxic misfolded SOD1 can be secreted out of motor neurons by chromogranin-mediated exocytosis[73-75]. Chromogranins are the components of the secretory vesicles. Mutant SOD1 has been shown to bind to chromogranin and secreted out in the extracellular space. Extracellular mutant SOD1 can further activate surrounding glial cells, mainly astrocytes and microglia. This results in astrocytosis and microgliosis which can further lead to motor neuron death.

1.2.2 Non cell autonomous toxicity of mutant SOD1 in FALS pathogenesis

As motor neuron is the primary cell type affected in ALS, it was considered that the presence of mutant SOD1 within the motor neurons was the main cause of the motor neuron death. This notion was first challenged when mice expressing mutant SOD1 exclusively in motor neurons failed to show the signs of ALS[76,77]. Arguing that the
level of mutant SOD1 expression in the motor neurons of these mice was not high enough to cause the disease, another study showed that mice expressing higher levels of mutant SOD1 in motor neurons develop some signs of motor neuronopathy[78]. However, these mice also failed to develop the entire spectrum of ALS symptoms. ALS is not a cell-autonomous disease was further proved when the Cre-mediated reduction of mutant SOD1 exclusively from motor neurons resulted in extension of the median survival of ALS mice by only delaying the disease onset[30]. Mutant SOD1 reduction in motor neurons did not alter the disease progression, suggesting that the non-neuronal cells other than motor neurons play critical role in this phase of the disease. In corollary experiments, it was shown that chimeric mice with mutant SOD1 expressing-motor neurons, surrounded by wild type non-neuronal cells did not develop the symptoms of ALS[79]. However, ALS pathology was evident in mice with wild-type motor neurons surrounded by mutant SOD1 expressing non-neuronal cells, suggesting the critical role of non-neuronal cells in the final disease outcome. The concept of non-cell autonomous toxicity of mutant SOD1 was further corroborated with the use of transgenic reduction of mutant SOD1 from different non-neuronal cells.

1.2.2.1 Role of astrocytes in ALS pathogenesis

Astrocytes are star shaped glial cells present in brain as well as spinal cord. These are one of the most abundant cell types present in the CNS. Astrocytes play a crucial role
in maintaining structural as well as functional homeostasis in CNS[80]. Along with endothelial cells, astrocytes take active part in the formation of blood brain barrier (BBB) that shields the CNS from the peripheral components. Astrocytes have also been shown to provide support for growth and maintenance of neurons by secreting neurotrophic factors like BDNF, GDNF. Astrocytes are actively involved in maintaining synaptic transmissions. Astrocytes control the glutamate concentration at the synapses with the help of glutamate transporter, EAAT2 that uptakes the residual glutamate released from the presynaptic termini. Recent studies have also suggested that astrocyte function is crucial in the nervous system repair. Upon injury to nerve cells in brain or spinal cord, astrocytes become activated and form a glial scar by filling up the space at the expense of the damaged cells[66,80,81].

In ALS mouse models as well as human patients, astrocytes become activated resulting in astrogliosis. Astrogliosis is evident even before the actual disease onset, suggesting that astrocytes play an important role in ALS pathogenesis. The definitive contribution of astrocytes in the pathogenesis of ALS was proven by transgenic removal of mutant SOD1 from astrocytes in lox SOD1\textsuperscript{G37R} mice carrying GFAP-Cre recombinase[82]. Here, reduction of mutant SOD1 exclusively from astrocytes resulted in significant extension in the life span of ALS mice by delaying specifically the progression phase of the disease. On the other hand, it was also shown that the restricted expression of mutant SOD1 only in astrocytes did not result in the development of ALS-like symptoms in mice but only caused astrogliosis[83], suggesting that the presence of
mutant SOD1 in both neuronal as well as non-neuronal cell types is required for full culmination of the disease phenotype. However, in a reciprocal study, transplantation of mutant SOD1 expressing astrocyte precursors in spinal cords of wild-type animals resulted in motor neuron degeneration and ALS like symptoms[84]. Direct effects of ALS astrocytes on motor neurons have been extensively studied using in vitro coculture models[85-90]. Several studies have shown that primary or NPC derived mouse astrocytes, carrying mutant SOD1, are toxic to motor neurons in vitro. This motor neuron selective toxicity was also demonstrated when post-mortem spinal cord NPC derived astrocytes from familial ALS patients were cocultured with wild-type mouse motor neurons[87]. Importantly, it has been shown that the motor neuron toxicity of ALS astrocytes is not limited to the presence of mutant SOD1 as post-mortem spinal cord NPC derived astrocytes from sporadic ALS patients were equally toxic to the motor neurons in vitro as astrocytes from fALS patients. Moreover, shRNA mediated knockdown of SOD1 in both familial as well as sporadic ALS astrocytes rescued the motor neuron death, in coculture, suggesting the disparate pathogenic mechanisms in familial and sporadic astrocytes might converge at SOD1. Although human post mortem NPC derived astrocytes provided an excellent model system to study the human disease in petri dish, this model system has two main drawbacks: limited availability of the starting material and representation of only end-stage of the disease. This criticism was resolved when induced astrocytes (i-astrocytes), derived by direct conversion of fibroblasts from familial as well as sporadic ALS patients, were shown to be equally toxic to the motor neurons in
in vitro coculture systems[90]. These studies have provided a very strong consensus about the role of astrocytes in the non-cell autonomous toxicity towards the motor neurons in ALS.

Although the exact mechanisms by which ALS astrocytes kill motor neurons are still under investigation, there are two schools of thought proposed: a) reduction in the neurotrophic support and b) secretion of neurotoxic factors[91-94]. Reduced expression of neurotrophic factors like BDNF, GDNF, VEGF, IGF-1 in ALS astrocytes has been reported earlier. Also, replenishment of these factors either by exogenous delivery or viral mediated overexpression has been shown to rescue the motor neuron death and ameliorate the disease phenotype in vitro as well as in vivo[95-103]. Considering the role of astrocytes in maintaining the synaptic levels of glutamate, reduced levels of glial glutamate transported EAAT2 has been correlated with the increased motor neuron death by excitotoxicity[48,49,104]. Small molecule mediated activation or viral mediated overexpression of EAAT2 within ALS astrocytes has been shown to provide some neuroprotection in ALS mouse models[105-108]. There is an increasing amount of evidence suggesting that secretion of neurotoxic molecules by ALS astrocytes is one of the mechanisms of astrocyte-mediated motor neuron toxicity. This idea was confirmed by studies involving the conditioned media experiments where culture medium collected from mouse ALS astrocytes was shown to be toxic to the motor neurons in vitro[88,109]. Similar phenomenon was also reported for the post mortem spinal cord derived astrocytes from human ALS patients[87]. Although exact identity of these soluble toxic factors is
not confirmed, it is postulated that it includes reactive oxygen species and several inflammatory molecules like prostaglandin 2, interferon-\(\gamma\). Nevertheless, the toxic factor released from astrocyte have been shown to induce the motor neuron toxicity by activating pro-apoptotic cascades involving Bax, p53, NF-\(\kappa\)B and even via activation of voltage-gated sodium channels and nitoxidative stress. Recent studies have proposed that the ALS astrocytes possess a capacity of inducing necroptosis in surrounding motor neurons in vitro which finally leads to motor neuron death[89].

Even though mutant SOD1 expressing astrocytes have the inherent ability to kill the motor neurons, it is considered that their neurotoxic potential increases over time. This change can be attributed to misdialogue between motor neurons and astrocytes as well as astrocytes and microglia during the disease course. Several studies have shown specific gene expression changes within astrocytes throughout the disease progression that correlate well with their neurotoxic activity[110,111]. It is also suggested that a subpopulation of astrocytes with higher neurotoxic potential may be responsible for the accelerated progression of the disease[112]. Such phenotypically aberrant astrocytes were isolated from spinal cords of SOD1\(^{G93A}\) rats at the symptomatic stage of the disease. The aberrant astrocytes differ from the canonical astrocytes in their marked proliferative capacity and lack of replicative senescence. Also, the aberrant astrocytes were shown to secrete soluble factors that are almost 10 fold more toxic than their neonatal counterparts. These studies suggest that appearance of such astrocytic population with enhanced proliferative and neurotoxic capacity may be responsible for the fast progression of ALS.
With the unprecedented role in motor neuron toxicity and the profound effect on driving the disease progression, ALS astrocytes can be considered as one of the most plausible therapeutic strategy in slowing the disease progression and ameliorating the disease phenotype in ALS.

1.2.2.2 Role of Microglia in ALS pathogenesis

Microglia are the primary immune cells of the nervous system. They serve as the first line of defense against pathogens in CNS. Their regular function involves constant surveillance of the central nervous system and execution of an appropriate response against different environmental stimuli like pathogens, damaged cells and cell debris to maintain homeostasis. CNS is an immune-privileged organ, thus microglia are extremely sensitive to any small pathological changes in the nervous tissue. Presence of such stimuli results in microglial activation, also called as microgliosis. Activated microglia are involved in clearing out pathogens, infected cells and even debris via phagocytosis from the CNS. Activated microglia also act as antigen presenting cells for infiltrating T lymphocytes. Microglia, thus play a crucial role in immune response and maintaining homeostasis in CNS.

Neuroinflammation is considered to be one of the most important pathogenic mechanisms for the non-cell autonomous toxicity of motor neurons in ALS[113-117]. It has been demonstrated that microglial activation is the major cause of this
neuroinflammation in ALS. Microglial activation has been detected in spinal cords of ALS mouse models as well as ALS patients. It has been shown that the microgliosis initiates even before the disease onset in ALS mice and the number of activated microglia in the ventral horn of spinal cords increases with the disease progression. This suggested that microglia may have an active role in the disease progression of ALS[30,118-121]. This notion was supported by an important study involving transgenic reduction of mutant SOD1 specifically from microglia in LoxSOD1G37R mice[30]. Reduction of mutant SOD1 from microglia in these mice resulted in significant delay in the disease progression and subsequent extension in the median survival. The involvement of mutant SOD1 carrying microglia in pathogenesis of ALS was further corroborated when the resident microglia from ALS mice were ablated by crossing these mice with PU.1 knockout mice (unable to develop myeloid and lymphoid cells)[118]. Replenishing the microglial population in the PU.1 crossed ALS mice by bone marrow transplantation from wild-type mice resulted in slower motor neuron death, prolonged disease progression and extended median survival. Although microglia are involved in the disease pathogenesis, similar to motor neurons and astrocytes, expression of mutant SOD1 in exclusively in microglia did not lead to motor neuron symptoms and ALS like phenotype in mice, suggesting that microglia mediated cytotoxicity is not the root cause of the disease.

The inherent sensitivity to pathogenic stimuli has been considered as the major attribute to the microglial activation in ALS. Microglial activation is not always a bad
sign. There are two types of activated microglia that can be present in the CNS. Classically activated microglia, also called as M1 microglia are cytotoxic in nature due to the secretion of reactive oxygen species like H$_2$O$_2$, nitric oxide as well as pro-inflammatory cytokines and interleukins like TNF-α, Il1-β. On the other hand, M2 microglia are alternatively activated microglia that are mainly involved in the restoration and repair process by producing high levels of anti-inflammatory cytokines like IL-4 and neurotrophic factors. An intricate balance between these two types of activated microglia results in the final outcome of microglial response in CNS. In vitro studies have shown that treatment of ALS mouse derived microglia with mutant SOD1 or misfolded wild-type SOD1 can polarize them towards classically active M1 phenotype[121]. Classical activation has been shown to cause increased production of superoxide radicals, nitric oxide, TNF-α from M1 microglia and subsequent killing of motor neurons in coculture system[119]. As mentioned earlier, microglial activation is a hallmark of ALS pathogenesis. As the disease progresses, there is an increase in the levels of M1 markers in the spinal cords of ALS mice, suggesting that ALS microglia adopt the cytotoxic M1 phenotype which results in the motor neuron death and thus contributes to disease progression[119,120]. In vivo studies have also shown that ALS microglia do not have the M1 phenotype all throughout the disease process. At early progression phase of the disease, ALS microglia show elevated expression of M2 markers which changes to elevated expression of M1 markers at the late progression phase. This suggests that there is a switch between the two microglial phenotypes that might be time dependent. In vitro studies have also shown that treatment of ALS microglia with M2 inducing agents like Il-
4 or IGF-1 results in downregulation of M1 markers and enhanced motor neuron survival in co-cultures[122]. These studies suggest that ALS microglia do possess the plasticity to shift between the neurotoxic and neuroprotective phenotypes based on the extracellular signals present in the microenvironment. In vivo studies have shown that treatment of ALS mice with minocycline results in reduced microglial activation and enhanced median survival of ~3 weeks in SOD1\(^{G93A}\) and SOD1\(^{G37R}\) mice[123-126]. Viral vector mediated delivery of IGF-1 has also shown to reduce the microglial activation and enhance the survival by ~2 weeks in SOD1\(^{G93A}\) ALS mice[127]. Although these and several other anti-inflammatory compounds have been shown to be protective in ALS mouse studies, most of these treatments have failed in human clinical trials[128,129]. Recent study from the Kaspar laboratory has shown that the microglia-mediated motor neuron death is dependent on the NF-κB pathway activation[119]. NF-κB is the main signaling pathway that controls the expression of pro-inflammatory cytokine production. Both in vitro and in vivo studies have shown that downregulation of NF-κB pathway in ALS microglia results in the reduced expression of downstream proinflammatory cytokines like TNF-α, IL-10. NF-κB downregulation also results in the reduced motor neuron death and significant extension in the median survival of ALS mice. The definitive role of NF-κB pathway in the microglia mediated motor neuron death was shown by enhanced capacity of wild type microglia with constitutively active NF-κB pathway to kill the motor neurons in coculture. As NF-κB is the most upstream activator of most of the pro-inflammatory signaling cascades, development of therapeutic
approaches targeting this central pathway may provide the best possible outcome in reducing the microglia mediated non-cell autonomous toxicity in ALS pathogenesis.

### 1.2.2.3 Involvement of other non-neuronal cell types

Cell types other than astrocytes and microglia are also shown to be involved in the pathogenesis of ALS. Schwann cells play a crucial role in the support of neurons and their myelination in the peripheral nervous system. Mutant SOD1 reduction from Schwann cells using Cre-mediated excision actually resulted in acceleration of the disease process in ALS mice [130,131]. Oligodendrocytes that are involved in the support and myelination of CNS neurons have also been investigated for their contribution to ALS disease pathogenesis[132,133]. Several studies, focused on the muscle involvement in ALS, have suggested that muscle is not the primary target for the therapy development in ALS. Downregulation of mutant SOD1 from muscles had shown no improvement in the median survival of ALS mice[134].

### 1.2.3 Involvement of wild-type SOD1 in SALS pathogenesis

With mutated SOD1 considered to be the main culprit of familial ALS, several studies have proposed the role of wild-type SOD1 in the pathogenesis of sporadic ALS[87,135-137]. Mutations in SOD1 gene result in changes in the conformation of the protein which gets misfolded and further aggregated. Almost all the mouse models of
SOD1-linked ALS show presence of misfolded SOD1 throughout the spinal cord, detected by variety of antibodies targeted against misfolded SOD1[138-140]. Misfolded SOD1 has been detected in the spinal cord of familial ALS patients suggesting that observations in mouse study complement nicely with the human ALS condition[141]. Similar to mutant SOD1, wild-type SOD1 has also been shown to undergo the same conformational changes as a result of oxidation, forming misfolded wild-type SOD1 protein with higher propensity of aggregation. Interestingly, several studies have shown that the misfolded conformation specific antibodies could detect the presence of misfolded and aggregated SOD1 in the post mortem spinal cords of sporadic ALS patients[140,142-146]. In vitro study involving the co-culture of post-mortem spinal cord NPC derived astrocytes with mouse motor neurons have shown that reduction of wild-type SOD1 in a subset of sporadic astrocytes rescues the motor neuron death, suggesting shared mechanism of pathogenesis between FALS and SALS via SOD1[87,146]. Like mutant SOD1, misfolded wild-type SOD1 has been proposed to exert its adverse effects within the motor neurons by increase in ER stress, oxidative damage, damage to the axonal transport. Misfolded SOD1 has been shown to be secreted out in the extracellular environment where it can activate astrocytes and microglia. This can result in increased expression and secretion of proinflammatory cytokines from these cells that can further kill motor neurons in the vicinity[57,142,147-149]. Thus, reduction of misfolded SOD1, mutant or wild-type, seems to be the best therapeutic intervention for the better disease outcome of familial as well as sporadic ALS.
1.3 Current therapeutic inventions in ALS

With the plethora of pathogenic mechanisms proposed for mutant SOD1, various therapeutic strategies have been implemented to alleviate the neurotoxicity in different ALS mouse models. These strategies are primarily based either on targeting the different signaling cascades that are postulated as effector pathways of SOD1-mediated toxicity or reducing the main upstream player of the neurotoxicity, mutant SOD1. The current therapeutic strategies can be broadly divided into 3 major categories on the basis of mode of intervention as a) small molecule based therapy, b) gene therapy and c) cell based therapy.

1.3.1 Small molecule based therapy

As the name suggests, small molecule based therapies comprise of administration of a low molecular weight organic compound that helps modulate a biological process. These small molecules range from chemical drug compounds to small biological components like proteins (growth factor, antibodies). Once administered in the patients, these compounds can get absorbed by the cells and modulate the intracellular signaling pathways or act extracellularly by binding to the cell membrane receptors. With the knowledge of a variety of pathogenic mechanisms involved in ALS, large scale, high-throughput screens of vast libraries of small molecule compounds have been performed to obtain the most effective therapeutic candidates. These candidate compounds target the
pathways that are most anticipated to influence the pathogenesis of ALS[129,150].

As the misfolding and aggregation is the first and foremost result of mutations in SOD1, the majority of the therapeutic treatments have been focused on removal of aggregated mutant SOD1 protein by upregulation of protein degradation pathways. Small molecules that enhance heat shock protein expression (molecular chaperons that play crucial role in protein folding) have been shown to delay the disease progression and extend the survival of SOD1\textsuperscript{G93A} mice by 7-10 days[151,152]. Lithium has been shown as one of the promising candidates to reduce the mutant SOD1 aggregates in mouse models of ALS conferring delay in disease onset, progression and ~38 days extension in the median survival[153]. However, lithium has also shown to be a failure in human clinical trials due to safety and efficacy issues[154-156].

Higher vulnerability of motor neurons to glutamate-induced excitotoxicity has attracted quite an attention for therapy development. Riluzole is the only FDA approved drug for the treatment of ALS. Riluzole enhances the glutamate transporter function, reduces the extracellular glutamate levels and reduces the calcium influx by blocking sodium and calcium channels[157,158]. Although Riluzole is in clinics, it has very modest effect on the survival of ALS patient[12-16,51]. Alternatives to Riluzole like Ceftriaxone and Memantine are now being tested for their effect on the glutamatergic excitotoxicity. Ceftriaxone, a β-lactam antibiotic, has been shown to reduce the glutamate excitotoxicity and prolong the survival in SOD1\textsuperscript{G93A} mice by ~10 days[159]. Memantine has also been shown to prolong the survival of ALS mice but without any motor
benefits[160-162]. Unfortunately, Ceftriaxone failed to provide any functional and survival benefits in human clinical trials for ALS[163] while memantine showed no efficacy in ALS patients with the survival benefits yet to be determined[164]. With their anti-excitotoxic, anti-inflammatory and neuroprotective nature, HDAC inhibitors like Valproic acid and sodium phenyl butyrate have also been shown to extend the survival of ALS mice when administered before or after symptoms[165-167].

Owing to the important role in cellular energy productions, calcium homeostasis and intrinsic apoptosis pathway, mitochondria have shown to be involved in the mutant SOD1 mediated damages in ALS[168-170]. Dexpramipexole, a compound that enhances ATP production, reduces oxidative stress and apoptosis, has been shown to marginally improve survival in SOD1<sup>G93A</sup> mice by 7 days[171]. Mitochondria control the process of apoptosis by release of cytochrome C from the inter-membrane space to the cytoplasm which further activates caspases. This release is controlled by mitochondrial permeability transition pore (mPTP). Compounds like olesoxime, notrityline that act on mPTP have been shown to extend the survival of ALS mice[129]. However, most of them failed completely in human clinical trials.

As transgenic studies involving the deletion of proteins involved in apoptosis proved to have beneficial effects on survival, small compounds that regulate the apoptotic pathways have also been implemented in ALS therapy development[129,172]. Melatonin which is an anti-apoptotic agent as well as an antioxidant has been shown to delay the disease onset and improve the survival of ALS mice by 10 days. Erythropoetin,
another inhibitor of apoptosis, prevents neuronal cell death. A variety of broad spectrum Caspase inhibitors have also been shown to delay the disease onset and prolong survival in ALS mice. However, there implementation in human is limited due to their extreme toxicity.

Neuroinflammation plays an important role in the pathogenesis of ALS. Activated non-neuronal cells, especially microglia and astrocytes, elevate the expression and release of proinflammatory cytokines like nitric oxide, TNF-α, IL-10 which are toxic to the motor neurons[118,120,173-176]. Based on this evidence, several anti-inflammatory compounds have been tested in ALS mice for their effect on the disease outcome. Minocyclin, an inhibitor of microglial activation, was effective in delaying the disease onset and prolonging the survival in ALS mice[125,126,177,178]. Copaxone, another anti-inflammatory drug, showed beneficial effects in the mouse model of ALS. Administration of Thalidomide and its analogue Lenolidomide to SOD1G93A mice extended the survival of mice by inhibiting the expression of TNF-α and other cytokines by destabilizing their mRNAs[179,180]. However, despite being beneficial in the mouse studies, all of these anti-inflammatory drugs failed to provide any therapeutic benefits in the human clinical trials[129,181].

Passive immunization with antibodies specifically targeted towards the misfolded mutant SOD1 has also been shown to have beneficial effects by clearing out the burden of the toxic species from the extracellular environment. It was shown that the administration of D3H5 and B8H10 antibodies via intra-cerebroventricular route resulted...
in the clearance of mutant SOD1 in spinal cord of SOD1\textsuperscript{G93A} mice and prolonged the survival by 6 days\cite{182}. Immunization with the SEDI peptide which comprises of an epitope only exposed in misfolded SOD1 has also shown to improve the motor performance, delay disease onset and extend the survival of SOD1\textsuperscript{G37R} mice by 40 days\cite{183}. Based on this study, unique antibodies targeting specific epitopes on the misfolded SOD1, sparing the wild-type SOD1, are being generated and tested in mouse studies since then. Active immunization with SOD1 protein itself has also shown to exert beneficial effects in mutant SOD1 transgenic mice\cite{184,185}. Administration of the misfolded mutant SOD1 protein itself or wild-type protein with high propensity to misfold, has been shown to elicit an immune response followed by the clearance of the mutant protein in SOD1 ALS mice\cite{184}.

Even though small molecule based therapies are the most preferred therapies for ALS patients for the ease of administration, they suffer from the limitations of dosage levels, distribution and bioavailability. These 3 parameters are interconnected and all of them need to be optimized to achieve the maximum beneficial effect. Dosage level of the agent needs to be optimized to obtain the effective therapeutic dose, without exerting any other toxic side-effects. This effective therapeutic dose is dependent on the proper distribution of the agent to its target site. Route of administration influences the biodistribution of the therapeutic agent. For CNS disorders like ALS, the therapeutic agent needs to reach the affected areas of motor cortex, brainstem and mainly spinal cord.
Oral or intravenous administration of the agent does not result in its widespread distribution in CNS due to the presence of blood brain barrier (BBB). BBB restricts almost 98% of small molecules from reaching the CNS. This can be overcome by changing the route of administration to intra-cerebroventricular or intrathecal. As only a subset of cells in CNS can readily absorb these small molecules, even these routes of administrations do not guarantee widespread targeting of all the cell types in CNS. Bioavailability, the systemically available fraction of the compound, is directly proportional to its distribution. Hence, the therapeutically effective concentration of the drug in CNS could only be achieved by increasing the administered dose, which itself is a limiting factor when it comes to the human patients. Due to these and other limitations on the small molecule administration, other avenues of targeted therapy like gene therapy and cell-based therapies are being considered as more viable options for ALS.

1.3.2 Gene therapy

As per its conventional definition, gene therapy involves introduction of a DNA molecule in the patient cells to replace the non-functional, mutated gene. However, gene therapy can also be utilized to provide the regulatory elements to control the expression of specific target genes as well as to introduce the transgenes themselves that can in turn regulate the downstream signaling cascades. To achieve this, different types of DNA molecules are delivered to the target cell by packaging in a vector. Gene therapy approach is mainly divided into two broad categories: non-viral vector mediated gene
therapy and viral-vector mediated gene therapy. Both these vector systems have been utilized extensively in the development of ALS therapy.

1.3.2.1 Non-viral vector mediated gene therapy

As the name suggests, non-viral vector mediated gene therapy comprises of delivery of the therapeutic agent without the help of viral expression systems. Here, the therapeutic molecule is delivered either by itself or with the help of chemical formulations. Non-viral vector mediated gene therapy mainly includes delivery of oligonucleotides to the target cells. Oligonucleotides used in non-viral mediated gene therapy are involved in the regulation of specific gene expression in the target cell via RNA interference pathway[186]. These include small interfering RNAs (siRNA), micro RNAs (miRNA) and antisense oligonucleotides. siRNA and miRNA 19-25 nucleotide long, synthetically generated RNA molecules that specifically bind to the mRNA of the specific gene in the target cells. The binding of these molecules to the mRNA can lead to either degradation of the transcript with the help of RNA induced silencing complex (RISC) or blockage of translation. Antisense oligonucleotides are the synthetically generated DNA molecules that can exert the same effects of mRNA degradation via RNase H-mediated pathway as well as translational block. These molecules are delivered to the cells, tissues or organs either as naked oligonucleotides or encapsulated in chemical formulation. Due to the large size and negative charge, entry of the naked
oligonucleotides into the target cell is dependent on endocytic pathway[187]. This limits the bioavailability and biodistribution of these oligonucleotides. To facilitate this process, oligonucleotides are administered encapsulated in liposomal formulations. Liposomes are artificially synthesized spherical vesicles made up of lamellar phase lipid bilayer. Due to the same properties as the cell membrane, liposomes can fuse with the cell membrane and empty their contents inside the cells, thus avoiding endosome-lysosome pathway. Administration of naked as well as encapsulated oligonucleotides have been widely studied in targeting specific pathways as treatment for various diseases including cancer, cardiovascular diseases, infectious diseases and neurodegenerative diseases[188-190].

Administration of siRNA oligonucleotides targeting human mutant SOD1, has a beneficial effect on the disease outcome in ALS rodent models[191,192]. Mutant SOD1 can also be silenced by utilizing antisense oligonucleotides which interfere with the translation of the mutant protein. A study involving the intraventricular delivery of antisense oligonucleotides against mutant SOD1 has shown successful reduction in mutant SOD1 expression in brain as well as lumbar regions of the spinal cord[193]. It also resulted in the significant delay in the disease progression but very modest effect on the median survival by increasing the lifespan of SOD1^{G93A} rats by 10 days. Due to the simplicity and safety of this treatment in mice, antisense oligonucleotide therapy is currently being tested in human clinical trials. Undertaken by ISIS pharmaceuticals, Phase 1 clinical trial for the same antisense oligonucleotide against human SOD1, administered intrathecally in FALS patients, has shown that the antisense treatment is
safe and well tolerated[190], thus indicating the oligonucleotide treatment can be a feasible treatment for ALS in near future. However, a significant delay in disease progression and median survival extension has to be achieved by ISIS antisense oligonucleotide administration to be implemented as a therapy.

With the advantages of efficient downregulation and easy administration, oligonucleotide based therapies still suffer from a number of limitations. As discussed further in Chapter 3, main disadvantages of oligonucleotide therapy include short stability and bioavailability, non-specificity for the target cells and toxicity. All of these factors affect the long-term effects of oligonucleotide based downregulation of the target gene. To overcome these shortcomings, viral vector based delivery methods are preferred.

1.3.2.2 Viral vector mediated gene therapy

Viruses have an inherent property to infect the cells and transfer their genetic material inside the infected cells. To be used as a viral vector for gene therapy, viruses are modified to remove the parental pathogenic elements, for example, deletion of part of the viral genome required for viral replication and packaging. These parts can then be replaced with the therapeutic genetic material that needs to be delivered into the diseased cells. Viral production is achieved with the help of exogenous helper plasmids to produce the transgene containing virions which are utilized as gene therapy vectors. Different types of viruses have been utilized so far for the gene therapy. The choice of the viral
vector depends upon the tropism of the virus towards the particular cell type involved in disease process and its transduction efficiency. Here, we will discuss two of the most commonly used viral vectors for gene therapy studies, Lentiviruses and Adeno Associated Viruses.

Lentiviruses (LV) belong to the Retroviridic family of viruses. These are RNA viruses which carry a single-stranded RNA genome which, after infection, gets transcribed to DNA with the help of viral reverse transcriptase. Lentiviruses are one of the most commonly used vector systems for the delivery of short hairpin RNAs as well as complete transgenes due to their following advantages\[194,195\]: a) Lentiviruses can infect dividing as well as non-diving cells. b) They have a large cloning capacity (~10 kb). c) Lentiviral transduction results in integration of the transgene in the host genome, thus confirming a stable transgene expression. Minimal lentiviral vectors based on Human Immunodeficiency Virus (HIV), Simian Immunodeficiency Virus (SIV), Equine Infectious Anemia Virus (EIAV) and Feline Immunodeficiency virus (FIV) have been generated by deletion of the accessory and regulatory genes from the viral genome. Lentiviral vectors based on HIV and EIAV with VSV-G envelope are the most commonly used vector systems for gene therapy of neurological disorders. These vectors have higher tropism towards neuronal cells and result in higher neuronal and glial cell transduction.\[195\]. Intramuscular, intraspinal administration of lentiviral vectors have successfully resulted in amelioration of several neurological disorders like Parkinson’s disease, Huntington’s disease and ALS \[195,196\].
Adeno Associated Viruses (AAV) belongs to the Parvoviridie family of viruses. These are small, replication defective, non-enveloped viruses. AAV are single stranded DNA viruses that can infect both dividing and non-dividing cells. In the gene therapy AAV vector, viral rep and cap genes are replaced by the expression cassette of the desired transgene which is flanked by 2 inverted terminal repeats (ITRs)[197,198]. After infection, the single stranded AAV genome is converted into double stranded AAV DNA which remains in the episomal form due to loss of viral rep-cap genes. Single stranded AAV vectors have cloning capacity of only 4.8 kb and hence are not suitable for delivering larger genes. As the traditional AAV can package only single-stranded DNA and hence is dependent upon the second strand synthesis, specialized AAV vector have been constructed for quicker and more efficient transgene expression. Self-complementary AAV (scAAV) vectors package two shorter strands that are complementary to each other, thus avoiding the second-strand synthesis.[199-201]

However, this strategy results in reducing the cloning capacity of the scAAV by half to 2.4 kb. Despite these limitations, Adeno Associated Viruses are currently the most desired gene therapy vectors because of the following advantages: AAV vectors can infect dividing as well non-dividing cells with the equal efficiency. Random integration of AAV DNA into host genome occurs at very low frequency. Most importantly, AAV vectors are relatively non-pathogenic. They have very low immunogenicity with restricted capacity to induce only neutralizing antibody response.

Eleven different serotypes of AAV have been identified so far[197,198,202-204].
Each serotype differs in the structure of the capsid protein which also determines the tropism of each serotype. AAV2, AAV4, AAV6, AAV8 and AAV9 are extensively used in gene therapy of neuronal disorders.

Mutant SOD1 being the upstream regulator of all the pathogenic mechanisms leading to motor neuron toxicity in ALS, presents as the most effective target for therapeutic interventions[66,170]. Transgenic studies have already proven the beneficial effects of mutant SOD1 removal from various cell types [30,82], encouraging the necessity of therapeutic approaches for the removal of mutant SOD1 from ALS patients. These strategies are mainly focused on downregulating the expression of mutant SOD1 by utilizing RNA interference. First evidence of beneficial effects of SOD1 downregulation by gene therapy was obtained by intramuscular administration of a lentiviral vector carrying shRNA against SOD1 in SOD1\textsuperscript{G93A} mice[205,206]. This treatment resulted in reduced mutant SOD1 expression as well as enhanced survival of motor neurons in brainstem and spinal cord of the injected mice. Intramuscular injection of lentiviral SOD1 shRNA delayed disease onset and extended the median survival significantly. Here, the aim was to target certain motor neuron population innervating the injected muscles via retrograde axonal transport of the viral particles. A subset of studies utilizing the intramuscular route of administration has shown beneficial improvement in the disease outcome in ALS mice. However, it is also observed that this treatment has limited success due to the correction of SOD1 mediated toxicity in only one cell type[207,208]. As the intramuscular injections only target the motor neurons, mutant
SOD1 expression in other non-neuronal cells still drives the disease pathogenesis to unfavorable outcome. To avoid this problem, studies involving administration of the viral particle directly in the CNS have been tried. Intraspinal injections of viral particles carrying SOD1 shRNA have been shown to have modest effect on the disease onset and progression but no change in the survival of SOD1\textsuperscript{G93A} mice ALS mice[209].

The other avenue of gene therapy, introduction of a therapeutic gene product, has also been extensively studied in the field of ALS. The first successful ALS gene therapy was achieved as the result of virus mediated introduction of neurotrophic factor IGF-1 in ALS mice. Intramuscular administration of AAV encoding IGF-1 resulted in the retrograde delivery of the neurotrophic factor to the motor neurons in the spinal cord of ALS mice[102]. AAV IGF-1 treatment resulted in improved motor performance, delayed the disease onset, slowed disease progression and significantly extended the survival of SOD1\textsuperscript{G93A} mice by ~37 days. Interestingly, intramuscular delivery of IGF-1 also resulted in reduced astrogliosis. Subsequent studies with intramuscular delivery of IGF-1 also demonstrated beneficial effects in ALS mouse models[95]. Intraspinal delivery of IGF-1 also resulted in efficient motor neuron transduction and elevated expression of IGF-1 which led to the enhanced motor neuron survival in ALS mice. Improvement of motor function, delay in the disease onset and 10 days extension of median survival were observed as a result of intraspinal IGF-1 delivery in SOD1\textsuperscript{G93A} mice,[127,210,211]. Since these promising findings, various genes encoding neurotrophic factors, inflammation inhibitors, apoptosis inhibitors have been introduced in ALS mice by viral mediated
delivery systems and have shown to improve the disease outcome to varying levels with maximum survival of less than 30 days achieved in the fast progressing SOD1^{G93A} mice\[212,213\].

Although the viral mediated delivery has some obvious advantages over the small molecule based therapies, this route still suffers from limitations of its own. The major disadvantage of viral vector based therapies is the delivery to the central nervous system. Most of the currently available viruses lack the ability to cross the blood brain barrier and achieve widespread transduction of CNS cells. In the previous studies, affected motor neurons could be targeted only via the retrograde delivery of the virus from muscle. This limits the delivery of the therapeutic agent to only certain motor neurons. Considering the non-cell autonomous nature of the disease, it is extremely important to simultaneously target multiple cell types for better prognosis of the disease. Even the intraventricular, intrathecal or intraparenchymal routes of vector delivery do not result in the widespread transduction of CNS due to selective tropism of different viruses towards specific cell types. Hence, to achieve the most effective gene therapy, it is imperative to find the vector system with the maximum accessibility to the CNS and show higher tropism for different cells types within the CNS.

Recent studies from our lab as well as other groups have shown that Adeno-Associated Virus Serotype 9 (AAV9) has the unique capacity of crossing the blood brain barrier and target the cells in CNS\[214\]. It has been shown unequivocally that the intravenous administration of AAV9 in mice results in the widespread transduction of
different cell types in the CNS. AAV9 possesses a selective tropism for the cells transduced in the mouse CNS which depends on the time of delivery. Neonatal injection of AAV9, via facial vein, results in transduction of the majority of motor neurons (>70%) in the spinal cord as well as brain. However, intravenous injection of AAV9 via tail vein in adult mice results in a major shift in the viral tropism, transducing a majority of astrocytes (~70%) and some motor neurons (<10%) throughout the CNS. Another study, however, shows that intravenous delivery of AAV9 results in significant motor neuron transduction (~28%) in adult mice and even in adult cats, along with some astrocyte transduction.[215]. Nonetheless, it was also shown that a single injection of AAV9 can result in the persistent expression of the transgene in the transduced cells for up to 5 months. To translate the AAV9 approach to humans, AAV9 transduction has been studied in non-human primates (NHP)[216]. NHP studies revealed that intravenous administration of AAV9 at time points from birth to 3 year old adult Cynomolgus monkeys results in widespread transduction of motor neurons and some glial cells. This data suggests that AAV9 tropism in primates is different from rodents where unlike rodents, in NHP both motor neurons and primary glial cells can be targeted even at the later stages of adult life in NHP. Systemic delivery of AAV9 in primates also resulted in efficient transduction of tissues outside the central nervous system like skeletal muscles and multiple organs. As the systemic transduction may not be helpful in all paradigms, intraventricular, intrathecal as well as intracisternal delivery of AAV9 has been studied. Intrathecal and intracisternal delivery of AAV9 in monkey as well as pig models resulted in similar transduction patterns in brain and spinal cord [203,216-223], corroborating the
usefulness of these delivery routes in concentrating the viral bioavailability in the target tissue and minimizing the effective dose of administration.

Several studies have been performed utilizing the AAV9 mediated delivery as a therapeutic approach[202,222]. One milestone was the complete rescue of survival in mouse models of spinal muscular atrophy (SMA) following the neonatal delivery of AAV9-SMN[224,225]. Subsequent GLP-toxicology studies have shown that AAV9 administration is safe and well tolerated in mice. The efficient motor neuron transduction in non-human primate studies also corroborated the efficacy of this treatment approach for human cases[216]. Currently, intravascular delivery of AAV9-SMN is in phase 1 human clinical trials. This successful transition of AAV9 mediated delivery approach from benchside to bedside has opened new avenues for the AAV9 mediated therapeutic strategies in other CNS disorders.

As AAV9 not only targets motor neurons within the CNS but can also transduce non-neuronal cells efficiently, it can be considered better delivery vehicle for disorders like ALS. Also, persistent expression of the transgene in the AAV9 transduced cells suggests that a single injection of AAV9 is sufficient to last for the whole disease course. Most of the other viruses suffer from the challenge of naturally occurring antibodies which results in the faster clearance of virus from the system, which in turn affects its bioavailability. Much lower titers of naturally occurring antibodies against AAV9 have been documented in humans, making it the most desirable gene therapy vector[226-228]. In Chapter 2, we determine the efficacy and feasibility of AAV9 SOD1 shRNA mediated
downregulation of mutant SOD1 in two different mouse models of ALS while in Chapter 3, we demonstrate successful design of the clinical AAV SOD1 shRNA vector that can be used in the human clinical trials of ALS.

1.3.3 Cell mediated therapy

Stem cell therapy is a relatively new and exciting field of therapeutic intervention in the treatment of neurodegenerative disorders[229]. Here, the crucial property of stem cells to differentiate into the required cell type, based on the extracellular microenvironment, is being used to modify disease pathogenesis, provide nutritional support to neurons and even replace the damaged cells. Different types of stem cells can be utilized for the therapeutic strategy[230]. The selection is based on the ability of the cells to survive, migrate, engraft and then differentiate into the required cell types. 4 different types of stem cells available to use are embryonic stem cells, mesenchynal stem cells, neural stem cells and induced pluripotent stem cells. These stem cells differ from each other in their plasticity and ability to differentiate into one or more types of mature cells. Use of the particular type of stem cells for the clinically translatable therapy thus depends upon the availability of the cells and propensity of the particular stem cell to differentiate into target cells. Bone marrow derived stem cells are considered to be the most favorable source of stem cell for therapy development. Success of the stem cell therapy is also based on the route of administration of these stem cells and capacity of cell migration to the target site. For CNS disorders, intravenous, intrathecal as well as
intraparenchymal routes of administration have been previously tested\cite{230}. For ALS, intrathecal and intravenous administration are considered as the effective routes for delivery of stem cells as both of them are minimally invasive and are capable of achieving widespread distribution in the CNS. Several \textit{in vivo} studies in mouse models of ALS have shown the beneficial effects of bone-marrow derived stem cells as well as spinal cord derived neural stem cells\cite{231}. Multiple clinical trials involving the delivery of autologous bone marrow derived stem cells, hematopoietic stem cells as well as spinal cord- neural stem cells are in Phase I/II clinical trials in US as well as other countries\cite{231,232}.

1.4 \textbf{Crosstalk between neuronal and non-neuronal cell types in ALS}

Unlike other neurodegenerative diseases, in ALS, motor neurons do not die alone but require the participation of surrounding non-neuronal cells in the motor neuron toxicity\cite{66,170}. This is apparent from studies where only ubiquitous expression of mutant SOD1 resulted in development of the complete spectrum of ALS symptoms in mice while over-expression of mutant SOD1 exclusively in individual cell types like motor neurons, astrocytes and microglia did not result in ALS\cite{28,30,82}. Interestingly, reduction of mutant SOD1 from individual cell types improves the final disease outcome in mice by extending the median survival \cite{233} where different cell types affect different phases of the disease. Removal of mutant SOD1 from motor neurons mainly delays the disease onset while removal of mutant SOD1 from astrocytes or microglia only delays
the disease progression. Recent studies have shown that the presence of mutant SOD1 not only causes the intrinsic changes within individual cell types but also affects the crosstalk between each other through cell surface ligand-receptors as well as secretory molecules[110,111]. The final outcome of these interactions can be context-dependent neuroprotection or neurotoxicity.

1.4.1 Motor neuron- microglial crosstalk

Motor neurons can communicate with microglia directly via ligand receptor interactions. Two ligand proteins namely fractalkine and CD200, expressed by motor neurons, have been shown to be neuroprotective[233]. Fractalkine on motor neurons binds to its receptor CX3CR1 on microglial surface which leads to neuroprotective signaling in microglia. Deficiency of CXCR1 on microglia has been shown to cause neurotoxicity in vivo. Also, transgenic removal of this receptor from ALS mice has been shown to enhance motor neuron toxicity and exacerbate the disease outcome[234]. Recent studies on the correlation of the presence of different CX3CR1 variants and the progression rate of disease in sporadic and familial ALS patients revealed that CX3CR1 could be a disease modifying gene in ALS[235]. A neuronal glycoprotein, CD200 has also been shown to bind to the CD200R receptor on myeloid cells, including microglia, to confer neuroprotection in vivo[236-238].

Along with the generic neuroprotective mechanisms, ALS motor neurons also
have the ability to trigger neurotoxic response from microglia, owing to their ability to secrete mutant or misfolded SOD1. Chromogranins are one of the components of neurosecretory vesicles that play an important role in folding and packaging of the protein cargo in the vesicles[73-75]. Chromogranins have been shown to interact with mutant forms of SOD1 in vitro as well as in vivo. It is suggested that mutant SOD1 is released into the extracellular environment with the help of chromogranins. Neuronal overexpression of Chromogranin A in ALS mice results in exacerbation of the disease phenotype with acceleration of disease onset[75]. This effect is accompanied by the increased levels of misfolded SOD1 in the spinal cords of these mice. Both in vitro and in vivo studies have shown that extracellular mutant or misfolded SOD1 have capacity to polarize microglia towards the neurotoxic M1 phenotype[120,121]. It is also shown that the mutant SOD1 binds to CD14, TLR2 or TLR4 receptors on the microglial membrane and activates the proinflammatory signaling cascades that result in the secretion of neurotoxic cytokines like TNF-α, nitric oxide and superoxide anions in vitro[122,233,239-242]. This interaction between the mutant SOD1 and microglial receptors was proven by rescue of motor neuron toxicity when blocking antibodies against CD14, TLR2 and TLR4 were utilized in vivo. Transgenic removal of CD14 from ALS mice did not result in beneficial effects on the disease outcome, suggesting that this may not be the only pathway by which mutant SOD1 can activate the microglial cells in vivo.
1.4.2 Motor neuron-astrocyte crosstalk

Although no specific motor neuronal molecule has been identified to interact with ALS astrocytes directly, studies have shown that ALS astrocytes undergo gene expression changes in the presence of motor neurons in the microenvironment[110,111]. Gene expression analysis of motor neurons as well as astrocytes from spinal cords of SOD1<sup>G93A</sup> mice revealed that there is a deregulation of TGF-β signaling between motor neurons and astrocytes in these mice. As TGF-β is an immuno-modulatory agent which regulates the activation of astrocytes, it is considered that misregulation of this pathway in motor neurons leads to reactive astrogliosis and subsequent motor neuron loss. Exogenous TGF-β administration has been shown to improve motor performance in SOD1<sup>G93A</sup> mice[243]. Glutamate, released from motor neurons has also been shown to dysregulate lactates release and pro-NGF secretion from SOD1<sup>G93A</sup> astrocytes in vitro[110]. Interestingly, the presence of mutant SOD1 in astrocytes seemed to be a requirement for the impaired lactate secretion and increased pro-NGF secretion. Impaired lactase secretion causes energy deficits in the motor neurons, leading to motor neuron death. Pro-NGF released from SOD1 astrocytes can bind to p75 receptor on the motor neurons and activate apoptotic pathways involving NF-kB, p53 and Bax. Enhanced motor neuron survival was obtained when p75 receptor was blocked using antibodies. Along with the different neurotoxic factors secreted by ALS astrocytes, mutant SOD1 itself was shown to be secreted by ALS astrocytes via exosome mediated secretion[147,244]. In vitro studies have shown that mutant SOD1 containing exosomes are toxic to mouse
spinal motor neurons.

1.4.3 Microglia-astrocyte crosstalk

Interplay between microglia and astrocytes is very crucial in maintaining the homeostasis of the immune responses in CNS[245,246]. Different secretory molecules from both cell types control the extent of neuroinflammation in CNS, one of the major components of the non cell autonomous toxicity in ALS.

In several neurodegenerative disorders like multiple sclerosis (MS), experimental autoimmune encephalitis (EAE) and Alzheimer’s disease (AD), microglia activation has been considered to occur before astrogliosis[245]. In the field of ALS, there is no consensus whether microgliosis precedes the astrocyte activation or vice versa. The main reason behind this debate is the lack of simultaneous expression of different activation markers utilized to define the initiation of activation in these two cell types. The majority of the studies, however, show an early initiation of microgliosis followed by reactive astrogliosis in the spinal cord of both mouse models and human ALS patients[30,118-120,176]. Reactive microglia have the ability to activate the surrounding astrocytes by secretion of pro-inflammatory cytokines like IL-1, IL-18, TNF-α[245]. In vitro studies have shown that treatment of astrocytes with IL-1 results in the activation of astrocytes which further release neurotoxic molecules like NO and free radicals. IL-1 and TNF-α released from microglia have also been shown to bind to connexin 43 receptor on astrocyte cell membrane. This interaction opens the connexin channel leading to Ca2+ influx and
reciprocal ATP efflux. Increased extracellular ATP can directly cause motor neuron toxicity[247]. Another cytokine, IL-18 secreted by microglia can also activate astrocytes[248]. Prostaglandin 2, produced by activated microglia has been shown to bind its receptors DP1 and DP2 on astrocyte cell membranes and cause enhanced astrogliosis and demyelination in the central nervous system[249].

Activated astrocytes do possess the ability of further activating the surrounding microglia by releasing ATP in the extracellular environment[245]. This occurs as a result of Ca^{2+} influx that takes place in astrocytes. The released ATP can bind to the P2Y12 and P2Y6 receptors on microglia, thus triggering the potassium currents and secretion of proinflammatory cytokine that are neurotoxic. Treatment of ALS microglia with mutant SOD1 has been shown to cause microglia activation and enhance their neurotoxic potential[121]. Although not studied yet, astrocyte mediated mutant SOD1 release may contribute to the microglia activation in ALS.

Activated astrocytes can also have inhibitory effects on microglial activation[245,250]. Activated astrocytes have been shown to downregulate microglial production of pro-inflammatory cytokines via TGF-β signaling cascade[251]. Binding of TGF-β to its receptor on microglia reduces the production of NO, ROS and TNF-α from microglia. *In vitro* studies have shown that TGF-β treatment of activated microglia results in deactivation of NF-kβ pathway, the major pathway regulating the production of pro-inflammatory cytokines[252,253]. It is also shown that, Nrf2 activation in astrocytes can result in controlling the inflammatory response in microglia by reduction of ROS and NO
production[250]. Very recent studies have suggested that lectin-binding proteins, especially Galectin-1 plays a pivotal role in modulating the microglial activation in the central nervous system[254].

1.5 Involvement of Galectin 1 in neurodegenerative disorders

Galectin 1 belongs to a family of lectins, carbohydrate binding proteins, that have an affinity towards β-galactosides[255,256]. Galectins are phylogenetically conserved throughout animal evolution. These are 130 amino acid proteins that share a consensus carbohydrate recognition domain (CRD) that is responsible for binding to β-galactosides. So far, 15 galectin proteins have been identified from mammalian cells. Galectins are divided into three types based on their biochemical structure: a) Prototype galectins that are present as monomers or noncovalent homodimers of CRD, b) Chimera type galectins and c) Tandem repeat type galectins. Galectin 1 is a prototype galectin which occurs as a monomer as well as a non-covalent homodimer consisting of subunits of one CRD. These two forms have different biological activities. Galectin 1 is present intracellularly as well as extracellularly, carrying out different functions based on its location. Intracellularly, Galectin 1 can be present in cytosole, cell nucleus as well as on the intracellular side of the cell membrane. Galectins lack the classical signal sequence required for protein secretion via the vesicle mediated secretory pathway. However, most of the galectins are secreted out in to the extracellular environment by an unorthodox
secretory mechanism. It is postulated that secretion of Galectin 1 in the extracellular space is dependent on activity of a sodium pump for its export. Conventionally, Galectins have affinity towards its minimum saccharide ligand N-acetylgalactosamine which is a diasaccharide present in many cellular glycoproteins. However, based on the certain structure of different galectins and their specificity towards different tissues, galectins are also engaged in protein-protein interactions. It has been demonstrated that extracellular functions of Galectin 1 require its lectin binding activity while intracellular functions are based on protein-protein interactions.

### 1.5.1 Role of Galectin 1 in immunomodulation

Several studies have shown that Galectin 1 plays a crucial role in maintaining the homeostasis of inflammatory processes by modulating initiation as well as resolution of the inflammatory cascades. Galectin 1 influences both adaptive as well as innate immune responses in periphery as well as CNS[255,257-259]. Galectin 1 has been shown to regulate the cell growth and survival of T lymphocytes[255]. Galectin 1 can affect the proliferation of activated T cells and also reduce the clonal expansion of CD8+ T cells. Galectin 1 has also been shown to induce apoptosis of murine as well as human T cells during development. The effects of Galectin 1 on T lymphocytes have been attributed to its binding to 3 different glycoproteins that are present on the cell surface of T cells namely, CD45, CD43 and CD7[255]. Binding of Galectin 1 to CD45 results in the dimerization of this receptor on the cell surface which further leads to Caspase-
independent apoptosis of the T cell[260]. CD45, a tyrosine phosphatase is involved in various surface receptor signaling cascades via its dephosphorylation activity. However, the Galectin1-CD45 mediated apoptotic cascade in T cells operates with a completely different signaling cascade. Galectin1 binding to CD7 also results in the T cell death. Galectin1 can also regulate T cell receptor signaling, acting as a partial ligand for the TCR and leading the cells towards apoptosis[261]. Galectin 1 has also been shown to influence the inflammatory profile of T effector cells[255]. Galectin 1-mediated modulation of the inflammatory immune response has been shown to provide beneficial effects in various models of T-cell mediated immune disorders. Binding of Galectin1 to T cells reduces the secretion of proinflammatory cytokines like interleukin-2 while upregulating the secretion of anti-inflammatory cytokine, IL-10. Galectin1 can shift the immune response to a T helper 2 (Th2)-polarized response, accompanied with the decreased production of IFN-γ and increased IL-5 production[262]. Galectin 1 has also shown to affect components of the innate immune response. Galectin 1 binding to neutrophils results in decreased extravasation of these cells[263]. Importantly, Galectin 1 can bind to macrophages and cause reduced arachidonic acid and nitric oxide secretion while increasing the Arginase 1 activity[263,264]. Thus, Galectin 1 can shift the macrophages from neurotoxic M1 phenotype to neuroprotective M2 phenotype.

1.5.2 Role of Galectin 1 in cancer

Role of Galectin1 in the immunomodulation has been the main focus of research
in the field of cancer. Higher Galectin1 expression is considered as a poor prognosis for the tumor metastasis[265-267]. This notion is mainly based on the phenomenon of tumor immune escape by Galectin 1 expressing cancer cells. Several studies have suggested that the expression of Galectin 1 in cancer cells themselves or in the tumor microenvironment leads to tumor cell evasion from the anti-tumoral immune response. There also appears to be a positive correlation between the expression levels of Galectin 1 and tumor cell aggression. This can be attributed to the detrimental effects of secreted Galectin 1 on the proliferation and survival of anti-tumor T effector cells infiltrating the tumor microenvironment and reduction in their proinflammatory cytokine secretion[265,268]. Galectin1 has also been shown to play an important role in tumor proliferation, transformation and tumor metastasis by activating different intracellular signaling cascades like Ras activation, AKT/ERK pathway activation, cytoskeletal reorganization by activation of CDC42, RhoA, PKC and notch signaling[265]. Extracellular Galectin1 also improves tumor angiogenesis by promoting the proliferation of vascular endothelial cells in the tumor microenvironment via activation of ERK and JNK pathways[269].

Taking into account the pivotal role of Galectin1 in the cancer development and progression, Galectin1 inhibition is an important area of research for cancer therapeutics.

### 1.5.3 Role of Galectin1 in CNS

In the nervous system, Galectin 1 has been shown to play an important role in the
development and axonal growth of sensory as well as motor neurons[258]. In vivo studies with Galectin1 knockout mice show that the absence of endogenous Galectin1 affects the axonal targeting of olfactory as well as sensory DRG neurons, suggesting that Galectin 1 plays a crucial role in cell autonomous regenerative pathways in neurons[270-273]. Extracellular Galectin 1, especially in its oxidized form, has been implemented in the post-injury axonal growth. In vivo studies have shown that exogenous delivery of oxidized Galectin 1 results in the growth of peripheral axons[274-277]. Unlike endogenous Galectin 1, the effects of extracellular Galectin1 are considered to be non-cell autonomous as the direct addition of oxidized Galectin1 to monocultures of neurons did not induce the neurite outgrowth. It is postulated that oxidized Galectin1 binds surrounding macrophages which leads to the secretion of unidentified factors from these macrophages that promote the axonal regeneration as well as Schwann cell migration. Galectin 1 also plays a crucial role in the differentiation of astrocytes which further leads to the production and secretion of neurotrophic factor, BDNF[278,279]. Galectin 1 mediated BDNF production from astrocytes has been shown to provide beneficial effects in the rat model of cerebral ischemia. This was further proved by the successful improvement of motor deficits in the gerbil model of brain ischemia by transplantation of Galectin1 expressing human neural stem cells/progenitor cells[280].

Recent studies in mouse models of experimental autoimmune encephalitis (EAE) show that Galectin 1 plays a crucial role in the deactivation of classically activated microglia in the CNS[254]. Classically activated (M1) microglia are the key players of
inflammation-induced neurodegeneration in multiple sclerosis (MS) and its mouse model, EAE. *In vitro* studies revealed that Galectin1 has a higher affinity towards the classically activated M1 microglia where binding of Galectin1 to M1 microglia results in the reduced expression of proinflammatory genes like TNF-α, iNOS, reduced secretion of neurotoxic, proinflammatory cytokines as well as enhanced neuron survival in coculture systems. It was also shown that Galectin 1 binding results in the upregulation of anti-inflammatory genes like arginase-1 which are characteristic of neuroprotective M2 phenotype. This immunomodulation of CNS microglia from M1 to M2 phenotype was attributed to the binding of exogenous Galectin 1 to CD45 receptor on microglia which results in retention of this phosphatase on the cell membrane, augmenting its phosphatase activity and inhibitory function. This leads to the downregulation of proinflammatory signaling cascade activators namely CREB, p38MAPK and NF-kB pathways. It was also shown that Galectin1 binding to CD45 on activated microglia did not affect the proliferation of these cells. Interestingly, astrocytes are the main source of Galectin 1 in the central nervous system. Stimulation of astrocyte with TGF-β or Galectin 1 itself can result in the upregulation of Galectin-1 expression as well as its secretion from astrocytes. Treatment of M1 microglia with recombinant Galectin 1 or the conditioned media from stimulated astrocytes resulted in reduction of proinflammatory profile of these microglia and subsequent motor neuron toxicity in *in vitro* cocultures. Astrocyte mediated modulation of microglia was further confirmed in a mouse model when stimulated astrocytes were transplanted in the brains of EAE mice. Adoptive transfer of Galectin 1
secreting astrocytes suppressed EAE symptoms via downregulation of microglial inflammatory cascades. These results suggest that Galectin 1-glycan interactions function in modulating the microglial activation in the CNS and thus reducing the inflammation and neurodegeneration in a therapeutic manner. Importantly, astrocytes can be utilized to target the surrounding microglial cells, thus eliminating the need for direct targeting of the CNS microglia themselves.

1.5.4 Role of Galectin 1 in ALS

Previous studies have investigated the role of Galectin 1 in ALS[281]. Galectin 1 is found accumulated in spinal cord neurofilamentous lesions in both familial and sporadic patients[282]. Owing to the axonal regeneration property, it is considered that the accumulated Galectin 1 in the axonal spheroids leads to the axonal swelling and may contribute to neuronal degeneration in ALS. Further studies showed that oxidized form of Galectin 1 has beneficial effects on ALS mice[283,284]. Intramuscular injections of oxidized Galectin 1 resulted in the preservation of a higher number of motor neurons in the lumbar and cervical region of the spinal cord than in control mice. Administration of oxidized Galectin 1 also delayed the disease onset, improved motor performance and extended the median survival by 2 weeks in SOD1^{H46R} mice, suggesting that oxidized Galectin 1 can be considered as a therapeutic target in ALS. Very recent studies revealed that the absence of Galectin 1 within the motor neurons of ALS mice results in its reduced accumulation in the neurofilamentous lesions which leads to reduced axonal

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swelling and increased motor neuron survival[285]. In this study, SOD1\textsuperscript{G93A} mice were crossed with Gal1\textsuperscript{-/-} knockout mice. Ubiquitous removal of Galectin 1 from ALS mice resulted in a delay in the disease onset, owing to correction of the motor neuron phenotype in these mice. However, there was no change in the median survival, suggesting that the progression phase of the disease was accelerated in these mice. With reference to the previous studies, progression of ALS can be attributed to the surrounding non-neuronal cells. Hence, it is postulated that the removal of Galectin 1 from non-neuronal cells, especially astrocytes, has adverse effects on the motor neuron survival. This study thus provides with the two possible opposite roles of Galectin 1 in motor neurons and surrounding glial cells. This suggests the need for selective downregulation of Galectin 1 in motor neurons while maintaining or upregulating the Galectin 1 expression selectively in the glial cells.

In Chapter 4, we sought to determine the role of extracellular Galectin 1 on modulation of inflammatory response in ALS microglia and its effect on the reduction of microglia mediated motor neuron toxicity in ALS.

1.6 Summary and Significance

Amyotrophic Lateral Sclerosis is one of the most common, adult-onset neurodegenerative disorders, characterized by progressive and fatal loss of motor neurons in the spinal cord, motor cortex and brainstem resulting in muscular paralysis and
ultimate respiratory failure leading to death. Dominant mutations in Superoxide Dismutase 1 (SOD1) gene are one of the frequent causes of familial ALS. Mutant SOD1 confers cell autonomous as well as non cell autonomous toxicity towards motor neurons in ALS. Therefore, along with motor neurons, surrounding non-neuronal cells like astrocytes, microglia and oligodendrocytes play important roles in the pathogenesis of familial ALS. Non cell autonomous toxicity towards the motor neurons is also evident in sporadic ALS cases. Therefore, the reduction of motor neuron toxicity from multiple cell types within the CNS is required to achieve the maximum therapeutic benefits in ALS.

Transgenic removal of mutant SOD1 from motor neurons and astrocytes significantly delays the disease onset and progression of ALS mice with significant extension in survival. In Chapter 2, we determined the feasibility and efficacy of post-natal downregulation of mutant SOD1 via AAV9 mediated shRNA delivery in two distinct mouse models of ALS. Our results show that AAV9 mediated delivery of SOD1 shRNA before or even after the disease onset results in significant extension in the lifespan of SOD1<sup>G93A</sup> and SOD1<sup>G37R</sup> mice. We also show that AAV9 SOD1 shRNA administration is safe and well-tolerated in wild-type mice. Moreover, widespread spinal cord transduction and efficient SOD1 reduction in non-human primates provide strong basis for the translation of AAV9 mediated SOD1 shRNA delivery approach to the human settings.

In Chapter 3, we discuss the design of the clinical AAV9 SOD1 shRNA vector in accordance with the guidelines established by the Food & Drug Administration (FDA),
USA. We show successful construction of clinical AAV SOD1 shRNA plasmid with non-coding stuffer sequence, replacing the GFP expression cassette. We show that \textit{in vitro} administration of clinical AAV SOD1 shRNA vector into human cells results into efficient downregulation of SOD1 protein levels with lack of GFP expression as well as any unnatural transcript production from the stuffer. Here, we show successful generation of clinical AAV SOD1 shRNA vector that can be utilized directly in human clinical trials for ALS.

Microglia are one of the key players involved in non cell autonomous toxicity in ALS pathogenesis. Transgenic removal of mutant SOD1 from microglia has confirmed their role in disease progression. Transgenic inhibition of NF-\(\kappa\)B pathway in ALS microglia has also proven their role in neuroinflammation induced motor neuron death in ALS. However, none of the currently available approaches are able to target the microglia post-natally. In Chapter 4, we show that conditioning of endstage microglia from SOD1\(^{G93A}\) mice with exogenous Galectin 1, a lectin binding carbohydrate, results in a reduction of their neurotoxic potential towards motor neurons. We also show that Galectin 1 treatment of ALS microglia reduces the secretion of proinflammatory cytokines like TNF-\(\alpha\). Finally, our results show that Galectin 1 exerts its immunomodulatory effects on ALS microglia via inhibition of NF-\(\kappa\)B pathway. As astrocytes act as a source of endogenous Galectin 1 and remain to be one of the cell types readily targeted by AAV9, our results suggest a plausible approach for post-natal immunomodulation of ALS microglia by for overexpression of exogenous Galectin 1.
Chapter 2

AAV9 mediated therapeutic suppression of mutant SOD1 extends survival in mouse models of inherited ALS

This work has been published as: Therapeutic AAV9-mediated suppression of mutant SOD1 slows disease progression and extends survival in models of inherited ALS. Likhite S, Foust KD, Salazar D, Ferraiuolo L, Ditsworth D, Ilieva H, Meyer K, Schmelzer L, Braun L, Cleveland DW, Kaspar BK. *Nature Molecular Therapy* 2013;21(12):2148-2159.

2.1 Introduction

Amyotrophic lateral sclerosis (ALS) is an adult-onset, rapidly progressive, and fatal neurodegenerative disease, characterized by selective degeneration of both upper and lower motor neurons. ALS is the most prominent motor neuron disease, responsible for one in every 2,000 deaths. Most of the cases have no clear genetic linkage and are referred to as sporadic but in 10% of the instances, disease is familial with dominant inheritance. Twenty percent of the familial cases are caused by mutations in superoxide dismutase 1 (SOD1), with over 140 distinct mutations identified to date [17,286]. Many
efforts to identify how mutations alter the function of SOD1 have produced a consensus view that SOD1 mutants acquire one or more toxicities, whose nature still remains controversial[66]. However, there is clear evidence that a proportion of mutant SOD1 is misfolded which subsequently aggregates[287,288]. SOD1 aggregates are, in fact, one of the histological hallmarks of SOD1-related ALS cases[287].

In the past 20 years, multiple animal models expressing mutant forms of human SOD1 have been generated. These models recapitulate the hallmarks of ALS, developing age-dependent motor axon degeneration and accompanying muscle denervation, glial inflammation, and subsequent motor neuron loss. Selective gene excision experiments have determined that mutant SOD1 expression within motor neurons themselves contributes to disease onset and early disease progression, as does mutant synthesis in NG2+ cells[133] that are precursors to oligodendrocytes. However, mutant SOD1 protein expression in microglia and astrocytes significantly drives rapid disease progression[30,82], These findings have led to the conclusion that ALS pathophysiology is non cell autonomous[66].

Furthermore, astrocytes have been found to be toxic to motor neurons in multiple in vitro models where mutant forms of human SOD1 were overexpressed[85,86,289]. In a recent study, our group derived astrocytes from postmortem spinal cords of ALS patients with or without SOD1 mutations. In all cases, astrocytes from sporadic ALS patients were as toxic to motor neurons as astrocytes carrying genetic mutations in SOD1, but neither sporadic nor familial ALS glia were toxic to GABAergic neurons[87]. Even
more strikingly, reduction of SOD1 in astrocytes derived from both sporadic and familial ALS patients decreased astrocyte-derived toxicity toward motor neurons. This finding, along with the reports that misfolded SOD1 inclusions are found in the spinal cords of familial as well as some sporadic ALS patients[136,140,145], has provided strong evidence for a pathogenic role of wild-type SOD1 in sporadic ALS.

Despite the insights that mutant SOD1 expressing animal models have provided for understanding the mechanisms involved in motor neuron degeneration, their utility for the development of therapeutic approaches has been questioned[290], as no drug with a reported survival benefit in mutant SOD1$^{G93A}$ mice has been effective in clinical trials with sporadic ALS patients. We note, however, that in all but one case, the drugs taken to human trial had been reported only to extend mutant SOD1 mouse survival when applied presymptomatically and even then to provide a survival benefit solely by delaying disease onset with no benefit in slowing disease progression. The one exception to this was riluzole, which similar to the human situation, modestly extended survival of mutant SOD1$^{G93A}$ mice by slowing disease progression[291]. Recognizing that success at human trial will require slowing of disease progression, the SOD1-mutant mice have perfectly predicted the success of riluzole and the failure of efficacy of each other drug attempted in human trial. Therefore, development of additional strategies that affect the disease progression in these mice is of the extreme importance for future therapy development for ALS.
Previous studies have established that Adeno-Associated Virus 9 (AAV9) can cross the blood–brain barrier and efficiently target neurons and astrocytes in the brain and spinal cord when injected systemically\([214,215]\). We hypothesized that these attributes of AAV9 could be used to deliver SOD1 shRNA to slow disease progression in models of ALS. We have now tested this hypothesis in two mouse models using a single, peripheral injection of AAV9-encoding SOD1 shRNA. Furthermore, we also tested if intrathecal delivery of AAV9-encoding SOD1 shRNA and Green Flourescent Protein (GFP) into nonhuman primates could efficiently target all levels of the spinal cord and significantly reduce the levels of SOD1.

2.2 Materials and methods

2.2.1 Vectors

AAV CB GFP plasmid was obtained from Merion Scientific. This is a self-complementary AAV vector with mutated AAV2 ITR and one wild-type AAV2 ITR. 2 ITR flank a reporter GFP transgene under chicken β-actin promoter and CMV enhancer. GFP is transgene is followed by modified SV40 intron and bGH Poly A terminator sequence. The plasmid also carries pBR322 origin of replication and Ampicillin resistance gene.

shRNA constructs targeting human SOD1 were generated and obtained from the Life Technologies design tool. These constructs were first cloned in pSilencer 3.1
(Genscript, Piscataway, NJ) at BamH1/HindIII sites, under the human H1 promoter and tested \textit{in vitro}. shRNA 130 along with H1 promoter was PCR amplified and further cloned into the AAV CBA GFP vector at Kpn1 site, along with a reporter GFP under chicken \(\beta\)-actin promoter to identify the transduced cells.

shRNA 130 along with the H1 promoter was PCR amplified from AAV9 SOD1 shRNA vector and cloned into the lentiviral vector pBOB-GFP using Xba1 sites. pBOB-GFP vector contains GFP transgene under CMV promoter followed by WPRE and bGH Poly A terminator. The shRNA cassette and GFP expression cassette are flanked by lentiviral Inverted terminal repeats used for the lentiviral packaging.

Scrambled shRNA construct was generated using Life technologies design tool as well. The shRNA construct was first cloned into pRNA-H1 neo vector under human H1 promoter and tested \textit{in vitro}. Scrambled shRNA SOD1 along with the H1 promoter was then PCR amplified and cloned into the AAV CBA GFP vector carrying GFP under chicken \(\beta\)-actin promoter.

\textbf{Table 2.1 Primers used for cloning}

<table>
<thead>
<tr>
<th>Construct</th>
<th>Primer</th>
<th>Application</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOD1 shRNA</td>
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<td>Cloning</td>
</tr>
<tr>
<td></td>
<td>R 5’-AATTGGTACCACAGGCTGCGCACTGTGTTGGG-3’</td>
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<td>Scr SOD1</td>
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<tr>
<td>shRNA</td>
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</table>
2.2.2 Virus production

Self-complementary AAV9-SOD1-shRNA was produced by transient transfection procedures using a double-stranded AAV2-ITR–based CB-GFP vector, with a plasmid encoding Rep2Cap9 sequence as previously described along with an adenoviral helper plasmid pHelper (Stratagene, Santa Clara, CA) in 293 cells.

2.2.3 Cells

HEK-293 cells were maintained in Iscove's modified Dulbecco's media containing 10% FBS, 1% L-glutamine, and 1% penicillin/streptomycin. Upon reaching ~60% confluence, cells were transfected with pSilencer 3.1 containing the SOD1 shRNAs or pRNA H1 neo containing scrambled SOD1 shRNA. Protein lysates were prepared 72 hours post-transfection and analyzed for SOD1 levels by western blot.

Cos-7 cells were maintained in Dulbecco's Modified Eagle Medium with 10% FBS and 1% penicillin/streptomycin. Cells were infected with a lentiviral vector expressing SOD1 shRNA 130 under the H1 promoter and RFP under cytomegalovirus promoter. RNA was extracted from infected and noninfected cells 72 hours postinfection using an RNAeasy Kit (Qiagen, Valencia, CA). cDNA was prepared using RT2 First strand synthesis kit (SABiosciences, Valencia, CA). SOD1 transcript levels were analyzed by quantitative reverse transcriptase polymerase chain reaction (RT-PCR).
2.2.4 Animals

All procedures performed were in accordance with the NIH Guidelines and approved by the Research Institute at Nationwide Children’s Hospital (Columbus, OH), University of California (San Diego, CA), or Mannheimer Foundation (Homestead, FL) Institutional Animal Care and Use Committees.

High-copy SOD1\textsuperscript{G93A} mice were obtained from Jackson Laboratories (Bar Harbor, ME) and bred in the Kaspar laboratory. Animals were genotyped before the treatment to obtain SOD1\textsuperscript{G93A}-expressing mice and their wild-type littermates. Only female mice were included in the SOD1\textsuperscript{G93A} experiments. loxSOD1\textsuperscript{G37R} ALS mice, carrying a human-mutant SOD1\textsuperscript{G37R} transgene flanked by lox p sites under its endogenous promoter were maintained in the Cleveland laboratory, as previously described.\textsuperscript{8}

One-year-old Cynomolgus macaques (Macaca fascicularis) with average body weight of 2 kg were used for this study at the Mannheimer Foundation. Regular monitoring of overall health and body weight was performed before and after the injections to assess the welfare of the animals.

2.2.5 Injections

For neonatal mouse injections, P1–P2 SOD1\textsuperscript{G93A} pups were used. Total volume
of 50 μl containing $5 \times 10^{11}$ (3.6 × 10$^{14}$ vg/kg) DNase-resistant viral particles of AAV9-SOD1-shRNA (Virapur LLC, San Diego, CA) was injected through temporal vein as previously described. A correct injection was verified by noting blanching of the vein. After the injection, pups were returned to their cage. For adult tail vein injections, animals were placed in a restraint that positioned the mouse tail in a lighted, heated groove. The tail was swabbed with alcohol and then injected intravenously with AAV9-SOD1-shRNA. SOD1$^{G93A}$ mice were injected ~21 days and ~85 days of age with 200 μl or 300 μl viral solution containing $2 \times 10^{12}$ or $3 \times 10^{12}$ DNase-resistant viral particles, for an average dose of $1.7 \times 10^{14}$ vg/kg at P21 or $1.6 \times 10^{14}$ vg/kg at P85.
Figure 2.1 Schematic of intravenous administration of AAV9 CB GFP/AAV9 SOD1 shRNA in SOD1^{G93A} mice. For P1 group, 1 day old SOD1^{G93A} pups were administered with 3.6x10^{14}vg/kg particles of AAV9 CB GFP or AAV9 SOD1 shRNA via facial vein. For P21 and P85 groups, SOD1^{G93A} mice were injected with AAV9 CB GFP or AAV9 SOD1 shRNA via tail vein injection at the doses of 1.7x10^{14}vg/kg and 1.6x10^{14}vg/kg particles respectively. For all the groups, non-injected SOD1^{G93A} mice were used as control. Injected as well as control mice were carefully monitored till the end stage. Throughout the disease process, all the mice were subjected to behavioral analysis to determine motor performance and coordination (Hindlimb grip strength and Rotarod). At the endstage, mice were euthanized and subjected to spionial cord analysis.
Mutant lox SOD1<sup>G37R</sup> mice were injected at ~215 days of age with 300 μl containing 3 × 10<sup>12</sup> viral particles of AAV9-SOD1-shRNA (average dose of 1.1 × 10<sup>14</sup> vg/kg) or AAV9-GFP or empty capsid (average dose of 1.0 × 10<sup>14</sup> vg/kg).

For nonhuman primate injections, three anesthetized cynomolgus monkeys received intrathecal injections of 1 × 10<sup>13</sup> vg/kg AAV9-SOD1-shRNA and one received 1 × 10<sup>13</sup> vg/kg AAV9-CB-GFP. The injection was performed by lumbar puncture into the subarachnoid space of the lumbar thecal sac. AAV9 was resuspended with omnipaque (GE, Fairfield, CT) (iohexol), an iodinated compound routinely used in the clinical setting. Iohexol is used to validate successful subarachnoid space cannulation and was administered at a dose of 100 mg/kg. The subject was placed in the lateral decubitus position and the posterior midline injection site at ~L4/5 level identified (below the conus of the spinal cord). Under sterile conditions, a spinal needle with stylet was inserted and subarachnoid cannulation was confirmed with the flow of clear CSF from the needle. In order to decrease the pressure in the subarachnoid space, 0.8 ml of CSF was drained, immediately followed by injection with a mixture containing 0.7 ml iohexol (300 mg/ml formulation) mixed with 2.1 ml of virus (2.8 ml total).

2.2.6 Perfusion and tissue processing

Control and treated SOD1<sup>G93A</sup> mice were killed at either 21 days postinjection or at end stage for immunohistochemical analysis. Animals were anesthetized with
xylazene/ketamine cocktail, transcardially perfused with 0.9% saline, followed by 4% paraformaldehyde. Spinal cords were harvested, cut into blocks of tissue 5–6 mm in length, and then cut into 40-µm thick transverse sections on a vibratome (Leica, Bannockburn, IL). Serial sections were kept in a 96-well plate that contained 4% paraformaldehyde and were stored at 4 °C. End-stage loxSOD1<sup>G37R</sup> mice were anesthetized using isoflurane and perfused with 4% paraformaldehyde. Spinal cord segments, including cervical, thoracic, and lumbar segments were dissected. Following cryoprotection with 20% sucrose or 4% paraformaldehyde overnight, spinal cords were frozen in isopentane at −65 °C, and serial 30-µm coronal sections were collected free floating using a sliding microtome.

For safety studies, P1- and P21-treated mice and control wild-type mice were killed at 180 days of age. Animals were anesthetized using xylazene/ketamine cocktail and perfused with 0.9% saline. Different tissues were removed and stored in 10% buffered formalin. These tissues were further processed, blocked, and mounted for hematoxilin & eosin staining by the Nationwide Children’s Hospital Morphology Core.

Cynomolgus monkeys injected with virus were killed 2 weeks postinjection. Animals were anesthetized with sodium pentobarbital at the dose of 80–100 mg/kg intravenously and perfused with saline solution. Brain and spinal cord dissection were performed immediately and tissues were processed either for nucleic acid isolation (snap frozen) or postfixied in 4% paraformaldehyde and subsequently cryoprotected with 30%
sucrose and frozen in isopentane at −65 °C. Coronal sections of 12 µm were collected from lumbar cord using a cryostat for free floating immunostaining.

2.2.7 Immunohistochemistry

Mouse spinal cords were stained as floating sections. Tissues were washed three times for 10 minutes each in Tris-buffered saline (TBS), then blocked in a solution containing 10% donkey serum, 1% Triton X-100, and 1% penicillin/streptomycin for 2 hours at room temperature (RT). All the antibodies were diluted with the blocking solution. Primary antibodies used were as follows: rabbit anti-GFP (1:400; Invitrogen, Carlsbad, CA), chicken anti-GFP (1:400; Abcam, Cambridge, MA), rabbit anti-SOD1 (1:200; Cell signaling, Danvers, MA), goat anti-ChAT (1:50; Millipore, Billerica, MA), mouse anti-GFAP (1:200; Millipore), chicken anti-GFAP (1:400; Abcam, Cambridge, MA), and rabbit anti-Iba1 (1:400; Wako, Richmond VA). Tissues were incubated in primary antibody at 4 °C for 48–72 hours and then washed three times with TBS. After washing, tissues were incubated for 2 hours at RT in the appropriate fluorescein isothiocyanate-, Cy3-, or Cy5-conjugated secondary antibodies (1:200; Jackson Immunoresearch, Westgrove, PA) and DAPI (1:1,000; Invitrogen). Tissues were then washed three times with TBS, mounted onto slides, and then coverslipped with PVA-DABCO. All images were captured on a Zeiss laser-scanning confocal microscope.
For 3,3'-diaminobenzidine staining, monkey spinal cord sections were washed three times in TBS, blocked for 2 hours at RT in 10% donkey serum and in 1% Triton X-100. Sections were then incubated overnight at 4 °C with rabbit anti-GFP primary antibody (1:1,000; Invitrogen) diluted in blocking buffer. The following day, tissues were washed with TBS three times, incubated with biotinylated secondary antibody antirabbit (1:200; Jackson Immunoresearch) in blocking buffer for 30 minutes at RT, washed three times in TBS, and incubated for 30 minutes at RT with ABC (Vector, Burlingame, CA). Sections were then washed for three times in TBS and incubated for 2 minutes with 3,3'-diaminobenzidine solution at RT and washed with distilled water. These were then mounted onto slides and covered with coverslips in mounting medium. All images were captured with the Zeiss Axioscope.

**Table 2.2 Primary Antibodies for IHC**

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Species</th>
<th>Dilution</th>
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</tr>
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<tbody>
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<td>Invitrogen</td>
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IHC (Immunohistochemistry); ICC (Immunocytochemistry)
Table 2.3 Secondary Antibodies for IHC

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<th>Conjugation</th>
<th>Dilution</th>
<th>Application</th>
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<td>Cy-5</td>
<td>1:200</td>
<td>IHC/ICC</td>
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IHC (Immunohistochemistry); ICC (Immunocytochemistry)

2.2.8 Motor neuron and astrocyte quantification

For motor neuron quantification, serial 40-μm thick lumbar spinal cord sections, each separated by 480 μm, were labeled as described for GFP and ChAT expression. Stained sections were serially mounted on slides from rostral to caudal, then coverslipped. Sections were evaluated using confocal microscopy (Zeiss, Munich, Germany) with a ×40 objective and simultaneous fluorescein isothiocyanate and Cy3 filters. The total number of ChAT-positive cells found in the ventral horns with defined soma was tallied by careful examination through the entire z-extent of the section. GFP-labeled cells were quantified in the same manner, while checking for colocalization with
ChAT. For astrocyte quantification, as with motor neurons, serial sections were stained for GFP, GFAP and then mounted. Using confocal microscopy with a ×63 objective and simultaneous fluorescein isothiocyanate and Cy5 filters, random fields in the ventral horns of lumbar spinal cord sections from tail vein-injected animals were selected. The total numbers of GFP- and GFAP-positive cells were counted from a minimum of at least 24 fields per animal while focusing through the entire z extent of the section. Spinal cord sections of three animals per group were examined for motor neuron and astrocyte quantification.

2.2.9 Immunoblot analysis

Spinal cords were harvested from P1- and P21-injected mice and control SOD1<sup>G93A</sup> mice 21 days postinjection and from treated and control monkeys 2 weeks postinjection of AAV9-SOD1-shRNA. Spinal cords were homogenized and protein lysates were prepared using T-Per (Pierce, Rockford, IL) with protease inhibitor cocktail. Samples were resolved on SDS-PAGE according to the manufacturer’s instructions. Primary antibodies used were rabbit anti-SOD1 (1:750; Cell signaling) mouse anti-SOD1 (1:750; Millipore), rabbit anti-SOD1 (1:1,000; Abcam), rabbit anti-Actin (1:1,000; Abcam) and mouse anti-GAPDH (1:1,000, Millipore). Secondary antibodies used were anti-rabbit HRP (1:10,000–1:50,000) and anti-mouse HRP (1:10,000). Densitometric analysis was performed using Image J software.
Table 2.4 Primary Antibodies for Immunoblotting

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Species</th>
<th>Dilution</th>
<th>Application</th>
<th>Source</th>
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</table>

WB (Western blot)

Table 2.5 Secondary Antibodies for Immunoblotting

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<th>Conjugation</th>
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<td>Jackson Immuno-research</td>
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<td>HRP</td>
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WB (Western blot)

2.2.10 Laser capture microdissection

Lumbar spinal cord frozen sections of 12 µm were collected onto PEN membrane slides (Zeiss) and stained with 1% Cresyl violet (Sigma, St Louis, MO) in methanol. Sections were air dried and stored at −80 °C. After thawing, motor neurons were collected within 30 minutes from staining using the laser capture microdissector PALM Robo3 Zeiss) using the following settings: cut energy: 48, laser pressure catapulting
energy: 20, cut focus: 80/81, laser pressure catapulting focus: 1, position speed: 100, cut speed: 50. Approximately 500 motor neurons were collected per animal. Non-neuronal cells from the ventral horn were collected from the same sections after collecting the motor neurons.

2.2.11 Quantitative RT-PCR

RNA from laser-captured cells or whole spinal cord sections from the cervical, thoracic, and lumbar segments was isolated using the RNaqueous Micro Kit (Ambion, Grand Island, NY) according to the manufacturer’s instructions. RNA was then reverse transcribed into cDNA using the RT2 HT First Strand Kit (SABiosciences). RNA of 12.5 ng was used in each Q-PCR reaction using SyBR Green (Invitrogen) to establish the relative quantity of endogenous monkey SOD1 transcript in animals who had received the AAV9-SOD1-shRNA compared with the animals who had received only AAV9-GFP. Each sample was run in triplicate and relative concentration calculated using the ddCt values normalized to endogenous actin transcript.

Table 2.6 Primers for qRT-PCR

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<td>qRT-PCR</td>
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<td></td>
<td>R 5'-CACCTTTGCCCCAAGTCATCT-3'</td>
<td>qRT-PCR</td>
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<tr>
<td>β-Actin (Monkey)</td>
<td>F 5'-ATCAATTCAGCTCCTCATCAGGCG-3'</td>
<td>qRT-PCR</td>
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<tr>
<td></td>
<td>R 5'-ACTCTTGCTTGCTGATCCAC-3'</td>
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2.2.12 Behavior and survival analysis

Treated and control SOD1\textsuperscript{G93A} mice were monitored for changes in body mass twice a week. loxSOD1\textsuperscript{G37R} mice were weighed on a weekly basis. Motor coordination was recorded using a rotarod instrument (Columbus Instruments, Columbus, OH). Each weekly session consisted of three trials on the accelerating rotarod beginning at 5 rpm/minute. The time each mouse remained on the rod was registered. Both SOD1\textsuperscript{G93A} and loxSOD1\textsuperscript{G37R} mice were subjected to weekly assessment of hindlimb grip strength using a grip strength meter (Columbus Instruments). Each weekly session consisted of three (SOD1\textsuperscript{G93A} mice) or five (loxSOD1\textsuperscript{G37R} mice) tests per animal. Survival analysis was performed using Kaplan–Meier survival analysis. End stage was defined as an artificial death point when animals could no longer “right” themselves within 30 seconds after being placed on its back. Onset and disease progression were determined from retrospective analysis of the data. Disease onset is defined as the age at which the animal reached its peak weight. Disease duration is defined as the time period between disease onset and end stage. Early disease duration is the period between peak weight and loss of 10% of body weight while late disease duration is defined as the period between 10% loss of body weight until disease end stage. Due to shorter life span of SOD1\textsuperscript{G93A} animals, we did not assess the distinction between the early and late progression.

For toxicity analysis following injection at P1- or P21-treated mice and control WT mice were subjected to behavioral analysis starting at ~30 days of age and monitored
up to 6 months. Body mass was recorded weekly while rotarod performance and hindlimb grip strength were recorded biweekly.

2.2.13 Hematology and serum studies

Blood samples were collected in (dipotassium ethylene diamine tetraacetic acid) EDTA microtainer tubes (Becton Dickinson, San Jose, CA) from treated and control wild-type mice at 150 days of age by mandibular vein puncture. The same animals were bled at 180 days of age, and blood was collected in serum separator microtainer tubes. The blood was allowed to clot for an hour and was then centrifuged at 10,000 rpm for 5 minutes. The clear upper phase (serum) was collected and frozen at −80 °C. Hematological and serum analysis were conducted by AniLytics, Gaithersburg, MD.

2.2.14 Statistical analysis

All statistical tests were performed using the GraphPad Prism (San Diego, CA) software package. Kaplan–Meier survival analyses were analyzed by the log-rank test. Comparisons of median disease durations and survival times were analyzed by the Wilcoxon signed-rank test.
2.3 Results

2.3.1 AAV9 transduction pattern and persistence in SOD1\textsuperscript{G93A} mice

We first evaluated the efficiency of AAV9 transduction in the SOD1\textsuperscript{G93A} mouse model that develops fatal paralytic disease. Animals were injected intravenously at postnatal day 1 or day 21 (to be referred to as P1 and P21, respectively) with self-complementary AAV9-expressing GFP from the cytomegalovirus enhancer/β-actin (CB) promoter (AAV9-CB-GFP; n = 3 per group). Three weeks post-injection, animals were killed and spinal cords were examined for GFP expression (Figure 2.2a–u). Transduction efficiency was high in SOD1\textsuperscript{G93A} astrocytes with GFP expressed in 34 ± 2 and 54 ± 3% of P1- and P21-injected spinal gray matter astrocytes (defined by immunoreactivity for glial fibrillary acidic protein (GFAP)). This efficiency was similar to our previous report of 64 ± 1% in P21-injected wild-type animals\cite{214}. Motor neurons were a prominent cell type transduced at all levels of the spinal cords of P1-injected SOD1\textsuperscript{G93A} animals (62 ± 1%), compared with significantly lower targeting to motor neurons in P21-injected animals (8 ± 1%).

Although we have previously reported that transduced astrocytes in wild-type spinal cords persist with continued GFP expression for at least 7 weeks post-injection\cite{214}, longevity of mutant SOD1 astrocytes (and their continued synthesis of genes encoded by the AAV9 episome) during active ALS-like disease was untested. Therefore, SOD1\textsuperscript{G93A} mice were injected at P1 and P21 with AAV9-CB-GFP and followed to end stage (~P130; n = 3 per group) (Figure 2.2c,d,h,i,m,n,r,s).
Immunofluorescent examination of the end-stage SOD1\textsuperscript{G93A} spinal cords from animals injected at P1 and P21 showed a comparable number of GFP-expressing astrocytes as were found 21 days after AAV9 injection (P1: 42 ± 2%, P21: 61 ± 2%). These data are consistent with the survival of transduced astrocytes for the duration of disease (~110 days postinjection at P21) in SOD1\textsuperscript{G93A} mice and that AAV9-encoded gene expression is maintained.

Furthermore, considering mutant SOD1 mediated damage, including astrocytic and microglial activation and early changes in the blood–brain barrier develop during disease in SOD1-mutant mice\cite{292}, we tested if this damage affected AAV9 transduction. SOD1\textsuperscript{G93A} mice were injected at P85 with AAV9-CB-GFP and killed at end stage (n = 3; Figure 2.2e,j,o,t). Analysis of the spinal cords revealed that the transduction pattern seen in P85 animals was similar to P21-treated animals with astrocytes as the predominant cell type transduced at all levels (51 ± 6% GFP+/GFAP+ cells in lumbar gray matter).
Figure 2.2 AAV9 transduction pattern and persistence in SOD1<sup>G93A</sup> mice. SOD1<sup>G93A</sup> mice were injected intravenously with AAV9-CB-GFP at P1 and P21 and euthanized 21 days postinjection (n = 3 per time point). Spinal cords were examined for GFP, ChAT (motor neuron marker), and GFAP (astrocyte marker) expression. (a,f,k,p) Temporal vein injection of AAV9-CB-GFP at P1 resulted in efficient transduction of
motor neurons and glia in SOD1<sup>G93A</sup> mice. (b,g,l,q) Tail vein injection at P21 predominantly targeted astrocytes with few GFP-positive motor neurons. To test the persistence of transduced cells, AAV9-CB-GFP was intravenously injected at P1 and P21 in SOD1<sup>G93A</sup> animals that were killed at end stage (~P130). (c,d,h,i,m,n,r,s) Immunofluorescence analysis of lumbar ventral horn demonstrated that GFP expression was maintained in astrocytes throughout the disease course. To determine whether SOD1-mediated inflammation and damage would affect AAV9 transduction, we intravenously injected SOD1<sup>G93A</sup> mice at P85 and harvested their spinal cords at end stage. There was no difference observed in the transduction pattern of SOD1<sup>G93A</sup> mice treated at P21 or P85. Insets in (r–t) show colocalization between GFP and GFAP signal. (u) Quantification of transduced cells in ALS spinal cords (for each group tissues were analyzed from three animals). GFP, ChAT, and GFAP columns show numbers of cells counted. Bars = 100 µm. AAV, adeno-associated virus; GFP, green fluorescent protein; ChAT, choline acetyltransferase; GFAP, glial fibrillary acidic protein; P1, postnatal day 1; P21, postnatal day 21; P85, postnatal day 85.
2.3.2 Development of an shRNA sequence specific for human SOD1

To specifically target the human SOD1 mRNA, we generated four shRNA constructs that had a minimum of four base mismatches compared with the mouse mRNA sequence (Figure 2.3a). The base numbers for the human sequences shown correspond to record number CCDS33536.1 in the NCBI CCDS database. Each shRNA sequence was inserted into an expression cassette to place it under control of the H1 promoter. Human 293 cells were transfected with each cassette, lysates were harvested 72 hours post-transfection and SOD1 levels were analyzed by immunoblotting. All four sequences reduced SOD1 protein levels by >50% (Figure 2.3b,c). shRNA130 was selected because it produced the most consistent knockdown across three separate transfection experiments. It was further cloned into a self-complementary AAV9 vector that also contained a GFP gene as a reporter to identify transduced cells (referred to as AAV9-SOD1-shRNA). To confirm that the shRNA could suppress accumulation of human SOD1, SOD1\(^{G93A}\) mice (n = 3) were injected intravenously with AAV9-SOD1-shRNA at either P1 or P21. Animals were killed 3 weeks post-injection and the spinal cords were harvested and analyzed by immunoblotting for both human (mutant) and murine (wild-type) SOD1 protein. P1- and P21-injected spinal cords showed 60 and 45% reductions in mutant SOD1 protein, respectively (Figure 2.3d,e). Murine SOD1 levels remained unchanged in response to human SOD1 knockdown.
Figure 2.3 shRNA constructs show efficient reduction of human SOD1 protein *in vitro* and *in vivo*. (a) Sequence alignments between human and mouse SOD1 for the regions targeted by the four different shRNA constructs tested. (b) shRNA sequences were cloned into an H1 expression construct and transiently transfected into 293 cells. Lysates were collected 72 hours posttransfection and analyzed by western blot. (c) Quantification of *in vitro* suppression of human SOD1 from three separate transient transfections showed >50% reduction in SOD1. (d) shRNA 130 was packaged into AAV9 and injected into SOD1<sup>G93A</sup> mice at either P1 or P21. Spinal cords (n = 3 per time point) were harvested 3 weeks postinjection and analyzed by western blot for human SOD1 protein levels. (e) Quantification of *in vivo* suppression of human SOD1 within the
spinal cord of ALS mice. P1- and P21-injected spinal cords showed 60 and 45%
reductions in mutant SOD1 protein. GAPDH, glyceraldehyde 3 phosphate
dehydrogenase; hSOD1, human superoxide dismutase 1; mSOD1, mouse superoxide
dismutase 1; P1, postnatal day 1; P21, postnatal day 21.

2.3.3 AAV9-SOD1-shRNA is safe and well tolerated in wild-type mice

To determine whether high-dose AAV9-SOD1-shRNA would be safe, wild-type
mice of both sexes were intravenously injected at P1 or P21 (P1: 5 males, 5 females at
3.6 × 10^{14} vg/kg; P21: 5 males, 5 females at 1.7 × 10^{14} vg/kg (vector genomes per kg
body weight)) and then monitored up to 6 months of age. Both P1- and P21-injected mice
showed a steady increase in body mass similar to untreated mice (Figure 2.4). Weekly
behavioral tests observed no significant differences between injected and control groups
in motor skills (measured by rotarod) as well as in hindlimb grip strength.
**Figure 2.4 AAV9-shRNA-SOD1 administration is well tolerated in WT mice.** Female and male WT animals were injected with scAAV9 SOD1 shRNA at P1 or P21 and monitored up to 6 months of age. (a,b) Both male and female treated mice showed steady increase in body mass as compared to control animals. (c,d) Rotarod performance and (e,f) hind limb grip strength were not affected by P1 or P21 treatment in both groups as compared to respective controls. n = 5/group. WT, wild type; P1, postnatal day 1; P21, postnatal day 21.
Figure 2.4 AAV9-shRNA-SOD1 administration is well tolerated in WT mice.
At 150 and 180 days of age, blood samples were collected by mandibular bleed. Complete and differential blood counts of both treated and untreated groups showed similar blood chemistry parameters (Figure 2.5). Serum samples from both groups showed no significant differences in the levels of alkaline phosphatase, creatinine, blood urea nitrogen, potassium, sodium, and chloride. Finally, all the animals were euthanized at the age of 180 days. Histopathological analyses by a pathologist blinded to treatment group revealed no significant alterations in the AAV9-SOD1-shRNA treated animals compared with the uninjected controls (data not shown). We conclude that both administration of AAV9 and sustained shRNA expression were apparently safe and well tolerated.
Figure 2.5 Hematology and serum chemistry of AAV9-SOD1-shRNA-treated WT animals. (a-m) Blood was collected from P1 (green) or P21 (red) treated and control (gray) WT animals at 150 days of age for hematology studies. No significant differences

Continued
Figure 2.5 continued

were observed between treated and control animals. (n-w) Serum samples collected at 180 days of age from the same mice showed no significant differences in serum chemistry profile. Mean ± SEM. n = 5/group. RBC, red blood cell count; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MPV, mean platelet volume; WBC, white blood cell count; AST, aspartate transaminase; ALT, alanine transferase; GGT, gamma-glutamyl transpeptidase; Alk Phos, alkaline phosphatese; BUN, blood urea nitrogen; CPK, creatin phosphokinase; P1, postnatal day 1; P21, postnatal day 21.
2.3.4 Extended survival of SOD1\textsuperscript{G93A} mice from AAV9-mediated reduction in mutant SOD1 even when initiated mid-disease

To test the efficacy of AAV9-mediated SOD1 reduction, we treated cohorts of SOD1\textsuperscript{G93A} mice with a single intravenous injection of AAV9-SOD1-shRNA before (P1: $3.6 \times 10^{14}$ vg/kg, n=6 and P21: $1.7 \times 10^{14}$ vg/kg, n=9) or after (P85: $1.6 \times 10^{14}$ vg/kg, n=5) disease onset, recognizing that many astrocytes, but few motor neurons, would be transduced at the two later time points. Onset of disease (measured by weight loss from denervation-induced muscle atrophy) was significantly delayed by a median of 39.5 days (Figure 2.6a,c; uninjected, 103 days; P1: 142.5 days; $P < 0.05$, Wilcoxon signed-rank test) in the P1-injected cohort but was not affected by either of the later injections (P21: 110 days; P85: 105 days). P1- and P21-treated animals maintained their weights, had better rotarod performance and hindlimb grip strength when compared with the age-matched controls, indicating treated animals maintained muscle tone and motor function during their prolonged survival (Figure 2.6f–h). Survival was significantly extended by AAV9 injection at all three ages, yielding survival times 30–51.5 days beyond that of uninjected SOD1\textsuperscript{G93A} mice (uninjected: 132 days; P1: 183.5 days; P21: 171 days; P85: 162 days; log-rank test, $P \leq 0.0001$, $P \leq 0.0003$, and $P \leq 0.001$, respectively) (Figure 2.6b,e). Defining disease duration as the time from onset to end stage revealed that the P21 treatment group had significantly increased duration, indicative of slowed disease progression, compared with the uninjected controls (uninjected: 29.5 days; P21: 49 days; Wilcoxon signed-rank test, $P = 0.01$), with trends
toward slowed disease progression in animals injected at the other two ages (P1: 41 days; P85: 40 days; P = 0.06 and P = 0.12, respectively) (Figure 2.6d). The lower percentage of targeted non-neuronal cells at P1 versus those targeted at P21 (Figure 2.2u) suggests that a minimum percentage of non-neuronal cells must be targeted to slow disease progression in the fast-progressing SOD1\textsuperscript{G93A} model (Figure 2.2u).
Figure 2.6 Intravenous delivery of AAV9-SOD1-shRNA improves survival and motor performance in SOD1^{G93A} mice. SOD1^{G93A} mice received a single intravenous injection of AAV9-SOD1-shRNA at P1 (n = 6, green), P21 (n = 9, red), or P85 (n = 5, blue). Treated mice were monitored up to end stage and compared with noninjected control SOD1^{G93A} mice (n = 15, gray). (a,c) AAV9-SOD1-shRNA injection into P1 SOD1^{G93A} mice significantly delayed median disease onset 39.5 days compared with control animals (uninjected: 103 days; P1: 142.5 days; P < 0.05). Injection in P21 (red) or P85 (blue) ALS animals had no effect on disease onset (P21: 110 days; P85: 105 days). However, AAV9-SOD1-shRNA administered at P1, P21, or P85 all significantly extended median survival (b,e) (uninjected: 132 days; P1: 183.5 days; P21: 171 days; P85: 162 days; all comparisons to control P < 0.001). The P21 group had a significant extension in median disease duration (d) indicating a slowing of disease (uninjected: 29.5 days; P1: 41 days; P21: 49 days; P85: 40 days; Wilcoxon signed-rank test, P = 0.06, P = 0.01, and P = 0.12, respectively). (f-h) P1- and P21-treated animals maintained their weights, had better hindlimb grip strength and rotarod performance as compared with age-matched controls, indicating treated animals retained muscle tone and motor function during their prolonged survival. Lines between bars in (c–e) indicate statistically significant differences. *P < 0.05. P1, postnatal day 1; P21, postnatal day 21; P85, postnatal day 85.
Figure 2.6 Intravenous delivery of AAV9-SOD1-shRNA improves survival and motor performance in SOD1$^{G93A}$ mice.

Continued
Figure 2.6 Intravenous delivery of AAV9-SOD1-shRNA improves survival and motor performance in SOD1\textsuperscript{G93A} mice.
To confirm that the therapeutic benefits obtained after intravenous administration of AAV9 SOD1 shRNA in SOD1^{G93A} mice were indeed from the specific downregulation of human mutant SOD1 by shRNA 130 and not due to any unintended off-targeting, we generated a scrambled shRNA control vector. *In vitro* transfection of HEK293 cells with AAV scrambled shRNA plasmid revealed no change in SOD1 protein levels (Figure 2.7).
Figure 2.7 AAV scrambled shRNA has no effect on human SOD1 protein levels *in vitro*. HEK293 cells were transfected with AAV scrambled shRNA using Calcium phosphate. Cell lysates collected 72hrs post transfection were analyzed by western blot. (a) AAV scrambled shRNA treatment did not reduce human SOD1 levels in 293 cells, unlike AAV SOD1 shRNA treated cells. (b) Quantification of the *in vitro* knockdown efficiency of AAV scrambled shRNA.
Next, AAV9 scrambled shRNA was administered intravenously into cohorts of SOD1\textsuperscript{G93A} mice at P1 (3.6 \times 10^{14} \text{ vg/kg, } n = 5) and P21 (1.7 \times 10^{14} \text{ vg/kg, } n = 5) and monitored throughout the disease course. Compared to non-injected controls, P1 and P21 treated mice did not show any significant alterations in the disease onset (Figure 2.8a,c; uninjected, days; P1, days; P21, days). No significant change in the median survival of the treated and nontreated SOD1\textsuperscript{G93A} mice was observed (Figure 2.8b,e). Finally, neither P1 nor P21 treatment of SOD1\textsuperscript{G93A} mice with intravenous AAV9 scrambled shRNA resulted in significant extension of the progression phase of the disease. (Figure 2.8e; uninjected, days; P1, days; P21, days). Behavior analysis of AAV9 scrambled shRNA treated mice also showed no improvement in motor strength and coordination as compared to the non-injected controls. These results corroborated our previous data that the specific downregulation of human mutant SOD1 in CNS is the basis of improved disease outcome in AAV9 SOD1 shRNA treated ALS mice.
Figure 2.8 Improved survival and motor performance in SOD1\textsuperscript{G93A} mice is solely dependent on AAV9 mediated human mutant SOD1 downregulation. SOD1\textsuperscript{G93A} mice were treated with a single intravenous injection of AAV9 scrambled shRNA at P1 (n=5, green) or P21 (n=5, red) and monitored throughout the disease course. (a,c) AAV9 Continued
Figure 2.8 continued

scrambled shRNA treatment into P1 or P21 SOD1^{G93A} mice did not alter the disease onset as compared to non-injected control mice (n=9, gray). (b,e) No significant change in the median survival was observed between AAV9 scrambled shRNA treated group and noninjected controls. (d) Neither P1 nor P21 treated mice showed significant extension in the disease duration phase. (f,g) HIIndlimb grip strength and rotarod analysis revealed no improvement in motor performance and coordination in P1 and P21 treated mice versus non-injected controls. , postnatal day 1; P21, postnatal day 21
2.3.5 Reduction of mutant SOD1 in AAV9-infected cells in treated SOD1\textsuperscript{G93A} mice

Indirect immunofluorescence with an antibody that recognizes human, but not mouse SOD1, was used to determine accumulated mutant SOD1 levels in end-stage spinal cords of treated and control mice. Human SOD1 levels in end-stage spinal cord sections inversely correlated with increased survival (Figure 2.9a–d). At end stage, P1 (Figure 2.9b), P21 (Figure 2.9c), and P85 (Figure 2.9d), AAV9-SOD1-shRNA-injected animals had lower levels of mutant SOD1 when compared with the uninjected SOD1\textsuperscript{G93A} animals (Figure 2.9a). SOD1 expression within transduced motor neurons (identified by GFP- and choline acetyltransferase (ChAT)-expressing cells) was reduced compared with the surrounding neurons that had not been transduced to express viral-encoded GFP (Figure 2.9h, l, p, t; arrows versus arrowheads).
Figure 2.9 Intravenous injection of AAV9-SOD1-shRNA reduces mutant protein in spinal cords of SOD1\textsuperscript{G93A} mice. (a–d) Images of lumbar spinal cord sections from

Continued
Figure 2.9 continued

(a) uninjected, (b) P1-injected, (c) P21-injected, and (d) P85-injected mice were captured with identical microscope settings to qualitatively show SOD1 levels at end stage. SOD1 levels inversely correlate with survival. (e–t) Colabeling for GFP, ChAT, and SOD1 shows that AAV9-transduced motor neurons had reduced SOD1 expression (arrows) while cells that lacked GFP maintained high levels of mutant protein (arrowheads). As described in Figure 1u, higher motor neuron transduction and corresponding SOD1 reduction was observed in (i–l) P1-injected mice as compared with (m–p) P21-injected and (q–t) P85-injected mice. Bar = 100 µm. ChAT, choline acetyltransferase; GFP, green fluorescent protein; P1, postnatal day 1; P21, postnatal day 21; P85, postnatal day 85; SOD1, superoxide dismutase 1.
A subset of mutant SOD1 is present in the non-native, misfolded form in rodent models as well as human ALS patients as shown by the immunostaining with confirmation specific antibodies. B8H10 antibody has been successfully used to confirm the presence of misfolded SOD1 in mouse models as well as human patients. Misfolded SOD1 being the common link between the pathogenesis of familial and sporadic ALS, we determined whether AAV9 SOD1 shRNA treatment also reduces the burden of misfolded SOD1 in SOD1\(^{G93A}\) mice. Immunostaining of lumbar spinal cord sections of endstage SOD1\(^{G93A}\) mice revealed that human mutant SOD1 can be detected by B8H10 antibody (Figure 2.10a-d). Similar analysis of endstage spinal cords from P1- and P21-treated SOD1\(^{G93A}\) mice showed overall reduction in misfolded SOD1 protein levels as compared to uninjected mice (Figure 2.10e-p).
Figure 2.10 Intravenous injection of AAV9-SOD1-shRNA reduces misfolded SOD1 protein spinal cords of SOD1G93A mice. (a-d) Immunostaining of lumbar spinal cord from endstage SOD1G93A mice with B8H10 antibody revealed that a subset of mutant SOD1 is present in the misfolded confirmation. (e-p) Intravenous injection of AAV9 SOD1 shRNA at P1 as well as P21 results in the reduction of the misfolded SOD1 in

Continued
Figure 2.10 continued

lumbar spinal cords of SOD1<sub>G93A</sub> mice as compared to uninjected mice. Bar = 100 µm.
ChAT, choline acetyltransferase; GFP, green fluorescent protein; P1, postnatal day 1; P21, postnatal day 21; SOD1, superoxide dismutase 1.

Moreover, immunofluorescence imaging of end-stage spinal cords revealed corresponding reduction in astrogliosis (Figure 2.11a-d) but no difference was observed in microgliosis in AAV9-SOD1-shRNA-treated animals versus controls (Figure 2.11e-h).
**Figure 2.11** AAV9-SOD1-shRNA treatment in SOD1\textsuperscript{G93A} mice reduces astrogliosis. Endstage sections from control and scAAV9 SOD1 shRNA treated animals were harvested and stained for GFAP, an astrocyte activation marker. P1 (b) and P85 (d) injected mice showed reduced levels of astrogliosis as compared to control (a) mice while P21(c) injected mice showed the maximum reduction. This was consistent with the percent astrocyte transduction achieved in these mice (Figure 3.1u). However, no effect was observed on microglia reactivity (e-h). Bar = 100\textmu m. GFAP, glial fibrillary acidic protein; Iba1, ionized calcium-binding adapter molecule 1; P1, postnatal day 1; P21, postnatal day 21; P85, postnatal day 85.
2.3.6 Therapeutic slowing of disease progression with peripheral injection of AAV9 after onset

To determine if AAV9-mediated mutant SOD1 reduction would slow disease progression, a cohort of SOD1$^{G37R}$ mice[30] were injected intravenously with AAV9-SOD1-shRNA after disease onset (average age at treatment = 215 days versus median onset of 197 days in treated animals; log-rank test, $P = 0.46$; Figure 3.11a). A combination of AAV9-CB-GFP (n = 9) and uninjected (n = 12) littermates were used as controls. Post hoc analysis showed no differences between GFP and uninjected animals; therefore, the groups were compiled as “control” and are shown in Figure 2.12. Animals were evaluated weekly for body weight and hindlimb grip strength and monitored until end stage. AAV9-SOD1-shRNA treatment after disease onset significantly extended median survival by 86.5 days over control animals (control, n = 21, 392 days; SOD1 shRNA, n = 25, 478.5 days; log-rank test, $P < 0.0001$). Early disease duration, defined by the time from peak weight to 10% weight loss, was significantly slowed (control: 89 days; SOD1 shRNA-treated mice: 162 days; Wilcoxon signed-rank test, $P < 0.01$; Figure 2.12c). A continuing trend toward slowing of later disease (10% weight loss to end stage) was also seen (control: 63 days; SOD1 shRNA-treated mice: 81 days; Wilcoxon signed-rank test $P = 0.1389$; Figure 2.12d). Overall disease duration following AAV9-SOD1-shRNA therapy rose to 239 days after disease onset versus 173 days in control mice (Wilcoxon signed-rank test, $P < 0.0001$; Figure 2.12e). Consistent with the slowed disease progression, AAV9 therapy maintained grip strength relative to control
SOD1-mutant animals (Figure 2.12g). The 86.5-day extension in survival surpassed the 62-day extension seen in transgenic studies that used astrocyte-specific Cre expression to inactivate the mutant SOD1$^{G37R}$ transgene[82], presumably reflecting efficient AAV9 transduction of astrocytes after peripheral delivery and the possible transduction of other cell types (especially microglia)[30] whose synthesis of mutant SOD1 accelerates disease progression.
Figure 2.12 AAV9-SOD1-shRNA improves survival and motor performance in SOD1<sup>G37R</sup> mice treated after disease onset. (a) There was no difference in median disease onset between AAV9-SOD1-shRNA– and control-treated mice (average age at treatment = 215 days versus median onset of 194 days control and 197 days treated; log-rank test, $P = 0.46$). (b,f) Median survival of AAV9-SOD1-shRNA–treated
SOD1\textsuperscript{G37R} mice \((n = 25)\) was significantly extended versus control mice \((n = 21)\) (control, \(n = 21, 392 \) days; SOD1 shRNA, \(n = 25, 478.5 \) days; log-rank test, \(P < 0.0001\)).

The early phase of disease was significantly slowed by 73 days in treated mice as compared with control mice (control: 89 days; SOD1 shRNA: 162 days; \(P < 0.0001\); Wilcoxon signed-rank test) while the late phase of disease showed a nonsignificant slowing (control: 63 days; SOD1 shRNA: 81 days; \(P = 0.14\), Wilcoxon signed-rank test). Together this amounted to a 66-day increase in median disease duration (control: 173 days; SOD1 shRNA: 239 days; \(P < 0.0001\); Wilcoxon signed-rank test). A trend to improved hindlimb grip strength appeared in AAV9-SOD1-shRNA–treated mice compared with control mice.

Histological examination of end-stage SOD1\textsuperscript{G37R}-treated animals revealed similar levels of intraspinal cell transduction in animals treated with AAV9-SOD1-shRNA or AAV9-GFP (Figure 2.13). GFP expression was predominantly observed within motor neurons and astrocytes of both groups, and SOD1 expression was detectably decreased only in animals those received AAV9-SOD1-shRNA (Figure 2.13k,o).
Figure 2.13 Intravenous injection of AAV9 in adult SOD1<sup>G37R</sup> mice targets astrocytes and motor neurons within the spinal cord. (a–h) Immunofluorescence analysis revealed neuronal as well as glial transduction in both (a–d) AAV9-CB-GFP– and (e–h) AAV9-SOD1-shRNA–treated mice. (i–p) Human SOD1 levels appeared reduced in (o) AAV9-SOD1-shRNA treated mice compared with (k) AAV9-GFP treated mice. Bar = 100 µm. ChAT, choline acetyltransferase; GFP, green fluorescent protein; GFAP, glial fibrillary acidic protein; SOD1, superoxide dismutase 1.
Immunoblotting of whole spinal cord extracts from end-stage SOD1\textsuperscript{G37R} mice revealed an 80% reduction in hSOD1 protein levels in AAV9-SOD1-shRNA-treated animals compared with controls (Figure 2.14).

![Image](image_url)

**Figure 2.14** Intravenous injection of AAV9-SOD1-shRNA efficiently reduces levels of mutant SOD1 protein in spinal cords of SOD1\textsuperscript{G37R} mice. (a) SOD1\textsuperscript{G37R} mice were injected after the disease onset with a single, intravenous injection of AAV9 SOD1 shRNA or AAV9 CB GFP. Spinal cords were harvested at the end stage (4 mice per group) and analyzed by western blot for human SOD1 protein. (b) Quantification of in vivo suppression of human SOD1 within the spinal cord of SOD1\textsuperscript{G37R} mice. AAV9 SOD1 shRNA administered mice showed 80% reductions in mutant SOD1 protein. GAPDH, glyceraldehyde 3 phosphate dehydrogenase; hSOD1, human superoxide dismutase 1.
2.3.7 AAV9-mediated suppression of SOD1 in nonhuman primates

To test whether SOD1 levels could be efficiently lowered using AAV9 in the nonhuman primate spinal cord, AAV9 was injected intrathecally through lumbar puncture. This method was chosen over systemic delivery to decrease the amount of virus required and to minimize any effects from reduction of SOD1 in peripheral tissues. Sequencing of cDNA copied from mRNA isolated from African Green Monkey (COS cells) and the Cynomolgus macaque verified that the 130 shRNA had a single base mismatch to either sequence (Figure 2.15a). The 130 shRNA expression cassette was inserted into a lentiviral vector which was then used to transduce COS cells. Quantitative RT-PCR of total RNA harvested 72 hours post-infection revealed that the monkey SOD1 mRNA was reduced by ~75% in 130 shRNA-transduced cells compared with the mock-transduced control cells (Figure 2.15b).
Figure 2.15 shRNA 130 efficiently reduces the levels of monkey SOD1 in vitro. (a) Sequence alignment of the region targeted by SOD1 shRNA 130 and a single mismatch with the monkey sequence. Monkey sequence corresponds to SOD1 sequence from Rhesus monkey (NM 001032804.1), Cynomolgus monkey (sequenced in-house) and African green monkey. (B) The shRNA 130 expression cassette was cloned into lentiviral vector and used to infect Cos-7 cells. Lysates were analyzed 72 hours post infection by qRT PCR for SOD1. shRNA 130 efficiently reduced the SOD1 transcripts levels in Cos-7 cells.

The AAV9-SOD1-shRNA virus ($1 \times 10^{13}$ vg/kg) was infused along with contrast agent through lumbar puncture into the subarachnoid space of the three male Cynomolgus macaques and one control subject was injected with AAV9-CB-GFP.
(1 × 10^{13} \text{ vg/kg}) (Figure 2.16a). No side effects from the treatments were identified. Two weeks post-injection, spinal cords were harvested for the analysis of GFP expression and SOD1 RNA levels. GFP expression was seen broadly in neuronal and astrocytic cells throughout the gray and white matter of the lumbar spinal cord, the area closest to the site of injection (Figure 2.16b–e). Immunoblotting of extracts of lumbar spinal cord revealed 87% reduction in monkey SOD1 protein levels (Figure 2.16f,g). Laser capture microdissection was then used to isolate total RNA from motor neurons as well as from glia in the nearby neuropil. Analysis by quantitative RT-PCR using primers specific for monkey SOD1 (and normalized to actin) confirmed a 95 ± 3% knockdown in the motor neuron pool and a 66 ± 9% knockdown in the neuropil pool as compared with the samples from a control animal (Figure 2.16h).
Figure 2.16 Intrathecal infusion of AAV9-SOD1-shRNA in nonhuman primates leads to efficient reduction in SOD1 levels. (a) A myelogram shortly after intrathecal infusion of AAV9-SOD1-shRNA mixed with contrast shows proper delivery into the subarachnoid space of a *Cynomolgus macaque*. Arrows show diffusion of the contrast agent along the entire spinal cord. (b) Lumbar spinal cord sections from treated monkeys (*n* = 3) were harvested 2 weeks postinjection and stained for GFP using 3,3′-diaminobenzidine staining. Sections had widespread GFP expression throughout the gray and white matter. (c–e) Immunofluorescence analysis of the lumbar spinal cord sections showed (c) robust GFP expression within (d) ChAT-positive cells indicating (e, merge) motor neuron transduction. (f) Western blot analysis of the lumbar spinal cords showed significant reduction in SOD1 levels in AAV9-SOD1-shRNA–injected animals as compared with controls. (g) *In vivo* quantification of SOD1 knockdown in monkey lumbar spinal cord homogenate (*n* = 3) showed an 87% reduction in animals that received AAV9-SOD1-shRNA compared with uninjected controls. (h) Laser capture microdissection was used to collect motor neurons or surrounding neuropil from injected and control lumbar monkey sections. Collected cells were analyzed for SOD1 levels by quantitative reverse transcriptase polymerase chain reaction. Motor neurons collected from AAV9-SOD1-shRNA animals (*n* = 3) had a 95 ± 3% reduction in SOD1 RNA. Non-neurons had a 66 ± 9% reduction in SOD1 RNA in AAV9-SOD1-shRNA treated animals. Scale bars: b = 100 μm; e = 50 μm. SOD1, superoxide dismutase 1.
Figure 2.16 Intrathecal infusion of AAV9-SOD1-shRNA in nonhuman primates leads to efficient reduction in SOD1 levels.
Figure 2.16 continued

Figure 2.16 Intrathecal infusion of AAV9-SOD1-shRNA in nonhuman primates leads to efficient reduction in SOD1 levels.
Next, we examined the level of cell transduction throughout the spinal cord including cervical, thoracic, and lumbar segments. GFP was found to be expressed broadly within all sections analyzed (Figure 2.17a–c). Motor neuron counts revealed a caudal to rostral gradient in cell transduction, with the cervical region showing more than 50% of GFP/Chat+ motor neurons, increasing to 65% in the thoracic region and reaching 80% in the lumbar region (Figure 2.17d). In order to determine the overall level of SOD1 knockdown achieved with this transduction pattern, quantitative RT-PCR for SOD1 was performed on whole-section homogenates from cervical, thoracic, and lumbar cord segments. The results confirmed robust SOD1 reduction at all three spinal cord levels, ranging from a 60% decrease in the cervical segment, a 70% decrease in the thoracic region, and an 88% decrease in the lumbar region (Figure 2.17e), consistent with the proportion of cells transduced in each region.
Figure 2.17 Lumbar intrathecal infusion of AAV9-SOD1-shRNA leads to efficient transduction of motor neurons and non-neuronal cells in the cervical, thoracic, and lumbar cord resulting in reduction of SOD1. (a–c) Immunofluorescence analysis of the three segments of the spinal cord; (a) cervical, (b) thoracic, (c) and lumbar, showed robust GFP (green) expression within ChAT (red)-positive cells indicating motor neuron transduction. (d) GFP+/Chat+ cell counts show a caudal to rostral gradient of motor neuron transduction ranging from 85% of transduced cells in the lumbar region to more than 50% in the cervical region. (e) SOD1 mRNA levels in cervical, thoracic, and lumbar

Continued
Figure 2.17 continued

cord section homogenates analyzed by quantitative reverse transcriptase polymerase chain reaction show significant reduction in SOD1 transcript, consistently with motor neuron transduction. SOD1 levels were normalized to β-actin– and AAV9-SOD1-shRNA–injected animals were compared with an AAV9-CB-GFP–injected control. (a–c) Scale bars: = 50 µm; (d–e) error bars: = SD.
2.4 Discussion

Only one drug is currently approved by Food and Drug Administration as a therapy for ALS, providing a modest survival benefit[51]. For the 20% of familial cases caused by mutation in SOD1, attempts at improving therapy by reducing synthesis of SOD1 have been the focus of multiple therapeutic development approaches. Antisense oligonucleotides and virus-delivered RNA interference were tested in rat[193] and mouse models[205,206,209] that develop fatal paralysis from overexpressing human SOD1G93A. Antisense oligonucleotides infused at disease onset produced SOD1 reduction and a modest slowing of disease progression[193]. Direct cerebrospinal fluid (CSF) infusion of antisense oligonucleotides has been tested clinically[190], leading to encouraging results in terms of tolerability and safety but without significant reduction in SOD1 levels at the low dosages used. In each of the prior viral studies[205,206,209], SOD1 knockdown was achieved before disease onset by direct injection into the nervous system or taking advantage of axonal retrograde transport when a virus was injected intramuscularly[206,209]. These studies led to varying degrees of success in extending survival or improving motor performance, depending on the time of treatment as well as level of SOD1 knockdown achieved in the spinal cord. Although these studies provided important proof of principle, the approaches were far from being readily translated into clinical strategies. Indeed, there have been controversial reports surrounding these initial virus-mediated SOD1 suppression studies[206-209,293].
Here, we have tested the therapeutic potential of the groundbreaking finding that AAV9 crosses the blood–brain barrier and successfully transduces motor neurons and astrocytes[214,215]. Our results also show that intravenous administration of AAV9-SOD1-shRNA is safe and well tolerated in wild-type mice, with the absence of adverse effects after long-term assessment. Remarkably, our efforts with this approach have achieved one of the longest extensions in survival ever reported in the rapidly progressive SOD1\textsuperscript{G93A} mouse model of ALS (increasing survival by 39% when treatment is initiated at birth). Even more encouraging, markedly slowed disease progression is seen even when AAV9 therapy to reduce mutant SOD1 synthesis is applied after disease onset in SOD1\textsuperscript{G37R} mice, thereby significantly extending survival. Therefore, the vascular delivery paradigm in mice represents a proof of concept that mutant SOD1 knockdown after disease onset can be beneficial in both rapid and more slowly progressive models of ALS at clinically relevant points in disease. Together, these data show that robust targeting and suppression of SOD1 levels through AAV9-mediated delivery of shRNA is effective in slowing disease progression in mouse models of ALS, critically even when treatment is initiated after onset.

The significant reduction in SOD1 protein levels at end stage in both SOD1\textsuperscript{G93A} and SOD1\textsuperscript{G37R} mouse models (Figure 2.9 and Figure 2.14) exceeded expectations, given the range in transduction efficiency of the virus seen at 3 weeks postinfection (Figure 2.2u), and the level of reduction in SOD1 levels in both cell culture (Figure 2.3c) and in mice at 3 weeks postinfection (Figure 2.3d). This raises the
possibility that in the central nervous system, cells with reduced SOD1 levels might exhibit a survival advantage over time, consistent with data from Ralph et al.[206] using lentiviral-mediated knockdown of SOD1 and from Reaume et al.30 in SOD1 deficient mice. Although contrasting with previous evidence that SOD1 knockdown leads to senescence in fibroblasts or cancer cells[294], together these findings suggest that sensitivity to SOD1 reduction may be cell type specific. Although not yet directly tested elsewhere in a mammalian system in vivo, the sensitivity of certain cells to SOD1 reduction may depend on metabolic status or proliferative potential, as reduction of respiration in yeast prevented the rapid viability loss in SOD1-mutant strains[295].

A metabolic link underlying cell type-specific sensitivity to SOD1 reduction would also be consistent with another approach that has shown significant lifespan extension in the SOD1\(^{G93A}\) mouse model[296,297] and in the SOD1\(^{G37R}\) mouse model.6ALS-associated mutations in SOD1 have been shown to the increase activity of NADPH-oxidase 2 by binding to Rac1, resulting in an overproduction of extracellular reactive oxygen species released from microglia[296,297]. Genetic deletion of NADPH-oxidase 2 or inhibition with apocynin treatment slowed both the onset and progression of disease[297]. However, the significant neuroprotection conferred by apocynin treatment starting at P14 has not been reproduced when initiated at P21 and therefore, controversy remains[298]. Most recently, Nox activation in microglial cells has also been linked to an induction of protein disulphide isomerase expression by the unfolded protein response to mutant SOD1 at an early symptomatic phase in the SOD1\(^{G93A}\) mouse model[299]. In
vivo studies testing pharmacological inhibition of protein disulphide isomerase or genetic depletion in any available SOD1 mouse models would clarify the potential of these alternate therapeutic approaches targeting downstream consequences of misfolded SOD1.

Whether SOD1 is involved in sporadic disease remains controversial. Although some reports have argued against any such involvement[141,300,301], multiple recent studies have brought forward the hypothesis that wild-type SOD1 may contribute through misfolding to the pathogenic mechanism(s) that underlie sporadic ALS through a pathway similar to that triggered by mutant SOD1.[87,135-137], . Included in this body of evidence is our own demonstration that astrocytes produced from sporadic ALS patients are toxic to cocultured motor neurons and that toxicity is alleviated by siRNA-mediated reduction in wild-type SOD1[27]. Several additional reports have provided data supporting SOD1 misfolding in sporadic disease[87,135,140,143-146,302]. Although no consensus has yet emerged, the evidence creates the potential that a proportion of sporadic ALS patients could also benefit from an AAV9-mediated SOD1 reduction approach that we have demonstrated to be effective in slowing disease progression in mice that develop fatal, ALS-like disease from expressing ALS-causing mutations in SOD1.

Finally, for translation of an AAV9-mediated suppression of SOD1 synthesis to the human setting, we have determined that infusion directly into the CSF at the lumbar level in a nonhuman primate produces substantial SOD1 reduction by targeting both motor neurons and non-neuronal cells. This outcome provides strong support for
extending these efforts to an adult human by direct injection into CSF, as previously proposed[216,228] so as to limit the cost of viral production, to reduce the possibility that chronic suppression of SOD1 in the periphery may have deleterious consequences, and to reduce viral exposure to the peripheral immune system[216]. These data strongly suggest that AAV9-SOD1-shRNA is a valid candidate for clinical trials in ALS and also opens the opportunity for delivering genes to non-neuronal cells in other disorders, given the recently established contribution of astrocytes in Rett syndrome and age-related decline in neurogenesis[303,304].
Chapter 3

Development of clinical AAV9 SOD1 shRNA vector for human clinical trials in ALS

3.1 Introduction

The phenomenon of RNA interference was discovered in 1998 as an endogenous cellular pathway for innate immune defense against invading viruses and transposable elements [186]. RNAi comprises of a mechanism of post-transcriptional regulation of gene expression by either degradation of specific messenger RNA or blockage of its translation. This process is carried out by a variety of small RNAs like endogenous microRNA (miRNA) and exogenous small interfering RNA (siRNA), small hairpin RNA (shRNA) or antisense oligonucleotides. siRNAs are synthetic, short, double stranded RNA constructs that mediated the degradation of specific messenger RNA via RNA induced silencing complex (RISC). Antisense oligonucleotides, on the other hand, are single stranded DNA molecules that can bind to the specific mRNA to either block its
translation or direct it to RNAsse-H mediated degradation. Both of these constructs are applied directly to cells, tissues or organisms where the cellular entry is mediated via endocytosis. Owing to its potential to efficiently downregulate specific genes that are “untouchable” otherwise, RNA interference has gained a tremendous attraction as a novel therapeutic tool to target disease genes for treatment. Currently, there are more than 25 ongoing clinical trials to test the effectiveness and safety of siRNA/antisense oligonucleotide based gene silencing in various diseases like bacterial infections, cardiovascular diseases, cancer and neurodegenerative diseases including ALS[188,189].

In spite of efficient and specific gene silencing, siRNA/antisense approach suffers from a number of limitations[186,305]. Both siRNA and antisense oligonucleotides have a short half-life which results in only transient gene silencing[305]. Another important limitation of siRNA based therapies is the induction of innate immune response to the double stranded RNA via interaction with RNA binding proteins like Toll like receptors and protein kinase receptors[306-308]. Finally, the main drawback with current RNAi therapeutics is the delivery of these molecules to the target cells. Due to the negative charge and larger size, siRNA/ antisense oligonucleotides cannot readily enter the target cells. The only way by which these molecules enter the target cells is endocytosis. The subsequent endosome-lysosomal pathway by which these molecules are finally released within the cells limits their effective dose[187]. Various other strategies that exclude the endosomal pathway are being investigated for the therapeutic delivery of siRNA/antisense oligonucleotides[309].
Viral mediated delivery of small hairpin RNA (shRNA) is one of the best alternatives to the oligonucleotide based delivery\[186,310]. shRNAs are transcribed from an external expression vector by RNA polymerase II or III into a short double stranded DNA sequence with hairpin loop. This transcript is further processed by Drosha and Dicer and incorporated into RISC complex. Due to the constant synthesis of the shRNA construct, this method results in long-lasting, durable gene silencing. Thus, unlike siRNA, shRNA based gene silencing does not suffer from limitations on stability and bioavailability. Targeted delivery of shRNA expression cassette can be achieved by utilizing the natural tropism of different viruses toward different cell types and also by \textit{in vitro} engineering of the viral capsid proteins\[194,197,310-312]. Utilizing the same strategy, we have previously shown that AAV9 mediated downregulation of human SOD1 resulted in significant extension in the survival of ALS mice\[313]. A single, intravenous injection of AAV9 carrying human SOD1 shRNA and a reporter GFP transgene, either before or after the disease onset resulted in persistent and widespread transduction of spinal cord with efficient downregulation of mutant human SOD1 protein. We also showed that intrathecal lumbar infusion of AAV9 SOD1 shRNA in non-human primate results in widespread transduction throughout the spinal cord with efficient SOD1 reduction, thus setting the stage for the future clinical trials.

Although our study showed that AAV9 SOD1 shRNA administration is safe and well-tolerated in wild-type mice, several other groups have reported the presence of GFP mediated toxicity \textit{in vitro} as well as \textit{in vivo}\[314-322]. The toxic effects of GFP range
from interference with the intracellular pathways to induction of immune response to the foreign transgene. These results strongly recommend excluding the GFP transgene as well as its regulatory elements from the vector to be used in the clinic. Here, we have designed an alternative expression cassette for the delivery of SOD1 shRNA in humans, which is devoid of any foreign transgene but still maintains the size of the total cassette optimal for its efficient packaging into viral capsids. We show that administration of this newly designed clinical AAV-SOD1 shRNA cassette results in efficient downregulation of human SOD1 in vitro. We also show unequivocally the lack of GFP expression as well as any other coding and non-coding transcript expression from the newly designed shRNA cassette. These results strongly encourage the administration of the clinical AAV9-SOD1 shRNA as potential therapeutic approach for human clinical trials.

3.2 Materials and methods

3.2.1 Vectors

AAV CB GFP plasmid was obtained from Merion Scientific. This is a self-complementary AAV vector with mutated AAV2 ITR and one wild-type AAV2 ITR. 2 ITR flank a reporter GFP transgene under chicken β-actin promoter and CMV enhancer. GFP is transgene is followed by modified SV40 intron and bGH Poly A terminator sequence. The plasmid also carries pBR322 origin of replication and Amipicillin resistance gene.
Clinical SOD1 shRNA construct, containing SOD1 shRNA 130 under H1 promoter and the stuffer sequence, was synthesized from Genscript. The entire construct was cloned into pJet1.2/Blunt vector at EcoRV site.

To generate the clinical AAV SOD1 shRNA vector, pJet SOD1 shRNA plasmid was double digested with Kpn1 and Sph1 which released the entire construct from this plasmid. The destination vector, AAV.CB.MCS was cleaved with Kpn1/SPh1 as well to remove all the elements of the expression cassette. Finally, the clinical SOD1 shRNA construct was ligated into the Kpn1/SPh1 cut AAV.CB.MCS backbone to obtain Clinical AAV SOD1 shRNA clone.

3.2.2 Cells

HEK-293 cells were maintained in Iscove's modified Dulbecco's media containing 10% FBS, 1% L-glutamine, and 1% penicillin/streptomycin. Upon reaching ~60% confluence, cells were transfected with AAV SOD1 shRNA (the same one in Chapter 2), pJet SOD1 shRNA containing the SOD1 shRNA 130 and the stuffer sequence or clinical AAV SOD1 shRNA using Calcium phosphate method. Protein lysates were prepared 72 hours post-transfection and analyzed for SOD1 levels by western blot. RNA was extracted from transfected cells 72 hours postinfection using an RNAeasy Kit (Qiagen, Valencia, CA). cDNA was prepared using RT2 First strand synthesis kit (SABiosciences, Valencia, CA) and used for ORF analysis.
3.2.3 Immunoblot analysis

HEK293 cells, transfected with AAV SOD1 shRNA, PJet SOD1 shRNA and clinical AAV SOD1 shRNA, were harvested 72 hrs post-tranfection and protein lysates were prepared using T-Per (Pierce, Rockford, IL) with protease inhibitor cocktail. Samples were resolved on SDS-PAGE according to the manufacturer’s instructions. Primary antibodies used were rabbit anti-SOD1 (1:750; Cell signaling), rabbit anti-Actin (1:1,000; Abcam) and mouse anti-GAPDH (1:1,000, Millipore). Secondary antibodies used were anti-rabbit HRP (1:10,000–1:50,000) and anti-mouse HRP (1:10,000). Densitometric analysis was performed using Image J software.

Table 3.1 Primary Antibodies for Immunoblotting

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WB (Western blot)

Table 3.2 Secondary Antibodies for Immunoblotting

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WB (Western blot)
3.2.4 ORF analysis

RNA from transfected HEK293 cells was isolated at 72hrs post-transfection, using the RNaqueous Micro Kit (Ambion, Grand Island, NY) according to the manufacturer’s instructions. RNA was then reverse transcribed into cDNA using the RT2 HT First Strand Kit (SABiosciences). cDNA of 12.5 ng was used in each PCR reaction using Choice Taq polymerase to determine the presence of transcriptionally active putative ORFs in cells who had received the pJet SOD1 shRNA or Clinical AAV SOD1 shRNA (both with stuffer sequence) compared with the cells that received AAV SOD1 shRNA. PCR products were analysed by agarose gel electrophoresis. The experiment was repeated in triplicate.

3.2.5 Statistical analysis

All statistical tests were performed using the GraphPad Prism (San Diego, CA) software package. Efficiency of SOD1 knockdown was analyzed by One-way ANOVA.

3.3 Results

3.3.1 Development of clinical SOD1 shRNA construct

Our current AAV SOD1 shRNA vector carries shRNA against human SOD1 sequence under the H1 promoter (Figure 3.1a). The same vector also contains a GFP
expression cassette which expresses GFP under CBA promoter. The other regulatory elements present in this cassette include CMV enhancer, SV40 intron and bGH PolyA terminator sequence. We have previously shown that AAV9 SOD1 shRNA administration results in efficient SOD1 downregulation along with robust expression of GFP \textit{in vitro} as well as \textit{in vivo}. No significant alterations were observed after the long term assessment of wild-type mice administered with AAV9 SOD1 shRNA\textsuperscript{[313]}. These results suggested that there are no evident off-target effects due to the long-term expression of SOD1 shRNA as well as overexpression of GFP. Although we did not find GFP toxicity in our mice, several reports have shown the adverse effects of GFP overexpression \textit{in vitro} as well as \textit{in vivo}\textsuperscript{[314-321]}. Therefore, to eliminate the possibility of GFP toxicity altogether, we modified our current SOD1 shRNA construct by replacing the GFP expression cassette with a non-coding stuffer sequence while maintaining the size of the total DNA construct flanked by the ITRs (figure 3.1b). This is important as the distance between the two ITR sequences greatly affects the packaging capacity of the flanked construct into AAV9 capsids\textsuperscript{[323-326]}.

Although several RNAi based human clinical trials are ongoing in the United States, none of these trials utilize viral mediated delivery of shRNA\textsuperscript{[188,189]}. Currently preferred method for post-transcriptional gene silencing is the direct siRNA administration. As siRNA administration does not need the whole expression cassette, none of the FDA approved stuffer sequences are currently available. However, there are several human clinical trials in process to study the therapeutic effects of DNA vaccines...
against various diseases[327]. DNA vaccine plasmid is made up of two main structure, the plasmid backbone and expression cassette for the therapeutic transgene[328]. There are several plasmid backbones that are approved by FDA for the human administration. Here, we picked the small DNA fragments from these plasmids which do not correspond to any essential DNA sequences necessary for selection and replication of the plasmid or the elements of the transcriptional units. The plasmid backbones used in this study are listed in Table 3.3. These DNA elements from different plasmids were arranged in tandem to generate 1329bp stuffer sequence. Finally, a DNA construct containing the SOD1 shRNA expression cassette, followed by the stuffer sequence was synthesized from Genscript.
Figure 3.1 Design of clinical SOD1 shRNA construct. (a) Original AAV SOD1 shRNA construct contains shRNA sequence against human SOD1 under H1 promoter followed by the expression cassette for GFP which includes CMV enhancer, CBA promoter, modified SV40 intron, and GFP transgene sequence followed by bGH PolyA terminator. SOD1 shRNA expression cassette and GFP expression cassette are flanked by AAV2 ITRs which ensures the packaging of the complete flanked sequence in AAV9 capsid. (b) In clinical SOD1 shRNA construct, GFP expression cassette is replaced by a stuffer element that contains tandem, noncoding sequences from FDA approved DNA vaccines.
Figure 3.1 continued.

ITR: inverted terminal repeats; shRNA, small hairpin RNA; SOD1, superoxide dismutase 1; CMV, cytomegalo virus enhancer; CBA, Chicken β-actin promoter; GFP, green fluorescent protein; bGH pA, bovine growth hormone poly A terminator.
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Table 3.3 Plasmid backbones utilized in the construction of stuffer sequence for clinical SOD1 shRNA construct. These plasmids have been approved by FDA to be used as DNA vaccines in human clinical trials. Small non-coding DNA fragments that do not correspond to the bacterial or therapeutic elements in these plasmids were utilized to generate the stuffer sequence for clinical SOD1 shRNA construct.
Clinical SOD1 shRNA construct has shRNA against human SOD1 under H1 promoter which is followed by the non-coding stuffer sequence. This construct is designed in such a way that multiple shRNA expression cassettes can be added to the final vector by simultaneous removal of the stuffer sequence\[^{329}\]. Restriction endonuclease sites have been added to the stuffer sequence so that a part of the stuffer can be removed when another shRNA expression cassette is added. This simultaneous removal and addition of DNA sequences would help maintaining the optimal size of the whole construct between the ITRs (~2.0kb) to achieve efficient packaging (Figure 3.2).

**Figure 3.2 Schematic of clinical SOD1 shRNA construct.** Different restriction sites are placed in the clinical SOD1 shRNA construct that would allow the cloning of multiple shRNA expression cassettes while maintaining the total distance between the 2 ITRs.
Clinical SOD1 shRNA construct from Genscript was cloned into pJet1.2 plasmid via EcoRV. This parental clone was screened using various restriction endonucleases designed within the construct to confirm the correct clone (Figure 3.3). Kpn1/Sph1 double digestion of pJet SOD1 shRNA confirmed the presence of the complete construct (2023 bp) while Xba1 digestion confirmed the presence of SOD1 shRNA expression cassette (414bp) and the stuffer element (1588bp), along with pJet backbone (~3000bp). EcoRV/Pme1 double digestion also revealed the presence of stuffer element (1588bp).
Figure 3.3 Restriction endonuclease screening of pJet SOD1 shRNA plasmid.

Presence of full length construct (2023bp) in pJet SOD1 shRNA plasmid was confirmed by double digestion with Kpn1/Sph1 restriction enzymes. Xba1 digestion and of the plasmid confirmed the presence of shRNA expression cassette (414bp) which includes H1 promoter and shRNA 130 sequence and 1588bp fragment from pJet SOD1 shRNA plasmid that corresponds to the stuffer sequence. Presence of stuffer element (1588bp) was further ascertained by EcoRV/Pme1 double digest.
3.3.2 Clinical SOD1 shRNA efficiently reduces human SOD1 protein levels *in vitro*

To determine the efficacy of the de novo synthesized SOD1 shRNA construct to downregulate SOD1 levels, HEK293 cells were transfected with pJet SOD1 shRNA plasmid using Calcium Phosphate method. AAV SOD1 shRNA plasmid was used as a positive control. Immunofluorescence analysis of HEK293 cells, 72 hrs post transfection revealed the lack of native GFP fluorescence from pJet SOD1 shRNA transfected cells as compared to AAV9 SOD1 shRNA transfected cells (Figure 3.4a-h). Immunoblot analysis of cell lysates from these cells further confirmed the successful replacement of GFP from pJet SOD1 shRNA plasmid. Importantly, pJet SOD1 shRNA resulted in efficient downregulation of SOD1 protein levels (>50%), similar to AAV SOD1 shRNA plasmid (Figure 3.4i,j).
Figure 3.4 *In vitro* transfection of clinical SOD1 shRNA construct efficiently reduces human SOD1 protein in HEK293 cells. Representative microscopic fields showing bright-field images of non-transfected control (a), AAV SOD1 shRNA transfected (b) and pJet SOD1 shRNA transfected (c,d) HEK 293 cells, 72 hrs post transfection. Corresponding fluorescence images reveal the lack of GFP fluorescence from pJet SOD1 shRNA transfected HEK 293 cells (g,h) as compared to AAV SOD1 shRNA transfected HEK 293 cells.
Figure 3.4 continued

cells (f). (i) Western blot analysis of the cell lysates confirms the efficient knockdown of human SOD1 protein in pJet SOD1 shRNA transfected cells as compared to the non-transfected control cells. Immunoblot analysis also confirms removal of GFP transgene from pJet SOD1 shRNA construct. (j) Quantification of the in vitro downregulation of SOD1 by pJet SOD1 shRNA. pJet SOD1 shRNA reduces the protein levels of human SOD1 by almost 50% in HEK293 cells as compared to control. This reduction is similar to that achieved with AAV SOD1 shRNA construct.
3.3.3 **ORF analysis of clinical SOD1 shRNA construct reveals presence of naturally occurring ORFs in human genome**

To determine whether the stuffer sequence contains any potential protein coding sequences, Open reading frame (ORF) analysis was performed on the entire clinical SOD1 shRNA construct. This analysis identifies parts of a reading frame that does not contain stop codon. ORF analysis only predicts the evidence of protein coding region in the DNA sequence but the presence of an ORF does not necessarily mean that the region is ever translated. As shown in Figure 3.5a, ORF analysis of clinical SOD1 shRNA construct revealed 11 different ORFs across 6 reading frames. We excluded ORF 4 and 9 from further analysis as they correspond to the region of SOD1 shRNA construct. In order to determine if these ORFs are functionally active, antibodies that can specifically detect the ORF encoded sequences are required. As these antibodies are not readily available, we utilized an indirect method to test the activation status of these ORFs. Total mRNA was collected from non-transfected control HEK293 cells and AAV SOD1 shRNA or pJet SOD1 shRNA 1 transfected cells, 72hrs post transfection and converted to cDNA. PCR analysis was performed on these mRNAs using primers that amplify predicted ORFs within the stuffer sequence (Figure 3.5b,c). pJet SOD1 shRNA transfected cells showed amplification of cDNA for all the ORFs tested. Interestingly, AAV SOD1 shRNA transfected cells and even non-transfected control cells showed the presence of amplicons corresponding to the predicted ORFs. A plausible reason for amplification of the cDNA corresponding to these ORFs from the cells that did not even
receive the stuffer containing plasmid is that the genetic elements corresponding to the above tested ORFs occur naturally in HEK293 cells. BLAST analysis of the stuffer sequence revealed that these DNA elements align with the components of the transposon system. Several studies have already shown the presence of transposon elements present in the human genome[330,331]. Here, we show that that administration of clinical SOD1 shRNA in human cells might just increase the abundance of naturally occurring transcripts in human cells. Although long-term safety assessment of clinical SOD1 shRNA in non-human primates is necessary, our results strongly support the use of clinical SOD1 shRNA in human clinical trials for ALS.
Figure 3.5 ORF analysis of clinical SOD1 shRNA construct. (a) 11 different ORFS across 6 reading frames were obtained by analyzing the clinical SOD1 shRNA sequence via ORF finder (www.ncbi.nlm.nih.gov/gorf/gorf.html). (b) Primers corresponding to multiple ORFs were generated and used for the PCR analysis to determine the presence of transcribed mRNA corresponding to the predicted ORFs. (c) PCR analysis of cDNA obtained from total mRNA of non-transfected control, AAV SOD1 shRNA transfected and pJet SOD1 shRNA transfected HEK293 cells revealed presence of the predicted ORF transcripts in all the samples.
Figure 3.5 ORF analysis of clinical SOD1 shRNA construct
3.3.4 Generation of clinical AAV SOD1 shRNA

Clinical SOD1 shRNA construct was further cloned into AAV.CB.MCS vector using Kpn1/Sph1 sites to generate clinical AAV SOD1 shRNA plasmid (Figure 3.6). AAV.CB.MCS was generated from previously used AAV.CB.GFP plasmid by replacing GFP with a multiple cloning site (MCS). Cloning of clinical SOD1 shRNA construct at Kpn1/Sph1 sites puts it between the two AAV ITRs which would facilitate the future packaging of the construct in AAV9 viral capsids.

Figure 3.6 Schematic of cloning strategy for clinical AAV SOD1 shRNA vector. Clinical SOD1 shRNA construct was cloned into AAV CB MCS vector using Kpn1/SPh1 sites. Kpn1/SPh1 double digest of AAV CB MCS plasmid results in the release of the complete transgene expression cassette from this vector which is further replaced with clinical SOD1 shRNA construct carrying SOD1 shRNA expression cassette and stuffer sequence.
Clinical AAV SOD1 shRNA plasmid was screened with restriction endonucleases to confirm the presence of SOD1 shRNA expression cassette (Xba1 digest), stuffer sequence (EcoRV/Pme1 double digest) and also intact ITR sequences (Sma1 digest) (Figure 3.7).

Figure 3.7 Restriction endonuclease screening of clinical AAV SOD1 shRNA vector.

Sma1 digest of clinical AAV SOD1 shRNA vector confirmed the presence of intact ITRs in the final vector that are crucial for its packaging into the viral capsids. Kpn1/Sph1 double digest confirmed the presence of the complete clinical SOD1 shRNA construct while Xba1 and EcoRV/Pme1 digests confirmed the presence of SOD1 shRNA expression cassette and stuffer sequence respectively.
Clinical AAV SOD1 shRNA efficiently reduces human SOD1 protein levels \textit{in vitro}

Clinical AAV SOD1 shRNA plasmid was transfected in HEK293 cells to determine its knockdown efficiency. Similar to the pJet SOD1 shRNA plasmid, clinical AAV SOD1 shRNA transfected cells were devoid of any GFP expression as evident by immunofluorescence (Figure 3.8f) and immunoblot assay (Figure 4.8g). More importantly, clinical AAV SOD1 shRNA efficiently reduced human SOD1 protein levels in HEK293 cells by more than 50% (Figure 3.8g,h). Altogether, these results confirmed the successful generation of clinical AAV SOD1 shRNA vector with functional SOD1 shRNA expression cassette and complete removal of the transgene expression cassette.
Figure 3.8 Clinical AAV SOD1 shRNA efficiently reduces human SOD1 levels in vitro. HEK293 cells were transfected with clinical AAV SOD1 shRNA plasmid by Calcium phosphate method. Representative microscopic fields showing brightfield images of non-transfected control, AAV SOD1 shRNA and Clinical AAV SOD1 shRNA

Continued
transfected cells respectively, 72hrs post-transfection (a-c). Successful removal of GFP from clinical AAV SOD1 shRNA was confirmed by lack of GFP expression in Clinical AAV SOD1 shRNA transfected cells (f,g). (g) Western blot analysis of cell lysates, harvested 72hrs post-tranfection confirmed efficient downregulation of SOD1 in clinical AAV SOD1 shRNA transfected cells as compared to control. AAV SOD1 shRNA was used as a positive control. (h) Quantification of the *in vitro* knockdown of SOD1 by clinical AAV SOD1 shRNA.
3.4 Discussion

Post transcriptional gene silencing is the most widely used approach for the treatment of various diseases. The goal of this approach is to reduce the levels of the culprit protein involved in the disease pathogenesis by either degradation of its mRNA transcript or by blockage of the translation. Out of the different methods available, RNA interference based approach is the most preferred one for the post-natal gene silencing[186]. This is mainly because of the specificity that can be achieved with the use of small oligonucleotides targeting the particular gene sequence. This method outweighs the use of pan inhibitors of the protein transcription and translation. siRNAs and antisense oligonucleotides have been extensively used in treating major diseases like cancer, cardiovascular disorders, infectious diseases as well as neurodegenerative diseases like HD, Alzheimer’s disease and even ALS[188-190].

Although siRNA based gene silencing leads to efficient downregulation of the target gene expression, this approach also suffers from variety of limitations[186,305]. siRNA as well as antisense oligonucleotides are short lived molecules. The stability and hence bioavailability of these single or double stranded nucleic acids is compromised due to intracellular nuclease-mediated degradation. Various modifications of the nucleotides used in the generation of these synthetic oligonucleotides have shown to increase the intracellular half-life of these molecules[305]. The bioavailability of these oligonucleotides is also affected by the route of delivery. Systemic delivery of these molecules has shown to result in the higher accumulation of these molecules in the
kidney and their subsequent excretion[332-337]. Administration of foreign nucleic acids has also shown to elicit an innate immune response in vivo which further results in the increased clearance of these molecules from the system and hence affects their therapeutic potential[306-308]. Finally, all of these RNAi therapeutics enter the target cells only by endocytosis[187]. Hence, the potential therapeutic effect exerted by these molecules mainly depends on their escape from the endosome-lysosomal pathway which can degrade these small oligonucleotides by prolonged exposure to very low pH. In order to bypass the endosome-lysosomal pathway, several strategies like liposomes based delivery, adjuvant mediated delivery are being tested for efficient targeting of siRNA/antisense oligonucleotides to the diseased cells[309].

Most of the limitations of siRNA/antisense based strategies can be alleviated with the use of shRNA mediated post-transcriptional gene silencing[186,310]. In this case, a complete expression cassette capable of transcribing the shRNA sequence specific to the target gene is delivered to the diseased cells. The continuous expression of the shRNA within the affected cells results in long-lasting and durable gene silencing. Moreover, these expression cassettes can be delivered to the affected cells by packaging within viral capsids[194,197,310-312]. Due to the inherent property of viruses to infect the mammalian cells and introduce their genomic content within, the viral mediated delivery of shRNA to the diseased cells is more efficient than siRNA administration. Also, based on the tropism of the virus used, cell, tissue or organism specificity can be achieved in the viral mediated delivery. Although viral mediated delivery also encounters the vector
clearance due to the presence of humoral immune response of the host, this can be
minimized by the use of viruses with lower immunogenicity. Several studies have shown
that Adeno-associated viruses are the best available vectors for the gene therapy of
neurological diseases[197,202,214,216,222-225,310-312,338]. We have previously
showed that AAV9 mediated delivery of an shRNA against human SOD1 results in the
widespread transduction of neuronal as well as glial cells in mouse models of ALS
carrying the mutant human SOD1[313]. The efficient downregulation of mutant SOD1
significantly extends the survival of ALS mice. We also show that AAV9 mediated
shRNA delivery to the nonhuman primates results in efficient SOD1 downregulation,
thus suggesting that AAV9 mediated SOD1 shRNA delivery can be translated to the
human settings.

As the presence of shRNA in the transduced cells cannot be readily visualized,
AAV9 SOD1 shRNA vector used in this study also carried GFP expression cassette
which acted as a reporter[313]. GFP expression analysis thus enabled us to identify the
transduced cells in CNS of ALS mice as well as non-human primates and indirectly
suggested the coexpression of SOD1 shRNA which results in downregulation of human
SOD1 in these cells. Thus, we showed that AAV9 mediated delivery of SOD1 shRNA
targeted the two crucial players in ALS pathogenesis namely, motor neurons and/or
astrocytes, based on the time of delivery. Although the use of such reporter systems is
very common, several studies have also suggested that the overexpression of GFP can be
toxic to the cells as well as the organism. In vitro overexpression of GFP has been shown

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to interfere with the ubiquitine-proteasome pathway, resulting in cellular toxicity[315]. In vivo studies with the administration of AAV6 CMV renilla Enhanced GFP in mice have shown the dose dependent toxicity in mouse muscles[314,321]. This GFP mediated toxicity was evident with massive inflammatory lesions near the injection site which can be attributed to the overexpression of the foreign transgene which would induce the host immune response. Lower doses of the GFP vector were well tolerated in these mice. However, the subtoxic doses of the vector coexpressing GFP and shRNA rendered the vector practically useless with respect to transduction as well as target gene silencing. Recent study also demonstrated that AAV9 mediated expression of a foreign, n (GFP), but not self-recognized, protein triggered complete immune response in nonhuman primates, irrespective of the route of administration[322]. These results corroborate the necessity of removal of GFP from any gene therapy vector to be used for the human clinical trials.

Considering the adverse effects of GFP, it seems pretty obvious just to remove the transgene from the vector construct. However, deleting the GFP transgene is not enough as it would drastically reduce the size of the total cassette placed between AAV ITRs. It is important to maintain the optimal size of the cassette to achieve efficient packaging. As mentioned earlier, there is an upper size limit of 2.7kb for the transgene cassette placed between the ITRs where packaging efficiency reduces as the cassette size increases[323-325]. It is also shown the efficiency of packaging also reduces when the cassette is truncated with the minimum cassette size for efficient packaging to be around 1500bp[326]. This was confirmed by determining the expression of eGFP under neuron
specific promoter placed in expression cassettes that range from 1.3kb to 2.0kb. It was observed that highest eGFP expression levels were obtained when the expression cassette of 2.0kb was placed between the ITRs while eGFP expression was reduced to almost 30% when the cassette size was less than 1.5kb. Although these results were obtained with a different promoter than the one used in our study, these results strongly support the need of maintaining the optimum size of the expression cassette between the AAV ITRs to achieve the maximum efficiency in packaging.

To maintain the optimum size of our total construct to be around 2.0kb to achieve its efficient packaging into AAV9 capsids, we sought to replace the GFP expression cassette with stuffer sequence. According to the FDA guidelines for administration of nucleic acids in treatment of human diseases, the proposed DNA molecule needs to meet certain criteria, the first and foremost requirement being lack of immunogenicity. FDA guidelines recommend that the proposed macromolecule itself must not elicit innate or adaptive immune response. Any foreign transgenes, including the therapeutic product and others encoded by the macromolecule must not activate autoimmunity. The therapeutic agent must not elicit any local reactogeneity and systemic toxicity. All of these parameters have been met by a variety of plasmid DNA vaccines that are currently being used in human clinical trials in US[327]. DNA vaccines include purified preparations of plasmid DNA that are administered directly in humans as therapeutic agents. DNA vaccines utilize several plasmids as a backbone for the expression of the therapeutic transgene. Here, we utilized the DNA fragments from these FDA approved plasmid
backbones that do not code for any protein involved in bacterial replication and selection or the therapeutic effect. These plasmids include pVax1, pcDNA3, pBLCAT3, pUCMV3, pBK-CMV, pUC18, pGA2. The DNA sequences from these plasmids were put together to generate a long stretch of the stuffer DNA sequence. This method of stuffer design was chosen over random DNA sequence generation as unlike the random sequence, all of these DNA fragments have been already cleared for the administration into human by FDA. This would maximize the suitability of the total stuffer sequence for the human administration. However, the stuffer construct will still be assessed for its immunogenicity as a whole.

This is the first report of generation of such stuffer sequence that will be used for the viral mediated delivery of shRNA for the treatment of disease. Here, we have successfully constructed a non-coding stuffer sequence that can be utilized for the coexpression of shRNA against SOD1 via AAV9 mediated delivery. To comply with FDA’s recommendation of establishing a test to identify and distinguish the final product, we have shown that the newly synthesized clinical AAV9 SOD1 shRNA construct contains only SOD1 shRNA expression cassette and the stuffer sequence by restrictionendonuclease digestion. To meet the FDA guideline of developing the potency assay, we also show that clinical AAV SOD1 shRNA construct is devoid of any foreign transgenes and can efficiently reduces the SOD1 protein levels in vitro. These results strongly encourage the use of clinical AAV9 SOD1 shRNA for further long-term toxicity studies and in ultimate human clinical trials for ALS patients.
Chapter 4

Galectin-1 mediated immunomodulation of microglia improves motor neuron survival in ALS

4.1 Introduction

Neuroinflammation is the hallmark of the ALS disease pathogenesis and microglia are considered to be the most crucial player involved in the neuroinflammation induced motor neuron toxicity in ALS[113-117]. It has been shown that in ALS mice, microglossis occurs even before disease onset. Role of mutant SOD1 in the activation of microglia has been confirmed by in vitro as well as in vivo studies[30,118-121]. Both intracellular and extracellular mutant SOD1 can activate ALS microglia. Removal of mutant SOD1 from ALS microglia has been shown to rescue to the motor neuron toxicity in vitro and extend the median survival in ALS mice by delaying the disease progression. As the disease progresses, ALS microglia adopt the classically activated M1 microglial
phenotype which is characterized by the secretion of proinflammatory cytokines like nitric oxide, TNF-α as well as IL-1, IL-10 that are capable of causing motor neuron toxicity[120,173-176,339]. Microgliosis has also been detected in post mortem spinal cord sections of human ALS patients, both familial and sporadic[115,117,340-342], suggesting that neuroinflammation is a common mechanism for microglia mediated motor neuron degeneration. Hence, a lot of focus has been concentrated on developing therapeutic strategies to control the neuroinflammation in central nervous system in ALS.

Several strategies have been already tested in ALS mice to attenuate the inflammation in CNS[129]. Administration of small compounds like minocycline, thalidomide etc. via intraperitoneal, intravenous routes has been shown to reduce microglial activation, improve disease symptoms and extend the median survival in ALS mice. Utilization of viral vectors expressing neurotrophic factors like IGF-1 has been shown to affect the microgliosis and improve the disease outcome as well. Passive immunization with anti-IFN-γ antibodies have also shown to modulate the neuroinflammation in ALS mice with significant extension in the lifespan. In spite of achieving successful therapeutic effects in the mouse models, all of these strategies have failed miserably in the human clinical trials with minimum to no benefits at all to the human ALS patients. One of the reasons for the failure of these agents could be the involvement of multiple signaling cascades in the neuroinflammation. As it is impossible to predict which pathway will predominate in a particular patient, modulation of a specific inflammatory pathway by a single agent might not confer sufficient therapeutic
benefits. Hence, it is important to target the most upstream regulator of these disparate inflammatory cascades for global modulation of neuroinflammation.

A recent study from our lab has shown that NF-κB in microglia can be considered as this most upstream target for the modulation of neuroinflammation in ALS[119]. NF-κB is a key regulator of several inflammatory cascades. NF-κB activation occurs at the disease onset itself and the level of NF-κB activation in microglia is positively correlated with the microglial activation and disease progression in ALS. Downregulation of NF-κB in ALS microglia results in the reduced expression of anti-inflammatory cytokine expression, reduced secretion of TNF-α, NO as well as enhanced motor neuron survival in coculture studies. Similarly, in vivo downregulation of NF-κB, specifically in microglia, results in reduced microglial activation in SOD1G93A mice which is accompanied by significant extension in the median survival of these mice by delay in the disease progression. These results confirm a definitive role of NF-κB activation in microglial mediated motor neuron toxicity in ALS, thus providing a better therapeutic target for the modulation of global neuroinflammation in ALS. Although NF-κB seems to be the most suitable therapeutic target for the treatment of ALS, downregulating this pathway specifically in microglia is still not possible. Neither any of the currently available agents have the capacity to downregulate the NF-κB pathway nor there are any viral vectors available that can target the microglia in CNS.

Recent studies show that the deactivation of classically activated microglia by Galectin-1 results in the improvement of disease phenotype in the experimental
autoimmune encephalitis (EAE) mouse model of Multiple Sclerosis[254]. Extracellular Galectin1 can bind to the CD45 receptor on M1 microglia and activate the downstream signaling cascade that results in the downregulation of multiple key regulators of inflammation including p38MAPK, pCREB and especially NF-κB. Interestingly, Galectin-1 is exclusively produced by astrocytes in the CNS which is then secreted out and can exert a paracrine effect on the surrounding microglia. This crucial role of astrocytes was further confirmed by adoptive transfer of Galectin-1 secreting astrocytes in the brain of EAE mice which resulted in reduced activation of microglia and improvement of disease phenotype in the EAE mice. Thus, astrocytes can be utilized to modulate the microglial activation by downregulating NF-κB pathway via Galectin-1 in CNS.

In the proposed study we wanted to determine the efficacy of Galectin-1 in modulating ALS microglia activation and its effects on the microglia mediated motor neuron toxicity. Here, we show that the treatment of ALS microglia with extracellular Galectin 1 results in enhanced motor neuron survival in the coculture system. We also show that Galectin 1 conditioning of ALS microglia results in reduction of their M1 activation. This is evident by reduced expression of the M1 markers like CD68, CD86 and iNOS. Galectin-1 treated microglia also show reduced secretion of inflammatory cytokines like TNF-α. Importantly, reduced microglial activation and enhanced motor neuron survival is accompanied by downregulation of the NF-κB pathway activation. These results suggest that Galectin 1 can be considered as a possible therapeutic agent
for modulating the ALS microglia in vivo.

4.2 Materials and methods

4.2.1 Vectors

In order to obtain mouse Galectin-1 CDS, total RNA from mouse lumbar spinal cord tissue was obtained using RNAeasy kit. The RNA was converted to cDNA using Qiagen RT first strand synthesis kit. Mouse Galectin 1 was PCR amplified from the lumbar spinal cord cDNA and further cloned into a lentiviral vector pBOB-empty under CMV promoter using XbaI site.

<table>
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<tr>
<th>Construct</th>
<th>Primer</th>
<th>Application</th>
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<tr>
<td>Ms Galectin 1</td>
<td>F 5’-AATTATCTAGAATCATGGCCTGTGTGTCGC-3’</td>
<td>Cloning</td>
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<td></td>
<td>R 5’-AATTATCTAGAGCTTCACTCAAAGGCCACGCAC-3’</td>
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</tr>
<tr>
<td></td>
<td>R 5’-CTCTTGTAGTGCACGCAGATTTC-3’</td>
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4.2.2 Cells

HEK-293 cells were maintained in Iscove's modified Dulbecco's media containing 10% FBS, 1% L-glutamine, and 1% penicillin/streptomycin. Upon reaching ~60% confluence, HEK-293 cells were transfected with pBOB-Galectin1 or pBOB-RFP plasmids in Iscove's modified Dulbecco's media containing 10% FBS, 1% L-glutamine,
and 1% penicillin/streptomycin. 24 hrs post transfection, the medium was changed to Iscove's modified Dulbecco's media containing 2% FBS, 1% L-glutamine, and 1% penicillin/streptomycin. The supernatant was collected at every 24 hr interval for 3 days post transfection. Supernatant was filtered through 0.2µ filter and stored frozen at -80°C. Microglia were pre-incubated with HEK-293 supernatants mixed with microglia medium in a ratio of 1:1, for 3 days before the coculture.

4.2.3 Isolation and culture of adult primary microglia

Adult microglia were isolated from brains of SOD1\textsuperscript{G93A} and WT littermates as previously described (Moussaud and Draheim, 2010) with minor modifications. 4-month old SOD1\textsuperscript{G93A} and WT littermate mice were deeply anesthetized and perfused transcardially with ice-cold Ringers solution (Fisher Scientific). Brains that appeared to not be fully exsanguinated were discarded. Brains were fragmented with a scalpel and incubated with an enzymatic solution containing papain for 60 minutes at 37°C, 5% CO2. The papain solution was quenched with 20% FBS in HBSS and centrifuged for 4 minutes at 200g. The pellet was resuspended in 2ml of 0.5 mg/ml DNase I (Worthington Biochemical) in HBSS and incubated for 5min at room temperature. The brain tissue was gently disrupted with fire-polished Pasteur pipettes and then filtered through a 70 micron cell strainer (Fisher Scientific) and centrifuged at 200g for 4 minutes. The resulting pellet was then resuspended in 20ml of 20% isotonic Percoll (GE healthcare) in HBSS. 20mL
of pure HBSS was carefully laid on top the percoll layer and centrifugation was
performed at 200g for 20 min with slow acceleration and no brake. The interphase layer
containing myelin and cell debris was discarded, and the pellet containing the mixed glial
cell population was washed once with HBSS and suspended in Dulbecco’s modified
Eagle’s/F12 medium with GlutaMAX™ (DMEM/F12) supplemented with 10% heat
inactivated FBS, antibiotic-antimycotic (all from Life Technologies) and 5 ng/ml of
carrier-free murine recombinant granulocyte and macrophage colony stimulating factor
(GM-CSF) (R&D systems). The cell suspension from four mouse brains was plated on a
15cm² plate (Corning) coated with poly-l-lysine (Sigma) and maintained in culture at
37°C in a 95% air/5% CO2. The medium was replaced every 3 days until the cells
reached confluency (after approximately 2 weeks). After the glial layer becomes
confluent, microglia form a non-adherant, floating cell layer that can be collected,
replated, and cultured for an extended period of time. After collecting the floating layer,
microglia were incubated for 3 days without GM-CSF before re-plating for co-culture
with MNs. Collected microglia were characterized by immunocytochemistry.

4.2.4 MN differentiation

Mouse embryonic stem cells expressing GFP driven by the Hb9 promoter (HBG3
cells, kind gift from Tom Jessell) were cultured on primary mouse embryonic fibroblasts
(Millipore) and differentiated to MNs with the addition of 2 μM retinoic acid (Sigma) and
2 μM purmorphamine (Calbiochem). After 5 days of differentiation, the embryoid bodies were dissociated and sorted for GFP on a FACS Vantage/DiVa sorter (Becton Dickinson).

4.2.5 Microglia/MN Coculture

Culture adult microglia were conditioned with supernatants of HEK-293 cells transfected with pBOB-Galectin1 or pBOB-RFP mixed 1:1 with fresh microglia medium, for 3 days before the coculture. Hb9-GFP+ MNs were plated in 96-well plates coated with poly-ornithin (10 μg/ml, Sigma) and laminin (5 μg/ml, Invitrogen) at a density of 6,000 cells per well in 100 μl MN medium containing DMEM:F12 (Invitrogen), 5% horse serum, 2% N2 (Invitrogen), 2% B27 (Invitrogen) + GDNF (10 ng/ml, Invitrogen), BDNF (10 ng/ml, Invitrogen), and CNTF (10 ng/ml, Invitrogen). The day after, preconditioned microglia were plated on top of MNs at a density of 35,000 cells per well in 100 μl MN media. The coculture plate was imaged each day by the IN Cell Analyzer 6000 (GE Healthcare). Images were processed and analyzed using IN Cell Developer Toolbox 1.9 and IN Cell Analyzer Workstation 3.7 software (GE Healthcare) to quantify number of surviving GFP+ MNs per well. Depending on the assay, culture medium or cell lysates were prepared after 3 day coculture.
Figure 4.1 Experimental setup for Coculture of Galectin 1 conditioned microglia and motor neurons. HEK 293 cells were transfected with LV-Galectin1 or LV-RFP plasmids and supernatants were collected and filtered over 72hrs post-transfection. Adult microglia were cultured from endstage ALS mice and age-matched WT mice. Microglial cells were conditioned with supernatants from LV-Galectin 1 or LV-RFP transfected HEK293 cells for 3 days prior to coculture. Conditioned microglia were plated on top of MS ES cell derived motor neurons and cocultured for 72 hrs.
4.2.6 Immunohistochemistry/Immunocytochemistry

Mouse spinal cords were stained as floating sections. Tissues were washed three times for 10 minutes each in Tris-buffered saline (TBS), then blocked in a solution containing 10% donkey serum, 1% Triton X-100, and 1% penicillin/streptomycin for 2 hours at room temperature (RT). All the antibodies were diluted with the blocking solution. Primary antibodies used were as follows: rabbit anti-Galectin 1 (1:200; Abcam, Cambridge, MA), goat anti-ChAT (1:50; Millipore, Billerica, MA), mouse anti-GFAP (1:200; Millipore), chicken anti-GFAP (1:400; Abcam, Cambridge, MA), and rabbit anti-Iba1 (1:400; Wako, Richmond VA). Tissues were incubated in primary antibody at 4 °C for 48–72 hours and then washed three times with TBS. After washing, tissues were incubated for 2 hours at RT in the appropriate fluorescein isothiocyanate-, Cy3-, or Cy5-conjugated secondary antibodies (1:200; Jackson Immunoresearch, Westgrove, PA) and DAPI (1:1,000; Invitrogen). Tissues were then washed three times with TBS, mounted onto slides, and then coverslipped with PVA-DABCO. All images were captured on a Zeiss laser-scanning confocal microscope.

MN-microglia cocultures or cell-monolayers grown on Polyornithin/Laminin coated coverslips were fixed with 4% Paraformaldehyde solution for 20 min, followed by 3 washes with TBS. Cocultures or coverslips were then blocked in a solution containing 10% donkey serum, 1% Triton X-100, and 1% penicillin/streptomycin for 2 hours at room temperature (RT), followed by incubation with primary antibody for 24-48 hrs at 4 °C. After washing with TBS for 3 times, cocultures/coverslips were incubated for 2 hrs
with appropriate secondary antibody at RT. Cocultures were finally washed with TBS and stored in TBS at 4 °C. Coverslips were washed with TBS and then mounted on slides with PVA-DABCO. Images were captured on an Olympus fluorescence microscope and a Zeiss laser-scanning confocal microscope.

Table 4.2 Primary Antibodies for IHC and ICC

<table>
<thead>
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<th>Species</th>
<th>Dilution</th>
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IHC (Immunohistochemistry); ICC (Immunocytochemistry)

Table 4.3 Secondary Antibodies for IHC and ICC

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IHC (Immunohistochemistry); ICC (Immunocytochemistry)
4.2.7 ELISAs

TNFα Quantikine ELISA kit (R&D Systems) was used according to manufacturer instructions to quantify the TNFα concentration in co-culture medium. Nitric oxide levels in the co-culture medium were determined using the Total Nitric Oxide and Nitrate/Nitrite Parameter Kit (R&D Systems) according to manufacturer instructions. Co-culture medium was collected, centrifuged for 2 minutes at 200g, and 50μL of medium was added to each well for analysis. Phospho-p65 and Total p65 ELISA kits were used according to manufacturer instructions to quantify NF-κB activation in cell lysates (Cell Signaling). All conditions were tested in triplicate.

4.2.8 M1 and M2 marker analysis

RNA from cocultured microglial cells was isolated using the RNaqueous Micro Kit (Ambion, Grand Island, NY) according to the manufacturer’s instructions. RNA was then reverse transcribed into cDNA using the RT2 HT First Strand Kit (SABiosciences). RNA of 12.5 ng was used in each Q-PCR reaction using SyBR Green (Invitrogen) to establish the relative quantities of CD68, CD86, iNOS (M1 markers) and Arginase1, IL-10 (M2 markers) transcripts in wild-type and SOD microglia that were preconditioned with Gal1 containing medium compared with the microglia preconditioned with Lv-RFP medium. Each sample was run in triplicate and relative concentration calculated using the ddCt values normalized to endogenous actin transcript.
Table 4.4 Primers for M1 and M2 expression analysis

<table>
<thead>
<tr>
<th>Construct</th>
<th>Primer</th>
<th>Application</th>
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<tr>
<td>CD68 (Mouse)</td>
<td>F 5'-TTCTCCAGCTGTTACCTTGACCT-3'</td>
<td>qRT-PCR</td>
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<tr>
<td></td>
<td>R 5'-GTTGCAAGAGAAACATGGCCCGAA-3'</td>
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<tr>
<td>CD86 (Mouse)</td>
<td>F 5'-CCGTGCCCATTTACAAAGGCTCAA-3'</td>
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<td></td>
<td>R 5'-TCTCTGTGCCCAATAGTCTCGT-3'</td>
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<td>F 5'-CTGCTGGTGTTGACACAACATTT-3'</td>
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<tr>
<td></td>
<td>R 5'-ATGTCATGACAAAGGCGCAGA-3'</td>
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<tr>
<td>Arginase 1</td>
<td>F 5'-AACACGGCAGTGGCTTAACCTTG-3'</td>
<td>qRT-PCR</td>
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<tr>
<td>(Mouse)</td>
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<td>IL-10 (Mouse)</td>
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<td>β-Actin (Mouse)</td>
<td>F 5'-GGTGGCCCGGCTAGGCACCA-3'</td>
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<td></td>
<td>R 5'-CTCTTTTGATGTCAGCAGATTTC-3'</td>
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4.2.9 Statistical analysis

All statistical tests were performed using the GraphPad Prism (San Diego, CA) software package. Motor neuron survival, ELISAs, M1/M2 marker expression were analyzed by One-way ANOVA.

4.3 Results

4.3.1 Galectin-1 levels are elevated only at the endstage in SOD1<sup>G93A</sup> mice

To determine the role of Galectin 1 in modulating neuroinflammation, we first determined the changes in the expression levels of Galectin 1 in SOD1<sup>G93A</sup> mice as the disease progresses (Figure 4.2). Immunohistochemical analysis of Galectin 1 expression
in the spinal cords collected from SOD1<sup>G93A</sup> mice at presymptomatic, disease onset, symptomatic, late symptomatic and endstage of the disease revealed that Galectin 1 protein levels were elevated only at the endstage of the disease. Colabeling with motor neuron marker (ChAT: Choline acetyltransferase) and astrocyte marker (GFAP: Glial fibrillary acidic protein) confirmed that the Galectin 1 expression was confined to the astrocyte population in the spinal cord. These results corroborate the previous mRNA expression results where Galectin-1 mRNA was elevated only at disease endstage (126 days). As NF-κB is activated in microglia as early as the disease onset and it gradually increases with the disease progression, the lower levels of Galectin 1 present in the spinal cord initially thus can be considered insufficient to modulate this microglial activation during the progression phase of the disease. Galectin-1 expression has been reported to be regulated by NF-κB activation itself. In T cells, NF-κB activation leads to increase in the Galectin-1 expression which in turn inactivates NF-κB. This intricate loop seems to be crucial for the modulation of the immune response. Massive upregulation of Galectin 1 towards the endstage of the disease can thus be attributed to the high NF-κB activation in the astrocytes itself. However, at this stage, this late response of Galectin 1 activation might be too late to modulate the microglia mediated motor neuron toxicity.
Figure 4.2 Galectin 1 expression is elevated only at the endstage of the disease in SOD1\textsuperscript{G93A} mice. Immunofluorescence analysis of lumbar spinal cords from SOD1\textsuperscript{G93A} mice at different stages of ALS disease course showed that Galectin 1 protein expression remains at the basal levels up to late symptomatic stage and elevate only at the endstage. This acute increase in Galectin 1 expression is in contrast with the progressive NF-κB activation seen in ALS microglia and subsequent reduction in motor neuron survival. Bar = 10μm. Gal-1, Galectin 1.
Figure 4.2 Galectin 1 expression is elevated only at the endstage of the disease in SOD1$^{G^{93}A}$ mice.
4.3.2 Galectin 1 can modulate the microglia mediated motor neuron toxicity

To gain insight into the direct effects of Galectin 1 on ALS microglia, we determined whether Galectin 1 conditioning of ALS microglia can rescue the motor neuron toxicity in vitro. Galectin 1 lacks a classical signal sequence for vesicle mediated protein secretion and is secreted in the extracellular environment by an unorthodox secretory mechanism. To assess the role of secreted Galectin 1, HEK 293 cells were transfected with mouse Galectin-1 expressing plasmid as described in section 4.2. A reporter plasmid carrying RFP was used as a control plasmid. The supernatant was collected from the transfected cells for up to 72 hrs post transfection, filtered and frozen until the experiment. Overexpression of Galectin1 in HEK 293 cells was confirmed by immunocytochemistry (Figure 4.3a-h) while the secretion of Galectin 1 from HEK 293 was confirmed by Galectin 1 ELISA on the HEK 293 supernatants post transfection (Figure 4.3j).
Figure 4.3 Overexpression of Galectin 1 results in increased levels of secreted Galectin 1 in vitro. HEK293 cells were transfected with pBOB-RFP or pBOB-Galectin 1. (a-h) Overexpression of Galectin 1 and RFP was confirmed by immunostaining of transfected cells 72hrs post-transfection. (j) Culture supernatant was collected over 3 days post-transfection while cell lysates were prepared 72hrs post-transfection. Galectin 1 ELISA confirmed the overexpression of Galectin 1 in pBOB-Galectin1 transfected cells as compared to pBOB-RFP transfected cells. Corresponding increase in the secreted Galectin 1 was also found in supernatant from Galectin 1 transfected cells.
Figure 4.3 Overexpression of Galectin 1 results in increased levels of secreted Galectin 1 \textit{in vitro}.
Figure 4.3 continued

Figure 4.3 Overexpression of Galectin 1 results in increased levels of secreted Galectin 1 *in vitro*.
To determine the effect of Galectin 1 on microglia mediated motor neuron toxicity, an *in vitro* coculture system of cultured adult microglia and wild-type HB9:GFP motor neurons was established as described previously. Cultured microglia from endstage SOD1<sup>G93A</sup> mice and age-matched wild type mice were conditioned with the Galectin-1 or RFP containing HEK supernatant, mixed 1:1 with fresh microglial medium for 3 days before coculture. Conditioned microglia were then washed and plated on the monolayer of HB9:GFP motor neurons differentiated from wild-type ES cells using RA/Puromorphamine or SAG. After 72 hrs in coculture, ALS microglia resulted in around 40% reduction in motor neuron survival as compared to the wildtype microglia. ALS microglia also affected the axonal length of the cocultured motor neurons as compared to the wild-type microglia. Preconditioning of ALS microglia with Galectin-1 resulted in almost complete rescue of the motor neuron death (Figure 4.4). Galectin 1 conditioning of ALS microglia also rescued the axonal length defect exerted by the ALS microglia. To determine if the coincubation of ALS microglia and MN with Galectin 1 would affect the motor neuron survival, we preconditioned the microglia with Galectin-1 supernatant but also added recombinant Galectin 1 (10ug/ml) in the coculture medium itself. However, coincubation of Galectin-1 did not exert any additive effect on the motor neuron survival.
Figure 4.4 Extracellular Galectin 1 reduces ALS microglia mediated motor neuron toxicity in vitro. Cultured microglia from endstage SOD1<sup>G93A</sup> mice and age matched wildtype mice were treated with HEK293 condition medium containing Galectin 1 or RFP for 3 days before the coculture. (a) Representative microscopic fields of HB9 GFP<sup>+</sup> motor neurons after 1 day (24hrs) and 3 days (72hrs) in coculture with WT or SOD1<sup>G93A</sup> microglia, conditioned with RFP or Galectin-1. (b,c) Quantification of the HB9:GFP<sup>+</sup> MN at the end of coculture. SOD1<sup>G93A</sup> microglia treated with RFP containing medium showed Galectin 1 conditioning of SOD1<sup>G93A</sup> microglia resulted in rescue of the motor neuron toxicity.
Figure 4.4 Extracellular Galectin 1 reduces ALS microglia mediated motor neuron toxicity in vitro
Figure 4.4 continued

Figure 4.4 Extracellular Galectin 1 reduces ALS microglia mediated motor neuron toxicity *in vitro.*
4.3.3 Galectin-1 modulates ALS microglia activation via NF-κB pathway

Motor neuron toxicity of ALS microglia has been attributed to their activation to the pro-inflammatory M1 phenotype. M1 activated microglia have been shown to secrete pro-inflammatory cytokines like TNF-α, NO in coculture experiments. Reduction of these proinflammatory cytokines in ALS microglia have been shown to reduce their neurotoxic potential. Here, we wanted to determine whether enhanced motor neuron survival after Galectin 1 preconditioning of ALS microglia was due to the modulation of neuroinflammatory status of ALS microglia. To determine this, coculture supernatants were screened for the TNF-α levels. Galectin 1 conditioning of ALS microglia resulted in the reduced secretion of TNF-α as compared to non-conditioned ALS microglia (Figure 4.5).
Figure 4.5 Conditioning of ALS microglia with Galectin 1 results in reduced secretion of TNF-α. (a-b) ELISA based quantification of TNF-α levels in the media collected from RFP- or Galectin 1-conditioned wildtype or SOD1<sup>G93A</sup> microglia, cocultured with HB9:GFP<sup>+</sup> MN. Galectin-1 conditioning reduced the levels of TNF-α secreted from SOD1<sup>G93A</sup> as well as wild-type microglia.

We also determined the expression levels of other M1 activation markers like CD68, CD86 and M2 markers (Arginase 1, IL-10) by qRT PCR. Galectin1 conditioned ALS microglia did not show significant reduction in expression levels of all of these markers. However, Galectin1 conditioning resulted in significant increase in Arginase 1 in ALS microglia. Higher Arginase 1 expression is the indication of anti-inflammatory, neuroprotective M2 phenotype of microglia. Thus, Galectin1 conditioning may not result in the complete reduction of neurotoxic M1 phenotype of ALS microglia but may play
important role in counterbalancing the expression of neuroprotective M2 gene expression.

Figure 4.6 Galectin 1 conditioning of ALS microglia affects M1 and M2 marker expression levels *in vitro*. qRT-PCR based quantification of expression levels of CD68, CD86 (M1 markers) and Arginase 1, IL-10 (M2 markers) was performed on RFP or
Figure 4.6 continued

Galectin 1 conditioned wildtype or SOD1<sup>G93A</sup> microglia, cocultured with HB9:GFP<sup>+</sup> MN. Although only modest changes in M1 marker expression were observed, Galectin 1 conditioning significantly increased Arginase 1 expression (M2 marker) in SOD1<sup>G93A</sup> microglia. Similar trend was also observed with IL-10 expression.

Finally, to determine if Galectin1 can directly affect the NF-κB activation in ALS microglia, protein extracts from galectin1 conditioned microglia, cocultured with motor neurons, were prepared and analyzed for levels of phospho-p65 protein. It was found that Galectin 1 conditioned ALS microglia showed significantly reduced levels of phospho-p65/Total p-65 as compared to non-conditioned ALS microglia (Figure 4.6). This confirms that the Galectin1 conditioning of ALS microglia results in the reduced activation and reduced motor neuron toxicity which can be attributed to reduced NF-κB pathway activation.
Figure 4.7 Galectin 1 conditioning modulated NF-κB activation in ALS microglia. (a-b) NF-κB activation in RFP- or Galectin 1-conditioned wild-type and SOD1\(^{G93A}\) microglia in coculture with HB9:GFP\(^{+}\) MN was determined by performing ELISA based quantification of phospho-p65 and Total p-65. Galectin-1 conditioning of SOD1\(^{G93A}\) microglia resulted in lower levels of phospho-p65/Total p-65 as compared to RFP treated SOD1\(^{G93A}\) and wild-type microglia, suggesting reduced NF-κB activation.
4.4 Discussion

Neuroinflammation has been a center of attraction for therapy development in ALS[129]. Microglial activation is the major contributor to this neuroinflammation as seen in rodent models as well as human ALS patients. As the microglial activation goes hand in hand with disease progression in ALS, several strategies to reduce this microglial activation have been implicated with the anticipation of slowing the disease progression. Activated microglia have been shown to produce several pro-inflammatory cytokines like nitric oxide, TNF-α as well as interleukins like IL-1B which are shown to be neurotoxic. Intraperitoneal administration of Minocyclin, an inhibitor of microglial activation, has been shown to improve the motor performance and extend the median survival in ALS mice[123-126]. Thalidomide and lenalidomide have been shown to reduce the TNF-α production, reduce the motor neuron loss and significantly extend the survival of SOD1<sup>G93A</sup> mice when administered before disease onset [179,180]. Post onset administration of lenalidomide has also shown to provide beneficial effects in ALS mice[180]. Cyclooxygenase-2 is a key molecule in the inflammatory pathways. COX-2 inhibitor CELEBREX has been shown to provide significant neuroprotection and reduced astrogliosis and microgliosis in ALS mice with delay in disease onset and prolonged survival[343]. Based on these highly successful proof-of-principle mouse studies, all of the aforementioned compounds and their derivatives have been tested in the human clinical trials for ALS.
Unfortunately, all of the above mentioned compounds and their derivatives have failed to provide any beneficial effects on the disease outcome of ALS patients[128,129]. The translational failure might be explained in part by following reasons: a) Biodistribution and bioavailability of the therapeutic agent significantly affects its beneficial effects. Both of these qualities are dependent on the time interval of drug administration and the stability of the drug itself. Considering the smaller size, clear disease manifestation and shorter time span of the disease duration, it is easier to estimate the correct dosage, time interval and route of administration for these therapeutic agents in mice. However, it is extremely difficult to achieve the same when it comes to human patients. Therefore, therapeutic strategies that can provide local and continuous production of the therapeutic agent within the central nervous system itself may be more effective in providing beneficial effects. b) Each of these compounds only target a particular pathway from the milieu of cascades involved in the inflammatory signaling. Even though modulating such individual pathways provides significant therapeutic benefits in the mouse studies, whether the same mechanism will predominate in any particular patient is unpredictable. Owing to the non-cell autonomous nature of ALS[66,81,344], it is also possible that the modulation of a particular pathway in one particular cell type may be overtaken by another neurotoxic signaling mechanism in the same or surrounding cells, thus negating the beneficial effects of the treatment on the final disease outcome. Therefore, a neuroprotective agent that can target multiple neurotoxic pathways in multiple cell types may provide the best therapeutic effect in ALS.
Here, we determined the efficacy of one such factor, Galectin-1 in modulating the neuroinflammation and motor neuron toxicity in ALS. Our results show that conditioning of ALS microglia with Galectin1 results in reduced motor neuron toxicity in in vitro coculture assays. We also show that Galectin 1 conditioning of ALS microglia results in the reduced secretion of proinflammatory cytokines like TNF-α. This effect is also accompanied with increased expression of M2 markers like Arginase 1. Remarkably, our studies show that this immunomodulation of ALS microglia by Galectin1 is dependent on the downregulation of NF-κB activation which is the key pathway that controls multiple downstream inflammatory pathways. Together, these data show that Galectin1 mediated deactivation of ALS microglia results in enhanced motor neuron survival by regulating NF-κB activation.

The importance of NF-kB pathway in neuroinflammation is well appreciated[119,345-349]. Its definitive role in ALS pathogenesis has been proven with transgenic expression of a dominant negative IKKB, specifically in microglia in SOD1G93A mice[119]. NF-κB downregulation in ALS microglia has been shown to delay the disease progression and significantly extend the lifespan of SOD1G93A mice. In vivo as well as in vitro studies have shown that downregulation of NF-κB in ALS microglia directly affects their neurotoxic potential by reducing the production and secretion of proinflammatory cytokines and interleukins. Based on these results, NF-kB downregulation in microglia can be considered as a suitable therapeutic strategy for ALS. However, the main obstacle in this endeavor is the inability to downregulate NF-κB in
ALS microglia, post-nataly and specifically. Currently available anti-inflammatory agents do not modulate NF-κB specifically in microglia. As NF-κB is involved in other essential cellular processes, global downregulation of this pathway using pan NF-κB inhibitors is extremely risky and highly unrecommended. Also, none of the available delivery approaches, including viral vectors can target microglial cells in vivo. Even, intravenous and intrathecal administration of AAV9 fail to transduce microglial cells in CNS[214,221,223]. Hence, to date, the post-natal downregulation of NF-κB pathway directly in the ALS microglia is practically unachievable.

Although direct regulation of NF-κB in adult microglia is difficult, it is shown that other non-neuronal cells in CNS can be utilized to achieve the same goal. Studies in EAE mouse model have shown that Galectin1, expressed and secreted by astrocytes, have the capacity to downregulate multiple key regulators of inflammation in microglia, including NF-κB[254]. Adoptive transfer of Galectin1 secreting astrocytes in EAE mouse brains resulted in the deactivation of classically activated M1 microglia in CNS, followed by amelioration of disease symptoms. This study suggests that Galectin1 mediated crosstalk between astrocytes and microglia can be utilized in immunomodulation of the later partner in ALS as well. Our recent study shows that intravenous as well as intrathecal administration of AAV9 results in efficient transduction of astrocytes in ALS mice[313], even when AAV9 is administered after the disease onset. Hence, AAV9 mediated delivery approach can be utilized to overexpress Galectin 1 in ALS astrocytes during the disease progression. As astrocytes have the inherent capacity to secret the
Galectin 1, overexpression of Galectin 1 in astrocytes will result in the continuous and local secretion of Galectin 1 in ALS spinal cord, thus affecting the microglial cells in the extracellular environment. Thus, immunomodulation of ALS microglial can be achieved indirectly by AAV9 mediated targeting of astrocytes. Elevated levels of galectin-1 in ALS spinal cords throughout the disease progression will thus help in reducing the microglial activation and subsequent motor neuron toxicity and provide the beneficial effects on the disease outcome.

Neuroprotective effects of galectin 1 are not solely limited to the immunomodulation of microglial activation in CNS. Galectin 1 has been shown to act on multiple cell types in CNS and periphery to achieve neuroprotection[256,350]. Galectin 1 has been shown to provide axon regenerative neuroprotection by activating the macrophages to secrete neurotrophic factors in mouse models of peripheral nerve axotomy[277]. Most importantly, Galectin-1 has been shown to induce the production and secretion of BDNF in astrocytes and provide neuroprotection in ischemic rat brains[278,279]. Intramuscular administration of oxidised Galetin-1 has also been shown to improve neuroprotection and extend the life span of SOD1$^{G93A}$ mice[281,284]. A very recent study also showed that the transgenic removal of Galectin 1 from ALS astrocytes exacerbated the disease progression in SOD1$^{G93A}$ mice, confirming the neuroprotective role of Galectin 1 in ALS astrocytes[285].

Finally, by utilizing the AAV9 mediated delivery approach, we can modulate the two important players in the cell-autonomous toxicity of ALS. Post-natal suppression of
mutant SOD1 combined with overexpression of Galectin 1 in astrocytes may provide the maximum therapeutic benefits by direct reduction of mutant SOD1 mediated toxicity from ALS astrocytes and indirect reduction of the microglia mediated motor neuron toxicity by Galectin 1 mediated immunoregulation.
Chapter 5

Summary & Future directions

5.1 Summary of results

Amyotrophic Lateral sclerosis is one of the most debilitating neurodegenerative disorders, affecting thousands of people in the United States itself[2]. It not only affects the patients suffering with it but also the other individuals who are constantly involved in taking care of the patient with progressive ALS symptoms. With the tremendous physical, psychological and financial burden ALS puts on the patients as well as caregivers, it is imperative to develop successful therapeutic treatment for this dreadful disease. The goal of the study presented here is to determine the efficacy of novel therapeutic approaches for the amelioration of ALS which can be clinically translated for the treatment of human ALS patients.
With almost 90% of cases being sporadic, most of the knowledge regarding the pathogenesis ALS has been obtained from studying the mouse models of familial ALS, especially the ones harboring human mutant SOD1[31,32]. Some of the pioneering studies in these mice have proven that mutant SOD1 is the main culprit of the non-cell autonomous motor neuron toxicity in ALS[82,170]. Is it shown unequivocally that the transgenic removal of this toxic protein from different cell types results in survival benefits in different mutant SOD1 ALS mouse models. Interestingly, misfolded, wildtype SOD1 is also shown to be involved in similar motor neuron toxicity in sporadic ALS, suggesting that SOD1 removal can be considered as a common therapeutic treatment for both familial and sporadic ALS[87,142,144]. Here, we show the suitability of AAV9 mediated gene therapy approach for treatment of familial ALS patients. Our study shows that intravenous administration of AAV9 in neonatal as well as adult mice results in widespread CNS transduction. As seen in earlier studies, neonatal administration of AAV9 in ALS mice primarily transduces motor neurons while administration in adult mice predominates astrocyte transduction. We show that downregulation of mutant SOD1 by AAV9 mediated shRNA delivery provides significant extension in the median survival of fast progressing SOD1^G93A mice. In accordance with the previous transgenic studies, removal of mutant SOD1 from motor neurons by neonatal AAV9 shRNA SOD1 injection results in delay of the disease onset as well as disease progression while adult injection of AAV9 shRNA SOD1 affects the disease progression phase due to predominant astrocytes involvement. Additionally, our study shows that the intravenous administration of AAV9 SOD1 shRNA in a slow progressing SOD1^G37R, even after the
disease onset, results in significant extension of median survival by prolonging the late disease progression phase. With our long-term safety assessment studies, we also show that AAV9 shRNA SOD1 administration is safe and well-tolerated in wild-type mice. Finally, translating our therapeutic regimen to the human settings, we show that intrathecal delivery of AAV9 shRNA SOD1 to non-human primates results in widespread CNS transduction, accompanied with efficient SOD1 knockdown throughout the spinal cord, thus setting the stage for the potential human clinical trial for AAV9 mediated shRNA SOD1 delivery as a therapeutic approach for ALS.

Neuroinflammation plays a crucial role in driving the progression phase of ALS. Reactive microglia are shown to be the key players responsible for this overwhelming neuroinflammation and subsequent motor neuron death[113-117]. Multitude of studies have shown that the modulation of microglial inflammatory response using variety of small molecule compounds results in enhanced motor neuron survival and prolonged lifespan in SOD1 transgenic mice. However, most of these exogenously administered compounds failed to provide any therapeutic benefits in the human clinical trials[129,181]. Here, in the second part of the study, we sought to determine the efficacy of an endogenous ligand, astrocyte-secreted Galectin 1, in modulating the activation of ALS microglia and subsequent motor neuron toxicity. In consensus with the previous results, we show that Galectin 1 expression remains at minimum throughout the disease progression and only elevates at the end stage of the disease. This suggests the lack of this immuno-modulatory molecule in CNS may be one of the reasons for the
overwhelming neuroinflammation in ALS spinal cords. Our results also demonstrate the neuroprotective role of extracellular Galectin 1 on microglia-mediated motor neuron toxicity *in vitro*. We show that Galectin 1 can reduce the secretion of proinflammatory cytokines like TNF-α from ALS microglia while upregulating the expression of Arginase 1, suggesting that Galectin 1 conditioning may affect the polarization of ALS microglia from M1 to M2 phenotype. We show that effects of Galectin 1 on microglia are mediated via suppression of the proinflammatory signaling cascades. Finally, we also show that Galectin 1 treatment of ALS microglia results in the downregulation of NF-kB activation, thus inhibiting one of the key regulatory pathways involved in the neuroinflammation in ALS.

5.2 SOD1 targeted therapies

Since the development of first SOD1 transgenic mouse model of ALS, various studies have been conducted to determine the role of mutant SOD1 in the pathogenesis of ALS. Although there is still no consensus regarding the exact mechanism of pathogenesis, the two decades-long research endeavor has well proven the simultaneous expression of mutant SOD1 within multiple cell types as a requirement for the complete manifestation of ALS in mice[66,170]. Although motor neuron is the primary cell type affected in ALS, presence of mutant SOD1 in other non-neuronal cells like astrocytes, microglia has shown to be critical in motor neuron toxicity in ALS. Milestone studies with transgenic ALS mice have shown that pre-natal removal of mutant SOD1 from any
of these cell types results in amelioration of the disease symptoms and extension of the median survival of ALS mice[30,82]. In vitro studies have corroborated the SOD1 dependent motor neuron toxicity exerted by ALS astrocytes and microglia[87,119]. With such overwhelming evidence, post-natal downregulation of mutant SOD1 has been one of the most studied and the most anticipated therapeutic[207,293]. Since its discovery, RNA interference technique has gained tremendous attention for the downregulation of particular gene expression[186,187]. Small hairpin RNAs or small interfering RNAs designed to bind to a specific sequence in the target gene mRNA can cause downregulation of the target gene expression by either degradation of the transcript signal or halting the translational processing of the target mRNA. In either case, the subsequent protein expression of the target gene is reduced. shRNA based downregulation of mutant SOD1 has been extensively studied in ALS transgenic mice. Viral vector based delivery systems have been used to carry shRNA constructs to target cells in the CNS[195-197,202,310]. Viral vector-mediated SOD1 shRNA delivery in ALS mice resulted in efficient knockdown of mutant SOD1, accompanied by improvement of motor performance and significant extension of life span[205,206,209]. In spite of providing therapeutic benefits in ALS mice, these shRNA carrying vectors are postulated to be less effective in human clinical trials due to their poor transduction efficiency and biodistribution in the CNS. Each of the previously used viruses for SOD1 shRNA delivery in ALS mice differs in their individual transduction efficiencies, an inherent property of a virus to infect particular cell types[310]. Most of the previously used viruses have predominant neuronal tropism in CNS which limits the expression of the
therapeutic agent confined to neuronal cells. Biodistribution of the virus is mainly based in the route of delivery in ALS mice. Intramuscular route of delivery of LVs or AAVs in ALS mice provides limited transduction capacity by targeting only a small population of motor neurons via retrograde transport. Similarly, intraspinal and even intreparenchymal injections of AAV2 and AAV6 with shRNA SOD1 failed to achieve a widespread viral transduction in CNS. These studies have demonstrated the need for a viral vector with efficient CNS transduction and ease of administration. Here, we show that AAV9 can be utilized to achieve such widespread CNS transduction in ALS mice. Intravenous administration of AAV9, in neonatal or adult mice, results in robust transduction of motor neurons or glial cells based on the time of delivery. Overall, intravenous AAV9 SOD1 shRNA administration results in more than 50% knockdown of mutant SOD1 protein levels in the spinal cords of SOD1\textsuperscript{G93A} and SOD1\textsuperscript{G37R} mice. Finally, we show that the AAV9 SOD1 shRNA mediated knockdown of mutant SOD1 results in the improvement of motor performance and extension of median survival in both SOD1\textsuperscript{G93A} and SOD1\textsuperscript{G37R} mice.

From previous studies, it is clear that reduction of mutant SOD1 in ALS mice results in significant therapeutic extension in the life span of these mice\cite{30,82}. It is important to notice that the SOD1 reduction from individual cell type in the CNS affects the disease pathogenesis in unique ways. Reduction of mutant SOD1 from motor neurons in the spinal cord mainly delays the disease onset while mutant SOD1 downregulation from astrocytes or microglia mainly delays the progression phase of the disease.
Considering the human ALS patient situation where the patient presents himself in the clinic most probably at or after the onset of clinical symptoms, it is extremely crucial to have a therapeutic approach that can alter the progression phase of the disease[2,7,351,352]. Most of the previously employed strategies have provided therapeutic benefits in ALS mice only by delaying the disease onset with no effect on the rate of disease progression. Such strategies are bound to fail in the human clinical trials. With ALS being an adult onset disorder and a longer time required to confirm the diagnosis in the clinic, the pre-onset window for therapeutic intervention is already lost. Hence, the only treatments that can be administered at or post disease onset and can significantly extend the progression phase have a fighting chance to provide therapeutic benefits in human ALS patients. In our study, we show that AAV9 SOD1 shRNA administration in adult mice provides therapeutic survival benefits in SOD1$^{G93A}$ mice by slowing down the progression phase of the disease. Adult injection of AAV9 SOD1 shRNA results in efficient transduction of astrocytes in the spinal cords of ALS mice. Transgenic reduction of mutant SOD1 from these non-neuronal cells has already been shown to affect the disease progression in ALS mice. Here, we show that post-natal administration of AAV9 SOD1 shRNA in SOD1$^{G93A}$ mice, via intravenous route, at the time of disease onset resulted in efficient astrocyte transduction with delayed disease progression and a massive 25% increase in the overall lifespan of the mice. We also show that in slowly progressing SOD1$^{G37R}$ mice, intravenous delivery of AAV9 SOD1 shRNA, even after disease onset, results in a similar extension in the median survival by prolonging the progression phase of the disease. Together, these results suggest that the
AAV9 SOD1 shRNA approach has better potential in achieving clinically beneficial therapeutic effect in human clinical ALS trials due to its ability to prolong the disease progression.

Interestingly, our study also demonstrated that beneficial outcome of a therapeutic treatment in ALS is not only dependent on the transduction of a specific cell type involved in the disease but also on the timing of the intervention. Intravenous delivery of AAV9 SOD1 shRNA in adult SOD1\(^{G93A}\) mice resulted in the predominant transduction of astrocytes in the spinal cord. However, despite the similar levels of transduced astrocytes, administration of AAV9 SOD1 shRNA at P21 provided better protection than administration at P85. These results suggest that the maximum therapeutic benefits of the AAV9 SOD1 shRNA approach can be achieved by administering the treatment as early as possible during the disease course.

The key players, motor neurons, astrocytes and microglia, involved in the pathogenesis of ALS undergo progressive cellular and molecular changes throughout the duration of the disease\([110,111,173]\). Transcriptome analysis has shown that motor neurons and astrocytes from SOD1\(^{G93A}\) mice exhibit different gene expression profiles at different stages of the disease. Higher expression levels of apoptotic genes have been detected in motor neurons from the symptomatic stage as compared to presymptomatic stages while elevated levels of proinflammatory gene profiles were obtained from symptomatic astrocytes as compared to presymptomatic astrocytes. Another study has also proposed the presence of aberrant astrocytes that are prevalent only in the
symptomatic phase of the disease in ALS mice and are mainly responsible for the motor neuron toxicity[112]. Similar changes in microglia are also reported where ALS microglia undergo a shift from neuroprotective M2 phenotype to the classically activated neurotoxic M1 phenotype as the disease progresses[120]. It has been shown that these changes are dependent on the mutant SOD1 as the removal of mutant SOD1 from these cell types alleviates these changes[29,30,64,77,82,144,146,291,301,353]. Studies have shown that there is a gradual accumulation of misfolded mutant SOD1 in various cell types in the spinal cord as the disease manifests in ALS mice. It is not known if the steady increase in the mutant SOD1 accumulation simultaneously causes the molecular changes in different cell types or a threshold of the accumulated SOD1 toxicity needs to be achieved within individual cell types for these changes to occur. If the changes in these different cell types occur gradually throughout the disease process, they might be reversible to a certain extent after mutant SOD1 removal. However, if there occurs a molecular switch that controls the toxic phenotype of neuronal or nonneuronal cells in CNS, it may be irreversible even after the SOD1 knockdown. In this case, SOD1 knockdown could be supplemented with modulation of other downstream pathways involved in disease pathogenesis as discussed further in section ***.

One of the major concerns regarding the gene therapy approach is the safety of the treatment. Even if the therapeutic treatment results in the amelioration of the disease phenotype, it is extremely important to ascertain that the same treatment does not have any off-target effects. Viral vector mediated delivery possesses a dual possibility of
unaccounted complications, first off-target effects of the foreign DNA construct like a transgene or an shRNA and second the immune response against the viral capsid itself.[310,354] It has been shown that overexpression of shRNA can lead to the adverse effects[355]. It has been shown that viral mediated shRNA delivery in Huntington mouse models worsened the disease symptoms, increased microglial activation and resulted in demise of the animals[356]. Similar effects have been shown with AAV mediated shRNA delivery for targeting Neuropilin 1 and 2 in CNS injury models[357]. One of the possible mechanisms for shRNA toxicity is the downregulation of an off-target gene by the overexpressed shRNA. Second and the most important speculation for the potential shRNA toxicity is the saturation of endogenous miRNA pathway due to overexpression of the exogenous shRNA. Not only the shRNA but even the exogenous transgenes have been shown to cause toxicity in in vivo systems. Transgene overexpression can oversaturate the transcription-translation machinery. It can also affect protein folding pathways leading to ER stress. Accumulation of misfolded protein can oversaturate the protein degradation pathway thus affecting cellular protein homeostasis. These adverse effects are exaggerated in the already stressed out disease cells thus leading to even greater damage. Several studies have stated the toxic effects of prolonged overexpression of reporter genes like GFP in mice[314,316,317,319-321,358]. It is important to determine if the administration of the gene therapy vector exerts such adverse effects and thus undermines or even masks the actual therapeutic potential of the treatment. In our study, we designed an shRNA specifically against human SOD1 sequence to limit the
off-target effect. We also determined the effects of constitutive overexpression of shRNA and GFP transgene by administering AAV9 SOD1 shRNA in wild type mice. Long-term assessment of injected mice revealed that intravenous AAV9 SOD1 shRNA administration is safe and well tolerated. The successful non-GLP toxicology study thus provides strong evidence for pushing this treatment towards clinical trials.

Due to the ease of availability, maintenance and reasonable recapitulation of human disease condition, mouse models are extensively used for the development of therapeutic approaches. For ALS, several therapeutic compounds and gene therapy vectors have been successfully tested in different mouse models of ALS[129,213,231]. However, most of these compounds and vectors have failed in human clinical trials. This failure can be partially attributed to the phylogenetic distance between rodents and humans. In spite of having gross similarities in the anatomy and physiology between these two systems, there are definite differences at the cellular and molecular levels. To bridge the gap between these two, use of a non-human primate model has been highly encouraged. The genetic closeness and the phenotypic similarities to humans make the NHP models highly useful for preclinical studies. To translate our AAV9 mediated approach to humans, we administered the AAV9 SOD1 shRNA intrathecally in 1 year old Cynomolgus monkeys. Our results show that intra lumbar infusion of AAV9 SOD1 shRNA results in widespread spinal cord and brainstem transduction in monkeys with greater than 90% SOD1 knockdown achieved by a single injection. It is worth noting that unlike mice, intrathecal delivery of AAV9 in adult monkeys results in widespread
transduction of motor neurons as well as other non-neuronal cells, the percent transduction of these cells types is dependent on the diffusion of the viral particles from the injection site. These results suggest that unlike mice, AAV9 administration in monkey and eventually in humans will result in targeting two of the key players involved in motor neuron toxicity, hence providing even a better therapeutic effect than that observed in mouse studies.

5.3 Future of SOD1 targeted therapies

Here, we have developed an AAV9 mediated approach to downregulate mutant SOD1 expression in motor neurons and astrocytes of ALS mouse models. We have shown that a single intravenous injection of AAV9 SOD1 shRNA results in improvement of motor performance along with a significant extension in the lifespan of ALS mice. We also show that this extension in survival can still be achieved when AAV9 SOD1 shRNA is administered even after the disease onset. Finally, we have shown that AAV9 SOD1 shRNA administration is safe and well tolerated and can be translated to the non-human primates. All these results point towards the obvious future direction towards the human clinical trials.

Indeed, we have already initiated the efforts for the clinical translation of our AAV9 SOD1 shRNA approach. First, to be suitable for human administration, we have modified our construct to eliminate the nonessential components including GFP
transgene and its regulatory elements. Simple deletion of these elements is not enough as an optimal distance between the AAV ITRs needs to be maintained for the efficient packaging of the complete shRNA construct in AAV9 capsids. Hence, we have generated a stuffer DNA sequence by combining various nonessential, non-coding elements from other vectors that have been approved by FDA and are currently used in the human clinical trials. This newly generated clinical construct has been successfully retested for its efficiency to downregulate human SOD1 \textit{in vitro}. We have also determined the presence of any unwanted transcripts derived from the stuffer sequence itself by ORF analysis. Next, we plan to perform a formal toxicology study under Good Laboratory Practices (GLP) for the long term assessment of our clinical AAV9 SOD1 shRNA vector in mice as well as non-human primates to ensure the safety of AAV9 SOD1 shRNA administration in humans.

In addition to modifying the current strategy of RNAi based downregulation of SOD1 using the AAV9 mediated delivery approach in humans, it is also important to incorporate multiple mechanisms to target mutant SOD1 to achieve the maximum benefits of mutant SOD1 removal. One such strategy includes passive immunization using antibodies against mutant or misfolded SOD1. Previously, it was shown that the passive immunization by administered antibodies specific towards misfolded SOD1 provided therapeutic benefits in SOD1 ALS mice[182,183,185]. Two very recent studies have shown that expression of these misfolded SOD1 specific antibodies within the CNS itself can provide much better therapeutic effect on motor performance, disease
progression and median survival[359]. We propose to combine these two approaches by designing an AAV9 vector carrying the shRNA against mutant SOD1 and an overexpression cassette with a transgene encoding misfolded SOD1 antibody. We hypothesize that administration of such vector will provide a two tiered defense system against mutant SOD1 toxicity not only by downregulating mutant SOD1 expression itself, but also by clearing the residual mutant SOD1 protein from CNS.

Considering the plethora of studies establishing the involvement of different non-neuronal cell types in ALS pathogenesis, it is extremely important to develop strategies to target these cell types. Even though AAV9 is the best available vector for efficient transduction of the CNS, it still has limited tropism towards different CNS cell types. As shown in the current study, AAV9 SOD1 shRNA fails to target microglial cells in SOD1^G93A^ mice[214]. This leaves the motor neurons vulnerable to the microglia-mediated toxicity in ALS. Extensive efforts are being focused on engineering of the existing AAV capsids to expand the repertoire of vectors with multivalent tropism. Newly engineered recombinant AAV (rAAV) vectors have been shown to transduce microglial cells in vivo[221]. It is important to determine the feasibility and efficacy of such vectors to deliver SOD1 shRNA and SOD1 antibodies to multiple cell types within the CNS of ALS mice. Integration of these various vector systems to deliver the therapeutic agents and targeting as many cell types as possible in the central nervous system of ALS patients will certainly help us develop the best therapeutic strategy for the amelioration of this dreadful disease.

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5.4 Non-SOD1 targeted therapies

Development of ALS mouse models expressing human mutant SOD1 protein has brought to light several mechanisms involved in the pathogenesis of ALS[66,170]. Transgenic studies have not only confirmed the adverse effects of mutant SOD1 within motor neurons, but also the surrounding non-neuronal cells like microglia and astrocytes have been shown to actively participate in disease pathogenesis[30,82]. In vitro studies have shown that the presence of mutant SOD1 within microglia and astrocytes is sufficient to kill motor neurons in coculture studies[87,119,120]. It has been demonstrated repeatedly that neuroinflammation caused by activated microglia and astrocytes in CNS plays a crucial role in driving disease progression in ALS mice. Evidence of activated microglia and astrocytes has also been found in the post-mortem spinal cord tissues of sporadic ALS patients, suggesting that neuroinflammation can be considered a common pathogenic mechanism in familial as well as sporadic ALS[113-117]. Therefore, tremendous efforts have been made to correct the neuroinflammatory signaling cascades as a way to rescue motor neuron toxicity in ALS. Recent studies have shown that inhibition of the NF-kB pathway, a key regulator of inflammation, specifically in microglia results in their reduced activation leading to enhanced motor neuron survival, better motor performance and delays disease progression leading to prolonged survival of SOD1\textsuperscript{G93A} mice. [119]. Similar inhibition of this pathway in ALS astrocytes, however, did not result in any therapeutic benefits[119,360]. More importantly, constitutive activation of NF-kB pathway in wild-type microglia resulted in
the motor neuron toxicity by the wild-type microglia in coculture studies. These results confirm that microglia-induced motor neuron death is mediated by activation of NF-kB pathway which can be either SOD1 dependent or independent. As neuroinflammation is the hallmark of both familial and sporadic ALS, controlling the microglial activation by inhibiting the NF-kB pathway will certainly have significant therapeutic effects on the disease progression and survival of ALS patients. As the mouse studies involved prenatal downregulation of NF-kB using transgenic mice, it is crucial to design an approach to target microglial activation post-nataly. Here, we sought to determine the efficacy of a lectin binding protein Galectin-1, in controlling the ALS microglial activation post-nataly. Our results show that exogenous treatment of adult ALS mouse microglia with Galectin 1 can rescue the motor neuron toxicity in vitro. We also show that conditioning of ALS microglia with Galectin-1 reduced the secretion of proinflammatory cytokines like TNF-α. Finally, we show that immunomodulatory effects of Galectin-1 can be partially attributed to the downregulation of NF-kB pathway activation in ALS microglia. These studies show that Galectin1 can be utilized as a potential candidate for modulating the microglia activation in ALS.

With the overwhelming evidence of neuroinflammation in ALS, several anti-inflammatory compounds have been tested in ALS mouse models for their therapeutic effects[129]. Most of these compounds have shown to reduce the microglial activation in vitro as well as in vivo, resulting in prolonged survival of ALS mice. With the success in mouse studies, a number of compounds have also been tested in the human clinical trials.
However, almost all of the compounds have failed miserably in the human clinical trials[129,181]. A few key factors can be attributed to the failure of anti-inflammatory therapeutic strategies applied in ALS clinical trials so far. Most of the compounds used in these clinical trials target a very specific pathway in the entire neuroinflammatory response. For example, enhanced expression and secretion of TNF-α has been reported in ALS mice as well as human ALS samples. A variety of compounds that inhibit the TNF-α production have been tested successfully in ALS mouse models. However, all these compounds failed to provide significant therapeutic benefits in the human clinical trials. Considering the complex nature of ALS pathogenesis, correcting a single pathway may not be sufficient to change the disease course in human ALS patients. Hence, compounds that act on the upstream regulators of the inflammatory process (p38-MAPK, pCREB, NF-kB) and thus targeting multiple signaling cascades at the same time may provide better disease outcome in ALS.

The second most important reason for the failure of anti-inflammatory compounds in ALS clinical trials can be their inability to reach the target cells in the CNS. Central nervous system is protected from the peripheral environment by blood brain barrier (BBB). The blood brain barrier limits the molecules that can pass from the blood to the central nervous system. Almost 98% of the compounds as well as various viruses can not cross this barrier to enter the CNS. Most of the anti-inflammatory compounds tested in ALS mice were administered via subcutaneous, intraperitonal or intravenous routes[129]. Although these compounds were therapeutically successful in mice,
considering the anatomical and physiological differences between mice and humans, it is possible that the administered drug does not get the access to the CNS in humans. This may result in failure to achieve the optimal concentration of the drug within the CNS required for the therapeutic effect. Maintaining the optimal concentration in the target area is also dependent on the half-life of the drug administered. Hence, time interval for the dose administration is equally important. Along with the concentration, equal distribution of the administered drug throughout the CNS is crucial to achieve the required therapeutic effect. All of these parameters are interlinked and are basically dependent on how much dose of the compound has been administered. This itself can be limited due to the safety concerns. Hence, the maximum tolerable dose may not be sufficient enough to achieve optimum bioavailability and biodistribution of the drug required for therapeutic benefits. With all these limitations on administering the anti-inflammatory compounds and their complete failure to provide any benefits in human clinical trials, the foremost question that emerges is whether Galectin 1 is a better alternative to existing anti-inflammatory compounds and why.

The answers to these questions lie in the immuno-modulatory effects of Galectin 1 and its site of production. Galectin 1 is a 14kd lectin binding protein which is involved in modulation of adaptive as well as innate immune response[259,263,361]. Galectin 1 can induce the apoptosis of Th-1 cells, induce IL-10 secretion, inhibit T cell trafficking and reduce the production of NO from macrophages. The function of Galectin 1 has been extensively studied in the cancer field as elevated levels of Galectin 1 in tumor cells has
been related to increased immune escape and poor prognosis[265-267]. Galectin 1 has been shown to have a neuroprotective role by inducing production of neurotrophic factors from macrophages in the periphery [276,362]. In the CNS, Galectin 1 can induce the expression of BDNF in astrocytes which is shown to be neuroprotective in ischemic neuronal death[278,279]. A very recent study has shown that Galectin 1 can deactivate the classically activated microglia in EAE mouse model by inhibiting the major regulators of the inflammatory process like p38-MAPK, pCREB and NF-kB[254]. Exogenous Galectin 1 reduces the neurotoxic M1 phenotype of microglia and provided enhanced neuron survival. This overall effect of Galectin 1 on reducing the global inflammatory response makes it a potential candidate for diseases with inflammation induced neuronal toxicity like ALS. As Galectin-1 is produced and secreted out by astrocytes in the CNS, this provides a continuous source for the production of Galectin 1 within the CNS itself. This eliminates the issue of bioavailability and biodistribution faced by prior therapeutic agents. Adoptive transfer of stimulated astrocytes, secreting Galectin-1, has been shown to inhibit microglial activation and rescue disease phenotypes in EAE mice. Thus, the capacity to achieve global downregulation of the inflammatory response and the ability to be continuously produced in the disease microenvironment makes Galectin 1 a potential candidate for targeting neuroinflammation in ALS.

Role of activated ALS microglia in motor neuron toxicity is very well documented[30,118-121]. Primary microglia isolated from mutant SOD1 expressing mice at symptomatic as well as endstage have been shown to induce motor neuron death in
coclure systems. Interestingly, ALS microglia isolated from presymptomatic stages were not neurotoxic to the motor neurons in culture. It was shown that ALS microglia undergo a shift in their phenotype from neuroprotective M2 phenotype to neurotoxic, classically activated M1 phenotype. Several anti-inflammatory compounds have been tested before to reduce the microglia mediated toxicity towards the motor neurons[129]. Here, we utilized Galetin-1, an endogenously expressed lectin binding protein to treat the endstage microglia for their effect on motor neuron survival. We show that treatment of ALS microglia with exogenous Galectin 1 results in the reduction of motor neuron death in coculture system, suggesting that Galectin 1 may provide similar therapeutic benefits in vivo.

Although Galectin 1 provided neuroprotection from ALS microglia, it remains to be determined the mechanism of this Galectin 1 mediated effect. Previous studies have shown that Galectin 1 has the potential to downregulate the expression and secretion of proinflammatory cytokines in activated microglia while upregulating the anti-inflammatory gene expression[254]. Here, we show that Galectin-1 conditioning of ALS microglia results in the reduced secretion of TNF-a when cocultured with motor neurons. We also show that Galectin 1 affects the expression of certain M1 markers like CD68, CD86 and iNOS while upregulating Arg1, an M2 marker. Finally, we also show that conditioning of ALS microglia with Galectin 1 results in reduced activation of NF-kB pathway as evident from the reduced phopho-p65 levels compared to unconditioned ALS microglia. These results are consistent with previous findings where Galectin 1
downregulated the expression of upstream regulators of the inflammatory pathway in classically activated microglia in EAE. These results suggest that unlike previous compounds, Galectin 1 can affect the key regulators of the inflammatory process in ALS microglia and hence is a suitable candidate to pursue for regulation of neuroinflammation in ALS.

5.5 Future of non-SOD1 targeted therapies

In the present study, we have shown the beneficial effect of Galectin 1 on microglia-mediated motor neuron toxicity in vitro. We show that Galectin 1 conditioning of cultured microglia from end-stage ALS mice reduces their neurotoxic potential, resulting into enhanced motor neuron survival in a coculture system. We also show that this Galectin 1 mediated effect on ALS microglia is due to the deactivation of activated ALS microglia as evident by reduced secretion of pro-inflammatory cytokines, downregulation of expression of M1 markers and upregulation of M2 markers. Finally, we also show that inactivation of NF-kB pathway in ALS microglia by Galectin-1 conditioning might be the basis of this enhanced neuroprotection from ALS microglia. Although transient pre-conditioning of ALS microglia with Galectin 1 resulted in reduction of their motor neuron toxicity, this experimental setup does not recapitulate the ALS spinal cord microenvironment. There exists a complex crosstalk between the motor neurons and non-neuronal cells within ALS spinal cord throughout the disease
course[110,111,246]. Therefore, it is important to determine if co-conditioning of ALS microglia with Galectin 1 will still have the similar beneficial effects. To test this, there needs to be a continuous source of Galectin 1 production. Considering astrocytes are the primary source of Galectin 1 expression and secretion, a triple coculture system needs to be established with motor neurons, ALS microglia and Galectin 1 overexpressing ALS astrocytes. Different combinations of these three cell types with respect to cell-cell contacts can further reveal if the direct contact is required for the desired therapeutic effect.

Even after knowing the definitive role of microglia in ALS pathogenesis, none of the therapeutic strategies to target these mechanisms specifically in microglia are currently available. All of our knowledge regarding the direct and indirect role of mutant SOD1 in ALS microglia in driving disease progression comes from transgenic studies. None of the currently available viral vectors have the capacity to target microglia in vivo[195,202,212]. This presents a hurdle against downregulating the disease specific mechanism specifically in microglia. However, recent studies regarding the Galectin 1-mediated crosstalk between astrocytes and microglia can be utilized to downregulate the inflammatory responses in microglia[254]. Our previous studies show that astrocytes can be efficiently targeted by intravenous administration of AAV9 even after the disease onset[313]. Here, we propose to utilize AAV9 mediated delivery of Galectin 1 to ALS astrocytes. This would result in the overexpression and secretion of Galectin 1 from the endogenous ALS astrocytes within the ALS spinal cord. We show that endogenous
Galectin 1 expression within the ALS spinal cord remains at basal levels throughout the disease course with an acute elevation at the end stage. We hypothesize that AAV9 mediated overexpression of Galectin 1 in ALS astrocytes, at or even after the disease onset will result in increased local expression and secretion of Galectin 1 within the CNS, throughout the progression phase of the disease. With the continuous bioavailability and widespread distribution, AAV9-mediated overexpression of Galectin 1 will result in the reduction of overall inflammatory response in ALS microglia. This will further result in inhibition or much delayed polarization of ALS microglia towards neurotoxic M1 phenotype, providing therapeutic benefits on motor performance and survival by prolonging the disease progression.

With its diverse role in CNS, Galectin 1 expression in astrocytes itself has been shown to exert neuroprotection in vivo[278,279]. Increased expression of Galectin 1 within the astrocytes induces expression and secretion of BDNF from the astrocytes. Enhanced production and release of this neurotrophic factor from astrocytes provides beneficial effects in mouse models of neurodegeneration. The protective role of BDNF in motor neuron degeneration is well studied in mouse models of axotomy. Intramuscular injection of adenovirus expressing BDNF in mice has been shown to transduce the motor neuron via retrograde transport and provide enhanced neuroprotection in axotomized facial motor neurons[363]. Intraparenchymal or intraventricular administration of recombinant BDNF as well as lentiviral expression of BDNF has been shown to be neuroprotective in mouse models of cerebral palsy[364,365]. Importantly, BDNF has
been postulated to provide neuroprotection against excitotoxic insults to the neurons. 
Excitotoxicity being one of the major contributors to the motor neuron toxicity in ALS, several clinical trials have been conducted using subcutaneous delivery of BDNF[366]. However, none of these studies were able to ameliorate patient’s condition or survival. One of the major reasons for lack of beneficial effects of BDNF is considered to be its inability to cross BBB and reach CNS. However, the role of BDNF is still ambiguous as intrathecal delivery of BDNF directly in cerebrospinal fluid also failed to alter the disease outcome, suggesting that BDNF on its own may not be able to provide sufficient therapeutic benefits in ALS. Here, we propose that AAV9 mediated expression of Galectin 1 within ALS astrocytes will result in modulation of ALS microglia in the surrounding microenvironment, along with increasing the neurotrophic potential of transduced astrocytes by increasing BDNF production and release. We hypothesize that these combined effects will have a substantial effect on disease severity.

Another reason to provide attention to astrocyte-mediated Galectin 1 overexpression in ALS is the mechanism of Galectin 1 secretion. Several studies have shown that the presence of mutant SOD1 in motor neurons as well as astrocytes exerts a lot of stress on the protein metabolism. It is also shown that mutant SOD1 causes downregulation of genes involved in the canonical protein secretion pathways[244]. Defects in the secretory system may thus obstruct the neuroprotective dialogue between motor neurons and non-neuronal cells. It also suggests that exogenous expression of paracrine molecules like neuroprotective factors or anti-inflammatory molecules in
astrocytes may experience difficulties in exerting their effect due to defective secretory mechanisms. Unlike other proteins, Galectin 1 does not have a canonical secretory signal peptide. Galectin 1 is secreted outside the cells by a non-conventional secretory pathway[256,367]. Hence, in spite of the defective secretory pathways, Galectin 1 would be secreted from ALS astrocytes in the extracellular environment where it can bind to and deactivate the surrounding ALS microglia.

5.6 Concluding remarks

ALS is both cell autonomous and non-cell autonomous disease where multiple cell types have been shown to drive disease pathogenesis. Presence of mutant SOD1 in motor neurons has been shown to dictate the disease onset while mutant SOD1 expressing astrocytes and microglia have been shown to affect the progression phase of the disease. Targeting these individual cell types directly or indirectly has proven to be effective in changing the overall disease course in mice. However, the maximum therapeutic benefits can be achieved only when multiple cell types are targeted simultaneously. With the currently available approaches, motor neurons and astrocytes can be efficiently targeted using viral vectors. Here, we have shown that AAV9-mediated post-natal downregulation of mutant SOD1 in motor neurons and astrocytes results in significant extension in the lifespan of ALS mice. We also show that AAV9 mediated SOD1 shRNA delivery approach can be successfully translated to the non-human prmates. We have also designed a clinical AAV SOD1 shRNA vector for the
administration in humans for clinical trials. Microglia mediated neuroinflammation is considered to be the major cause of motor neuron toxicity in ALS. None of the currently available approaches can specifically target ALS microglia. Owing to the secretory property of astrocytes in the CNS, however, microglial cells can be targeted indirectly by utilizing astrocytes as the source of the therapeutic agent. Our study shows that Galectin 1 treatment can reduce the motor neuron toxicity in vitro by modulating the inflammatory responses in ALS microglia. Therefore, it will be of utmost importance to combine these two approaches by utilization of AAV9 mediated delivery of SOD1 shRNA and Galectin 1 transgene in ALS mice. This approach will enable us to simultaneously target all the key players involved in the non-cell autonomous toxicity towards the motor neurons in ALS (Figure 5.1).
Figure 5.1 Proposed mechanism of AAV9 mediated dual targeting of astrocytes and microglia in ALS. Intravenous administration of self-complementary AAV9-Gal1-SOD1 shRNA in ALS mice at or even after disease onset would result in efficient transduction of astrocytes within the CNS. This would result in shRNA-mediated downregulation of mutant SOD1 within the astrocytes and subsequent reduction in astrocyte-induced motor neuron toxicity. Simultaneous overexpression of Galectin 1 within astrocytes will result in secretion of the Galectin 1 and subsequent immunomodulation of surrounding microglial cells which would ameliorate microglia-mediated motor neuron toxicity. Thus, administration of AAV9-Gal1-SOD1shRNA may provide even better neuroprotection and improved disease outcome by modulating two key players in ALS pathogenesis.
Appendix: Plasmid maps

AAV CB GFP
AAV SOD1 shRNA
AAV Scrambled shRNA
LV SOD1 shRNA
pJet SOD1 shRNA
Clinical AAV SOD1 shRNA
LV RFP
LV Galectin 1
## AAV CB GFP

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## AAV SOD1 shRNA

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## AAV Scrambled shRNA

![AAV Scrambled shRNA diagram](attachment:image.png)

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LV SOD1 shRNA

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Clinical AAV SOD1 shRNA

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References


95. Dodge JC, Treleaven CM, Fidler JA et al. AAV4-mediated expression of IGF-1 and VEGF within cellular components of the ventricular system improves


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