Development of new therapeutic approaches in mouse models of Amyotrophic Lateral Sclerosis

Thèse

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Résumé

La sclérose latérale amyotrophique (SLA) est une pathologie neurodégénérative caractérisée par une perte progressive des neurones moteurs et par de l'atrophie musculaire menant à de la paralysie. Bien que plusieurs mécanismes pathologiques aient été élucidés, la SLA reste un mystère médical puisqu'il n'existe toujours pas de traitement efficace. Nous avons développé deux stratégies dans le but de traiter la SLA. La première stratégie fut de cibler la protéine SOD1 mal repliée (chapitre 2) et la deuxième consistait à traiter la neuroinflammation présente dans la maladie (chapitre 3). Dans le chapitre 2, nous avons tenté de diminuer le niveau de la protéine SOD1 mal repliée présent dans le système nerveux de souris transgéniques développant un phénotype de SLA. Pour ce faire, nous avons testé une nouvelle approche thérapeutique basée sur l'utilisation d' « adeno-associated virus (AAV) ». Ce virus contient une séquence d'ADN qui encode pour un anticorps à chaîne unique variable (scFv). Cet anticorps est composé d'une des deux chaînes légères et lourdes de l'anticorps D3H5 ciblant de manière spécifique la protéine SOD1 mal repliée. Une injection intra-thécale unique de l'AAV encodant l'anticorps à chaîne unique dans des souris $SOD1^{G93A}$ repousse le début de la maladie et augmente la survie des souris de 28%. Nous avons démontré que la Withaferin A (WA), un inhibiteur du facteur NF-κB, diminuait le phénotype neurologique retrouvé chez le modèle de souris transgénique TDP-43. Donc, nous avons testé la Withaferin A sur deux autres lignées de souris transgéniques exprimant des mutations dans la protéine SOD1, soit la lignée de souris $SOD1^{G93A}$ et la lignée de souris $SOD1^{G37R}$. L'effet bénéfique de la WA chez les souris $SOD1^{G93A}$ était accompagné d'un soulagement de la neuroinflammation, d'une diminution du niveau de protéine SOD1 mal repliée dans la moelle épinière et d'une baisse de la mortalité des neurones moteurs. En considérant ces résultats, l'utilisation d'AAV encodant un anticorps à chaîne unique contre la protéine SOD1 mal repliée ainsi que l'utilisation de Withaferin A devrait toutes les deux être considérées comme des approches pour traiter la SLA.

Abstract

Amyotrophic lateral sclerosis (ALS) is a progressive neurodegenerative disease associated with motor neuron degeneration, muscle atrophy and paralysis. Although numerous pathological mechanisms have been elucidated, ALS still remains a medical mystery in the absence of any effective therapy. Riluzole is the only therapeutic drug approved for ALS with regard to prolonging survival. Here, we have developed two strategies for treatment of ALS, first targeting the misfolded SOD1 (chapter 2) and other targeting neuroinflammation (chapter 3). In chapter 2, we aimed to reduce the level of misfolded SOD1 species in the nervous system. We tested a novel therapeutic approach based on adeno-associated virus (AAV)-mediated tonic expression of a DNA construct encoding a secretable single chain fragment variable (scFv) antibody composed of the variable heavy and light chain regions of a monoclonal antibody (D3H5) binding specifically to misfolded SOD1. A single intrathecal injection of the adeno-associated virus encoding the single chain antibody in $SOD1^{G93A}$ mice delayed disease onset and extended the life span by up to 28%, in direct correlation with scFv titers in the spinal cord. Our second treatment strategy which is aimed to target neuroinflammation is based on previous reports from our lab where it has been shown that Withaferin A (WA), an inhibitor of NF-κB activity was efficient in reducing disease phenotype in TDP-43 transgenic mouse model of ALS. We tested WA in mice from two transgenic lines expressing different ALS-linked SOD1 mutations, $SOD1^{G93A}$ and $SOD1^{G37R}$. The beneficial effects of WA in $SOD1^{G93A}$ mice model was accompanied by alleviation of neuroinflammation, decrease in level of misfolded SOD1 species in spinal cord, a reduction in loss of motor neurons, resulting in delayed disease progression and mortality. Based on these evidences, AAV encoding a secretable scFv against misfolded SOD1 and WA should be considered as a potential treatment for ALS.

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List of Abbreviations

AAV: Adeno associated virus
ALS: Amyotrophic lateral sclerosis
AMPA: α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
ANG: Angiogenin
APEX1: Apurinic/ Apyrimidinic endonuclease
ASOs: Antisense oligonucleotides
ATP: Adenosine triphosphate
BiP: Binding immunoglobulin protein.
BMAA: (β-N-methylamino-L-alanine)
CCS: Copper chaperone for SOD1
CgA: Chromogranin A
CNS: Central Nervous system.
CSF: Cerebrospinal fluid
DCTN1: Dynactin
EAAT2: Excitatory amino acid transporter 2
ER: Endoplasmic reticulum
ERAD: ER-associated degradation
FALS: Familial amyotrophic lateral sclerosis
FUS: Fused in sarcoma.
GAP-43: Growth associated protein-43
G-CSF: Granulocyte-colony stimulating factor
GDNF: Glia derived neurotrophic factor
GFAP: Glial fibrillary acidic protein
GLT-1: Glutamate transporter1
GluR2: Glutamate receptors 2
GOF: Gain of function
GPx: Glutathione peroxidases
HAMA: Human anti- mouse antibody
Hsp: Heat shock factor
ICV: Intracerebroventricular
Ig-G: Immunoglobulin G
IPC: Insoluble protein complexes
KA: Kainate
LBIs: Lewy body hyaline inclusions
LMN: Lower motor neuron
LOF: Loss of function
MN: motor neuron
MVBs: Multivesicular bodies
NEFH: Neurofilament heavy chain

NF-κB: Nuclear factor κB
NMDA: N-methyl-D-aspartate
NOS: Nitrogen species
Nrf2: Nuclear erythroid 2-related factor 2
PDI: Protein disulphide isomerase
PON: Paraoxonase
PRPH: Peripherin
Prxs: Peroxiredoxins
RNAi: RNA interference
ROS: Reactive oxygen species
SALS: Sporadic amyotrophic lateral sclerosis
scFv: Single-chain variable fragment
SEDI: SOD1-exposed-dimer-interface
siRNAs: Small interfering RNAs
SMN1: Survival motor neuron
SOD1: Superoxide dismutase 1
SQSTM1: **S**equestosome 1
TARDBP: TAR DNA binding protein.
UMN: Upper motor neuron
UPR: Unfolded protein response
UPS: Ubiquitin proteasome system
UPS: Ubiquitin-proteasome system
VEGF: Vascular endothelial growth factor
VEGF: Vascular endothelial growth factor
VL: Variable light
VH: Variable heavy
WA: Withaferin A

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Foreword

Chapter 2 and 3 are published manuscripts.

Chapter 2:

Priyanka Patel, Jasna Kriz, Mathieu Gravel, Geneviève Soucy, Christine Bareil, Claude Gravel and Jean-Pierre Julien. Adeno-associated virus-mediated delivery of a recombinant single-chain antibody against misfolded superoxide dismutase for treatment of amyotrophic lateral sclerosis. Molecular Therapy (2014) Mar; 22(3):498-510.

In this paper, I am responsible for generation of all data in figure 1, 2 3, 5, 6 and 7. I analyzed the figures and wrote the manuscript. Mathieu Gravel is responsible for generation of data in figure 4. Claude Gravel helped me in generation of AAV-scFv-D3H5 construct. Technical assistance was provided by Genevieve Soucy and Christine Bareil.
Jasna Kriz provided GAP-43-luciferase mice and also provided access to live imaging system. I and JP Julien wrote the paper.

Chapter 3:

Priyanka Patel, Jean-Pierre Julien and Jasna Kriz. Early stage treatment with Withaferin A reduces levels of misfolded superoxide dismutase 1 and extends the lifespan in mouse model of ALS. Neurotherapeutics DOI 10.1007/s13311-014-0311-0 (2014).
I am responsible for the generation of all data and figures. Priyanka Patel, JP Julien and Jasna Kriz wrote the paper.

Chapter 1: Introduction

1.1 Amyotrophic lateral sclerosis

Amyotrophic lateral sclerosis (ALS) is the most common motor neuron disease in human adults, described as early as 1869 by a French neurobiologist and physician, Jean-Martin Charcot. Since its first comprehensive clinico-pathological description by Jean Martin Charcot, there has been years of research, millions of dollars spent and tremendous efforts have been made to make new treatments available. Despite of all these efforts, ALS has remained an elusive therapeutic target. To date, there is still a paucity of treatment options available.

ALS is a chronic degenerative process of the central nervous system (CNS) that primarily affects upper and lower motor neurons in the brain and spinal cord as well as descending neuronal pathways. The term “amyotrophy” refers to progressive atrophy of muscle fibers, which lose their efferent support as a result of neuronal cell degeneration in the anterior horn of the spinal cord or brain stem motor nuclei. Consequent to the loss of axonal projections from the affected areas of the brain or spinal cord, anterior and lateral corticospinal tracts undergo fibrotic and sclerotic changes. This degenerative process is termed “lateral sclerosis.

1.1.1 Epidemiology

The incidence of the disease varies from 1.47 to 1.75 per 100,000 per year in Europe and North America (Worms, 2001; Pradas et al., 2013). According to several reports, over the last few years the incidence of ALS has been on the rise, which may be attributed to an overall increase in the population age, use of better diagnostic criteria, and seemingly stronger contributing influence of various environmental factors (Worms, 2001).The most common age at the initial presentation of ALS varies between 55 and 65 years (Haverkamp et al., 1995; Cudkowicz et al., 1997). Denervation of the respiratory muscles and diaphragm, with consequent respiratory insufficiency is generally the main fatal event that occurs within 2-5 years after onset (Rowland and Shneider, 2001). Gender probably plays a

role in ALS, as the number of men developing the disease is higher than that of women. Initial epidemiological studies that focused on gender differences in ALS demonstrated a ratio of men to women close to 2:1 (Kahana et al., 1976; Kurtzke, 1982; Annegers et al., 1991; Guidetti et al., 1996). However, recent population based analyses reveal an apparent tendency for the gender ratio that approaches unity (Worms, 2001; Abhinav et al., 2007; Logroscino et al., 2008), even though there remains a slight predominance of ALS in men – 1.3:1. Several reasons have been proposed to explain existing discrepancy in the gender distribution of ALS, among them protective influence of female sex hormones and an increased likelihood of exposure to environmental risk factors in men.

There are few factors that may play a role in developing ALS, including occupation, diet, toxic exposure, family history of other neurodegenerative disorders (Wijesekera and Leigh, 2009). However, no single environmental component appears to be reliably associated with the risk of developing ALS and in fact, the etiology of the disease most likely constitutes a complex interplay of endogenous, exogenous, and genetic contributors.

1.1.2 Genetics of ALS

Two distinct forms of ALS have been identified, a familial form (FALS) and a sporadic form (SALS). FALS accounts for about 10% of all cases of ALS, of these, 20% are caused by mutations in the superoxide dismutase 1 (SOD1) gene (Rosen et al., 1993) of which more than 150 mutations have been identified, spanning all exons of the SOD1 gene (Cleveland, 1999; Valentine and Hart, 2003; Dion et al., 2009). Most of these SOD1 mutations follow an autosomal dominant pattern of inheritance (Andersen et al., 1996; Hentati et al., 1998; Hadano et al., 2001; Gros-Louis et al., 2006). In the remaining 90% no genetic linkage has been implicated and these are therefore characterised as sporadic. Besides a lower mean age of onset, the disease pathogenesis in FALS and SALS cases is remarkably similar.

1.1.2.1 Sporadic ALS

Approximately 90% of ALS cases are sporadic forms with unknown cause. Different hypotheses, listed as below, have been proposed as risk factors for this type of disease:

1) Environmental toxicants such as heavy metals, pesticides and chemicals appear to be a risk factor for ALS. Neurotoxins and cytotoxins, like BMAA (β-N-methylamino-L-alanine), saxitoxin, curacin, microcystins and cylindrospermopsin, associated to cyanobacteria have been also proposed as ALS inducing factors (Rowland and Shneider, 2001; Oliveira and Pereira, 2009). The role of toxic agents has received renewed attention because of the emerging hypothesis that exposure to some environmental toxicants might have played a relevant role in triggering the neurodegenerative process in genetically predisposed subjects belonging to some communities with an increased risk of ALS, such as the Chamorro indigenous people of Guam and the Veterans of the 1991 Gulf War (Cox and Sacks, 2002).

2) Autoimmunity may have a role in the sporadic pathogenesis since IgG antibodies against both motor neurons and voltage-gated calcium channels, involved in the degeneration of motor neurons, have been observed in patient spinal cord (Appel et al., 1995).

3) Genetic factors : Although a number of genes have been identified as causative of familial ALS, genetic studies have also been performed in sporadic ALS, finding candidate genes which confer susceptibility to sporadic ALS. Repeated expansion in *C9orf72* gene accounts ~7% of apparently sporadic ALS cases in people of European ancestry (Majounie et al., 2012). Mutations in the *SOD1,* and *TARDBP* genes account for 1% and 1-5% of SALS cases respectively (Chio et al., 2008; Rutherford et al., 2008; Sreedharan et al., 2008). Other candidates include neurofilament heavy chain (*NEFH)* ,vascular endothelial growth factor *(VEGF),* angiogenin (*ANG),* Apurinic/ Apyrimidinic endonuclease (*APEX1),* Peripherin *(PRPH),* Dynactin *(DCTN1),* Survival motor neuron (*SMN1)* Paraoxonase

(PON). However, mutations in these genes account only for a small percentage of sALS cases (Renton et al., 2014).

SQSTM1 encodes p62, a major pathologic protein deposited in neurodegeneration. P62 regulates ubiquitin binding and activation of nuclear factor kappa-B signaling. A candidate gene screening approach identified missense and deletion variants in ~1% of ALS cases (Fecto et al., 2011).

Chromogranins (CgA and CgB), which are major constituents of secretory large dense-core vesicles in neurons, may act as chaperone-like proteins that promote secretion of mutant SOD1 (Urushitani et al., 2006) and also constitutes a major risk factor. SOD1 secretion may activate microglia and lead to neuronal death. Gros-Louis et al in 2009 reported that P413L Chromogranin B is associated with ALS susceptibility and age at onset in a population of 289 French (French or French Canadian origins) ALS patients (Gros-Louis et al., 2009). In these combined populations, the presence of this CHGB variant conferred a 2.2-times greater risk to develop ALS. For the population of French origin, the P413L variant conferred a 3.3-fold greater risk to develop ALS.

1.1.2.2 Familial ALS

90% ALS are sporadic cases with no clear genetic linkage. However, the remaining 10% of cases show familial inheritance. Most of cases are associated with an autosomal-dominant inheritance, although recessive and X-linked transmission has been described (Maruyama et al., 2010). Among those 20% of FALS cases are caused by the mutation in SOD1 gene, 4-5% of FALS cases are the results of mutations in TARDBP and FUS genes, more than 30% of FALS cases are associated with C9orf72 mutations (Renton et al., 2014). Pathogenic mutations in several other genes have also been discovered. Table 1.1 summarizes the mutations linked to familial ALS cases.

Table 1.1 Up to date, more than 20-ALS genes have been identified in FALS.

Genetic subtype	Chromosomal locus	Gene	Protein	Onset	Inheritance	Clinical feature	Other diseases caused by the gene
ALS1	21q22.1	SOD1	Cu/Zn SOD-1	Adult	AD/AR	Typical ALS	NA
ALS2	2q33-2q35	Alsin	Alsin	Juv	AR	Slowly progressive, predominantly UMN signs like limb, & facial spasticity	PLS IAHSP
ALS3	18q21	Unknown	Unknown	Adu	AD	Typical ALS with limb onset especially lower limb	NA
ALS4	9q34	SETX	Senataxin	Juv	AD	Slowly progressive, distal hereditary motor neuropathy with pyramidal signs	SCAR 1 and AOA2
ALS5	15q15-21	SPG 11	Spatacsin	Juv	AR	Slowly progressive	HSP
ALS6	16p11.2	FUS	Fused in Sarcoma	Juv/Adu	AD/AR	Typical ALS	NA
ALS8	20q13.3	VAPB	VAPB	Adu	AD	Typical and atypical ALS	SMA
ALS9	14q11.2	ANG	Angiogenin	Adu	AD	Typical ALS, FTD and Parkinsonism	NA
ALS10	1p36.2	TARDBP	DNA-binding protein	Adu	AD	Typical ALS	NA
ALS11	6q21	FIG 4	Phosphoinositide-5phosphatease	Adu	AD	Rapid progressive with prominent corticospinal tract signs	CMT 4 J
ALS12	10p13	OPTN	Optineurin	Adu	AD/AR	Slowly progressive with limb onset and predominant UMN signs	Primary Open Angle Glaucoma
ALS14	9p13.3	VCP	VCP	Adu	AD	Adult onset, with or without FTD	IBMPFD
ALS15/ ALSX	Xp11	UBQLN2	Ubiquilin 2	Adu/Juv	XD	UMN signs proceeding LMN signs	NA
ALS16	9p13.2-21.3	SIGMAR1	SIGMAR1	Juv	AR	Juvenile onset typical ALS	FTD
ALS-FTD1	9q21-22	unknown	unknown	Adu	AD	ALS with FTD	FTD
ALS-FTD2	9p21	C9ORF72	C9ORF72	Adu	AD	ALS with FTD	FTD
NA	2p13	DCTN1	Dynactin	Adu	AD	Distal hereditary motor neuropathy with vocal paresis	NA
Other rare-occurring ALS genes							
ALS3	18q21	Unknown	Unknown	Adu	AD	Typical ALS with limb onset especially lower limb	NA
ALS7	20ptel-p13	Unknown	Unknown	Adu	AD/AR	Typical ALS	NA
NA	12q22-23	DAO	DAO	Adu	AD	Typical ALS	NA

These genetic mutations represent different molecular pathways of motor neuron degeneration. Abbreviations for Table 1.1: PLS Primary Lateral Sclerosis, IAHSP Infantile onset ascending hereditary spastic paralysis, SCAR 1 Autosomal Recessive Spino-cerebellar ataxia, AOA2 Ataxia Ocular Apraxia 2, HSP Hereditary spastic paraplegia, VAPB Vesicle associated membrane protein associated protein B, SMA Spinal Muscular Atrophy, CMT 4 J Charcot-Marie Tooth disease type 4 J, VCP Valosin Containing Protein, IBMPFD Inclusion body myopathy with Pagets disease and fronto temporal dementia, SIGMAR1 Sigma Non Opiod Intracellular Receptor, C9ORF72 Chromosome 9 open

reading frame 72, PD Parkinson disease, DAO D-Amino Acid Oxidase, FTD Frontal-temporal dementia, AD Autosomal dominant, AR Autosomal recessive. (Taken from (Chen et al., 2013))

1.1.3 Clinical and neuropathological features

Initially, the site of onset determines the clinical features. In 75-80% of patients, symptoms begin with limb involvement and results in weakness of leg and arms, while 20-25% of patients present with bulbar symptoms involving initial swallowing and speech difficulties. In lower motor neuron (LMN) involvement, fasciculations may occur early on in the disease, particularly in the tongue and limbs. Upper motor neuron (UMN) involvements generally are hyperreflexic and stiff, reflexes may be diminished due to LMN involvement. UMN symptoms may include spasms and sudden, uncontrolled straightening movements of the lower limbs. Over few years it has been recognised that also alterations in cognition are a common factor in ALS patients (Wilson et al., 2001). Thus, frontal and temporal lobe atrophy can be demonstrated in a subset of ALS patients by CT-scanning (Kato et al., 1993). However, early manifestations of frontotemporal lobe dysfunction can only be visualised with more sensitive dynamic neuroimaging techniques (Strong et al., 1999). The major neuropathological features correlate with the principal clinical manifestation, namely the degeneration of motor neurons in the spinal cord, brainstem, and motor cortex. However, the neuropathology of ALS also includes degeneration of at least 30% of the small interneurons in the motor cortex and spinal cord, in addition to reactive gliosis (Murayama et al., 1991; Kawamata et al., 1992; Ekblom et al., 1994; Schiffer et al., 1996).

An established hallmark of ALS is the presence of various inclusion bodies in degenerating neurons and surrounding reactive astrocytes (McGeer and McGeer, 2002; Barbeito et al., 2004; Turner et al., 2004). Ubiquitinated inclusions are the most specific and the most common type of inclusions in ALS and are found in lower motor neurons of the brainstem, the spinal cord (Matsumoto et al., 1993) and in corticospinal upper motor neurons (Sasaki

and Maruyama, 1994). These inclusions are classified as 'Lewy body-like' and 'Skein-like (Kawashima et al., 1998; He and Hays, 2004). Additionally, Bunina bodies (BBs), which are cystatin C-containing inclusions, are found in the cell bodies of motor neurons in ALS (Okamoto et al., 1993; Sasaki and Maruyama, 1994), although these are now thought to be less specific for ALS than the ubiquitinated and neurofilamentous inclusions, as they are similar to structures found in neurons of aged rats and humans (Kusaka, 1999). Other neuropathological features seen in ALS include fragmentation of the Golgi apparatus (Fujita et al., 2000; Fujita et al., 2002), mitochondrial vacuolisation (Okamoto et al., 1990) and ultrastructural abnormalities of synaptic terminals (Sasaki and Iwata, 1996).

1.2 Cu-Zn superoxide dismutase

There are three isoforms of SOD in human, each encoded by a separate gene: Copper-zinc SOD (CuZnSOD or SOD1) located in cytoplasm, manganese SOD (MnSOD or SOD2), located in mitochondria and extracellular SOD (SOD3). The SOD1 gene is composed of 5 exons and 4 introns and is spread over 11 kb on chromosome 21q22.1. Each SOD1 monomer contains two metal ions, one each of copper and zinc, playing structural and catalytic roles in the enzyme.

The SOD1 protein homodimer has robust antioxidant activity and protects the cell by transforming highly reactive superoxide anions into hydrogen peroxide that is catalyzed to H_2O by catalase, peroxiredoxins (Prxs), or glutathione peroxidases (GPx) (Valentine et al., 2005; Hart, 2006). The catalysis is a two-step process, where, one molecule of superoxide first reduces the cupric ion to form dioxygen and then a second molecule of superoxide reoxidizes the cuprous ion to form hydrogen peroxide as shown in figure 1.1 below (Adapted from (María Clara Franco et al., 2013),

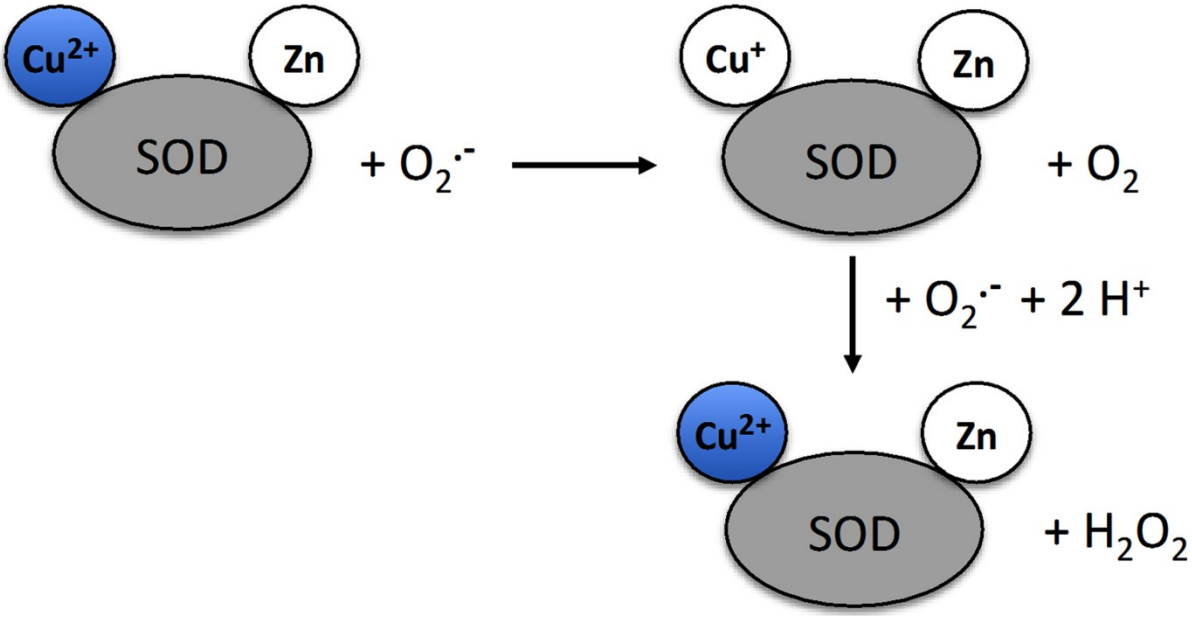

Figure 1.1 Physiological function of SOD1 enzyme

The disproportionation of superoxide is a two-step oxidation-reduction reaction that involves the cycling of the copper atom in SOD1 from Cu2+ to Cu+ and back to Cu+2. The zinc does not participate in this reaction but is essential for the structure of the active site.(Adapted from (María Clara Franco et al., 2013))

1.2.1 SOD1 Structure

According to the crystal structure of human apo Cu, Zn-Superoxide dismutase found by x-ray diffraction, SOD1 consist of four identical chains (homodimer), each chain containing 153 residues. The chains are held together by hydrophobic and electrostatic interactions (Figure 1.2). SOD1 is made up of eight anti parallel beta-pleated sheets, ~5% alpha helices and seven loops (Strange et al., 2003). Each subunit has a Cu and Zn ion bonded to it, and a disulfide bridge between Cys57 and Cys146, both of which constitute the active site and increases SOD1 stability.

1.2.2 Post-translational processing of SOD1

SOD1 is subject to at least four post-translational maturation steps in addition to N-terminal acetylation, copper insertion, zinc insertion, dimerization and disulfide bond formation. Failure or alteration of one or more of these processes could result in a build up of immature SOD1 which will not be properly folded since the native protein requires each of these modifications to maintain its structure and stability. Thus, a mutation in a gene that controls SOD1 post-translational modification may mimic a SOD1 mutation in FALS by causing improper folding of SOD1 (Khare et al., 2003). The presence of an intramolecular (within each subunit) disulfide bond in SOD1 is somewhat unexpected since it is present at high concentration within the reducing environment of the cytoplasm. The stability of SOD1 is dependent upon proper disulfide bond formation (Hornberg et al., 2007; Perry et al., 2010).

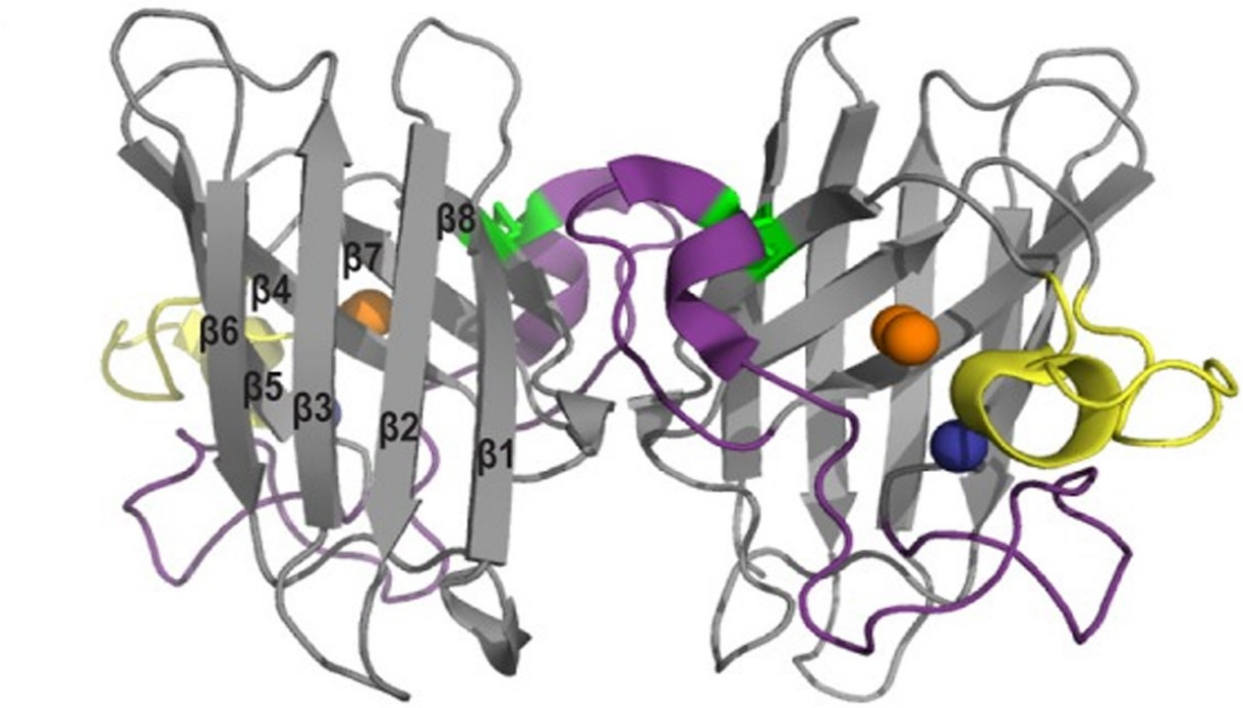

Figure 1.2 Crystal structure of metal bound dimeric human Cu/Zn superoxide dismutase (pdb code 2C9V).

SOD1 consists of eight beta sheets that form the beta barrel core. The major functional loops are the zinc binding loop (Purple; residue 49-81) and electrostatic loop (yellow; residue 124-139). An intramolecular disulfide bonds (C57-C146; green) stabilizes the protein structure (Adapted from (Rotunno and Bosco, 2013)).

1.3 Mutated SOD1 based animal models for ALS and mechanism of toxicity

Mutation in the gene for SOD1 is the most common known cause of ALS and is by far the most studied. Since 1993, when Rosen *et al*. discovered a link between SOD1 mutations and FALS (Rosen et al., 1993), over 150, mainly missense, mutations in the SOD1 gene have been reported to cause FALS (Turner and Talbot, 2008) (Figure 1.3). These disease-associated mutations are located throughout the protein structure including the dimer interface and metal binding sites (Valentine et al., 2005). The structural diversity of fALS-associated mutations in SOD1 suggests that this disease occurs as a result of a general toxic mechanism, but not a site-specific mechanism. Shortly after the discovery of SOD1 mutations in FALS (Rosen et al., 1993), a transgenic mouse model ($SOD1^{G93A}$) of SOD1–ALS was developed, expressing approximately 20–24 copies of the human coding sequence with the G93A mutation, under control of the human SOD1 promoter (Gurney et al., 1994). Since the development of this model, over twenty other SOD1 models have been created (Table 1.2) and SOD1 transgenic rodents have been used as the primary rodent models of ALS

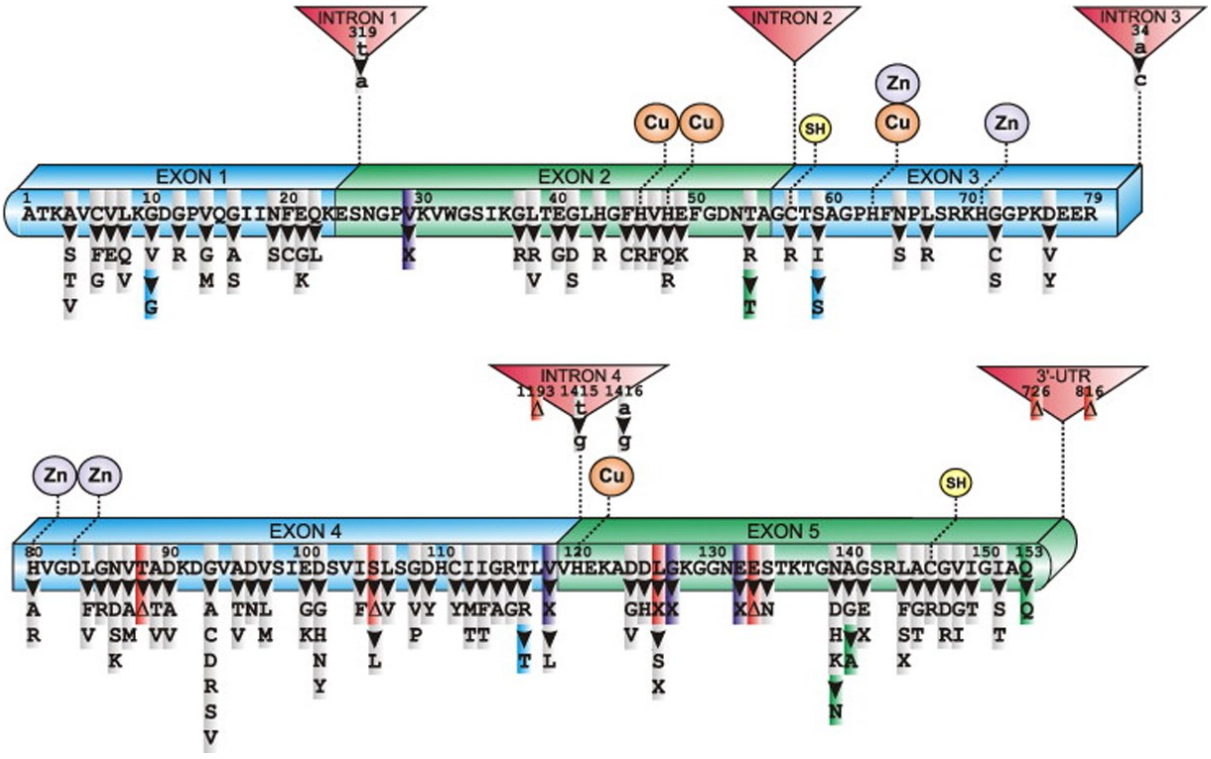

Figure 1.3 Schematic representation of human SOD1 primary sequence.

Primary sequence of SOD1 with exons, introns, Cu and Zn binding domains, intramolecular disulfide bond (SH) and mutations linked to sporadic and familial ALS. Mutation legend: grey, missense; purple, insertion; red, deletion; blue/green, silent; (Δ) inframe deletion; X, truncation. These mutations are scattered throughout the gene but have the highest prevalence in exons 4 and 5. (Taken from (Turner and Talbot, 2008))

Table 1.2 Mutant SOD1 transgenic mice models.

SOD1 mutant	SOD1 activity (fold)	Disease onset (months)	Disease duration (months)	Reference
$SOD1^{A4V}$	n.d.	-	-	
$SOD1^{G37R}$	**14.5**	**4 to 6 (10)**	**n.d. (2.5)**	**(Wong et al., 1995)**
$SOD1^{H46R}$	n.d.	5	1	(Chang-Hong et al., 2005)
$SOD1^{H46R/H48Q}$	0	4 to 6	n.d.	(Wang et al., 2002a)
$SOD1^{H46R/H48Q/H63G/H120G}$	0	8 to 12	n.d.	(Wang et al., 2003)
$SOD1^{L84V}$	n.d.	5 to 6	1	(Tobisawa et al., 2003)
$SOD1^{G85R}$	0	8 to 14	0.5	(Bruijn et al., 1997)
$SOD1^{G86R}$	0	3 to 4	1	(Ripps et al., 1995)
$SOD1^{D90A}$	6 to 8	12	2	(Jonsson et al., 2006)
$SOD1^{G93A}$	**11**	**3 to 4 (3)**	**1 to 2 (1,5)**	**(Gurney et al., 1994)**
$SOD1^{I113T}$	n.d.	12	2	(Kikugawa et al., 1997)
$SOD1^{L126X}$	n.d.	7 to 9	n.d.	(Wang et al., 2005a)
$SOD1^{L126X}$	n.d.	11	0.75	(Deng et al., 2006)
$SOD1^{L126delTT}$	0	15	1	(Watanabe et al., 2005)
$SOD1^{G127X}$	0	8	0.25	(Jonsson et al., 2004)

At present, 12 different human SOD1 mutations have been expressed in mice. These include nine missense and three C- terminally truncated variants (L126X,G127X and $SOD1^{L126delTT}$) and lastly the SOD1 $^{H46R/H48Q/H63G/H120G}$ is a transgenic with artificial mutations in the copper binding domains. In bold are models used in this thesis. Time of onset and duration of disease of mutant SOD1 mice can vary due to environmental factors. Hence, average times of onset and duration for the lifespan of SOD1 ^{G93A} and $SOD1^{G37R}$ mice in the laboratory of Dr Jean-Pierre Julien are included in parenthesis for reference to the current work. Adapted from (Turner and Talbot, 2008).

Figure 1.4 below presents an overview of the time course and certain pathological events that occur in the $SOD1^{G93A}$ mice model. In the present study we used this mice model (Gurney et al., 1994) since it constitutes the most widely used and the best-characterized mouse model of ALS, as illustrated in figure 1.4. These animals carry a high copy number (25±1.5) of the human SOD1 gene (transgene) with a point mutation responsible for a glycine to alanine substitution at position 93. These mice develop hind limb tremors and muscle weakness around 90 days detected by locomotor deficits, progressing to paralysis and premature death at around 120-140 days (Gurney et al., 1994). Pathologically, neuromuscular junctions degenerate at around 47 days of age, in a manner that is selective for fast-fatigable motor units (Fischer et al., 2004; Pun et al., 2006). Moreover, proximal axonal loss is prominent after 80 days of age, coinciding with motor impairment. This is followed by about 50% loss of lower motor neurons after 100 days of age (Fischer et al., 2004). Pathological features of spinal motor neurons include mitochondrial vacuolization (Dal Canto and Gurney, 1995), Golgi apparatus fragmentation (Mourelatos et al., 1996) neurofilament-positive inclusions (Tu et al., 1996) and cytoplasmic SOD-immunoreactive aggregates (Johnston et al., 2000). Spinal cords are also characterized by astrocytosis and microgliosis around symptom onset (Hall et al., 1998). Similar features are observed in transgenic $SOD1^{G37R}$ mice and in transgenic $SOD1^{G85R}$ mice, but with a much later disease onset and much shorter disease duration for $SOD1^{G85R}$ mice (Bruijn et al., 1997).

The generation of these transgenic mice models of ALS gave hope for testing and developing efficacious therapeutic compounds. Yet, understanding the toxicity of mutant SOD1 has been particularly challenging and the generation of therapies hurdled with failures.

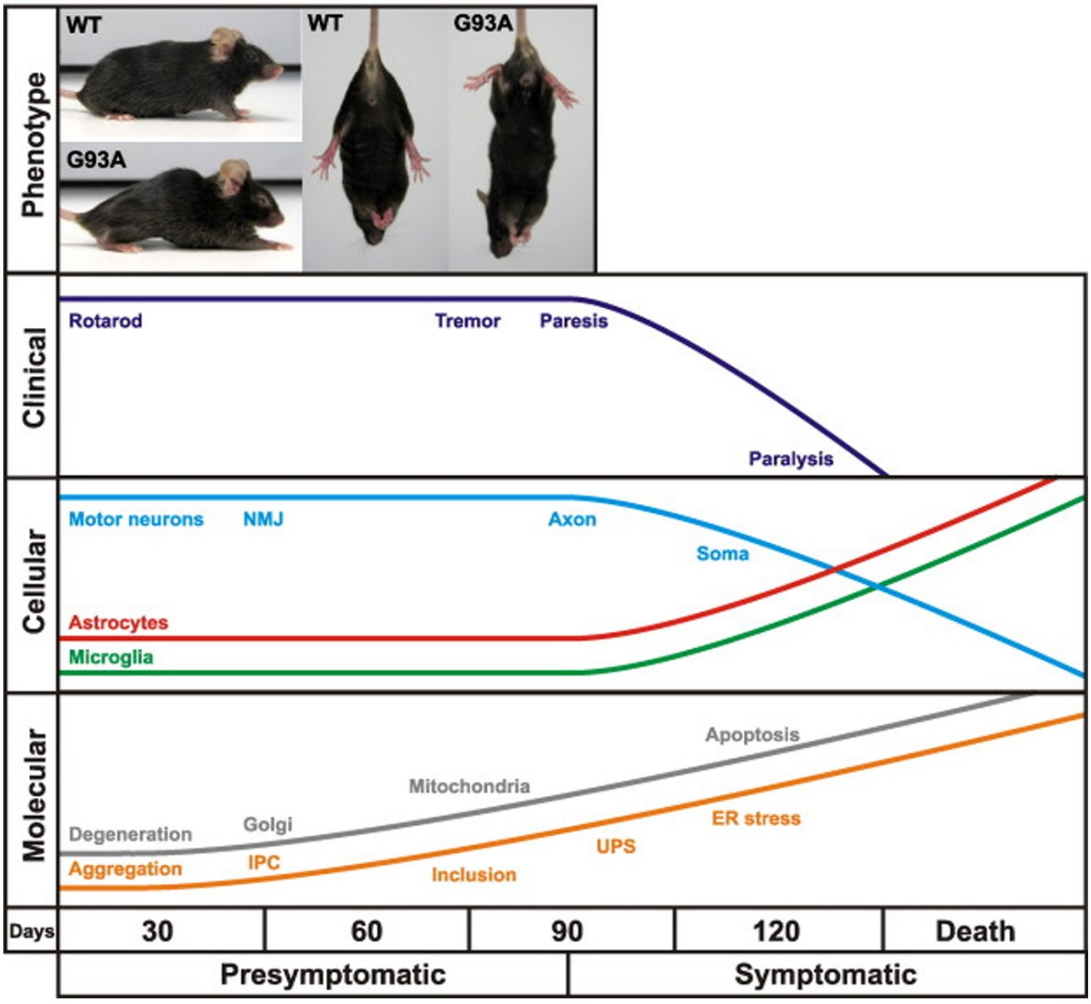

Figure 1.4: Time course of clinical and neuropathological events in high copy number transgenic SOD1^{G93A} mice.

Mice develop hind limb tremor, weakness and locomotor deficits at about 3 months which is preceded by distal synaptic and axonal degeneration. This progresses into fatal paralysis about 1 month later concomitant with spinal motor neuron loss and reactive gliosis. A sequence of mutant SOD1 aggregation into insoluble protein complexes (IPC), inclusion bodies modified the ubiquitin-proteasome system (UPS) and subcellular degeneration in motor neurons may underlie the phenotype (Adapted from (Turner and Talbot, 2008)).

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1.3.1 Mutant superoxide dimutase 1: mechanism of toxicity

1.3.1.1 Loss of function

The finding of loss of dismutase activity in patients with ALS and the distribution of ALS-causative mutations spread throughout the SOD1 gene initially suggested loss of function as a mechanism (Deng et al., 1993; Rosen et al., 1993). However, evidence for a gain of function mechanism was quick to follow from analysis of mutant SOD1 transgenic (tgSOD1) mouse models as they show increased dismutase activity and remarkable heterogeneity in respect to catalytic activity, polypeptide half-life and resistance to proteolysis (Ratovitski et al., 1999; Shaw and Valentine, 2007).

Thus a loss of function mechanism became less favoured compared with gain of function because: (i) in humans, a lack of correlation was found between SOD1 dismutase activity and aggressiveness of clinical phenotypes (Ratovitski et al., 1999). (ii) in mice, a lack of overt ALS-like phenotype was found in Sod1 null (Sod1-/-) animals, the first of which was published by Reaume et al (Reaume et al., 1996); whereas (iii) transgenic mouse models over-expressing mutant human SOD1 have increased SOD1 activity and a loss of motor neurons that mimics human ALS.

The experimental data published by the Cleveland laboratory (Bruijn et al., 1998) where they analysed survival time in mice carrying a mutant SOD1 transgene (tgSOD1^{G85R}) on a normal mouse background (i.e. with two copies of the endogenous mouse Sod1 gene) compared with the same transgene on a Sod1-/- background showed no change in survival of the mice, thus concluding that survival was entirely due to a gain of function mechanism, and independent of mouse SOD1 loss of function. These findings essentially ended the debate for a role of loss of function as a contributor to SOD1- familial ALS (Bruijn et al., 1998). The causative gain of function is indisputable and several mechanisms by which this occurs have been proposed and comprehensively reviewed (Turner and Talbot, 2008; Ilieva

et al., 2009; Rothstein, 2009). However, it also has been shown that SOD1 knockout mice have an age related peripheral axonopathy, denervation muscle atrophy and accelerated sarcopenia which confers locomotor deficits as well as increased vulnerability after axotomy and cerebral ischemia (Reaume et al., 1996; Kondo et al., 1997; Flood et al., 1999; Shefner et al., 1999; Muller et al., 2006). The most affected motor units in Sod1-/- mice are fast-twitch, which is in accordance with observations in ALS models (Frey et al., 2000). Further, in Sod1-/- mice, motor neurons are preferentially affected compared to sensory neurons, recapitulating the selectivity observed clinically and pathologically in ALS.

A reduction in SOD1 activity is not causative for ALS (which is certainly what the mouse data show), however, it may modify disease (Figure 1.5) and such modifying effects would come through an increased susceptibility to neurodegeneration either directly through, for example the increased susceptibility to axonal damage seen in Sod1+/- mice, or indirectly through, for example, effects on respiration in high energy consumers such as motor neurons.

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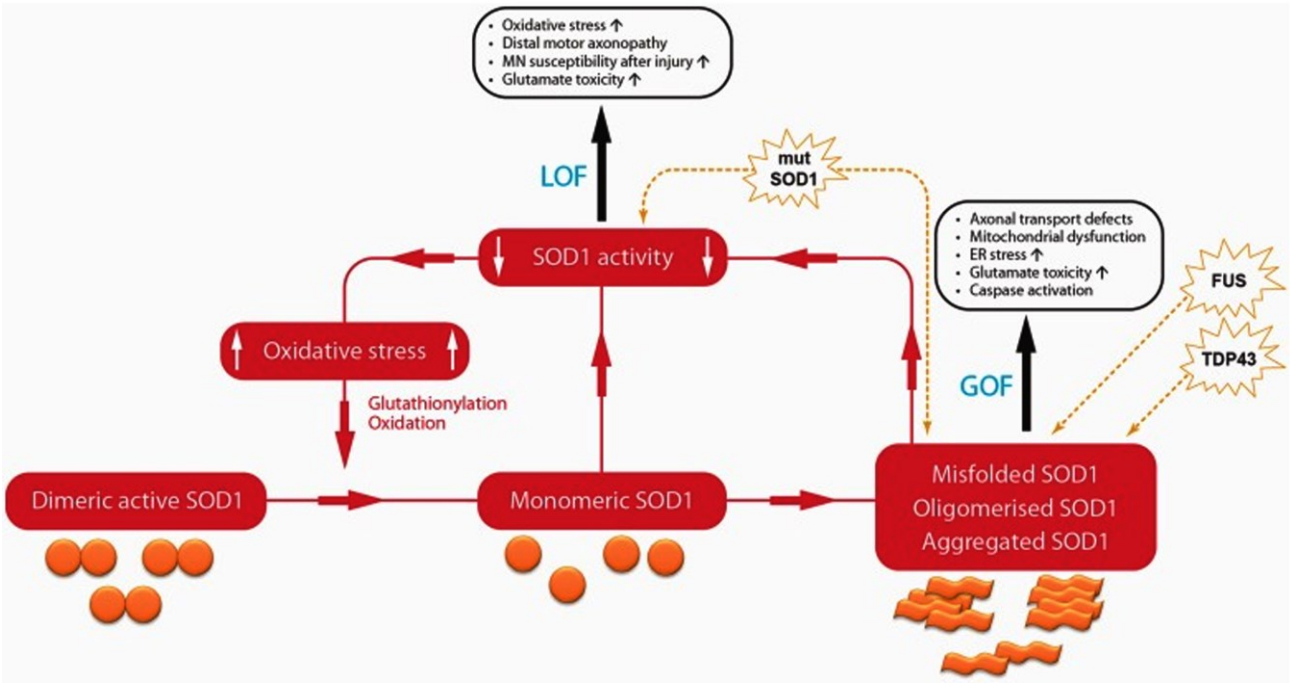

Figure 1.5 The cycle of SOD1 loss of function.

Schematic representation of a potential co-operation between SOD1 loss and gain of function in SOD1–familial ALS pathogenesis. SOD1 loss of function (LOF) increases levels of oxidative stress, which through glutathionylation and oxidation, can facilitate the monomerisation of dimeric SOD1. Once monomerized, SOD1 is more prone to become misfolded, oligomerized and aggregated. The monomerization of previously active dimeric SOD1 and the recruitment of SOD1 into aggregates further enhance the loss of function, feeding back to the beginning of the loop. In this way the gain of function (GOF) effects of misfolded, oligomerized and aggregated SOD1, which are known to cause motor neuron degeneration, are amplified by the loss of function circle. Mutant SOD1 (mutSOD1) has both a direct effect on reduction of SOD1 activity and induces SOD1 misfolding and aggregation. Mislocalisation of both TDP43 and FUS result in misfolding of SOD1. ER = endoplasmic reticulum; MN = motor neuron. Adapted from (Saccon et al., 2013)

1.3.1.2 Gain of Function

Several hypotheses have been proposed to provide an explanation for the toxic gain of function of SOD1 mutants and to explain how a multitude of mutations in SOD1 can give rise to the same ALS-like phenotype. Some of these hypotheses are discussed below.

1.3.1.2.1 *Oxidative hypothesis*

According to this hypothesis, in addition to its dismutase activity, SOD1 can also act as a peroxidase by using the hydrogen peroxide produced in the conventional dismutase reaction as a substrate. In this way, SOD1 can catalyse the reverse reaction of the dismutation, and can directly promote the generation of reactive oxygen and nitrogen species (ROS, NOS) (Hodgson and Fridovich, 1975; Yim et al., 1997). More appropriately, misfolding of SOD1 mutants could allow the use of inappropriate enzymatic substrates and the generation of peroxynitrite and/or hydroxyl radicals (Figure 1.6) (Bruijn et al., 2004)

Mutations in SOD1 may result in increased peroxynitrite levels through two hypothesised mechanism. The first hypothesis assumes that the mutant SOD1 activity is lower than normal SOD1 activity, causing a shift in the steady-state equilibrium towards higher superoxide concentrations. Since superoxide reacts with nitric oxide more rapidly than it does with native SOD1, there would be a concomitant increase in peroxynitrite levels, which in turn reacts with SOD1 to form a nitronium-like intermediate that nitrates tyrosine residues on cellular proteins (Beckman et al., 1993). Although increased nitrotyrosine levels have been reported in SOD1-ALS patients (Beal et al., 1997; Tohgi et al., 1999) and mutant SOD1 transgenic mice (Ferrante et al., 1997; Casoni et al., 2005), it is now understood that many SOD1 mutants retain dismutase activity (Borchelt et al., 1994; Valentine et al., 2005) so this mechanism could only occur in mutants where dismutase activity is reduced. The importance of peroxynitrite synthesis in mutant SOD1 toxicity remains controversial. If such a mechanism was central to the toxic gain of function, it would be hypothesised that reduction of nitric oxide levels by inhibition of neuronal NO synthase would reduce mutant SOD1 toxicity to motor neurons. Although this was found in

one study using cultured motor neurons (Estevez et al., 1999), inhibition of neuronal NO synthase had no effect on survival of mutant SOD1 transgenic mice (Facchinetti et al., 1999). Similarly, if increased peroxynitrite was a major mechanism leading to neurodegeneration, increased levels of tyrosine nitration would be expected. Elevated levels of free 3-nitrotyrosine have been found in $SOD1^{G37R}$ and $SOD1^{G93A}$ transgenic mice compared to mice expressing human wild type SOD1 (Bruijn et al., 1997; Ferrante et al., 1997) and also in both sporadic and SOD1-ALS patients (Beal et al., 1997). However, levels of protein-bound nitro-tyrosine were unchanged at all ages in $SOD1^{G37R}$ and $SOD1^{G85R}$ mice, and also in end-stage of sporadic or mutant SOD1-mediated human ALS (Bruijn et al., 1997).

All of the proposed mechanisms for the mutant SOD1 gain of toxic function require a copper ion to be present within the active site. In support of this, neither loss of dismutase activity nor reduced copper binding is a common property shared by ALS-associated SOD1 mutants (Corson et al., 1998; Valentine et al., 2005). However, deletion of the copper chaperone for SOD1 (CCS), which led to a reduction in copper loading into SOD1 of at least 90% and a marked reduction of SOD1 activity, had no effect on disease onset, progression or pathology of G93A, G37R or G85R mutant mice (Subramaniam et al., 2002). Moreover, mutations in SOD1 that disrupt some or all the copper coordinating residues do not eliminate toxicity (Wang et al., 2003).

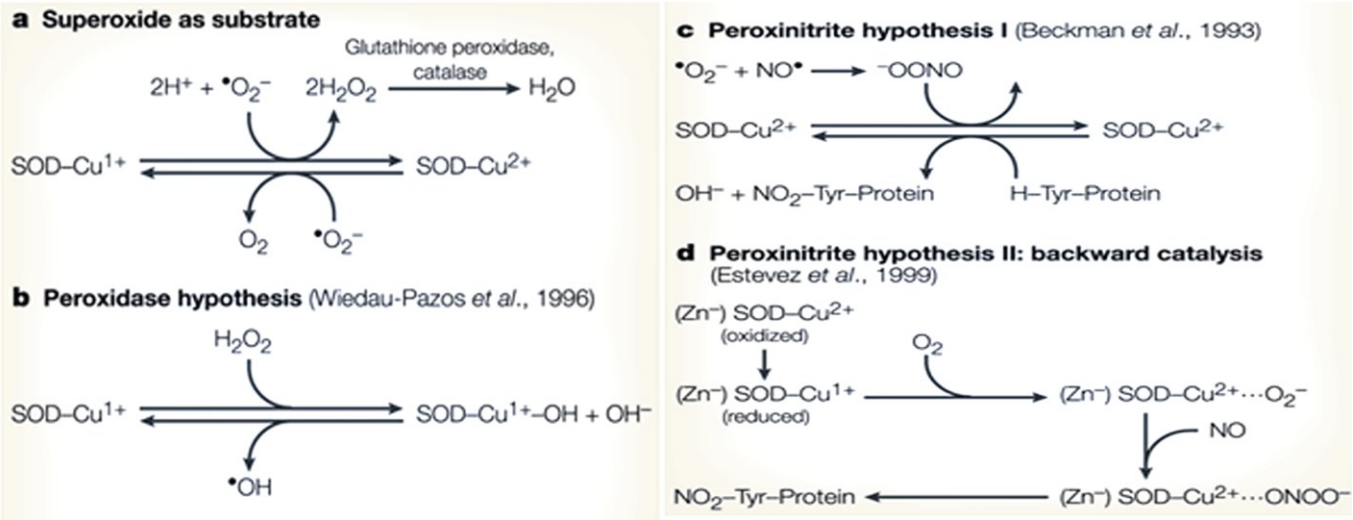

Figure 1.6 Proposed mechanisms of mutant SOD1 oxidative mediated damage.

(Normal SOD1 chemistry: a) SOD1-mediated dismutation of superoxide in two asymmetric steps. b-d) Proposed toxic chemistries arising from the use of aberrant substrates. b) The formation of hydroxyl radicals by the use of hydrogen peroxide as an aberrant substrate by the reduced form of the enzyme. c, d) Two models for protein nitration: c) the use of diffusible -ONOO as an aberrant substrate and d) a zinc-depleted SOD1 mutant generating superoxide from O2 •. Adapted from (Cleveland and Rothstein, 2001)

1.3.1.2.2 Aggregation hypothesis

Neurodegenerative diseases are diverse and accumulation of insoluble proteinaceous material in intra- or extracellular aggregates within the CNS has been implicated in the pathogenesis of many different neurodegenerative diseases including Alzheimer's, Parkinson's, Huntington's, and prion disease (Prusiner and DeArmond, 1991; Chiti et al., 2003; Ross and Poirier, 2004; Saxena and Caroni, 2011). Similarly, SOD1-containing aggregates have been found in SALS and FALS patients (Okamoto et al., 1991; Shibata et al., 1996a; Shibata et al., 1996b; Kato et al., 2001) and also in mutant SOD1 transgenic mice models (Kato, 2008; Turner and Talbot, 2008).

However, whether protein aggregation itself is toxic or a harmless byproduct of oxidative damage has been argued and debated. According to the aggregation hypothesis the toxic gain of function of mutant SOD1 resides in the polypeptides increased propensity to oligomerize with itself or with other proteins and thereby to form an aggregate species (Shaw and Valentine, 2007). In support of this theory, it has been found that high molecular weight complexes containing mutant SOD1 are detectable in spinal cord extracts from transgenic mice before motor neuron pathology is apparent (Johnston et al., 2000). Interestingly, others have reported that SOD1 aggregation coincides with the onset of neurodegeneration in transgenic mouse models of ALS (Wang et al., 2002a). Additionally, a study investigated the appearance and abundance of mutant SOD1 aggregates, throughout the disease course in SOD1 fALS mice models (G93A, G37R and H46R/H48Q) (Karch et al., 2009). This work reported that small aggregates (*e.g.,* soluble oligomers) form in the early stages of the disease but larger aggregates of mutant SOD1 form primarily in the later stages of the disease, concurrent with the appearance of rapidly progressing symptoms (Karch et al., 2009). Taken together, small aggregates of mutant SOD1, rather than large and mature aggregates, appear to be more toxic. However, the mechanisms that connect SOD1 aggregation to motor neuron toxicity still remain unknown. Various hypotheses have been proposed to explain the cytotoxicity of SOD1 aggregates including aggregate-mediated inhibition of the proteasome machinery, impaired chaperone activity,

deregulation of organelle function such as mitochondria, and microglia (Boillee et al., 2006a), overwhelming of protein chaperones, perturbations in mitochondrial function and calcium homeostasis (Figure 1.7). These mechanisms are in accordance with evidence gathered form a number of studies investigating mutant SOD1 toxicity. For example, misfolded proteins are targeted for disposal *via* the proteasome. Therefore, the proteasome machinery can be adversely affected by accumulation of ubiquitinated and/or misfolded proteins. In the case of ALS, ubiquitinated proteins have been confirmed as part of the inclusions found in patients, implicating that impaired proteasomal machinery is prominent in ALS. More specifically, the insoluble forms of mutant SOD1 in the spinal cords of G93A mice models clearly show both mono- and oligo-ubiquitinated proteins (Basso et al., 2006) . Misfolded and aggregates of SOD1 can also interact improperly with folding chaperones, abolishing their normal functions. In fact, inclusions from SALS and FALS patients, and transgenic mice models, have been shown to contain Hsp 70 (Watanabe et al., 2001) and Hsp 25 (Wang et al., 2003). Thus, it can be postulated that mutants of SOD1 may devastate the cell's defense mechanism through diminishing the protective effects of the proteasome and chaperone mechanisms, thereby causing the disease.

There is growing evidence that SOD1 mutants cause ALS by interfering with a different cell type and organelles, the mitochondria (Vande Velde et al., 2008) and microglia (Urushitani et al., 2006). Misfolded SOD1 has been found to be associated with the cytoplasmic face of the outer mitochondrial membrane in spinal cord tissues, but not other tissues, of mice models (Sturtz et al., 2001). Velde *et al.* proposed that the mutant proteins bound to mitochondria might be responsible for the malfunction of protein import, ionic homeostasis, mitochondrial mobility, mitochondrial fission/fusion, or mitochondrial regulation of apoptosis (Sturtz et al., 2001).

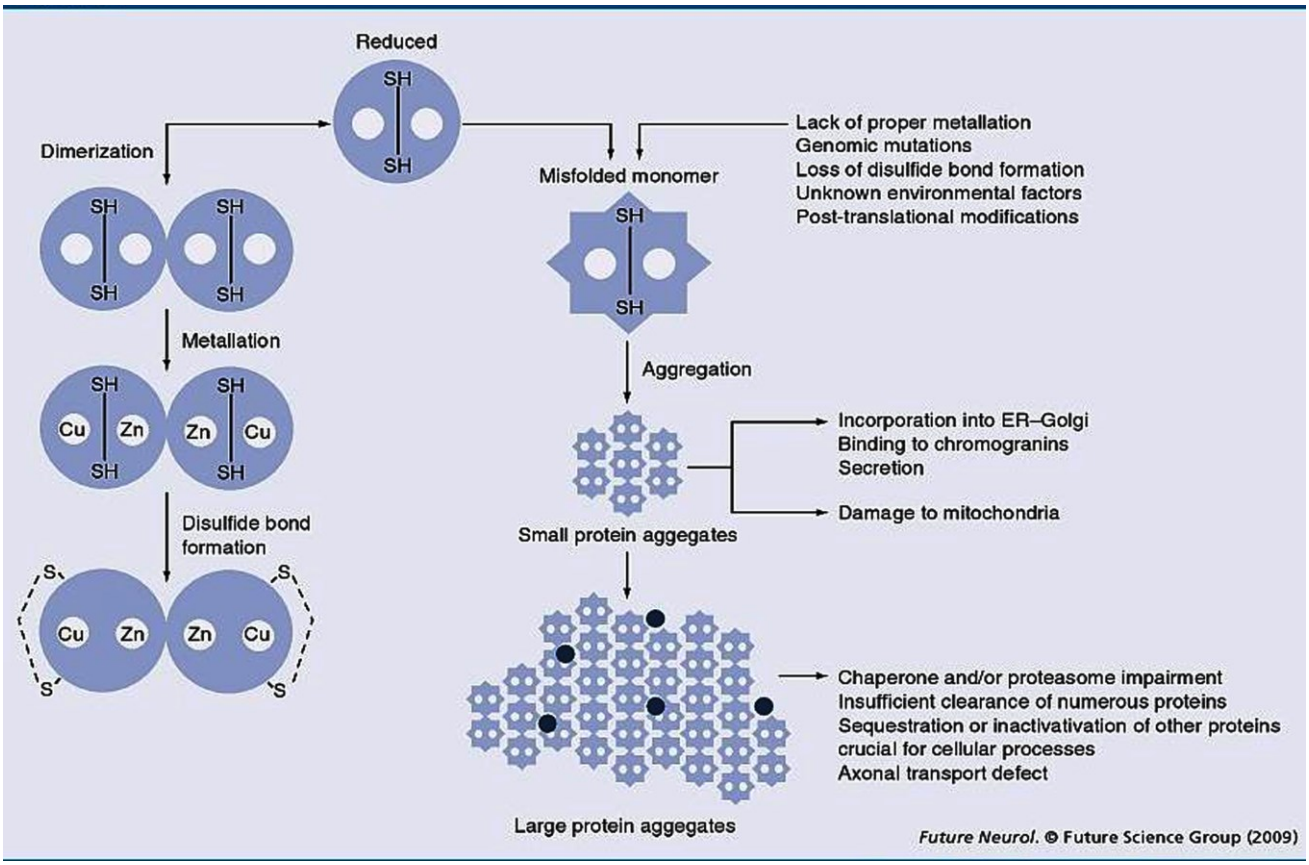

Figure 1.7 Schematic representation of toxicity model based on misfolding and aggregation of mutant superoxide dismutase.

In normal healthy conditions, wild-type SOD1 is correctly processed to acquire metal ions and via disulfide bond, which prevents SOD1 from aggregation. Binding of copper and zinc ions and the formation of a highly conserved intramolecular disulfide bond gives stability to SOD1. Holo-SOD1 with a disulfide bond has been known as one of the most stable proteins. Loss of copper and zinc ions destabilizes it to apo form , which is more prone to aggregation.(Adapted from Gros-Louis et al, 2009). ER: Endoplasmic reticulum; SOD: Superoxide

1.3.1.2.3 Oxidation and aggregation hypothesis

Current research indicates that the two hypotheses may not be mutually exclusive, because there is evidence to show that oxidative damage to SOD1 enhances aggregation of the protein (Rakhit et al., 2002), and that aggregates from patients contain oxidative modifications (Kato et al., 2000). SOD1's normal role as a free radical scavenger puts it at risk of oxidative damage, which can trigger misfolding primed by mutation. ALS-causing mutations have the common property, not of promoting global SOD1 unfolding, but of priming SOD1 to populate aggregation-competent native-like misfolded states, in response to denaturational stresses or covalent damage (Rakhit et al., 2004; Rakhit and Chakrabartty, 2006; Mulligan et al., 2012). Elevated oxidative stress in disease-affected tissues, partially as a result of mitochondrial malfunction, is a known hallmark of ALS (Beal et al., 1997; Andrus et al., 1998); furthermore, oxidative modifications to proteins, including SOD1, have been reported in mouse ALS models (Andrus et al., 1998). This hypothesis is attractive, since, if it is correct, one would expect SOD1 to be most prone to oxidation-induced misfolding and aggregation in cells in which its half-life is longest (*i.e.* in which it has the greatest lifetime risk of being damaged). Since motor neurons are some of the physically longest cells in the body, with axons up to 1 m long, the slow transport processes that carry SOD1 from the cell body to the end of the axon necessitate an SOD1 half-life on the order of hundreds of days (Rakhit et al., 2002) much longer than in most cell types, which often have cellular lifespan shorter than this. Greater propensity to misfold and aggregate would also be expected in highly metabolically active cells in which the mitochondria produce more free radical species.

Also, certain studies have demonstrated that aggregation of mutant SOD1 may be dissociable from the toxic events that drive disease onset (Witan et al., 2008; Prudencio et al., 2009). Co-expression of CCS with SOD1 ^{G93A} greatly accelerated disease onset while reducing aggregation (Son et al., 2007). Others have also demonstrated that the aggregation potential of SOD1 *per se* does not correlate with the increased toxic properties in a cell

culture and *Caenorhabditis elegans* nematode model (Witan et al., 2008). In their paper, Witan *et al,* propose that aberrant enzyme activity may be the initial step in the pathogenic cascade possibly leading to the formation of oxidized SOD1 which, in turn, causes protein oligomerization, aggregation and aberrant subcellular localization and toxicity.

1.4 Wild-Type Cu, Zn-Superoxide Dismutase: Pathological role in ALS

In initial studies, the chronic increase in the level of wild type human SOD1 (and the dismutase activity) did not seem to affect the progression of motor neuron disease in transgenic mice expressing mutant $SOD1^{G85R}$ (Bruijn et al., 1998) and neither did it accelerate it (Jaarsma et al., 2000). Also, human SOD1wt over expression did not affect the lifespan of mice over expressing mouse $SOD1^{G86R}$ (Audet et al., 2010). However later on various group reported that over expression of human wt SOD1 caused dramatic exacerbation of disease in mice expressing different SOD1 mutants, including two SOD1 mutants ($SOD1^{G85R}$ and $SOD1^{L126Z}$) that express highly unstable and enzymatically inactive SOD1 (Deng et al., 2006; Deng et al., 2008; Jaarsma et al., 2008). Recent studies have focused the attention on possible role of wt SOD1 as a modulator of disease initiation. Here we will summarize recent developments in our understanding of roles of wt SOD1 in ALS.

1.4.1 Inherent propensity of wild type SOD1 to form aggregates

SOD1 is a homodimer in which each monomer binds one Cu atom and one Zn atom. Copper ion bound in SOD1 serves as an active site for dismutation of superoxide anion, and the structural stability of SOD1 is significantly increased upon binding of a zinc ion and the formation of a disulfide bond (Furukawa and O'Halloran, 2005). Mutation is believed to increase the conformational flexibility of SOD1, giving rise to a misfolded SOD1 population with novel cytotoxic properties.

In normal physiologic conditions, wild-type SOD1 undergoes proper post translational processing to acquire metal ion and disulfide bonds which prevents SOD1 to form aggregates (Furukawa et al., 2010). However, reports have suggested that SOD1 can form amyloid-like fibrillar aggregates in disulfide reduced and apo state (Furukawa et al., 2008; Furukawa et al., 2010). These observations confirm that wild type SOD1 is also susceptible to aggregation and can become pathogenic when the post translational processes are disrupted. Recent studies have demonstrated the possible role of wt SOD1 as a modulator of disease initiation.

It has been reported that in transgenic mice, co-expression of high levels of wild type human SOD1 (wt hSOD1) with mutant hSOD1 (A4V, G85R, G93A, T116X or L126Z) accelerated the course of disease by decreasing the age to onset and paralysis with no obvious change in the rate of progression from disease onset to paralysis (Deng et al., 2006; Deng et al., 2008; Wang et al., 2009c). However, in cultured cell models, wt hSOD1 did not promote the aggregation of mutant SOD1, but rather seems to slow the process (Prudencio et al., 2009; Witan et al., 2009). In vitro, small amounts of immature mutant SOD1 is capable of acting as the seed of aggregation of wt hSOD1 (Chattopadhyay and Valentine, 2009). Collectively, these studies indicate a potential for wt hSOD1 to play a role in the aggregation of mutant SOD1 and to modulate the course of disease.

1.4.2 Wild type SOD1 with aberrant conformation in sporadic ALS cases.

Recently, mutations in genes other than *sod1* have been identified in a subset of FALS cases, which include genes encoding TDP-43 (Gitcho et al., 2008), FUS (Kwiatkowski et al., 2009), and optineurin (Maruyama et al., 2010). Recent evidence also supports SOD1 as a common toxic factor in a subset of Familial as well as sporadic ALS cases. These reports

are based on the finding that oxidation, demetallation and other alteration in post translational modifications, cause wt SOD1 to acquire the similar toxic functions that are observed in Familial ALS cases (Ezzi et al., 2007; Bosco et al., 2010; Guareschi et al., 2012). Shibata et al in 1996, first pointed out the involvement of wt SOD1 in pathological inclusions. They found Lewy body hyaline inclusions (LBHIs) in 10 out of 20 sporadic ALS cases. Staining the spinal cord with rabbit antisera raised against human SOD1 gave intense immunoreactivity to some of these LBHIs (Shibata et al., 1996a). Interestingly, the proportion of LBHIs with SOD1 immunoreactivity varied from case to case (7–60 %). They however, did not verify whether their SALS cases had mutations in *sod1* gene. Care must be needed to interpret the SOD1 immunoreactive inclusions in sporadic ALS cases because mutations in sod1 gene had been found in 5% of total sporadic ALS cases.

Recently, misfolded wt SOD1 has been detected in patients with sporadic ALS and there has also been report that reducing SOD1 levels in astrocytes derived from sporadic patients inhibits astrocytes mediated toxicity on motor neurons, suggesting that wt SOD1 may acquire toxic properties similar to familial ALS linked mutant SOD1, perhaps through post translational modifications (Bosco et al., 2010; Haidet-Phillips et al., 2011). Guareschi et al in 2012 used patient's lymphoblast and showed that wt SOD1 is over oxidized in a subset of sporadic ALS patients with bulbar onset and that through this oxidation; modified SOD1 acquires toxic properties similar to those induced by disease-causing genetic mutations in patient-derived cells (Guareschi et al., 2012). Thus, misfolded SOD1 could be involved in disease pathogenesis in both FALS and SALS patients.

1.4.2.1 Immunodetection of wild type SOD1 with aberrant conformation by conformation specific antibodies

Native SOD1 exist as a dimer, and the monomerization is often considered to be involved in the misfolding/aggregation pathway of SOD1 (Watanabe et al., 2001). For detection of

misfolded SOD1 in affected tissues, different conformation specific antibodies have been generated which have proved to be very useful in providing detailed insight into the misfolded nature of FALS-linked mutant SOD1. Mutation induces some degree of misfolding and it exposes linear sequences or conformational epitopes that are normally buried in intact native protein. Most of the existing conformational specific antibodies selectively recognize these exposed epitopes on mutant SOD1 (Rakhit et al., 2007; Urushitani et al., 2007; Bosco et al., 2010; Gros-Louis et al., 2010; Grad et al., 2011; Broering et al., 2013). Thus all these antibodies are specific only for misfolded SOD1 and not wild type SOD1. The misfolded SOD1 conformation specific antibodies namely, B8H10, D3H5, A5C3 and C4F6 were generated against the apo-SOD1^{G93A} antigen (Gros-Louis et al., 2010). These antibodies reacts differently with multiple FALS linked mutant SOD1 proteins from spinal cord lysate of different transgenic mice model (G93A, G37R,G85R,G127X and D90A), but not with wild type SOD1 proteins (Urushitani et al., 2007; Gros-Louis et al., 2010). Any change in post translational modification or oxidation of wild type SOD1 may acquire some toxic properties observed for FALS-linked SOD1 mutants such as enhanced propensity to misfold (Rakhit et al., 2002; Ezzi et al., 2007). B8H10, A5C3 and D3H5 are able to recognize any such oxidized or modified WTSOD1 (Gros-Louis et al., 2010).

Likewise, C4F6 is a mouse monoclonal antibody raised against recombinant apo-SOD1 with G93A mutation (Urushitani et al., 2007). The C4F6 antibody recognizes a conformation-dependent epitope common to both mutant (G37R, G85R, G93A, A4V) and wild-type SOD1 proteins associated with ALS pathology. But the recognition is strong only when these proteins are in their native state but exhibits weaker reactivity for denatured SOD1 proteins (Urushitani et al., 2007; Brotherton et al., 2012). In *in vitro* experiments, an epitope of C4F6 was found to include specific conformations present in SOD1^{G93A} but not wild-type SOD1. However, treatment of WT SOD1 protein with H_2O_2 changed its structure, which became detectable with C4F6 (Bosco et al., 2010). Also C4F6 antibody could detect misfolded wild-type SOD1 species associated with SALS (Bosco et al., 2010). Notably, positive C4F6 staining was also observed in 4 out of 9 sALS cases. The diffused

staining patterns of C4F6 antibody in SALS cases suggested that the pathological forms of wildtype SOD1 remain relatively soluble. Immunoreactivity of D3H5 (Gros-Louis 2010) and C4F6 (Brotherton et al., 2012) for $SOD1^{G93A}$ protein, directly correlates with disease progression in the transgenic $SOD1^{G93A}$ mice model, which indicates that these antibodies are able to report the presence of toxic misfolded forms of SOD1 protein.

Another group of antibody are the ones which have been generated against sequences that are predicted to become exposed only upon SOD1 misfold.. To detect misfolded SOD1 in affected tissues of ALS, it is quite reasonable to make an antibody that specifically recognizes monomeric SOD1. For that purpose Rakhit et al, raised an antibody called SEDI (SOD1-exposed-dimer-interface) to an epitope that is normally buried in the SOD1 native homodimer interface and inaccessible in the dimeric state but becomes exposed upon monomerization (Rakhit et al., 2007). SEDI reactivity was specific for mutant SOD1 over native wtSOD1 and it detected the monomer misfolded SOD1 in G37R, G85R and G93A transgenic SOD1 animal models as well as in human post mortem tissues harboring $SOD1^{A4V}$ mutation. The SEDI antibody thus, report on misfolding events within the C-termini, which are induced by ALS-linked mutations. Indeed, the SEDI antibody has successfully immunostained hyaline inclusions in motor neurons of the FALS cases with *sod1*mutation (A4V, A4T); however the SEDI antibody could not detect the inclusions in all of 13 SALS cases and 1 non-SOD1 FALS case, where no mutations in *sod1* gene were confirmed (Liu et al., 2009) .

Another antibody (USOD) has also been generated by rabbit immunization with a SOD1 fragment from Leu^{42} to His^{48}, which constitutes an internal hydrophobic core of the native structure (Kerman et al., 2010). This region (Leu^{42}–His^{48}) is supposed to become exposed only when SOD1 is extensively misfolded; therefore, the USOD antibody specifically binds extensively misfolded SOD1 but not native dimeric as well as folded monomeric states. Similar to the SEDI antibody, the USOD antibody has detected pathological inclusions in FALS cases with SOD1 mutations (A4V, ΔG27/P28) but not in

sALS cases without *sod1* mutations. These results suggest that, wild-type SOD1 protein may participate in the pathogenesis of ALS and some antibodies specific for misfolded SOD1 can also detect the pathological modifications of wild type SOD1 in sporadic ALS cases.

1.4.2.2 Prion like activity of Cu-Zn superoxide dismutase in ALS

A well-studied consequence of SOD1 mutation and/or oxidation is a propensity of the protein to misfold and aggregate (Hart, 2006). Classically, protein misfolding disease are considered as errors in proteostasis, where the burden of misfolded protein species eventually overwhelms the compensatory mechanism that normally keep their concentration in check (Hart, 2006).

The alternate view is that, a pathologically disordered protein may recruit and induce misfolding of natively folded isoforms, by seeded polymerization or template assistance (Horwich and Weissman, 1997). In its mutated or modified state SOD1 has a higher tendency to misfold and form oligomers and aggregates. Under denaturing conditions wild-type and mutant forms of SOD1 can spontaneously form aggregates and fibrils in vitro, (Chia et al., 2010) where the relative propensity for aggregation is dependent upon the variant of SOD1 (Prudencio et al., 2009). Co-expression of misfolded human SOD1 mutants can confer a misfolded conformation on endogenous wtSOD1 that is revealed by conformation- specific antibodies whose epitopes are only accessible when the protein is misfolded (Grad et al., 2011; Pokrishevsky et al., 2012). A key feature to the prion mechanism of misfolded SOD1 is the ability of the protein to transfer its misfold properties onto wild-type protein, which in turn can provide new template for further rounds of misfolding (Fig 1.8). Induced misfolding of SOD1 was shown to occur even in the absence of the misfolded “seed” in cell culture (Grad et al., 2011). It suggests that newly misfolded

polypeptide can act as template for subsequent cycles of SOD1 misfolding, a property that has been found in pathological prion protein. Intercellular transmission of misfolded SOD1 has also been seen in ALS. When neurons die, they release their protein aggregates to extracellular milieu, these are further taken up by healthy neighbouring cells and thus intercellular spread occurs in ALS (Munch et al., 2011; Grad et al., 2014). Direct exposure of misfolded SOD1 to the extracellular space is an important characteristic as it allows for access of potential therapeutic molecules to recognize and bind directly to pathological protein, thereby blocking its contact with native SOD1 substrate or targeting it for subsequent destruction (Grad et al., 2014).

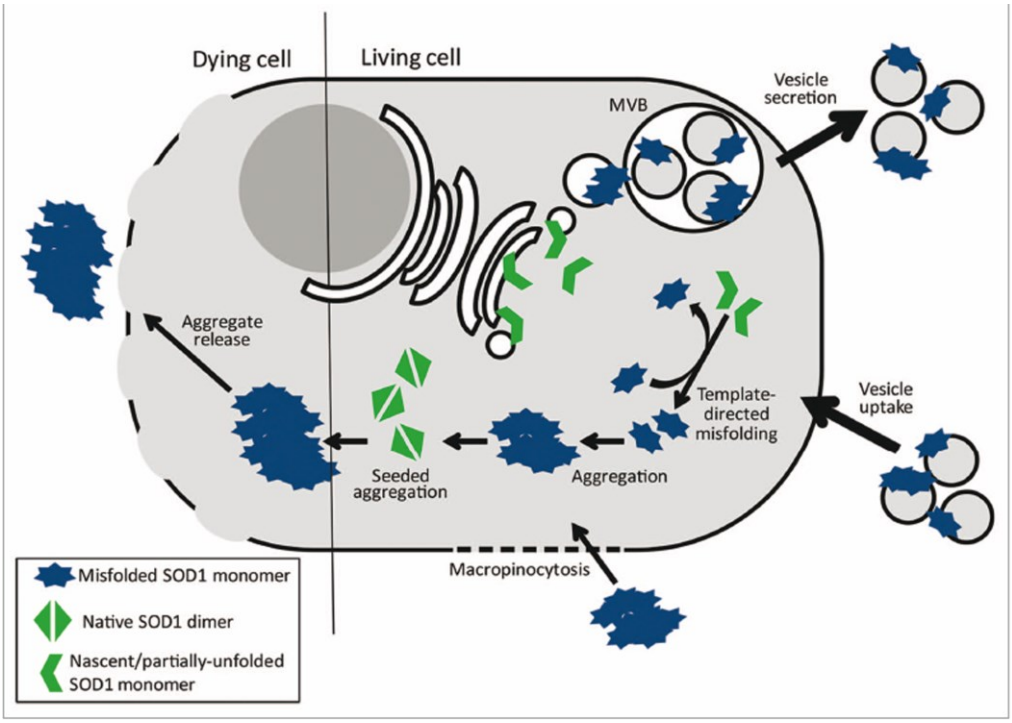

Figure 1.8 Intermolecular conversion and intercellular transmission of misfolded SOD1.

Intermolecular conversion of wild-type SOD1 may occur via two possible mechanisms. Newly-translated SOD1 that is still partially unfolded are substrates for template-directed conversion by pathological forms of SOD1. Conversion of nascent SOD1 to a misfolded isoform provides template for further rounds of native SOD1 conversion. Additionally, homodimeric wild-type SOD1 (or soluble forms of misfolded SOD1) can be misfolded and incorporated into pre-existing insoluble aggregates of misfolded SOD1 to form larger complexes. For intercellular transmission of misfolded SOD1 isoforms, soluble forms of misfolded SOD1 (likely monomers and small oligomers) are packaged via the endocytic pathway into multivesicular bodies (MVBs); MVBs fuse with the plasma membrane thus releasing vesicles, such as exosomes, to the extracellular environment. (Adapted from (Grad et al., 2014))

1.5 Pathogenic factors in ALS

1.5.1 Cell autonomous and non-cell autonomous toxicity in ALS

The selective death of motor neurons initially led researchers to believe that the unique properties of motor neurons are responsible for their vulnerability in ALS associated injury. However, genetic and chimeric mice studies showed that non-cell autonomous processes might be involved in motor neuron loss in these models and hence potentially in ALS. Here, we will review some of the extrinsic or intrinsic mechanistic pathways (Figure 1.8) that are proposed to contribute to ALS pathology caused by mutant SOD1, these factors may not be mutually exclusive.

1.5.2 Intrinsic pathways to motor neuron degeneration

Human motor neurons might possess various molecular and neurochemical features that may render this cell group differentially vulnerable to degenerative process occurring to ALS.

Motor neurons are large cells with approximately somatic diameter of 50-60 um and very long axonal processes with long axonal caliber as well which needs a higher mitochondrial activity compared to other neuronal groups (Shaw and Eggett, 2000). Intrinsic neuronal properties that may predispose them to degeneration in ALS may include elevated vulnerability to mitochondrial dysfunction, weak calcium buffering capacity, excessive neurofilament content, increased AMPA mediated inward calcium current and reduced GABA mediated chloride current (Ferraiuolo et al., 2011). Here we will briefly review the intrinsic pathways responsible for motor neuron degeneration in ALS.

1.5.2.1 Excitotoxicity and calcium homeostasis

Glutamate is the main excitatory neurotransmitter in the mammalian CNS and exerts its effect in many aspects of normal brain function. Glutamate transporters are responsible for the bulk transport of glutamate across the plasma membrane of cells and act to buffer synaptically released glutamate (Tzingounis and Wadiche, 2007). To perform these tasks, glial cells express transporters in abundance, whereas neurons express fewer transporters, and they appear to be precisely located near or at the synapses. By far the most important glutamate transporter is EAAT2/GLT-1 as it is widely expressed in astrocytes throughout the central nervous system (CNS) and as it has the highest affinity for glutamate. The group of Rothstein et al. in 1992, found defects in glutamate signaling in neuronal tissue from patients who died of ALS (Rothstein et al., 1992). Another group reported that increased cerebrospinal fluid glutamate levels were apparent in approximately 40% of nearly 400 patients with sporadic ALS and correlated with disease severity (Spreux-Varoquaux et al., 2002).With regard to ALS this phenomenon was later attributed to the selective loss and malfunction of the astrocytic glutamate transporter EAAT2, which clears glutamate from the synaptic cleft after firing and thus prevents repetitive firing and stops excitotoxicity (Maragakis et al., 2004). Both familial and sporadic cases of human ALS and mutant SOD1 mice, have decreased levels of functional EAAT2 protein and increased circulating glutamate in the cerebrospinal fluid (CSF). $SOD1^{G85R}$ mutant mice have 50% less levels of EAAT2 protein in the spinal cord at the end stage of disease (Bruijn et al., 1997). In case of $SOD1^{G93A}$ transgenic rats, EAAT2 expression in the ventral horn is reduced presymptomatically and further almost completely abolished by end-stage disease (Howland et al., 2002).

There are two category of glutamate receptors namely ionotropic and metabotropic.
The ionotropic receptors are further subdivided into three classes according to their preferred synthetic agonist, AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid), NMDA (N-methyl-D-aspartate) and KA (kainate) receptors (Seeburg, 1993). Functionally, AMPA receptors are considered to be the most important glutamate receptors

to mediate fast excitatory neurotransmission (Collingridge and Lester, 1989). Excitotoxicity mediated neuronal injury occurs through disruption of intracellular calcium homeostasis (Choi, 1987). In spinal motor neurons, calcium entry occurs through AMPA receptors. Excessive influx of Ca2+ causes stress to the Ca2+ buffering systems in the cell, in particular the mitochondria. When the mitochondria are overly stressed by cytosolic Ca2+ levels, an apoptotic cascade can be activated concomitant with increased ROS production (Van Den Bosch et al., 2000; Urushitani et al., 2001). In addition large influx of Ca2+ ions can result in the activation of several enzymes, such as lipases, phospholipases, proteases, endonucleases, protein phosphatases, protein kinase C (Carriedo et al., 1996).

The calcium permeability of the AMPA receptor complex is largely determined by the GluR2 subunit, (Williams et al., 1997) Further defects in editing of GluR2 subunit has also been shown to have implications in ALS because a significant reduction in RNA editing of GluR2 at the Q/R site occurred specifically in motor neurons of five patients with sporadic ALS (Kwak and Kawahara, 2005; Kawahara et al., 2006). Surprisingly transgenic mice that lack the GluR2 subunit do not suffer from a motor neuron disease (Jia et al., 1996) suggesting that a low GluR2 level is not sufficient to cause ALS, but it could be a exacerbating factor for motor neuron degeneration in ALS (Heath and Shaw, 2002).

In line with this evidence P. Van Damme and colleague show that GluR2 deficiency significantly accelerated the motor neuron degeneration and shortened the life span of $SOD1^{G93A}$ transgenic mutant mice (Van Damme et al., 2005). In contrary, the over expression of edited GluR2 significantly delayed neurodegeneration in an ALS mouse model (Tateno et al., 2004).

The association between glutamate excitotoxicity and neuron death is strengthened by the fact that anti-glutamate treatment, riluzole, is the only treatment that modestly extends the life span of ALS patients (Miller et al., 2012). However, it is not clear whether these

dysfunctions in the glutamate-transport system are a primary disease mechanism or whether they are more a secondary event consequential to primary pathologic insults. So the question of temporal correlation between glutamate transporter dysfunction and pathology must be addressed more thoroughly.

1.5.2.2 Oxidative stress

Oxidative stress arises from an imbalance between the generation of reactive oxygen species (ROS) and from a reduced ability of the biological system to remove or repair ROS-induced damage. Oxidative damage to DNA, proteins and lipids and other macromolecules accumulates over time and contribute significant amount of endogenous damage, leading to ageing.

The accumulation of oxidative stress within non-dividing cells such as neurons during aging may be cumulative and cellular injury by ROS is a major potential cause of the age related damage in neuronal function that occurs in neurodegenerative disease. SOD1 encodes a major anti-oxidant defense protein and mutation in SOD1 accounts for 20% of FALS cases which makes the role of oxidative stress in ALS very interesting.

Several pathological studies have reported evidence of increased oxidative stress in ALS post-mortem tissue. Studies on CSF and human post-mortem CNS tissue have shown the presence of biochemical changes which reflects the effect of free radical damage in ALS cases than in controls (Smith et al., 1998; Mitsumoto et al., 2008). Protein carbonyl levels have been found to be elevated in both spinal cord (Simpson et al., 2004) and motor cortex (Tohgi et al., 1999) from sporadic ALS cases. Immunoreactivity to 3-nitrotyrosine has been found to be increased within large ventral horn neurons whereas markers for protein and lipid oxidation have been found to localise in motor neurons, reactive astrocytes and

microglia/macrophages in the grey matter neuropil of sporadic ALS patients, as compared to the control (Shibata et al., 2001).

Oxidative damage to RNA species has also been reported in mutant SOD1 mouse model as well as human CNS (Chang et al., 2008). Evidence of oxidative damage are also present in cellular and murine models of SOD1-related ALS (Barber and Shaw, 2010) and in particular the SOD1 protein itself appears to be highly susceptible to oxidative post-translational modification (Andrus et al., 1998). Familial SOD1 mouse models has been thoroughly investigated to find out source of oxidative stress and several different aberrant oxidative reactions have been proposed to be associated with the disease (Barber et al., 2006). A number of aberrant oxidative reactions catalyzed by mutant SOD1 have been proposed to contribute to the toxic function which suggests that mutant SOD1 may induce oxidative stress via a mechanism beyond its own catalytic activity (Tu et al., 1997). In vitro studies in motor neuron cell lines expressing mutant SOD1 reported down-regulation of genes involved in the antioxidant response, including the transcription factor Nrf2 (nuclear erythroid 2-related factor 2), several members of the glutathione S-transferase family, and two peroxiredoxins (Kirby et al., 2005). Recent evidence has shown reduced Nrf2 messenger mRNA and protein expression in spinal cord neurons of ALS patients (Sarlette et al., 2008).

A mechanism for the role of microglial mSOD1 has been reported within microglia according to which mSOD1 increases microglial superoxide production by NADPH oxidase (Nox) enzymes Nox enzymes have important role in inflammation, host defense and cell death. However increased Nox activity can be pathogenic and Nox-2 has been found to be upregulated in SALS patients and $SOD1^{G93A}$ mutant mice. Likewise deletion of either Nox1 or Nox2 extended survival in $SOD1^{G93A}$ mice (Wu et al., 2006; Marden et al., 2007).

1.5.2.3 Mitochondrial dysfunction

Mitochondria have major fundamental roles in eukaryotic cells. They provide bulk of adenosine triphosphate (ATP) required for most cellular functions and are therefore one of the main source of energy. They also play an important role in intermediate metabolism, in maintaining cellular calcium homeostasis and regulation of intrinsic apoptotic pathway (Hervias et al., 2006). Several line of evidence indicates a potential involvement of mitochondria in pathogenesis of SALS, FALS as well as in mutant SOD1 mice model of disease (Hirano et al., 1984; Dal Canto and Gurney, 1995; Kong and Xu, 1998; Sasaki and Iwata, 2007). Observations of mitochondrial morphology in cell or animal models of familial ALS showed aggregated, swollen, vacuolated or fragmented mitochondria (Bendotti et al., 2001; Jaarsma et al., 2001; Coussee et al., 2011). The mitochondrial swelling and extensive vacuolization of the motor neuron cytoplasm in mutant SOD1 mice increases with age and accompanies massive motor neuron damage (Bendotti et al., 2001). Changes in the activity of the different complexes of the electron transport chain have been described in tissues obtained from sALS patients (Wiedemann et al., 1998; Borthwick et al., 1999; Vielhaber et al., 2000). Similarly in motor neurons of mutant $SOD1^{G93A}$ mice a reduction in activity of complex I was observed before disease onset and progressing to inhibition of complex IV at later stages (Jung et al., 2002). Also, complexes II and IV activity were decreased in a cell culture model of FALS (Menzies et al., 2002). It has been reported that calcium buffering is altered in mitochondria purified from the CNS of mSOD1 mice, and could increase the susceptibility of motor neurons to the altered calcium homeostasis (Damiano et al., 2006).

Depletion of mitochondrial calcium-buffering capability is specifically deleterious to neurons and skeletal muscle, whose normal functioning involves frequent influxes of calcium to generate action potentials (Wong and Martin, 2010). Caspase-1 activation is an early event in SOD1 mutants, occurring prior to neuronal cell death and damage to mitochondrial function is likely to contribute this process (Guegan et al., 2002). Reports also show that misfolded SOD1 conformers are associated with the cytoplasmic face of spinal cord mitochondria but not mitochondria from other tissues and that specific factor could favour the association of misfolded SOD1 with spinal mitochondria and thus, induce

toxicity (Vande Velde et al., 2008). In conclusion, many aspects of mitochondrial functioning are affected in ALS. They may exhibit interactive properties and contribute to the overall pathogenesis of the disease. Many of the described changes may be causative or consequential during neuronal degeneration and, in conjunction with other cellular and molecular mechanisms, present a complex picture of progressive deterioration that leads to cell death.

1.5.2.4 Neurofilament abnormalities and defects in axonal transport:

Neurofilament proteins constitute a major component of the cytoskeleton of neurons and play key role in the maintenance of cell shape and axonal calibre as well as in axonal transport (Xu et al., 1993). Neurofilament subunits are assembled in the cell body of the motor neuron and are transported down the axon. Abnormal assembly and accumulation of neurofilaments in the cell body and proximal axons of motor neurons is a hallmark of the pathology of ALS (Carpenter, 1968; Hirano et al., 1984; Hays et al., 2006). Transgenic mice with overexpression of neurofilament subunits or point mutations display neurofilament accumulation and selective motor neuron dysfunction (Lee et al., 1994).

Over expression of any of the wild-type neurofilament subunits can induce the formation of perikaryal neurofilament accumulation, but no motor neuron degeneration (Julien, 2001). High-level expression of human NF-H proteins causes large perikaryal neurofilament inclusions associated with motor axon atrophy, axonal transport defects, altered axon conductance, motor dysfunction but not with motor neuron death (Kriz et al., 2000). Hyperphosphorylation of neurofilaments also contributes to defective transport by causing their detachment from motor complexes and promoting aberrant self-association (Ackerley et al., 2003). In ALS, this phenomenon is attributable to overactivation of p38 MAP kinase and Cdk5, which phosphorylate NF-M and NF-H (Ackerley et al., 2004). Abnormal

phosphorylation of neurofilament or disruption of the neurofilament network could therefore lead to defect in axonal transport or the sequestration of organelles such as mitochondria and thereby induce motor neuron degeneration.

1.5.2.5 Deficient protein quality control and endoplasmic reticulum stress

Cellular functions that may be impaired by mutant aggregates include, the heat shock protein (HSP) pathway and proteosome-mediated proteolysis pathways (Shinder et al., 2001; Wood et al., 2003). The protein-directing chaperone mechanisms of the HSP pathway may be affected by direct sequestration, depletion or decreased expression of HSPs induced by the aggregates (Bruening et al., 1999). Reduced expression of HSP-25 precedes the appearance of SOD1 aggregates and the onset of motor neuron death in SOD1 transgenic mice, suggesting that impaired HSP expression may facilitate neuronal death (Maatkamp et al., 2004).

The presence of protein aggregates in spinal cords of FALS and SALS patients correlates with the activation of stress signaling pathway emerging from the endoplasmic reticulum (ER), a cellular reaction named the “unfolded protein response” (UPR). Protein misfolding elicits the ER stress response pathway (Kaufmann and Mitsumoto, 2002). ER stress engages the unfolded protein response (UPR), an integrated signal transduction pathway that re-establish homeostasis by increasing the protein folding capacity and quality control mechanisms of the ER. The ubiquitin proteasome system (UPS), in which ubiquitin-tagged proteins are targeted for proteasomal degradation, is one such mechanism of misfolded protein clearance. Ubiquitin- and ubiquitin ligase-positive intraneuronal inclusion bodies are found in FALS mouse models (Bruijn et al., 1997) and post-mortem spinal cord of SALS patients,(Watanabe et al., 2001; Sasaki et al., 2010) indicating UPS activity and sequestration in both forms of the disease. Studies of SOD1 mutant transgenic mice reveal

that protein disulphide isomerase (PDI), an ER-resident chaperone and a marker of the UPR, is activated in mSOD1 mice (Atkin et al., 2006) and in biosamples from patients with SALS (Atkin et al., 2008), where it co localizes with mSOD1 inclusions. The ER-associated SOD1 aggregates bind to the ER-luminal polypeptide chain binding protein (BiP) (Kikuchi et al., 2006), a chaperone that regulates the activation of ER stress transducers such as IRE1, PERK, and ATF6. Protein quality control by ER-associated degradation (ERAD) is also impaired in ALS, leading to stress signaling that can directly induce motor neuron death via activation of apoptosis. Dysfunction or overloading of ERAD results in accumulation of unfolded proteins and triggers the unfolded protein response (UPR) (Kozutsumi et al., 1988).

1.5.2.6 A role of non-neuronal cells in motor neuron death

Initial efforts assumed that ALS was cell autonomous; meaning that damage within a selective population of affected neurons alone suffices to produce disease. Accordingly, attempts were made to generate disease from selective mutant SOD1 expression only in motor neurons.

However, several studies using cell-specific expression of mutant SOD1 support a prominent role of non-neuronal cells in promoting cell death. The most striking evidence against cell-autonomous motor neuron death is the reported lack of ALS phenotype of transgenic mice expressing mutant SOD1 under a neuron-specific promoter NF-L or Thy1 (Pramatarova et al., 2001; Lino et al., 2002). These observations put a question mark on neuronal cells as the primary pathological site of injury in ALS and implied a role for non-neuronal cells in the disease.

A later study succeeded with mutant synthesis largely restricted to neurons, using mutant SOD1 expression driven by neuron-specific Thy1.2 promoter (Jaarsma et al., 2008). In this

latter paradigm, however, even animals with the highest level of mutant synthesis developed disease only at very late ages and disease progressed slowly without reaching the same degree of paralysis relative to lines expressing the same mutant SOD1 ubiquitously. The animals showed different pathological changes compared to transgenics ubiquitously expressing FALS mutant SOD1. Symptoms onset occurred later, were diffused rather than focal, and lacked certain morphological hallmarks such as mitochondrial vacuolization. These studies supported a role for intrinsic pathways to motor neuron degeneration in ALS but later evidence for role of non-neuronal cells in ALS has also emerged.

More substantial evidence came from construction and analysis of chimeric mice that were mixtures of hSOD1 mutant-expressing cells and normal cells (Clement et al., 2003). The use of chimeric mice showed that damage to motor neurons could derive from toxicity of mutant SOD1 in cell types other than motor neurons. In chimeric mice expressing mSOD1 in specific cell types, normal motor neurons developed signs of ALS pathology when surrounded by mSOD1-expressing glia. They also demonstrated that the proportion of non-neuronal cells that did not harbour the transgene correlated positively with the proportion of surviving mSOD1-expressing motor neurons and the lifespan of the chimeric mice. The loss of motor neurons was more prominent when surrounded by mSOD1 expressing non-neuronal cells as compared to wild type non-neuronal cells. Wild type motor neurons also showed signs of injury e.g., intracellular ubiquitination, when surrounded by mSOD1 expressing nonneuronal cells. (Clement et al., 2003)

To determine the contribution of astrocytes and microglia, double transgenic mice with a floxed SOD1 ^{G37R} gene have been generated which express the Cre-Lox recombination system to exclude the mutant SOD1 from motor neuron and cells of myeloid lineage (Boillee et al., 2006a). Excision of the floxed mutant SOD1 gene exclusively within motor neurons delayed the disease onset but there was no alteration in disease course, once initiated. In contrast, diminishing mutant SOD1 levels from microglia altered the disease onset and disease progression was extended by nearly 50%. Slowed progression after

reduced mutant SOD1 synthesis in microglia was further confirmed by an analogous approach in a different mutant transgenic line (Wang et al., 2009b). Taken together, it was concluded that mutant SOD1 expression in motor neurons determines the initial timing of disease onset but very surprisingly does not contribute much to later disease progression. The restricted mutant SOD1 expression in astrocytes failed to produce the disease (Gong et al., 2000), but selective reduction of mutant SOD1 in astrocytes slowed disease progression and doubled the length of disease duration after onset (Yamanaka et al., 2008). Rodent derived primary astrocytes expressing mSOD1 exerts toxic effects on cultured primary motor neurons as well as embryonic mouse and human stem cell-derived motor neurons (Clement et al., 2003; Di Giorgio et al., 2008). Advancing the theory of the non-cell autonomous contribution of astrocytes in ALS, the Kaspar group generated astrocytes from post-mortem tissue from both fALS and sALS patients. They showed, in co-culture and conditioned medium, that these astrocytes from both patient groups were similarly toxic for motor neurons (Haidet-Phillips et al., 2011). The group also showed that the knock-down of SOD1 expression significantly attenuated astrocyte-derived motor neuron toxicity. This clearly indicates that mutant SOD1-expressing astrocytes exert toxic effects and are unable to provide any trophic support to motor neurons. Mutant SOD1 expression in oligodendrocytes and Schwann cells alters the properties of these cells but it does not seem to be sufficient to cause motor degeneration (Lobsiger et al., 2009; Turner et al., 2010). Hence, it is clear that mutant SOD1 expression within motor neurons determines the onset of disease and it confers vulnerability to the degenerative process which can be exacerbated by the presence of mutant SOD1 in surrounding glial cells.

1.5.2.7 Neuroinflammation

Neuroinflammation is a pathological hallmark of neurodegenerative diseases including ALS. The presence of activated microglia, astrogliosis and infiltrating lymphocytes indicates existing inflammatory components in CNS pathology in ALS (Henkel et al., 2004). Alexianu et al. (2001) reported that levels of a marker of astrocytic activation, glial acidic fibrillary protein (GFAP), were increased at the terminal phase of the disease in

$SOD1^{G93A}$ mice, whereas microglial activation, as measured by protein levels of CD11b, occurred at 80 days of age during the late pre-symptomatic stage (Alexianu et al., 2001). In contrast, dendritic cells, another type of antigen presenting cell, are significantly increased in number during the late symptomatic period of ALS in transgenic mice (Henkel et al., 2006).

An increased presence of pro-inflammatory molecules like MCP-1 and MCS-F has been detected in spinal cord of of ALS patients (Henkel et al., 2004). Marked increased in COX-2 expression has beed reported in postmortem tissue of patients (Yasojima et al., 2001) as well as SOD1 mice (Almer et al., 2001). However, no significant difference in other cytokines such as IL-1~, IL-6, RANTES, IFN-γ and TNF-α was observed in SOD1 mice. Although other groups reported an increase in TNF-α and IFN-γ in serum samples and of IL-1 in spinal cord of ALS patients (Babu et al., 2008).

T cells have also been reported in spinal cord tissues of ALS patients in late stage of disease (Kawamata et al., 1992). A reduction in circulating T cells numbers has also been reported to occur in ALS patients. Reduced blood CD4+ and CD25+ regulatory T (Treg) and monocytes (CD14+) counts have been found early in ALS. Recently, evidence has established a neuroprotective role of Treg cells in attenuating inflammation by stimulating secretion of anti-inflammatory cytokines (Kipnis et al., 2004). Interestingly, adoptive transfer of activated T lymphocytes proved to be protective (Banerjee et al., 2008) and knock-out for CD4 (CD4-/-), developed a more aggressive phenotype, reversible by bone marrow transplantation (Beers et al., 2008). In a similar observation lack of functional T cells via the use of various knock-out mouse models ($RAG^{-/-}$, $TCR^{-/-}$) was shown to accelerate motor neuron degeneration and diminish survival of mutant SOD1 mice (Beers et al., 2008; Chiu et al., 2008). Surprisingly, T cell ablation also resulted in a decrease in microglial cell expression of neurotrophic factors such as IGF-1 (Beers et al., 2008) .

Astrocyte activation plays a central role in inflammation and reports suggest that mSOD1 astrocytes secrete inflammatory mediators including prostaglandin E2, leukotriene B4, iNOS, and NO under both basal and activated conditions (Hensley et al., 2006). Reports also suggest that mutant SODl-expressing astrocytes release toxic factors that can selectively kill both wildtype and mutant motor neurons in culture (Di Giorgio et al., 2007; Nagai et al., 2007; Di Giorgio et al., 2008).Taken together these studies suggest that microglia and astrocytes may represent a necessary component of the deleterious cascade ultimately resulting in neuron cell death. Therefore, modulating inflammatory response in ALS has become an attractive and worthwhile pursuit that may ultimately become an essential component of therapeutic management.

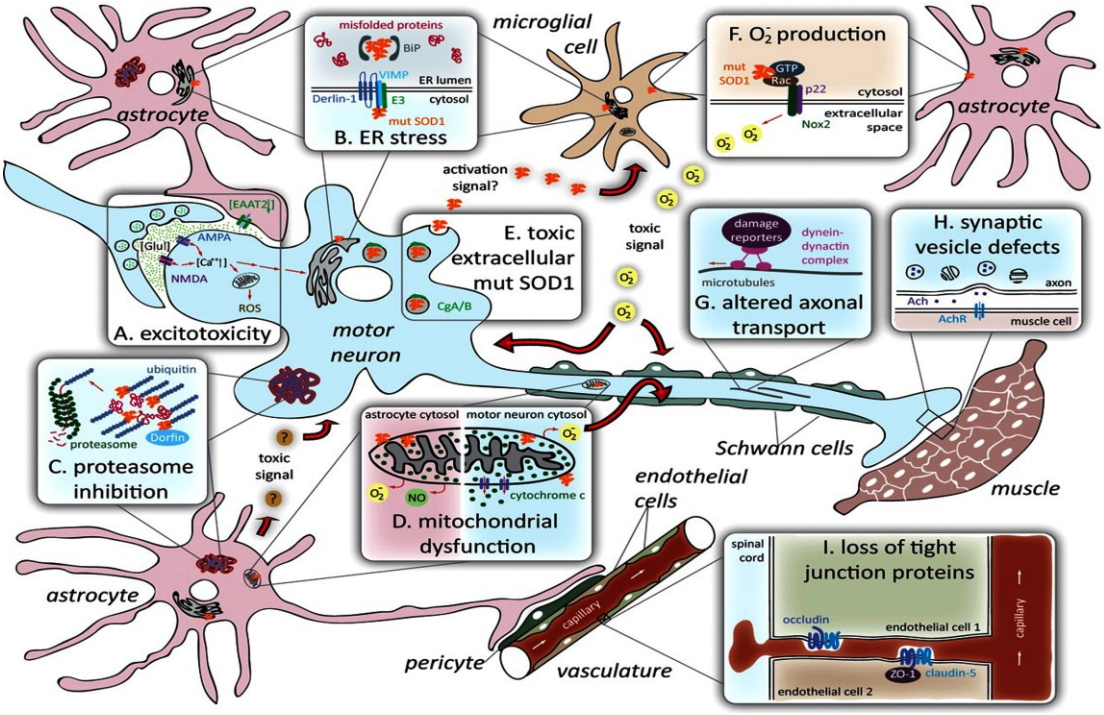

Figure 1.9 The pathophysiological mechanisms underlying neurodegeneration in ALS.

ALS is a multifactorial disease with a complex interplay between molecular and genetic pathways. (A) Excitotoxicity is the hyperactivation of motor neurons resulting from failure to rapidly remove neurotransmitter glutamate from synapses due to deficiency in the glutamate transporter EAAT2 in the neighbouring astrocytes. (B) Abnormal interactions of mutant SOD1 with ER associated proteins induce ER stress. (C) Overload of the proteasome degradation pathway with ubiquitinated misfolded proteins may lead to proteasome inhibition. (D) Mutant SOD1 deposition on mitochondrial membrane induces release of cytochrome c in motor neurons. (E) Toxic intracellular mutant SOD1 is secreted from motor neurons and astrocytes after interaction with components of neurosecretory vesicles. (F) Altered axonal transport (H) Synaptic vesicle defects (I) Loss of tight junction proteins within capillary endothelial cells results in disruption of the blood-spinal cord barrier, which results in microhemorrhages within the spinal cord before disease onset. (Adapted from (Ilieva et al., 2009))

1.6 ALS pathogenic pathways as therapeutic target

1.6.1 Pharmacologic approach

As stated above, ALS is a multifactorial disease and several pathogenic mechanisms may lead to motor neuron death in ALS. Consequently, many different therapeutic approaches have been evaluated for possible treatment of ALS patients (Bruijn, 2002; Morrison, 2002; Strong, 2003; Zinman and Cudkowicz, 2011; Morren and Galvez-Jimenez, 2012; Gordon et al., 2013). To facilitate the study of pathogenic mechanisms and to explore new treatments, transgenic animals expressing mutant SOD1 have been widely used and remain the most used and best described animal models. This section will focus on different therapeutic strategies aimed to limit the pathological mechanism in ALS disease models and ALS patients.

1.6.1.1 Anti-oxidants

Neuropathological studies have shown evidence of oxidative stress in SALS and FALS cases with SOD1- mutations (SOD1-FALS) (Barber et al., 2006).

Based on previous evidence of protective effect of vitamin E in ALS, in 2001, the ALS riluzole--tocopherol study group published results from their randomized, double blind, placebo controlled trial of vitamin E, 500 mg twice daily. There was no effect on the primary outcome measure and survival was not influenced over the 12-month trial period (Desnuelle et al., 2001). Likewise, treatments with N-acetyl-L-cysteine, which is an essential cofactor for the electron transport chain within mitochondria produced promising results in ALS transgenic mouse models. (Matthews et al., 1998) but failed to show any benefits in human trial (Louwerse et al., 1995; Henderson et al., 1996). CoQ10 at 2700 mg/day for 9 months did not show sufficiently favorable results to pursue a Phase III trial (Kaufmann et al., 2009). Manganese porphyrin-labeled AEOL 10150 (SOD mimetic) is a small-molecule compound which catalytically neutralizes hydrogen peroxide, superoxide,

peroxynitrite and also inhibits lipid peroxidation. Daily administration of AEOL 10150 (2.5 mg/kg) by the intraperitoneal or subcutaneous route from symptom onset (~90 days) in the $SOD1^{G93A}$ mice led to a significant increase in survival (Crow et al., 2005). Currently this drug is up for phase II/III clinical trial.

1.6.1.2 Glutamate modulators

Impeding excitotoxicity is an appealing therapy target for ALS. This approach is so promising that currently the only approved treatment for ALS, , is riluzole, which is thought to inhibit glutamate release from nerve endings by stabilizing the inactive state of voltage-dependent sodium channels (Doble, 1996). In preclinical studies, riluzole was found to modulate the transmission of glutamate in hippocampus slices (Bellingham, 2011). After clinical studies in ALS, riluzole was approved by the Food and Drug Administration (FDA) in 1995 for treatment. The clinical benefits are modest, extending ventilator-free survival by approximately 3 months, (Bensimon et al., 1994) but it remains the only FDA approved disease-modifying drug for ALS.

Other antiglutamate drugs have been evaluated in clinical trials. Most of them are antiseizure medications that have antiglutamate properties, and include topiramate, gabapentin, and lamotrigine, but none have showed any survival benefit (Eisen et al., 1993; Miller et al., 2001; Maragakis et al., 2003). Ceftriaxone was shortlisted from screening over 1000 FDA-approved compounds and it stimulated EAAT2 expression which was found to be neuroprotective in various ALS models (Rothstein et al., 2005). However the three-stage study determined that Ceftriaxone a third-generation cephalosporin, does not significantly increase the survival time or significantly decrease the rate of decline in function for subjects with ALS (Zhao et al., 2014).

1.6.1.3 Mitochondrial protectants and ER stress reducers

Different therapeutic approaches have been targeted to mitochondrial dysfunction and increased reactive oxygen species (ROS), thereby trying to protect mitochondria against the pro-apoptotic effects of mutant SOD1. Coenzyme Q10, cofactor of the electron transport chain was demonstrated to significantly extend survival in a transgenic mouse model of ALS (Beal, 2002). Oral administration of the energy-buffering compound creatine resulted in a dose dependent increase in survival of SOD1 transgenic mice (Klivenyi et al., 1999). However, creatine failed to show efficacy in ALS patients (Groeneveld et al., 2003).

The tetracycline derivative, minocycline might be a multifunctional drug that affects different molecular pathways involved in ALS pathogenesis. Minocycline inhibits cytochrome c release from the mitochondria and it was shown to delay the disease onset and extend survival in SOD1-transgenic mice (Kriz et al., 2002; Zhu et al., 2002). However, later on Keller et al in 2009 showed that when administered at later stages of disease, once microglial cells are chronically reactive, minocycline may not have anti-inflammatory properties, and contrary to expectations, may alter astrocyte reactivity and increase microgliosis (Keller et al., 2011). Clinical trial with minocycline (up to 400 mg daily) was also performed but unexpectedly the treatment increased deterioration rate of patients (Gordon et al., 2007). Dexpramipexole, reduces the generation of reactive oxygen species, and suppress apoptosis. It showed neuroprotection in vitro and in vivo, including the G93A mouse model (Gribkoff and Bozik, 2008) . However, dexpramipexole also failed in a phase III trail (Cudkowicz et al., 2013).

ER stress has been previously implicated as a likely contributor to ALS onset and progression, and the new findings suggest that therapies designed to reduce ER stress potentially may offer some benefit in ALS. The compounds named *salubrinal*, *guanabenz* and *phenazine*, as well as the previously identified *methylene blue*

suppressed toxicity caused by mutant TDP43 protein in worm and zebra fih model of ALS (Vaccaro et al., 2013). Salubrinal was also found to be effective in SOD1^{G93A} mouse model of ALS (Saxena et al., 2009).

1.6.1.4 Anti-inflammatory agents:

High levels of Cyclooxygenase-2 (COX-2) has been detected in ALS spinal cord (Almer et al., 2001). Expressed in spinal astrocytes and neurons, COX-2 catalyzes prostaglandin 2, which stimulates glutamate release from astrocytes via a calcium-dependent pathway (Bezzi et al., 1998). The COX-2 inhibitor and anti-inflammatory agent celecoxib significantly delayed the onset and prolonged survival of ALS mice; preserved spinal neurons with reduced astrogliosis and microglial activation (Drachman and Rothstein, 2000).When evaluated in a clinical trial of 300 ALS patients, celecoxib was ineffective (Cudkowicz et al., 2006).

TNF-α activates microglia and cause neuronal apoptosis, and elevated levels of TNF-α have been found in the spinal cords of SOD1^{G93A} mice and serum human ALS cases (Robertson et al., 2001). Immunomodulatory agents, thalidomide and its analog lenalidomide inhibited TNF-α production, enhanced motor performance and significantly increased survival in SOD1^{G93A} mice when administered prior to onset of disease (Kiaei et al., 2006).

1.6.1.5 Anti-aggregation agents

Few chemical compounds are capable of reducing SOD1 aggregates in models of ALS. Arimoclomol is an amplifier of HSP expression, which physiologically increases the availability of HSPs in response to acute or chronic stress. Arimoclomol, delayed disease progression and extends the lifespan of pre- or early symptomatic stages in transgenic

$SOD1^{G93A}$ mice (Kieran et al., 2004). Late-stage treatment improved muscle function (Kalmar et al., 2008). Arimoclomol reduces ubiquitin aggregates in the spinal cord of $SOD1^{G93A}$ mice. Ongoing phase II/III clinical trials indicated arimoclomol's good safety and tolerability (Cudkowicz et al., 2008). Transgenic mice overexpressing a subset of Hsp genes have been generated to investigate the effects of increased expression of Hsps in models of ALS (Liu et al., 2005). Genetic up-regulation of a single Hsp was found to be not as effective in models of ALS. For example, genetic over-expression of Hsp70 levels to approximately 10-fold higher than normal mutant SOD1 mice did not affect either disease onset or survival (Liu et al., 2005). These results suggest the fact that even up-regulation of a single, specific Hsp, as effective at targeting protein aggregation as Hsp70, is not sufficient to enhance the overall protein quality control machinery. Instead, targeting a broader and more complex chaperone network could be beneficial. For example, Patel et al showed that in an in vitro cell model expressing variants of mutant SOD1, over-expression of Hsp27 and Hsp70 together exert greater cytoprotective effect against apoptotic stimuli than observed following individual expression of these Hsps (Patel et al., 2005). However, Yerbury et al, showed that co-incubation of mutant SOD1 recombinant protein with Hsp27 resulted in reduced aggregation of SOD1 in a cell free system (Yerbury et al., 2013).The $SOD1^{G93A}$/Hsp27 double transgenic mice over expressing Hsp27 showed, delayed decline in motor strength, a significant improvement in the number of functional motor units and increased survival of spinal motor neurons compared to single transgenic $SOD1^{G93A}$ mice during early phase of disease (Sharp et al., 2008). This suggests that pharmaceutical agents that up-regulate Hsp expression are a more attractive therapeutic strategy than those based on genetic manipulation.

1.6.2 Gene and Anti-sense therapy

Systemic delivery of proteins to the CNS is only possible if specific uptake sites in the blood brain barrier (BBB) are present. A disadvantage of systemic delivery of therapeutic proteins is the occurrence of some systemic side effects and their rapid degradation. Direct delivery of therapeutic proteins into the CNS is likely to circumvent these limitations.

Therefore, direct delivery techniques have been developed including gene therapy, which allows long-term, localised, regulated expression of therapeutic genes. To introduce therapeutic genes into the CNS, both ex vivo gene therapy and in vivo gene therapy can be exploited.

1.6.2.1 Delivery of factors for neuroprotection

The rationale for gene delivery of neurotrophic factors for ALS comes from animal proof-of-principle data demonstrating that secreted neurotrophic factors can support MN survival in a diseased milieu and thus prevent progression of degeneration.

Kaspar and co-workers used the retrograde transport ability of AAV to deliver insulin growth factor-1 (IGF-1) to motoneurons in high-copy mSOD1 ALS mice by injections into the respiratory- and motor limb-muscles. IGF-1 treatment started at 60 days of age delayed onset by 31 days and the median survival was increased by 37 days compared to control mice. Interestingly, when AAV-IGF-1 was applied at the time of onset (i.e.injections at 90 days of age) the life-span of IGF-1 treated animals was still increased by 22 days compared to the control group (Kaspar et al., 2003).

In another study in which AAV injections into the gastronemicus and triceps brachii muscles were used to deliver Glia derived neurotrophic factor (GDNF) to the spinal cord, treatment starting at 9 weeks of age delayed onset and survival (Wang et al., 2002b). Similarly, intramuscular injection of an EIAV-based lentiviral vector expressing VEGF resulted in prolonged survival in the same mouse model (Azzouz et al., 2004). In 2010, Henriques et al.used AAV to directly target G-CSF expression to the spinal cord, showing that intraspinal delivery improved motor function, delayed disease progression, and increased survival by 10% (Henriques et al., 2010; Henriques et al., 2011). All these observations demonstrate that sustained neurotrophic factor delivery and activity in the periphery and CNS significantly rescue ALS neurodegeneration. Currently, MoNuDin®

(an EIAV-based lentiviral vector system for the delivery of VEGF) is the only gene therapy technology currently in the stage of preclinical development for the treatment of ALS.

1.6.2.2 Gene silencing

A non-pharmacological approach to the interception of damage is to silence the synthesis of specific genes coding for proposed mediators in the pathogenesis of ALS.
Small interfering RNAs (siRNAs) provide a powerful research tool to decrease the expression of a single gene. Recent studies have investigated the therapeutic potential of using siRNA in mouse models of ALS (Miller et al., 2005; Ralph et al., 2005; Raoul et al., 2005).

Treatment of $SOD1^{G93A}$ mice with EIAV-based lentiviral vector mediating expression of RNAi molecules specifically targeting the human SOD1 gene resulted in an efficient and specific reduction of SOD1 expression and delayed the onset by more than 100% and an extension in survival by nearly 80% of their normal life span (Ralph et al., 2005). Raoul et showed that intraspinal injection of a lentiviral vector that produces RNAi-mediated silencing of *SOD1* substantially retards both the onset and the progression rate of the disease (Raoul et al., 2005).

In a similar approach siRNA targeting overall SOD1, when delivered to spinal motor neurons through retrograde transport of adeno-associated virus (AAV-2) injected into muscles substantially decreases an abundant target protein (SOD1) and produces a functional impact, delaying loss of grip strength in a mouse model of ALS (Miller et al., 2005) .Additionally, intramuscular delivery of AAV6.shRNAs.SOD1 in newborn mice has also failed to stop disease progression (Towne et al., 2008). Recent study by Foust et al showed that peripheral injection of AAV9 encoding an shRNA to SOD1 into significantly

reduced the amount of SOD1, delayed the disease onset and increased survival in $SOD1^{G93A}$ mice (Foust et al., 2013).

However currently there is no ongoing clinical trial with AAV vector encoding siRNA. But a phase I safety trial of the antisense technique to inhibit the production of SOD1 has been initiated by Isis Pharmaceuticals. The antisense oligonucleotides are delivered via an external pump and intrathecal delivery into the CSF. This marks the first antisense-based therapy for ALS.

1.6.3 Immunotherapy in ALS

The idea that immunological approach could be used to target misfolded toxic proteins is relatively new and is an emerging field of neurotherapeutics. It is very well known that toxicity of mutant SOD1 forms is related to misfolding/aggregation (Bruijn et al., 1998) . Moreover, the discovery of a pathogenic mechanism based on the toxicity of secreted SOD1 mutant proteins provided the idea of considering immunization as a potential approach to treat familial ALS caused by SOD1 mutations (Urushitani et al., 2007).

Both active and passive immunization approaches have been tried in order to decrease the load of misfolded SOD1 protein in mouse model of ALS (Figure 1.10) .The basic concept of active immunization is to prime the immune system to recognize an antigen as a foreign protein in order to mount a response against it. Utilizing this approach, the group of Dr. Jean-Pierre Julien targeted the extracellular SOD1 by vaccinating two mouse models of ALS by recombinant human SOD1 mutant. Vaccination with mutant SOD1 was effective in alleviating disease symptoms and delaying mortality in $SOD1^{G37R}$ model that moderately overexpress mutant SOD1.Thus, the vaccination delayed onset of disease and extended life span of $SOD1^{G37R}$ mice by about 4 weeks. This immunotherapy approach also conferred neuroprotection by the attenuation of motor neuron loss at the end stage of disease. However, vaccination against high-copy $SOD1^{G93A}$ mice was not effective, which

is not very surprising considering the excessive levels of mutant SOD1 molecules in this animal model (Urushitani et al., 2007). A peripheral immunization approach with a low BBB translocation of anti-SOD1 antibodies is obviously not ideal for clearance or neutralization of extreme amount of SOD1 mutant molecules in the CNS. Later on group of Urushitani et al in 2010, choose low copy $SOD1^{G93A}$ animal model and used two types of immunogens, G93A-apo-SOD1 and also WT-apo-SOD1, considering its misfolded nature of later. WT-apo SOD1 protein prolonged the life span and delayed the disease onset in low copy number $SOD1^{G93A}$ mice. The $SOD1^{G93A}$ vaccine also delayed the onset significantly and showed a trend to prolonging the life span (Takeuchi et al., 2010).

Liu et al in 2012, used an active immunization strategy using the SEDI antigenic peptide, displayed on a branched peptide dendrimer to target monomer/misfolded in $SOD1^{G37R}$ and $SOD1^{G93A}$ mutant SOD1 transgenic mice. Immunization delayed disease onset and extended disease duration, with survival times increased by an average of 40 d in $SOD1^{G37R}$ mice (Liu et al., 2012).

However, active immunization approaches based on directing the immune system against a normal human protein like SOD1 are probably not the most appropriate approach. Considering this, Gros-Louis et al in 2010 developed passive immunization strategies for ALS (Gros-Louis et al., 2010). Collection of monoclonal antibodies was generated that recognize specifically misfolded forms and not the intact wild type SOD1. Two monoclonal antibodies, namely, D3H5 and A5C3 were intracerebroventricularly infused into $SOD1^{G93A}$ mice by osmotic mini pump. One antibody succeeded in reducing the level of misfolded SOD1 by 23% in the spinal cord and in prolonging the lifespan of $SOD1^{G93A}$ mice in proportion to the duration of treatment. However another antibody (A5C3) failed to confer protection which indicates that not all anti-SOD1 antibodies might be suitable for immunotherapy. A key finding indicated in this study was that the Fab fragment of D3H5 monoclonal antibody was sufficient to confer protection in $SOD1^{G93A}$ mice (Gros-Louis et

al., 2010). Partial dispensability of the Fc fragment raised up the possibility of generating recombinant single-chain variable fragment (scFv) antibody.

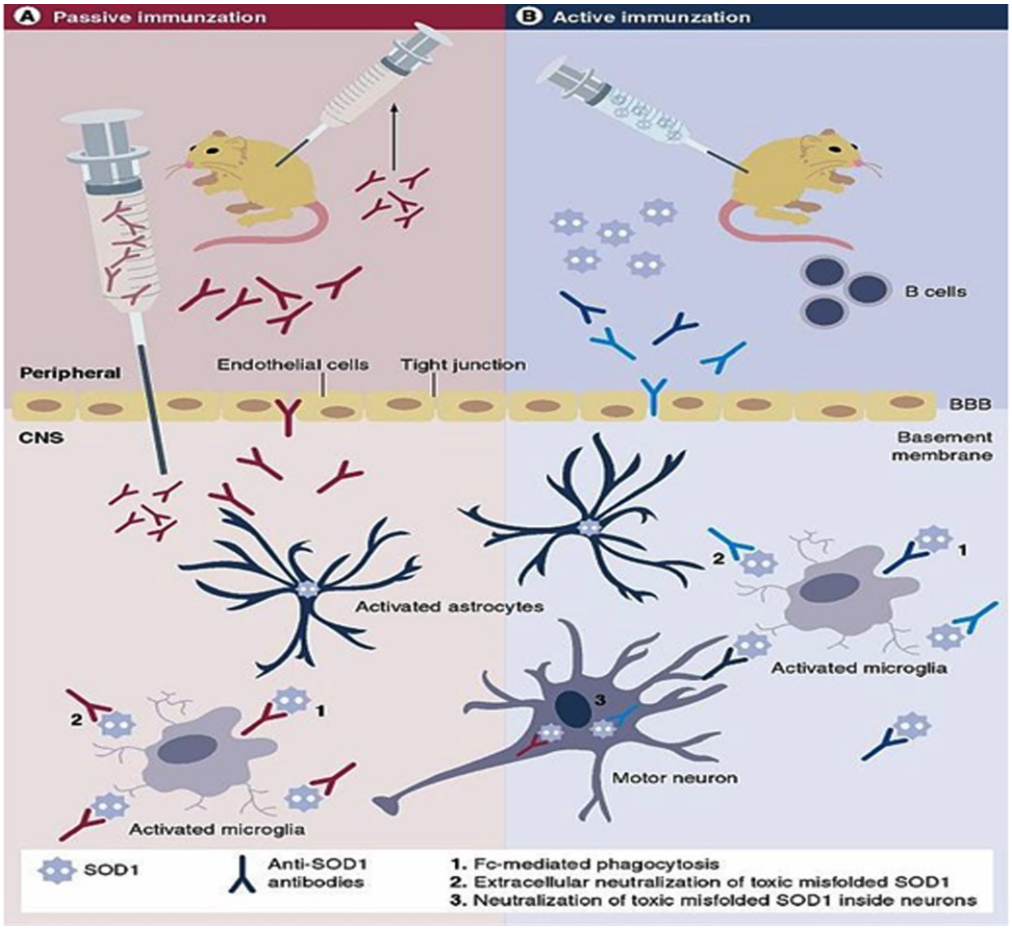

Figure 1.10 Specific immune reaction resulting from different immunization mechanisms that result in clearance of misfolded SOD1.

(A) Passive immunization with specific antibodies against misfolded SOD1 can be administered either at the periphery or directly into the CNS via intrathecal injections or infusion with mini-osmotic pumps. (B) Active immunization with either SOD1 peptides or misfolded unmetallated SOD1 protein can be administered to trigger humoral response and production of antibodies through B cells. The blood brain barrier (BBB) is created by the tight apposition of endothelial cells lining blood vessels in the brain preventing easy passage of large macromolecules and pathogens between the circulation and the brain. In both modes of immunization, a

small fraction of peripheral antibodies can cross the BBB to enhance the clearance of misfolded SOD1 species through various potential mechanisms.

1.6.4 Therapeutic approaches for TDP-43 transgenic mice model of ALS

From drug discovery and treatment perspective, TDP-43 is a target for both fALS as well as sALS. But lack of any TDP-43 mice model that truly mimic human amyotrophic ALS is a limiting factor towards development of therapeutic approach. Recently our lab generated three transgenic mouse models ($hTDP^{WT}$ overexpressor, TDP-43 ^{G348C} and TDP-43 ^{A315T}) which develop many age related pathological and biochemical changes occurring in human ALS and these animal models can be valuable for testing therapeutics (Swarup and Julien, 2011) .

Studies on these mice models suggested that TDP-43 deregulation in ALS may contribute to pathogenic pathways through abnormal activation of P65 NF-κB .The transcription factor NF-KB is a key regulator of hundreds of genes involved in innate immunity, cell survival and inflammation. TDP-43 interacts with and colocalizes with p65 in glial and neuronal cells from ALS patients and mice expressing wild-type and mutant TDP-43 transgenes, but not in cells from healthy individuals or nontransgenic mice. TDP-43 acted as a co-activator of p65, and glial cells expressing higher amounts of TDP-43 produced more proinflammatory cytokines and neurotoxic mediators after stimulation with lipopolysaccharide or reactive oxygen species (Swarup et al., 2011). The finding that TDP-43 acts as a co activator of P65 suggested a key role of NF-KB signalling in ALS pathogenesis and opened door for targeting NF-KB as a possible therapeutic strategy for treatment. Treatment of TDP-43 transgenic mice with Withaferin A, an inhibitor of NF-κB activity, reduced denervation in the neuromuscular junction and ALS disease symptoms. Overall, pharmacological inhibition of NF-κB by WA treatment attenuated disease phenotypes in TDP-43 transgenic mice models.

1.6.5 Single chain antibodies as therapeutic tools

Antibodies are a special class of proteins that play an important role in maintaining our immunity. These proteins are produced by B lymphocytes as part of the humoral immune system. Antibodies like Ig-G (Immunoglobulin G) are Y shaped molecules that have heavy and light chains which contain either variable or constant regions (Holliger and Bohlen, 1999). Variable regions differ between each antibody and confer the specificity that is required. Specific regions within the variable parts called the complementarily determining regions or CDR regions have different sequences enabling unique antigen binding.

Recombinant antibody technology has become a major player in the therapeutic pipeline for cancer, infectious diseases, and autoimmunity and are beginning to realize the promise enclosed in their earlier denomination "magic bullets".

Monoclonal antibodies have found applications in diagnosis and in treatment of various diseases, including cancer. Approx. 20 of them have the approval for therapeutic use in humans such as RituxanR for lymphoma and HerceptinR for breast cancer. However monoclonal antibodies face several difficulties, as they are almost exclusively murine in origin thus could create human anti-mouse antibody (HAMA) when introduced to human therefore limits their clinical applications (Watkins and Ouwehand, 2000). Added to this, monoclonal antibody producing technology is very laborious and time consuming. So, to improve this technology smaller antibodies have been engineered which exhibit better tissue penetration and enable binding specificity encoded by a single polypeptide gene. Among these novel antibodies, single chain antibodies are the smallest one with sizes of approx between 28 to 30 kDa versus 150 kDa for entire immunoglobulins. A scFv comprises the variable domain of the heavy and light chains (VH and VL) of a monoclonal antibody joined by a linker peptide (Figure 1.11). Variable heavy chains alone are found in some recombinant antibodies like Nanobodies (approximately 15 kD). Bispecific antibodies are formed when two scFv fragments are combined. ScFv may also be combined to form triabodies (trivalent approximately 75 kD) and tetrabodies (tetravalent

approximately 100 kD). The advantages of scFvs compared to Igs make them interesting tools for therapy of neurodegenerative diseases: (i) they can be easily selected and expressed in bacterial systems, (ii) they should better penetrate brain tissues, (iii) they can be delivered by gene therapy, and (iv) they do not induce immune responses due to the lack of the Fc part. ScFvs already proved great potential in several publications and clinical trials predominantly in the cancer field. Nevertheless, new scFvs are emerging for neurodegenerative diseases. Liu et al in 2004 developed scFvs directed against ß amyloids as an alternative to monoclonal antibodies that provoke dangerous side effects in patients. These scFvs can eliminate toxic effect of aggregated Aß-peptides causing Alzheimer's disease in cells (Liu et al., 2004). ScFvs directed against huntingtin have been engineered and might be helpful to treat Huntington disease (Khoshnan et al., 2002).

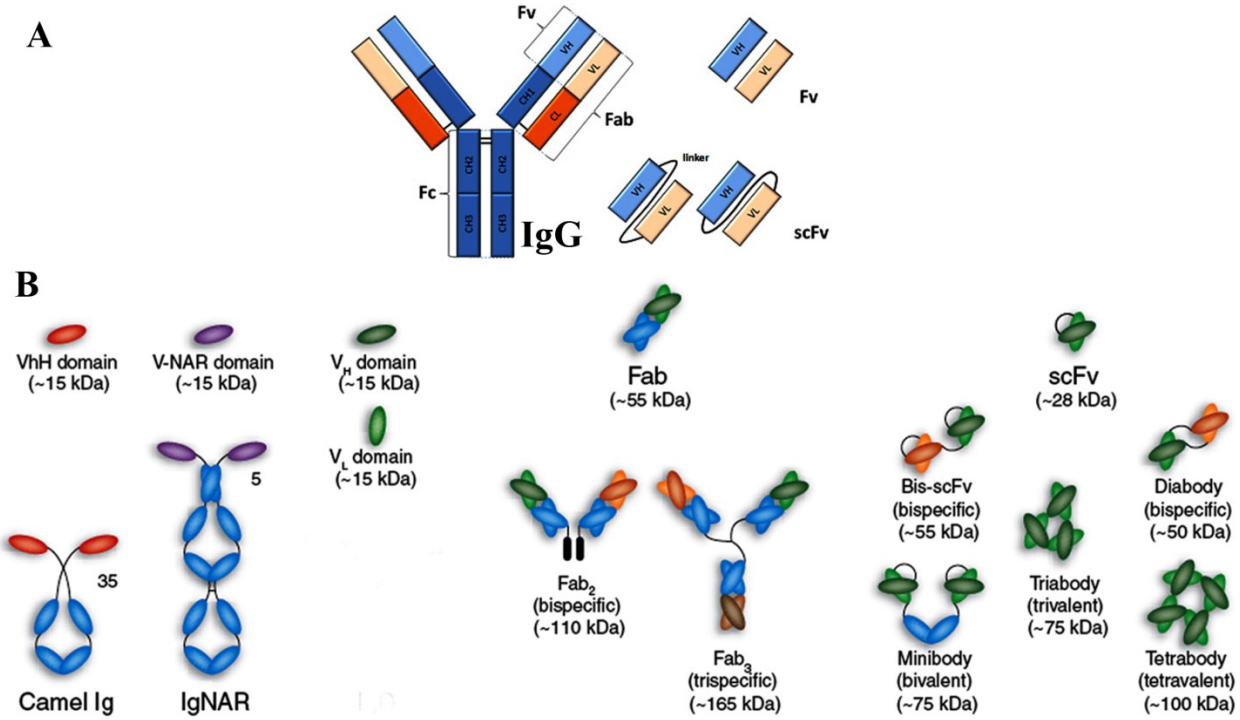

Figure 1.11 Schematic representation of different antibody formats.

(A) showing intact 'classic' IgG molecules alongside a single-chain variable fragment (scFv) which is a fusion protein of the variable regions of the heavy (V_H) and light chains (V_L) of IgG, connected with a short linker peptide of ten to about 25 amino acids.

(B) Below are the Camelid VhH-Ig and shark Ig-NAR immunoglobulins. Camelid VhH-Ig and shark Ig-NARs are unusual immunoglobulin -like structures comprising a homodimeric pair of two chains of V-like and C-like domains (neither has a light chain), in which the displayed V domains bind target independently. Shark Ig-NARs comprise a homodimer of one variable domain (V-NAR) and five C-like constant domains (C-NAR). A variety of antibody fragments are depicted, including Fab, scFv, single-domain VH, VhH and V-NAR and multimeric formats, such as minibodies, bis-scFv, diabodies, triabodies, tetrabodies and chemically conjugated Fab multimers (sizes given in kilodaltons are approximate). (Taken from (Holliger and Hudson, 2005))

1.7 Objectives of the thesis

The research described in this thesis focuses on the use of single chain antibody-mediated gene therapy and Withaferin A as therapeutic options in ALS.

In chapter 2, we demonstrated the feasibility of a new gene therapy modality, where we generated an AAV vector encoding a secretable scFv antibody (AAV-scFv) to target misfolded SOD1. A single intrathecal injection of this AAV viral vector in adult $SOD1^{G93A}$ mice led to sustained production of secretable scFv antibodies in the spinal cord and it significantly delayed disease onset and mortality. This therapeutic approach may be applicable to ALS cases with SOD1 mutations and perhaps to subset of sporadic ALS cases given that misfolded and aggregated SOD1 species have been detected in sporadic ALS.

In chapter 3, we tested effect of Withaferin A in mice from two transgenic lines expressing different ALS-linked SOD1 mutations, $SOD1^{G93A}$ and $SOD1^{G37R}$. Intraperitoneal administration of WA at a dosage of 4mg/kg resulted in improved survival in both mice model of ALS. The beneficial effects of WA in $SOD1^{G93A}$ mice model was accompanied by alleviation of neuroinflammation, a reduction in loss of motor neurons and decrease in levels of misfolded SOD1 species in spinal cord. Interestingly, WA treatment triggered robust induction of heat shock protein 25. The therapeutic effects of WA in various ALS mouse models suggest that WA should be considered as a promising lead compound for drug development aiming to treat ALS.

Chapter 2: Adeno-associated virus-mediated delivery of a recombinant single chain antibody against misfolded superoxide dismutase for treatment of amyotrophic lateral sclerosis

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2.1 Resume

Des études récentes démontrent que le mauvais repliement de l'enzyme superoxyde dismutase 1 (SOD1) peut représenter un mécanisme pathogénique commun à la fois pour les formes sporadique et familiale de la sclérose latérale amyotrophique (SLA). Pour réduire les dommages causés par la SOD1 mal repliée dans le système nerveux, nous avons testé une nouvelle approche thérapeutique basée sur l'expression ponctuelle et bénéfique d'un virus adéno-associé (AAV) contenant une construction d'ADN codant pour le fragment d'anticorps unique, variable et sécrétable (scFv) composé des régions variables de chaînes lourdes et légères (VH et VL) de l'anticorps monoclonal D3H5, se liant spécifiquement à la SOD1 mal repliée. Une injection intrathécale unique du virus adéno-associé codant pour l'anticorps à chaîne unique chez la souris SOD1G93A à 45 jours d'âge a donné lieu à l'expression prolongée d'anticorps à chaîne unique dans la moelle épinière et a retardé l'apparition de la maladie et a prolongé la durée de vie jusqu'à 28%, en corrélation directe avec des titres de scFv dans la moelle épinière. Le traitement a provoqué une atténuation des signaux de stress neuronal et la réduction des niveaux de la protéine SOD1 mal repliée dans la moelle épinière des souris SOD1G93A. Basé sur ces résultats, nous proposons qu'une immunothérapie basée sur l'inoculation intrathécale d'AAV codant pour un scFv sécrétable contre la protéine SOD1 mal repliée soit considérée comme traitement potentiel pour la SLA, en particulier pour les individus porteurs de mutations SOD1.

2.2 Abstract

There is emerging evidence that the misfolding of superoxide dismutase 1(SOD1) may represent a common pathogenic event in both familial and sporadic amyotrophic lateral sclerosis (ALS). To reduce the burden of misfolded SOD1 species in the nervous system, we have tested a novel therapeutic approach based on adeno-associated virus(AAV)-mediated tonic expression of a DNA construct encoding a secretable single chain fragment variable (scFv) antibody composed of the variable heavy and light chain regions of a monoclonal antibody (D3H5) binding specifically to misfolded SOD1. A single intrathecal injection of the adeno-associated virus encoding the single chain antibody in $SOD1^{G93A}$ mice at 45 days of age resulted in sustained expression of single chain antibodies in the spinal cord and it delayed disease onset and extension of life span by up to 28%, in direct correlation with scFv titers in the spinal cord. The treatment caused attenuation of neuronal stress signals and reduction in levels of misfolded SOD1in the spinal cord of $SOD1^{G93A}$ mice. From these results, we propose that an immunotherapy based on intrathecal inoculation of AAV encoding a secretable scFv against misfolded SOD1 should be considered as potential treatment for ALS, especially for individuals carrying SOD1 mutations.

2.3 Introduction

Amyotrophic lateral sclerosis (ALS) is an adult-onset neurodegenerative disorder characterized by the selective loss of upper and lower motor neurons (Tandan and Bradley, 1985; Albert and Lomen-Hoerth, 2008). Approximately 20% of familial ALS cases are caused by mutations in the Cu, Zn superoxide dismutase 1 (SOD1) (Gurney, 1994; Pramatarova et al., 1995; Eisen et al., 2008). Although the mechanism by which SOD1 mutations cause selective degeneration of motor neurons is not fully understood, many lines of evidence suggest that the toxicity of mutant SOD1 is related to its propensity to misfold and to aggregate (Reaume et al., 1996; Bruijn et al., 2004). Furthermore, some studies suggest a possible involvement of SOD1 abnormalities in sporadic ALS cases with no SOD1 mutations (Ezzi et al., 2007; Gruzman et al., 2007; Kabashi et al., 2007; Bosco et al., 2010; Pokrishevsky et al., 2012; Graffmo et al., 2012). For instance, oxidation of wild type (WT) SOD1 generates misfolded proteins that may acquire the binding and toxic properties of mutant SOD1 (Ezzi et al., 2007; Bosco et al., 2010).

The finding that mutant SOD1 can be secreted and evidence of toxicity of extracellular mutant SOD1 (Urushitani et al., 2006) provided a rationale for testing immunization approaches for ALS treatment. An active immunization approach with recombinant mutant or WT SOD1 as immunogen was found to delay disease onset and to increase life span of $SOD1^{G37R}$ mice and $SOD1^{G93A}$ mice expressing moderate levels of mutant SOD1 (Urushitani et al., 2007; Takeuchi et al., 2010) . Similar results have been obtained with active immunization using an antigenic peptide that targets the dimer interface of SOD1 using $SOD1^{G37R}$ or $SOD1^{G93A}$ mice (Liu et al., 2012). However, because of potential adverse effects of immune responses to active vaccination approaches, passive immunization strategies appear more appropriate for future human ALS clinical trials. Some monoclonal antibodies recognizing the misfolded forms of SOD1 have been tested in $SOD1^{G93A}$ mice (Gros-Louis et al., 2010). Intracerebroventricular injection of one of those monoclonal antibodies in $SOD1^{G93A}$ mice, named the D3H5 antibody, caused reduction in

levels of misfolded SOD1 in the spinal cord and prolonged the life span of $SOD1^{G93A}$ mice in relation to duration of treatment. The monoclonal D3H5 antibody was shown to react against various human SOD1 mutants besides $SOD1^{G93A}$ including SOD1 G37R, G127X, G85R and D90A (Gros-Louis et al., 2010). In addition, the D3H5 antibody also detected wild-type SOD1 after treatment with metal chelators that induce protein misfolding (Gros-Louis et al., 2010). So, the D3H5 antibody acts as a probe for SOD1 misfolding whether it is caused by mutations or other alterations such as copper or zinc depletion. The activity of D3H5 antibody against CNS tissue from sporadic cases of ALS remains to be investigated.

Interestingly, intracerebroventricular injection of the variable Fab fragment of the same anti-SOD1 antibody (D3H5) also slowed down disease in $SOD1^{G93A}$ mice raising the possibility to engineer a single chain fragment of variable regions (scFv) from this antibody to neutralize the toxicity of misfolded SOD1. Such scFv antibody should offer some advantages such as small size and low immunogenicity. Moreover, scFv antibodies can be used in gene delivery systems. Recombinant adeno-associated viruses (AAV) are presently vehicles of choice for gene transfer in the nervous system (Towne et al., 2010). AAV vectors provide stable and safe gene expression with minimal immune responses and broad cell type tropism. In recent years, adeno-associated virus (AAV) has been used with success for gene delivery in treatment of human genetic disorders, especially in retinal disease (Bennicelli et al., 2008; Maguire et al., 2008) .When injected into the cerebrospinal fluid (CSF), AAV vectors were reported to confer widespread and sustained transgene expression in the central nervous system (CNS) (Snyder et al., 2011).

Here, we report the generation an AAV vector encoding a secretable scFv antibody (AAV-scFv) to target misfolded SOD1. A single intrathecal injection of this AAV viral vector in adult $SOD1^{G93A}$ mice led to sustained production of secretable scFv antibodies in the spinal

cord and it significantly delayed disease onset and mortality. This therapeutic approach may be applicable to ALS cases with SOD1 mutations and perhaps to subset of sporadic ALS cases given that misfolded and aggregated SOD1 species have been detected in sporadic ALS (Ezzi et al., 2007; Gruzman et al., 2007; Kabashi et al., 2007; Bosco et al., 2010; Pokrishevsky et al., 2012).

2.4 Materials and methods

Generation of recombinant scFvD3H5 antibody

mRNA was isolated from D3H5 hybridoma cell line (Gros-Louis et al., 2010) using an mRNA isolation kit (Qiagen, Chatsworth, CA). cDNA was synthesized using Superscript First Strand, catalog #12371-019 (Invitrogen,Carlsbad, CA, USA) with oligodT priming according to manufacturer's instructions . The variable regions of heavy chain (VH) and light chain (Vκ) were amplified separately from first-strand cDNA by using a mixture of universal PCR primers and Platinum Pfx DNA polymerase (Invitrogen, USA). The PCR products for heavy chain and light chain were cut with restriction enzymes *Pst*I/*BstE*II and SacI/XhoI respectively and agarose gel-purified. The cDNA inserts corresponding to VL and VH were cloned into the pBZUT7 vector and sequenced using M13 forward primer (5'-GTAAAACGACGCCCAG-3') and reverse primer (5'-CAGGAAACAGCTATGAC-3'). The VH and VL domains were assembled and linked together by PCR to yield the full-length scFv gene . The scFv gene that was constructed in a VH-linker-VL format together with a standard flexible 20-amino acid linker (Gly4Ser)3 was then subcloned upstream of the Myc-tagged Psw1 scFVD1.3 Tag1 expression vector to generate scFvD3H5. Each scFv contained a murine immunoglobulin (Ig) κ-secretory signal for efficient secretion and a human c-myc epitope to facilitate detection.

Expression and secretion of scFv vector in cultured cells

HEK-293 cells (Human embryonic Kidney cell line) cells were cultured in Dulbecco's modified Eagle's medium (DMEM). The plasmids were transiently transfected with Lipofectamine (Invitrogen) into cells. After 48 hours, cells were harvested and culture media was collected, centrifuged at 15,000*g* for 15 minutes at 4 °C and the proteins in the media and cells were separated by 14% SDS-PAGE. After electrotransfer to polyvinylidene difluoride (PVDF) membranes (Immobilon-P, Millipore, Bedford, MA), scFvs were visualized by an HRP-conjugated antimyc antibody (Invitrogen).

ScFvD3H5 binding to SOD1

The D3H5 monoclonal antibody has been shown to be specific only for misfolded SOD1 and it does not detect WT SOD1 (Gros-Louis et al., 2010). Western Blot analysis and ELISA were performed to evaluate the immunoreactivity of scFvD3H5 to SOD1. 14% SDS-PAGE was carried out with spinal cord lysates of $SOD1^{G93A}$ mice. Culture media from HEK-293 cells transfected with scFv construct were used as a primary antibody source. After electrotransfer to polyvinylidene difluoride (PVDF) membranes (Immobilon-P, Millipore, Bedford, MA), scFvs were visualized with an anti-myc antibody (Invitrogen). For ELISA, plates were coated with 3ug/ml of recombinant $SOD1^{G93A}$ protein. The harvested culture media was added at different dilutions to the coated plates and incubated at 4°C overnight. On the next day, wells were washed and incubated with anti-myc-HRP antibody for 1 hr at room temperature to detect the scFv.

AAV-scFvD3H5 construction and preparation

The AAV serotype-1/2 vector (AAV2 terminal repeats in AAV1 capsids) encoding misfolded SOD1-scFv was prepared by standard method (Rabinowitz et al., 2002). Briefly, to generate an AAV viral vector capable of conferring the capacity to produce and secrete the mabD3H5 scFv upon infection, the fragment encompassing the scFv expression cassette in pScFv-D3H5 was excised using HindIII and EcoRV, cloned into the plasmid BluescriptII KS (+) (Stratagene, Canada). It was then recloned as a SalI/NotI fragment into the XhoI/NotI digested plasmid AAV-CMV-GFP (McCarty et al., 2001) replacing the EGFP-encoding sequence and creating the pscAAV-D3H5 plasmid to be used in the production of AAV recombinant viruses. For the production of scAAV recombinant viruses, the 293T cell line was used . Cells were cultured in DMEM (Gibco Canada) supplemented with 10% normal bovine serum, 100 U/ml of penicillin G, and 100 μg/ml of streptomycin. Plasmids used for cotransfection of 293T cells were an ITR-containing plasmid derived from scAAV-CMV-GFP containing a scFv-encoding transgene (AAV-scFv or AAV-D1.3), and plasmids pXR1 and pxx-6 as packaging and helper plasmids respectively (McCarty et al., 2001; Rabinowitz et al., 2002).

Generation of GAP-43-luc/gfp; SOD1^{G93A} transgenic mice

Transgenic mice over-expressing the SOD1^{G93A} mutations (B6SJL-TgN_ [SOD1-G93A] _1 Gur) were purchased from the Jackson Laboratory (Bar Harbor, ME, USA) and were genotyped in accordance with Jackson Laboratory protocols. . To confirm that the transgene copy number of SOD1G93A was not altered in the mice used for this study, we evaluated genomic SOD1 levels by Quantitative RT-PCR using genomic DNA isolated from tail tissue. Analysis of the mouse housekeeping gene encoding glyceraldehyde-3-phosphate dehydrogenase was used for normalization purposes. Oligoprimer pairs (used at concentration of 300nm) were designed by GeneTool 2.0 software (Biotools Inc, Edmonton, AB, CA) and their specificity was verified by blast in the GenBank database. Standard cycling conditions were used.

Mice were maintained heterozygous in the C57BL/6 background. The transgenic GAP-43-luc/gfp reporter mice were generated as described previously (Gravel et al., 2011). These mice were crossed with the SOD1G93A transgenic mice (C57/BL6, Jackson labs to generate double transgenic GAP-43-luc/gfp/ SOD1^{G93A} mice. To avoid the effects of genetic background, all experiments were performed on age-matched littermates. Double transgenic mice were genotyped according to the following procedure. The presence of GAP-43-luc/gfp transgene was assessed by PCR of the luciferase reporter gene with the following primers: (5′-GGCGCAGTAGGCAAGGTGGT and (5′-CAGCAGGATGCTCTCCAGTTC) as described previously (Lalancette-Hebert et al., 2009). The transgenic TLR2-LUC-Ac-GFP mice were generated and genotyped as described previously (Lalancette-Hebert et al., 2009). The presence of the SOD1^{G93A} mutant transgene was assessed by PCR as previously described (Gowing et al., 2006). The use and maintenance of the mice described in this article was performed in accordance to the Guide of Care and Use of Experimental Animals of the Canadian Council on Animal Care.

In Vivo Bioluminescence Imaging

As previously described, images were gathered using IVIS 200 Imaging System (Xenogen, Alameda, CA)(Lalancette-Hebert et al., 2009). Twenty minutes prior to imaging session the mice received intraperitoneal injection of D-luciferine, a luciferase substrate (150 mg/kg, Xenogen, Alameda, CA) dissolved in 0.9% saline. The mice were then anesthetized with 2% isoflurane in 100% oxygen at a flow rate of 2 L/ min and placed in the heated, light-tight imaging chamber. Images were collected using high sensitivity CCD camera with wavelengths ranging from 300–600 nm. Exposition time for imaging was 1 minutes using different field of views and F/1 lens aperture. The bioluminescence emission was normalized and displayed in physical units of surface radiance, photons per second per centimeter squared per steradian (photons/s/cm2/sr). The light output was quantified by determining the total number of photons emitted per second using the Living Image 2.5 acquisition and imaging software (Xenogen, Alameda, CA). Region-of-interest (ROI) measurements on the images were used to convert surface radiance (photons/s/cm2/sr) to source flux or total flux of photons expressed in photons/s. Three-dimensional images were created using diffuse luminescent imaging tomography (DLIT) algorithms, to reconstruct for the position, geometry and strength of the internal light sources. The modifiable parameters were analysis across the wavelengths, source spectrum and tissue properties (Living Image 3D Analysis Software, Xenogen, Alameda, CA).

Intrathecal injection of AAV-scFvD3H5 in SOD1^{G93A} mice

Intrathecal injection of AAV-scFvD3H5 vector of vehicle in SOD1^{G93A} mice or GAP-43-luc/gfp/SOD1^{G93A} mice was carried out at postnatal day 45 (P45). A total of $3x10^9$ particles were injected in a 10 ul volume by a Hamilton syringe which was slipped under the dura and the vector was slowly released into the CSF. The syringe was removed after 1 min to minimize CSF and vector leakage. For all surgeries, mice were anesthetized with 2% isoflurane and post treated with subcutaneous buprenorphine (0.05 mg/kg) for pain. As a control, age matched SOD1^{G93A} mice received either same amount of vehicle or AAV vector encoding a lysozyme specific single chain fragment D1.3 (AAV-scFvD1.3) (n=20

for all group). After surgery, animals were housed in cage with free access to food and water till end point.

Analysis of disease progression

The onset of weight loss was determined as the time when mice started to exhibit a decline of body weight after reaching a peak. The survival was defined as the age when the animal could not right itself within 30 s when placed on its side. Measurements of body weight, hind limb reflex, and rotarod performance were used to score the clinical effects of $SOD1^{G93A}$ mice. The extensibility and postural reflex of the hind limbs when mice were held up with their tails were scored as described previously (Urushitani et al., 2006). The $SOD1^{G93A}$ reflex score and body weight were measured every 2 days, beginning at 90 days. Scoring was performed in a blind manner by animal technicians who had no information about the genotype but had experience in grading $SOD1^{G93A}$ mice paralysis. Analysis of $SOD1^{G93A}$ mice disease progression was performed with an accelerated rotarod, starting at 4 rpm with a 0.25 rpm/s acceleration, and time was noted when the mice fell off the roll. Three trials were done per animal, and the mean value was calculated for statistics and graphs. Rotarod tests for $SOD1^{G93A}$ mice were performed once a week.

ELISA for determining scFvD3H5 titer

The levels of scFvD3H5 in the spinal cord lysate were determined by ELISA. Spinal cord tissues were homogenized in lysis buffer (137x10-3 mol/l NaCl, 20x10-3 mol/l Tris [pH 8.0], 1% NP40, and 10% glycerol, supplemented with Protease Inhibitor Cocktail Tablets Complete Mini (Roche Applied Science, USA), ultrasonicated, and then centrifuged at 4°C. ultrasonicated, and then centrifuged for 15 min at 8,000 *g* 4°C. The supernatant was diluted in bicarbonate/carbonate coating buffer and 100 ul of that was used to coat the 96 well ELISA plate (Peprotech) overnight at 4°C, followed by washing and blocking overnight again. The plates were then incubated sequentially with biotinylated 9E10 to c-myc (Abcam, Cambridge MA) overnight, washed followed by incubation in streptividin-HRP

(BD Pharmingen, Canada) for 1 hr at RT, washed again followed by incubation in tetramethylbenzidine solution for color development. The reaction was stopped with hydrochloric acid and the absorbance at 450 nm was read. Spinal cord lysates from vehicle-injected mice were used as a control.

Immunoprecipitation and western blotting

At the end point, the spinal cord was dissected out, rapidly frozen in liquid nitrogen and stored at -/80°C for ELISA, immunoprecipitation and western blot analysis. Whole protein lysates from spinal cord were extracted by homogenization of the tissues in TNG-T lysis buffer (50 mM Tris–HCl pH: 7.4; 100 mM NaCl; 10% Glycerol; 1% Triton X), and centrifugation for 20 min at 9000 g at 4°C. The soluble protein was quantified by the Lowry method. Immunoprecipitation of misfolded SOD1was performed as previously described (Gros-Louis et al., 2010). Briefly beads were coated with mouse monoclonal anti misfolded-SOD1 antibody B8H10 for immunoprecipitating misfolded-SOD1 and incubated overnight with whole spinal cord lysate, washed and fractioned on 14% SDS-PAGE. Immunoprecipitation of spinal cord extracts expressing scFvD3H5 was performed by immunoprecipitating with anti c-myc tag as described earlier (Wuertzer et al., 2008).

Immunohistochemistry

Mice were anesthetized by intraperitoneal injection of 4% chloral hydrate and perfused intracardially with PBS, followed by 4% paraformaldehyde, pH 7.4. Spinals cords were removed, post-fixed for 1 h and then frozen in Tissue-Tek OCT embedding compound (Sakura Finetek, Torrance, CA, USA), permeabilized in 0.25% Triton-X for 10 min, blocked in 3% normal goat serum for 1 h, and then incubated with the monoclonal antibodies for 16 h at 20°C. Sections were then incubated overnight at 20°C using primary antibodies, 1:100 mouse monoclonal anti-myc 9E10 (Abcam, Cambridge, MA,USA), 1:250 rabbit anti-choline acetyltransferase (ChAT) (Millipore Corporation, Bedford, MA, USA), 1:500 rabbit polyclonal anti-glial fibrillary acidic protein (GFAP) (Dako, Carpinteria,CA,

USA), 1:500 rabbit anti-Iba1 (Wako Chemicals USA,Richmond, VA, USA), 1:50 rabbit polyclonal ATF3 (Santacruz Biotechnology, CA,USA), 1:500 mouse monoclonal NEU N (Millipore Corporation, CA,USA), 1:100 mouse monoclonal B8H10 and C4F6 (Medimabs, Montreal, Canada).Visualization was made by incubating the slides with Alexa-Fluors 488 or 594 rabbit anti-mouse secondary antibody (Invitrogen). Dissected dorsal root ganglia (DRG) were post fixed in a solution of 3% glutaraldehyde for a period of 48 h, washed in PBS, treated with 1% osmium tetra oxide for 2 h, and dehydrated through graded alcohol solutions. Before Epon plastic embedding, DRG were further dissected to ensure that all ventral root (VR) axons would be sampled at a distance of 3 mm from the DRG cell body. Semi-thin cross sections were stained with Toluidine Blue, rinsed, and cover slipped. To quantify the immunoreactivity score of immunofluorescent sections, we measured the optical densities of each staining with ImageJ software (NIH).

Statistical analyses

Data were analyzed using Prism 5.0 software (GraphPad Software,LaJolla, CA, USA). Behavioral data were computed by performing two-way ANOVAs (except when specified) followed by Bonferroni post-tests and survival data using Mantel-Cox log-rank tests. VR axon Counts and immunoreactivity scores for Iba1, GFAP and anti misfolded SOD-1 specific antibody were compared using two-tailed Student's *t*-tests. Data are expressed as mean_SEM; $P<0.05$ was considered statistically significant.

2.5 Results

2.5.1 Generation of a scFv from the D3H5 hybridoma

Based on our finding that the Fc region of mAbD3H5 was dispensable for extending the survival of transgenic SOD1^{G93A} mice when delivered into CSF (Gros-Louis et al., 2010), we planned to test whether tonic delivery of the binding domain of the antibody via virus-mediated gene transfer in the spinal cord would be effective in blocking the toxicity of misfolded human SOD1. Thus, a scFv-encoding plasmid was generated by cloning and assembly of the coding sequences of the variable immunoglobulin heavy and light chains of mAbD3H5 into a single open-reading frame encoding a hybrid protein bearing both variable immunoglobulin regions attached by a short flexible peptide linker. The DNA sequences and deduced amino acid sequences of the cloned hypervariable regions of mAbD3H5 light and heavy chains are shown in figure 2.1 A.

The scFv DNA construct was subcloned into an expression vector under the control of CMV promoter that provided a murine immunoglobulin (Ig) κ-secretory signal and a human c-myc epitope to facilitate detection. We analyzed expression of scFvD3H5 after DNA transfection in HEK293T cells by western blotting and ELISA. The scFv was detected in the 1% Triton cell lysate and in the conditioned media after transient transfection in HEK293T cells (Fig. 2.1 *B*). A 28 kDa band was detected by immunoblotting after SDS-PAGE using an antibody against the human c-myc tag in the recombinant scFv protein. To test whether the scFvD3H5 antibody was able to detect misfolded SOD1, we used conditioned media from cells transiently transfected with the D3H5scFv encoding plasmid as a primary antibody source for immunoblotting detection after SDS-PAGE of spinal cord lysate from SOD1^{G93A} mice. The scFvD3H5 in the media detected specifically SOD1 species by immunoblotting as revealed by an anti-myc antibody, whereas no signal was seen with conditioned medium from mock- transfected cells (Fig. 2.1C). In addition, when conditioned media containing scFvD3H5 antibodies

was loaded on ELISA plate coated with recombinant human SOD1, the bound scFvD3H5 protein could be detected using HRP conjugated anti-myc (Fig. 2.1D).

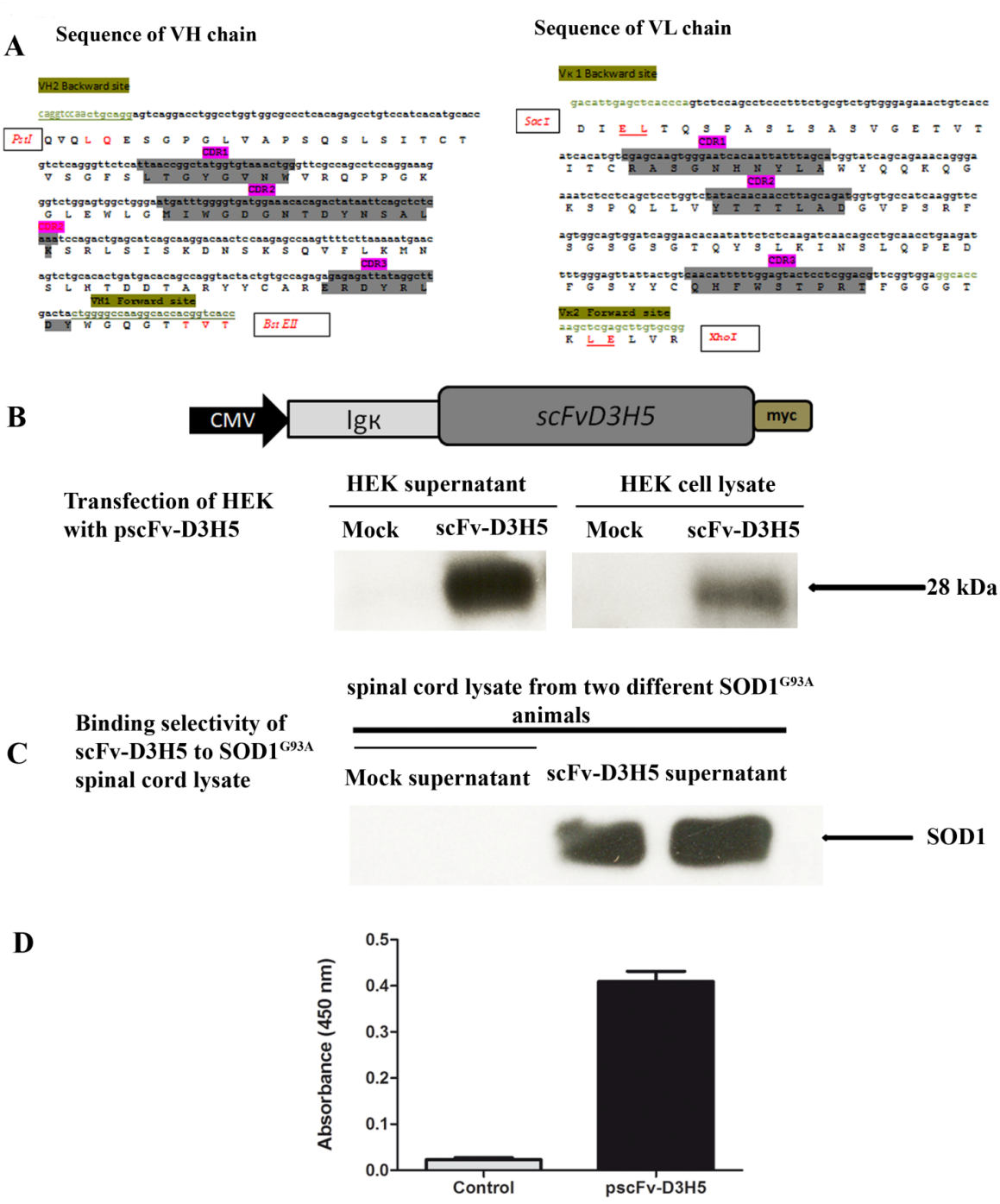


Figure 2.1 Generation of recombinant scFv antibody against misfolded SOD1.

Figure 2.1 Generation of recombinant scFv antibody against misfolded SOD1

(A) Nucleotide and deduced amino acid sequences for the scFv heavy chain variable, light chain variable are shown along with complementarity determining regions (CDRs), positions of restriction sites for enzymes and primers. **(B)** A cytomegalovirus (CMV) enhancer containing promoter drives the expression of scFv, which harbors an amino terminal immunoglobulin (Igκ) secretory signal and carboxy-terminal human c-myc epitope. pscFvD3H5 was transiently transfected into HEK cells and mock transfected HEK cells (without any vector plasmid) was used as a control. Culture media (CM) was harvested 48 hours following transfection. Conditioned media and 1% Triton lysate was analyzed by western blot, detected with anti c-myc, showing expression of anti-mSOD1scFv. **(C)** Secreted scFv antibodies in conditioned media from transfected HEK cells were used as primary antibodies source to probe SOD1G93A spinal cord lysates by western blot, showing binding selectivity of scFv-D3H5. **(D)** Ninety-six well plates were coated with recombinant G93A polypeptide and the harvested CM containing scFvD3H5 was subsequently added to well. Unbound proteins were washed away, and anti-myc-HRP antibody was used to detect the scFv that remained bound to well. An unpaired t-test was performed to determine significant differences ($p< 0.0001$).

2.5.2 AAV-mediated transduction of scFv anti-SOD1 in the spinal cord of SOD1^{G93A} mice

As described in Methods, we then generated an AAV viral vector encoding the secretable scFvD3H5 protein. To test the therapeutic effect of scFvD3H5 in ALS pathogenesis, single intrathecal injection of $3x10^9$ particles of the AAV 1/2 encoding scFvD3H5 was carried out in SOD1^{G93A} mice at 45 days of age. At 5 weeks after injection, spinal cord sections were examined by immunostaining using an anti-myc antibody for detection of scFvD3H5. Robust scFvD3H5 immunostaining was observed in the ventral spinal cord of lumbar and sacral regions. The scFvD3H5 antibody was detectable over an extensive portion of the spinal cord. Double immunostaining was also performed using anti-myc tag and anti-choline acetyltransferase (ChAT) antibodies. The myc tag immunoreactivity was detected in some of the ChAT-positive cells in ventral horn spinal cord of SOD1^{G93A} mice injected with AAV-scFvD3H5 (Fig 2.2 A, B). No staining was detected in vehicle-injected SOD1^{G93A} mice. The myc tag immunoreactivity remained detectable at end stage of mice (100 days or more after AAV injection) indicating a stable transduction of scFvD3H5 by AAV-infected cells in SOD1^{G93A} mice (Fig. 2.2 C, D). The presence of scFvD3H5 antibody in the spinal cord was also confirmed by immunoprecipitation of scFvD3H5 antibody from total spinal cord lysate using a monoclonal anti-c-myc as described previously (Wuertzer et al., 2008) and then by detection of immunoprecipitated protein on immunoblot after SDS-PAGE with a second polyclonal anti-c-myc by immunoblotting. The immunoblots further confirmed the presence of scFvD3H5 in the spinal cord at five weeks post-injection of AAV-scFvD3H5 in SOD1^{G93A} mice (Fig. 2.2 E).

One potential concern associated with AAV-mediated gene delivery is the host immune response. To investigate any possible immune response of AAV-scFvD3H5 into mice, we took advantage of a TLR2-LUC-AcGFP (Toll like receptor 2 –luciferase Aequorea coerulescens Green Fluorescent Protein) mouse model where transcriptional activation of TLR2 as reporter of inflammation can be visualized in live animals using biophotonic

molecular imaging (Lalancette-Hebert et al., 2009). Single intraperitoneal injection of LPS at rate of 5mg/kg in mice, induced a strong induction of bioluminescense signal in spinal cord after 24 hours (Supplementary figure 2.1, A-C). We injected intrathecally $3x10^9$ particles of the AAV 1/2 encoding scFvD3H5 or same amount of saline in TLR2-LUC-AcGFP mice. Live imaging of the spinal cord of injected mice was carried out by up to three weeks. Our imaging analysis did not reveal induction of inflammatory response after intrathecal injection of AAV-scFvD3H5 or saline in TLR2-LUC-AcGFP transgenic mice (Supplementary figure 2.1, D-J).

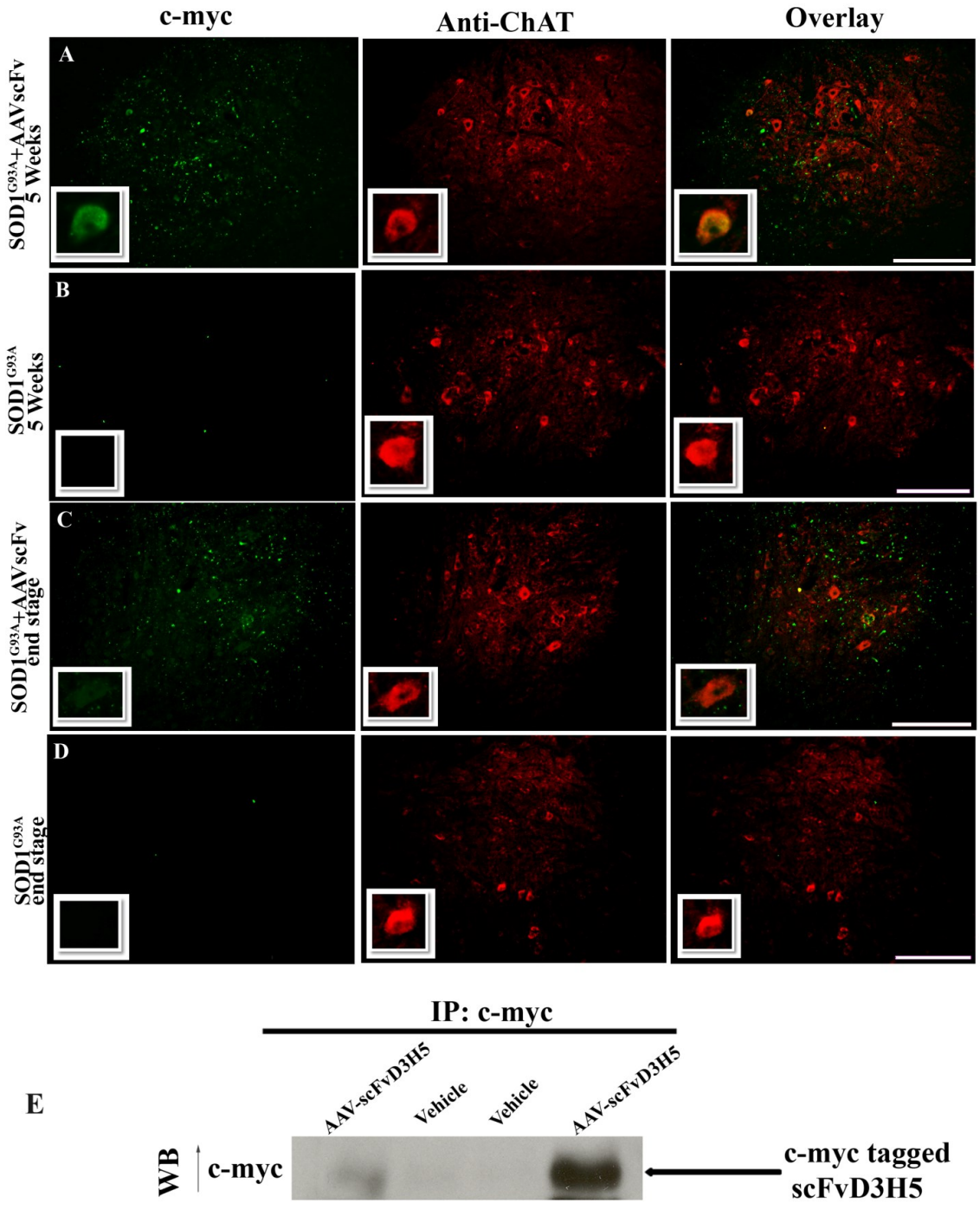
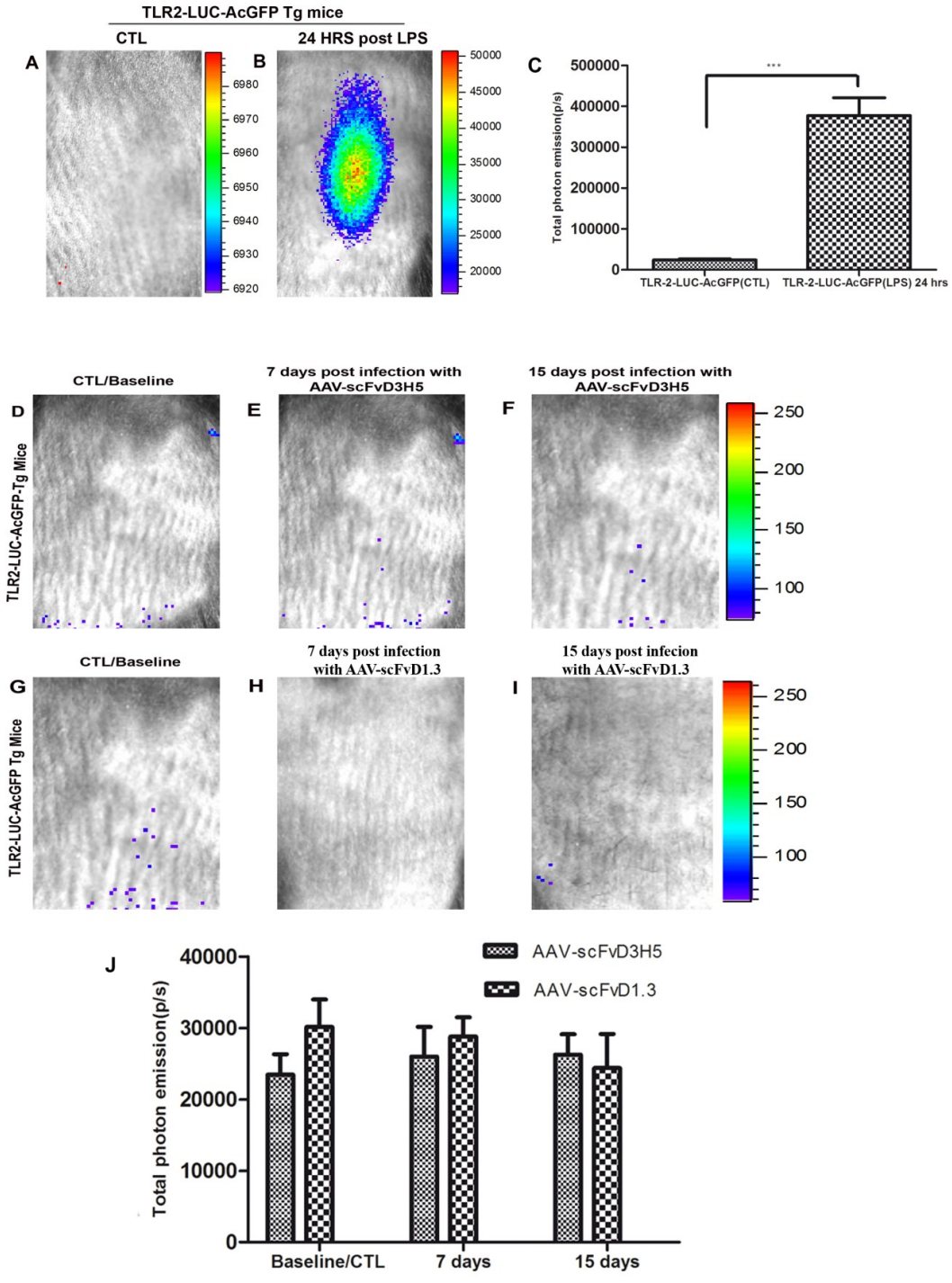


Figure 2.2 Immunodetection of scFv antibody with anti-myc antibody in spinal cord of SOD1^{G93A} mice.

Figure 2.2 Immunodetection of scFv antibody with anti-myc antibody in spinal cord of SOD1^{G93A} mice. Postnatal day 45 (P45) mice were intrathecally injected with AAV-scFvD3H5. The presence of the scFv protein was visualised with antibodies recognizing the human c- myc tag present in the protein in spinal cord tissues collected 5 weeks after injection of the viral vector, or at the end point of mice (100 days or more after injection). Myc tag immunoreactivity was widely distributed in spinal cord sections at both 5 weeks and much later at the mice end stage **(A, C).** Double immunofluorescence staining with anti-ChAT and anti-c-myc tag antibodies reveals co- localization of myc tag and ChAT immunoreactivities in some motor neurons of spinal cord ventral horn of AAV-scFvD3H5 injected mice (anti-c-myc and ChAT co-localization is shown in inlay magnification of a representative cell in the boxed panel). No myc tag immunoreactivity was seen in control mice **(B, D**), scale bar 50 um. **(E)** Immunoprecipitation (IP) from tissue expressing scFvD3H5. The spinal cord of AAV-scFvD3H5 injected mice was harvested at five weeks post delivery, homogenized and immunoprecipitated with an anti-human myc monoclonal antibody. A second anti-myc polyclonal antibody was used for western blotting. Immunoprecipitates from different samples were loaded in each well.



Supplemental Figure 2.1 Real time imaging of TLR2 induction after LPS/AAV-scFvD3H5 or vehicle injection in TLR2-LUC-Ac-GFP transgenic mice.

Supplemental Figure 2.1 Real time imaging of TLR2 induction after LPS/AAV-scFvD3H5 or vehicle injection in TLR2-LUC-Ac-GFP transgenic mice: In vivo imaging of TLR-LUC-Ac-GFP mice reveals an activation of TLR2 promoter 24 hrs post intraperitoneal injection of LPS (5 mg/kg). Representative images of TLR2 induction, before (CTL) and 24 hrs after LPS injection **(A, B).**

(C) Quantification of luciferase activity by the count of total photon emission (p/s) in spinal cord shows significant induction of the TLR2 promoter 24 hrs post LPS injection. (mean±SEM, n=7, $p<0.001$). **(D-F)** Bioluminescence imaging shows no induction of TLR2 signal in spinal cord before (D) and at 7 days (E) and 15 days (F) after intrathecal administration of AAV-scFvD3H5. **(G-I)** Representative images of TLR2 signal before (G) and after 7 days (H) and 15 days (I) of saline administration. **(J)** Quantification of luciferase activity by the count of total photon emission (p/s) in spinal cord shows no significant induction of the TLR2 promoter after 7 and 15 days post AAV-scFvD3H5 or vehicle administration.($n=5$, $p<0.05$)

2.5.3 Single intrathecal injection of AAV-scFvD3H5 delayed disease onset and increased life span of SOD1^{G93A} mice

We have examined the effects on life span of SOD1^{G93A} mice of a single intrathecal injection of AAV-scFvD3H5 and vehicle at 45 days of age. AAV-scFvD3H5 injected mice had a significant extension of life span by an average of 16 days (p value of 0.0001, median survival of 159 days) when compared to SOD1^{G93A} mice injected with vehicle (median survival of 143 days (Fig. 2.3 A). No adverse effect or premature death occurred in normal non-transgenic mice injected with AAV-scFvD3H5. Moreover the administration of AAV-scFvD3H5 significantly delayed the onset of disease as assessed by rotarod performance tests, reflex score and body weight, compared with control virus AAV-scFvD1.3 (AAV encoding lysozyme specific single chain fragment D1.3) or vehicle injected SOD1^{G93A} mice (Fig. 2.3 B-D). Rotarod tests demonstrated that AAV-scFvD3H5 treated SOD1^{G93A} animals maintained their ability to coordinate their movement for a longer period than both of control group animals from 110 days of age onward until end stage ($P < 0.05$ from 110 days of age onward, $n = 10$ animals/group) (Fig. 2.3 B). In addition, reflex score measurements demonstrated that AAV-scFvD3H5 treated animals maintained significantly ($P < 0.05$) higher reflex of hind limb from 106 days of age through 135 days of age when compared with those animals having received AAVscFvD1.3 or vehicle injection (Fig. 2.3 C). Body weight measurements revealed a significantly slower loss of body weight in AAV-scFvD3H5 treated transgenic mice from 113 days up to 127 days ($P < 0.05$) (Fig. 2.3 D). The mean delay in the combined assessments for rotarod test and body weight was of 15 days. In all motor function tests there was no difference between vehicle-treated controls and AAV-scFvD1.3-treated SOD1^{G93A} mice.

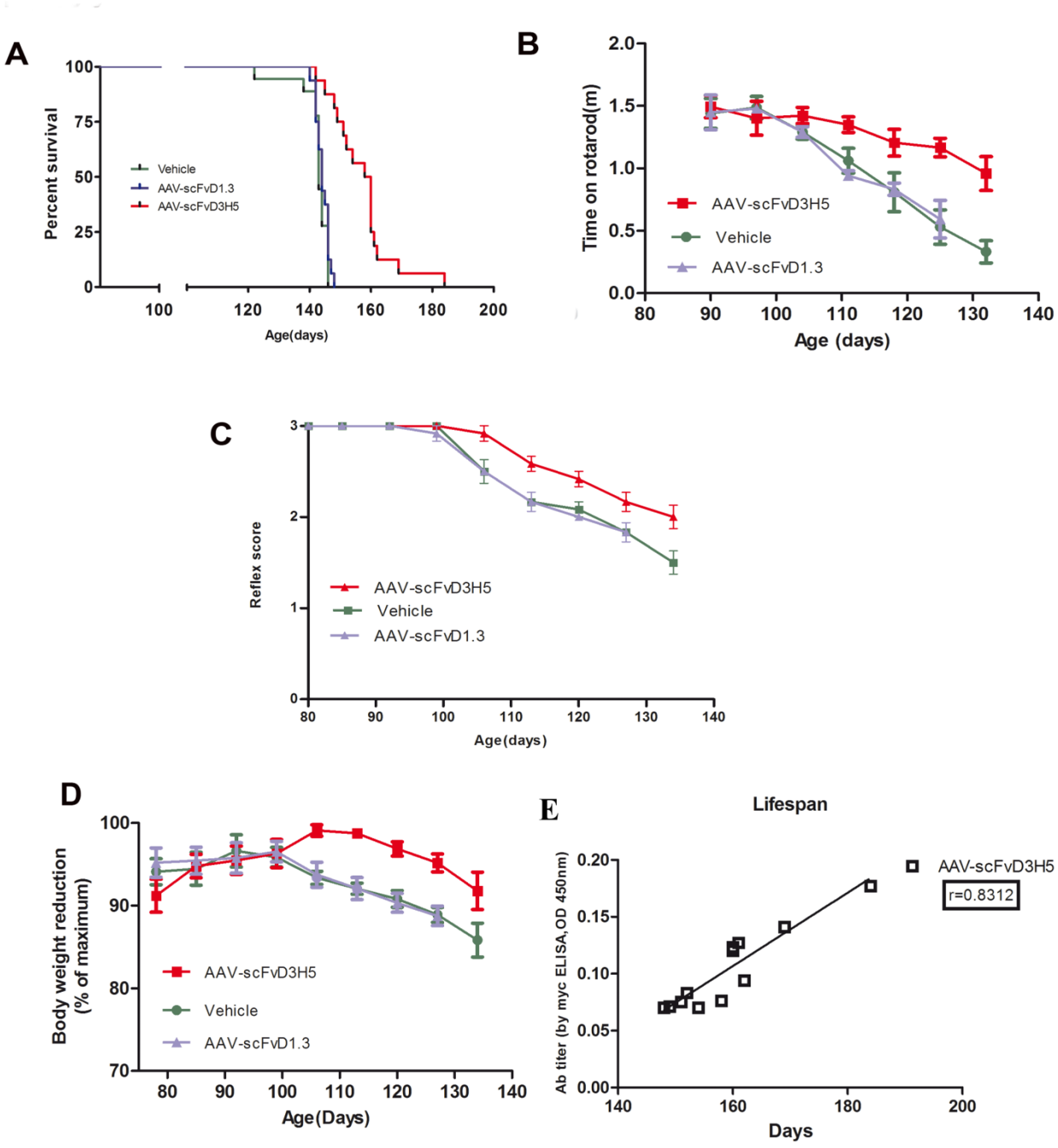


Figure 2.3 Delayed disease onset and extension of life span in AAV-scFvD3H5 treated SOD1^{G93A} mice.

Figure 2.3 Delayed disease onset and extension of life span in AAV-scFvD3H5 treated SOD1^{G93A} mice. (A) Intrathecal administration of AAV-scFvD3H5 increased the life span of SOD1^{G93A} mice. Kaplan-Meier curve for survival is shown (n=16 for AAV-scFvD3H5 and n= 18 for vehicle, $p<0.0001$ by long rank test). **(B)** Rotarod test: the time on rotarod was determined for SOD1^{G93A} mice injected with AAV-scFvD3H5, AAV-scFvD1.3 and with vehicle (n=6 for all). AAV-scFvD3H5 treatment significantly improved motor performance ($p< 0.001$ by posthoc test) when compared to AAVscFvD1.3 or vehicle administered mice. Each point indicates average ± SEM .Also AAV-scFvD3H5 injection delays onset as defined by prolonged maintenance of reflex score in the treated mice **(C)** and reduction in body weight loss **(D)** ($p<0.05$) AAVscFvD3H5 treated mice are shown in red, AAVscFvD1.3 treated mice are shown in blue and vehicle treated mice are shown in green. **(E)** Antibody titer positively correlated with life span of AAV-scFvD3H5 injected mice. ELISA for c-myc tag in spinal cord lysates from AAV-scFvD3H5-injected mice (end stage) was carried out to measure scFv titers. Absorbance (450 nm) from ELISA for c-myc tag and life span was plotted on a scatter diagram, shows a direct correlation of titer and longevity ($p <0.0001$ and Spearman r = 0.8312).

2.5.4 SOD1^{G93A} mediated disease is associated with onset of early neuronal stress

Synthesis of mutant SOD1 within motor neurons has been found a primary determinant of early neuronal stress, disease onset and motor neuron injury (Saxena et al., 2009). To visualize these events from live animals and to analyze in real time the effects of treatment of SOD1^{G93A} with scFvD3H5 on neuronal stress, we took advantage of the GAP-43-luc/gfp reporter mice recently generated and validated in our laboratory (Gravel et al., 2011).

Recent studies using laser capture dissection and gene array analysis revealed that GAP-43 is up regulated in the motor neurons of SOD1^{G93A} mice in the pre-symptomatic and early symptomatic stage of disease (Perrin et al., 2005) suggesting that GAP-43 may represent a good biomarker to visualize early neuronal stress/damage in ALS mice. Double transgenic GAP-43-luc/gfp;SOD1^{G93A} mice were generated by crossing heterozygous mice carrying the mutant SOD1^{G93A} transgene with the heterozygous GAP-43-luc/gfp mice co-expressing reporter transgene, luciferase (luc) and green fluorescent protein (gfp), driven by the murine GAP-43 promoter. In this mouse model, a GAP-43 upregulation (luciferase expression detectable as a bioluminescence/photon emission and gfp expression detectable by confocal microscopy) can be followed longitudinally in live animals using bioluminescence/biophotonic imaging and a high sensitivity/ high-resolution CCD camera (Fig. 2.4 A).

The quantitative analysis of biophotonic signals revealed significant early and pre-symptomatic upregulation of the GAP-43 signals starting at 8 weeks /56days and reaching the peak at 11 weeks/77 days of age in the spinal cord of SOD1^{G93A} mutant mice when compared with wild-type littermates (Supplementary figure 2.2 A). Fluorescence microscopy of gfp and anti-NeuN has been carried out to identify neurons as being the cells expressing induced GAP-43-luc/gfp transgene (Supplemental Fig. 2.2 B). Furthermore, as previously reported (Saxena et al., 2009), the activating transcription factor 3 ATF3 is upregulated in spinal motor neurons SOD1^{G93A} (Fig. 2.4 B). The detection of ATF3 in

GFP-positive motor neurons in the spinal cord sections of GAP-43-luc/gfp/SOD1^{G93A} mice further confirmed the validity of the GAP-43-luc/gfp transgene for live analysis of neuronal stress in this mouse model (Fig. 2.4 B).

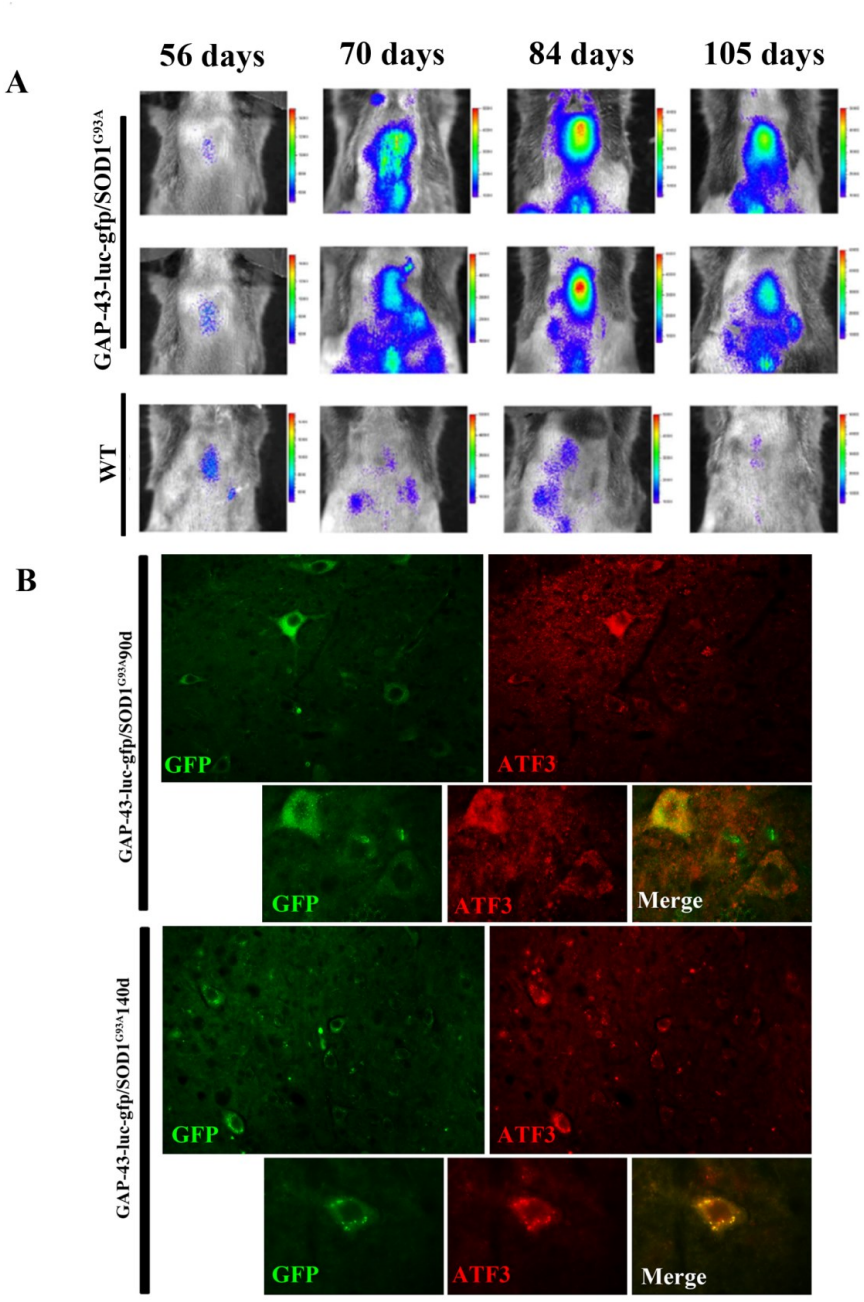
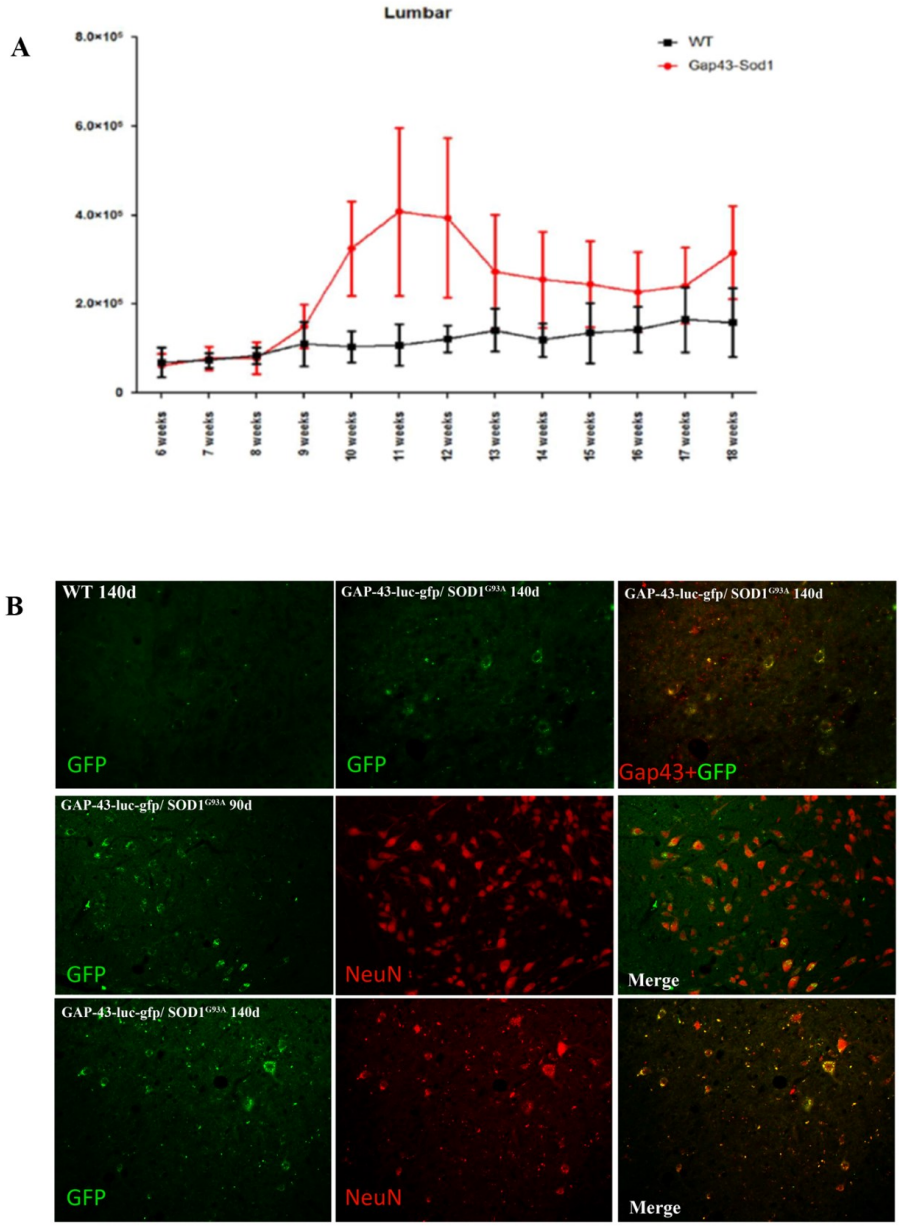


Figure 2.4 GAP-43-luc-gfp;SOD1^{G93A} double transgenic mice as a model for live imaging of neuronal stress.

Figure 2.4 GAP-43-luc-gfp;SOD1^{G93A} double transgenic mice as a model for live imaging of neuronal stress (A) Representative images of in vivo bioluminescence imaging of spinal cord of GAP-43-luc-gfp/SOD1^{G93A} mice and WT mice at various time points, which shows the GAP-43 signal intensity as a measure of neuronal damage. **(B)** Representative immunofluorescence images of spinal cord sections of double transgenic mice showing co localization of GFP and ATF3 at lower and higher magnifications.



Supplemental Figure 2.2 Longitudinal quantitative analysis of total photon GAP-43 signal/ bioluminescence in GAP-43-luc/gfp/SOD1^{G93A} double transgenic mice and WT mice in spinal cord.

Supplemental Figure 2.2 Longitudinal quantitative analysis of total photon GAP-43 signal/ bioluminescence in GAP-43-luc/gfp/SOD1^{G93A} double transgenic mice and WT mice in spinal cord. (A) Total photon GAP-43 signal shows early neuronal stress starting as early as 63 days/9 weeks and reaching a peak at 77 days/11 and 84 days/12 weeks in the double transgenic mice. **(B)** Representative immunofluorescence images of spinal cord sections from double transgenic and WT mice in the first panel showing expression and co localization of GFP and GAP-43 in -gfp/SOD1^{G93A} at 90 and 140 days which is absent in WT mice.

2.5.5 AAV-scFvD3H5 treatment reduces neuronal stress

Series of live imaging experiments and quantification of signals in GAP-43-luc/gfp;SOD1^{G93A} mice injected with AAV-scFvD3H5 or vehicle revealed that scFvD3H5 expression resulted in a significantly weaker bioluminescence signal in the spinal cord at five and six weeks after injection (11 and 12 weeks of age) compared to vehicle-treated double transgenic mice (Fig. 2.5 A,B). The reduction of neuronal stress in mice was further confirmed by examination of spinal cord sections from AAV-scFvD3H5-treated and untreated SOD1^{G93A} mice after immunofluorescence processing to detect the presence of cyclic AMP-dependent transcription factor ATF-3, a marker for neuronal stress (Nascimento et al., 2000) (Fig. 2.5 C). Thus, the ATF3-like immunofluorescence was much weaker in the spinal neurons of scFvD3H5-treated SOD1^{G93A} mice at six weeks after injection as compared to untreated mice. The combined data from GAP-43-luc/gfp transgene and ATF-3 expression signals demonstrate that treatment with AAV-scFvD3H5 reduced the neuronal stress associated with ALS pathogenesis in SOD1^{G93A} mice.

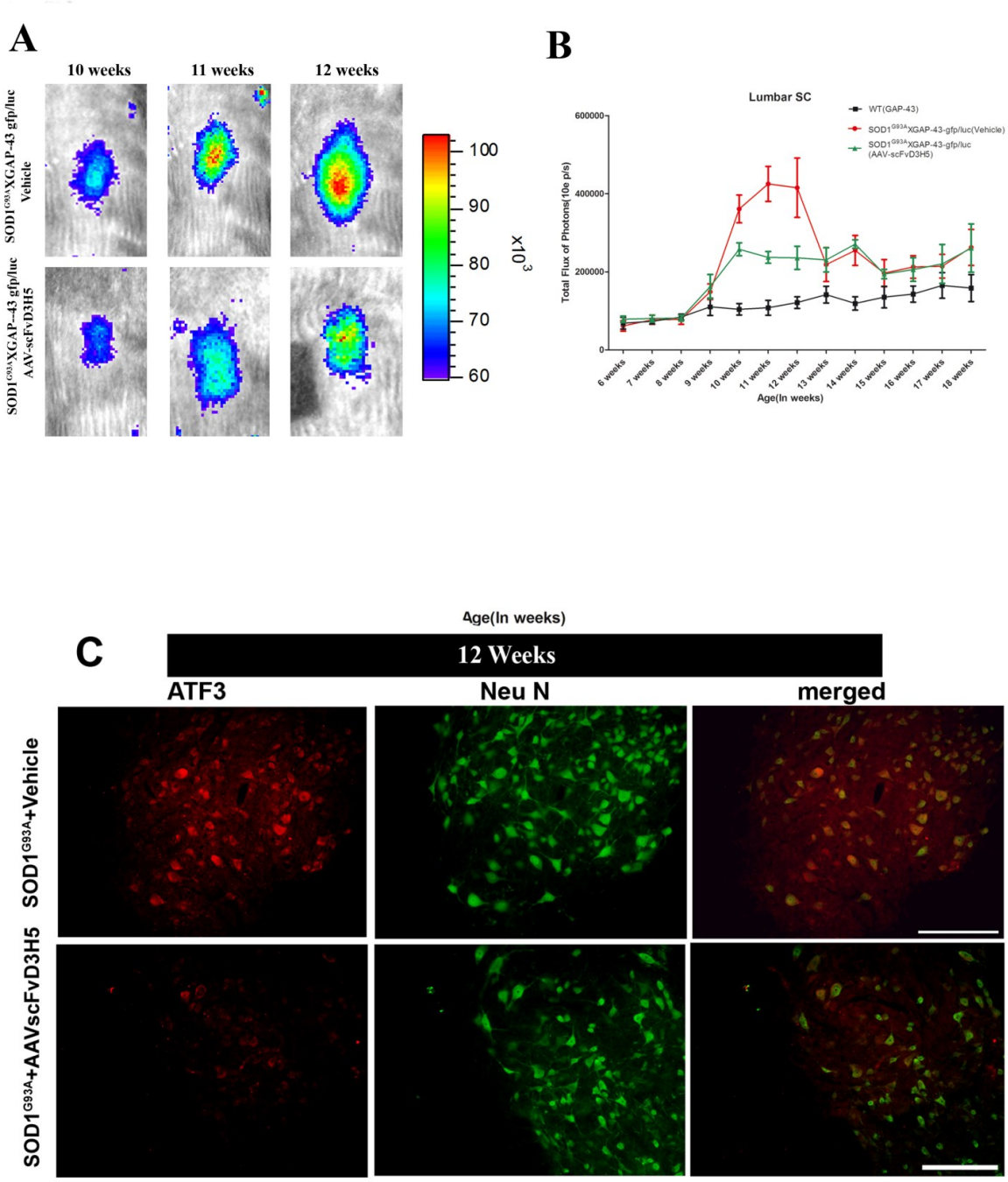


Figure 2.5 AAV-mediated delivery of scFvD3H5 antibody in GAP-43-luc/gfp;SOD1^{G93A} mice reduced neuronal stress.

Figure 2.5 AAV-mediated delivery of scFvD3H5 antibody in GAP-43-luc/gfp;SOD1G93A mice reduced neuronal stress. (A) In vivo bioluminescence imaging of GAP-43 induction was analyzed at various time points in spinal cord of GAP-43-luc/gfp;SOD1G93A mice. Typical sequence of representative images of spinal cord area obtained from double transgenic mice after AAV-scFvD3H5 or vehicle injection at different time points (10, 11 and 12 weeks) by in vivo imaging (n=6 each group) are shown. **(B)** Longitudinal quantitative analysis of total photon GAP-43 signal of bioluminescence in GAP-43-luc/gfp;SOD1^{G93A} mice in spinal cord is shown. Two-way ANOVA revealed a statistically significant reduction in neuronal stress between treated and untreated group at 11 and 12 weeks (p<0.05). Error bar represents mean ± SEM. **(C)** Immunofluorescence using ATF3 (a marker for neuronal stress) and NeuN antibody was performed in spinal cord of AAV-scFvD3H5 mice and vehicle treated mice at 12 weeks of age. ATF3 signal was found to be comparatively less in treated mice than non treated mice. Merging of both signals shows co localization in motor neurons of spinal cord ventral horn. Scale bar 50um.

2.5.6 Alleviation of gliosis and reduction in burden of misfolded SOD1

In addition to motor neuron loss, gliosis in the spinal cord is a prominent pathological feature in ALS patients and in rodent models of ALS (Fischer et al., 2004; Pasinelli and Brown, 2006). Reactive astrocytes and microglia are characterized by an up regulation of Iba1 (Ionized calcium binding adaptor molecule 1) and of GFAP (glial fibrillary acidic protein) in the disease process (Fischer et al., 2004). We therefore examined the effect of the scFvD3H5 treatment on microglial and astroglial activation in $SOD1^{G93A}$ mice at 120 days of age. As shown in Fig. 2.6 A and B, Iba 1 immunofluorescence in spinal cord samples from scFvD3H5-treated mice was 29 % lower than in control samples (significant difference, p=0.0438). Similarly, astrocyte reactivity as determined by GFAP immunodetection was 38% weaker in $SOD1^{G93A}$ mice injected with AAV-scFvD3H5, also a statistically significant difference (p=0.0369) (Fig. 2.6 C, D).

To examine the effect of the scFvD3H5 treatment on the levels of misfolded SOD1 species in affected tissues, we carried out immunoprecipitation of misfolded SOD1 from total spinal cord extract at postnatal day 120 , using the anti-misfolded SOD1-specific B8H10 antibody (Gros-Louis et al., 2010) followed by SDS-PAGE and immunoblotting using a polyclonal anti-SOD1 antibody. The immunoblots revealed a 19% reduction (p=0.0293) in levels of misfolded SOD1 species immunoprecipitated by the B8H10 antibody from spinal cord extracts of AAV-scFvD3H5 injected SOD1G93A mice in comparison to levels detected in vehicle-injected $SOD1^{G93A}$ mice (Fig. 2.6 E, F). In addition, comparison of immunoreactivity scores of spinal cord sections measured by fluorescence microscopy revealed marked reduction of anti-misfolded SOD1 antibody signals (B8H10 and C4F6) from samples of AAV-scFvD3H5 injected mice when compared to controls (n=3, p=0.01) (Fig. 2.6 G,H). From these results, we conclude that the AAV-mediated delivery of scFvD3H5 antibodies succeeded in reducing levels of misfolded SOD1.

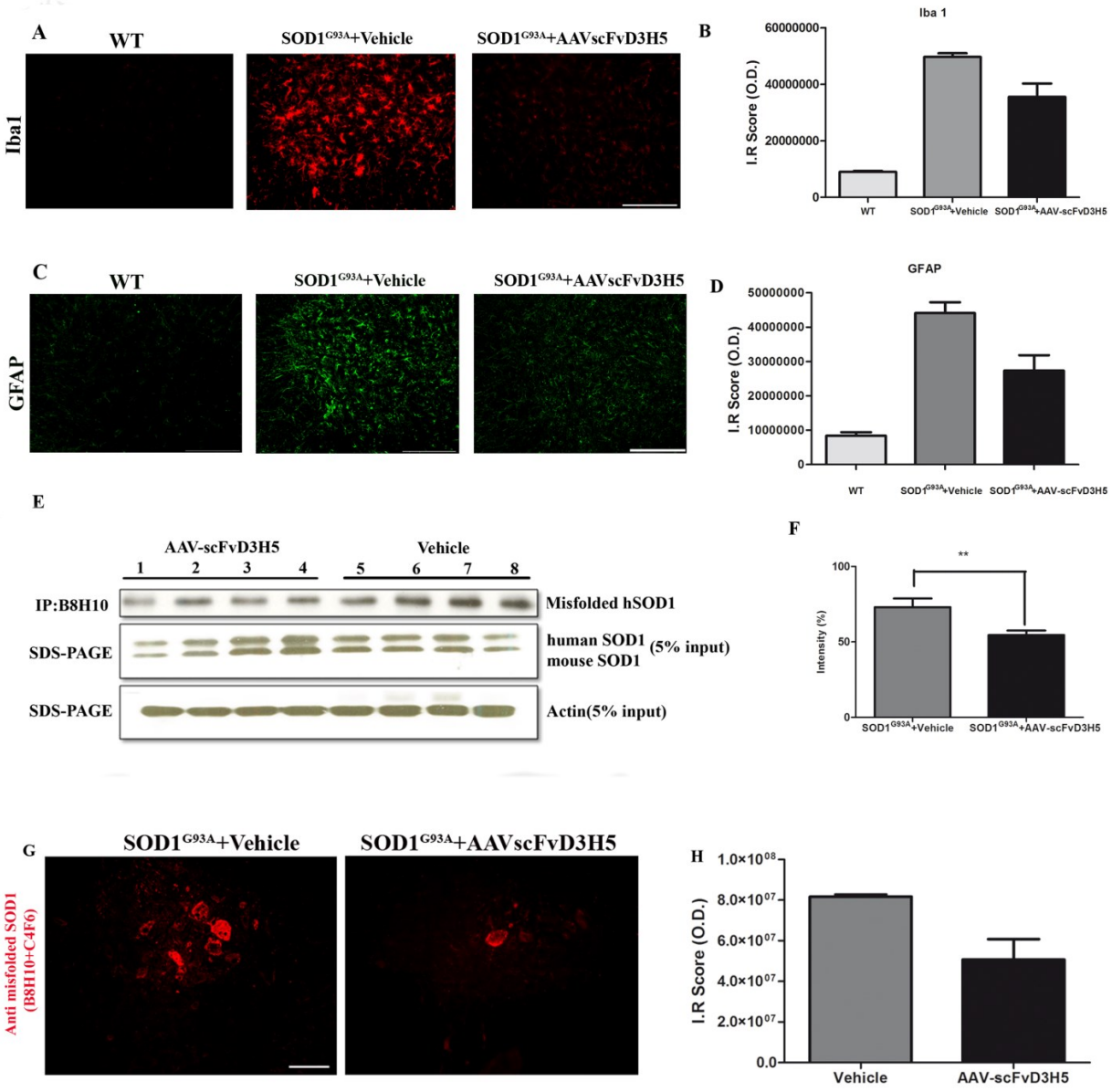


Figure 2.6 AAV-scFvD3H5 treatment resulted in decreased glial activation and reduced the burden of misfolded SOD1 in SOD1^{G93A} mice.

Figure 2.6 AAV-scFvD3H5 treatment resulted in decreased glial activation and reduced the burden of misfolded SOD1 in SOD1^{G93A} mice. (A) Lumbar spinal cord section from WT mice, SOD1^{G93A} mice treated with vehicle (middle) and SOD1^{G93A} mice treated with AAV-scFvD3H5 (right) as stained with anti-Iba1 antibody. **(B)** Quantification of immunoreactivity score (IR) for Iba1 showed statistically significant reduction of Iba1 signals in treated mice compared to untreated (n=3 for each group, $p<0.05$). **(C)** Representative pictures of GFAP immunofluorescence in the anterior horn of spinal cord from WT mice, SOD1^{G93A} control (middle) and AAV-scFvD3H5 treated SOD1^{G93A} (right). **(D)** The graph represents immunoreactivity quantification for GFAP, which was statistically reduced in treated mice. (n=3 for each group). Data are mean ± SEM for all quantification (scale bar, 50 um for all images). **(E)** Reduced level of misfolded SOD1 in the spinal cord of SOD1^{G93A} mice treated with AAV vector. Intrathecal injection of AAV-scFvD3H5 led to reduction of about 19% in the levels of misfolded SOD1 species as detected by B8H10 antibody *(p*=0.0293). Equal amount of proteins was used as shown on western blots after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with an actin antibody. Commercial SOD100 polyclonal antibody revealed equal amount of SOD1 protein in all samples. Data represents the mean±SEM. The *p* value was derived from student's t-test. All images are from P120 mice. **(G)** Representative pictures for immunofluorescence detection in spinal cord sections of misfolded SOD1 species with C4F6 and B8H10 antibodies (H) Quantification of immunoreactivity score for misfolded SOD1 protein showed significant reduction of signal intensity in SOD1^{G93A} mice treated with AAV vector (n=3 for each group, $p<0.05$, scale bar 50 um).

2.5.7 Attenuation of motor neuron loss in SOD1^{G93A} mice injected with AAV-scFvD3H5

Motor neuron loss correlates with disease severity in transgenic mice expressing mutant SOD1 (Gurney, 1994). Therefore, we investigated the effect of the scFvD3H5 treatment on motor neuron survival in SOD1^{G93A} mice by quantitative evaluation of the number of large Nissl-positive neurons in the ventral horn region of lumber spinal cord sections (Fig. 2.7 A). At 120 days of age, the SOD1^{G93A} mice injected at 45 days with the AAV-scFvD3H5 vector exhibited 28% more motor neurons in the lumbar spinal cord than the vehicle-injected SOD1^{G93A} mice (38.67 ± 0.67 per hemi section in AAV-scFvD3H5-treated mice vs 27.67 ± 2.19 in vehicle-treated mice, p=0.0086) (Fig. 2.7 B).

Progressive axonal degeneration is also a hallmark of mutant SOD-1 expression in transgenic mice (Zhang et al., 1997). Transverse sections from the L5 ventral root of AAV-scFvD3H5 and vehicle injected SOD1^{G93A} mice at 120 days of age were collected and analyzed for the number of myelinated axons (Fig. 2.7 C). In sections from AAV-scFvD3H5-treated SOD1^{G93A} mice, 30% more myelinated axons were detected when compared to sections from vehicle-treated SOD1^{G93A} mice (Fig. 2.7 D) (582.3 ± 57.75 vs 407.0 ± 10.12 in AAVscFvD3H5-treated and vehicle-treated mice, respectively p=0.0403).

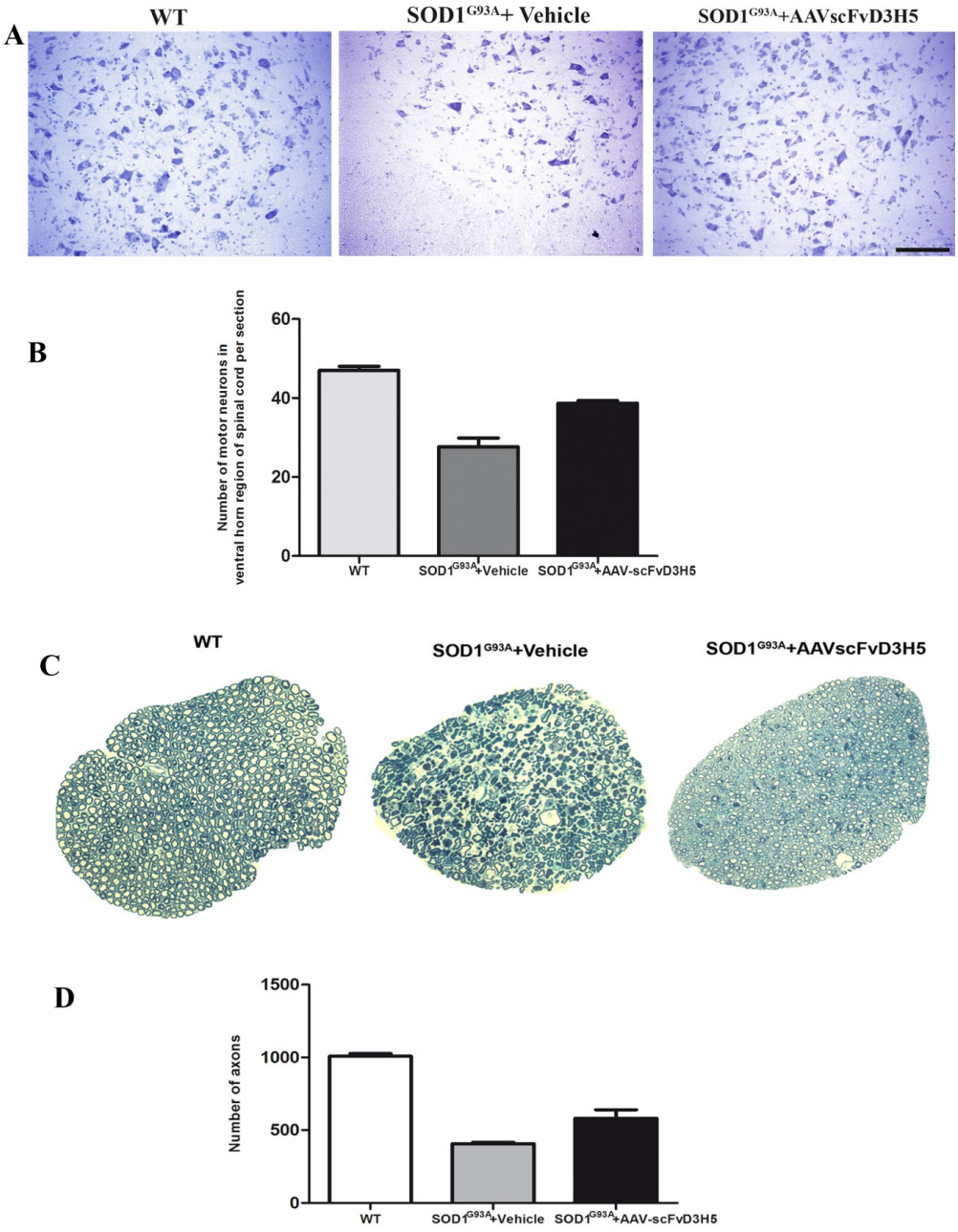


Figure 2.7 AAV-scFvD3H5 attenuated loss of motor neurons and ventral root axons in SOD1^{G93A} mice.

Figure 2.7 AAV-scFvD3H5 attenuated loss of motor neurons and ventral root axons in SOD1^{G93A} mice. (A) Cross sections of cresyl violet stained hemi-lumbar spinal cord in WT mice, control SOD1^{G93A} mice and AAV-scFvD3H5 mice at P120. **(B)** AAV-scFvD3H5-treated SOD1^{G93A} mice contained 28% more motor neurons (38.6±0.6687, n=3, *p*=.0086) compared with vehicle-treated SOD1^{G93A} mice (26.67±2.186, n=3). Data are mean± SEM. **(C)** Representative cross section pictures of ventral roots of spinal cord lumbar segment from WT mice, control SOD1^{G93A} mice and AAV-scFvD3H5 mice at P120. **(D)** Quantification of myelinated axons in the ventral root of spinal cord is shown. The total number of myelinated axons in control SOD1^{G93A} mice (407.0±10.12) was about 30% less compared to AAV-scFvD3H5-treated mice (582.3±57.75, p=0.0203, n=3 each group). Data are mean ± SEM.

2.6 Discussion

Here, we report the testing of a novel gene therapy approach for ALS based on AAV delivery of recombinant secretable scFv antibody specific to misfolded SOD1 species. This secretable scFv antibody was derived as described below from a hybridoma cell line expressing a monoclonal antibody called D3H5 which is specific to misfolded SOD1 (Gros-Louis et al., 2010). A single intrathecal injection of AAV-scFvD3H5 in $SOD1^{G93A}$ mice at 45 days of age yielded sustained expression of scFv molecules in the spinal cord. The treatment extended survival by an average of 16 days and by up to 40 days in direct correlation with levels of antibody detected in tissue. It is also noteworthy that the approach succeeded in delaying disease onset and in attenuating gliosis.

Recent studies reported that intrathecal injection of AAV vectors yielded strong levels of biodistribution and motor neuron transduction in the spinal cord (Snyder et al., 2011). Here we tested this method to achieve high level and sustained production of secretable scFv antibodies in the spinal cord. Our results show that five weeks after single intrathecal injection of AAV-scFvD3H5 into $SOD1^{G93A}$ mice, there was widespread immunodetection of scFv antibodies throughout the spinal cord including within ChAT-positive motor neurons (Fig. 2.2). The expression of scFv antibodies was persistent until end stage of disease, as revealed by anti-myc immunofluorescence and ELISA (Fig. 2.2). We noticed variation in scFv antibody titers in the spinal cord from one mouse to another (Fig. 2.3 E), a phenomenon due in part to inherent technical difficulty to inject intrathecally in a small animal constant amount of AAV vectors. Nonetheless, the data revealed that longevity of $SOD1^{G93A}$ mice correlated directly with the scFv antibody titer measured by ELISA in whole spinal cord lysates at end stage of disease. It is noteworthy that this immunotherapy approach via AAV delivery system extended survival of $SOD1^{G93A}$ mice by up to 40 days. This would rank among the best therapeutic interventions accomplished so far in this mouse model of ALS with high copy number of mutant SOD1 transgene.

The success of this therapeutic strategy lies in the fact that scFv antibodies are targeting misfolded SOD1 species, the source of toxicity in the disease (Bruijn et al., 1997; Bruijn et al., 1998; Wang et al., 2009a). Immunoprecipitation experiments from spinal cord lysates at end stage of disease mice confirmed that treatment led to a reduction in amount of misfolded SOD1 species (Fig. 2.6 E). The results also suggest a direct beneficial effect of scFv immunotherapy antibody on motor neurons. Bioimaging analysis of GAP-43/luc/gfp; $SOD1^{G93A}$ mice treated with AAV-scFvD3H5 revealed reduction of neuronal stress at presymptomatic stage (Fig. 2.5). Symptoms monitoring also indicated a postponing of disease onset in $SOD1^{G93A}$ mice injected with AAV-scFvD3H5 (Fig. 2.3 C-E). Such alleviation of motor neuron damage by scFvD3H5 antibody associated with delayed disease onset is consistent with previous studies on genetic ablation of mutant SOD1 within various cell types which concluded that toxicity of mutant SOD1 exerted within motor neurons constitute a primary determinant of disease onset (Boillee et al., 2006b).

A common characteristic of ALS is the occurrence of a neuroinflammatory reaction consisting of activated glial cells, mainly microglia and astrocytes. Previous studies have demonstrated that activated microglia induces the production of neurotoxic factors such as superoxide, nitric oxide and proinflammatory cytokines whereas reactive astrocytes express inflammatory markers such as iNOS (Barbeito et al., 2004; Moisse and Strong, 2006; Block et al., 2007). It is now well established that the surrounding cells are active players in motor neuron dysfunction and death in mouse model of ALS as well as in FALS and in SALS (Clement et al., 2003; Yamanaka et al., 2008). Here, the scFv treatment succeeded in attenuating the activation of microglia and astrocytes in the spinal cord of $SOD1^{G93A}$ mice (Fig. 2.6 A, B), an effect that may contribute to neuroprotection.

These results suggest that AAV-based delivery of scFv antibodies against misfolded SOD1 species should be considered for treatment of ALS patients bearing SOD1 mutations. Unlike other drugs that act on deleterious pathways or cell survival, such immunotherapy-based approach can target specifically misfolded SOD1 species, the primary cause of toxicity in the disease. Moreover, there is evidence that aggregated forms of SOD1 can seed misfolding and aggregation of native wild type SOD1 protein (Chia et al., 2010; Grad et al., 2011; Munch and Bertolotti, 2011) Thus, the beneficial effects of antibodies might come not only from clearance of pathogenic SOD1 molecules but also from neutralization of toxic epitope exposed by misfolded SOD1 or from interference in formation and propagation of misfolded SOD1 species (Grad et al., 2011). An immunotherapeutic approach is more selective than an antisense oligonucleotide therapy which aims to reduce mRNA levels encoding both mutant SOD1 and WT SOD1 (Smith et al., 2006). Moreover, another advantage of the AAV-delivery approach is that it can achieve high level and sustained expression of secreted scFv antibodies without repeated injections of antibodies. Finally, the small size of scFv antibodies lacking Fc fragment allows them to thoroughly penetrate CNS tissues without adverse immune system response.

An immunotherapy based on intrathecal inoculation of AAV to transducer in the CNS secretable scFv antibodies against misfolded SOD1 would be applicable for treatment of human ALS. Recombinant AAV vectors are vehicles of choice for gene transfer in the nervous system (Towne et al., 2010; Salegio et al., 2012; Samaranch et al., 2012) . AAV can provide stable and safe gene expression with minimal immune responses. In recent years, AAV delivery of genes has been used with success in treatment of human genetic disorders, especially in retinal disease (Bennicelli et al., 2008; Maguire et al., 2008). When injected into the cerebrospinal fluid (CSF), AAV vectors can confer widespread and sustained transgene expression in the CNS (Towne et al., 2010; Snyder et al., 2011). Intrathecal injection in human ALS patients would require less AAV vectors than systemic injection and it would target production of scFv antibodies to the CNS, an immunoprivileged location. In the gene therapy approach described here, the question of what CNS cell types express the scFv antibody is not of crucial importance because the

scFv protein has been engineered with an immunoglobulin secretion signal. The crucial point in this approach is the amount of scFv antibodies being secreted in the spinal cord milieu in order to maximize the benefits. As shown in Fig. 2.3, there is a direct correlation between the amount of scFv antibodies in the spinal cord milieu and therapeutic effects. Thus, the transduction of secretable scFv molecules in various cell types would be advantageous.

An AAV-based immunotherapy to target misfolded SOD1 would be applicable for treatment of ALS patients carrying SOD1 mutations. A prophylactic treatment would even be conceivable for individuals carrying ALS-linked SOD1 mutations since the approach is capable to delay the onset of disease. Moreover, potential application of such immunotherapy to subsets of sporadic ALS cases cannot be excluded at this time as recent studies suggest the existence of misfolded/aggregated forms of SOD1 in sporadic ALS cases with no SOD1 mutations (Forsberg et al., 2010; Bosco et al., 2010; Guareschi et al., 201; Pokrishevsky et al., 2012).

2.7 Acknowledgement

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Chapter 3: Early stage treatment with Withaferin A reduces levels of misfolded superoxide dismutase 1 and extends the lifespan in mouse model of ALS.

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3.1 Resume

Environ 20% des cas familiaux de sclérose latérale amyotrophique sont causés par une mutation dans le gène produisant la protéine Cu/Zn superoxide dismutase (SOD1). Des études récentes ont démontré que l'utilisation de la Withaferin A (WA), un inhibiteur de l'activité du facteur nucléaire-κB (NF-κB), était efficace pour réduire le phénotype de la maladie dans des souris transgéniques TDP-43. Ces résultats nous ont encouragés à déterminer l'efficacité de la WA dans deux autres modèles de souris transgéniques exprimant des mutations dans la protéine SOD1, soit les lignées de souris $SOD1^{G93A}$ et $SOD1^{G37R}$. Nous avons réalisé des injections intrapéritonéales de WA à des doses de 4mg/kg dès l'âge de 40 jours (P40) et ce, jusqu'au décès, dans les souris $SOD1^{G93A}$ et dès l'âge de 9 mois dans les souris $SOD1^{G37R}$. L'effet bénéfique de la WA chez les souris SOD1 se caractérisait par une baisse de la neuroinflammation normalement présente, par une baisse du niveau de SOD1 mal-repliée dans la moelle épinière et par une baisse de la mortalité des motoneurones, retardant la progression de la maladie et la mortalité des souris. Le traitement des souris avec la WA induisait une activité importante de la protéine heat shock protein 25 (Hsp-25, un analogue murin de Hsp-27), ce qui peut expliquer la diminution du niveau de SOD1 mal-repliée dans la moelle épinière et la baisse du dommage neuronal infligé par SOD1. Le niveau de dommage neuronal a été déterminé par l'utilisation d'imagerie bio-photonique en temps réel des souris $SOD1^{G93A}$ exprimant un transgène luciférase sous le contrôle du promoteur GAP-43. Ces résultats suggèrent l'utilisation potentiel de la withaferin A dans le but de traiter de manière efficace la sclérose latérale amyotrophique.

3.2 Abstract

Approximately 20% cases of familial amyotrophic lateral sclerosis (ALS) are caused by mutations in the gene encoding Cu/Zn superoxide dismutase (SOD1). Recent studies have shown that Withaferin A (WA), an inhibitor of NF-κB activity was efficient in reducing disease phenotype in TDP-43 transgenic mouse model of ALS. These findings led us to test WA in mice from two transgenic lines expressing different ALS-linked SOD1 mutations, $SOD1^{G93A}$ and $SOD1^{G37R}$. Intraperitoneal administration of WA at a dosage of 4mg/kg of body weight was initiated from postnatal day 40 (P40) till end stage in $SOD1^{G93A}$ mice and from 9 months until end stage in $SOD1^{G37R}$ mice. The beneficial effects of WA in $SOD1^{G93A}$ mice model was accompanied by alleviation of neuroinflammation decrease in levels of misfolded SOD1 species in spinal cord, a reduction in loss of motor neurons, resulting in delayed disease progression and mortality. Interestingly, WA treatment triggered robust induction of heat shock protein 25 (Hsp-25, a mouse orthologue of Hsp-27), which may explain the reduced level of misfolded SOD1 species in the spinal cord of $SOD1^{G93A}$ mice and the decrease of neuronal injury responses as revealed by real-time imaging of biophotonic $SOD1^{G93A}$ mice expressing a luciferase transgene under the control of GAP-43 promoter. These results suggest that WA may represent a potential lead compound for drug development aiming to treat ALS.

3.3 Introduction

Amyotrophic lateral sclerosis (ALS) is a fatal progressive degenerative disorder characterized by progressive muscle weakness, muscle atrophy and eventual paralysis, leading to death within 2 to 5 years. About 5–10% of patients inherit the disease, typically in an autosomal dominant manner (familial ALS, FALS). In 20% of FALS, missense mutations have been identified in the gene coding for superoxide dismutase 1 (SOD1) (Rosen et al., 1993; Cudkowicz et al., 1997; Cohen et al., 2010).Various hypotheses have been proposed to explain the toxicity of SOD1 mutants including protein aggregation (Chattopadhyay and Valentine, 2009; Ticozzi et al., 2010), oxidative stress (Barber and Shaw, 2010), mitochondrial dysfunction (Pizzuti and Petrucci, 2011) and excitotoxicity (Bogaert et al., 2010). TAR DNA binding protein 43 (TDP-43) is another protein detected in pathological inclusions of ALS and frontotemporal lobar degeneration with ubiquitin inclusions (FTLD-U) cases (Arai et al., 2006; Neumann et al., 2006). Dominant mutations in *TARDBP*, which codes for TDP-43, were reported by several groups as a primary cause of ALS (Gitcho et al., 2008; Kabashi et al., 2008; Sreedharan et al., 2008; Van Deerlin et al., 2008; Corrado et al., 2009; Daoud et al., 2009) and may account for ~3% of familial ALS cases and ~1.5% of sporadic cases.

Previously we showed that treatment of TDP-43 transgenic mouse model of ALS with Withaferin A (WA), an inhibitor of nuclear factor -κβ (NF-κβ) activity, ameliorated disease symptoms and pathological phenotypes such as reduction of denervated neuromuscular junctions and attenuation of neuroinflammation (Swarup et al., 2011). These findings led us to test WA in mice from two transgenic lines expressing different ALS-linked SOD1 mutations, $SOD1^{G93A}$ and $SOD1^{G37R}$. Importantly, recent studies by Frakes and colleagues have demonstrated that in $SOD1^{G93A}$ mouse model of ALS, motor neuron death is induced by activated microglia in NF-κB dependent manner (Frakes et al., 2014). WA is a steroid lactone found in the medicinal plant *Withania somnifera*. Semi purified root extract of *Withania Somnifera* consisting of withanolides and withanosides reversed behavioral deficits, plaque pathology, accumulation of β-amyloid peptides (Aβ) and oligomers in the brains of APP/PS1 Alzheimer's disease transgenic mice (Sehgal et al., 2012). WA exhibits

a variety of beneficial effects including anti-tumor, anti-inflammatory and immunomodulatory properties (Mishra et al., 2000). It has also been reported to be an inducer of heat shock proteins (Khan et al., 2012).

Here, we investigated the effects of WA treatment on disease progression and on pathological changes in two ALS mouse models expressing either $SOD1^{G93A}$ or $SOD1^{G37R}$ mutants. We report that when started early in disease pathogenesis, at time of onset of initial motor function deficits (Vinsant et al., 2013b, a), treatment with WA significantly extended the life span of $SOD1^{G93A}$ mice and of $SOD1^{G37R}$ mice. WA treatment was associated with a reduction of neuronal stress, inflammation, upregulation of heat shock proteins 25, (Hsp-25; mouse orthologue of Hsp-27), Hsp-70 and decreased levels of misfolded SOD1 species.

3.4 Materials and Methods

Generation of GFAP-luc/SOD1^{G93A} and GAP-43-luc/gfp;SOD1^{G93A} transgenic mice

The transgenic GFAP-luc mice (FVB/N background) were obtained from Xenogen (Caliper Life Sciences, Hopkinton, MA, USA). As previously described, (Keller et al., 2009) the GFAP-luc mice were crossed with the transgenic SOD1^{G93A} transgenic mouse (C57/BL6, Jackson labs) (Gurney et al., 1994; Gurney et al., 1996) to generate double transgenic GFAP-luc/SOD1^{G93A} mice. Double transgenic mice were genotyped according to the following procedure. The presence of GFAP-luc transgene was assessed by PCR with HotStar Taq Master mix Kit (Quiagen, Mississauga, ON, Canada) in 15 mM $MgCl_2$ PCR buffer with the following primers: 5′GAAATGTCCGTTCGGTTGGCAGAAGC and 5′CCAAAACCGTGATGGAATGGAACAACA. The presence of the SOD1^{G93A} mutant transgene was assessed by PCR as previously described (Gowing et al., 2006). To confirm that the transgene copy number of SOD1^{G93A} was not altered in the mice used for this study, we evaluated genomic SOD1 levels by Quantitative RT-PCR using genomic DNA isolated from tail tissue. Analysis of the mouse housekeeping gene encoding glyceraldehyde-3-phosphate dehydrogenase was used for normalization purposes. Oligoprimer pairs (used at concentration of 300 nm) were designed by GeneTool 2.0 software (Biotools Inc, Edmonton, AB, CA) and their specificity was verified by blast in the GenBank database. Standard cycling conditions were used.

The transgenic GAP-43-luc/gfp reporter mice were generated as described previously (Gravel et al., 2011). The mice were crossed with the SOD1^{G93A} transgenic mice (C57/BL6; Jackson) (Gurney et al., 1994; Gurney et al., 1996) to generate double transgenic GAP-43-luc/gfp; SOD1^{G93A} mice. To avoid the effects of genetic background, all experiments were performed on age-matched littermates. Double transgenic mice were genotyped according to the following procedure. The presence of GAP-43-luc/gfp transgene was assessed by PCR of the luciferase reporter gene with the following primers:
(5′-GGCGCAGTAGGCAAGGTGGT and (5′-CAGCAGGATGCTCTCCAGTTC) as described previously (Lalancette-Hebert et al., 2007)

All experimental procedures were approved by the Laval University Animal Care Ethics Committee and are in accordance with The Guide to the Care and Use of Experimental Animals of the Canadian Council on Animal Care.

Analysis of clinical symptoms

The onset of weight loss was determined at the time when mice started to exhibit a decline of body weight after reaching a peak. The survival was defined as the loss of Righting reflex, the age when the animal could not right itself within 30 s when placed on its side. Measurements of body weight and the loss of hind limb reflex were used to score the clinical effects of $SOD1^{G93A}$ mice. The extensibility and postural reflex of the hind limbs when mice were held up with their tails were scored as described previously (Urushitani et al., 2006). The $SOD1^{G93A}$ reflex score and body weight were measured every 2 days, beginning at 45 days. Scoring was performed in a blind manner by animal technicians who had no information about the genotype but had experience in grading $SOD1^{G93A}$ mice paralysis.

***In vivo* bioluminescence imaging**

As previously described, images were gathered using IVIS 200 Imaging System (Xenogen, Alameda (Cordeau et al., 2008; Keller et al., 2009). Twenty minutes prior to imaging session the mice received intraperitoneal injection of D-luciferine, a luciferase substrate (150 mg/kg, Xenogen, Alameda, CA) dissolved in 0.9% saline. The mice were then anesthetized with 2% isoflurane in 100% oxygen at a flow rate of 2 L/ min and placed in the heated, light-tight imaging chamber. Images of lumbar spinal cord region of interest were collected using high sensitivity CCD camera with wavelengths ranging from 300–600 nm. Exposition time for imaging was 1 minute using different fields of view and F/1 lens aperture. The bioluminescence emission was normalized and displayed in physical units of surface radiance, photons per second per centimeter squared per steradian (photons/s/cm2/sr) (Maysinger et al., 2007; Cordeau et al., 2008). The light output was quantified by determining the total number of photons emitted per second using the Living Image 4.1 acquisition and imaging software (PerkinElmer, MA, USA). Region-of-interest

(ROI) measurements on the images were used to convert surface radiance (photons/s/cm2/sr) to source flux or total flux of photons expressed in photons/s.

Withaferin A administration

The drug used in this study was Withaferin A, obtained from Enzo Life sciences (Farmingdale, NY). Withaferin A was first dissolved in DMSO and diluted in 0.9% saline. The final concentration of DMSO was 10%. The drug was made fresh every two weeks and was protected from light. Male and female transgenic mice and their transgenic littermates were divided randomly into following two groups (n= 15 per group): (A) Transgenic controls, which received vehicle (0.9% Saline with 10% DMSO) and (B) Transgenic WA treatment group, which received an intraperitoneal injection of WA at the rate of 4 mg/kg body weight, twice a week.

The treatment was performed from early symptomatic stage (40 days old) as recently proposed by Vinsant et al. (2013) to end point stage. End-stage of the disease was scored as complete paralysis of both hind limbs and the inability of the animals to right themselves after being placed on their side.

Tissue collection and immunofluorescence microscopy

Mice were anaesthetized by intraperitoneal injection of chloral hydrate (10 mg/ml) and transcardially perfused with 30 ml of 0.9% NaCl, followed by ice-cold PBS 1x buffered 4% paraformaldehyde (PFA) at pH 7.4. Tissue sample were then post-fixed overnight in 4% PFA and equilibrated in phosphate-buffered 20% sucrose. Spinal cords were cut at 25 μm of thickness. The double immunofluorescence analysis was performed according to the following procedure. After 1 to 2 h air drying, sections were blocked in PBS containing 10% goat

serum and 0.25% Triton X-100 for 30 min. Spinal cord sections were incubated with either 1 : 500 rabbit polyclonal anti-glial fibrillary acidic protein (GFAP) (Dako, Carpinteria,CA, USA), 1 : 500 rabbit anti-Iba1 (Wako Chemicals USA,Richmond, VA, USA), 1:50 rabbit polyclonal ATF3 (Santacruz Biotechnology, CA,USA), 1:500 mouse monoclonal NEU N

(Millipore Corporation, CA,USA), Slides were washed in PBS containing 5% goat serum and 0.25% triton X-100 and incubated with appropriate fluorescent-conjugated secondary antibodies (Alexa, Molecular Probes Inc, Eugene, OR, USA) for 2 h at room temperature. A final wash was performed in PBS and slides were coverslipped with Fluoromount medium (Electron Microscopy Sciences, Hatfield, PA, USA). Observations were made with classical fluorescent microscope (Leica, Germany).

Stereological counts of motor neurons

Sections of horizontal spinal cord were Nissl Stained to identify motor neurons in the lumbar spinal cord. The L3 to L5 spinal cord sections were individually traced with a 40X microscopic observation and sampled under a 400X observation. The density of labeled cells were estimated by optical fractionator method using Stereo Investigator software (MBF Biosciences, Williston, USA). The counting parameters were the distance between counting frames (150 μm), the counting frame size (150 μm×150 μm), the dissector height (10 μm) and the guard zone thickness (1 μm). Motor neurons were identified on the basis of their correct anatomical location (ventral horn/laminae 9), required a distinct nucleolus within the plane of the optical dissector and had a cross sectional area ≥ to 250 μm^2. Results are expressed as the total number of motor neuron/mm^3.

Immunoprecipitation and western blotting

At P120, spinal cord were dissected out, rapidly frozen in liquid nitrogen and stored at -80°C for cytokine array, immunoprecipitation and western blot analysis. Whole protein lysates from mouse spinal cords were extracted by homogenization of the tissues in TNG-T lysis buffer (50 mM Tris–HCl pH: 7.4; 100 mM NaCl; 10% Glycerol; 1% Triton X), sonicated and centrifuged for 20 min at 9000 g at 4°C. Blots were immunostained overnight at 4°C with primary antibodies, Hsp-25/27 (rabbit polyclonal antibody from Cell signalling MA,USA, 1:2500), Hsp-70, rabbit polyclonal antibody from Cell signalling, clone D69, detects endogenous level of total HSP-70 protein, MA,USA at dilution of 1:1000), Hsf-1 (rat monoclonal antibody from Thermo scientific, Ab-1, clone 4B4 at a dilution of 1:1000) Iba-1 (Wako chemical, Richmond,CA,USA, 1:1000),TLR2 (Abcam, AB16894, MA,USA

at a dilution of 1:1000). As previously described (Gros-Louis et al., 2010), immunoprecipitation experiments for misfolded SOD1 were done by using Dynabeads standard protocol from Invitrogen (Carlsbad, CA, USA). Briefly Dynabeads were washed and coated with the mouse monoclonal anti misfolded-SOD1 antibody B8H10, for two hours at room temperature, washed with PBST and BSA/HEPES-PBS, and incubated overnight with 300 ug of spinal cord lysate protein at 4° C with rotation. After incubation, the beads were washed and fractioned on 14% SDS-PAGE.

Cytokine array

The expression profile of inflammatory cytokines were performed with a Mouse Cytokine Antibody Array (Raybio Mouse Inflammation Antibody Array 1 ; Cat#AAM- INF-1, RayBiotech, Norcross, GA, USA) as previously described in details (Lalancette-Hebert et al., 2007). Protein samples were obtained by homogenization of WA injected and vehicle injected $SOD1^{G93A}$ spinal cord (N=3) at P120 in 1x cell lysis buffer with Protease inhibitor cocktail (Sigma #P8340, St. Louis, MO, USA) included in the RayBiotech kit (RaybioWMouse Inflammation Antibody Array 1.1, Ray Biotech, #AAM-INF- 1 L). After extraction, samples were spun down at 13000 rpm for 10 minutes at $4^{O}C$ and supernatant was used for the experiment. For each group (three mice per group) samples were pooled together and incubated with the array membrane overnight at 4°C. After washing in the washing buffer (included in the RayBiotech kit), membranes were incubated with biotin-conjugated antibodies overnight. After washing, membranes were incubated with horseradish peroxidase conjugated streptavidin diluted in the blocking buffer for 2 h. Signal detection was performed according to RayBiotech protocol, by exposing membranes to x-ray film (Biomax MR1; #8701302; Kodak, Rochester, NY, USA), and the obtained results analyzed with image J software. Cytokine expression assays were quantified by measuring the optical density of each cytokine spot on the membrane with ImageJ software, as previously described (Lalancette-Hebert et al., 2007).

Cytokines were presented on membranes as duplicates and the analysis was performed twice. The background values were subtracted from cytokine expression values. Data were

expressed in arbitrary units relative to appropriate positive control. Further, data were averaged and analyzed by a two-tailed unpaired Student's t-test.

Flow cytometry analysis

Blood was collected from the submandibular vein of WA injected and Vehicle injected mice at 112 and 125 days as previously done by (Golde et al., 2005) and sent for flow cytometry analyses (The CHUL Hospital Research Institute's Core Flow Cytometry Laboratory). The panel of antibodies (BD Biosciences) used to evaluate the leucocytes from mice included CD4 (APC Rat Anti-Mouse CD4, clone RM4-5), CD8 (PE-CF594 Rat Anti-Mouse CD8a, Clone 53-6.7), CD25 (FITC Rat Anti-Mouse CD25, clone 7D4), CD45 (V500 Rat Anti-mouse CD45,clone 3O-f11), FoxP3 (V450 Rat Anti-Mouse FOXP3, clone MF23), IL-4 (PE-Cy^{TM}7 rat Anti-Mouse IL-4, clone 11B11) and IL-10 (PE Rat Anti-Mouse IL-10, clone JEs5-16E3). Samples were afterwards analyzed on a flow cytometer (BD LSRII) immediately after the end of the staining protocol by a 'blinded' individual.

Statistical analysis

Data were analyzed using Prism 5.0 software (Graph Pad Software, LaJolla, CA, USA). Behavioral data were computed by performing two-way ANOVAs (except when specified) followed by Bonferroni post-tests and survival data using Mantel-Cox log-rank tests. Immunoreactivity scores for Iba1, GFAP antibody and quantification of western blots were done by Image J and were compared using two-tailed Student's *t*-tests. Data are expressed as mean_SEM; $p<0.05$ was considered statistically significant.

3.5 Results

3.5.1 WA extends survival in transgenic mice over expressing $SOD1^{G93A}$ or $SOD1^{G37R}$ mutants

We examined the effect of WA in transgenic mice overexpressing $SOD1^{G93A}$ or $SOD1^{G37R}$ mutants. WA was dissolved in 10% DMSO and injected intraperitoneally into $SOD1^{G93A}$ mice twice a week from the postnatal day 40 until death at a dose of 4 mg/kg body weight per each injection. The $SOD1^{G37R}$ mice were injected twice a week with the same dose starting at 9 months of age until end point. The treatment with WA significantly extended survival of $SOD1^{G93A}$ mice. Namely, the mean survival of vehicle-treated $SOD1^{G93A}$ mice was 145 days (N=15) whereas treatment with WA increased life span of $SOD1^{G93A}$ mice to 153 days (N=16), $p<0.05$ (a difference of 8 days) (Fig. 3.1A). In the mouse model with slow progressing disease, the $SOD1^{G37R}$ model, the mean survival of WA-treated $SOD1^{G37R}$ mice was 397 days (N=8) compared to controls (379 days ; N=8; $p<0.0001$; a difference of 18 days) (Fig. 3.1B). Furthermore, treatment with WA significantly delayed the loss of motor function observed in the motor function tests (Fig. 3.1C) and prevented the loss of body weight (Fig. 3.1D).

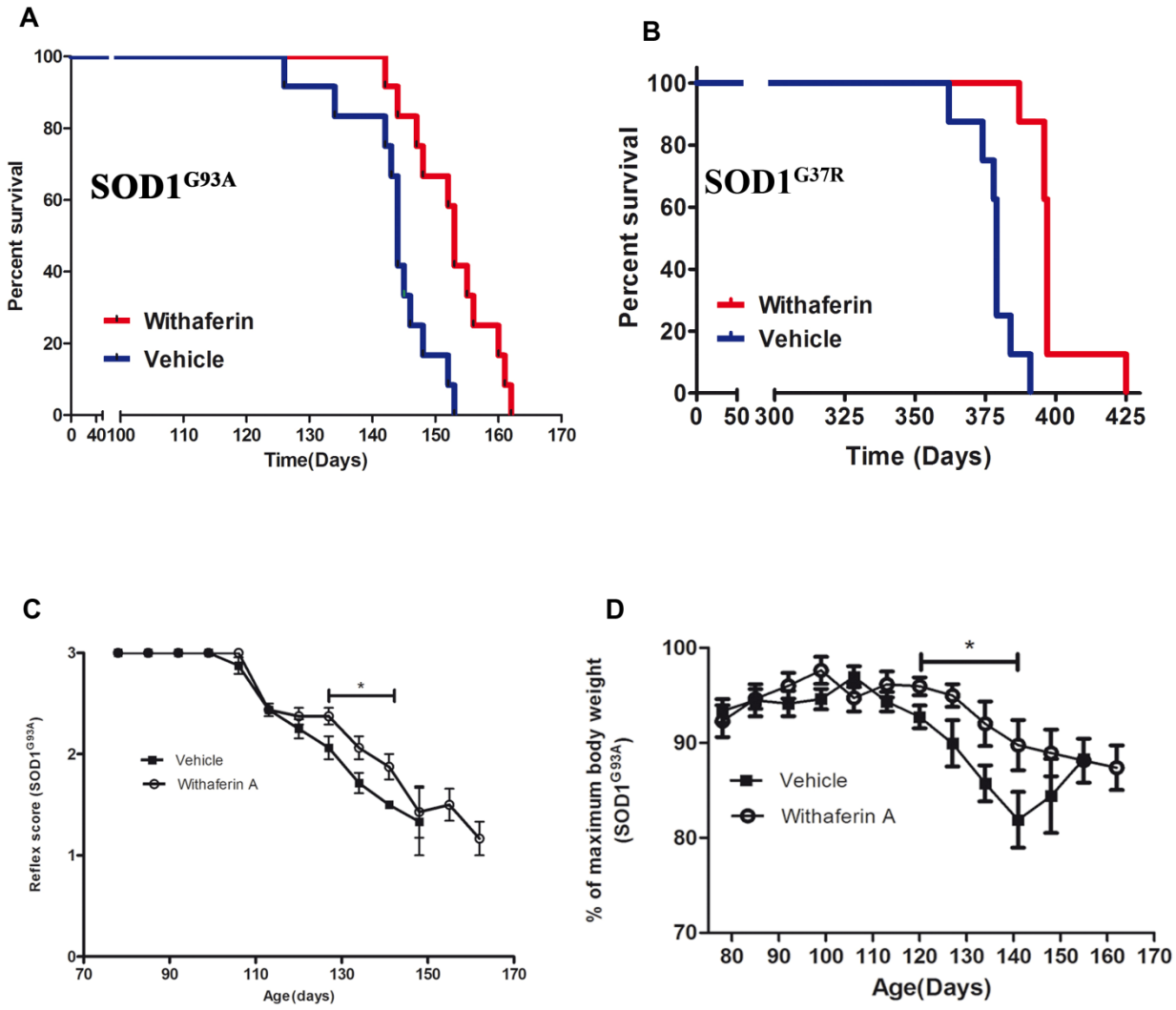

Figure 3.1 Withaferin A extended survival of SOD1^{G93A}mice.

Figure 3.1 Withaferin A extended survival of SOD1^{G93A}mice

To examine whether WA can alleviate the mutant SOD1-induced neurotoxicity in vivo, SOD1^{G93A} and SOD1^{G37R} mice were intraperitoneally injected with WA, 4mg/kg or vehicle (Saline + 10% DMSO) twice a week from day 40 until end stage and then statistically analyzed by Kaplan-Meier method. **(A)** Kaplan-Meier survival curve shows that vehicle treated SOD1^{G93A} (N=12) transgenic mice had a mean survival of 144 days whereas WA treated mice (N=12) lived for 153 days. Long rank test shows a statistically significant value of (p=0.0017). **(B)** Kaplan-Meier survival curve shows that vehicle treated SOD1^{G37R} (N=8) transgenic mice had a mean survival of 379 days whereas WA treated mice (N=8) lived for 397 days. Long rank test shows a statistically significant value of (p=0.0001). **(C)** Hind limb reflex score analysis showed prolonged maintenance of reflex score for particular time points in WA treated mice. Difference is significant for marked time period. **(D)** Disease onset was determined by the initial loss of body weight (age of peak body weight). The difference was significant for the marked time period. Each point indicates the average ± SEM. The data were analyzed by unpaired t-test.

3.5.2 Reduction of early neuronal injury response biophotonic signals by WA treatment in GAP-43-luc/gfp; SOD1^{G93A} mice

Treatment with WA extended survival in two different SOD1 mutant mouse models. Therefore by using live imaging approach and cell type specific reporter mouse, we further investigated potential therapeutic mechanisms and its cellular targets. To visualize the effects of WA treatment in SOD1^{G93A} mice in real time, we took advantage of the GAP-43-luc/gfp reporter mice recently generated and validated in our laboratory (Gravel et al., 2011). Importantly, the results of our recent study revealed that the GAP-43 biophotonic signals imaged from the spinal cords of live SOD1^{G93A} mice may serve as a valid biomarker to assess early neuronal injury response in SOD1 mutant mediated disease (Patel et al., 2013). Moreover, immunofluoresence analysis revealed almost perfect co-relation between GAP-43 driven gfp transgenic and ATF-3, known to be up regulated in affected and injured neurons (Saxena et al., 2009; Malaspina et al., 2010; Patel et al., 2013). Double transgenic GAP-43-luc/gfp;SOD1^{G93A} mice were generated by crossing heterozygous mice carrying the mutant SOD1^{G93A} transgene with the heterozygous GAP-43-luc/gfp mice co-expressing reporter transgene, luciferase (luc) and green fluorescent protein (gfp), driven by the murine GAP-43 promoter. In this mouse model, a GAP-43 upregulation (luciferase expression detectable as a bioluminescence/photon emission and gfp expression detectable by confocal microscopy) can be followed longitudinally in live animals using bioluminescence/biophotonic imaging and a high sensitivity/ high-resolution CCD camera.

To determine the *in vivo* effect of WA treatment on early neuronal injury response, the bioluminescence imaging of the spinal cord was carried out longitudinally on GAP-43-luc/gfp;SOD1^{G93A} double transgenic mice (Fig. 3.2). WA treatment resulted in significant reduction of the GAP-43 bioluminescence signal in the spinal cord at sixteenth and seventeenth weeks of age compared to vehicle-treated double transgenic mice (Fig. 3.2D-J). The signal was lower even at eighteenth week of age (Fig. 3.2F,I). Reduction of neuronal injury response signal was further confirmed by immunofluorescence microscopy of spinal cord sections from WA-treated and control SOD1^{G93A} mice for detection of cyclic AMP-dependent transcription factor ATF-3, marker of neuronal injury (Nascimento et al., 2011) (Fig. 3.2K).

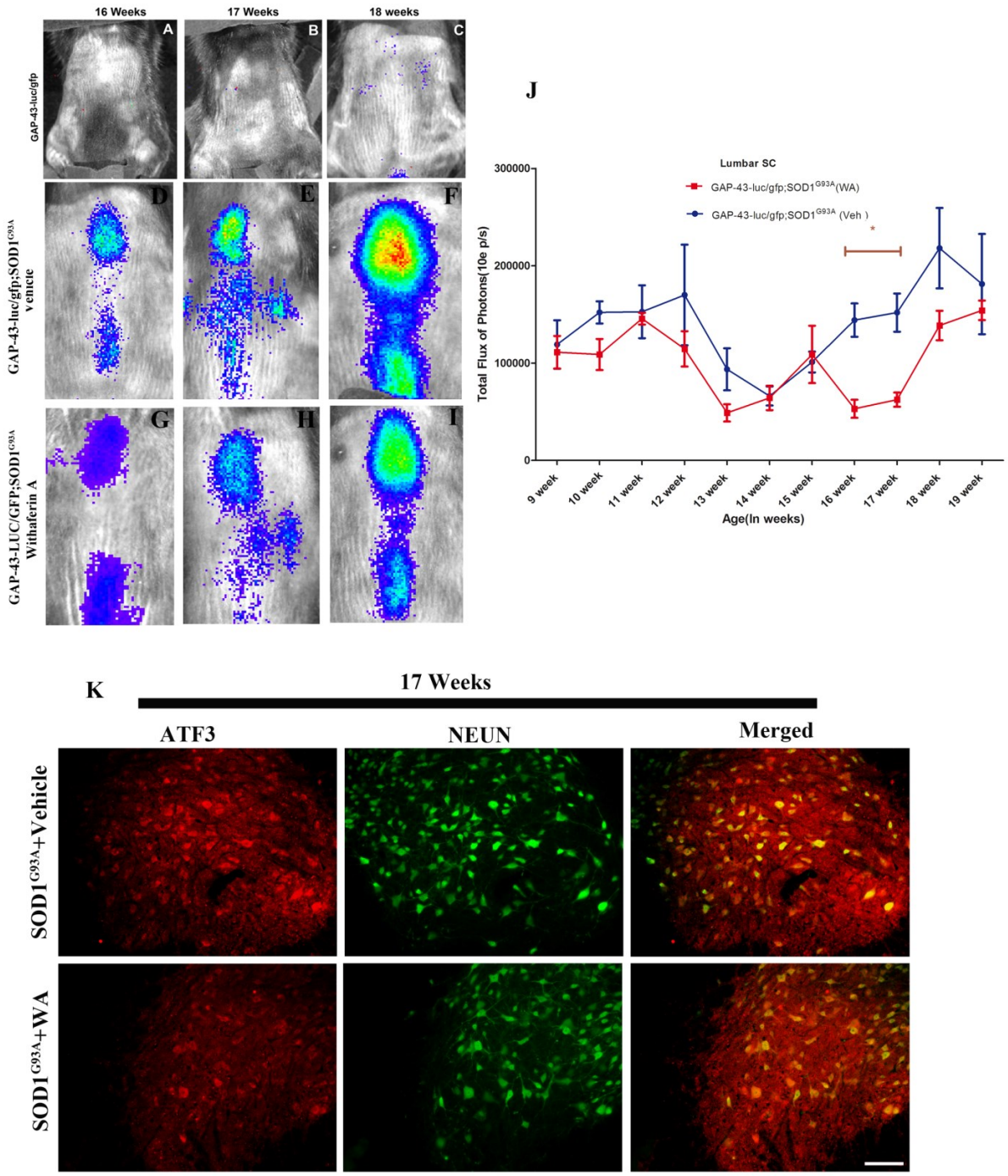

Figure 3.2 WA treatment in GAP-43-luc/gfp;SOD1^{G93A} mice reduced neuronal injury biophotonic signals.

Figure 3.2 WA treatment in GAP-43-luc/gfp;SOD1^{G93A} mice reduced neuronal injury biophotonic signals.

In vivo bioluminescence imaging of GAP-43 induction was analyzed at various time points in spinal cord of GAP-43-luc/gfp;SOD1^{G93A} mice. Typical sequence of representative images of spinal cord area obtained from double transgenic mice treated with WA and vehicle at different time points (16 and 17 weeks) by in vivo imaging (N=6 each group) are shown **(A-I). (J)** Longitudinal quantitative analysis of total photon GAP-43 signal/ bioluminescence in GAP-43-luc/gfp;SOD1^{G93A} mice in spinal cord is shown. Two way ANOVA revealed a statistically significant reduction in bioluminescence signal in WA treated group at 16 and 17 weeks (p<0.05) We also observed a slight reduction in bioluminescence signal at 18 weeks of age in WA treated mice. Error bar represents mean ± SEM. (K) Immunofluorescence microscopy using ATF3 and NeuN antibody was performed in spinal cord WA treated and vehicle treated mice at 17 weeks of age. ATF3 signal was found to be comparatively less in WA treated mice than non treated mice. Merging of both signals shows co localization in motor neurons of spinal cord ventral horn. Scale bar 50 um.

3.5.3 WA reduced level of the misfolded SOD1 species and induced upregulation of Hsps in SOD1^{G93A} mice

The misfolded SOD1 species, detectable with specific monoclonal antibodies, have been reported to be among the earliest pathological features in mutant SOD1 mice (Gros-Louis et al., 2010; Brotherton et al., 2012; Saxena et al., 2013) and are a common hallmark of familial and sporadic ALS (Bosco et al., 2010; Forsberg et al., 2010; Brotherton et al., 2012; Pokrishevsky et al., 2012). Moreover, previous reports suggest that misfolded SOD1 species, detected primarily in affected motor neurons, may serve as a valid biomarker of disease progression (Bosco et al., 2010; Forsberg et al., 2010; Brotherton et al., 2012; Pokrishevsky et al., 2012). The effects of WA treatment on levels of misfolded SOD1 in SOD1^{G93A} mice were examined by using a specific antibody (B8H10) against misfolded SOD1 species. Whole protein fractions of spinal cord lysates prepared from WA-and vehicle-injected SOD1^{G93A} mice at 120 days of age and were processed for immunoprecipitation, followed by SDS-PAGE and immunoblotting using a polyclonal anti-SOD100 antibody. Remarkably, WA treatment starting at 40 days of age resulted in a 39% reduction in levels of misfolded SOD1 in the spinal cord of SOD1^{G93A} mice (Fig. 3.3A,B). It is noteworthy that WA, in addition to its anti-inflammatory properties, is known to induce a variety of heat shock family proteins (Hsps) (Khan et al., 2012) and roles of Hsps as intracellular chaperons in protein unfolding/aggregation have been widely established (Sherman and Goldberg, 2001). Hence, we investigated whether observed decrease in level of misfolded SOD1 species in WA treated mice is associated with increase in level of Hsps. Although crossing the SOD1^{G93A} mutant mice with Hsp25/27 over-expressors did not significantly affect the course of disease (Krishnan et al., 2008), previous work has demonstrated that co-incubation of SOD1^{G93A} with Hsp 25/27 can significantly reduce insoluble aggregate formation in cell models of SOD1 aggregation (Yerbury et al., 2013). Moreover, administration of either heat shock protein Hsp25/27 or Hsp70 had a neuroprotective effects against SOD1 disease associated mutant-induced cell death (Patel et al., 2005). Therefore, to assess the effects of WA on different elements of cellular stress response we examined the effects of this compound on the levels of different Hsp family proteins, namely Hsp-25 and Hsp-70. The levels of Hsp-25 and Hsp-70 (known to be

affected in ALS) (Maatkamp et al., 2004; Robinson et al., 2005) were quantified from spinal cord extracts from treated and non-treated $SOD1^{G93A}$ mice at 120 days of age. Western blot analyses revealed a significant, 2.6- fold upregulation in levels of Hsp-25 and a 2.2- fold upregulation in levels of Hsp-70 in $SOD1^{G93A}$ mice treated with WA (Fig. 3.3C, D). Next, we examined the activation of heat shock transcription factor- 1 (Hsf-1) in the spinal cord of WA treated and untreated mice. Activation of Hsf-1 is characterized by shift in Hsf-1 band in western blot as Hsf-1 becomes phosphorylated (Sarge et al., 1993). Western blot analysis of WA treated spinal cord lysate showed a shift in Hsf-1 band which was not observed in vehicle treated mice (Fig. 3.3E). Hence, WA induced increased in survival was associated with significant reduction in SOD1 misfolded species, marked increase in Hsp- 25, Hsp-70 levels and as revealed by *in vivo* imaging, a marked decrease in neuronal early injury response marker Gap-43.

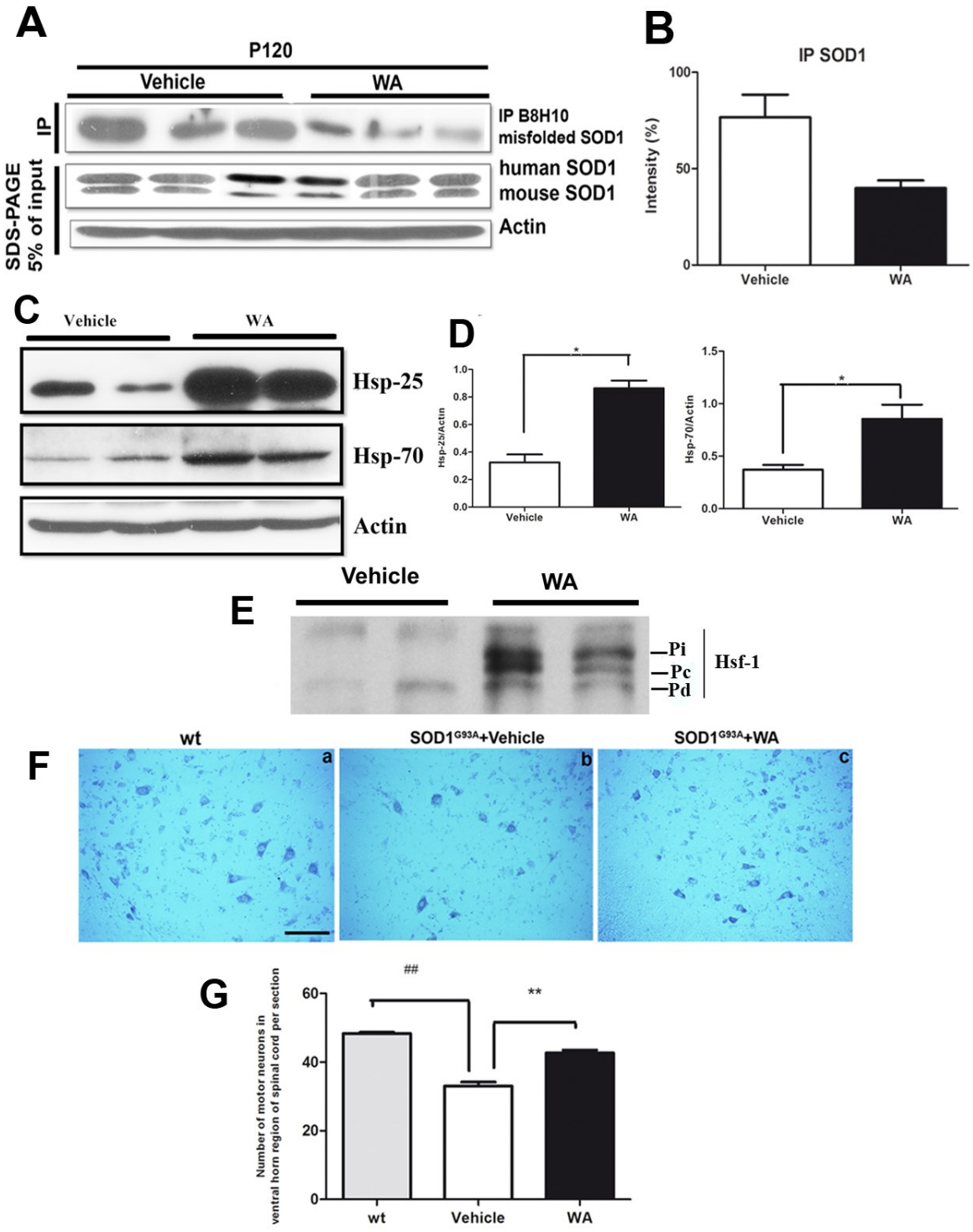

Figure 3.3 WA reduced the level of misfolded SOD1, induced upregulation of heat shock proteins and attenuated loss of motor neurons.

Figure 3.3 WA reduced the level of misfolded SOD1, induced upregulation of heat shock proteins and attenuated loss of motor neurons.

(A) Reduced level of misfolded SOD1 in spinal cord of mice. Intraperitoneal injection of WA led to reduction in the levels of misfolded SOD1 species as detected by immunoprecipitation with B8H10 antibody. Equal amount of proteins was used as shown on western blots after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with an actin antibody. Commercial SOD100 polyclonal antibody shows amount of SOD1 protein in all samples **(B)** Quantitative densitometric analysis showed a reduced level of misfolded SOD1 protein in WA treated mice. **(C)** The protein levels of Hsp25 and Hsp70 in the spinal cord lysates subjected to SDS-PAGE and immunoblotting were compared in the vehicle and WA injected $SOD1^{G93A}$ mice on P120. Representative immunoblots for HSP-25, HSP-70 are shown. **(D)** Quantitative densitometric analysis of western blot showed a significant upregulation in the level of Hsp-25 in the WA treated mice (WA: 0.8634 ± 0.05656 ; Vehicle: 0.325 ± 0.05718; p=0.0108; N=2). There was also an increase in the level of Hsp-70 protein (WA: 0.854 ± 0.13; Vehicle: 0.372 ± 0.45; p=0.038; N=2). Data represents mean ± SEM. p value were derived from student's t-test. All images are from P120 mice. **(E)** Western blot analysis of Hsf-1 expression in spinal cord tissue from, vehicle treated $SOD1^{G93A}$ and WA-treated $SOD1^{G93A}$ mice. Normally, the Hsf-1 monomer is present between 65–75 kDa, but is activated and shifted by 8–10 kDa in the WA-treated $SOD1^{G93A}$ mice. Pi, inducible phosphorylated Hsf-1; Pc, constitutively phosphorylated Hsf-1; Pd, dephosphorylated Hsf-1 *= p<0.05 by students t test.

(F) Cross sections of cresyl violet stained hemi-lumbar spinal cord in wt, control $SOD1^{G93A}$ and WA- treated $SOD1^{G93A}$ mice at P120 are shown.

(G) Quantification of number of motor neurons showed that WA- treated $SOD1^{G93A}$ mice contained more motor neurons (42.6 ± 0.8; N=3) compared with vehicle treated $SOD1^{G93A}$ mice (33.00 ± 1.1; N=3) (p=0.0027). Data are mean± SEM.

3.5.4 Withaferin A treatment suppressed neuroinflammatory signals in SOD1^{G93A} mice.

Progressive increase in neuroinflammatory signals is a hallmark of chronic neurodegenerative disorders including ALS. Namely, the substantial activation of microglial cells and astrocytes is one of the first microscopic finding in the spinal cord sections of ALS patients and SOD1 mutant mice (McGeer et al., 1991; Hall et al., 1998). Our previous work using biophotonic/bioluminescence imaging demonstrated that one of the first sign of disease in SOD1^{G93A} mice is an early induction of the biophotonic GFAP signal (Keller et al., 2009). Here it is noteworthy that the GFAP gene promoter activity is a target of activated NF-κB and we previously showed and validated its sensitivity to WA treatments (Swarup et al., 2011). The *in vivo* effect of WA treatment on astrogliosis was assessed by bioluminescence imaging of luciferase activity driven by the GFAP promoter in live GFAP-luc;SOD1^{G93A} mice. We injected GFAP-luc;SOD1^{G93A} double transgenic mice with 4 mg/kg body weight of WA twice a week , starting at P40, till end stage. The analysis of signal emitted from the spinal cord revealed marked decrease in the luciferase signal in WA-treated GFAP-luc;SOD1^{G93A} mice at 8 to 10 weeks when compared to non-treated controls (p<0.05) (Fig. 3.4A ,B). Another significant decrease in luciferase signal was observed at 17 and 18 weeks of age (p<0.05) in WA-treated mice (Fig. 3.4B). In line with the obtained *in vivo* imaging results, the immunofluorescence analysis of the GFAP staining in spinal cord sections (ventral horn area) from WA-treated SOD1^{G93A} mice at 17 weeks revealed significant reduction of the signal when compared to vehicle-treated SOD1^{G93A} age-matched littermates. (p<0.05) (Fig. 3.4C, D). In addition, fluorescence analysis of the Iba-1 immunoreactivity revealed a significant reduction in spinal cord sections from treated mice as compared to control group thus suggesting decrease in microglial activation (Fig. 3.4E, F) (p<0.05). This was further confirmed by Western blot analysis. As shown in figure 3.4G, WA treatment resulted in decreased levels of Iba1 and TLR2 expression (Fig. 3.4G). Taken together our data suggest that WA exerted marked anti-inflammatory effects in SOD1-mutant model resulting in decreased astrogliosis and microgliosis.

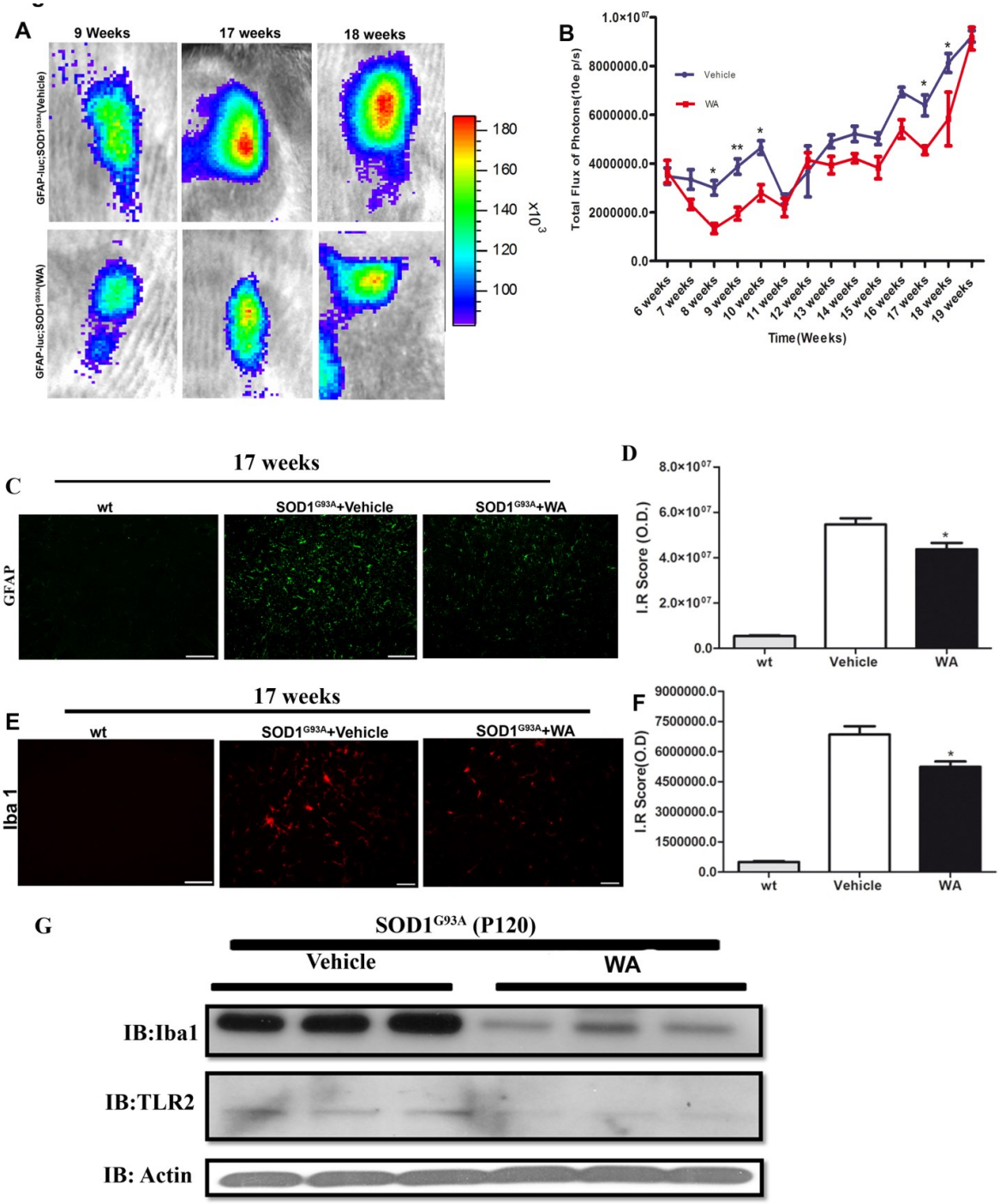

Figure 3.4 Bioluminescence imaging of astrocyte activation in the spinal cord of GFAP-luc;SOD1^{G93A} mice.

Figure 3.4 Bioluminescence imaging of astrocyte activation in the spinal cord of GFAP-luc;SOD1^{G93A} mice. (A) Typical sequence of images of spinal cord area obtained from GFAP-luc;SOD1^{G93A} mice at different time points (9, 17 and 18 weeks). **(B)** Quantitative analysis of the total GFAP-signal/bioluminescence (total flux of photon/s) in GFAP-luc;SOD1^{G93A} control mice (blue, N=8), GFAP-luc;SOD1^{G93A} treated (red, N=8) at P 40 revealed that early treatment with WA reduced GFAP-signal at 8, 9 and 10 weeks. A second reduction in GFAP signal after treatment was observed at later stage at 17 and 18 weeks of age. Two way ANOVA revealed a statistically significant reduction in the GFAP signal between treated and untreated group at (p<0.05 at 8,10 and 17, 18 weeks, p<0.05 at 9 weeks). Error bar represents mean ± SEM. **(C)** Photomicrograph of GFAP immunostaining in ventral horn of the spinal cord from wt, Vehicle and WA treated SOD1^{G93A} mice at 120 days (N=3 for all groups). **(D)** Graph represents quantitative analysis of GFAP labeling by measure of optical density (p=0.0491, N=3). **(E)** Photomicrograph of Iba1 staining in ventral horn of spinal cord from wt, Vehicle and WA treated SOD1^{G93A} mice at 120 days. **(F)** Graph represents quantitative analysis of Iba1 labeling by measure of optical density (p=0.034, N=3). **(G)** Lumber spinal cord lysate from Vehicle treated SOD1^{G93A} and WA treated SOD1^{G93A} mice at P120 were subjected to immunoblotting against Iba1 and TLR2 (N=3). Actin was used as an internal control. (*****= $p \leq 0.05$; ******= $p \leq 0.01$ by t test.)

3.5.5 WA alters cytokine profiles in spinal cord

There is evidence of alterations in expression levels of pro-inflammatory factors such as IFNγ, TNFα, IL-1β and GM-CSF in ALS patients and in mouse models of ALS (Sekizawa et al., 1998; Poloni et al., 2000; Elliott, 2001; Nguyen et al., 2001; Hensley et al., 2003). Previous findings from different experimental paradigms suggest that treatment with WA decreases levels of phospho-p65 and thus attenuates NF-κB dependent production of proinflammatory cytokines (Oh and Kwon, 2009; Swarup et al., 2011; SoRelle et al., 2013). To examine effects of WA treatment on expression profiles of pro-inflammatory cytokines, we used a standard array of mouse cytokine antibodies to measure over 40 different cytokines from spinal cord extracts of WA-treated and vehicle-treated $SOD1^{G93A}$ mice at P120 (Lalancette-Hebert et al., 2007). To our surprise, quantitative analysis revealed no significant changes in the levels of pro-inflammatory cytokines IL-1β and TNFα (Fig. 3.5A, B). WA-treated group exhibited significant increase in the levels of IL-6 (0.0064 ± 0.0008; N=3) when compared to levels of IL-6 (0.0020± 0.0002; N=3) in the vehicle-treated controls (Fig. 3.5C). Interestingly however, we observed significant increase in the levels of key anti-inflammatory cytokine IL-10 (0.0089 ± 0.0000016; N=3) when compared to levels of IL-10 in the vehicle treated controls (0.0056± 0.0003; N=3) (Fig. 3.5D).There was no change in the level of IL-4 and MCP-1 (Fig. 3.5E-F, I). No major changes were observed in colony stimulated factors such as G-CSF and M-CSF, while there was a decreased level of GM-CSF in the WA-treated mice (0.0157 ± 0.0003; N=3) when compared to vehicle-treated mice (0.0198 ± 0.0008; N=3) (Fig. 3.5H). Given that WA treatment did not produce marked changes in pro-inflammatory cytokine profile, taken together, our results suggest that the observed anti-inflammatory effects of WA are rather due to an increase in the levels of anti-inflammatory cytokine IL-10.

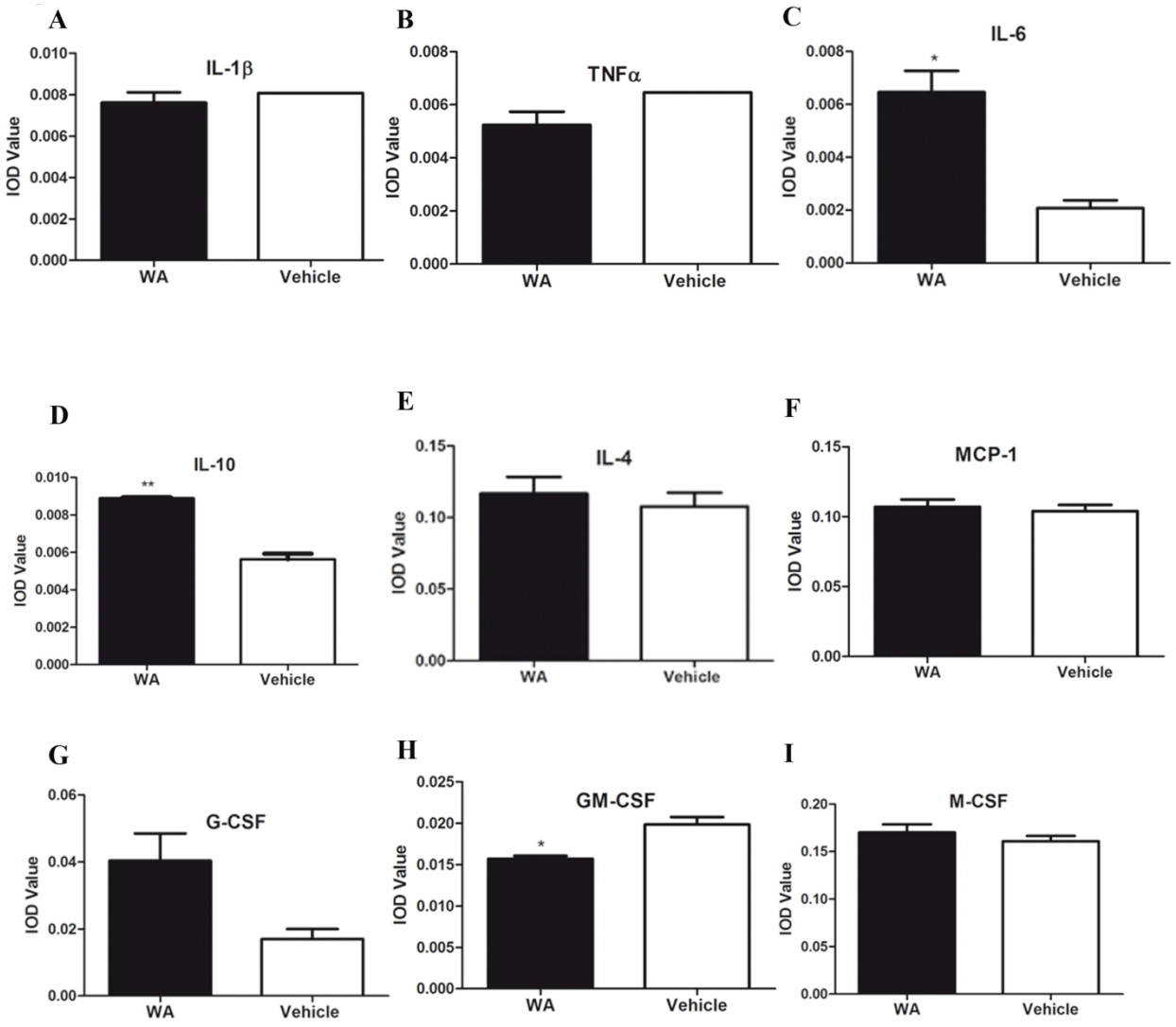

Figure 3.5 Expression analysis of cytokines as analyzed with cytokine array.

Figure 3.5 Expression analysis of cytokines as analyzed with cytokine array (A) IL-1β (WA, 0.0076 ± 0.0005; Vehicle, 0.0080 ± 0.0000000009; N=3; *p=0.447*). **(B)** TNF-α (WA, 0.005 ± 0.0005 N=3; Vehicle ,0.006 ± 0.000000002; N=3; *p=0.134*). Both IL-1β and TNF-α, on protein levels revealed no significant difference between the treated and nontreated group. **(C)** IL-6 (WA, 0.006 ± 0.0008; Vehicle, 0.002 ± 0.0002; N=3; *p=0.036*). **(D)** IL-10 (WA, 0.0089 ± 0.0000017; Vehicle, 0.0056 ± 0.00029; N=3; *p=0.0082*) showed a significant increase in the WA treated group. **(E)** IL-4 (WA, 0.12 ± 0.01; Vehicle, 0.1 ± 0.009; N=3; *p=0.607*). **(F)** MCP-1(WA,0.1070 ± 0.005 ;Vehicle 0.1040 ± 0.004; *p*=0.7). **(G)** G-CSF (WA, 0.04 ± 0.008; Vehicle, 0.017 ± 0.003; N=3; *p*=0.113). **(H)** GM-CSF (WA, 0.01 ± 0.0003; Vehicle, 0.019 ± 0.00089; N=3; *p=0.049*). **(I)** M-CSF (WA, 0.1700 ± 0.008481; Vehicle, 0.1608 ± 0.005628; N=3; *p=0.461*). *****, $p \leq 0.05$; ******, $p \leq 0.01$ by *t* test.

3.5.6 No effect on proliferation and polarization of peripheral immune cell population

Evidence suggests that at periphery WA may affect ratio and polarization properties of peripheral myeloid cells including macrophages and T-cells. One of the particular concerns was the potential effects of WA on the Tregs subpopulation (Sinha and Ostrand-Rosenberg, 2013). Namely, it has been well documented that there is an alteration in population of T-lymphocytes (specifically Tregs) in the blood of ALS patients as well as in ALS mouse models (Shi et al., 2007; Mantovani et al., 2009; Lincecum et al., 2010; Seksenyan et al., 2010; Zhao et al., 2012). Tregs are critically involved in suppressing inflammation induced by neurotoxic T-lymphocytes and microglia/macrophages and they play a prominent role in slowing the rate of disease progression in ALS mice (Banerjee et al., 2008; Beers et al., 2008; Chiu et al., 2008; Beers et al., 2011). Therefore we analyzed the number of Tregs in the blood by FACS in the WA-treated and non-treated $SOD1^{G93A}$ mice at two time points, P112 and P125. As Tregs can express anti-inflammatory cytokines, we also measured the levels of IL-4 and IL-10. The Treg transcription factor FoxP3 is currently the most reliable marker for identifying Tregs. Therefore, $CD4^+$ $CD25^+FoxP3^+$ Tregs from the WA-treated and control $SOD1^{G93A}$ mice were quantified. As shown in figure 3.6 (A, B and C) there was no significant difference in the number of Treg cells from the groups of animals at 112 or 125 days. Likewise, there was no difference in the levels of IL-10 (Fig. 3.6D-F) and IL-4 (Fig. 3.6G-I) between WA treated and control group. In addition, we also investigated for possible shift in $CD4,^+$ $CD8^+$ as well as $CD11b^+$ population. The quantitative FACS analysis revealed no changes in $CD4^+$ and $CD8^+$ T lymphocyte population at any mentioned time point (Fig. 3.6J, K), while at initial time point at 112 days we observed slight and transient increase in $CD11b^+$ population. However, the observed changes did not reach statistical significance (Fig. 3.6L).

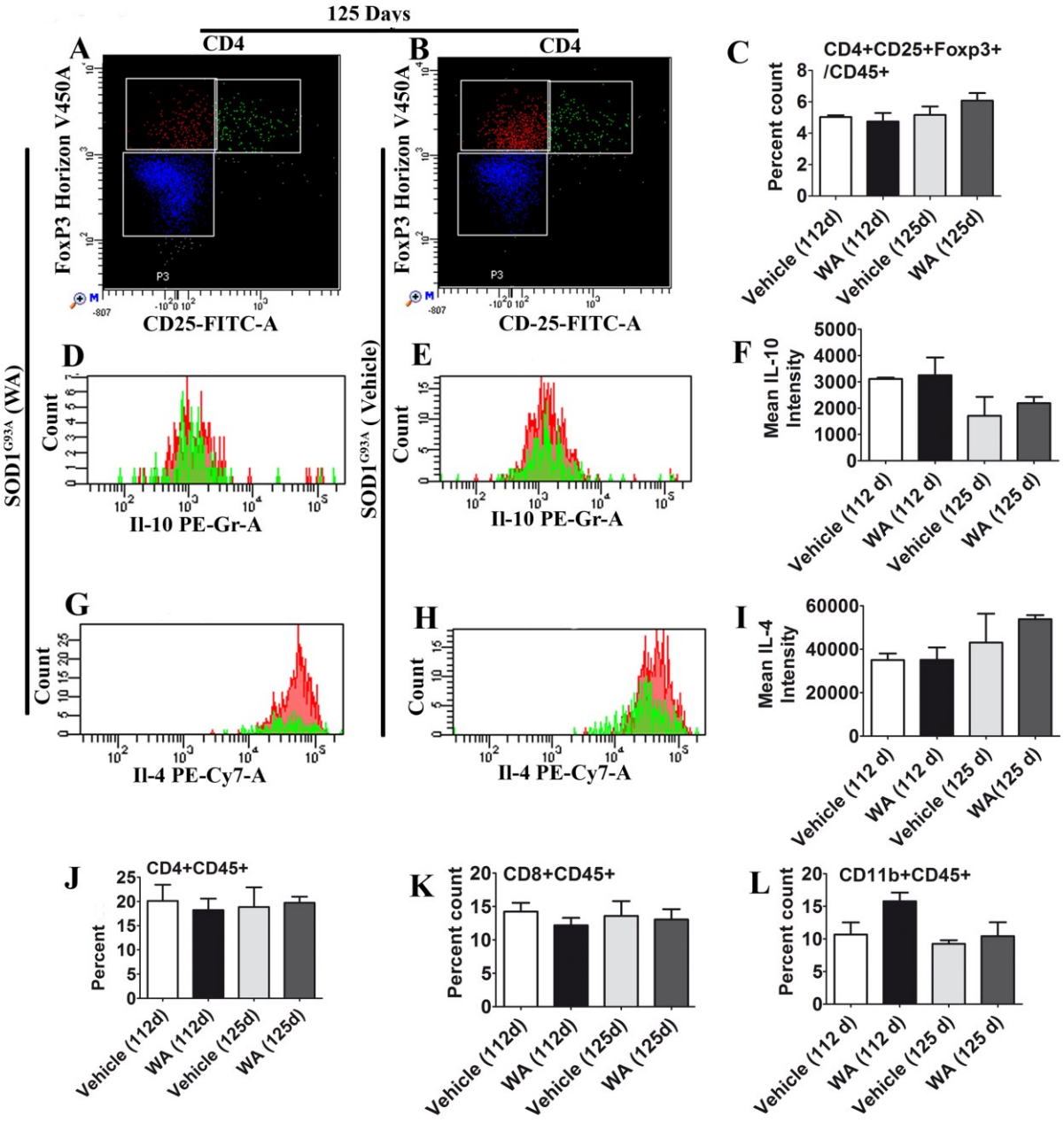

Figure 3.6 No change in proliferation and polarization of peripheral immune cells in SOD1^{G93A} mice after WA treatment.

Figure 3.6 No change in proliferation and polarization of peripheral immune cells in SOD1^{G93A} mice after WA treatment.

Topographic representation of CD4+CD25+Foxp3+ Treg cells over CD45+ population, of WA treated **(A)** and control group **(B)**, as analyzed by using flow cytometry. **(C)** Flow cytometry analysis showed no difference in Treg population between the treated and control group at 112 days or 125 days. Topographic representation of mean IL-10 **(D, E)** and IL-4 **(G, H)** intensity in Tregs of WA treated and control SOD1^{G93A} mice. Analysis of IL-10 **(F)** and IL-4 **(I)** mean intensity in Treg cells in WA treated and control group showed no difference at any mentioned time point. No change in $CD4^+$ and $CD8^+$ T lymphocyte population at any mentioned time point **(J, K)**. FACS analysis showed tendency for higher $CD11b^+$ population in WA treated group at 112 days (WA, 15.8 ± 1.3; Vehicle 10.69 ± 1.8; p=0.08) but not at 125 days (WA, 10.42 ± 2.1; Vehicle, 9.2 ± 0.5; p=0.73) **(L)**.

3.5.7 No beneficial outcome with late initiation of WA treatment

We next investigated whether initiation of WA treatment at later time points would also provide neuroprotection and extend survival in SOD1-mutant mice. We have carried out the same injection protocol of WA in $SOD1^{G93A}$ mice with initiation of treatment at 90 days of age. Our results revealed no significant difference in the mean survival between the vehicle-treated (150 days; N=12) and WA-treated group of $SOD1^{G93A}$ mice (148 days; N=12; p=0.97) (Fig. 3.7A). Because neuroprotection with early treatment with WA correlated with an increase in Hsp-25 and Hsp-70 levels, we next asked whether same protective mechanisms are induced in our post-onset WA treatment protocol. Importantly, we did not observe significant changes and marked up-regulation in the levels of Hsp-25 or Hsp-70 in the spinal cord lysates if WA treatment is initiated after onset (Fig. 3.7B, C). Next, as shown in figure 3.7D, the poor Hsps response was clearly associated with the lack of activation of transcription factor Hsf-1, as demonstrated by evident lack of gel shift/Hsf-1phosphorylation in spinal cord samples obtained from mice treated with WA late in disease.

Finally, to assess the effects of late WA treatment on the cytokines profiles, we evaluated the levels of different pro and anti-inflammatory cytokines in the spinal cord of WA treated and vehicle treated mice. The cytokines were analyzed at end-stage of disease, at P120, while the treatment was initiated at P90. Our results confirmed an altered profile of cytokines but very surprisingly we observed changes in levels of both anti and pro inflammatory group of cytokines. Quantitative analysis revealed major changes in levels of pro-inflammatory cytokines such as IL-1β, TNF-α and IL-6, between the two experimental groups (Fig. 3.7E-G). Remarkably, quantitative analysis showed significant increase in levels of anti-inflammatory cytokines in WA treated group (Fig. 3.7H-J). In addition, levels of M-CSF was also significantly increased in WA treatment group with no significant change in levels of GM-CSF and G-CSF (Fig. 3.7K-M)

We next analysed the effect of late WA treatment on T-lymphocytes population in blood of $SOD1^{G93A}$ by flow cytometry. There was no significant difference in Tregs population, IL-10 and IL-4 levels in blood between WA treated and control group of animals (Fig. 3.7N-P) and quantitative analysis revealed no significant changes in $CD4^+$, $CD8^+$ and $CD11b^+$ populations at the periphery (Fig. 3.7Q-S). Taken together, our results suggest that when administered after disease onset WA lost its ability to significantly induce Hsp-25 and Hsp-70. Surprisingly, a post-onset initiation of WA treatment increases both, anti- and pro-inflammatory cytokine levels in the spinal cord tissue without significant impact on the peripheral immune cells/immune response.

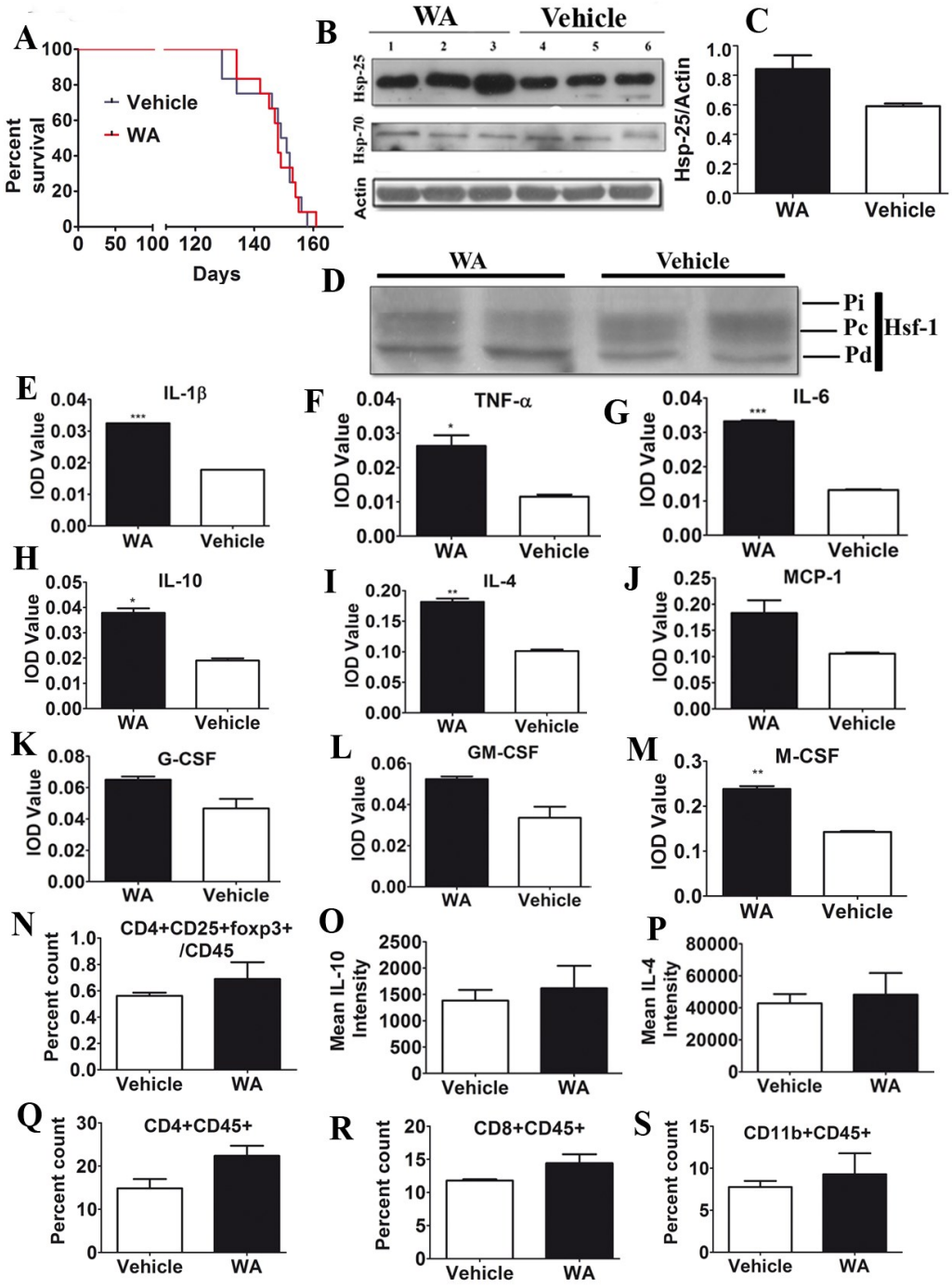

Figure 3.7 WA treatment at late stage of disease did not affect the course of disease and/or survival of SOD1^{G93A} mice.

Figure 3.7 WA treatment at late stage of disease did not affect the course of disease and/or survival of SOD1^{G93A} mice.

(A) The graph represents probability of survival of untreated SOD1^{G93A} mice (N=12) and WA treated with onset at 90 days (13 weeks; N=12) Median survivals were not significantly different (150 vs 148 days respectively p=0.9791). **(B)** The protein levels of Hsp-25 in the spinal cord lysates subjected to SDS-PAGE and immunoblotting were compared in the WA and vehicle injected SOD1^{G93A} mice on P120. Representative immunoblots for Hsp-25 and Hsp-70 and Actin as loading control are shown. **(C)** Quantitative densitometric analysis of western blots did not show a significant upregulation in the level of either Hsp-25 or Hsp-70 in the WA treated mice ($p>0.05$). **(D)** Representative western blot of Hsf-1 expression in spinal cord tissue from WA-treated SOD1^{G93A} mice and untreated SOD1^{G93A} mice. Pi, induced phosphorylated Hsf-1; Pc, constitutively phosphorylated Hsf-1; Pd, dephosphorylated Hsf-1

(E-M) Expression analysis of different cytokines on protein levels between WA and vehicle treated SOD1^{G93A} mice spinal cord Il-1β **(E)** , TNF-α **(F)** and IL-6 **(G)** levels were significantly increased after WA treatment (IL-1β: WA, 0.03 ± 0.00000015, Vehicle, 0.017 ± 0.00000003 ; TNF-α: WA,0.026 ± 0.003,Vehicle, 0.011 ± 0.0006; IL-6 : WA,0.033 ± 0.0002 Vehicle, 0.013 ± 0.0002). Levels of IL-10 **(H)** and IL-4 **(I)** were also increased in WA treated animals (IL-10: WA, 0.037 ± 0.0018, Vehicle 0.019 ± 0.0008; IL-4: WA, 0.18 ± 0.005, Vehicle 0.1 ± 0.0026) There was no change in levels of MCP-1 **(J)**. Levels of G-CSF **(K)** and GM-CSF **(L)** were not significantly changed while levels of M-CSF **(M)** were significantly increased in WA treated group (WA, 0.23 ± 0.006, Vehicle 0.14 ± 0.0017). FACS analysis showed no change in Treg population **(N)** and mean intensity of IL-10 **(O)**, IL-4 **(P)** in blood lymphocytic population between WA and Vehicle treated groups. Analysis of CD4+ **(Q),** CD8+ **(R)** lymphocytes and CD11b+ **(S)** population in blood by FACS showed no significant change.

3.6 Discussion

A previous study revealed beneficial effects of WA involving a reduction of inflammation and amelioration of motor deficits in a mouse model of ALS based on over expression of human TDP-43 transgene (Swarup et al., 2011). Here, we report that WA treatment conferred neuroprotective effects with extension of life span in two mouse models of ALS with over expression of different mutant SOD1, $SOD1^{G93A}$ or $SOD1^{G37R}$ mutants (Fig. 3.1). WA was effective only when treatment was initiated early in disease pathogenesis, at time of onset of initial motor function deficits (Vinsant et al., 2013b, a). Our analyses of $SOD1^{G93A}$ mice suggests that WA may exert protective effects through multiple pathways.

First, it is well established that WA exerts potent anti-inflammatory effects (Kaileh et al., 2007; Sabina et al., 2008; Min et al., 2011) and our results confirmed that WA can reduce neuroinflammation in $SOD1^{G93A}$ mice when treatment is initiated at early stage of disease. For instance, we took advantage of double transgenic GFAP-luc/$SOD1^{G93A}$ mice in which astrocyte activation can be visualized throughout disease progression (Keller et al., 2009). Our results of *in vivo* imaging revealed an attenuation of astrogliosis by WA treatment at 8 to 10 weeks of age and then at 17 and 18 weeks of age in $SOD1^{G93A}$ mice (Fig. 3.4A, B). Immunofluorescence microscopy and immunoblotting further confirmed a decrease of GFAP and of Iba1 signals in 17 week old $SOD1^{G93A}$ mice treated with WA (Fig. 3.4C, D). As activated astrocytes and microglia can produce a variety of cytokines with some having harmful effects (Elliott, 2001), we have further determined the effect of WA treatment on cytokine expression pattern in the spinal cord of $SOD1^{G93A}$ mice . Interestingly, WA treatment resulted in a significant increase in IL-10 levels (Fig. 3.5D) in lumbar spinal cord of $SOD1^{G93A}$ mice at 120 days of age. IL-10 is known to confer beneficial effects in several neuroinflammatory disease models including experimental autoimmune encephalomyelitis (EAE), traumatic or excitotoxic spinal cord injuries, stroke and Parkinson's disease (Bethea et al., 1999; Brewer et al., 1999; Cua et al., 2001; Frenkel et al., 2005; Qian et al., 2006). Conversely, WA caused a down regulation in levels of GM-CSF in spinal cord of $SOD1^{G93A}$ mice (Fig. 5H). GM-CSF is a pro-inflammatory cytokine which is up-regulated in various neurological disorders like AD, vascular dementia,

multiple sclerosis (Tarkowski et al., 2001; Mellergard et al., 2010; Parajuli et al., 2012). Thus, the reduction of inflammation by WA treatment in SOD1^{G93A} mice may be in part due to an upregulation of anti-inflammatory cytokine IL-10 and by a down regulation of pro-inflammatory cytokine GM-CSF. Moreover, we carried out FACS analysis of the blood to examine the effect of WA treatment on lymphocyte population, specifically regulatory T lymphocytes (Tregs). A previous study on SOD1^{G93A} mice revealed that the numbers of Treg cells are increased at early slowly progressing stages, augmenting IL-4 expression, and are then decreased when the disease rapidly accelerates possibly through the loss of FoxP3 expression (Beers et al., 2011). In ALS patients, the numbers of Tregs and expression levels for FOX-3 and IL-4 were inversely correlated with disease progression rates (Henkel et al., 2013). However, our FACS analysis revealed no effect of WA on the number of Tregs in the blood or in their levels of IL-10 or IL-4 cytokines (Fig. 3.6F-I). Taken together, our data provided no evidence of protective inflammatory responses through a modulation of peripheral Tregs.

The accumulation of misfolded SOD1 species is a valuable indicator of disease progression (Saxena et al., 2013). To date, a reduction in levels of misfolded SOD1 species by different therapeutic approaches including the treatment with monoclonal antibodies has been proven effective in delaying disease onset and progression (Gros-Louis et al., 2010; Patel et al., 2013). An upregulation of heat shock proteins constitutes another mechanism by which WA may confer neuroprotection in SOD1^{G93A} mice. As shown in Fig 3.3 (C- D), WA treatment significantly increased the amount of Hsp-25 (a mouse orthlogue of Hsp-27) in the spinal cord of SOD1^{G93A} mice, which is line with a report that WA is an inducer of heat shock proteins. Many reports have shown that Hsp-27/25 protects against neuronal damage induced by FALS-related SOD1 mutant (Benn et al., 2002; Patel et al., 2005; Sharp et al., 2006; An et al., 2008; Sharp et al., 2008; Yerbury et al., 2013). Moreover, Hsp-27/25 was found to inhibit the *in vitro* aggregation of SOD1^{G93A} proteins (Yerbury et al., 2013). Thus, an up-regulation of Hsp-25 in WA-treated SOD1^{G93A} mice may explain in part the reduction in levels of misfolded SOD1 species as determined by immunoprecipitation with

the specific B8H10 antibody (Fig. 3.3A, B) and increased number of surviving motor neurons (Fig. 3.3F, G).

The combined results revealed an effective therapeutic effect of WA when treatment was initiated at onset of motor deficits in SOD1^{G93A} mice, which has recently been reassessed to be at 30 to 40 days of age according to leaded grid test and treadmill gait analysis (Vinsant et al., 2013b, a) . However, when WA treatment was initiated at later stage of disease (90 days of age), at time coincident with detection of motor neuron death (Vinsant et al., 2013b, a), there was no beneficial effect on survival of SOD1^{G93A} mice (Fig. 3.7A). As shown in figure 3.7B, when administered after disease onset WA lost its ability to up-regulate Hsp-25 and Hsp-70. Interestingly, previous work by Maatkamp and colleagues revealed that in the SOD1^{G93A} mutant mice decrease in Hsp25 protein expression precedes degeneration of large motor neurons (Maatkamp et al., 2004) . Altogether these data suggest that a therapeutic intervention for ALS based on WA medication (and possibly some other therapeutic approaches) would need to be initiated early in the pathogenic process while cellular responses to stress and injury such as Hsps induction, as well as adequate inflammatory responses are still functional. For example, a late-onset initiation of WA administration in SOD1^{G93A} mice caused an increase in both anti and pro inflammatory cytokines (Fig. 3.7E-M) suggesting a marked deregulation of immune system responses in late stage disease. If started at early disease stage, WA should be effective in attenuating deleterious neuroinflammatory responses and in conferring neuroprotection through an up-regulation of Hsp-25 and reduction of misfolded protein species. However, no evidence of therapeutic value was observed when WA treatment was initiated at advanced stage of ALS disease in SOD1^{G93A} mice. WA is a steroid lactone present in a medicinal plant *Withania somnifera*, which has been used for centuries in Ayurvedic medicine. The therapeutic effects of WA in various ALS mouse models suggest that WA should be considered as a promising lead compound for drug development aiming to treat ALS.

3.7 Acknowledgement

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Chapter 4: General discussion and conclusion

4.1 Treatment strategies for multifactorial disease such as ALS

Although a lot of research has been carried out to understand the pathophysiology of ALS, still clarity in terms of viable drug targets is elusive. In fact, the situation is more complicated because many mechanisms are involved in the pathogenesis of ALS (Eisen, 1995).

However, knowledge about dominant pathological mechanisms underlying the biology of ALS and delivery methods for drugs in the nervous system has resulted in a number of potential therapeutic strategies. Based on their target, these strategies can be subdivided into two main categories. The first strategy aims to directly target disease-causing genes or proteins, in order to avoid their expression and hence the pathogenic effects. The second strategy that has been widely approached, aims at one target factor or mechanism that influence pathological processes related to ALS. In the following section, I will discuss about the treatment strategies that has been developed during the research work presented in this thesis. Discussion will also focus on comparative analysis of present study to previous studies involving silencing of SOD1 gene for therapeutic purposes. Furthermore, I will also briefly discuss about another part of my research work which aims to target neuroinflammation via NFκB inhibition as a potential treatment option for ALS.

4.2 Treatments based on targeting the causal gene/protein

Around 20% of patients with familial ALS have a mutation in the gene encoding SOD1 (Rosen et al., 1993) that results in protein misfolding and an apparent gain in toxic function (Bruijn et al., 1998). The fact that mutant SOD1 causes disease by a toxic gain of function (Bruijn et al., 2004; Boillee et al., 2006a; Rothstein, 2009) suggested that lowering levels of mutant SOD1 could benefit patients with SOD1-linked ALS.

Two different strategies have been successfully utilized to obtain this goal. First, expression of genes can be stopped by using gene silencing methods. Silencing of the SOD1 gene by using antisense oligonucleotides (ASOs) or small interfering RNAs (siRNAs) have been effective in the SOD1 mouse model (Ralph et al., 2005; Raoul et al., 2005; Smith et al., 2006; Rizvanov et al., 2009; Towne et al., 2011). Second, mutant proteins can be cleared or their toxic epitopes can be neutralized by specifically designed antibodies. Passive and active immunization against misfolded SOD1 have shown therapeutic potential in mouse models (Urushitani et al., 2007; Gros-Louis et al., 2010; Liu et al., 2012).

Are these therapeutic strategies based on antisense method or the immunization method ideal for human ALS treatment? While the aforementioned therapeutic methods have their own advantages and disadvantages, it would be essential to critically evaluate each of them to judge their usefulness for their future testing in human ALS patients.

4.2.1 Silence the gene, save the cell: A therapeutic focus on SOD1

4.2.1.1 RNA interference

Strategies aimed at modulating gene expression are emerging as potential novel therapeutic options, particularly in light of significant advances in the understanding of the genetic causes of ALS (Gordon et al., 2013). Such strategies involve RNA interference (RNAi) and antisense therapy.

Recent studies using transgenic mice models of ALS with SOD1 mutations have indicated that SOD1 mutations induce the disease by their toxic properties, not by a loss of SOD1 activity (Cleveland, 1999). Therefore, lowering levels of mutant SOD1 is expected to provide a direct approach to therapy for SOD1 related familial ALS. The first report of

using siRNA for lowering SOD1 levels came from group of Dr. Azzouz et al (Ralph et al., 2005).This group generated a lentiviral vector to mediate expression of RNAi molecules specifically targeting the human SOD1 gene. Injection of this vector into various muscle groups of 7 days old $SOD1^{G93A}$ mice resulted in delayed disease onset and improved survival. Furthermore, it resulted in an efficient and specific reduction of SOD1 expression and improved survival of vulnerable motor neurons in the brainstem and spinal cord.
However, these strategies failed to slow disease progression after onset, despite treatment initiated in juvenile (7-day-old) mice. These findings may be explained by failure to suppress mutant action in the non-neuronal cells. Using the retrograde ability of viral particles for delivery by an intramuscular injection does not seem to be favourable approach because the effect would probably be limited to motor neurons. This might be a considerable drawback because expression of mutant SOD1 in both neuronal and non neuronal cells is thought to contribute to disease progression (Clement et al., 2003). In addition they used a siRNA that targets mutant as well as normal form of human SOD1. Nevertheless, reducing the normal physiological function of SOD1 may have potential deleterious effects.

The same year another study by Raoul et al, showed that intraspinal injection of a lentiviral vector (LV-shSOD1) that produces RNAi-mediated silencing of SOD1 substantially retards both the onset and the progression rate of the disease in $SOD1^{G93A}$ mice when injected at 40 days (Raoul et al., 2005). LV-shSOD1 also improved motor neuron survival by approximately 60%. Intraspinal injection allowed precise targeting of motor regions leading to motor neuron as well as glial cells transduction. Moreover the gene replacement technology was designed such that it allowed the knock down of all SOD1 forms while expressing a wild-type protein, which is refractory to RNAi-based silencing.

However, some of limitations of shRNA therapy is that in case of appearance of adverse effects, the silencing cannot be paused or stopped. Also, siRNAs in some cases can trigger

an immune response. Reports have shown that siRNA duplexes 23 nucleotides long can activate interferon responses and cause cell death in culture (Jackson et al., 2006).
Another drawback is the off-target reactions. Due to a great abundance of RNA sequences that may be partially complementary to engineered siRNAs, RNAi could provoke nonspecific gene silencing. i.e., suppression of genes other than the desired gene targets.

4.2.1.2 Antisense oligonucleotides

Another method of gene silencing is achieved by using antisense oligonucleotides. Antisense oligonucleotides (AOs) are short, synthetic nucleic acid sequences that selectively hybridize to target sequences in messenger RNA (mRNA). The mRNA in such a heteroduplex is a substrate for catalytic, intranuclear degradation by endogenous RNase H (Kuzmiak and Maquat, 2006).
Compared with siRNA, the oligonucleotide turnover is lower, allowing an important reduction of the administrated dose. Furthermore, since they can be administered to the CSF by use of osmotic pump, in case of any unforeseen side-effects, the rate of infusion can easily be adjusted or halted.

Antisense oligonucleotides targeting the SOD1 gene were first used by Smith et al in 2006 to down regulate the SOD1 protein in nervous system (Smith et al., 2006). Oligonucleotides were continuously administered to the lateral ventricle of a healthy rat and monkey. The main objective behind choosing ventricle as a site of administration was to ensure effective delivery to brain and spinal cord tissues. Cerebral ventricles are cavities within the brain that contain cerebrospinal fluid (CSF) that is produced by the choroid plexus (Rando and Fishman, 1992). Once produced, CSF circulates from the ventricles to all regions of the CNS. A 28 day-treatment resulted in a significant decrease in SOD1 mRNA expression and a 50% reduction in SOD1 protein concentration in the frontal cortex, cervical and lumbar spinal cord. The administration of antisense oligonucleotides to

presymptomatic (65 days) transgenic SOD1^{G93A} rats delayed the disease progression and extended survival by 37%. It did not however affect the disease onset (Smith et al., 2006).

However, the limitations of antisense drugs are defined by their pharmacokinetic and toxicological properties. Two main limitations are: the dose- dependent proinflammatory effects and the slow onset of action (24 - 48 h). Since antisense oligonucleotides do not cross an intact blood-brain barrier, nor do they enter skeletal muscle, their use in the treatment requires a direct administration to the CSF/brain parenchyma. Osmotic pumps can be used as a delivery system but long term use of these pumps in human cases does not seem to be very appropriate and feasible. Repeated intrathecal injections can also be tricky in case of human ALS patients. However recently intrathecal Baclofen therapy from Medtronic has been used in treatment of spasticity (Berman et al., 2014). This pump can be used to deliver the desired drug directly into cerebrospinal fluid within the spinal canal.

Recently, Isis Pharmaceuticals concluded a Phase 1 placebo-controlled, double-blind, dose-escalation, safety and tolerability clinical trial for their antisense drug ISIS-SOD1Rx. (http://www.clinicaltrials.gov/ct2/show/NCT01041222).

The oligo employed in this study, was modified antisense oligo targeted to the first exon (19th–38th bps) of SOD1 (regardless of mutation) and catalyzed RNase H1-mediated degradation (Miller et al., 2013). Participants were given 12h intrathecal infusions of ISIS oligos at varying concentrations, or placebo. No clinically significant adverse effects associated with oligo administration were reported.

4.2.1.3 Antibody mediated therapy

The idea that immunotherapy could be used to treat neurodegenerative diseases is a relatively new one. Why would one use a vaccine or an antibody to treat a non-infectious disease? The answer lies in the fact that in many neurodegenerative diseases, proteinopathy is a common toxic factor. This makes the rational very obvious- prevent the proteinopathy and the downstream cascade is prevented. In case of familial ALS, toxicity of mutant SOD1 forms is related to protein misfolding and aggregation. These aggregates in turn

induce neurodegeneration. This reinforces that therapeutic efforts could be designed to prevent the formation of these toxic aggregates.

Immunization is the process of providing specific protection against the most common and damaging pathogens or immunogenic agent in a disease. To orchestrate an immune response, the body first needs to be exposed to pathogens or foreign molecules. Antibodies are the primary defense against extracellular pathogens and they function by different mechanisms; first, neutralization through binding to the pathogen. Subsequently, antibodies can block the association of the pathogen with their targets. Secondly, they can stimulate removal of a pathogen by macrophages and other cells by coating the pathogen; and lastly they can trigger direct pathogen destruction by stimulating other immune responses such as the complement pathway that can result in lysis of certain bacteria and viruses.

Active immunization, engages the cellular and humoral immune system, including T cells and B cells, to promote the production of anti-antigen antibodies. Typically, an active immunogen is comprised of an antigen combined with an immune boosting adjuvant to ensure high antibody titers. Passive immunotherapy involves the direct injection of monoclonal antibodies (or fragments thereof) without requiring the immune system to generate an antibody response.

Both active and passive immunization strategies have shown to have therapeutic benefits in different mutant SOD1 transgenic mice models (Urushitani et al., 2007; Gros-Louis et al., 2010; Takeuchi et al., 2010; Liu et al., 2012).

The first reports of successfully using active immunization as a therapeutic strategy in ALS came from the group of Dr. Jean-Pierre Julien in 2007, where they used bacterially purified recombinant SOD1 protein as an immunogen (Urushitani et al., 2007). Previous studies have shown that a fraction of SOD1 can be translocated via the ER–Golgi network and that chromogranins, which are proteins abundant in motor neurons, interneurons and activated astrocytes, may act as chaperone-like proteins to promote secretion of misfolded SOD1 mutants (Turner et al., 2005; Urushitani et al., 2006; Urushitani et al., 2008). Moreover, extracellular mutant SOD1 can induce microgliosis and motor neuron death, suggesting a pathogenic mechanism based on toxicity of mutant SOD1 proteins (Urushitani et al., 2006). This evidence led to test immunization protocols aiming to reduce the burden of extracellular levels of mutant SOD1 proteins in nervous tissue of mice models of ALS. Recombinant metal-free human SOD1 mutant protein purified from bacteria was used to vaccinate $SOD1^{G37R}$ transgenic mice model which exhibits late onset of disease and harbors low level of mutant SOD1 proteins. The vaccination resulted in marked therapeutic effects as it delayed the disease onset and extended the life span of $SOD1^{G37R}$ mice by more than 4 weeks (Urushitani et al., 2007). The vaccination protocol attenuated the motor neuron loss. There was a reduction in levels of mutant SOD1 species in the spinal cord of vaccinated mice as detected with a monoclonal antibody specific for mutant SOD1 species. It is very reasonable that clearance of the extracellular SOD1 mutant occurred through microglial phagocytosis as anti-SOD1 antibodies were present in the spinal cord of vaccinated mice. Alternatively the internalization of antibodies in motor neurons might counteract some deleterious effects of mutant SOD1 or it could neutralize the formation of toxic mutant SOD1 oligomers.

The effectiveness of the immunization approach was tremendous considering that the final injection of mutant SOD1 in experimental protocol was administered in 6-month- old animals, several months before disease onset. However an active vaccination approach failed to confer significant protection in the $SOD1^{G93A}$ mice. This is not surprising, considering extremely higher levels of SOD1 mutant proteins expressed in these mice.

Lower translocation of anti- SOD1 antibodies across the blood brain barrier is not adequate to clear such an extreme amount of mutant SOD1 molecules in CNS. It could also be possible, however, that a combination of specific antigen and the host immune response can counteract the beneficial effects of vaccination. Thus it seems plausible that an immunotherapy approach would be suitable for low copy number mutant SOD1 mice, such as in the human ALS cases and also the choice of antigen can affect the desirable outcomes.

In 2010, the group of Dr. Makoto Urushitani compared the effects of G93A-apo SOD1 and WT-apo SOD1 vaccines on the survival and life span of low-copy $SOD1^{G93A}$ transgenic mice ($G93AGur^{dl}$) (Takeuchi et al., 2010). Their main objective was to develop a vaccine that is effective and not dependent upon the specific SOD1 mutation associated with the disorder. This is why, WT-apo SOD1 was chosen because the molecular behaviour of the non-metallated WT SOD1 protein is similar to the mutant SOD1 molecules (Furukawa et al., 2008).

Results showed that WT-apo SOD1 prolonged the life span of $SOD1^{G93A}$ mice. The main effect was delaying the disease onset rather than slowing its progression. The $SOD1^{G93A}$ vaccine also delayed the onset significantly and showed a trend towards prolonging the life span. SOD1 vaccination potentiated protective immunity in the spinal cord. Levels of IL-4 were markedly increased whereas levels of INFγ were decreased in vaccinated transgenic mice. WT-apo SOD1 vaccination induced a higher Th2/Th1 milieu as determined by the ratio of IgG (immunoglobulin G) isotypes. Th1 response produces proinflammatory cytokines and is critical in cell-mediated immunity, while Th2 response stimulates antibody production and plays an important role in humoral immunity. So, the results indicated that the Th1/Th2 milieu is affected by specific vaccinations and that antigenicity might counteract beneficial effects by enhancing Th1 immunity (Takeuchi et al., 2010).

Another report of using active immunization in familial ALS mice model came from Liu et al, where they showed the beneficial effects of an active immunization strategy using the SEDI (SOD1 exposed dimer interface) antigenic peptide (Rakhit et al., 2007) to target monomer/misfolded in $SOD1^{G37R}$ and $SOD1^{G93A}$ mutant SOD1 transgenic mice (Liu et al., 2012). Immunization delayed disease onset and extended disease duration, with increased survival times in $SOD1^{G37R}$ mice. The beneficial effects of immunization correlated with a reduction in accumulation of both monomer/misfolded and oligomeric SOD1 species in the spinal cord. SEDI peptide immunization also delayed disease onset by 12 days in the $SOD1^{G93A}$ transgenic mice.

However an active immunization does not seem to be an appropriate strategy to treat a disease like ALS because an active vaccine leads to a polyclonal antibody response, which means that it generates antibodies recognizing multiple, sometimes overlapping epitopes on the target protein. This may be helpful for broad coverage or, it may be less useful if the goal is to lower a specific form of a protein but not all forms. In case of ALS, it is important to decrease the load of only misfolded SOD1 protein and not the wild type protein. Secondly, an active vaccine also induces a T cell response that can increase the risk of a deleterious immune response (i.e., release of pro-inflammatory cytokines), especially, if the T cell recognizes the antigen as a self-protein. And, it takes time to "shut off" an active vaccine immune response. So it becomes important to make sure that a vaccine induces the protective immune response (Th2) and suppress the (Th1) response. For example, active immunization by beta amyloid peptide in Alzheimer's disease showed an immune response and cleared the amyloid plaque burden in transgenic mice (Schenk et al., 1999; Bard et al., 2000; Janus et al., 2000; Morgan et al., 2000). However this vaccination strategy when translated to Alzheimer's disease patients, 6% of subjects developed meningoencephalitis (Nicoll et al., 2003; Orgogozo et al., 2003; Gilman et al., 2005).

Therefore, considering the adverse immune responses associated with active-vaccination strategy, the use of passive immunization approach would probably be much safer.
Passive immunotherapy involves the direct injection of monoclonal antibodies (or fragments thereof) without requiring the immune system to generate an antibody response. Benefits of passive immunotherapy are that it can be stopped immediately if there are any adverse reactions and, that one can target specific epitopes or pathogenic conformations without disturbing the other forms of the protein of interest.

An ideal treatment for ALS would be to administer antibodies specific to pathogenic SOD1 forms without binding to the normal SOD1 form. In order to develop a passive immunization approach Gros Louis et al in 2010 generated a collection of monoclonal antibodies against SOD1 using standard hybridoma technology (Gros-Louis et al., 2010). Key feature of these antibodies was that they specifically recognized the misfolded form of mutant SOD1 (mSOD1) and not the intact wild type SOD1. Two monoclonal antibodies against mSOD1 namely, D3H5 and A5C3 was tested in a passive immunization approach involving intracerebroventricular (ICV) infusion in $SOD1^{G93A}$ mice. Whereas infusion of antibody D3H5, succeeded in delaying the onset and prolonging the lifespan of $SOD1^{G93A}$ mice, another monoclonal antibody A5C3 binding to a different SOD1 epitope failed to confer protection in these mice. Thus, not all anti-SOD1 antibodies might be suitable for immunotherapeutic approaches.

The exact mechanisms by which anti-SOD1 antibodies conferred protection were not totally understood. Results showed internalization of antibodies mainly within spinal motor neurons after ICV antibody infusion. This raised up the possibility of beneficial effects through neutralization of toxic SOD1 properties within motor neurons. Also it could be possible that microglial phagocytosis of IgG-SOD1 complex would have reduced the burden of toxic SOD1 molecules. The infusion of D3H5 antibody led to a reduction of about 23% in levels of mSOD1 species which was accompanied by reduced neuroinflammation in the spinal cord of treated transgenic $SOD1^{G93A}$ mice. Most

importantly extensions in life span of transgenic mice were directly correlated with the duration of treatment. This meant that a longer duration of treatment would be more effective in prolonging life span of these animals.

A chief finding of the study was that only the Fab fragment of D3H5 monoclonal antibody was sufficient to confer protection in $SOD1^{G93A}$ mice. Whereas the whole IgG molecule was more effective in prolonging survival time than the Fab fragment, suggesting that different mechanisms, such as Fc mediated microglial phagocytosis and neutralization of toxic epitopes, may work in concert to attenuate disease progression. However a less potent effect of Fab fragment in prolonging survival may also result from shorter half-life and increased clearance rate of antibody fragments. Yet the results showed that Fc fragment of D3H5 was partially dispensable for beneficial effects of antibodies and the antibodies conferred protection by neutralizing the toxicity of misfolded mutant SOD1 species. If the Fab fragment of an antibody is able to confer protection, this would raise the possibility of generating recombinant single-chain variable fragments (scFv) from the hybridomas. The use of a scFv fragment, which can neutralize the toxicity of mutant SOD1, should offer some advantages for immunotherapy owing to smaller molecular size, low immunogenicity and the possibility of achieving better nervous tissue penetration.

Single-chain fragment variable (scFv) -based immunotherapies have been developed to target abnormal proteins or various forms of protein aggregates including amyloid beta, alpha-synuclein, huntingtin, and PrP proteins (Lynch et al., 2008; Sakaguchi et al., 2009; Butler et al., 2012; Robert and Wark, 2012). Recently it has been shown that scFv generated against SOD1 if expressed as intrabodies within motor neuron cell line, interferes with mutant SOD1 in vitro aggregation and toxicity (Ghadge et al., 2013).

In chapter 2 we report the testing of a novel gene therapy approach for ALS based on AAV delivery of recombinant secretable scFv antibody specific to misfolded SOD1 species. This

secretable scFv antibody was derived from a hybridoma cell line expressing a monoclonal antibody called D3H5 which is specific to misfolded SOD1. Unlike the scFv developed by Ghadge et al, our scFv is specific to misfolded SOD1 and not to the WT SOD1. Our antibody contained a secretory signal, which means that the newly synthesized antibody will be secreted outside the cell and hence may effectively target the misfolded SOD1 species in the extracellular milieu. Furthermore, these antibodies could also be internalized by the neighbouring cells and thus could be effective in neutralizing the intracellular pool of misfolded SOD1. (Figure 4.1)

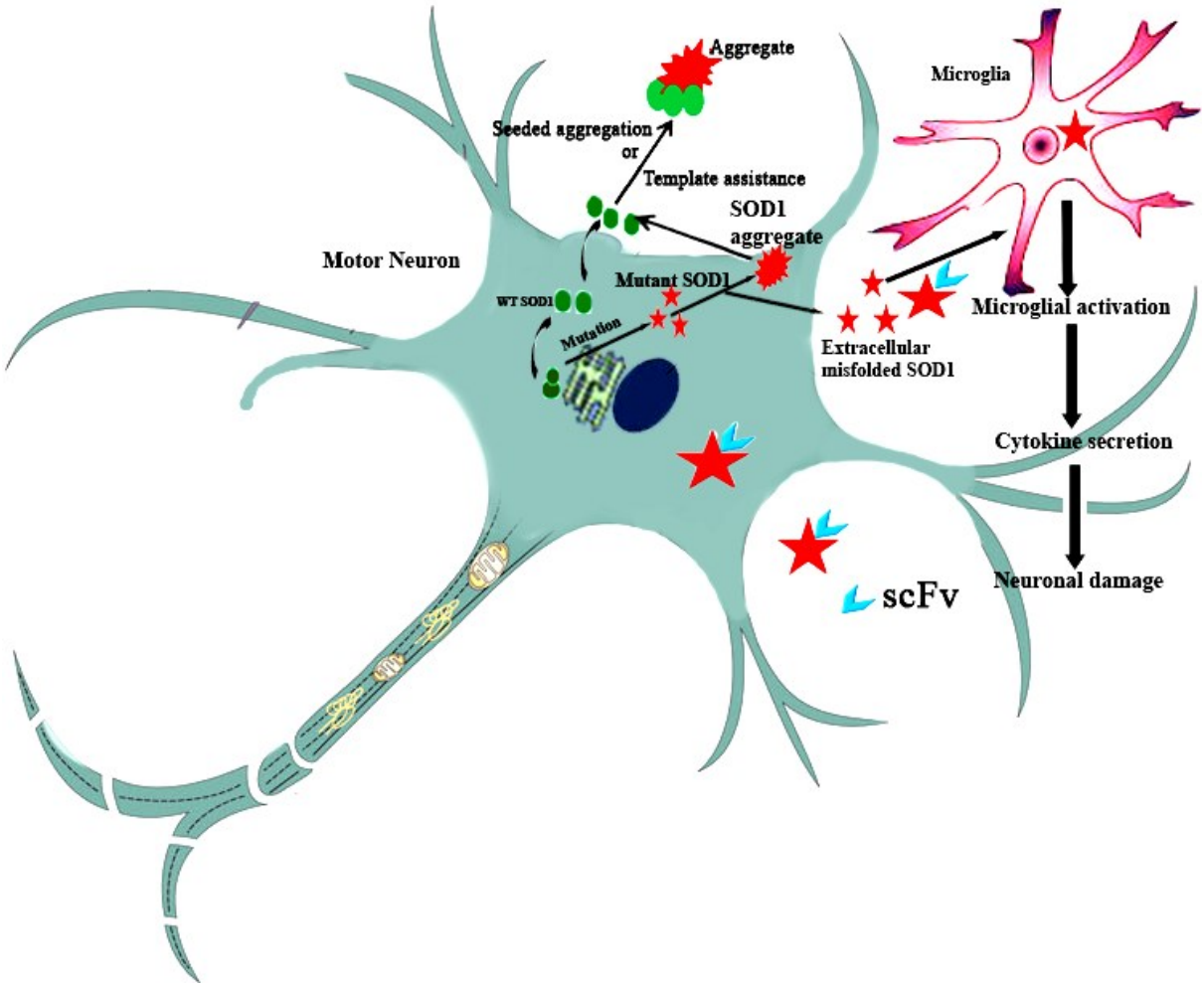

Figure 4.1 Toxicity of secreted misfolded SOD1 protein and mode of action of scFv

Wild type SOD1 protein is predominantly localized in cytoplasm but is also secreted outside the cell. Extracellular WT SOD1 increases the intracellular calcium levels and has protective effect on neurons. However, mutation in SOD1 can cause its misfolding.

Misfolded SOD1 is secreted by chaperon like protein, Chromogranin A and Chromogranin B. Extracellular mutant SOD1 can induce microgliosis and motor neuron death. In addition, a misfolded SOD1 protein may recruit and induce misfolding of a natively folded WT SOD1, by seeded polymerization or template assistance. For a therapeutic benefit, we can target the intra and extracellular pool of misfolded SOD1 by scFv. The scFv fragment can bind to the misfolded SOD1 and can neutralize it's toxicity or slow down propagation of aggregation.

In case of passive immunization, one needs repeated delivery of large amount of antibodies. This problem could easily be overcome by means of a viral vector based gene delivery system. AAV based gene delivery system offers a stable transgene expression in quiescent cells, has low pathogenicity and poor rate of integration in the host genome (Mandel and Burger, 2004; Daya and Berns, 2008). In addition when injected into the cerebrospinal fluid (CSF), AAV vectors were reported to confer widespread and sustained transgene expression in the central nervous system (CNS) (Snyder et al., 2010). Here we have shown that a single intrathecal injection of AAV-scFvD3H5 in $SOD1^{G93A}$ mice at 45 days of age yielded sustained expression of scFv molecules in the spinal cord starting after 5 weeks of injection to end stage of mice.

Intrathecal injection of AAV harbouring the scFv, seemed to be an efficient means of delivery because of reliably high titer delivery of virus directly to region of interest and potential ability to target delivery to more abundant motor neurons and neighbouring glial cells (Figure 4.2). The treatment extended survival by an average of 16 days and by up to 40 days in direct correlation with levels of antibody detected in tissues. This is a significant response in an aggressive mouse model like $SOD1^{G93A}$ which is known for its 20-fold SOD1 over expression. The data revealed that longevity of $SOD1^{G93A}$ mice correlated

directly with the scFv antibody titer. Immunotherapy approach via AAV delivery system extended survival of $SOD1^{G93A}$ mice by up to 40 days in direct correlation with levels of antibody detected in the tissues. This would rank among the best therapeutic interventions accomplished so far in this mouse model of ALS with high copy number of mutant SOD1 transgene. Bioimaging analysis of GAP-43/luc/gfp; $SOD1^{G93A}$ mice treated with AAV-scFvD3H5 revealed reduction of neuronal stress at presymptomatic stage. Motor function tests like rotarod and reflex score were also improved in treated animals. Such alleviation of motor neuron damage by scFvD3H5 antibody associated with delayed disease onset is consistent with previous studies on genetic ablation of mutant SOD1 within various cell types which concluded that toxicity of mutant SOD1 exerted within motor neurons constitute a primary determinant of disease onset (Boillee et al., 2006a).

The success of this therapeutic strategy lied in the fact that scFv antibodies targeted misfolded SOD1 species, the source of toxicity in the disease (Bruijn et al., 1997; Bruijn et al., 1998; Wang et al., 2009b). Immunoprecipitation experiments from spinal cord lysates of mice confirmed that treatment led to a reduction in amount of misfolded SOD1 species. Moreover, evidence suggest that aggregated forms of SOD1 can seed misfolding and aggregation of native wild type SOD1 protein (Chia et al., 2010; Grad et al., 2011; Munch et al., 2011) Thus, the beneficial effects of antibodies might come not only from clearance of pathogenic SOD1 molecules but also from neutralization of toxic epitope exposed by misfolded SOD1 or from interference in formation and propagation of misfolded SOD1 species. Our approach is more selective than an antisense oligonucleotide therapy because unlike the antisense oligonucleotide therapy which aims to reduce mRNA levels encoding both mutant SOD1 and WT SOD1 (Smith et al., 2006), the scFv only targets the misfolded SOD1 protein, the source of toxicity. Additional advantage is sustained expression of scFv antibodies without repeated injections and also the smaller size allows them to penetrate the tissues without any adverse immune system response. The scFv treatment succeeded in alleviating neuroinflammation which is another advantage because glial cells are considered to be active players in motor neuron dysfunction in ALS.

In conclusion these findings show than an AAV-based immunotherapy to target misfolded SOD1 could be considered for treatment of ALS patients bearing a SOD1 mutation. Additionally, such immunotherapy could also be applicable to subsets of sporadic ALS cases as recent studies suggest the existence of misfolded/aggregated forms of SOD1 in sporadic ALS cases with no SOD1 mutation (Bosco et al., 2010; Forsberg et al., 2010; Guareschi et al., 2012; Pokrishevsky et al., 2012).

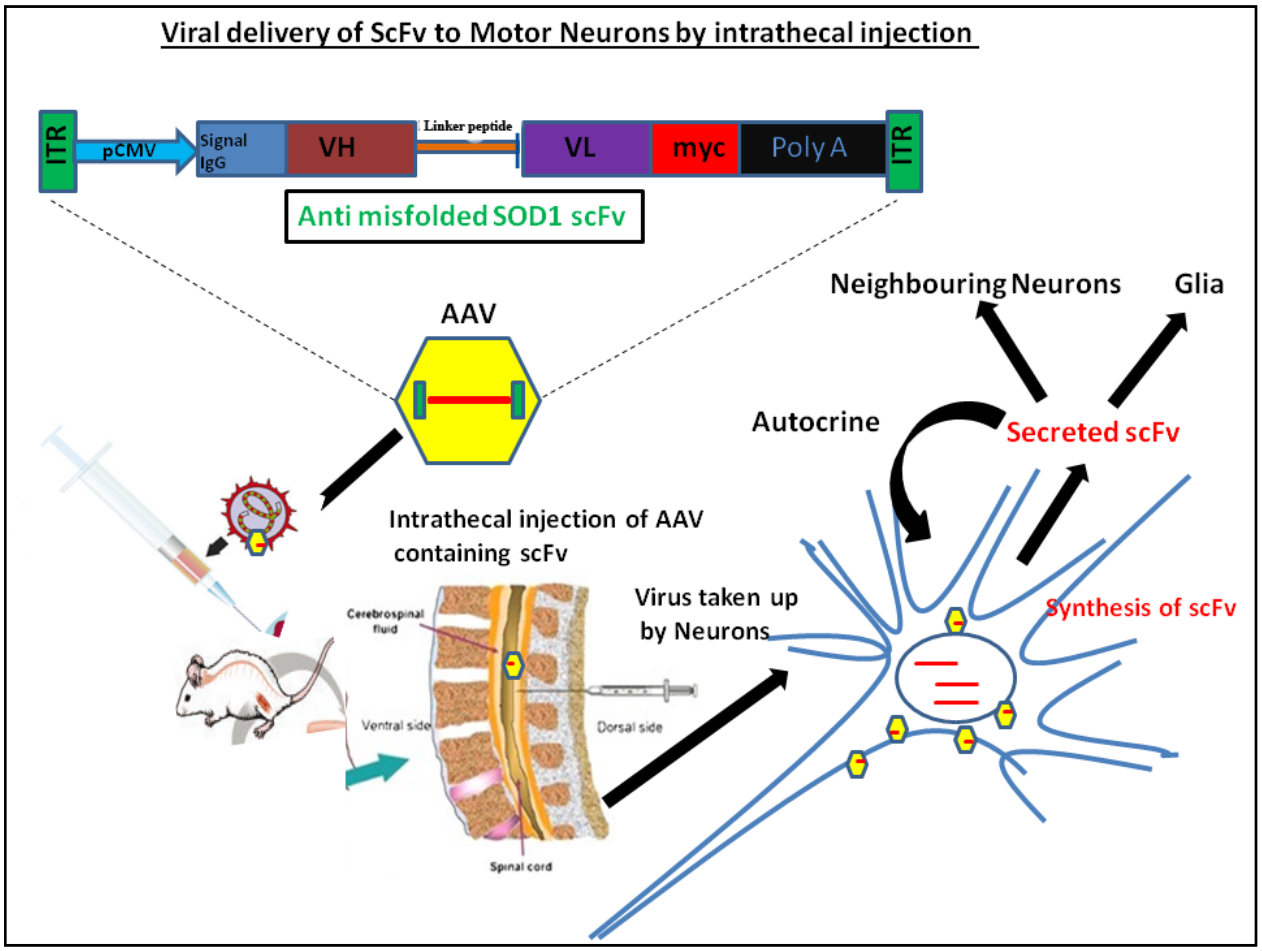

Figure 4.2 Viral delivery of scFv to motor neurons by intrathecal injection.

Injection of Adeno associated virus (AAV1/2) harbouring anti misfolded SOD1 scFv construct in intrathecal space of ($SOD1^{G93A}$) ALS mice model. Direct injection of viral particles into the spinal cord lead to transduction of motor neurons; where the scFv can be produced. The target cells for secreted scFv could be the motor neurons themselves, neighbouring neurons or glia.

4.3 Modulation of pathogenic pathways as possible treatment options

There are several pathological mechanisms such as oxidative damage, glutamate excitotoxicity, neuroinflammation, mitochondrial dysfunction etc that plays crucial role in disease process in ALS. The goal of a modifying treatment is to interfere with those pathological mechanisms. Modulating these different disease processes could have a positive effect on disease progression and survival.

4.3.1 Targeting neuroinflammation via NF-κB inhibition

Neuroinflammation is one of the pathogenic processes that are common in ALS patients with different etiology and hence can be targeted. The effect of such a modifying treatment might be smaller, but the target population will be larger as more ALS patients could benefit. Inhibition of inflammation by a variety of compounds has proven to be effective in mutant SOD1 transgenic mice.

Neuroinflammatory responses occur as a consequence of oxidative and excitotoxic neuronal damage and protein aggregation. Thus, it seems reasonable; drugs that modulate inflammation may combat disease progression. A recent study on TDP-43 mice model of ALS, published from our lab, showed that TDP-43 acts as co-activator of p65 suggesting a key role of NF-κB signalling in ALS pathogenesis. Pharmacological inhibition of NF-κB by Withaferin A treatment attenuated disease phenotypes in TDP-43 transgenic mice. Also, loss-of-function mutations in the gene optineurin, which is known to negatively regulate TNF-α-induced NF-κB activation, have been found in ALS patients (Maruyama et al., 2010). A recent study by Frakes et al showed that selective NF-κB inhibition in ALS astrocytes is not sufficient to rescue motor neuron death. However, deletion of NF-κB activity in microglia rescued MNs from microglial-mediated death in vitro and extended survival in ALS mice by impairing proinflammatory microglial activation (Frakes et al.,

2014). Considering the pathological role of NF-kB in different ALS mutations, it seems reasonable to target NF-κB, as a possible therapeutic option. As stated previously, WA, an inhibitor of NF-κB activity and has been proven effective in TDP-43 mice model of ALS. Hence we decided to test the effectiveness of WA in SOD1 mice model of ALS.

In chapter 3, we demonstrated the beneficial effect of Withaferin A in mouse models of ALS. Withaferin A is a steroidal lactone isolated from plant Withania somnifera. It exhibits an array of properties, including anti-tumor, anti-inflammatory, and immunomodulatory functions (Mishra et al., 2000).

We tested WA in mice from two transgenic lines expressing different ALS-linked SOD1 mutations, $SOD1^{G93A}$ and $SOD1^{G37R}$. WA treatment conferred neuroprotective effects with extension of life span in two mouse models of ALS with over expression of different mutant SOD1, $SOD1^{G93A}$ or $SOD1^{G37R}$ mutants. Our in vivo imaging results from double transgenic GFAP-luc/$SOD1^{G93A}$ mice in which astrocyte activation can be visualized throughout disease progression confirmed that WA can reduce neuroinflammation when treatment is initiated at early stage of disease. Further, analysis of cytokine expression in spinal cord revealed increased levels of IL-10 in WA treated mice. IL-10 is known to confer beneficial effects in several neuroinflammatory disease models including experimental autoimmune encephalomyelitis (EAE), traumatic or excitotoxic spinal cord injuries, stroke and Parkinson's disease (Bethea et al., 1999; Brewer et al., 1999; Cua et al., 2001; Frenkel et al., 2005; Qian et al., 2006). Also the level of pro-inflammatory cytokine GM-CSF level was decreased in spinal cord of WA treated mice. GM-CSF is a pro-inflammatory cytokine which is up-regulated in various neurological disorders like AD, vascular dementia, multiple sclerosis (Tarkowski et al., 2001; Mellergard et al., 2010; Parajuli et al., 2012). Thus it seems plausible that reduced inflammation by WA treatment

in SOD1^{G93A} mice may be in part due to an upregulation of anti-inflammatory cytokine IL-10 and by a down regulation of pro-inflammatory cytokine GM-CSF.

An important point our study revealed was reduction in levels of misfolded SOD1 species in spinal cord of WA treated mice. It has been shown previously that accumulation of misfolded SOD1 species is a valuable indicator of disease progression and different therapeutic approaches that target the misfolded SOD1 has been proved to be beneficial. Hence the observed beneficial effect of WA treatment might not only come from alleviation of inflammation but also from reduction in levels of misfolded SOD1 species. An upregulation of heat shock proteins (hsp) constitutes another mechanism by which WA may confer neuroprotection in SOD1^{G93A} mice.

As a type of chaperone protein, hsps facilitate nascent protein folding, and re-folding or degradation of un- or mis-folded protein. WA treatment significantly increased the amount of Hsp-25 (a mouse orthlogue of Hsp-27) in the spinal cord of SOD1^{G93A} mice. Many reports have shown that Hsp-27/25 protects against neuronal damage induced by FALS-related SOD1 mutant (Benn et al., 2002; Patel et al., 2005; Sharp et al., 2006; An et al., 2008; Sharp et al., 2008; Yerbury et al., 2013). Moreover, Hsp-27/25 was found to inhibit the *in vitro* aggregation of SOD1^{G93A} proteins (Yerbury et al., 2013). Thus, an up-regulation of Hsp-25 in WA-treated SOD1^{G93A} mice may explain in part the reduction in levels of misfolded SOD1 species as determined by immunoprecipitation with the specific B8H10 antibody and also increased number of surviving motor neurons. WA treatment also decreased the early neuronal injury response as evident by the bioluminescence imaging of the spinal cord on GAP-43-luc/gfp; SOD1^{G93A} double transgenic mice. WA treatment resulted in significant reduction of the GAP-43 bioluminescence signal in the spinal cord at sixteenth and seventeenth weeks of age compared to vehicle-treated double transgenic mice.

The combined results revealed an effective therapeutic effect of WA when treatment was initiated at onset of motor deficits in SOD1^{G93A} mice, which has recently been reassessed to be at 30 to 40 days of age according to leaded grid test and treadmill gait analysis (Vinsant et al., 2013a).

However, when WA treatment was initiated at later stage of disease (90 days of age), at time coincident with detection of motor neuron death (Vinsant et al., 2013b, a), there was no beneficial effect on survival of SOD1^{G93A} mice. When WA treatment was started after onset, no increase in Hsp proteins was observed. Additionally an increase in both anti and pro inflammatory cytokines was observed.

Altogether these data suggest that a therapeutic intervention for ALS based on WA medication would need to be initiated early in the pathogenic process while cellular responses to stress and injury such as Hsps induction, as well as adequate inflammatory responses are still functional. For example, a late-onset initiation of WA administration in SOD1^{G93A} mice caused an increase in both anti- and pro-inflammatory cytokines suggesting a marked deregulation of immune system responses in late stage disease. If started at early disease stage, WA should be effective in attenuating deleterious neuroinflammatory responses and in conferring neuroprotection through an up-regulation of Hsp-25 and reduction of misfolded protein species. However, no evidence of therapeutic value was observed when WA treatment was initiated at advanced stage of ALS disease in SOD1^{G93A} mice. The therapeutic effects of WA in various ALS mouse models suggest that WA should be considered as a promising lead compound for drug development aiming to treat ALS.

4.4 Targeting neuroinflammation: Lessons learned and path forward

Treating SOD1-mutant mice with variety of anti inflammatory drugs that might have effects on several aspects of the inflammatory response has proven to be efficacious. Administration of COX2 inhibitors (nimesulide, celecoxib), the immunosuppresant cyclosporine, thalidomide (inhibitor of TNF-α) and sulindac (NSAIO) suppressed inflammation and prolonged life in mutant SOD1 transgenic mice models (Drachman et al., 2002; Klivenyi et al., 2004; Kiaei et al., 2006). Also, the antibiotic minocycline is protective in both SOD1 ^{G37R} and SOD1 ^{G93A} transgenic mice (Kriz et al., 2002; Van Den Bosch et al., 2002; Zhu et al., 2002). Thus these results supported a role for the inflammatory response in ALS and the inhibition of inflammatory processes as a therapeutic target. However there are some points that should be taken in consideration before targeting neuroinflammation. For instance, many compounds exert effects on different pathways and do not selectively and exclusively target inflammatory processes (Wyss-Coray and Mucke, 2002). For example, thalidomide exerts its protective effect by inhibiting TNF-α; apart from this, it is a known teratogen and has potent anti-angiogenic effects and when used in therapeutic doses can lead to peripheral neuritis (Cavaliere and Schiff, 2006; Peltier and Russell, 2006). Moreover, most of the anti-inflammatory drugs failed in ALS clinical trials (Cudkowicz et al., 2006; Gordon et al., 2007; Stommel et al., 2009). If we look at the case of minocycline, it showed promising results from preclinical studies; but phase III trial reported failure in human clinical trials in patients with ALS, suggesting that treatment with minocycline may be harmful for some ALS patients (Gordon et al., 2007). Possible explanation for this adverse effect could be high doses of minocycline (400 mg) used in the clinical trial that could have exerted non-selective toxic effect (Carri, 2008; Leigh et al., 2008).

Overall few possible reasons for failed translation from animal studies to clinical trial could be: (1) The timing of initial drug administration: In preclinical experiments, treatment is most often started before appearance of phenotypic onset of the disease. This is not a feasible therapeutic strategy for sporadic ALS patients. As a consequence, it is important

that the administration of a compound in a preclinical trial is also performed at an age that is closer to onset or even after the onset of symptoms. (2) In treatment trials, very homogenous cohorts of mice (in terms of genetics, food exposure, and age) are used, whereas even in well designed studies of patients with ALS, the study population is heterogeneous. (3) In animal trials, beneficial effects are often related to disease onset and survival and many studies do not show benefit on disease progression. In case of ALS patients, who already have the disease, effect on disease progression is the relevant parameter that can be monitored to observe the effect of treatment.

Apart from validation in SOD1 model of ALS, it will be of high relevance to validate therapies in additional genetic models of ALS. Taking this point into consideration, Withaferin A could be an important candidate for clinical trials because, it has proven to be successful in ameliorating ALS related pathology in three mice models of ALS (ie, SOD^{G93A}, $SOD1^{G37R}$ and TDP-43 mice model of ALS).

While neuroinflammation occurs in ALS patients independent of the cause of the disease and thus qualifies to be a shared target, it is however likely that patients will respond differently to certain therapies depending on their progression rate and disease state when receiving the treatment. Thus, understanding when and which patients would benefit more or less from specific therapies is a crucial and a challenging task. For understanding this, it is important to develop biomarkers that meaningfully gauge disease activity and therefore allow trials to be conducted more efficiently.

4.5 Conclusion

Results presented in this thesis, revealed that an immunotherapy based on intrathecal inoculation of AAV encoding a secretable scFv against misfolded SOD1 should be considered as potential treatment for ALS, especially for individuals carrying SOD1 mutations. Moreover, this therapy could also be of potential application in sporadic ALS case as recent studies suggest the existence of misfolded/aggregated forms of SOD1 in sporadic ALS cases with no SOD1 mutations (Forsberg et al., 2010; Bosco et al., 2010). In addition, the therapeutic effects of WA in various ALS mouse models suggest that WA should also be considered as a promising lead compound for drug development aiming to treat ALS.

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Annex 1: Published manuscript with their links

Adeno-associated virus-mediated delivery of a recombinant single-chain antibody against misfolded superoxide dismutase for treatment of amyotrophic lateral sclerosis.

Patel P[1], Kriz J[1], Gravel M[1], Soucy G[1], Bareil C[1], Gravel C[1], Julien JP[1].

http://www.ncbi.nlm.nih.gov/pubmed/24394188

Early-stage treatment with withaferin A reduces levels of misfolded superoxide dismutase 1 and extends lifespan in a mouse model of amyotrophic lateral sclerosis.

Patel P[1], Julien JP, Kriz J.

http://www.ncbi.nlm.nih.gov/pubmed/25404049