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# CELLULAR AND MOLECULAR MECHANISMS OF MOTOR NEURON DEATH IN AMYOTROPHIC LATERAL SCLEROSIS

Topic Editor
Ricardo Tapia

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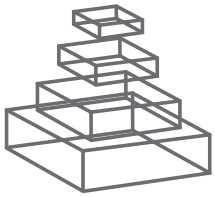

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# CELLULAR AND MOLECULAR MECHANISMS OF MOTOR NEURON DEATH IN AMYOTROPHIC LATERAL SCLEROSIS

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Amyotrophic lateral sclerosis (ALS), which was described since 1869 by Jean Martin Charcot, is a devastating neurodegenerative disease characterized by the selective and progressive loss of upper and lower motor neurons of the cerebral cortex, brainstem and the spinal cord. The cognitive process is not affected and is not merely the result of aging because may occur at young ages. The only known cause of the disease is associated with genetic mutations, mainly in the gene encoding superoxide dismutase 1 (familial ALS), whereas there is no known cause of the sporadic form of ALS (SALS), which comprises >90% of cases. Both ALS types develop similar histopathological and clinical characteristics, and there is no treatment or prevention of the disease. Because effective treatments for ALS, as for other neurodegenerative diseases, can only result from the knowledge of their cellular and molecular pathophysiological mechanisms, research on such mechanisms is essential. Although progress in neurochemical, physiological and clinical investigations in the last decades has identified several mechanisms that seem to be involved in the cell death process, such as glutamate-mediated excitotoxicity, alterations of inhibitory circuits, inflammatory events, axonal transport deficits, oxidative stress, mitochondrial dysfunction and energy failure, the understanding of the origin and temporal progress of the disease is still incomplete and insufficient.

Clearly, there is a need of further experimental models and approaches to discern the importance of such mechanisms and to discover the factors that determine the selective death of motor neurons characteristic of ALS, in contrast to other neurodegenerative diseases such as Parkinson's and Alzheimer's disease. Whereas studies in vitro in cell cultures, tissue slices or organotypic preparations can give useful information regarding cellular and molecular mechanisms, the experiments in living animal models obviously reflect more closely the situation in the human disease, provided that the symptoms and their development during time mimics as close as possible those of the human disease. It is necessary to correlate the experimental findings in vitro with those in vivo, as well as those obtained in genetic models with those in non-genetic models, aiming at designing and testing therapeutic strategies based on the results obtained.

# Table of Contents

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# Cellular and molecular mechanisms of motor neuron death in amyotrophic lateral sclerosis: a perspective

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**Keywords: motor neuron degeneration, Amyotrophic lateral sclerosis (ALS), spinal cord, muscle, skeletal, genetic expression, trophic factors, neuroinflammation**

Amyotrophic lateral sclerosis (ALS), which was described since 1869 by Jean Martin Charcot, is a devastating neurodegenerative disease characterized by the selective and progressive loss of upper and lower motor neurons of the cerebral cortex, brainstem and the spinal cord. The result of this loss is a progressive and irreversible paralysis leading to a complete incapacity of movements and finally to respiratory failure, but the cognitive functions are not affected and is not merely the result of aging because may occur at young adult ages. There is still no treatment, prevention or reliable biomarkers of ALS and it is clear that this could only be accomplished, as for other neurodegenerative diseases, through the knowledge of the cellular and molecular pathophysiological mechanisms involved. Research on such mechanisms is, therefore, essential. Although progress in neurochemical, physiological, genetic and clinical investigations in the last decades has identified several cellular processes and mechanisms that seem to be involved in the neuronal death, such as glutamate receptors-mediated excitotoxicity, disruption of spinal inhibitory circuits, inflammatory events, axonal transport deficits, oxidative stress, mitochondrial dysfunction, energy failure, intracellular $Ca^{2+}$ dishomeostasis, protein aggregation and misfolding, changes in gene expression, astrocytes alterations, and non-cell autonomous toxic factors, the understanding of the origin and temporal progress of the disease is still incomplete and insufficient. Clearly, there is a need of further experimental models and approaches to discern the importance of such mechanisms and to discover the factors that determine the selective death of motor neurons characteristic of ALS, in contrast to other neurodegenerative diseases such as Parkinson's and Alzheimer's disease in which other neuronal types located in other CNS regions are predominantly affected.

The only known cause of the disease is associated with genetic mutations, mainly in the gene encoding superoxide dismutase 1 (SOD1) which is the most frequent case of familial ALS (FALS), representing about 20% of cases of FALS, albeit over one hundred mutations have been described in FALS. In contrast, there is no known cause of the sporadic form of ALS (SALS), that comprises >90% of all cases, although several other mutations have recently been identified also in SALS. Both ALS types show similar histopathological and clinical characteristics, and in spite of numerous investigations using tissue from ALS patients and relevant advances in the design of several experimental models of motor neuron degeneration, it has not been possible to establish a clear cause-effect relationship regarding the loss of motor neurons or the motor alterations characteristic of the disease, such as fasciculations, spasticity, and progressive paralysis.

Studies *in vitro* in cell cultures, tissue slices or organotypic preparations have given useful information regarding the cellular and molecular mechanisms of motor neuron death. However, except for transgenic rodents expressing mutant forms of human SOD1 (mSOD1), which are the most extensively used model *in vivo* in spite of the low frequency of FALS as compared to SALS, experiments in living animals are scarce. These models should reflect more closely the situation in the human disease, provided that the symptoms and their development during time mimics as close as possible those of the human disease. It is therefore necessary to correlate the experimental findings *in vitro* with those *in vivo*, as well as those obtained in genetic models with those in non-genetic models, in order to design and test therapeutic strategies based on the results obtained. The aim of the original research articles and reviews of this Research Topic is to contribute to the progress in this important field.

Genetic alterations, including epigenetic and gene expression changes in both neurons and glial cells, mainly astrocytes, as well as studies *ex vivo* of nervous and muscular tissue from ALS patients and from mutant SOD1 transgenic mice, constitute the majority of the articles of this Research Topic. Such changes have been studied in whole tissue, microdissected isolated cells and subcellular fractions, mainly mitochondria, using several molecular biology techniques, not only in the CNS but also in muscle of mSOD mice, and have shown that there is a large variety of alterations in gene expression. These results are the subject of the articles by Heath et al. (2013); Crippa et al. (2013); de Oliveira et al. (2013), and Wong et al. (2013), which amply cover the literature on this subject and also provide new data, such as the relation of some of these genetic alterations with autophagy, epigenetic changes of mitochondrial DNA in the spinal cord and in muscle, and comparative analysis of early and late gene expression changes.

The role of astrocytes and microglia from mSOD mice in non-cell autonomous processes, including the release of toxic factors, is studied by Rojas et al. (2014) and Trias et al. (2013), with a useful *ex vivo* approach. Related studies by Staats et al. (2013)

and Bowerman et al. (2013) show the involvement of microglia and neuroimmunological events in the progress of ALS and suggest possible therapeutic strategies. The molecular modifications of mSOD1 protein, and how such alterations, like oxidation, aggregation or misfolding, result in its toxicity, are reviewed by Furukawa (2013); Rotunno and Bosco (2013), and Ogawa and Furukawa (2014). Again in mSOD1 mice but with a different approach, Mòdol et al. (2014) show a possible role of the potassium chloride cotransporter 2 in spasticity, including results on the role of spinal inhibitory interneurons.

A partial protective effect of the NMDA receptor antagonist gacyclidine on the motor deficits in mSOD1 mice is shown by Gerber et al. (2013), and the role of trophic factors in the modulation of motor neurons activity as well as their protective effect in several models of ALS, is reviewed by Tovar-y-Romo et al. (2014) and Lladó et al. (2013). It is emphasized in particular the potent protective action of vascular endothelial growth factor (VEGF). Other potential protective agents, reviewed by Lazo-Gómez et al. (2013), are activators or inhibitors of histone deacetylases, enzymes that regulate gene expression and may be involved in motor neuron degeneration.

A general panorama of the role of lipids in ALS is presented by Schmitt et al. (2014), with emphasis on energy and signaling mechanisms. In a more systemic approach, Garbuzova-Davis and Sanberg (2014) review the vascular changes that may alter blood brain barrier and thus be involved in the pathogenesis of ALS, as compared to the transgenic rodent models.

The large amount of information contained in this Research Topic, in both the comprehensive reviews and the original research articles, reflect the considerable advances made but at the same time the necessity for new experimental models and approaches for gaining clearer responses to several still unresolved questions, such as the following: why the clinical manifestations of motor neuron death in both FALS and SALS appear in young adulthood but are not age-related, particularly the former, in spite of the fact that genetic alterations are present since the beginning of individual life? Why the motor neuron degeneration progresses rapidly after the initial symptoms appear? What is the cause of SALS? It is now clear that the loss of motor neurons in ALS is multifactorial, but many of the deleterious factors seem to occur also in other neurodegenerative diseases like Parkinson's and Alzheimer' disease; then, maybe the crucial question to be answered is: what determines the predominant, if not totally selective characteristic death of motor neurons in ALS, sparing other neuronal types?

In spite of these unsolved questions, it is clear that the available information permits the design and testing of diverse therapeutic strategies that have already shown valuable results in delaying or preventing neuronal death in experimental models, for example the administration of VEGF. However, the translation of possibly effective treatments from experimental procedures to ALS patients represents different problems, such as the routes, doses and timing of administration and the potential undesirable collateral effects. Because we have no indication or biomarker of the susceptibility to ALS and of when will it manifest, it is extremely difficult to prevent or treat the disease. When symptoms have already started and the diagnosis is made it is probably too late to impede the progression of neuronal degeneration. A verifiable resolution of the above questions seems a requisite for efficient and reliable treatments or prevention of this dreadful disease.

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# Investigating cell death mechanisms in amyotrophic lateral sclerosis using transcriptomics

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Amyotrophic lateral sclerosis (ALS) is a motor neuron disease characterized by degeneration and loss of upper and lower motor neurons from the motor cortex, brainstem and spinal cord although evidence is suggesting that there is further involvement of other cell types in the surrounding tissue. Transcriptomic analysis by gene expression profiling using microarray technology has enabled the determination of patterns of cell death in the degenerating tissues. This work has examined gene expression at the level of the tissue and individual cell types in both sporadic and familial forms of the disease. In addition, further studies have examined the differential vulnerability of neuronal cells in different regions of the central nervous system. Model systems have also provided further information to help unravel the mechanisms that lead to death of the motor neurons in disease and also provided novel insights. In this review we shall describe the methods that have been used in these investigations and describe how they have contributed to our knowledge of the cell death mechanisms in ALS.

**Keywords: transcriptomics, cell death, amyotrophic lateral sclerosis, microarray**

## INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is described as a degenerative disease involving loss of the upper and lower motor neurons from motor cortex, brain stem and spinal cord leading to muscle denervation, wasting and death. There is now increasing evidence that other cell types are involved and the precise pattern of degeneration is unclear (Ince et al., 2011). Multiple mutually compatible mechanisms have been implicated in the pathogenesis of the disease including oxidative stress, excitotoxicity, ubiquitin and proteasome dysfunction, inflammatory activation, RNA processing dysregulation, mitochondrial dysfunction, cytoskeletal abnormalities and activation of apoptosis (Ferraiuolo et al., 2011). However, there remain many unanswered questions as to the origins of the disease and the precise pathogenetic mechanism. The use of microarray technology to explore gene expression at a particular time point and in particular cells is enabling us to unpick some of the specific changes undergone by cells or tissues involved in the disease and also to determine what processes act to protect cells from degeneration. The technology for different array types has been described and reviewed previously and we will not at this time extend this (Clark et al., 2007; Kirby et al., 2007). Whilst there are inconsistencies between the findings of transcriptomic studies and the precise biological mechanisms involved in a disease situation there is no doubt that properly designed studies which generate functionally verifiable results can be invaluable in assisting in the description of the disease process (Bolstad et al., 2004; Rosenfeld, 2010; Henriques and Gonzalez De Aguilar, 2011). For the purposes of this review we concentrated our attention upon articles that were found from a PubMed search using the search terms microarray and ALS.

## RNA QUALITY

A recurring dilemma in studies of gene expression in post mortem tissue has been the residual debate concerning the quality of RNA derived from such tissue and whether it is appropriate for these studies. However, it is now accepted that gene expression studies in post mortem tissue are possible as long as appropriate quality control measures are followed (Harrison et al., 1995; Trabzuni et al., 2011). For most model studies standardized procedures can be set in place in order to reduce potential factors that might cause RNA degradation but even in an established and well organized human brain banking situation there are variables relating to post mortem delay, agonal state and cause of death all of which can affect RNA quality (Tomita et al., 2004; Popova et al., 2008; Durrenberger et al., 2009; Trabzuni et al., 2011). As indicated a number of studies have examined the effect of multiple potential RNA degrading features but the overall consensus is that providing adequate care is taken and good experimental design is incorporated in order to eliminate gross differences between the material being used then a reliable study can be carried out. In order to monitor these potential confounding factors it is important to control for any RNase introduction by carrying out all experimental work in a clean and tidy environment using RNase free materials. In addition it is essential that quality control steps should be included to measure both quantity and quality of the RNA being extracted and used (Copois et al., 2007; Wilkes et al., 2010; Trabzuni et al., 2011). Spectrophotometric methods can be used to measure nucleic acid quantity by examining absorbance at 260 nm. This can give an indication of RNA quality and the 260/280 ratio provides a measure of protein contamination whilst the 260/230 ratio provides a measure of organic solvent

carryover. However, these metrics do not give a clear idea of RNA quality. For this, electrophoretic methods are required to examine the RNA directly. In the past a denaturing agarose gel (including formamide in the gel matrix) allowed the RNA to be visualized following staining with ethidium bromide. However, these methods required a large input of total RNA and have now been largely superseded by capillary based methodology such as the Agilent Bioanalyser and/or the TapeStation. In effect, both these devices drive the RNA sample through a capillary and over a laser which monitors absorbance at 260 nm over time hence providing a profile of the absorbance which can be compared to the profile of a molecular weight ladder run at the same time. If total RNA is the test sample then one would expect a small series of peaks at low molecular weight followed by the two ribosomal RNA peaks (the smaller peaks might be removed if a column based separation method is used). Software integral to the machine is then able to calculate an RNA integrity number (RIN) which is scaled from 0 to 10 and depends upon the ratio of the 28S to 18S rRNA peaks and the amount of degraded material that is present. It should be expected that for material derived from tissue culture experiments that a high RIN value is routinely achieved. However, post mortem human material is often quite degraded and RIN values of around 4 might be measured. It has been demonstrated in microarray, RNASeq and qRT-PCR that these values do not preclude a valid experiment (Preece et al., 2003). However, it is important to maintain equivalence in the experimental material and endeavor to compare samples of similar quality as well as support findings through appropriate validation methodologies.

## HUMAN STUDIES IN WHOLE TISSUE

Microarray studies on whole tissue generate an overview of the gene expression profile of the whole tissue at the time of the RNA collection. There have been a number of microarray studies which were designed to examine some aspect of gene expression changes in ALS by examining a particular tissue. A very early study by Malaspina et al. (2001), used gridded cDNA membrane arrays to examine the expression of 18,400 genes in the anterior horn of lumbar spinal cord from four ALS cases and four controls (with a further four patients and controls used as verification subjects). Fourteen transcripts were identified as being significantly differentially expressed, and these were shown to belong to processes relevant to ALS including; oxidative stress and neuro-protection, motor neuron function, lipid metabolism, neuroinflammation and anti-apoptotic factors. This was an important demonstration of the potential of this sort of study and whilst it depended upon a predefined group of cDNAs that were present upon the array, the results gave an overview of gene expression in the anterior horn of the lumbar spinal cord. It also confirmed that post mortem tissue could be used in such a study. Ishigaki et al. (2002) took a different approach. Using lumbar spinal cord from 8 sporadic ALS cases and controls they initially used one case and control for molecular indexing which identified 576 cDNA fragments in the sALS material that could be compared to the control to select identifiers for further analysis. In this way spotted microarrays were prepared for 84 cDNAs deemed to be differentially expressed between the sALS and control individuals that were further examined on a wider group of patients and controls. Ultimately six differentially expressed genes were identified; four corresponded to previously known genes, 30-kDa TATA-binding protein-associated factor (*TAFII30*), macrophage-inhibiting factor-related protein-8 (*MRP8*), metallothionein-3 (*MT-3*) and ubiquitin-like protein 5 (*UBL5*) whilst the remaining two were uncharacterized expressed sequence tags. This was an interesting approach but the limited numbers of genes identified made it difficult to identify processes related to cell death.

An early study by Dangond et al. (2004), used Affymetrix FL arrays to compare the expression profile of grey matter from the lumbar spinal cord of seven ALS cases and four controls. A robust analytical process identified 93 genes which discriminated between the two groups. There was some attempt to differentiate between sporadic and familial disease, however, the numbers in the familial group made this analysis less robust. The 93 significant genes were subdivided into groupings defined by known biological functions giving some 10 sub-groups including; transcription and RNA binding, excitotoxicity, cell survival and growth, neuronal function, inflammation, cell receptors and signaling and stress response. Wang et al. (2006), examined the motor cortex in five ALS cases compared to three controls using the newer Affymetrix U133A Gene Chips containing 14,500 genes for microarray analysis. They identified 275 significantly differentially expressed genes, of which 265 were downregulated and they were able to verify the changes in 22 of these genes by quantitative real time polymerase chain reaction (QRT-PCR). Interestingly different groups of genes seemed to be important in sALS motor cortex compared to the spinal cord, but there were similarities in that stress response and excitoxicity were featured in both groups, as were features of mitochondrial metabolism. Unfortunately, the different cell populations of the two regions might be having a profound impact upon the data. A further study of motor cortex (Lederer et al., 2007), used 11 sALS cases and 9 controls in an Agilent slide array based study. Fifty-seven genes were identified as differentially expressed following the filtering and pathway based analysis; 40 down and 17 upregulated. Such a small group of genes were readily verifiable by alternative methods and the list was compared to previous studies including that of Dangond et al. (2004), and 14 genes showed a similar response. Overall a very thorough analysis produced an interesting interacting pathway diagram examining the interplay of differentially expressed genes in the motor cortex of sALS individuals. It is important to note that without describing individual genes it is possible to discern similar patterns in the lists of genes differentially expressed in the whole tissue experiments described here. Recurrent themes are mitochondrial metabolism, excitotoxicity, calcium homeostasis, protein turnover and neuronal maintenance and signaling.

Using a different approach Offen et al. (2009) examined the gene expression profile in spinal cord of sALS patients and compared some of the changes to the Glycine 93 to Alanine (G93A) SOD1 mouse model of fALS. In the study of four sALS cases compared to four matched controls using the Clontech membrane arrays, 60 gene changes were identified in the 1176 probe sets available to be examined. On this occasion the genes identified

were grouped into biological processes such as; apoptosis, protein turnover, cell cycle functions, extracellular carrier/transport functions, cytoskeletal and intracellular proteins and proteins relating to transcription and translation. Six genes were further investigated in the G93A *SOD1* mouse model of fALS to determine if the changes seen post mortem were recapitulated during the lifespan of the mouse. These genes were; cathepsins *B* and *D*, apolipoprotein *E*, epidermal growth factor receptor (*EFGR)*, ferritin and lysosomal trafficking regulator (*LYST)*. These transcripts did seem to increase with disease progression in the mouse model suggesting some overlap between the human situation and the mouse model which does add some credence to the use of the model for human ALS.

Two recent studies have taken a quite different approach. Bernardini et al. (2013), concentrated upon mitochondrial related genes and Figueroa-Romero et al. (2012), tried to relate gene expression changes to methylation events in the genome. The Figueroa-Romero study was focussed on relating genome methylation events to gene expression changes found in sALS spinal cord. 12 sALS cases and 11 age and sex matched controls were used to determine the methylated genetic regions using Illumina Methylation 27 Bead Chips, whilst gene expression analysis was carried out using the Affymetrix Human U133 Plus 2 GeneChips. In the methylation chip assay 3574 regions associated with genes were found to be differentially regulated. In the gene expression study some 1182 genes were found to be differentially expressed in the sALS spinal cord. When comparing the two datasets 51 genes that were hypomethylated were upregulated and 61 genes that were hypermethylated were downregulated. These concordant "epigenes" could be functionally grouped into categories such as; immune response, defence response, neuron adhesion and plasma membrane. Overall this work suggested an epigenetic, or environmental, influence upon the pathogenesis of sALS which may be relevant to the process of cell survival. In the Bernardini study they concentrated upon skeletal muscle which was a new, but potentially relevant, tissue to examine. They hypothesized that muscle degeneration might be functionally important in the process of neurodegeneration and that differentially expressed genes in this tissue could be key in the pathogenic process. They examined quadriceps or biceps muscles in seven sALS cases and seven age and sex-matched controls. A focussed Affymetrix GeneChip array was used and using a fold change cutoff of 1 and *p*-value cutoff of 0.05, 96 differentially expressed genes were identified. Unlike in previous studies on this occasion more genes (80), were seen in the upregulated group. In total 13 genes related to muscle structure were distinguished and when ontology relationships were investigated using the DAVID software (Huang da et al., 2009) platform, other genes were found to be grouped in areas such as the actin cytoskeleton and glycogenesis, with mitochondrial function also identified. Further studies would be needed to determine the relevance of this work particularly with regard to further subtyping of the ALS cases. A study by Shtilbans et al. (2011) also looked at gene expression differences in muscle biopsies from ALS cases and controls. The study was hampered in that mixed groups of familial and sporadic cases were examined and different muscle groups were included in mixed proportions in each comparative group. The differences they found were in genes related to muscle structure, the cytoskeleton and cellular metabolism.

Two studies have used peripheral blood to look for gene expression differences with the underlying hypothesis that changes found in this tissue might reflect changes associated with the disease as has been seen in other neurodegenerative diseases. Saris et al. (2009) used whole blood and a designed analysis tool to uncover disease specific differences whereas Mougeot et al. (2011) isolated lymphocytes and examined these directly. In the Saris study, the weighted co-expression analysis found that genes in pathways related to apoptosis, the mitochondrion, stress response, calcium binding ubiquitination and vesicle transport were differentially expressed between the ALS patients and controls. In contrast, Mougeot used an ontology relationship tool called SAFE and found that a set of genes previously grouped into an ALS related KEGG pathway were differentially expressed in the lymphocytes. Genes in this pathway related to motor neuron degeneration in ALS (KEGG:05014), and included SOD1, caspases 1,3,9, neurofilament light, medium and heavy (*NFL, NFM* and *NFH)*, and N-methyl-D-Aspartate receptors *GRIN 1, 2A, 2B, 2C* and *2D*. In addition, an ontology analysis based upon individual genes identified changes in pathways related to DNA metabolism, RNA splicing, mitochondrial function, oxidation, endoplasmic reticulum,ubiquitin proteasome system, post-transcriptional modification and neurological function. The overall conclusion to be drawn from both studies in blood is that although it is a peripheral tissue, it does show gene expression changes and pathways previously implicated by the use of central nervous system (CNS) material, including apoptosis, mitochondria, RNA splicing and, surprisingly, neurological function.

The studies of whole tissue have reached different conclusions, perhaps as a result of using tissue from different stages of disease and location, but there are common themes involved in the processes that are found to be differentially expressed in the ALS tissue which have been associated to motor neuronal cell death. In particular these are apoptosis, oxidative stress, protein processing, mitochondrial function and the cytoskeleton.

## SINGLE CELL ANALYSIS OF HUMAN MOTOR NEURONS

The previous studies are valuable but they make it difficult to determine the precise input of each cell. In order to obviate this, several methods have been developed to examine the gene expression profile of individual cells isolated from tissue. Two recent reviews have discussed the available methods for cell specific transcriptome analysis (Kannanayakal and Eberwine, 2005; Okaty et al., 2011), and we shall not discuss these further. In terms of ALS, most studies seem to have adopted a laser capture microdissection approach. In 2005, Jiang et al. carried out a microarray study of laser captured motor neurons from the lumbar region of 14 sporadic ALS cases and 13 controls (Jiang et al., 2005). They used a PALM microdissector and applied the labeled RNA to BD Atlas glass microarrays. They found 52 up and 144 down regulated genes from the 4845 interrogated. Owing to the fact that they compared the isolated spinal motor neurons data to ventral horn spinal cord tissue data they were able to distinguish genes whose differential expression was restricted to the motor neurons and

which was not detected when the whole tissue was examined. It was found that genes associated with cell death and cell signaling were upregulated in the disease related motor neurons whereas those associated with the cytoskeleton and transcription were downregulated. This study provided information concerning gene expression changes that were specific to the spinal cord motor neurons and demonstrated the value of this approach over whole tissue studies. Our group has carried out a number of published studies examining the gene expression profile of motor neurons isolated by laser capture microdissection in several models. In her study in 2010, Cox et al. (2010) examined the differences between three individuals who had charged multivesicular body protein 2B (*CHMP2B)* mutations leading to an ALS phenotype and 7 matched normal controls. Following gene expression analysis using Affymetrix Human U133 Plus 2 GeneChips, 890 genes were downregulated and 55 upregulated at a fold change cutoff of 2 and *p*-value 0.05. These were categorized using DAVID software into ontological groupings and groups associated with axon guidance, regulation of actin cytoskeleton and soluble NSF attachment protein receptor (SNARE) interactions in vesicular transport, mammalian target of rapamycin (*mTOR*) signaling and regulation of autophagy, mitogen activated kinase (*MAPK*) signaling, calcium signaling, and cell cycle and apoptosis were found to be the most enriched. Further functional studies were able to confirm a dysfunction in the autophagy pathway in an *in vitro* model. A further study (Kirby et al., 2011), examined gene expression differences in cervical spinal motor neurons between three fALS cases carrying SOD1 mutations and seven normal controls using the Affymetrix Human U133 Plus 2 arrays. In total, 524 probe sets were found to be increased and 646 decreased. These were characterized using the DAVID software package and the major enriched categories were transcription, signaling and metabolism. Importantly further investigations demonstrated the relevance of the cell survival pathway involving *PTEN/AKT* in the motor neurons with anti-apoptotic genes being downregulated in the surviving motor neurons indicating an attempt by these cells to mount a pro-survival response. Comparison of these studies indicates that the different genetic variants have distinct gene expression changes which ultimately lead to motor neuron death.

In contrast to the case control scenario, Brockington et al. (2013), examined features that distinguish the motor neurons from the oculomotor nucleus and the lumbar spinal cord in normal individuals to determine those features that enable the oculomotor motor neurons to be selectively resistant to the cell death undergone by spinal motor neurons in ALS. Tissue from four neurologically normal individuals was collected and laser capture microdissection used to isolate motor neurons from the oculomotor nucleus and lumbar spinal cord. The labeled RNA from these was applied to the Affymetrix Human U133 Plus 2 GeneChip. 1521 gene expression differences were identified as being differentially expressed in the oculomotor neurons. Gene ontology analysis determined that genes involved in synaptic transmission, ubiquitin mediated protein degradation and mitochondrial oxidative phosphorylation were upregulated in oculomotor motor neurons; these pathways had shown decreased expression in the ALS spinal cord and motor cortex in previous studies. This work was supplemented by carrying out comparison studies with other gene expression data derived from an online database and the analysis showed that the differences observed in human oculomotor neurons were also found in two other species confirming that the oculomotor motor neurons had a particular profile of synaptic neurotransmitter receptors, particularly gamma aminobutyric acid (*GABA)* and glutamate which made them less vulnerable to excitotoxic cell death. Electrophysiological studies complemented and supported this conclusion.

Again some common features can be derived that seem to associate the differential expression of genes related to the mechanisms of cell death. These include; cell signaling, autophagy, phosphatase and tensin homologue/protein kinase B (*PTEN/AKT)* cell signaling pathway, ubiquitin and mitochondrial function, cytoskeleton, transcription and apoptosis.

## ANIMAL MODELS USING WHOLE TISSUE

Whilst human tissue can only be accessed at end stage, the use of animal models allows progression of the disease to be monitored. An early study by Yoshihara et al. (2002) examined the gene expression differences between tissue homogenates of lumbar spinal cord of G93A SOD1 versus non-transgenic littermates at three ages; 7, 14 and 17 weeks corresponding to presymptomatic, onset and end stage of disease. Using mouse Atlas arrays from Clontech they found an upregulation of inflammatory related genes associated with activated microglia and astrocytes. This was induced by 11 weeks of age and continued to advance up to the 17 week time point.

Fukada et al. (2007) examined the gene expression profile of whole spinal cord from two time points, presymptomatic (98 days) and post symptomatic (154/176 days), in a different mutant SOD1 transgenic model carrying the L126delTT mutation. They used the AceGeneMouse Oligo Chip. At the presymptomatic stage 11 genes were upregulated and two downregulated at a fold change of greater than two whilst at the post-symptomatic stage 54 were upregulated and four downregulated. With such small numbers of differences ontological relationships were hard to carry out and since whole spinal cord was examined the precise relationship of the gene changes to the disease process is difficult to disentangle.

It is thought that physical exercise might have a beneficial effect upon disease progression in ALS and two studies investigated this using microarrays and mouse models (Ferraiuolo et al., 2009; Hashimoto et al., 2009). Hashimoto et al. (2009) examined the response to exercise in normal mice by subjecting the mice to two exercise regimes; a single burst of 30 min exercise or 2 weeks of 30 min exercise per day on a treadmill. Whole spinal cord RNA was examined using the OpArray Mouse V4 array from Operon. Only a small number of changes in gene expression were identified; after the single burst of exercise 3 genes were upregulated and 29 downregulated whilst following the extended exercise regime 1 gene was found to be increased and 13 decreased. As seen in some previous studies the numbers are rather small for pathway analysis and since whole tissue has been studied it is difficult to dissect out precise effects. Ferraiuolo et al. (2009) examined the gene expression changes in gastrocnemius muscle as well as motor neurons isolated from lumbar spinal cord of three female

mice subjected to a voluntary exercise regime of 21 days on a running wheel compared to three sedentary mice. Data were also collected concerning the exercise behavior of the mice. In the muscle tissue, 194 genes were upregulated and 176 downregulated using the same parameters. These genes were functionally found to be associated with vascularization and myogenic processes involved with extracellular matrix reorganization. The profiles exhibited by the gastrocnemius muscle are representative of the mixed cell population of the whole tissue and indicate some overall changes in the muscle as a response to the exercise regime. Overall, 203 genes were upregulated and 241 down at a fold change of at least two and *p*-value of 0.05 or less in the motor neurons. Functionally these genes were categorized into signaling, cytoskeleton and transcription regulation. The response of the motor neurons to exercise was similar to that of hippocampal neurons to repetitive stimulation, akin to a long term potentiation. There is a body of evidence that links exercise to the processes of cell death observed in ALS. This work goes some way to highlight similarities between the physiological response of MNs and skeletal muscle to exercise and the pathophysiology of ALS.

Two recent studies from the same group (Chen et al., 2010; Hu et al., 2013), have used Mouse Exon 1 arrays from Affymetrix to examine gene expression changes and alternative splicing in the G93A SOD1 mouse model. In the first study they examined animals at onset versus litter mate controls and in the second study 30 day transgenic mice were compared to litter mate controls of the same age and 120 day transgenic mice. They examined both differential expression and alternative splicing events. There were only 202 differentially expressed genes with a *p*-value cutoff of 0.05 found when comparing the 30 day old transgenic to nontransgenic mice but only one of these was at a fold change of $> 2$. There were in total 2869 differentially expressed genes events found when comparing the 120–30 day transgenic mice with 263 up and 71 down when a fold change of two and *p*-value of $< 0.05$ was imposed. Gene ontology analysis of the differentially expressed genes revealed that pathways involving cytokine receptor, cell adhesion, haematopoesis, and cell signaling included the most genes. In the onset group 322 transcripts were differentially expressed with 309 upregulated in the transgenic group when of a fold change of two and $p < 0.05$ was applied. Similar pathways were identified in this study to the previous one. In both studies a splicing index analysis was carried out in order to determine the levels of alternatively spliced transcripts. Comparing the 120 day transgenic to 30 day control 563 transcripts were alternatively spliced, of which 537 were considered as exon inclusion events, whilst only 85 alternative splicing events were identified in the 30 transgenic versus 30 day control comparison and on this occasion 61 were classified as exon inclusion. When the splicing data was examined by pathway analysis it was interesting that similar pathways were highlighted as found in the differentially expressed genes analysis. The data was interpreted to demonstrate that as the disease progressed then an increased number of splicing changes were seen. To some extent this is borne out by the onset study where 333 probe sets showed an altered splicing index indicating possible alternative splicing. However, the comparison of 120 day transgenic mice versus controls is missing which would allow a better comparison relative to normal ageing effects. Whilst some qRTPCR validation was completed the studies represent early work in using the exon arrays to examine the changes invoked by the presence of the G93A *SOD1* mutation on alternative splicing events.

The studies discussed thus far have used mouse models but Hedlund et al. (2010) used the G93A SOD1 rat model for a study of selective motor neuron vulnerability. They quantified the extent of motor neuron loss in different motor neuron nuclei; oculomotor and trochlear (CN3/4), facial (CN7), trigeminal (CN5), hypoglossal (CN12) and cervical spinal cord in the transgenic model. This directed the laser capture studies where motor neurons were collected from the CN3/4, CN12 and cervical spinal cord of normal rats and applied the isolated RNA to whole genome rat microarrays (Rat 230 2 Affymetrix). The microarray analysis showed that the more vulnerable motor neurons of the cervical spinal cord and hypoglossal nerve were more similar than either group compared to the less vulnerable CN3/4 motor neurons. There were interesting differences in Hox gene expression between the motor neuronal groups which were concomitant to the relative positions of the neurons in the anterior to posterior axis. There were also differences in RNA processing between the groups which also seemed to relate to the known association of RNA processing genes with ALS (Baumer et al., 2010). This report highlighted the usefulness of alternative animal models for the study of ALS and took an interesting approach to uncovering some of the underlying differences between selectively vulnerable and resistant motor neurons.

Dupuis and Loeffler (2009) examined neuromuscular changes in transgenic models of ALS. This led from the observation that the first event in the disease process seen in the transgenic mouse model of ALS is the destruction of the neuromuscular junction. Hence, studies concentrating upon the motor neurons may be identifying effects rather than cause of disease. However, it must be borne in mind that the SOD1 mouse model represents an overexpression model of disease and that SOD1 mutations correspond to only 2% of all ALS.

Finally in this section, Kumimoto et al. (2013) have undertaken to study ALS in a Drosophila model. They used a GAL4-UAS promoter to drive expression of wild type Drosophila SOD1 or the human SOD1 mutation glycine 85 to arginine (G85R) in motor neurons and glia. They carried out microarray analysis upon whole flies 5 days old (young), and 45 days (old), using the Drosophila 2 GeneChips from Affymetrix. They used several analyses to examine gene expression changes at the different ages and in the different cell types as well as a meta-analysis to uncover general changes. The mutant *SOD1* G85R in motor neurons alone caused 58 gene expression changes (33 up) at 5 days and 102 transcripts (73 up) at 45 days whereas in glia alone these numbers were 65 (33 up) and 105 (57 up) respectively. When the transgene was expressed in both cell types simultaneously the numbers were; 70 (34 up), and 83 (38 up). The overexpression of G85R SOD1 in the cells of the Drosophila caused changes in gene expression in oxidative stress, mitochondria, lipid metabolism and neurodevelopmental and signaling genes. Since the microarray analysis was carried out upon whole flies it is again difficult to determine the precise cellular effect of the overexpression strategy

but there were commonalities in the pathways implicated with previous microarray studies and the impression is that the study offers a new model approach to understanding ALS. Since these studies were somewhat varied in the animal models, the tissue being investigated and the number of genetic changes identified it is difficult to describe common themes in the gene expression changes being seen and their relationship to cell death. However, cell signaling, mitochondrial dysfunction and oxidative stress continue to be implicated in the pathogenesis of ALS in animal models.

## SINGLE CELL ANALYSIS OF NON-HUMAN MODEL MOTOR NEURONS

Perrin et al. (2005) combined laser capture dissection with microarray to examine gene expression in the spinal cord motor neurons of the G93A *SOD1* mice. Lumbar spinal motor neurons from male mice at presymptomatic (60 days), onset (90 days) and end stage of disease (120 days) were analyzed using the Mouse 430 2 GeneChip from Affymetrix. At 60 days only 27 genes were differentially expressed with this number increasing to 150 at 90 days and more than 400 by 120 days. At all the time points more genes were found to be upregulated in the G93A SOD1 mice; 17, 95 and 389 respectively. Thirteen of these transcripts were found to be differentially expressed at all stages. A similar study from our group (Ferraiuolo et al., 2007), looked at the effect of both the G93A SOD1 and the wild type SOD1 compared to non-transgenic littermate controls on a homogeneous background of C57bl6. Few changes were seen in motor neurons isolated from the WTSOD1 mice but 252 genes were differentially expressed at 60 days (234 upregulated), 51 at 90 days (32 up) and 167 at 120 days (81 up), in the G93A SOD1 mice. These changes at the presymptomatic, symptomatic and end stage of the disease process in the mice indicate that at an early time point the motor neurons try to mount a metabolic response to the burgeoning disease process by upregulating several pathways. However, by the endstage, this process having failed, the cells appear to try to re-enter the cell cycle which is a doomed response. There are notable differences between the two studies in terms of the overall changes in gene expression profile, although both groups do see changes in the intermediate filament protein vimentin, it is likely that the differences might relate to the actual genetic background of the mice being used, the transgene copy number and also the analytical processes used in examining the data. A study by Saxena et al. (2009) took a different approach to examine the matter of selective vulnerability of motor neurons. They injected RITC-dextran into the gastrocnemius or soleus muscles and following retrograde transport they were able to identify and selectively isolate the motor neurons associated with the vulnerable phenotype which project to the fast fibre gastrocnemius muscle and those that are relatively resistant and project to the soleus muscle which has fatigue resistant or slow fibres. The RNA isolated from the selectively microdissected motor neurons was applied to the Mouse 430 2 arrays from Affymetrix. The longitudinal study showed that the vulnerable motor neurons are selectively prone to endoplasmic reticulum stress and a progressive failure to mount a proper unfolded protein clearance response.

This group of studies are more focussed and underline the importance of the single cell analysis at different stages of disease progression.

## SINGLE CELL ANALYSIS OF NON-HUMAN NON-MOTOR NEURON CELL TYPES

Using the G93A Mutant SOD1 mouse model Ferraiuolo et al. (2011) attempted to examine the relationship between motor neurons and astrocytes in this model of disease (Ferraiuolo et al., 2011). Astrocytes were collected from lumbar spinal cord of three male 60 day old mice and three non transgenic litter mate controls, and the labeled RNA applied to Mouse 430 2 Gene Chips from Affymetrix. 583 transcripts were found to be upregulated 526 downregulated in the G93A SOD1 astrocytes. Ontological analysis showed that multiple transcripts associated with carbohydrate metabolism were differentially expressed and an overall decline in the activity of the lactate shuttle was described in the mutant astrocytes indicating an inability to support the metabolic requirements of the motor neurons. The altered metabolism was further investigated and it was shown that non-transgenic motor neurons grown on G93A SOD1 astrocytes demonstrated an increase in the ratio between pro- nerve growth factor and mature nerve growth factor (NGF) which was associated with an over-expression of the p75 receptor for NGF. Hence it was shown that the G93A SOD1astrocytes provided reduced metabolic support to the motor neurons as a result of the downregulation of the lactate shuttle and the activation of the p75 receptor and these factors contributed to the toxicity of the mutant astrocytes to the co-cultured motor neurons.

## CELL CULTURE MODEL STUDIES

Two studies have examined the gene expression profile of the NSC 34 cells carrying different human SOD1 mutations. The first study (Kirby et al., 2002), utilized a designed membrane microarray from Clontech with cDNAs for just 588 genes. The experiment was carried out on cells carrying G93A, G85R or isoleucine 113 to threonine (I113T) SOD1 mutations at basal conditions and cells which had undergone stress exerted by serum withdrawal. At the basal condition differences were seen in the numbers of differentially expressed genes for the different SOD1 mutation but a combined analysis showed only 29 genes to be differentially expressed as a result of the SOD1 mutation with seven being downregulated. Very few genes were found to be differentially expressed following the induction of a stress imposed by serum withdrawal which might be a result of the analytical stringency being applied. In a subsequent study (Kirby et al., 2005), a comparison was made between NSC34 cells expressing G93A mutant SOD1 and those expressing wild type human SOD1. On this occasion the mouse U74Av2A GeneChip from Affymetrix was used. At a fold change of two with a $p$-value of 0.05, 268 transcripts were differentially regulated in the presence of mutant SOD1, of which 197 were downregulated. Following thorough ontological analysis it was found that the presence of the mutant SOD1 caused a significant down-regulation of the cells capacity to deal with oxidative stress. In particular the group of genes with an antioxidant response element in their promoter, termed

programmed cell life genes, which would be activated by the transcription factor nuclear factor (erythroid-derived) like 2 *(Nrf2)* were downregulated indicating a failure of the cells to mount a proper therapeutic antioxidant stress response. Subsequent work by Mead et al. (2013) has demonstrated that induction of the *Nrf2* response by S(+)-Apomorphine improves the motor function of the G93A *SOD1* mice underlining the importance of increasing the expression of the programmed cell life genes in a therapeutic strategy to treat ALS.

Vargas et al. (2008) isolated primary astrocytes from both G93A SOD1 mutant and normal littermate control embryonic rats and examined the profile of gene expression using Rat genome 230 2 arrays from Affymetrix. A total of 81 transcripts were differentially expressed by the mutant SOD1 bearing astrocytes with 55 being upregulated. The genes were characterized according to their biological function and were separated into groups; extracellular matrix and cell migration, signaling and receptor activity, transcription, cell proliferation, response to stress, oxidoreductase activity, catalytic activity and unknown. It is interesting to note that despite the fact that different cell types are being investigated there are some similarities in the effect of the *SOD1* mutation in astrocytes and motor neurons from mice and rats.

In the study by Boutahar et al. (2011) they derived primary cortical motor neurons from transgenic mice either with or lacking the G93A *SOD1* mutation. The cells were derived from E14 embryos and maintained in culture for a brief period until the presence of the transgene was confirmed. Some cultures were subjected to stress with hydrogen peroxide or NMDA treatment. Microarrays using Mouse 430 2 GeneChips from Affymetrix were carried out and the results analyzed to determine fold changes of two or more. In the untreated cells 260 transcripts were differentially regulated with 70 upregulated. Following hydrogen peroxide treatment the number was 163 with 59 upregulated and following NMDA treatment 181 with 62 upregulated. Few genes were shared between the three treatments. Gene ontological analysis showed an increase in transcripts associated with the proteasome, some associated with autophagy and cytoskeletal organization and axonal transport. There was some downregulation in transcripts associated with ion transport. It was noted that the presence of the transgene alone increased cell death but this process was mitigated when the additional stresses, hydrogen peroxide or NMDA, were introduced. Hence they identified a small group of genes which were upregulated in the untreated G93A cells but downregulated following the introduction of an additional stress. These genes were; Cathepsin H,(*CSTH*) and Autophagy related 4 homolog D (*ATG4D*) which are involved in protein degradation, tubulin beta-4B chain (*TUBB2C*) and Rho guanine nucleotide exchange factor 11 (*ARHGEF11*) which are cytoskeletal, cell division cycle 25 homolog (*CDC25*) which is involved in cell cycle regulation, solute carrier 7 (*SLC7A12*), DEAD box 43 (*DDX43*) and leucine rich recognition motif 4 (*LRRTM4*) which are involved in the transmembrane domain and transcripts *ACN9, SSBP9* and *TRIM36* involved in transcription regulation.

The studies in cellular culture models all seem to underline the importance of oxidative stress in the pathogenesis of ALS and the difficulty that cells containing mutant SOD1 have in dealing with this metabolic insult.

## SYSTEMS BIOLOGY

Some studies have taken a more broad brush approach to the analysis of microarray data and attempted to incorporate data from a number of sources. Kudo et al. (2010) developed an integrative approach to examine post mortem human material, mouse models and microarray methods. They used the mouse models of ALS, G93A SOD1 and frontotemporal dementia (FTD), P30L Tau and isolated both motor neurons and glia for microarray analysis. Using Agilent whole mouse genome microarrays 251 transcripts were identified as being differentially expressed in at least one of the four comparisons. Of these 186 corresponded to known genes and ontological analysis revealed that the most enriched biological processes were associated with protein modification/phosphorylation, signaling, regulation of muscle contraction, stress response, the immune system and cell communication. The data were confirmed both in further murine tissue by semi-quantitative PCR and in human tissue from sALS cases with tissue microarrays. Hence they used complementary methodology to identify common mechanisms in the SOD1 and TAU mouse models of motor neuron degeneration and human sALS. The genes *CNGA3, CRB1* and *OTUB2* were common to all the models and *CNGA3, CRB1, OTUB2, SLK, DDX58, RSPO2* and *MMP14* were related to sALS. In addition, 13 transcripts found differentially regulated in the SOD1 motor neurons were also found to be altered in their expression in the blood of G93A SOD1 mice.

Baciu et al. (2012) adopted a reanalysis approach where they performed an in depth analysis of a microarray experiment carried out using purified peripheral blood lymphocytes from 11 ALS patients and 11 age matched controls. The RNA had been interrogated using Agilent 4 × 44k human arrays and the authors used a modified BaFL pipeline and at the same time a severe TM4 statistical analysis to remove potential confounding probes sets and "purify" the dataset. The methodology was validated using a previous dataset carried out using similar microarrays and a coronary heart disease study. By carrying out the two different analytical methods the authors attempted to account for errors that might be inherent to either alone. Ultimately, a series of seven genes was identified as being upregulated in the sALS patients, were common to both methods and were verifiable by qRTPCR. These genes were; *B2M, ACTG1, DYNLT1, SKIV2L2, C12orf35, TARDBP* and *ILKAP* (which cross-hybridises with TARDBP).

A recent meta-analysis from Saris et al. (2013) looked to examine the datasets from a number of different microarray studies of both murine and human material. They included seven human studies; three of lumbar cord, two cervical cord and two motor cortex. In addition there were nine studies of transgenic mouse model material; seven of G93A SOD1, one L126delTT SOD1 and one study of the progressive motor neuropathy and wobbler mice. As the authors conclude, despite the variety of input material and different array platforms used, there was a level of consistency in the altered gene expression output. Hence they found altered gene expression in the areas of protein turnover, immune response and apoptosis in both human and murine studies. The mouse

studies also showed consistent differences in genes involved in the lysosome, metal ion binding and mitochondrial function. This study is an interesting new approach to attempt to summate all the data that has been produced thus far. There are likely to be further assays of this type which will take advantage of the large datasets in order to generate informative data with greater statistical power owing to the larger numbers of samples being included.

## CONCLUDING REMARKS

This short review has attempted to summarize the depth of study that has been carried out using transcriptomic technology to examine the gene expression changes that can be identified in comparisons of ALS tissue and controls. With the variety of studies that have been done it is quite difficult to accurately summarize the information but some overall differences can be seen. In terms of mechanisms that have been uncovered in these studies that are influential in the processes that underlie cell death in ALS it can be seen that certain pathways are repeatedly enriched as a consequence of the disease process. These include; oxidative stress, mitochondrial function, apoptosis, cytoskeleton, neuroinflammation and protein processing. It is interesting to note that when peripheral tissues have been used similar processes have been identified as distinguishing the material from individuals with disease. Whilst many of the studies delineated differences between disease and control at the level of the transcriptome an important development has been developing functional studies to demonstrate the validity of the identified changes and then subsequent studies to investigate mechanisms to ameliorate them.

It is likely that the use of the microarray technology is likely to become more of an adjunct to support a body of work as seen in the study from Egawa et al. (2012). In this case the transcriptomic element of the work was used to characterize human induced pluripotent stem cells derived from dermal fibroblasts of normal individuals and several TDP43 mutants. In the past we have seen the transcriptomic study as being the lead in the experimental approach but on this occasion the development of the induced stem cells was the primary goal and the microarray analysis was simply a tool to assist in the characterization of derived cells and define potential routes for drug intervention. It is likely that with the maturity and confidence that several years of transcriptomic research have generated this type of approach will become more widespread.

A further development in the examination of gene expression differences between tissues is going to be the use of next generation sequencing to examination gene expression directly rather than as an indirect measure based upon manufactured probes. The next generation sequencing methodology provides a more detailed examination of the transcriptome which allows for a more in depth examination of novel transcripts, splice junctions and non-coding RNAs in a single step rather than having to carry out multiple different array experiments. As with the early microarray work there is not yet a defined analytical pathway for interpreting the data from RNA Seq experiments but this is progressing rapidly. There is evidence that the two tools are being used in a combinatorial approach (Kogenaru et al., 2012).

Transcriptomics has been used successfully to investigate the mechanisms underlying the processes of cell death and has provided insights into the pathways that may be dysregulated. As the tools are becoming more established it is likely that more studies will use them to assist in the functional studies needed to develop new treatments for ALS.

## AUTHOR CONTRIBUTIONS

Paul R. Heath devised and produced the manuscript, Janine Kirby and Pamela J. Shaw contributed editing and revising of the manuscript.

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# Differential autophagy power in the spinal cord and muscle of transgenic ALS mice

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Amyotrophic lateral sclerosis (ALS) is a motoneuron disease characterized by misfolded proteins aggregation in affected motoneurons. In mutant SOD1 (mutSOD1) ALS models, aggregation correlates to impaired functions of proteasome and/or autophagy, both essential for the intracellular chaperone-mediated protein quality control (PQC), and to a reduced mutSOD1 clearance from motoneurons. Skeletal muscle cells are also sensitive to mutSOD1 toxicity, but no mutSOD1 aggregates are formed in these cells, that might better manage mutSOD1 than motoneurons. Thus, we analyzed in spinal cord and in muscle of transgenic (tg) G93A-SOD1 mice at presymptomatic (PS, 8 weeks) and symptomatic (S, 16 weeks) stages, and in age-matched control mice, whether mutSOD1 differentially modulates relevant PQC players, such as HSPB8, BAG3, and BAG1. Possible sex differences were also considered. No changes of HSPB8, BAG3, and BAG1 at PS stage (8 weeks) were seen in all tissues examined in tg G93A-SOD1 and control mice. At S stage (16 weeks), HSPB8 dramatically increased in skeletal muscle of tg G93A-SOD1 mice, while a minor increase occurred in spinal cord of male, but not female tg G93A-SOD1 mice. BAG3 expression increased both in muscle and spinal cord of tg G93A-SOD1 mice at S stage, BAG1 expression increased only in muscle of the same mice. Since, HSPB8-BAG3 complex assists mutSOD1 autophagic removal, we analyzed two well-known autophagic markers, LC3 and p62. Both LC3 and p62 mRNAs were significantly up-regulated in skeletal muscle of tg G93A-SOD1 mice at S stage (16 weeks). This suggests that mutSOD1 expression induces a robust autophagic response specifically in muscle. Together these results demonstrate that, in muscle mutSOD1-induced autophagic response is much higher than in spinal cord. In addition, if mutSOD1 exerts toxicity in muscle, this may not be mediated by misfolded proteins accumulation. It remains unclear whether in muscle mutSOD1 toxicity is related to aberrant autophagy activation.

**Keywords: amyotrophic lateral sclerosis, motoneurons, autophagy, HSPB8, BAG3, BAG1, protein quality control**

## INTRODUCTION

Autophagy is a fundamental intracellular degradative pathway activated to respond to the accumulation of aberrantly folded (misfolded) proteins (Mizushima and Komatsu, 2011). Autophagy is required for the cellular protein quality control (PQC) system, which also includes molecular chaperones and the ubiquitin-proteasome degradative system (UPS) (Carra et al., 2012). These systems work together protecting cells particularly sensitive to misfolded protein toxicity, such as motoneurons (Rusmini et al., 2010; Bendotti et al., 2012; Carra et al., 2013). Motoneurons are major targets of toxicity in diseases linked to mutant proteins prone to misfold, such as in amyotrophic lateral sclerosis (ALS) (Pasinelli and Brown, 2006; Rusmini et al., 2010; Sau et al., 2011; Strong and Yang, 2011; Carra et al., 2012, 2013; Robberecht and Philips, 2013). Most ALS cases appear in sporadic (sALS) forms; only about 10–15% have familial (fALS) history, and are clinically indistinguishable from sALS. fALSs have been associated with mutations in different genes, such as the superoxide dismutase 1 (SOD1), TAR DNA-binding protein 43 (TDP-43), fused in sarcoma/translocated in liposarcoma protein (FUS/TLS), optineurin (Robberecht and Philips, 2013), or the C9ORF72 gene (Dejesus-Hernandez et al., 2011; Renton et al., 2011; Ash et al., 2013; Lashley et al., 2013; Mori et al., 2013). Notably, also the wild type (wt) forms of the mutated fALS proteins may show aberrant behavior in sALS (e.g., oxydized wtSOD1, cleaved C-terminus of wtTDP-43, etc.) (Neumann et al., 2006; Daoud et al., 2009; Bosco and Landers, 2010; Bosco et al., 2010), suggesting the existence of a common pathological mechanism. An explanation for this is that these proteins (either the modified wt or the mutant forms) have the propensity to misfold and aggregate forming

insoluble inclusions that are a key neuropathological hallmark of ALS. Inclusions may alter several cellular functions, such as axonal transport, mitochondrial, and/or degradative activities, thereby leading or contributing to motoneuron death (Pasinelli and Brown, 2006; Cozzolino et al., 2008; Seetharaman et al., 2009).

In recent years, emerging evidences support the idea that also non-neuronal cells (e.g., surrounding astrocytes or Schwann cells, chemotactically attracted microglial cells, and target muscle cells) might contribute to disease onset and progression (Boillée et al., 2006), by making motoneuronal cells more sensitive to protein toxicity. For example, selective expression of mutant SOD1 (mutSOD1) in skeletal muscle induced atrophy associated with the loss of motoneurons in the anterior horn of the spinal cord (Dobrowolny et al., 2008a; Corti et al., 2009; Wong and Martin, 2010). However, mutSOD1 largely accumulates in spinal cord of transgenic mice expressing human G93A-SOD1 (Tg G93A-SOD1) (Cheroni et al., 2005, 2009; Basso et al., 2006, 2009; Bendotti et al., 2012), but not in skeletal muscle of the same mice at any stage of disease (Galbiati et al., 2012; Wei et al., 2012); thus, muscle cells better cope with misfolded mutSOD1 species than motoneuronal cells. This may be due to an higher muscular degradative capabilities compared to motoneurons (Onesto et al., 2011). In fact, in both cells, mutSOD1 clearance involves both UPS and autophagy, but both the proteasome and autophagy activities are higher in muscle cells than in motoneuronal cells.

The interplay between UPS and autophagy is finely regulated through a mechanism that involves the co-chaperones BAG1 and BAG3, which act as a switch between the two pathways (Luders et al., 2000; Carra et al., 2008a; Gamerdinger et al., 2009; Arndt et al., 2010; Zhang and Qian, 2011). BAG1 is expressed at relatively higher levels in young tissues, paralleled by higher UPS activity, while BAG3 is expressed at higher levels in aged tissues, characterized by higher autophagic activity (Gamerdinger et al., 2009), and the BAG3:BAG1 ratio determines the fate of client proteins to be degraded through either UPS or autophagy. Indeed, BAG1 routes substrates to UPS by interacting with a complex formed by the chaperone HSC70 and the ubiquitinating enzyme CHIP. UPS overwhelming or impairment, due to misfolded proteins, results in upregulation of BAG3 and its partner, the chaperone HSPB8 (a small heat shock protein involved in misfolded proteins recognition) (Wang et al., 2008; Du et al., 2009; Crippa et al., 2010b; Gentilella and Khalili, 2011; Carra et al., 2013). The increased BAG3:BAG1 ratio favors a stoichiometric HSC70/CHIP complex association to BAG3/HSPB8 complex, allowing the p62-mediated autophagic removal of CHIP-ubiquitinated substrates (Crippa et al., 2010b; Gamerdinger et al., 2011; Zhang and Qian, 2011; Carra et al., 2012, 2013). Interestingly, we found that mutSOD1 blocks the autophagic flux in motoneurons, but not in muscle cells (Onesto et al., 2011). This autophagic flux blockage can be counteracted by HSPB8, which participates together with BAG3/HSC70/CHIP in the autophagic clearance of several different misfolded proteins (polyQ containing proteins, mutSOD1, a truncated form of TDP-43) (Carra et al., 2005, 2008a,b, 2013; Crippa et al., 2010b). In addition, HSPB8 induces translation attenuation via phosphorylation of eIF2alpha (Carra et al., 2009), thereby decreasing the amount of aggregation-prone proteins to levels manageable by PQC.

In this study, we analyzed whether spinal cord, which comprises the two main cell targets of SOD1 toxicity, namely motoneuronal and astroglial cells, and skeletal muscle, differentially activate the HSPB8 mediated PQC system in response to mutSOD1 in tg G93A-SOD1 mice. The results demonstrate that the autophagic response of muscle tissue to the mutSOD1 expression is much higher than that found in the spinal cord of the same mice, suggesting that if mutSOD1 exerts toxicity in muscle, this may not be mediated by misfolded protein accumulation.

## MATERIALS AND METHODS

### ANIMALS

All the procedures involving animals and their care were carried out following the institutional guidelines and in accordance with national (D.L. no. 116, G.U. suppl. 40, Feb. 18, 1992), and international laws and policies (EEC Council Directives 86/609, OJ L 358, 1 DEC.12, 1987), and were approved by the Italian Institute of Technology Animal Care Committee. All the animals were kept under controlled temperature and humidity conditions with standardized dark/light cycles of 12 h each. Food (standard pellets) and water were supplied *ad libitum*. BL6JL Tg(SOD1)2Gur/J (Stock number 002297, Charles River, Wilmington, MA, USA) male mice or BL6JL-Tg(SOD1*G93A)2Gur/J (Stock number 002726, Charles River) male mice were crossed with wt female mice purchased (Stock number 100012, Charles River) or obtained in house by crossing C57Bl/6J female and SJL male mice. All the experiments were performed in mice coming from the F1 generation of the cross described above. Non-transgenic littermates were used as controls (NTg). Mice were genotyped by PCR on tail DNA as previously described (Gurney et al., 1994), using REDExtract-N-Amp Tissue PCR kit (Sigma-Aldrich, St. Louis, MO, USA). To evaluate disease stages, starting from the 8th week of age and twice a week, mice were tested for deficit by rotarod, and hanging wire by the same operator as previously described (Palazzolo et al., 2009). Body weight loss was also monitored. Disease onset was set as the time at which the mouse permanently starts to lose body weight. Four mice per group were anesthetized with isoflurane and sacrificed at 8 or 16 weeks of age, corresponding to presymptomatic (PS) or symptomatic (S) stage of disease. Quadriceps muscles and spinal cord were rapidly collected after the sacrifice, snap frozen in liquid nitrogen, and conserved at −80°C until RNA and protein extraction.

### RNA EXTRACTION

Total RNA from frozen spinal cords or muscles was extracted using the standard TRI Reagent protocol based on the method developed by Chomczynski and Sacchi (Chomczynski and Sacchi, 1987). RNA was subsequently extracted in accordance to manufacturer's protocol (Sigma-Aldrich). The precipitated RNA was dissolved in RNase-free water. Total RNA (1 μg) was treated for 15 min at room temperature with 1 U of DNaseI (Sigma-Aldrich). Samples were reverse-transcribed using the High-Capacity cDNA Reverse Transcription Kit (Life Technologies Corporation, Carlsbad, CA, USA) according to the manufacturer's instructions, in a 20 μL volume. Primers for selected genes

were designed via the Primer Express software (Life Technologies Corporation) and purchased from MWG Biotech (Ebersberg, Germany). Primer sequences were as follows: mouse HSPB8: 5′- ATA CGT GGA AGT TTC AGG CA -3′ (forward), 5′- TCC TTT GAC CTA ACG CAA CC -3′ (reverse); mouse BAG3: 5′- ATG GAC CTG AGC GAT CTC A -3′ (forward), 5′- CAC GGG GAT GGG GAT GTA -3′ (reverse); mouse BAG1: 5′- GAA ACA CCG TTG TCA GCA CT -3′ (forward), 5′- GCT CCA CTG TGT CAC ACT C -3′ (reverse); mouse MAP-LC3b: 5′- CGT CCT GGA CAA GAC CA -3′ (forward), 5′- CCA TTC ACC AGG AGG AA -3′ (reverse); mouse p62: 5′- AGG GAA CAC AGC AAG CT -3′ (forward), 5′- GCC AAA GTG TCC ATG TTT CA -3′ (reverse); mouse GAPDH: 5′-CCA GAA CAT CAT CCC TGC AT-3′ (forward), 5′-CAG TGA GCT TCC CGT TCA-3′ (reverse). The evaluated efficiency of each set of primers was close to 100% for both target and reference gene. Real-time PCR was performed using the CFX 96 Real Time System (Bio-Rad Laboratories, Hercules, CA, USA), in a 10 μL total volume, using the iTaq SYBR Green Supermix (BioRad), and with 500 nmol primers. PCR cycling conditions were as follows: 94°C for 10 min, 40 cycles at 94°C for 15 s, and 60°C for 1 min. Melting curve analysis was performed at the end of each PCR assay to control specificity. Data was expressed as Ct values and used for the relative quantification of targets with the ΔΔCt calculation to give N-fold changes in gene expression. Values were normalized to those of GAPDH. To exclude potential bias due to averaging data transformed through the equation $2^{-\Delta\Delta Ct}$, all statistics were performed with ΔCt values. Each experiment was carried out with four independent samples, and each sample was run in duplicate wells.

The BAG3:BAG1 relative ratio was obtained considering the group of 8 weeks-old NTg female mice as control for the basal physiological condition of the PQC system. In particular, for each sample a single BAG3:BAG1 ratio was determined [measuring the $(\text{BAG3}\ 2^{-\Delta\Delta Ct}/\text{BAG1}\ 2^{-\Delta\Delta Ct})_{\text{sample}}$] and the mean BAG3:BAG1 ratio of control was subtracted [$(\text{BAG3}\ 2^{-\Delta\Delta Ct}/\text{BAG1}\ 2^{-\Delta\Delta Ct})_{\text{control}}$]. Finally, the resulting values were normalized by the mean BAG3:BAG1 ratio of the control 8 weeks-old NTg female mice.

### WESTERN BLOTTING AND FILTER RETARDATION ASSAY (WB AND FRA)

Total proteins from frozen spinal cord or muscles were extracted in 1% SDS using the standard TRI Reagent protocol, in accordance with the manufacturer's protocol (Sigma-Aldrich). Protein concentration was determined with the bicinchoninic acid method (BCA assay, EuroClone, Pero, Milan, Italy). WB analysis was performed on 12% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis loading 15 μg of total proteins. Samples were then electrotransferred to Nitrocellulose membrane (Bio-Rad) using the Trans-Blot Turbo (Bio-Rad). The membranes were treated with a blocking solution containing 5% non-fat dried milk powder (EuroClone) in TBS-T for 1 h and then incubated with the primary antibodies: (1) home-made rabbit polyclonal anti-HSPB8 (kindly provided by Dr Jacques Landry, Quèbec, Canada, dilution 1:1000); (2) rabbit-polyclonal anti-BAG3 antibody (Abcam, Cambridge, UK; dilution 1:1000); (3) rabbit-polyclonal anti-BAG1 (Santa Cruz Biotechnology, Dallas, Texas, USA; dilution 1:1000); (4) rabbit polyclonal anti-LC3 (Sigma; dilution 1:1000); (5) rabbit polyclonal anti-SQSTM1/p62 (Abcam; dilution 1:1000); (6) rabbit-polyclonal anti-GAPDH (Santa Cruz Biotech; dilution 1:1000); (7) rabbit-polyclonal anti-SOD1 (SOD-100; StressMarq, Victoria, BC, Canada; dilution 1:1000). Immunoreactivity was detected using goat anti-rabbit (sc-2004, Santa Cruz Biotech, dilution 1:5000) secondary peroxidase-conjugated antibody. Immunoreactivity was then visualized using the enhanced chemiluminescence detection kit reagent (ECL prime Western Blotting Substrate, GE Healthcare, Maidstone, UK). The same membranes were subsequently processed with different antibodies to detect the levels of different proteins in the same samples loaded on the gel, after stripping for 10 min at room temperature (StripABlot, EuroClone). Each experiment was carried out twice, with four independent samples.

FRA was performed using a slot-blot apparatus (Bio-Rad). 3 μg of total proteins were loaded on a 0.2 μm cellulose acetate membrane (Whatman, GE Healthcare), pre-incubated with 20% methanol, washed with water and then incubated with 1% SDS solution. After slight vacuum filtration, slot-blot membranes were probed as described for WB.

A ChemiDoc XRS System (Bio-Rad) was used for the image acquisition of WB and FRA.

### STATISTICAL ANALYSIS

Statistical analysis was performed through two-tailed Student *t-test* for comparisons between 8 and 16 weeks mice of the same group (NTg/Tg wtSOD1/Tg G93A-SOD1) and two-way analysis of variance (ANOVA) for group comparisons, using the PRISM software (GraphPad, San Diego, CA, USA). Specific group pair(s) statistical difference was determined by the Bonferroni *post-hoc* test. Data were expressed as mean ± SD of four independent samples.

## RESULTS

Our study has been performed on 8 (presymptomatic stage, PS) and 16 weeks-old (symptomatic stage, S) Tg G93A-SOD1 mice, compared to age-matched non-transgenic (NTg) and transgenic human wild-type SOD1 (Tg wtSOD1) mice. In addition, since gender differences have been reported to affect the age of onset and disease progression both in ALS patients (Kurtzke, 1982; Giagheddu et al., 1983; Rudnicki, 1999; Manjaly et al., 2010; McCombe and Henderson, 2010; Lee et al., 2013a) and rodent models of ALS (Veldink et al., 2003; Suzuki et al., 2007), with male gender being more susceptible, we analyzed males and females separately. Based on our data on the protective role of HSPB8 in ALS, which is mediated by autophagy, we analyzed the expression of this small chaperone in the spinal cord and muscle of Tg G93A-SOD1 mice and control mice. We also analyzed the expression of the two co-chaperones BAG3 and BAG1, which may mediate the PQC activity by selecting the proper degradative system to be activated in cells in response to proteotoxicity. Finally, we analyzed two widely used autophagic markers, LC3 and p62. Using homogenates of spinal cord and skeletal muscle derived from Tg G93A-SOD1 mice and age-matched NTg or Tg wtSOD1 mice, we first compared the levels of wtSOD1 and mutSOD1 in both the soluble and insoluble fractions in 8 (corresponding to PS stage) and 16 weeks (corresponding to S stage) male and female mice. As

shown in **Figure 1**, soluble monomeric wtSOD1 and mutSOD1 were analyzed by western blot (WB; **A**: spinal cord; **B**: muscle). In the spinal cord of Tg mice the levels of wtSOD1 and mutSOD1 proteins were similar, while in skeletal muscle mutSOD1 levels were apparently lower as compared to wtSOD1 and no gender differences were observed. Using motoneuronal and muscle ALS models as well as tg G93A-SOD1 mice, we recently demonstrated that mutSOD1 accumulated both in motoneuronal cells and in spinal cord of tg mice (already at PS, increasing at S stage), but did not accumulate in ALS muscle cells and ALS muscle tissues at any age tested (Onesto et al., 2011; Galbiati et al., 2012). These data were recently confirmed and extended by Wei and coll. (Wei et al., 2013) using a different strain of tg G93A-SOD1 mice. Thus, here we repeated these experiments to provide a general view of mutSOD1 behavior in our mice. A representative filter retardation assay (FRA; **C**: spinal cord; **D**: muscle) illustrating the corresponding insoluble fractions of mutSOD1 is reported. The FRA analysis confirmed the presence of relevant amounts of mutSOD1 insoluble species in samples of S stage tg G93A-SOD1 mice. In muscle tissue, no mutSOD1 insoluble species were present, with the exception of samples obtained from tg G93A-SOD1 male mice at S stage (16 weeks), suggesting that muscle tissue may better cope with the misfolded fraction of mutSOD1 as compared to cells in the spinal cord.

Since we already showed that overexpression of HSPB8 prevents mutSOD1 intracellular accumulation by facilitating its clearance through autophagy (Crippa et al., 2010a,b), we analyzed HSPB8 mRNA levels in the spinal cord and skeletal muscle in response to wt or mutSOD1 expression. In spinal cord, we observed a significant increase of HSPB8 mRNA transcript levels in response to mutSOD1 expression in S stage (16 weeks) Tg G93A-SOD1 male mice as compared to age-matched NTg or Tg wtSOD1 mice (**Figure 2A**) (Crippa et al., 2010b). Interestingly, Tg G93A-SOD1 female mice did not show any significant increase of HSPB8 transcript level in response to mutSOD1expression, suggesting the existence of gender differences in the PQC system activated by misfolded proteins in spinal cord. In male 16-week-old NTg mice, the expression level of HSPB8 tended to decrease, even if it did not become statistically significant. However, we already shown that HSPB8 expression significantly decreases when older NTg mice (age-matched with Tg G93A-SOD1 mice at end stage of disease) were analyzed (Crippa et al., 2010a,b). In contrast to what we found in spinal cord, in the skeletal muscles of S stage (16 weeks) Tg G93A-SOD1 mice we observed a dramatic increase of HSPB8 mRNA (up to 8-fold) (**Figure 2B**). Importantly, this HSPB8 mRNA upregulation occurred in both sexes. HSPB8 mRNA levels were unaffected in both sexes in NTg and Tg wtSOD1 mice at all ages considered.

The robust induction of HSPB8 expression in response to mutSOD1 in muscle might serve to initiate and/or facilitate the clearance of aberrant mutSOD1 species. This may avoid the aggregation of misfolded protein and the accumulation to levels that cannot be managed by the PQC system. Considering that mutSOD1 species are degraded by the saturable UPS, but it can be preferentially degraded by the highly efficient autophagic system in presence of high levels of HSPB8 (Crippa et al., 2010b) and that the pro-degradative activity of HSPB8 is mediated by its partner BAG3, we analyzed the expression levels of BAG3 in both spinal cord and muscle (Carra et al., 2008b; Carra, 2009). We found that in the spinal cord of both male and female mutSOD1 mice BAG3 expression remains unchanged at PS stage (8 weeks), but it was significantly increased by approximately 3-fold compared to NTg or Tg wtSOD1 mice at S stage (16 weeks, **Figure 3A**). Furthermore, the mutSOD1-induced appearance of symptoms correlated with a great response of the HSPB8-BAG3-mediated PQC system also in muscle (**Figure 3B**). Curiously, in female mice, BAG3 expression differed from that of HSPB8, since it was upregulated by mutSOD1 both in spinal cord and in muscle of tg G93A-SOD1 mice at S stage.

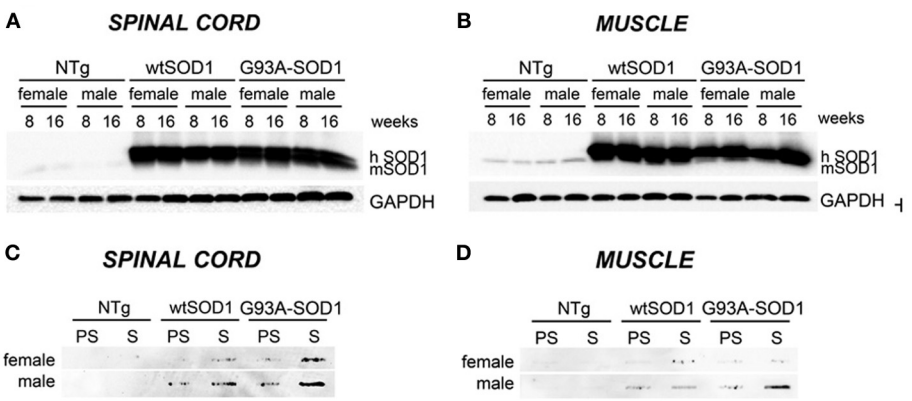


**FIGURE 1 | Biochemical properties of wtSOD1 and mutSOD1 in spinal cord and muscle of ALS animal models.** Western Blot (WB) analysis and Filter Retardation Assay (FRA) on proteins extracted from whole spinal cord (**A**, WB; **C**, FRA) or quadriceps muscles (**B**, WB; **D**, FRA) of female and male non-transgenic (NTg) mice, of mice expressing the wild type human SOD1 transgene (wtSOD1), and of mice expressing the G93A mutant form of human SOD1 (G93A-SOD1), at 8 (corresponding to presymptomatic stage, PS, in Tg G93A-SOD1 mice) or 16 weeks (corresponding to symptomatic stage, S, in Tg G93A-SOD1 mice). GAPDH was used to normalize protein loading. hSOD1, transgenic human SOD1; mSOD1, endogenous murine SOD1.

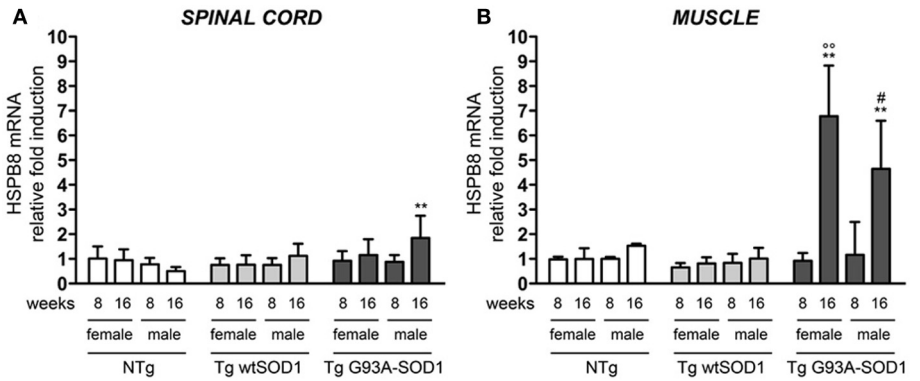


**FIGURE 2 | Changes in HSPB8 levels in response to wt or mutSOD1 expression in spinal cord and muscle of ALS animal models.** RT-qPCRs were performed on total RNA extracted from whole spinal cord **(A)** or quadriceps muscles **(B)** of female and male non-transgenic (NTg) mice, of mice expressing the wild type human SOD1 transgene (Tg wtSOD1), and of mice expressing the G93A mutant form of human SOD1 (Tg G93A-SOD1), at 8 (corresponding to presymptomatic stage, PS, in Tg G93A-SOD1 mice) or 16 weeks (corresponding to symptomatic stage, S, in Tg G93A-SOD1 mice). Data have been normalized to the amount of GAPDH mRNA, expressed relative to the levels determined in age-matched PS female NTg mice, taken as internal reference, and expressed as fold changes. Data are mean ± SD of four independent replicates. **(A)** $^{**}p < 0.01$ vs. age- and sex-matched NTg mice. **(B)** $^{**}p < 0.01$ vs. age- and sex-matched NTg mice and Tg wt SOD1 mice; $^{\circ\circ}p < 0.01$ vs. PS (8 weeks) female Tg G93A-SOD1; $^{\#}p < 0.05$ vs. PS (8 weeks) male Tg G93A-SOD1.

In light of our previous results showing that muscle cells possess a higher UPS activity with respect to motoneuronal cells (Onesto et al., 2011), we analyzed the expression of BAG1, which is involved in the re-routing of the misfolded proteins to proteasome-mediated substrates degradation. In spinal cord, BAG1 transcript levels were not affected by mutSOD1 expression (**Figure 3C**). On the contrary, BAG1 expression increased 2-folds in the skeletal muscle of S stage Tg G93A-SOD1 mice compared to all other groups analyzed (**Figure 3D**). This increase was similar in both sexes. These data suggest that proteasome-mediated substrate degradation is highly favored in muscle cells under proteotoxic conditions (e.g., misfolded mutSOD1 overexpression). We also estimated the ratio BAG3:BAG1 in each sample (**Figures 3E,F**) and found that this is always more elevated in muscle tissue, both in control and tg G93A-SOD1 mice, suggesting a predominance of the autophagic pathway, but it reaches higher levels at S stage of disease in response to mutSOD1. In the case of spinal cord, the BAG3:BAG1 ratio increases only at S stage (16 weeks) both in male and female tg G93A-SOD1 mice, suggesting that misfolded mutSOD1 could be re-routed to the autophagic system at this stage of disease (possibly not fully removed because of autophagic flux blockage).

Next, we asked whether the upregulation of the transcript levels of the genes described above results in an increase in protein expression. As shown in **Figure 4**, in the spinal cord **(A)**, HSPB8, BAG1, and BAG3 protein levels correlated with the data of gene transcription observed in the same mice. In skeletal muscle, we found that HSPB8, BAG3, and BAG1 were all up-regulated in Tg G93A-SOD1 mice at the S stage (16 weeks) of disease compared to Ntg and Tg wtSOD1 mice (**Figure 4B**). In the case of BAG3, the protein was up-regulated also at the PS stage (8 weeks). Little variation was observed in Tg G93A-SOD1 mice at S stage, while no major sex differences were noted. Also these data were perfectly in line and corroborate the mRNA expression analysis data reported above.

Since HSPB8 overexpression has beneficial effects in cell models of fALS, it is upregulated in human tissues from patients, and its mode of action requires the partner BAG3 and a functional autophagy system, we then analyzed the expression of two widely used markers of the autophagic flux: LC3 (which is overexpressed and associated with the autophagosome when autophagy is activated) and p62 (which is also upregulated during autophagy activation and is responsible for the insertion of ubiquitinated misfolded protein species into the autophagosomes). We found a moderate increase of LC3 mRNA selectively in the spinal cord of S stage (16 weeks) Tg G93A-SOD1 female mice (**Figure 5A**). In contrast, in skeletal muscle of Tg G93A-SOD1 mice (**Figure 5B**), LC3 expression was robustly increased at S stage both in male and female animals. p62 mRNA levels remained unchanged in spinal cord (**Figure 5C**), while they were increased in muscle of S stage female mice (**Figure 5D**). Notably, p62 levels increased also in Tg G93A-SOD1 male mice at S stage, even if this induction was not statistically significant.

Protein levels of LC3 and p62 as well as the conversion of free LC3-I to autophagosome-associated lipidated form of LC3-II were analyzed by WB (**Figure 6**). In spinal cord samples no major variations of p62 protein were observed, while the levels of LC3 protein were higher in Tg G93A-SOD1 mice (**Figure 6A**). Surprisingly, no conversion of LC3-I to LC3-II was observed, suggesting that the autophagic process is not massively activated in the spinal cord at these stages of disease. In skeletal muscle, p62 protein levels robustly increased in Tg G93A-SOD1 mice at both ages and in both sexes (**Figure 6B**). Moreover, at S stage (16 weeks), in skeletal muscle samples, the lipidated form of LC3 (LC3-II), which is the only one capable to associate with the newly formed autophagosomes, was increased in Tg G93A-SOD1 mice.

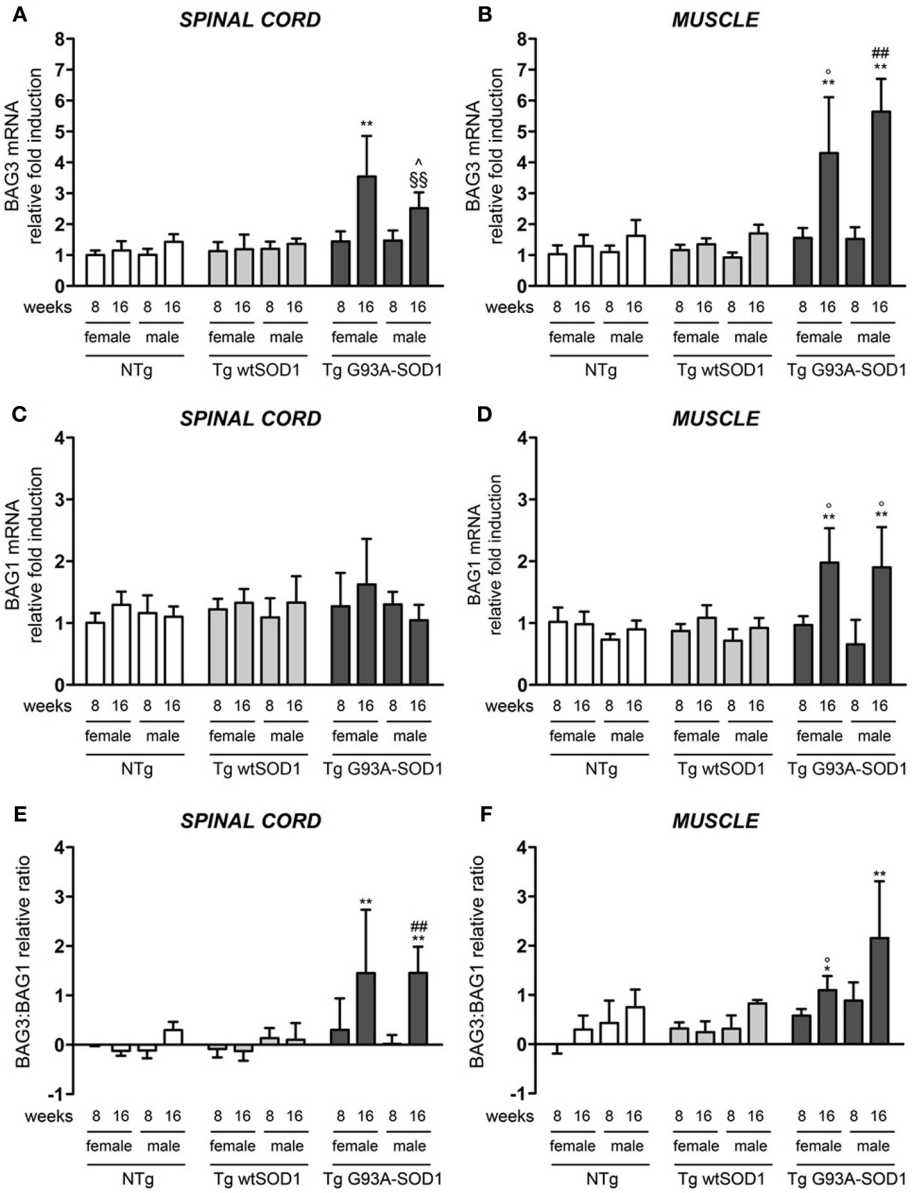


**FIGURE 3 | BAG3 and BAG1 expression in spinal cord and muscle of ALS animal models.** RT-qPCRs were performed on total RNA extracted from whole spinal cord **(A,C)** or quadriceps muscles **(B,D)** of female and male non-transgenic (NTg) mice, of mice expressing the wild type human SOD1 transgene (Tg wtSOD1), and of mice expressing the G93A mutant form of human SOD1 (Tg G93A-SOD1), at 8 (corresponding to presymptomatic stage, PS, in Tg G93A-SOD1 mice) or 16 weeks (corresponding to symptomatic stage, S, in Tg G93A-SOD1 mice). Data have been normalized to the amount of GAPDH mRNA, expressed relative to the levels determined in age-matched PS female NTg mice, taken as internal reference, and expressed as fold changes. Data are mean ± SD of four independent replicates. **(A)** RT-qPCR on BAG3 mRNA expression levels in whole spinal cord. $^{**}p < 0.01$ vs. age- and sex-matched NTg and Tg wtSOD1 mice; $^{\wedge}p < 0.05$ vs. age- and sex-matched NTg mice; $^{\S\S}p < 0.01$ vs. age- and sex-matched Tg wtSOD1 mice. **(B)** RT-qPCR on BAG3 mRNA expression levels in quadriceps muscles. $^{**}p < 0.01$ vs. age- and sex-matched NTg and Tg wtSOD1 mice; $^{\circ}p < 0.05$ vs. PS (8 weeks) female Tg G93A-SOD1; $^{\#\#}p < 0.01$ vs. PS (8 weeks) male Tg G93A-SOD1. **(C)** RT-qPCR on BAG1 mRNA expression levels in whole spinal cord. **(D)** RT-qPCR on BAG1 mRNA expression levels in quadriceps muscles. $^{**}p < 0.01$ vs. age- and sex-matched NTg and Tg wtSOD1 mice; $^{\circ}p < 0.05$ vs. sex-matched PS (8 weeks) Tg G93A-SOD1. **(E)** BAG3:BAG1 relative ratio of mRNA expression levels in whole spinal cord. Data represent variations of the relative levels of BAG3 and BAG1 normalized over the relative BAG3 and BAG1 levels of age-matched PS (8 weeks) female NTg mice (taken as internal reference, see Materials and Methods for details). $^{**}p < 0.01$ vs. age- and sex-matched NTg and Tg wtSOD1 mice; $^{\#\#}p < 0.01$ vs. PS (8 weeks) male Tg G93A-SOD1. **(F)** BAG3:BAG1 relative ratio of mRNA expression levels in quadriceps muscles. Data have been calculated as in **(E)**. $^{*}p < 0.05$ and $^{**}p < 0.01$ vs. age- and sex-matched NTg and Tg wtSOD1 mice; $^{\circ}p < 0.05$ vs. PS (8 weeks) male Tg G93A-SOD1.

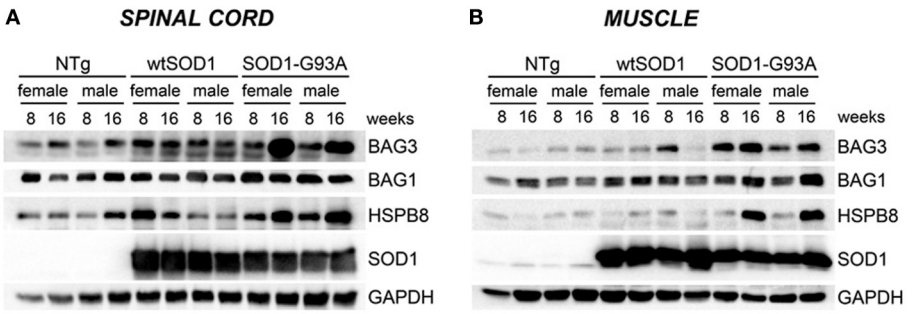


**FIGURE 4 | HSPB8-BAG1-BAG3 protein levels in spinal cord and muscle of ALS animal models.** Western Blot (WB) analysis on proteins extracted from whole spinal cord **(A)** or quadriceps muscles **(B)** of female and male non-transgenic (NTg) mice, of mice expressing the wild type human SOD1 transgene (Tg wtSOD1), and of mice expressing the G93A mutant form of human SOD1 (Tg G93A-SOD1), at 8 (corresponding to presymptomatic stage, PS, in Tg G93A-SOD1 mice) or 16 weeks (corresponding to symptomatic stage, S, in Tg G93A-SOD1 mice). GAPDH was used to normalize protein loading.

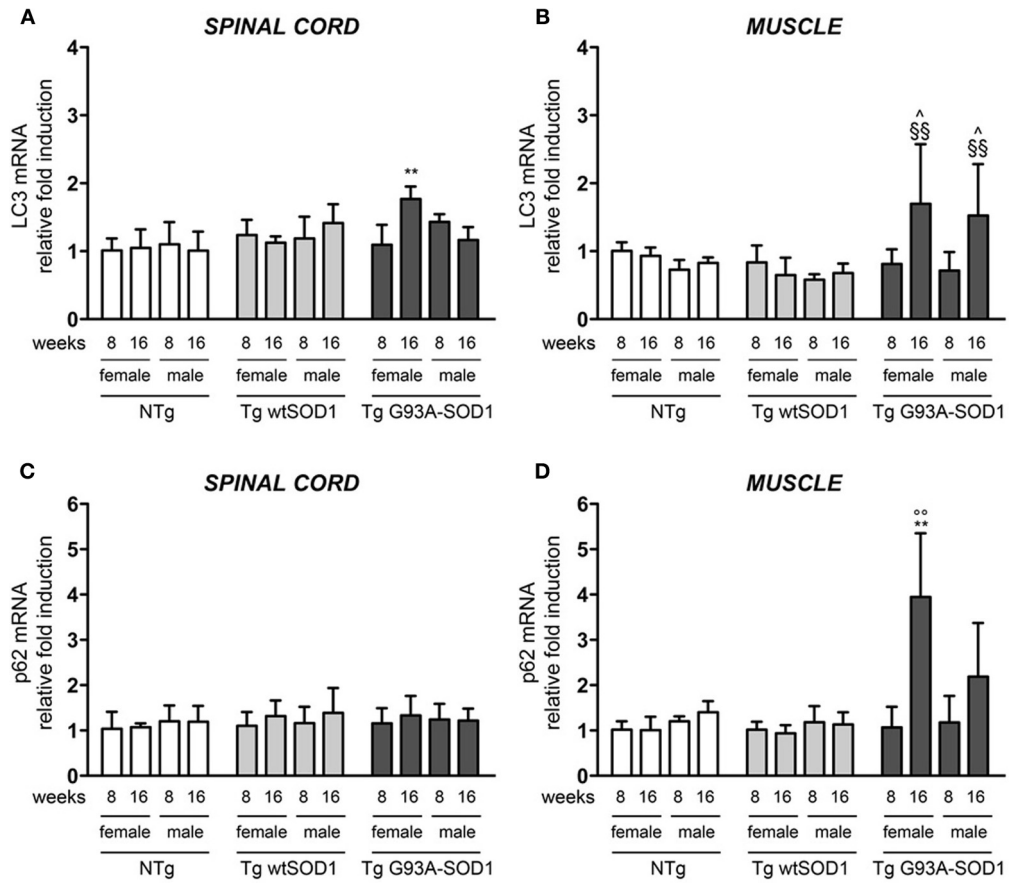


**FIGURE 5 | LC3 and p62 expression in spinal cord and muscle of ALS animal models.** RT-qPCRs were performed on total RNA extracted from whole spinal cord **(A,C)** or quadriceps muscles **(B,D)** of female and male non-transgenic (NTg) mice, of mice expressing the wild type human SOD1 transgene (Tg wtSOD1), and of mice expressing the G93A mutant form of human SOD1 (Tg G93A-SOD1), at 8 (corresponding to presymptomatic stage, PS, in Tg G93A-SOD1 mice) or 16 weeks (corresponding to symptomatic stage, S, in Tg G93A-SOD1 mice). Data have been normalized to the amount of GAPDH mRNA, expressed relative to the levels determined in age-matched PS female NTg mice, taken as internal reference, and expressed as fold changes. Data are mean ± SD of four independent replicates. **(A)** RT-qPCR on LC3 mRNA expression levels in whole spinal cord. $^{**}p < 0.01$ vs. age- and sex-matched NTg and Tg wtSOD1 mice. **(B)** RT-qPCR on LC3 mRNA expression levels in quadriceps muscles. $^{\wedge}p < 0.05$ vs. age- and sex-matched NTg mice; $^{\S\S}p < 0.01$ vs. age- and sex-matched Tg wtSOD1 mice. **(C)** RT-qPCR on p62 mRNA expression levels in whole spinal cord. **(D)** RT-qPCR on p62 mRNA expression levels in quadriceps muscles. $^{**}p < 0.01$ vs. age- and sex-matched NTg and Tg wtSOD1 mice; $^{\circ\circ}p < 0.01$ vs. PS female (8 weeks) Tg G93A-SOD1.

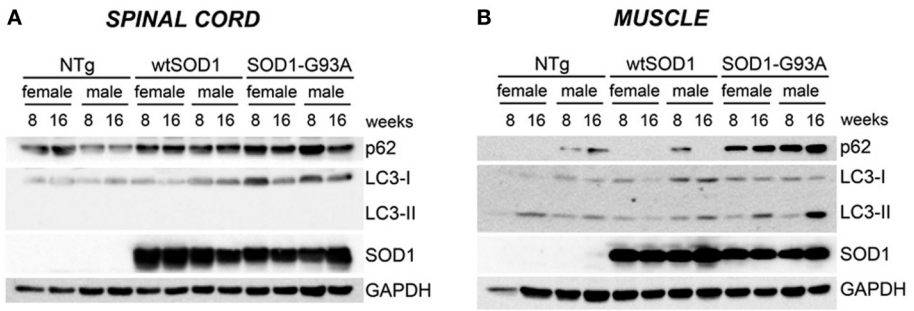


**FIGURE 6 | Autophagic-markers (p62 and LC-3) protein levels in spinal cord and muscle of ALS animal models.** Western Blot (WB) analysis on proteins extracted from whole spinal cord **(A)** or quadriceps muscles **(B)** of female and male non-transgenic (NTg) mice, of mice expressing the wild type human SOD1 transgene (Tg wtSOD1), and of mice expressing the G93A mutant form of human SOD1 (Tg G93A-SOD1), at 8 (corresponding to presymptomatic stage, PS, in Tg G93A-SOD1 mice) or 16 weeks (corresponding to symptomatic stage, S, in Tg G93A-SOD1 mice). GAPDH was used to normalize protein loading.

Collectively, these data strongly suggest that muscle, probably because of higher HSPB8, BAG1, and BAG3 expression, not only may better cope with misfolded mutant proteins, but also has a more intense autophagic response power compared to cells in the spinal cord.

## DISCUSSION

In a previous study, we showed that spinal cord and muscle differently cope with mutSOD1 (Onesto et al., 2011). In fact, in Tg G93A-SOD1 mice mutSOD1 tends to accumulate in the spinal cord, while no accumulation of high molecular weight (HMW) species was found in muscles (Galbiati et al., 2012). Thus, the specific ability of spinal cord and skeletal muscle to handle misfolded protein aggregation and clearance (Cheroni et al., 2005, 2009; Basso et al., 2006, 2009; Bendotti et al., 2012; Galbiati et al., 2012; Wei et al., 2012) could be related to differences in their response to proteotoxicity, mediated by the PQC system. In this study we further investigated this aspect and found that mutSOD1 poorly accumulates in skeletal muscle, and only a mild increase of SDS-insoluble species of mutSOD1 is detectable in this tissue in Tg G93A-SOD1 male mice at the S stage (16 weeks), but not in female mice. This suggests that differences in the PQC system may also exist between the two sexes. Consequently, we then evaluated whether the expression pattern of specific factors of the PQC system shows a different expression profile in the spinal cord and muscle of a tg ALS mice model also considering possible gender differences.

To this purpose, here, we evaluated whether modifications occur at the initial stages of the PQC system, by focusing our attention on the HSPB8-BAG3 mediated pathway, involved in the autophagic process as well as on BAG1 pathway, involved in UPS mediated PQC. We then further investigated the autophagic process, since mutSOD1 induced alterations of UPS have already been analyzed by several investigators, reviewed in Bendotti et al. (2012), including us (Onesto et al., 2011). Autophagic response was analyzed using two well-known autophagic markers: LC3 and p62.

Our data clearly demonstrated that in both spinal cord and muscle HSPB8, BAG1, and BAG3 expression levels are not affected at early stage of disease (PS). The HSPB8-mediated PQC response was induced at the S stage of disease, and this activation was lower in spinal cord than muscle. Therefore, in spinal cord, the presence of misfolded mutSOD1 might not be sufficient to induce the activation of the PQC system *per se*, unless the disease is in an advanced phase, when motoneurons loss and/or alterations of muscle structures are present (Crippa et al., 2010b). Considering BAG3 and BAG1 expression, we found that their behavior was similar to that observed for HSPB8 at S stage (16 weeks). However, the BAG3 response appeared higher than that of BAG1, and BAG3 induction was also present in the spinal cord at S stage. Thus, cells in spinal cord have a lower capability than muscle cells to activate the HSPB8/BAG3- and BAG1-mediated PQC responses, and this might explain the larger accumulation of mutant misfolded proteins in this structure. It is of note that mutSOD1 expression results in the induction of both HSPB8 and BAG3 in the same structures, since HSPB8 activity is enhanced by BAG3 recruitment. Once the HSPB8-BAG3 interaction occurs, the complex recognizes the misfolded proteins and recruits the constitutively expressed chaperone HSC70 bound to the CHIP ubiquitinating enzyme (Arndt et al., 2010), allowing misfolded proteins removal by autophagy (Carra et al., 2008a,b, 2012, 2013; Arndt et al., 2010; Crippa et al., 2010b). In fact, CHIP ubiquitinates the target misfolded protein, which is then recognized by the protein p62 for its delivery to the nascent autophagosome (Arndt et al., 2010). Thus, HSPB8, BAG3, and the autophagy pathway may represent key players in the stress response elicited by mutSOD1 expression. Therefore, changes in their expression levels may correlate with the efficiency with which spinal cord and muscle cope with aggregate-prone mutSOD1.

On the other hand, the complex HSC70/CHIP can also associate to BAG1 to re-route substrates to UPS. When the proteasome component of UPS is overwhelmed or impaired by the misfolded proteins, HSPB8 and BAG3 are both highly induced. UPS inhibition is indeed a condition which occurs in motoneurons and muscle cells expressing mutSOD1 (Onesto et al., 2011), and the increase in the BAG3:BAG1 ratio allows BAG3 to more efficiently direct the HSC70/CHIP–associated substrates to autophagy for degradation (Zhang and Qian, 2011). Our data

are in line with this working hypothesis, since in muscle we estimated that the BAG3:BAG1 ratio in tg G93A-SOD1 mice at S stage (16 weeks) is much higher than that observed in control mice. Conversely, in the spinal cord of tg G93A-SOD1 mice at S stage the BAG1 response is not detectable.

By following the auophagic response in muscle, our data here clearly show that at the S stage, muscle cells are still able to upregulate two essential players of the autophagic process, (a) p62, which recognizes the CHIP-ubiquitinated misfolded proteins in the HSPB8-BAG3-HSC70-CHIP complex, allowing their insertion into the autophagosome, and (b) LC3, which, after its activation to LC3, it is recognized by p62 and assists formation of autophagosomes. These steps are critical to initiate a correct autophagic flux required to clear the large excess of misfolded proteins from the cells during PQC response. This response does not efficiently take place in the spinal cord, since at S stage (16 weeks), misfolded mutSOD1 does not induce the overexpression of p62 and LC3, suggesting that the autophagic response in this structure is not as efficient as it is in muscle. In addition, in spinal cord very low basal levels of LC3-II form can be detected, suggesting that at these ages very few autophagosomes can be generated in response to mutSOD1. These data are consistent with previous reports obtained in the spinal cord of early symptomatic tg G93A-SOD1 mice, in which the LC3-I to LC3-II conversion, and thus, autophagy activation, occurs at very low levels, and it efficiently takes place only after the 17 week of age (Tian et al., 2011; Lee et al., 2013b). With disease progression, several autophagosomes appear in spinal cord motoneurons surviving at the end stage of disease (Li et al., 2008; Crippa et al., 2010b). This autophagic response can also be selectively activated in different cell types; for example LC3-I to LC3-II conversion can be visualized in cultured astrocytes in response to mutSOD1 (Kim et al., 2013).

With regards to the possible gender differences existing in the pathways here analyzed, we only found that, at S stage in tg G93A-SOD1 mice, the expression of p62 is significantly induced in skeletal muscle of female but not in that of male mice. These observations may help to explain why in skeletal muscle of tg G93A-SOD1 male mice mutSOD1 tends to accumulate in insoluble forms (see above). Interestingly, a mild increase of HSPB8 expression was present in spinal cord at S stage (16 weeks) of tg G93A-SOD1 male mice, but not in female mice. The molecular mechanism at the basis of this peculiar gender differences is unknown. Interestingly enough, there is a positive correlation between mutSOD1 accumulation and HSPB8 expression in the spinal cord of male tg G93A-SOD1 mice at S stage (16 weeks). This suggests that HSPB8 gene transcription increases in response to misfolded species accumulation. These data are in line with our previous results showing that an UPS impairment (which is know to result in a further mutSOD1 aggregation), lead to HSPB8 overexpression and stabilization (Crippa et al., 2010a,b; Bendotti et al., 2012; Carra et al., 2012, 2013). It remains to be determined why this HSPB8 overproduction fails to clear mutSOD1 insoluble species via autophagy (Crippa et al., 2010b; Rusmini et al., 2010).

By analysing the molecular and biochemical behavior of mutSOD1, we have previously elucidated several potential mechanisms of neurotoxicity associated with accumulation of misfolded proteins in motoneuronal cells (Sau et al., 2007, 2011; Crippa et al., 2010a,b; Onesto et al., 2011). For instance, the formation of HMW mutSOD1 species and aggregation correlated with an altered intracellular mutSOD1 distribution (Sau et al., 2007; Crippa et al., 2010b). In these conditions, mutSOD1 nuclear bioavailability was reduced in motoneuronal, but not in muscle cells (Sau et al., 2007; Crippa et al., 2010a,b; Onesto et al., 2011), an aberrant biochemical behavior that may result in a decreased nuclear protection from free radical species (Sau et al., 2007). In addition, by evaluating mitochondrial superoxide induced oxidation, using MitoSOX (a mitochondrial specific fluorogenic dye oxidized by superoxide) in motoneuronal NSC34 cell models of ALS, we found that mutSOD1 expressing motoneuronal cells are more sensitive to a superoxide-induced oxidative stress when compared to wtSOD1 expressing motoneuronal cells (Onesto et al., 2011). These mitochondrial alterations were not found in muscle C2C12 cells expressing either wt or mutSOD1 (Onesto et al., 2011). Therefore, mutSOD1 altered intracellular bioavailability, due to sequestration into aggregates, might result in a less efficient protection against free radical species damages in different subcellular compartments (Sau et al., 2007; Onesto et al., 2011).

Our results are in line with a recent report showing that protein misfolding, mitochondrial dysfunction and muscle loss are not directly dependent on soluble and aggregated mutSOD1 in skeletal muscle of ALS (Wei et al., 2012). Moreover, soluble mutSOD1 does not have direct effects on mitochondrial dysfunction as determined by quantifying the release of reactive oxygen species (ROS) in skeletal muscle mitochondria (Wei et al., 2012).

Interestingly, dysfunction of skeletal muscle and degeneration of neuromuscular junctions precede disease onset and motoneurons loss in tg ALS mice (Frey et al., 2000; Fischer et al., 2004). Notably, while the reduction of mutSOD1 expression in the skeletal muscle of tg ALS mice has no effect on disease progression (Miller et al., 2006), the selective expression of mutSOD1 in skeletal muscle induces atrophy and mitochondrial abnormalities in this tissue (Dobrowolny et al., 2008a; Corti et al., 2009; Wong and Martin, 2010). Moreover, mutSOD1-induced muscle damage leads to loss of mutSOD1-negative motoneurons in the anterior horn of the spinal cord, suggesting that mutSOD1 neurotoxicity can be exerted at different levels and its restricted expression in muscle can be sufficient to induce an ALS-like disease in mice (Dobrowolny et al., 2005, 2008a,b).

The data presented here clarify that mutSOD1 activates distinct pathogenetic pathways in muscle and motoneuron cells. In addition, the aggregation-independent mutSOD1 toxicity in muscle can be monitored at transcriptional levels. In fact, in our muscle cell models of ALS we observed specific alterations in the expression of typical genes controlling muscle pathways activated by nerve injury, or muscular atrophy [e.g., MyoD, myogenin (two myogenic regulatory factors), and TGFbeta1, which are markers for muscle fiber damage or atrophy] (Galbiati et al., 2012). Thus, muscle cells are able to better manage misfolded mutSOD1 species, even if misfolding is an intrinsic property of mutSOD1, and it does not depend on the cell environment (Dobson, 2003). It remains to be clarified whether the autophagic activation induced by mutSOD1 in muscle initially protective,

when excessive, may become one of the toxic events mediating the deleterious activity associated with this mutant protein (Nassif and Hetz, 2011; Zhang et al., 2011). This hypothesis has already been proved in a ALS-related motoneuron disease, the Spinal and Bulbar Muscular Atrophy (SBMA) linked to an expansion of a polyQ stretch in the androgen receptor (ARpolyQ) (Poletti, 2004; Rusmini et al., 2013). Even in this motoneuron disease the mutant ARpolyQ affects both spinal cord and muscle, but it has been shown that an enhanced autophagy exacerbated skeletal muscle atrophy (Yu et al., 2011). Indeed, the genetic inhibition of autophagy in muscle, through haploinsufficiency for Beclin-1, a component of the autophagic initiation complex, mitigates skeletal muscle atrophy and prolongs survival of a tg SBMA mouse model (Yu et al., 2011).

Therefore, the toxicity exerted by mutant misfolded proteins in muscle cells is probably not related to the classical mechanism of intracellular protein aggregation, but other mechanisms of toxicity may be involved.

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# Early gene expression changes in spinal cord from SOD1$^{G93A}$ Amyotrophic Lateral Sclerosis animal model

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Amyotrophic Lateral Sclerosis (ALS) is an adult-onset and fast progression neurodegenerative disease that leads to the loss of motor neurons. Mechanisms of selective motor neuron loss in ALS are unknown. The early events occurring in the spinal cord that may contribute to motor neuron death are not described, neither astrocytes participation in the pre-symptomatic phases of the disease. In order to identify ALS early events, we performed a microarray analysis employing a whole mouse genome platform to evaluate the gene expression pattern of lumbar spinal cords of transgenic SOD1$^{G93A}$ mice and their littermate controls at pre-symptomatic ages of 40 and 80 days. Differentially expressed genes were identified by means of the Bioconductor packages Agi4×44Preprocess and limma. FunNet web based tool was used for analysis of over-represented pathways. Furthermore, immunolabeled astrocytes from 40 and 80 days old mice were submitted to laser microdissection and RNA was extracted for evaluation of a selected gene by qPCR. Statistical analysis has pointed to 492 differentially expressed genes (155 up and 337 down regulated) in 40 days and 1105 (433 up and 672 down) in 80 days old ALS mice. KEGG analysis demonstrated the over-represented pathways tight junction, antigen processing and presentation, oxidative phosphorylation, endocytosis, chemokine signaling pathway, ubiquitin mediated proteolysis and glutamatergic synapse at both pre-symptomatic ages. *Ube2i* gene expression was evaluated in astrocytes from both transgenic ages, being up regulated in 40 and 80 days astrocytes enriched samples. Our data points to important early molecular events occurring in pre-symptomatic phases of ALS in mouse model. Early SUMOylation process linked to astrocytes might account to non-autonomous cell toxicity in ALS. Further studies on the signaling pathways presented here may provide new insights to better understand the events triggering motor neuron death in this devastating disorder.

**Keywords: ALS, SOD1$^{G93A}$, pre-symptomatic, spinal cord, microarray, laser microdissection, astrocytes**

## INTRODUCTION

Amyotrophic Lateral Sclerosis (ALS) is a fast disabling neurodegenerative disease characterized by upper and lower motor neuron loss of motor cortex, brainstem, and spinal cord leading to respiratory insufficiency and death (Turner et al., 2013). The incidence of ALS ranges from 1.7 to 2.3 cases per 100,000 population per year worldwide (Beghi et al., 2006). The mechanisms underlying neurodegeneration in ALS are multifactorial, and seem to involve neurons and non-neuronal cells (Boillee et al., 2006a,b; Yamanaka et al., 2008; Wang et al., 2011a) as well as several molecular pathways (Boillee et al., 2006a; Ferraiuolo et al., 2011b; Kiernan et al., 2011; Usuki et al., 2012). Approximately 5% of ALS cases are familial, and 20% of these have been linked to mutations in Cu/Zn superoxide dismutase 1 (SOD1) (Rosen et al., 1993; Andersen and Al-Chalabi, 2011). The first symptoms define the beginning of the clinical phase of the diagnosed cases of the more prevalent sporadic forms, consisting in muscle atrophy, weakness, fasciculations, and spasticity (Brooks et al., 2000). There is a lack of pathological studies on post mortem spinal cord from ALS patients that could add information about the triggering, initial time of motor neuron death and mechanisms of the disease. In fact, Fischer et al. (2004) reported the post-mortem evaluation in a patient with a short history of ALS, whose electromyography showed signs of acute and chronic denervation, coming out with an unexpected die without peripheral and central motor neuron death together with autolytic changes and a little axonal degeneration. Histological evaluations at the neuromuscular junctions and also electrophysiological analysis at the peripheral nerves in ALS patients have allowed authors to claim that motor neuron death correlates to the begging of clinical classical symptoms (Veugelers et al., 1996; Liu et al., 2013).

As the majority of familial ALS cases are linked to the mutations in SOD1 gene (Dion et al., 2009), transgenic mice expressing human mutant SOD1 (mSOD1) developing age-dependent clinical and pathological features of human ALS are current largely employed in the physiopathological studies of the disorder (Turner and Talbot, 2008). Using this mouse model, we previously described early behavior and electrophysiological alterations, prior the classical neurological symptoms and the beginning of motor neuron death (Alves et al., 2011). In fact, several early events demonstrated in animal

models seemed to precede the neuronal death, remarkably the activation of glial cells (microglia and astrocytes) close to motor neurons (Graber et al., 2010; Wang et al., 2011b; Gerber et al., 2012), retraction of motor neuron fibers and neuromuscular junction displacement (Fischer et al., 2004; De Winter et al., 2006; Narai et al., 2009). It is still unknown whether the most claimed pathogenic processes for ALS, for instance oxidative stress, mitochondrial and neurofilament dysfunction, excitotoxicity, inflammation, non-autonomous cell toxicity, protein misfolding and abnormal RNA processing (Rothstein et al., 1992; Bergeron et al., 1994; Boillee et al., 2006a; Lemmens et al., 2010; Bendotti et al., 2012; Richardson et al., 2013) are taking place at the pre-symptomatic period of the disease.

The central question in understanding ALS facing therapeutic target development involves a further knowledge about the toxic mechanisms that trigger motor neuron death (Boillee et al., 2006a). Until scientific technology approaches do not overstep ethical limitations of clinical studies, the mutant SOD1-expressing mouse model may offer opportunity for a detailed analysis of intra and intercellular signaling-related to motor neuron toxicity.

The profiling of gene expression using different platforms have been largely employed in the ALS model in several stages of the disease course (Olsen et al., 2001; Dangond et al., 2004; Malaspina and De Belleroche, 2004; Jiang et al., 2005; Perrin et al., 2005; Ferraiuolo et al., 2007, 2011a; Yamamoto et al., 2007; Offen et al., 2009; Brockington et al., 2010; D'arrigo et al., 2010; Guipponi et al., 2010; Saris et al., 2013b; Yu et al., 2013), including the early symptomatic phase (Olsen et al., 2001; Yoshihara et al., 2002; Ferraiuolo et al., 2007; Yu et al., 2013), however, there is a lack of information on differential gene expression taking place before classical clinical symptoms (Olsen et al., 2001; Yoshihara et al., 2002; Ferraiuolo et al., 2007; Guipponi et al., 2010). Olsen et al. (2001) inaugurated that issue by looking at patterns of gene expression from SOD1$^{G93A}$ spinal cord by means of a murine restricted platform of oligonucleotide microarray and by describing negligible changes in the transcript profile at the pre-symptomatic phases. Other authors that have examined gene profiling in pre-symptomatic phases of ALS disease employed restricted platforms of cDNA arrays, used animals with an uncommon symptom onset (Yoshihara et al., 2002; Guipponi et al., 2010) or evaluated gene profiling in specific spinal cord cells (Ferraiuolo et al., 2007, 2011a). Authors have encountered gene expressions related to inflammation, apoptosis, oxidative stress, ATP biosynthesis, myelination, axonal transport as candidates of biological processes taking place in the pre-symptomatic periods of ALS.

By means of a high-density oligonucleotide microarrays linked to specific tools capable to identify enriched pathways, the aim of this work was to identify early molecular changes in the pre-symptomatic stage in the spinal cord of the SOD1$^{G93A}$ mouse model. The data showed important alterations at early 40 days pre-symptomatic period of disease and in 80 days old pre-symptomatic mice.

## MATERIALS AND METHODS

### SAMPLES

Specific pathogen-free male SOD1$^{G93A}$ mice of preclinical 40 and 80 days old mice and their age-paired non-transgenic wild-type controls, 20–25 g body weight, from University of São Paulo Medical School (São Paulo, Brazil) were used in the experiments. A total of 5 animals were used in each group in microarray experiments, while in the verification experiments by quantitative polymerase chain reaction (qPCR) and laser microdissection, each group was comprised for 6 and 3 different animals, respectively. Animals were kept under standardized lighting conditions (lights on at 7:00 h and off at 19:00 h), at a constant temperature of 23°C and with free access to food pellets and tap water. The colony was derived from Jackson Laboratories (Bar Harbor, ME, USA) from G93A mutant mice with 25 ± 1.5 copies of the human SOD1 transgene (Gurney, 1994). Mouse identification (SOD$^{G93A}$ or WT) in our colony was performed by genotyping (Scorisa et al., 2010). Animals were killed by decapitation and their lumbar spinal cords were collected for molecular analysis. The study was conducted according protocols approved by the Animal Care and Use of Ethic Committee at the University of São Paulo and in accordance with the Guide for Care and Use of Laboratory Animals adopted by the National Institutes of Health.

### RNA EXTRACTION

Total RNA was isolated using the MiniSpin kit for RNA extraction (GE Healthcare, USA) according to the manufacturer's instructions. RNA quantity and integrity were assessed by spectrophotometry (Nanodrop, Thermo Scientific, USA) and microfluidics—based electrophoresis (Agilent 2100 Bioanalyzer, Agilent Technologies, USA), respectively. RNA samples with OD 260/280 of approximately 2.0 and RIN > 7.0 were used for microarray experiments and qPCR. A pool of RNAs from neonatal organs (heart, kidney, liver) was employed as reference sample. A representative eletropherogram from Bioanalyzer evaluation of RNA integrity is shown in supplementary material (Figure S1).

### MICROARRAY EXPERIMENTS

For samples and reference, respectively, 250 and 500 ng of RNA were reverse transcribed by the Low-input RNA Linear Amplification Kit (Agilent Technologies) and then transcribed to Cy3-labeled (samples) or Cy5-labeled (reference) cRNA according to the manufacturer. The labeled cRNA was purified (Minispin kit, GE Life Sciences), and the dye content and concentration of cRNA were measured by a NanoDrop ND-1000 spectrophotometer (Thermo Scientific). A total of 850 ng of Cy3-labeled cRNA was hybridized together with the same amount of Cy5-labeled reference to Whole Mouse Genome Oligo 4 × 44 K microarrays overnight at 65°C, and then the slides were washed and treated with Stabilizing and Drying Solution (Agilent Technologies) and scanned by Agilent Microarray Scanner. All steps were performed according to the manufacturer (Agilent Technologies).

The raw data from hybridizations and experimental conditions are available on the Gene Expression Omnibus website under accession number GSE50642.

### DATA ANALYSES

The Feature Extraction Software v9.1.3.1 (Agilent Technologies) was used to extract and analyze the assay signals and subsequently determine the signal-to-noise ratios from the microarray images. Microarrays without enough quality were taken out from further analysis. The analysis proceeded with 4 samples for each group. Microarray raw data (.txt files) were imported into R v. 3.0.1 (Team RDC, 2012) and analyzed with the Bioconductor (Gentleman et al., 2004) packages Agi4×44PreProcess and limma (Smyth, 2005). Briefly, after quality check, the microarray probes were filtered and their median foreground intensity was normalized within and between arrays according to Agi4×44Preprocess and limma user guides, respectively. Finally, the probes were tested for differential expression using a linear model followed by Bayes moderated *t*-test (Smyth, 2005) for the comparisons of interest. Genes with nominal $p < 0.05$ were accepted to be differentially expressed and further considered in the analysis.

### FunNet ANALYSIS

In order to further identify over-represented pathways and biological process, the lists with differentially expressed genes for both 40 and 80 days old mice were split into lists of up and down regulated genes and submitted to FunNet web based tool (Functional Analysis of Transcriptional Networks), using Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology (GO) annotations (Prifti et al., 2008).

### LASER MICRODISSECTION OF ASTROCYTES

The lumbar spinal cord of mice were rapidly removed and immediately frozen in ice cold isopentane at −45°C and stored at −80°C until use. The labeling procedure was performed as described previously (De Oliveira et al., 2009) and modified according to our experience. Frozen sections (5μm) were rapidly defrosted for 30 s and fixed with ice cold acetone, for 3 min. Sections were then incubated during 3 min in phosphate buffered saline (PBS) containing 3% Triton X-100 and then incubated with primary antibody, a polyclonal rabbit anti-glial fibrillary acidic protein (GFAP; Dako Cytomation; 1:100) diluted in 0.3% Triton X-100 containing 1% BSA for 5 min. Sections were then washed in PBS for 3 times of 15 s and then incubated with texas red-conjugated goat-anti-rabbit secondary antibody, in the same diluent than primary antibody, in a final concentration of 1:50 during 5 min in the dark and at room temperature. Sections were rinsed carefully three times with PBS for 15 s and immediately submitted to laser microdissection.

Around 200 astrocytes were isolated from each 40 and 80 days old mice lumbar spinal cords using P.A.L.M. Microlaser Technologies (Zeiss). RNA was extracted using PicoPure RNA isolation kit (Arcturus) and linear amplification of RNA was performed following Eberwine's procedure (Van Gelder et al., 1990) using the RiboampHSplus kit (Arcturus) according to the manufacturer's protocol. The quantity (NanoDrop 1000 Spectrophotometer) and quality (Agilent 2100 bioanalyser, RNA 6000 Pico LabChip) of amplified RNA was analyzed as described above. Also, the astrocytes enriched samples were submitted to PCRs in order to access contamination from other cell types. Protocol and results of astrocyte samples enrichment are presented in the supplementary material (Figure S2).

## QUANTITATIVE PCR

A proportion of genes identified as differentially expressed were selected for verification by qPCR, on the basis of robust microarray data confirming differential gene expression. The genes were chosen for verification based on their possible involvement in ALS related mechanisms. Verification addresses the possibility of false positive microarray signals, due to cross-hybridization with related genes, concern about the accuracy of array probe sets, and uncertainty about the hybridization kinetics of multiple reactions occurring on the miniature scale of an array chip. The qPCR verification of microarray results were performed on independent sample, as described above. cDNA was synthesized from 1 μg of total RNA treated with DNAse by a reverse transcription reagent kit (Applied Biosystems Life Technologies) according to manufacturer. qPCR reactions were carried out in duplicate with 40 ng cDNA, the DyNAmo ColorFlash SYBR Green qPCR kit (Thermo Scientific, USA) and 400 nM of each primer in a final volume reaction of 20 μl, by using the PikoReal Real-Time PCR System (Thermo Scientific). The information for SYBR primers can be found in **Table 1**. For astrocytes enriched samples, 1 μg of amplified RNA was reverse transcribed to cDNA by a reverse transcription reagent kit (Applied Biosystems Life Technologies) modified from original protocol in order to improve efficiency. Briefly, Oligo$(dT)_{16}$ primer was added to samples and incubated at 70°C during 5 min, then the other required reagents, such as reaction buffer, $MgCl_2$, dNTPs, RNAse inhibitor, in the same concentrations than manufacturer protocol, and 156,25 U of Reverse transcriptase (Multiscribe), were added to reaction and incubated at 37°C for 60 min followed by 95°C for 5 min. qPCR reactions were carried out in duplicate using Taqman master mix and the

**Table 1 | Information for primers used in SYBR qPCR experiments of 40 and 80 days old pre-symptomatic SOD1$^{G93A}$ and wild-type mice.**

| Gene ID | Primer sequences (5′-3′) | Amplicon (bp) |
|---|---|---|
| *Glg1* | F: GAGTGAGATTGCAGCCAGAG | 143 |
| | R:CAGGATGTAGTTCTTTGAGGGAG | |
| *Aqp4* | F: GCTCGATCTTTTGGACCCG | 112 |
| | R: AGACATACTCATAAAGGGCACC | |
| *Calca* | F: TGCAGATGAAAGCCAGGG | 149 |
| | R: CTTCACCACACCTCCTGATC | |
| *Eef2* | F: CATGTTTGTGGTCAAGGCATAC | 141 |
| | R:TTGTCAAAAGGATCCCCAGG | |
| *Nsg1* | F: AAGTGTACAAGTATGACCGCG | 128 |
| | R: GACAGTGTAAAATTTCTCCCGG | |
| *Syt10* | F: AGACCATTGGAACGAGATGC | 148 |
| | R: TGGAGGCTTTTATGGTGTGG | |
| **NORMALYZER** | | |
| *Gapdh* | F: GAGTAAGAAACCCTGGACCAC | 109 |
| | R: TCTGGGATGGAAATTGTGAGG | |

*The Glg1, Aqp4, Calca genes and Eef2, Nsg1, Syt10 genes were verified in 40 and 80 days mice, respectively, according to microarray results.*

following assays were used: *Ube2i* (Mm04243971_g1) and *Gapdh* (Mm99999915_g1).

For SYBR reactions the cycling was composed by an initial denaturation at 95°C for 10 min, templates were amplified by 40 cycles of 95°C for 15 s and 60°C for 30 s. A dissociation curve was then generated to ensure amplification of a single product, and absence of primer dimers. For each primer pair, a standard curve was generated to determine the efficiency of the PCR reaction over a range of template concentrations from 0.032 ng/μl to 20 ng/μl, using cDNA synthesized from mouse reference RNA. The efficiency for each set of primers was 100 ± 5%. For Taqman reactions, cycling was composed by an initial step of 50°C for 2 min, followed by denaturation at 95°C for 10 min, templates were amplified by 40 cycles of 95°C for 15 s and 60°C for 1 min. Gene expressions, normalized to *Gapdh*, could be determined using the ΔΔCt mathematical model (ABI PRISM 7700 Sequence Detection System protocol; Applied Biosystems). One-tailed unpaired *t*-test was used to determine the statistical significance of any differences in gene expression [GraphPad (San Diego, CA) Prism 5]. *Gapdh* was chosen as a housekeeping gene to normalize the qPCR values because the microarray analysis showed that its expression was stable across samples.

## RESULTS

### GENERAL FEATURES OF DIFFERENTIAL GENE EXPRESSION BETWEEN SOD1$^{G93A}$ AND WILD-TYPE MICE

Statistical analysis has pointed to 492 differentially expressed genes at the lumbar region of 40 days SOD1$^{G93A}$, compared to the age matched wild-type mice, being 155 up and 337 down regulated genes, respectively, while 1105 genes were found differentially expressed by 80 days old ALS mice compared to age matched controls, being 433 up and 672 down regulated genes, respectively. The whole list with differentially expressed gene for both age mice can be found in Tables S1 and S2 in the Supplementary material. Of interest, among differentially expressed genes, 66 are common to both ages; they are presented in the **Table 2**.

### VERIFICATION OF MICROARRAY RESULTS BY qPCR

The results of qPCR verification for the six representative genes are shown in Table S3 (Supplementary material). The up and down regulations of the verified genes in the 40 days and 80 days old SOD1$^{G93A}$ mice by means of qPCR were coincident and supported the microarray findings of correspondent animal ages (Table S3).

### FunNet ANALYSIS

KEGG terms which were significantly enriched (at level $p < 0.05$) amongst differentially expressed genes between SOD1$^{G93A}$ and wild-type mice were identified for both 40 days and 80 days old pre-symptomatic ALS mice. Over-represented KEGG pathways and respective genes taking part of them are given in **Tables 3**, **4**. Of importance, differentially expressed genes from 40 and 80 days old mice allowed to recognize 7 pathways common among both periods (**Figure 1**). Those were glutamatergic synapse, ubiquitin mediated proteolysis, chemokine signaling pathway, endocytosis, oxidative phosphorylation, antigen processing and presentation and tight junction. The number of transcripts in each pathway

**Table 2 | Differentially expressed genes common to both gene lists of 40 and 80 days old pre-symptomatic SOD1$^{G93A}$ and wild-type mice.**

| Gene symbol | Fold change 40 days | Fold change 80 days |
|---|---|---|
| *Ocel1* | −1.68 | −1.92 |
| *Fam32a* | −1.45 | −1.61 |
| *Trim37* | 1.22 | −1.34 |
| *Lsm6* | −1.3 | −1.29 |
| *Map1a* | 1.2 | −1.15 and −1.28 |
| *Bmpr2* | 1.21 | −1.26 |
| *Eif3j2* | 1.29 | −1.26 |
| *Foxn3* | 1.28 | −1.25 |
| *Plekha5* | 1.17 | −1.25 |
| *Rfxank* | −1.12 | −1.22 |
| *Malat1* | 1.1 | −1.21 |
| *Ddx6* | 1.3 | −1.19 |
| *Huwe1* | 1.12 | −1.19 |
| *Snx27* | 1.19 | −1.19 |
| *Srrm3* | 1.18 | −1.19 |
| *Dzip1* | 1.14 | −1.17 |
| *Hook3* | 1.14 | −1.17 |
| *Nemf* | 1.15 | −1.16 |
| *Pdlim5* | 1.09 | −1.16 |
| *Plvap* | −1.14 | −1.16 |
| *Thrap3* | 1.15 | −1.16 |
| *Srsf11* | −1.1 | −1.15 |
| *Azin1* | 1.12 | −1.14 |
| *Eif5b* | 1.19 | −1.14 |
| *Hspa4* | 1.1 | −1.14 |
| *Kras* | 1.19 | −1.14 |
| *Sp4* | 1.12 | −1.14 |
| *Tusc3* | −1.1 | −1.14 |
| *Vegfa* | 1.12 | −1.14 |
| *Fam133b* | 1.09 | −1.13 |
| *Fam81a* | 1.12 | −1.13 |
| *Prkrir* | −1.1 | −1.13 |
| *Ptrf* | −1.3 | −1.13 |
| *6330411E07Rik* | 1.09 | −1.12 |
| *Gria4* | 1.18 | −1.12 |
| *Zfp866* | 1.07 | −1.12 |
| *Marc-2* | −1.07 | −1.11 |
| *Hadh* | 1.12 | −1.11 |
| *Mtf2* | 1.1 | −1.11 |
| *Dhps* | −1.09 | −1.1 |
| *Nsd1* | 1.12 | −1.1 |
| *Mier1* | 1.13 | −1.09 |
| *U2surp* | 1.13 | −1.09 |
| *2610507B11Rik* | 1.07 | 1.1 |
| *Ncam1* | 1.17 | 1.12 |
| *Rtn1* | −1.17 | 1.12 |
| *Chd5* | 1.12 | 1.13 |
| *Dhcr7* | −1.08 | 1.15 |
| *Strbp* | 1.15 | 1.15 |
| *Synm* | −1.12 | 1.15 |
| *Map7d1* | 1.14 | 1.16 |
| *Specc1* | 1.1 | 1.17 |

*(Continued)*

**Table 2 | Continued**

| Gene symbol | Fold change 40 days | Fold change 80 days |
|---|---|---|
| *Maea* | 1.1 | 1.19 |
| *Ncl* | 1.1 and −1.1 | 1.19 |
| *Glg1* | 1.29 | 1.2 |
| *Dnajc27* | 1.17 | 1.21 |
| *Ncdn* | 1.14 | 1.21 |
| *Lpcat2* | 1.16 | 1.22 |
| *D17Wsu92e* | 1.11 | 1.24 |
| *Nisch* | 1.08 | 1.24 |
| *Tkt* | −1.1 | 1.25 |
| *Tmem59l* | 1.29 | 1.26 |
| *Mast3* | 1.24 | 1.28 |
| *Plac9a* | −1.45 | 1.35 |
| *Nsg1* | −1.17 | 1.22 and 1.35 |
| *Trappc3* | −1.2 | 1.32 and 1.41 |

*Map1a, Ncl, Nsg1, and Trappc3 genes were represented by an additional probe.*

is also shown in **Figure 1**. Moreover, other interesting pathways could also be identified to appear only in 40 days (**Table 3**) or 80 days old SOD1$^{G93A}$ mice (**Table 4**). Furthermore, among pathways common to both ages, ubiquitin mediated proteolysis, chemokine signaling pathway and endocytosis were over-represented by up regulated genes and oxidative phosphorylation was pointed by down regulated genes (**Figure 1**). Furthermore, glutamatergic synapse and tight junction were pointed by the genes that were up regulated in 40 days and also up or down regulated in 80 days gene expression lists (**Figure 1**). Finally, antigen processing and presentation was pointed for down regulated genes in 40 days and up regulated genes in 80 days lists (**Figure 1**).

Some pathways pointed by FunNet were omitted from table because they were composed by genes already presented in other pathways and also genes apparently not related to ALS. They were melanoma, measles, hepatitis C, melanogenesis, pathways in cancer and prostate cancer at 40 days and viral myocarditis and melanogenesis in 80 days results.

The results for GO enriched terms can be found in Tables S4 and S5 in Supplementary material.

### LASER MICRODISSECTION OF ASTROCYTES AND qPCR EXPERIMENT

The profile for GFAP immunofluorescence for specific identification of astrocytes can be found in **Figure 2**. Our protocol allowed easily identifying the astrocytic profiles (**Figure 2A**) to be microdissected (**Figure 2B**). The procedure allowed a complete microdissection of the desired cell type (**Figure 2C**), the astrocytes in our case. The results of qPCR for *Ube2i*, using the two cycle amplified RNA, from 40 and 80 days mouse laser microdissected astrocytes have shown increased gene expressions in transgenic mice of both pre-symptomatic ages (**Figure 3**). The *Ube2i* expression was increased by 5.53-fold in the astrocytes from 40 days old SOD1$^{G92A}$ mice and by 1.77-fold change in astrocytes from 80 days old SOD1$^{G92A}$ mice compared to respective age matched wild-type samples.

**Table 3 | KEGG pathways enriched amongst differentially expressed up or down regulated genes at 40 days old mice.**

| Gene ID | Gene symbol | Gene name |
|---|---|---|
| **PATHWAYS POINTED BY UP REGULATED GENES** | | |
| **Fructose and manose metabolism** | | |
| 170768 | *Pfkfb3* | 6-Phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 |
| 18640 | *Pfkfb2* | 6-Phosphofructo-2-kinase/fructose-2,6-biphosphatase 2 |
| 18642 | *Pfkm* | Phosphofructokinase, muscle |
| 230163 | *Aldob* | Aldolase B, fructose-bisphosphate |
| 54384 | *Mtmr7* | Myotubularin related protein 7 |
| **Tight junction** | | |
| 14677 | *Gnai1* | Guanine nucleotide binding protein (G protein), alpha inhibiting 1 |
| 16653 | *Kras* | v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog |
| 18176 | *Nras* | Neuroblastoma ras oncogene |
| 18417 | *Cldn11* | Claudin 11 |
| 192195 | *Ash1l* | Ash1 (absent, small, or homeotic)-like (Drosophila) |
| **Glutamatergic synapse** | | |
| 140919 | *Slc17a6* | Solute carrier family 17 (sodium-dependent inorganic phosphate cotransporter), member 6 |
| 14677 | *Gnai1* | Guanine nucleotide binding protein (G protein), alpha inhibiting 1 |
| 14802 | *Gria4* | Glutamate receptor, ionotropic, AMPA4 (alpha 4) |
| 14810 | *Grin1* | Glutamate receptor, ionotropic, NMDA1 (zeta 1) |
| 20511 | *Slc1a2* | Solute carrier family 1 (glial high affinity glutamate transporter), member 2 |
| **Axon guidance** | | |
| 12767 | *Cxcr4* | Chemokine (C-X-C motif) receptor 4 |
| 14677 | *Gnai1* | Guanine nucleotide binding protein (G protein), alpha inhibiting 1 |
| 16653 | *Kras* | v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog |
| 18176 | *Nras* | Neuroblastoma ras oncogene |
| 22253 | *Unc5c* | Unc-5 homolog C (*C. elegans*) |
| 56637 | *Gsk3b* | Glycogen synthase kinase 3 beta |
| **Ubiquitin mediated proteolysis** | | |
| 107568 | *Wwp1* | WW domain containing E3 ubiquitin protein ligase 1 |
| 17999 | *Nedd4* | Neural precursor cell expressed, developmentally down-regulated 4 |
| 22210 | *Ube2b* | Ubiquitin-conjugating enzyme E2B |
| 59026 | *Huwe1* | HECT, UBA and WWE domain containing 1 |
| 68729 | *Trim37* | Tripartite motif-containing 37 |
| 70790 | *Ubr5* | Ubiquitin protein ligase E3 component n-recognin 5 |
| **Chemokine signaling pathway** | | |
| 12767 | *Cxcr4* | Chemokine (C-X-C motif) receptor 4 |
| 14677 | *Gnai1* | Guanine nucleotide binding protein (G protein), alpha inhibiting 1 |

*(Continued)*

**Table 3 | Continued**

| Gene ID | Gene symbol | Gene name |
|---|---|---|
| 16653 | *Kras* | v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog |
| 18176 | *Nras* | Neuroblastoma ras oncogene |
| 18708 | *Pik3r1* | Phosphatidylinositol 3-kinase, regulatory subunit, polypeptide 1 (p85 alpha) |
| 56637 | *Gsk3b* | Glycogen synthase kinase 3 beta |
| 73178 | *Wasl* | Wiskott-Aldrich syndrome-like (human) |
| **Endocytosis** | | |
| 107568 | *Wwp1* | WW domain containing E3 ubiquitin protein ligase 1 |
| 12767 | *Cxcr4* | Chemokine (C-X-C motif) receptor 4 |
| 13854 | *Epn1* | Epsin 1 |
| 17999 | *Nedd4* | Neural precursor cell expressed, developmentally down-regulated 4 |
| 193740 | *Hspa1a* | Heat shock protein 1A |
| 26431 | *Git2* | G protein-coupled receptor kinase-interactor 2 |
| 78618 | *Acap2* | ArfGAP with coiled-coil, ankyrin repeat and PH domains 2 |
| **PATHWAYS POINTED BY DOWN REGULATED GENES** | | |
| **Nucleotide excision repair** | | |
| 19718 | *Rfc2* | Replication factor C (activator 1) 2 |
| 66979 | *Pole4* | Polymerase (DNA-directed), epsilon 4 (p12 subunit) |
| **DNA replication** | | |
| 19718 | *Rfc2* | Replication factor C (activator 1) 2 |
| 66979 | *Pole4* | Polymerase (DNA-directed), epsilon 4 (p12 subunit) |
| **Fatty acid metabolism** | | |
| 11363 | *Acadl* | Acyl-Coenzyme A dehydrogenase, long-chain |
| 74205 | *Acsl3* | Acyl-CoA synthetase long-chain family member 3 |
| **TGF-beta signaling pathway** | | |
| 12167 | *Bmpr1b* | Bmpr1b bone morphogenetic protein receptor, type 1B |
| 15902 | *Id2* | Inhibitor of DNA binding 2 |
| 19651 | *Rbl2* | Retinoblastoma-like 2 |
| **Antigen processing and presentation** | | |
| 12010 | *B2m* | Beta-2 microglobulin |
| 12317 | *Calr* | Calreticulin |
| 19727 | *Rfxank* | Regulatory factor X-associated ankyrin-containing protein |
| **ECM-receptor interaction** | | |
| 11603 | *Agrn* | Agrin |
| 12814 | *Col11a1* | Collagen, type XI, alpha 1 |
| 16773 | *Lama2* | Laminin, alpha 2 |
| **GnRH signaling pathway** | | |
| 12314 | *Calm2* | Calmodulin 2 |
| 16440 | *Itpr3* | Inositol 1,4,5-triphosphate receptor 3 |
| 16476 | *Jun* | Jun oncogene |
| 17390 | *Mmp2* | Matrix metallopeptidase 2 |

*(Continued)*

**Table 3 | Continued**

| Gene ID | Gene symbol | Gene name |
|---|---|---|
| **Parkinson's disease** | | |
| 104130 | *Ndufb11* | NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 11 |
| 12857 | *Cox4i1* | Cytochrome c oxidase subunit IV isoform 1 |
| 66576 | *Uqcrh* | Ubiquinol-cytochrome c reductase hinge protein |
| 67264 | *Ndufb8* | NADH dehydrogenase (ubiquinone) 1 beta subcomplex 8 |
| **Oxidative phosphorylation** | | |
| 104130 | *Ndufb11* | NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 11 |
| 12857 | *Cox4i1* | Cytochrome c oxidase subunit IV isoform 1 |
| 66576 | *Uqcrh* | Ubiquinol-cytochrome c reductase hinge protein |
| 67264 | *Ndufb8* | NADH dehydrogenase (ubiquinone) 1 beta subcomplex 8 |
| **Alzheimer's disease** | | |
| 104130 | *Ndufb11* | NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 11 |
| 12314 | *Calm2* | Calmodulin 2 |
| 12857 | *Cox4i1* | Cytochrome c oxidase subunit IV isoform 1 |
| 16440 | *Itpr3* | Inositol 1,4,5-triphosphate receptor 3 |
| 66576 | *Uqcrh* | Ubiquinol-cytochrome c reductase hinge protein |
| 67264 | *Ndufb8* | NADH dehydrogenase (ubiquinone) 1 beta subcomplex 8 |

## DISCUSSION

Gene-expression profiling studies have been conducted in the search of molecular pathways related to motor neuron death in ALS by employing animal models in different phases of the disease and human post mortem material at the very end stage of motor neuron degeneration (Olsen et al., 2001; Dangond et al., 2004; Malaspina and De Belleroche, 2004; Jiang et al., 2005; Perrin et al., 2005; Ferraiuolo et al., 2007, 2011a; Yamamoto et al., 2007; Offen et al., 2009; Brockington et al., 2010; D'arrigo et al., 2010; Guipponi et al., 2010; Saris et al., 2013b).

The analysis of the mechanisms that trigger motor neuron death in the ALS may include evaluation of the altered molecular pathways that are taking place in compromised regions before the occurrence of cell death. Previous works have attempted to describe gene profiling in the pre-symptomatic phases of ALS animal model by employing distinct methodologies (Ferraiuolo et al., 2007; D'arrigo et al., 2010; Guipponi et al., 2010). This is the first work to analyze gene expression profile in the whole lumbar spinal cord of early 40 and 80 days old pre-symptomatic $SOD1^{G93A}$ mouse in a whole genome array platform, which allowed depicting enriched pathways related to possible mechanisms of neuronal toxicity in ALS. Our analysis has pointed to up to 1105 differentially expressed genes in pre-symptomatic periods of $SOD1^{G93A}$ mouse model, a larger number of than described elsewhere (Perrin et al., 2005, 2006). It should be pointed that the average of fold change described in previous publications is about 3, which is higher than that found in our microarray analysis. However, it must be emphasized that

**Table 4 | KEGG pathways enriched amongst differentially expressed up or down regulated genes at 80 days old mice.**

| Gene ID | Gene symbol | Gene name |
|---|---|---|
| **PATHWAYS POINTED BY UP REGULATED GENES** | | |
| **Vascular smooth muscle contraction** | | |
| 104111 | *Adcy3* | Adenylate cyclase 3 |
| 12315 | *Calm3* | Calmodulin 3 |
| 14673 | *Gna12* | Guanine nucleotide binding protein, alpha 12 |
| 14674 | *Gna13* | Guanine nucleotide binding protein, alpha 13 |
| 18751 | *Prkcb* | Protein kinase C, beta |
| 213498 | *Arhgef11* | Rho guanine nucleotide exchange factor (GEF) 11 |
| 224129 | *Adcy5* | Adenylate cyclase 5 |
| 26413 | *Mapk1* | Mitogen-activated protein kinase 1 |
| **Antigen processing and presentation** | | |
| 14963 | *H2-Bl* | Histocompatibility 2, blastocyst |
| 14972 | *H2-K1* | Histocompatibility 2, K1, K region |
| 15006 | *H2-Q1* | Histocompatibility 2, Q region locus 1 |
| 15007 | *H2-Q10* | Histocompatibility 2, Q region locus 10 |
| 15013 | *H2-Q2* | Histocompatibility 2, Q region locus 2 |
| 15018 | *H2-Q7* | Histocompatibility 2, Q region locus 7 |
| 15039 | *H2-T22* | Histocompatibility 2, T region locus 22 |
| 15040 | *H2-T23* | Histocompatibility 2, T region locus 23 |
| 15481 | *Hspa8* | Heat shock protein 8 |
| 21355 | *Tap2* | Transporter 2, ATP-binding cassette, sub-family B (MDR/TAP) |
| **Tight junction** | | |
| 11465 | *Actg1* | Actin, gamma, cytoplasmic 1 |
| 13043 | *Cttn* | Cortactin |
| 13821 | *Epb4.1l1* | Erythrocyte protein band 4.1-like 1 |
| 13822 | *Epb4.1l2* | Erythrocyte protein band 4.1-like 2 |
| 14924 | *Magi1* | Membrane associated guanylate kinase, WW and PDZ domain containing 1 |
| 16897 | *Llgl1* | Lethal giant larvae homolog 1 |
| 17475 | *Mpdz* | Multiple PDZ domain protein |
| 18751 | *Prkcb* | Protein kinase C, beta |
| 67374 | *Jam2* | Junction adhesion molecule 2 |
| 71960 | *Myh14* | Myosin, heavy polypeptide 14 |
| **Chemokine signaling pathway** | | |
| 104111 | *Adcy3* | Adenylate cyclase 3 |
| 14083 | *Ptk2* | PTK2 protein tyrosine kinase 2 |
| 14688 | *Gnb1* | Guanine nucleotide binding protein (G protein), beta 1 |
| 14693 | *Gnb2* | Guanine nucleotide binding protein (G protein), beta 2 |
| 14697 | *Gnb5* | Guanine nucleotide binding protein (G protein), beta 5 |
| 14701 | *Gng12* | Guanine nucleotide binding protein (G protein), gamma 12 |
| 14708 | *Gng7* | Guanine nucleotide binding protein (G protein), gamma 7 |
| 18751 | *Prkcb* | Protein kinase C, beta |
| 224129 | *Adcy5* | Adenylate cyclase 5 |
| 26413 | *Mapk1* | Mitogen-activated protein kinase 1 |
| 277360 | *Prex1* | Phosphatidylinositol-3,4,5-trisphosphate-dependent Rac exchange factor 1 |
| **Ubiquitin mediated proteolysis** | | |
| 103583 | *Fbxw11* | F-box and WD-40 domain protein 11 |
| 15204 | *Herc2* | Hect (homologous to the E6-AP (UBE3A) carboxyl terminus) domain and RCC1 (CHC1)-like domain (RLD) 2 |
| 17237 | *Mgrn1* | Mahogunin, ring finger 1 |
| 19823 | *Rnf7* | Ring finger protein 7 |
| 217342 | *Ube2o* | Ubiquitin-conjugating enzyme E2O |
| 22192 | *Ube2m* | Ubiquitin-conjugating enzyme E2M |
| 22196 | *Ube2i* | Ubiquitin-conjugating enzyme E2I |
| 22213 | *Ube2g2* | Ubiquitin-conjugating enzyme E2G 2 |
| 229615 | *Pias3* | Protein inhibitor of activated STAT 3 |
| 50754 | *Fbxw7* | F-box and WD-40 domain protein 7 |
| 63958 | *Ube4b* | Ubiquitination factor E4B, UFD2 homolog (S. cerevisiae) |
| **Regulation of actin cytoskeleton** | | |
| 11465 | *Actg1* | Actin, gamma, cytoplasmic 1 |
| 14083 | *Ptk2* | PTK2 protein tyrosine kinase 2 |
| 14673 | *Gna12* | Guanine nucleotide binding protein, alpha 12 |
| 14674 | *Gna13* | Guanine nucleotide binding protein, alpha 13 |
| 14701 | *Gng12* | Guanine nucleotide binding protein (G protein), gamma 12 |
| 18717 | *Pip5k1c* | Phosphatidylinositol-4-phosphate 5-kinase, type 1 gamma |
| 192897 | *Itgb4* | Integrin beta 4 |
| 226970 | *Arhgef4* | Rho guanine nucleotide exchange factor (GEF) 4 |
| 227753 | *Gsn* | Gelsolin |
| 26413 | *Mapk1* | Mitogen-activated protein kinase 1 |
| 67771 | *Arpc5* | Actin related protein 2/3 complex, subunit 5 |
| 71960 | *Myh14* | Myosin, heavy polypeptide 14 |
| **Glutamatergic synapse** | | |
| 104111 | *Adcy3* | Adenylate cyclase 3 |
| 110637 | *Grik4* | Glutamate receptor, ionotropic, kainate 4 |
| 14645 | *Glul* | Glutamate-ammonia ligase (glutamine synthetase) |
| 14688 | *Gnb1* | Guanine nucleotide binding protein (G protein), beta 1 |
| 14693 | *Gnb2* | Guanine nucleotide binding protein (G protein), beta 2 |
| 14697 | *Gnb5* | Guanine nucleotide binding protein (G protein), beta 5 |
| 14701 | *Gng12* | Guanine nucleotide binding protein (G protein), gamma 12 |
| 14708 | *Gng7* | Guanine nucleotide binding protein (G protein), gamma 7 |
| 18751 | *Prkcb* | Protein kinase C, beta |
| 216456 | *Gls2* | Glutaminase 2 (liver, mitochondrial) |
| 224129 | *Adcy5* | Adenylate cyclase 5 |
| 26413 | *Mapk1* | Mitogen-activated protein kinase 1 |

*(Continued)*

**Table 4 | Continued**

| Gene ID | Gene symbol | Gene name |
|---|---|---|
| **Phagosome** | | |
| 11465 | *Actg1* | Actin, gamma, cytoplasmic 1 |
| 14963 | *H2-Bl* | Histocompatibility 2, blastocyst |
| 14972 | *H2-K1* | Histocompatibility 2, K1, K region |
| 15006 | *H2-Q1* | Histocompatibility 2, Q region locus 1 |
| 15007 | *H2-Q10* | Histocompatibility 2, Q region locus 10 |
| 15013 | *H2-Q2* | Histocompatibility 2, Q region locus 2 |
| 15018 | *H2-Q7* | Histocompatibility 2, Q region locus 7 |
| 15039 | *H2-T22* | Histocompatibility 2, T region locus 22 |
| 15040 | *H2-T23* | Histocompatibility 2, T region locus 23 |
| 15239 | *Hgs* | HGF-regulated tyrosine kinase substrate |
| 17113 | *M6pr* | Mannose-6-phosphate receptor, cation dependent |
| 21355 | *Tap2* | Transporter 2, ATP-binding cassette, sub-family B (MDR/TAP) |
| 22142 | *Tuba1a* | Tubulin, alpha 1A |
| 22151 | *Tubb2a* | Tubulin, beta 2A class IIA |
| **Protein processing in endoplasmic reticulum** | | |
| 100037258 | *Dnajc3* | DnaJ (Hsp40) homolog, subfamily C, member 3 |
| 108687 | *Edem2* | ER degradation enhancer, mannosidase alpha-like 2 |
| 12955 | *Cryab* | Crystallin, alpha B |
| 15481 | *Hspa8* | Heat shock protein 8 |
| 20014 | *Rpn2* | Ribophorin II |
| 20338 | *Sel1l* | Sel-1 suppressor of lin-12-like (C. elegans) |
| 216440 | *Os9* | Amplified in osteosarcoma |
| 22213 | *Ube2g2* | Ubiquitin-conjugating enzyme E2G 2 |
| 269523 | *Vcp* | Valosin containing protein |
| 50907 | *Preb* | Prolactin regulatory element binding |
| 54197 | *Rnf5* | Ring finger protein 5 |
| 56453 | *Mbtps1* | Membrane-bound transcription factor peptidase, site 1 |
| 56812 | *Dnajb2* | DnaJ (Hsp40) homolog, subfamily B, member 2 |
| 63958 | *Ube4b* | Ubiquitination factor E4B, UFD2 homolog (S. cerevisiae) |
| **Cell adhesion molecules (CAMS)** | | |
| 14963 | *H2-Bl* | Histocompatibility 2, blastocyst |
| 14972 | *H2-K1* | Histocompatibility 2, K1, K region |
| 15006 | *H2-Q1* | Histocompatibility 2, Q region locus 1 |
| 15007 | *H2-Q10* | Histocompatibility 2, Q region locus 10 |
| 15013 | *H2-Q2* | Histocompatibility 2, Q region locus 2 |
| 15018 | *H2-Q7* | Histocompatibility 2, Q region locus 7 |
| 15039 | *H2-T22* | Histocompatibility 2, T region locus 22 |
| 15040 | *H2-T23* | Histocompatibility 2, T region locus 23 |
| 17967 | *Ncam1* | Neural cell adhesion molecule 1 |
| 18007 | *Neo1* | Neogenin |
| 19274 | *Ptprm* | Protein tyrosine phosphatase, receptor type, M |
| 20340 | *Glg1* | Golgi apparatus protein 1 |
| 20970 | *Sdc3* | Syndecan 3 |
| 58235 | *Pvrl1* | Poliovirus receptor-related 1 |
| 67374 | *Jam2* | Junction adhesion molecule 2 |

*(Continued)*

**Table 4 | Continued**

| Gene ID | Gene symbol | Gene name |
|---|---|---|
| **Endocytosis** | | |
| 11771 | *Ap2a1* | Adaptor-related protein complex 2, alpha 1 subunit |
| 12757 | *Clta* | Clathrin, light polypeptide (Lca) |
| 13196 | *Asap1* | ArfGAP with SH3 domain, ankyrin repeat and PH domain1 |
| 13429 | *Dnm1* | Dynamin 1 |
| 14963 | *H2-Bl* | Histocompatibility 2, blastocyst |
| 14972 | *H2-K1* | Histocompatibility 2, K1, K region |
| 15006 | *H2-Q1* | Histocompatibility 2, Q region locus 1 |
| 15007 | *H2-Q10* | Histocompatibility 2, Q region locus 10 |
| 15013 | *H2-Q2* | Histocompatibility 2, Q region locus 2 |
| 15018 | *H2-Q7* | Histocompatibility 2, Q region locus 7 |
| 15039 | *H2-T22* | Histocompatibility 2, T region locus 22 |
| 15040 | *H2-T23* | Histocompatibility 2, T region locus 23 |
| 15239 | *Hgs* | HGF-regulated tyrosine kinase substrate |
| 15481 | *Hspa8* | Heat shock protein 8 |
| 16835 | *Ldlr* | Low density lipoprotein receptor |
| 18717 | *Pip5k1c* | Phosphatidylinositol-4-phosphate 5-kinase, type 1 gamma |
| 234852 | *Chmp1a* | Charged multivesicular body protein 1A |
| 243621 | *Iqsec3* | IQ motif and Sec7 domain 3 |
| 67588 | *Rnf41* | Ring finger protein 41 |
| 98366 | *Smap1* | Stromal membrane-associated protein 1 |
| **PATHWAYS POINTED BY DOWN REGULATED GENES** | | |
| **VEGF signaling pathway** | | |
| 11651 | *Akt1* | Thymoma viral proto-oncogene 1 |
| 16653 | *Kras* | v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog |
| 19056 | *Ppp3cb* | Protein phosphatase 3, catalytic subunit, beta isoform |
| 22339 | *Vegfa* | Vascular endothelial growth factor A |
| **Long-term depression** | | |
| 14678 | *Gnai2* | Guanine nucleotide binding protein (G protein), alpha inhibiting 2 |
| 14683 | *Gnas* | GNAS (guanine nucleotide binding protein, alpha stimulating) complex locus |
| 16653 | *Kras* | v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog |
| 18795 | *Plcb1* | Phospholipase C, beta 1 |
| 60596 | *Gucy1a3* | Guanylate cyclase 1, soluble, alpha 3 |
| **Gap junction** | | |
| 14678 | *Gnai2* | Guanine nucleotide binding protein (G protein), alpha inhibiting 2 |
| 14683 | *Gnas* | GNAS (guanine nucleotide binding protein, alpha stimulating) complex locus |
| 16653 | *Kras* | v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog |
| 18795 | *Plcb1* | Phospholipase C, beta 1 |
| 60596 | *Gucy1a3* | Guanylate cyclase 1, soluble, alpha 3 |

*(Continued)*

**Table 4 | Continued**

| Gene ID | Gene symbol | Gene name |
|---|---|---|
| **RNA degradation** | | |
| 104625 | *Cnot6* | CCR4-NOT transcription complex, subunit 6 |
| 13209 | *Ddx6* | DEAD (Asp-Glu-Ala-Asp) box polypeptide 6 |
| 66373 | *Lsm5* | LSM5 homolog, U6 small nuclear RNA associated (S. cerevisiae) |
| 72662 | *Dis3* | DIS3 mitotic control homolog (S. cerevisiae) |
| 78651 | *Lsm6* | LSM6 homolog, U6 small nuclear RNA associated (S. cerevisiae) |
| **Parkinson's disease** | | |
| 12866 | *Cox7a2* | Cytochrome c oxidase subunit VIIa 2 |
| 333182 | *Cox6b2* | Cytochrome c oxidase subunit VIb polypeptide 2 |
| 66142 | *Cox7b* | Cytochrome c oxidase subunit VIIb |
| 66495 | *Ndufb3* | NADH dehydrogenase (ubiquinone) 1 beta subcomplex 3 |
| 66916 | *Ndufb7* | NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 7 |
| 68202 | *Ndufa5* | NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 5 |
| **Oxidative phosphorylation** | | |
| 12866 | *Cox7a2* | Cytochrome c oxidase subunit VIIa 2 |
| 333182 | *Cox6b2* | Cytochrome c oxidase subunit VIb polypeptide 2 |
| 66142 | *Cox7b* | Cytochrome c oxidase subunit VIIb |
| 66495 | *Ndufb3* | NADH dehydrogenase (ubiquinone) 1 beta subcomplex 3 |
| 66916 | *Ndufb7* | NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 7 |
| 68202 | *Ndufa5* | NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 5 |
| **Tight junction** | | |
| 11651 | *Akt1* | Thymoma viral proto-oncogene 1 |
| 14678 | *Gnai2* | Guanine nucleotide binding protein (G protein), alpha inhibiting 2 |
| 16653 | *Kras* | v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog |
| 17888 | *Myh6* | Myosin, heavy polypeptide 6, cardiac muscle, alpha |
| 30960 | *Vapa* | Vesicle-associated membrane protein, associated protein A |
| 58187 | *Cldn10* | Claudin 10 |
| **Glutamatergic synapse** | | |
| 14678 | *Gnai2* | Guanine nucleotide binding protein (G protein), alpha inhibiting 2 |
| 14683 | *Gnas* | GNAS (guanine nucleotide binding protein, alpha stimulating) complex locus |
| 14702 | *Gng2* | Guanine nucleotide binding protein (G protein), gamma 2 |
| 14802 | *Gria4* | Glutamate receptor, ionotropic, AMPA4 (alpha 4) |
| 14805 | *Grik1* | Glutamate receptor, ionotropic, kainate 1 |
| 18795 | *Plcb1* | Phospholipase C, beta 1 |
| 19056 | *Ppp3cb* | Protein phosphatase 3, catalytic subunit, beta isoform |
| 216227 | *Slc17a8* | Solute carrier family 17 (sodium-dependent inorganic phosphate cotransporter), member 8 |

*(Continued)*

**Table 4 | Continued**

| Gene ID | Gene symbol | Gene name |
|---|---|---|
| **Huntington's disease** | | |
| 12866 | *Cox7a2* | Cytochrome c oxidase subunit VIIa 2 |
| 18795 | *Plcb1* | Phospholipase C, beta 1 |
| 333182 | *Cox6b2* | Cytochrome c oxidase subunit VIb polypeptide 2 |
| 66142 | *Cox7b* | Cytochrome c oxidase subunit VIIb |
| 66495 | *Ndufb3* | NADH dehydrogenase (ubiquinone) 1 beta subcomplex 3 |
| 66916 | *Ndufb7* | NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 7 |
| 68202 | *Ndufa5* | NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 5 |
| 69920 | *Polr2i* | Polymerase (RNA) II (DNA directed) polypeptide I |
| **Alzheimer's disease** | | |
| 11820 | *App* | Amyloid beta (A4) precursor protein |
| 12866 | *Cox7a2* | Cytochrome c oxidase subunit VIIa 2 |
| 18795 | *Plcb1* | Phospholipase C, beta 1 |
| 19056 | *Ppp3cb* | Protein phosphatase 3, catalytic subunit, beta isoform |
| 333182 | *Cox6b2* | Cytochrome c oxidase subunit VIb polypeptide 2 |
| 66142 | *Cox7b* | Cytochrome c oxidase subunit VIIb |
| 66495 | *Ndufb3* | NADH dehydrogenase (ubiquinone) 1 beta subcomplex 3 |
| 66916 | *Ndufb7* | NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 7 |
| 68202 | *Ndufa5* | NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 5 |
| **Ribosome** | | |
| 19951 | *Rpl32* | Ribosomal protein L32 |
| 19981 | *Rpl37a* | Ribosomal protein L37a |
| 19982 | *Rpl36a* | Ribosomal protein L36A |
| 20068 | *Rps17* | Ribosomal protein S17 |
| 20085 | *Rps19* | Ribosomal protein S19 |
| 22186 | *Uba52* | Ubiquitin A-52 residue ribosomal protein fusion product 1 |
| 57294 | *Rps27* | Ribosomal protein S27 |
| 66489 | *Rpl35* | Ribosomal protein L35 |
| 67945 | *Rpl41* | Ribosomal protein L41 |
| 68028 | *Rpl22l1* | Ribosomal protein L22 like 1 |
| 75617 | *Rps25* | Ribosomal protein S25 |

subtle changes in gene expression are exactly those that occur in initial stages of disease before the onset of clinical symptoms (Druyan et al., 2008). Moreover, some authors have argued that even small differences can be biologically relevant (Pedotti et al., 2008). Indeed, our qPCR verification analysis revealed higher fold changes than in the microarray, reaching values higher than 2 in the 80 days pre-symptomatic phase, which is closer to the symptom onset. The use of qPCR analysis to qualitatively verify the microarray results is largely accepted in the literature. However, it is well recognized that both methods have quantitative differences (Chuaqui et al., 2002), which are

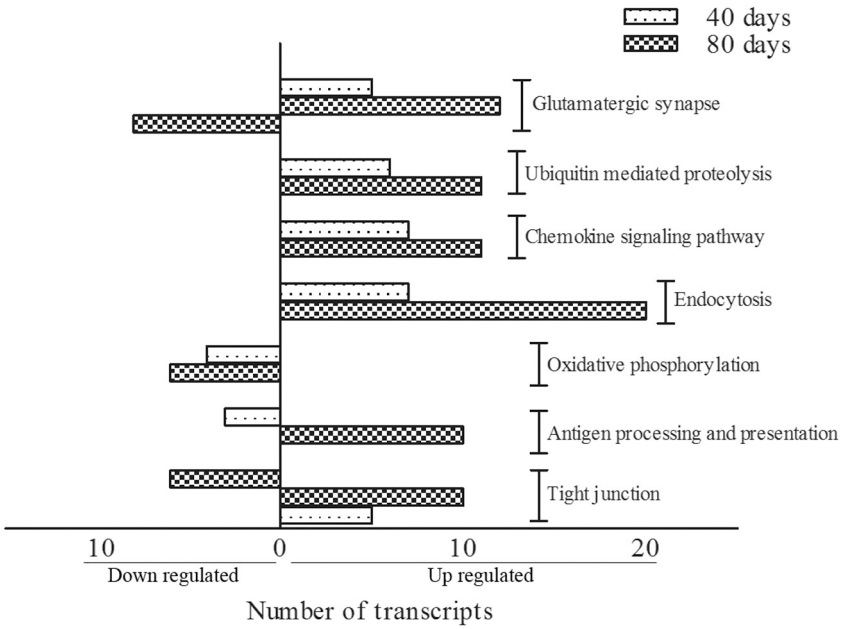


**FIGURE 1 | KEGG pathways classification showing the number of transcripts up regulated and down regulated per category in 40 and 80 days pre-symptomatic SOD1$^{G93A}$ mice in relation to age matched wild-types.** Bars on the left indicate the number of down regulated genes, and bars on the right indicate the number of up regulated genes, for each category.

thought to be related to the variation in the hybridization kinetics of the technologies, low fold changes or lack of concordance between transcripts accessed in each method. The number of genes employed in qPCR validation is comparable to that found by other studies (Dallas et al., 2005; Brockington et al., 2010).

The differentially expressed genes with a *p*-value lower than 0.05 were submitted to enrichment analyses based on GO and KEGG databases, which correlated genes to already described related pathways and processes. Modulated genes based on GO evidenced more general biological processes that might be implicated in the ALS mechanisms. Of interest, regulation of astrocyte differentiation, protein retention in endoplasmatic reticulum lumen, Golgi vesicle transport and fructose metabolism, among others, were pointed at the pre-symptomatic 40 days old transgenic mice. At later pre-symptomatic phase of 80 days, the pattern of gene expression identified the GO terms post-Golgi vesicle-mediated transport, tricarboxylic acid cycle (TCA) and mRNA processing, among others. GO database analyses have been largely employed in the ALS research in several phases of the disease (Ferraiuolo et al., 2007, 2011a; Brockington et al., 2010).

Authors have also used the KEGG database to identify over-represented pathways based on differentially expressed genes obtained by the microarray technique (Mougeot et al., 2011; Kalathur et al., 2012). The KEGG database analysis in the present work pointed to pathways that might be related to ALS mechanism at the pre-symptomatic ages of SOD1$^{G93A}$ mice. Some pathways were found to be common to both pre-symptomatic periods, emphasizing the putative toxic triggering that may last before the onset of classical ALS symptoms with possible significance to mechanisms of initiation of motor neuron degeneration. Those pathways are going to be discussed below. It should be mentioned that alternative splicing have been recently implicated in ALS mechanisms (Lenzken et al., 2011; Singh and Cooper, 2012), however we could not access this biological event because the present analysis employed a platform designed to gene expression studies on 3′UTR that does not allow evaluation of alternative splicing variants.

## GLUTAMATERGIC SYNAPSE

The microarray profiling study by means of KEEG enriched analysis pointed to the category of glutamatergic synapse pathway in the lumbar spinal cord of ALS SOD1$^{G93A}$. The large number of up regulated genes at 40 and 80 days underlines the excitotoxicity estate mediated by glutamatergic synapse of motor neurons in the pre-symptomatic condition of ALS disease (Bendotti et al., 2001; Gibb et al., 2007; Zhao et al., 2008; Jiang et al., 2009; Sunico et al., 2011). The modulation of GluR4, by means of *Gria4* findings in our microarray analysis, might reflect the dynamic state of the AMPA receptor subunit in the course of pre-symptomatic stages of ALS. At the early phases of the pre-symptomatic period, highly expressed *Gria4* gene might contribute to the AMPA receptor-mediated motor neuron toxicity, being a very early mechanism of the disease. The down regulation of the *Gria4* at the late pre-symptomatic stage could reflect a transient reactive mechanism to excitotoxic condition preceding motor neuron death. Reductions of GluR4 have been described at cellular level in the late disease stage of SOD1 mice, without alterations at the pre-symptomatic periods (Petri et al., 2005) thus, reflecting a disappearance of GluR4 containing neurons. In fact, imbalance of excitatory to inhibitory synaptic function precedes motor neuron degeneration

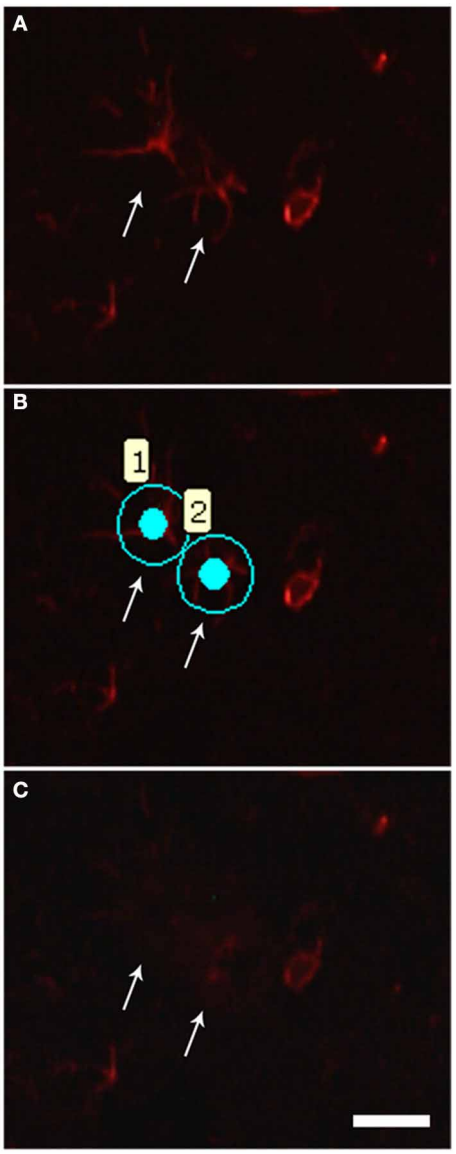


**FIGURE 2 | Photomicrographs illustrating astrocyte laser microdissection process. (A)** The quick GFAP immunofluorescence allows recognizing the astrocytic profiles (arrows). **(B)** Astrocytes (1 and 2) were then selected for microdissection. **(C)** After laser firing and microdissection, selected cells (arrows) can no longer be visualized in the tissue. Scale bars of 20 μm.

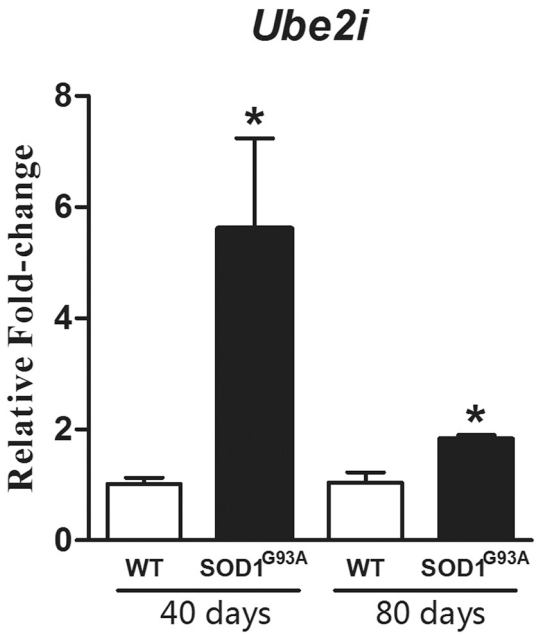


**FIGURE 3 | Graph shows relative fold change values for *Ube2i* in microdissected astrocytes from 40 and 80 days old SOD1$^{G93A}$ mice compared to the age matched wild-type controls (WT).** Significant increases are seen in both transgenic astrocytes enriched samples. Results are presented as means ± s.e.m. from 3 samples used for each group. *$p$-value < 0.05, according to unpaired $t$-test.

as described in the spinal cord motor neurons in the late stage of pre-symptomatic phase of SOD1 ALS model by means of cellular analyses (Schutz, 2005). It should be mentioned that $Ca^{2+}$ permeability of the AMPA receptor seems to occur mainly by the presence of the GluR2 subunit in the receptor complex. In fact, GluR2 deficiency clearly accelerated the motor neuron degeneration and shortened the life span of mutant SOD1$^{G93A}$ double transgenic mice (Tateno et al., 2004). Synaptic GluR1 increases/mRNA up regulation, and decreases of synaptic and total GluR2 were found at early ages prior to disease onset thus prompting motor neurons to a higher $Ca^{2+}$-permeable AMPA receptors -induced excitotoxicity (Zhao et al., 2008). The variant C-terminus of GluR4 (GluR4c), an alternative splicing isoform, stabilizes and locates AMPA receptors in the cell membrane, and also seems to potentate actions of GluR2 (Kawahara et al., 2004), thus highlighting the pivotal role of GluR4 subunit in regulating channel properties and trafficking of AMPA receptors. It must be then further clarified the role of GluR4 in the ALS mechanisms and possible dynamic interaction with that subunit with other AMPA receptor subtypes, especially GluR2.

The regulation of *Slc1a2* glial glutamate transporter (named EAAT2 or glial glutamate transporter GLT1) has not been evaluated in details. Excitotoxicity caused by a down-regulation of EAAT2 is thought to be a contributing factor to motor neuron death in ALS. Several mechanisms may account for impairment of EAAT2 function, for instance altered transcription/splicing, post-translational modifications, accelerated degradation, intracelular trafficking and inactivation by caspase-3 cleavage (Heath and Shaw, 2002; Boston-Howes et al., 2006) but not directly to gene regulation processes. It is possible that the impaired EAAT2 function could take place at the very early period of the pre-symptomatic stage, a matter that remains to be elucidated (Bendotti et al., 2001; Sasaki et al., 2001), thus, explaining the *Slc1a2* expression possibly related to motor neuron protection at those ages. The absence of this genomic process in the late pre-symptomatic period might potentiate loss of function of GLT1 thus culminating with the motor neuron death in ALS. Furthermore, the vesicular glutamate transporter 2 (VGLUT2), codified by *Slc17a6* gene, was found to be regulated and related

to neuronal death in the pre-symptomatic stage of ALS model (Schutz, 2005; Sunico et al., 2011). The genetic reduction of VGLUT2 protein level in the ALS mouse model accounted for motor neuron rescue without modifying functional impairment (Wootz et al., 2010). It is possible that the up regulation of the *Slc17a6* gene at the early pre-symptomatic stage of the 40 days old $SOD1^{G93A}$ mice potentiates the toxic state of motor neurons.

#### UBIQUITIN MEDIATED PROTEOLYSIS AND OXIDATIVE PHOSPHORYLATION

A recent meta-analysis study of the reported gene lists has described the evidences for a shared dysfunction in protein turnover in the ubiquitin-proteasome system in ALS mouse models and ALS patients (Saris et al., 2013a). Moreover, constitutive proteasome was decreased in motor neurons at the pre-symptomatic stage of $SOD1^{G93A}$ (Cheroni et al., 2005), an alternative processes to decrease aggregate formation, thus an attempt to neuroprotect motor neurons of preclinical $SOD1^{G93A}$ mice before the onset of clinical symptoms (Bendotti et al., 2012). That should be the case of *Nedd4* and *Fbxw7* expressions described here, whose encoded molecules have been already correlated to neuroprotection in ALS (Nateri et al., 2004; Matsumoto et al., 2011; Kwak et al., 2012). Moreover, it should be taken into attention the elevation of *Ubc12* in the spinal cord of $SOD1^{G93A}$ mice at the pre-symptomatic phase (Massignan et al., 2007). *Ubc12* is an ubiquitin E2 ligase that adds NEDD-8 to substrates. *Ubc12* elevation in pre-symptomatic ALS was correlated to a tentative response to protein aggregation (Massignan et al., 2007). Interestingly, *Nedd8* gene was down regulated in our microarray analysis only in 80 days old mice, possibly representing a failure of the above described process close to the period of clinical onset.

The down regulation of genes over-representing the oxidative phosphorylation category at both pre-symptomatic ages of ALS mice seen in this work may be related to the progressive deteriorations of mitochondrial function and oxidative phosphorylation system described at pre-symptomatic ALS phases (Lin et al., 2009; Chen et al., 2010; Martin, 2010, 2011; Koopman et al., 2013), thus triggering reactive oxygen species (ROS) production (Manfredi and Xu, 2005) and motor neuron vulnerability before the onset of clinical symptoms. It is also interesting to notice that the TCA was seen as an over-represented GO term (Table S5, Supplementary material) in the up-regulated 80 days gene expression list. The TCA cycle is responsible to provide substrate to oxidative phosphorylation (Koopman et al., 2013) and its up regulation was previously seen in laser microdissected motor neurons from a VEGF model of ALS already in the pre-symptomatic period (Brockington et al., 2010). All in all, a possible mechanism of oxidative phosphorylation in the astrocyte-neuronal unit taking place in pre-symptomatic ALS might amplify motor neuron vulnerability to ROS damage.

#### CHEMOKINE SIGNALING PATHWAY AND TIGHT JUNCTION

The up regulation of all genes in the category chemokine signaling pathway in the pathogenesis of ALS is in agreement to previous publications (Henkel et al., 2006; Zhang et al., 2006; Rentzos et al., 2007; Kuhle et al., 2009; Sargsyan et al., 2009; Tateishi et al., 2010; Gupta et al., 2012). The up regulation of *Cxcr4* and *Pik3r1* described in this work is an important finding because the genes might be involved in non-autonomous toxicity in the early phase of ALS (Shideman et al., 2006; Luo et al., 2007; Manzano et al., 2011). Furthermore, disruption of blood-brain barrier and blood-spinal cord barrier are described as early events in ALS, thus impairing neurovascular unit prior motor neuron degeneration (Garbuzova-Davis et al., 2011, 2012; Grammas et al., 2011; Miyazaki et al., 2011). Indeed, reduced levels of adhesion molecules and the tight junction proteins zona occludens-1, occludin and claudin-5 are shown in post mortem tissue from patients and in ALS animal models (Zhong et al., 2008; Arhart, 2010; Garbuzova-Davis et al., 2012).

Our KEGG enriched analysis also demonstrated the modulation of tight junction related genes. Of substantial interest, we might point out the up regulation of *Cldn11* at 40 days and the down regulation of *Cldn10* at 80 days pre-symptomatic ALS mice, in agreement to previous description on differential regulation of tight junction genes related to specific characteristics of ALS clinical evolution (Henkel et al., 2009).

It is also important to highlight the particular modulation of the *Kras* gene, which has been up regulated at the age of 40 days and down regulated at the age of 80 days. The *Kras* gene is an oncogene that was located in the tight junction category by the KEGG analysis probably due its relation to topography of invading/proliferating cells in the scenario of neurodegenerative processes. Moreover, Kras proteins regulate cell activities such as proliferation, differentiation, apoptosis, and cell migration, those taking place in neurodegenerative processes-induced astroglial/microglial activation as well as expression of inflammatory and neurotrophic/neurotoxic mediators (Rotshenker, 2009). There is a marked proliferation/activation of both microglia and astrocytes at specific disease stages in ALS mouse models (Hall et al., 1998; Weydt et al., 2002) leading to the production of neuroprotective or pro-inflammatory molecules, which can decrease or increase the rate of primary motor neuron degeneration, respectively. Taken all together, up regulation of *Kras* gene at the early pre-symptomatic phase is in line with the early glial proliferative and reactivity events that will initiate the toxic triggering of non-autonomous cells and also the glial neuroprotective mechanisms to maintain temporarily the motor neurons. Later in that period, still before neuronal degeneration taking place, *Kras* gene down regulation might allow glial cells to drive toxic insult.

#### ENDOCYTOSIS AND ANTIGEN PROCESSING AND PRESENTATION

Endocytosis was an additional over-represented pathway in the pre-symptomatic stage of ALS. Genes found before clinical onset pointing to endocytosis have been related to clathrin-dependent/independent endocytosis, autophagy and also neurotransmission (Massey et al., 2006; Luo et al., 2007; Kon and Cuervo, 2010; McMahon and Boucrot, 2011; Elmer and McAllister, 2012), thus, related to extracellular turnover, repair of molecular processes and neuroprotection (Le Roy and Wrana, 2005; Doherty and McMahon, 2009; McMahon and Boucrot, 2011; Polymenidou and Cleveland, 2011). Disruption of these processes has been implicated as a general feature in the pathogenesis of ALS (Otomo et al., 2012), whereas there is a lack of information on that issue in pre-symptomatic periods

(Morimoto et al., 2007; Tian et al., 2011). Clathrin-mediated endocytosis has a range of different physiological functions, remarkably the regulation of surface proteins, nutrition, activation of signaling pathways, protein trafficking and degradation of membrane components, in fact, mechanisms that might occur at the pre-symptomatic phases of ALS.

It is likely that the regulation of the genes for the heat shock proteins *Hspa1a* and *Hspa8* (also known as Hsp70-3 and Hsc70, respectively), described in our work is related to neuroprotective events before neurodegeneration, once treatment with recombinant human Hsp70 was able to both increase lifespan (Gifondorwa et al., 2007) and decrease neuromuscular junction denervation (Gifondorwa et al., 2012) in the SOD1$^{G93A}$ mouse model. This protective role of Hsp70 has been also supported by other authors (Bruening et al., 1999; Takeuchi et al., 2002; Kieran et al., 2004). Actually, the increase of Hsc70 in the spinal cord of transgenic mice at pre-symptomatic ages of disease (Basso et al., 2009) and the demonstration of ubiquitinated Hsc70-induced degradation of mutant SOD1 (Urushitani et al., 2004) emphasized the possible neuroprotective role of heat shock protein regulation described in our work.

Furthermore, antigen processing and presentation pathway was also pointed as enriched among down regulated genes in 40 days old and up regulated genes in 80 days old pre-symptomatic SOD1$^{G93A}$ mice. Genes presented in 40 and 80 days lists are mostly related to major histocompatibility complex (MHC) class I (*H2-Bl*, *H2-K1*, *H2-Q1*, *H2-Q10*, *H2-Q2*, *H2-Q7*, *H2-T22*, *H2-T23*—80 days ALS mice), molecules necessary for peptide loading (*Tap2*—80 days ALS mice) and to surface expression (*B2m*—40 days ALS mice) (Kimura and Griffin, 2000). *B2m* gene, possibly via cell surface MHC class I molecules, has been implicated in the synaptic plasticity at dendrites and axonal regeneration after peripheral nerve axotomy (Oliveira et al., 2004). It is possible that the down regulation of *B2m* in spinal cord from SOD1$^{G93A}$at pre-symptomatic ages is related to axonal and dendritic retractions and displacement of neuromuscular junction described as one of the earliest events faced by motor neurons in ALS models (Fischer et al., 2004). Our findings are in line with a description of down regulation of B2m protein reported in cerebrospinal fluid of ALS patients (Brettschneider et al., 2008), thus emphasizing the importance of its regulation in ALS. Additionally, *Rfxank* was down regulated at 40 days in our analysis, which is in agreement to a loss of MHC-II neuronal expression concurrent with abundant MHCII-positive microglia surrounding motor neurons in the pre-symptomatic SOD1$^{G93A}$ mice (Casas et al., 2013), thus, interfering with the neuroimmunemodulation mediated by microglia (Graber et al., 2010; Sanagi et al., 2010). All in all, dysregulation of genes related to antigen processing and presentation might account for a number of intercellular mechanisms able to amplify the harmful non-autonomous cell toxicity at the pre-symptomatic stages of ALS.

#### LASER MICRODISSECTION OF ASTROCYTES

We performed laser microdissection of GFAP positive astrocytes from lumbar spinal cord ventral horn of SOD1$^{G93A}$ transgenic and wild-type mice in the same pre-symptomatic ages of microarray analysis. The use of laser microdissection has been gained importance in recent years, once it allows specific cell enrichment from complex tissues, revealing to be a powerful tool in the study of neurodegenerative disorders in which individual cell types are known to be differentially involved in disease stages. The advantage of the methodology is the possibility to address molecular biology in the context of *in vivo* cellular analysis. The method is of substantial importance to evaluate changes in the astrocytes, the glial cell involved remarkably in toxic mechanisms of ALS. A previous study has employed laser microdissection of astrocytes to perform microarray experiments in ALS mouse model (Ferraiuolo et al., 2011a). The pattern of gene expression was first evaluated in the lumbar regions of the spinal cord in the present analysis, thus, taking into account all cell types from tissue. The depicted pathways represented the state of intercellular interaction in the pre-symptomatic studied periods of the ALS mouse model. The selected genes to be evaluated in type-specific cell, which is the case of *Ube2i* in the laser microdissected astrocytes described herein, would allow a closer analysis of astrocyte participation in the context of the neighbor cell toxicity. The *Ube2i* gene was then chosen for further evaluation in astrocytes by qPCR because astrocytes exert a non-autonomous cell toxicity to motor neurons and because SUMOylation pathway has gained importance in ALS mechanisms recently (for review, see Dangoumau et al., 2013). Increases of gene expression for *Ube2i* were found in enriched astrocytes samples from 40 and 80 days old pre-symptomatic mice, a regulation still not presented in the literature in that stage of disease, thus, entering in the context of ALS pathogenesis. In fact, conjugation of small ubiquitin-like modifier (SUMO) molecules involves a series of steps, being the ubiquitin conjugating enzyme E2, codified by *Ube2i* gene, responsible for the recognition of the target protein. SUMOylation is involved in the cellular response to oxidative stress, hypoxia, glutamate excitotoxicity and proteasome impairment, events that have been linked to motor neuron toxicity in ALS (Xu et al., 2011). Moreover, studies are required to determine the precise implication of the SUMO pathway in regulating the balance between cellular adaptive and neuroprotective response to stress (Fei et al., 2006; Dangoumau et al., 2013) with a special importance to motor neuron in the pre-symptomatic stage of ALS. Nevertheless, as discussed previously in this report, glutamate astroglial excitotoxicity faced by motor neurons in ALS is also hamfull by the cleavage of EAAT2 in the ventral horn of the spinal cord (Martin et al., 2007; Foran et al., 2011). The proteolytic fragments may be SUMOylated and accumulated in the nucleus of astrocytes (Boston-Howes et al., 2006; Foran et al., 2011) as described in SOD1$^{G93A}$ mice, worsening the gliotoxic effects of astrocytes to motor neurons (Foran et al., 2011). Taking together, SUMOylation process and expression of *Ube2i* might participate in complex events related to the astrocyte-neuron unit in ALS, and future works are required to address specific cellular events.

In conclusion, the present work gives further evidence about molecular events taking place in the spinal cord from ALS mouse model before the onset of classical symptoms. The gene expression changes reflect responses for both neuroprotection and toxicity at the spinal cord in the evaluated periods. Indeed, the study of Ube2i expression in astrocytes adds novel insights for the participation of this cell type on the early mechanisms in ALS.

## AUTHOR CONTRIBUTIONS

Gabriela Pintar de Oliveira and Chrystian J. Alves performed the experiments. All authors designed the study, analyzed the results and wrote the manuscript. All authors read and approved the final manuscript.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fncel.2013.00216/abstract

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# Mitochondrial DNMT3A and DNA methylation in skeletal muscle and CNS of transgenic mouse models of ALS

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Cytosine methylation is an epigenetic modification of DNA catalyzed by DNA methyltransferases. Cytosine methylation of mitochondrial DNA (mtDNA) is believed to have relative underrepresentation; however, possible tissue and cell differences in mtDNA methylation and relationships to neurodegenerative disease have not been examined. We show by immunoblotting that DNA methyltransferase 3A (Dnmt3a) isoform is present in pure mitochondria of adult mouse CNS, skeletal muscle, and testes, and adult human cerebral cortex. Dnmt1 was not detected in adult mouse CNS or skeletal muscle mitochondria but appeared bound to the outer mitochondrial membrane. Immunofluorescence confirmed the mitochondrial localization of Dnmt3a and showed 5-methylcytosine (5mC) immunoreactivity in mitochondria of neurons and skeletal muscle myofibers. DNA pyrosequencing of two loci (D-loop and 16S rRNA gene) and twelve cytosine-phosphate-guanine (CpG) sites in mtDNA directly showed a tissue differential presence of 5mC. Because mitochondria have been implicated in the pathogenesis of amyotrophic lateral sclerosis (ALS), but the disease mechanisms are uncertain, we evaluated mitochondrial Dnmt3a and 5mC levels in human *superoxide dismutase-1* (SOD1) transgenic mouse models of ALS. Mitochondrial Dnmt3a protein levels were reduced significantly in skeletal muscle and spinal cord at presymptomatic or early disease. Immunofluorescence showed that 5mC immunoreactivity was present in mitochondria of neurons and skeletal myofibers, and 5mC immunoreactivity became aggregated in motor neurons of ALS mice. DNA pyrosequencing revealed significant abnormalities in 16S rRNA gene methylation in ALS mice. Immunofluorescence showed that 5mC immunoreactivity can be sequestered into autophagosomes and that mitophagy was increased and mitochondrial content was decreased in skeletal muscle in ALS mice. This study reveals a tissue-preferential mitochondrial localization of Dnmt3a and presence of cytosine methylation in mtDNA of nervous tissue and skeletal muscle and demonstrates that mtDNA methylation patterns and mitochondrial Dnmt3a levels are abnormal in skeletal muscle and spinal cord of presymptomatic ALS mice, and these abnormalities occur in parallel with loss of myofiber mitochondria.

**Keywords: ALS, DNA pyrosequencing, Dnmt1, Dnmt3a, mitochondrial DNA, 5-methylcytosine, mitochondria, motor neuron**

## INTRODUCTION

Methylation of DNA on carbon five of cytosine (5-methylcytosine, 5mC) in cytosine-phosphate-guanine (CpG) dinucleotides is an epigenetic modification of DNA used by cells to regulate nuclear gene expression (Jones and Takai, 2001; Bird, 2002; Brenner and Fuks, 2006). Cytosine methylation signals through 5mC-binding proteins to remodel chromatin and downregulate gene expression. Cytosine methylation is mediated by a family of DNA methyltransferase (Dnmt) enzymes (Cheng, 1995). Dnmt1 is the most abundant isoform in proliferating cells and displays a preference for hemimethylated substrates and is targeted to replication forks, acting to maintain DNA methylation patterns during cell replication (Robertson, 2001), and to DNA repair sites (Mortusewicz et al., 2005). Recently, mutations in the *Dnmt1* gene have been identified as a cause of hereditary sensory and autonomic neuropathy type 1 (Klein et al., 2011) and autosomal dominant cerebellar ataxia, deafness, and narcolepsy (Winkelmann et al., 2012). Dnmt2 (also called tRNA methyltransferase-1) transfers methyl groups to RNA instead of DNA (Schaefer and Lyko, 2010; Motorin and Helm, 2011). DNA methyltransferase 3A (Dnmt3a) and Dnmt3b function as *de novo* methyltransferases because they methylate hemimethylated DNA and also completely unmethylated DNA (Okano et al., 1999; Xie et al., 1999). Dnmt3L functions as an essential regulatory cofactor for Dnmt3a (Jia et al., 2007). Nuclear DNA methylation attracts attention because it has been implicated in normal cell and tissue development and differentiation (Geiman and Muegge, 2010) and in human diseases, including cancer (Calvanese et al.,

2009), Rett syndrome (Amir et al., 1999), and, more recently, neurodegenerative diseases (Chestnut et al., 2011; Klein et al., 2011; Winkelmann et al., 2012; Martin and Wong, 2013).

Studies of mitochondrial DNA (mtDNA) cytosine methylation and the Dnmts that regulate mtDNA methylation are not as common as studies of nuclear DNA methylation; moreover, many mitochondrial-based mechanisms of disease have been implicated in amyotrophic lateral sclerosis (ALS) (Beal, 2005; Martin, 2010, 2012; Reddy and Reddy, 2011; Panov et al., 2012; Santa-Cruz et al., 2012), but mtDNA methylation and mitochondrial Dnmts have not been studied in ALS. Earlier work tends to minimize the occurrence and importance of mtDNA cytosine methylation. Some studies of cultured cells have found mtDNA cytosine methylation in mouse fibroblastoid cells (Pollack et al., 1984) and human fibroblasts (Shmookler Reis and Goldstein, 1983). In the latter study, ∼2–5% of CCGG sites were fully methylated. Other studies have found underrepresented or no mtDNA cytosine methylation (Maekawa et al., 2004). However, the earlier studies that failed to observe mtDNA cytosine methylation were done on mitochondria isolated from blood. More recently, it has been reported that Dnmt1 localizes to mitochondria in cultured mouse embryonic fibroblasts and human colon carcinoma cells (Shock et al., 2011) and that Dnmt3a localizes to mouse brain and spinal cord mitochondria (Chestnut et al., 2011). Studies have yet to examine comparatively mtDNA methylation in different adult tissue types *in vivo* to test the hypothesis that there is tissue specificity for mtDNA cytosine methylation. Moreover, the physiological significance and potential pathophysiological relevance of mtDNA methylation in ALS merit exploration in light of findings that Dnmt3 is localized to neuronal mitochondria and appears to be upregulated in human ALS neurons and mouse spinal motor neurons during their degeneration (Chestnut et al., 2011).

In the current study, we demonstrate that Dnmt3a and 5mC are in mitochondria of adult mouse and human tissue types that are mostly excitable tissues (nervous and muscle) and that tissue Dnmt3a levels are reduced and 5mC immunoreactivity accumulates in skeletal muscle myofibers and spinal cord motor neurons in mouse models of ALS. We also show directly by pyrosequencing that mtDNA from these tissues contains 5mC and that the levels of 5mC in the 16S rRNA gene are increased in transgenic mouse models of ALS.

## MATERIALS AND METHODS

### MICE

Wildtype and human *superoxide dismutase-1* (hSOD1) transgenic (tg) mice were used with approval from the institutional Animal Care and Use Committee. For studies of normal DNMT3a and 5mC localizations and distributions, non-tg adult C57BL/6 and SV129 mice ($n = 20$–25) were used at 2–6 months of age. Several different tg mouse lines were used. One line was hemizygous for a low copy number of hSOD1-G37R mutant allele driven by the endogenous human promoter (line 29) derived from a founder B6.Cg-Tg SOD1-G37R 29Dpr/J (stock # 008229, The Jackson Laboratory, Bar Harbor, MA). Another tg mouse line (hSOD1-wildtype) expressed normal wildtype human *SOD1* gene (B6SJL-TgN[SOD1] 2Gur, stock #002297, The Jackson Laboratory) at high levels (copy number ∼25) driven by the endogenous human promoter. These two tg lines thus expressed hSOD1 ubiquitously in many tissues (Gurney et al., 1994; Martin et al., 2007, 2009; Gertz et al., 2012). We also used tg mice with skeletal muscle-restricted expression of hSOD1-G37R, -G93A, and -wildtype variants (designated as $hSOD1^{mus}$) which have been described previously (Wong and Martin, 2010). All the lines of hSOD1 tg mice were studied at presymptomatic or early to middle stages of disease. Control mice were age-matched, non-tg littermates. Mouse genotypes were identified by PCR. Mitochondrial fractions were prepared from 5–10 mice/genotype.

### PREPARATION OF CRUDE MITOCHONDRIAL FRACTIONS FROM SKELETAL MUSCLE AND HEART

Tissue mitochondrial preparations were isolated from fresh tissues with no prior freezing or fixation. For fresh tissue harvesting, mice were deeply anesthetized and decapitated; organs and tissues were immediately removed, weighed, and placed in ice-cold phosphate-buffered saline (PBS). Muscle crude mitochondrial fractions were obtained using a modification (**Figure 1**) of a described method (Bhattacharya et al., 1991). The tissue was finely minced using a razor. The pieces were resuspended in 10 ml/g digestion buffer (100 mM sucrose, 10 mM EDTA, 100 mM Tris-HCl, 46 mM KCl, pH 7.4) with 0.1 mg/ml nagarse (Sigma-Aldrich) and were digested at room temperature for 10–20 min. The digest was placed in an equal volume of homogenization buffer consisting of digestion buffer with 5 mg/ml BSA and complete protease inhibitor (Roche Diagnostics). The tissue pieces were allowed to settle, the buffer was aspirated, and the tissue was resuspended in an equal volume of homogenization buffer. The tissue was homogenized using a PT 3100 Polytron (Kinematica) at 20,000 rpm on ice. The homogenate was centrifuged at 500 g for 10 min and the resulting supernatant was removed to a fresh tube. The supernatant was then centrifuged at 12,000 g for 20 min. The resulting pellet was the crude mitochondrial fraction.

### PREPARATION OF CRUDE MITOCHONDRIAL FRACTION FROM TISSUES OTHER THAN STRIATED MUSCLE

Crude mitochondrial fractions of organs were obtained using a described method (Okado-Matsumoto and Fridovich, 2001) with slight modifications (**Figure 1**). Brain, spinal cord, liver, kidney, colon, and spleen were homogenized in nycodenz homogenization buffer (210 mM mannitol, 70 mM sucrose, 10 mM Tris, 1 mM EDTA, and protease inhibitor, pH 7.5) at a 1:5 (w/v) ratio using a PT 3100 Polytron at 20,000 rpm on ice. The homogenate was centrifuged at 1300 g for 10 min and resultant supernatant removed to a fresh tube. The supernatant was then centrifuged at 17,000 g for 10 min. The resulting pellet was the crude mitochondrial fraction.

### GENERATION OF PURE MITOCHONDRIAL FRACTION

A highly enriched mitochondrial fraction was obtained by nycodenz (Axis-Shield) discontinuous gradient centrifugation (Okado-Matsumoto and Fridovich, 2001). Gradients were prepared by diluting 50% nycodenz solution (5 mM Tris, 3 mM KCl, 0.3 mM EDTA, 25 g nycodenz, pH 7.5, prepared in 50 ml of

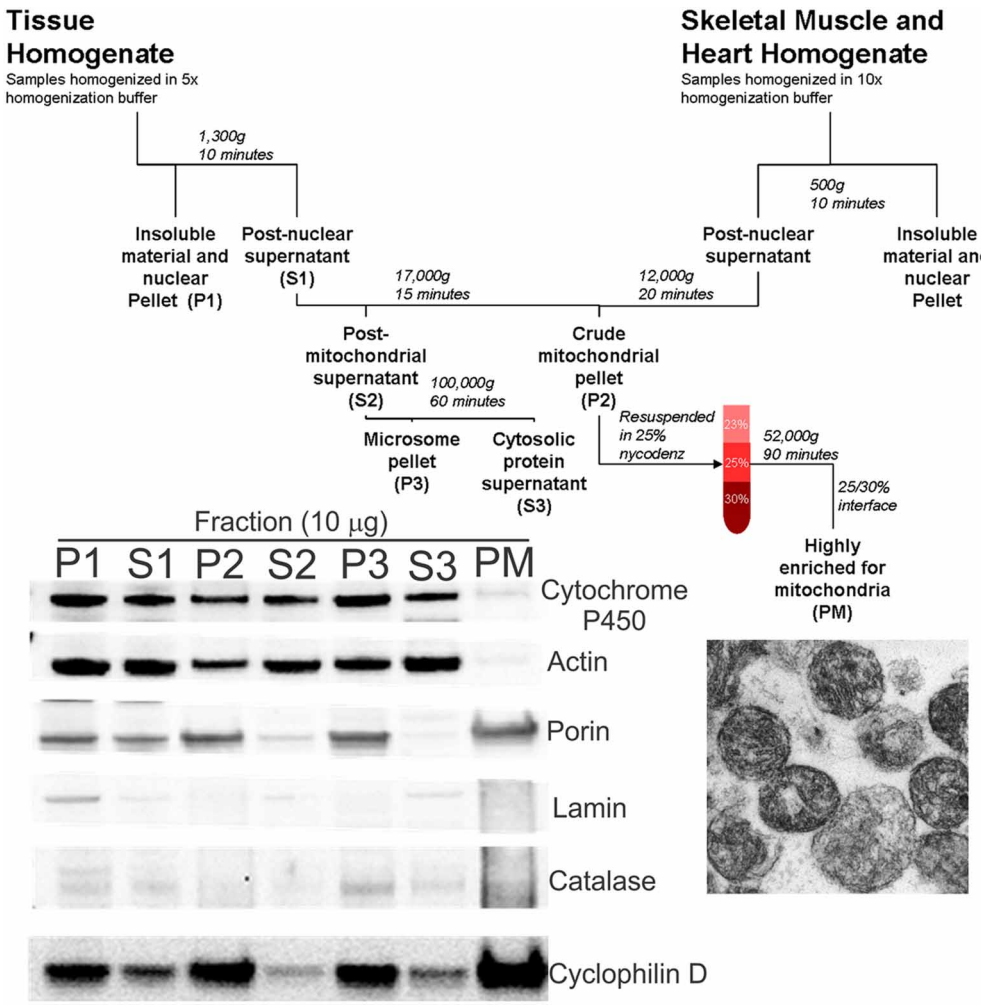


**FIGURE 1 | Schematic diagram of tissue fractionation and experimental verification of mitochondrial enrichment and purity.** Tissues were homogenized in appropriate buffer using a PT3100 polytron. After centrifugation to remove tissue and cellular debris, a crude mitochondrial pellet was obtained (fraction P2). The pellet was resuspended in 25% nycodenz and a highly pure mitochondrial fraction (fraction PM) was obtained by discontinuous gradient centrifugation. Bottom left panel: Immunoblotting of the various fractions confirms the pure mitochondria (PM) fraction to be highly enriched for mitochondria (porin and CyPD), with very little or no contamination by cytoplasmic (actin), microsomal (cytochrome P450), nuclear (lamin), and peroxisomal/lysosomal (catalase) compartments. Bottom right panel: Electron microscopy on PM fractions confirmed visually the very high enrichment of mitochondria.

double-distilled water) with the appropriate volume of dilution buffer (0.75 g NaCl, 5 mM Tris, 3 mM KCl, 0.3 mM EDTA, pH 7.5, prepared in 100 ml of double-distilled water) to obtain an iso-osmotic solution. The crude mitochondrial fractions were resuspended in 3 ml 25% nycodenz buffer, layered onto 2.5 ml 30% nycodenz buffer, and overlaid with 2.5 ml 23% nycodenz buffer. Samples were centrifuged at 52,000 g for 90 min in a swinging bucket rotor (Sorval TH641). The pure mitochondria (PM) fraction was isolated from the 25%/30% interface. This fraction was highly enriched in mitochondria (**Figure 1**, blot inset) as demonstrated by the absence of markers for nucleus (lamin), endoplasmic reticulum/microsomes (cytochrome P450 reductase), peroxisomes (catalase), and cytosol (actin), and the enrichment in mitochondrial markers, including porin (voltage-dependent anion channel, VDAC), cyclophilin D, and complex V (**Figures 1**, **2A–C,G**). Aliquots of freshly prepared PM samples were fixed in 2% glutaraldehyde, embedded in plastic, and thin-sectioned for electron microscopy (EM) to further validate the mitochondrial purification using this approach (**Figure 1**).

To confirm that Dnmt protein was localized within mitochondria rather than being bound to the surface of mitochondria, proteinase K digestion of freshly isolated intact mitochondria was performed. PBS-resuspended mitochondria were incubated in 5 mg/ml agarose-conjugated proteinase K (Sigma) for 30 min at 37°C, and then centrifuged and washed in PBS for several cycles to remove proteinase K.

### IMMUNOBLOTTING

Tissue subcellular fractions were separated by SDS-PAGE on 4–12% NuPage gels (Invitrogen) under denaturing and reducing

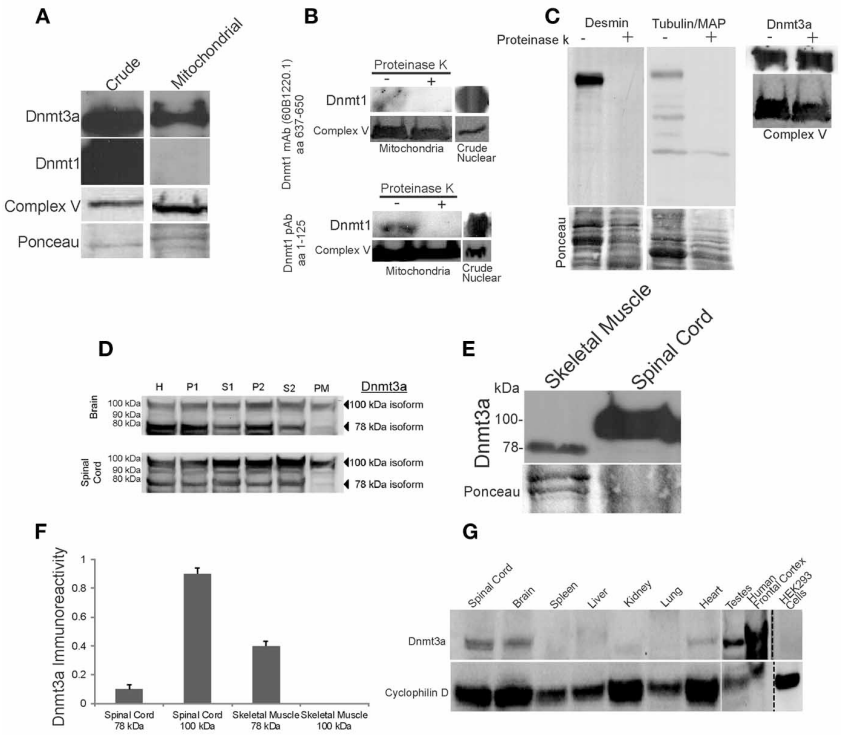


**FIGURE 2 | Dnmt3a is present in skeletal muscle and CNS mitochondria of adult mouse and human. (A)** Western blots showing that Dnmt3a, but not Dnmt1, is present in adult mouse skeletal muscle mitochondria. Dnmt1 was present in crude tissue fractions but was undetectable in pure mitochondrial fractions even with prolonged overexposures as shown. Western blot for the mitochondrial marker complex V is used to verify mitochondrial enrichment of the fraction. Ponceau S-stained membrane is used to show protein loading. **(B)** Experiments that establish that Dnmt1 is bound to the outer surface of mitochondria. Freshly isolated intact skeletal muscle mitochondria were treated (+) with agarose-conjugated proteinase K to digest surface-bound proteins or were not treated with proteinase K (−). Mitochondria were lysed and proteins were fractionated by SDS-PAGE and western blotted for Dnmt1 using two different antibodies that detect different amino acid (aa) domains of the protein. Protein loading was revealed by reprobing the blots for complex V. Crude nuclear fractions were used as a positive control. **(C)** Experiments that establish that Dnmt3a is within mitochondria and not merely bound to the outer surface of mitochondria. Freshly isolated intact skeletal muscle mitochondria were treated with agarose-conjugated proteinase K to digest surface-bound proteins. The efficacy of digestion is shown by the loss of desmin and tubulin/MAP immunoreactivities in the treated samples, but Dnmt3a immunoreactivity was not attenuated by proteinase K digestion. Protein loading is shown by Ponceau S staining of membranes. Mitochondrial enrichment is shown by complex V immunoreactivity. **(D)** Adult mouse brain and spinal cord were homogenized, fractionated and immunoblotted with antibody to Dnmt3a. Lanes were loaded with equivalent amounts of protein from the crude homogenate (H) and different fractions, including nuclear-enriched and insoluble material (P1), post-nuclear supernatant (S1), crude mitochondria (P2), post-mitochondrial supernatant (S2), and pure mitochondria (PM). See **Figure 1** for the validation of this fractionation method. The 100 kDa form of Dnmt3a was present in all fractions, including the pure mitochondria, while the 78 kDa form was present in all fractions except the mitochondria. **(E)** Western blot comparing the levels of Dnmt3a and isoform specificity in skeletal muscle (100 μg protein) and spinal cord (50 μg protein). Dnmt3a expression is greater in spinal cord compared to skeletal muscle. The 78 kDa isoform predominates in skeletal muscle while the 100 kDa isoform predominates in spinal cord. **(F)** Graph showing the relative levels of Dnmt3a isoform immunoreactivity determined by immunobloting of pure mitochondria isolated from adult mouse skeletal muscle and spinal cord. Values are mean ± SD. The 100 kDa isoform was not detected in skeletal muscle even after long exposures. **(G)** Immunoblot for Dnmt3a in pure mitochondria (PM) fractions from different types of adult mouse tissues, human brain, and a human cell line. The 100 kDa isoform of Dnmt3a was present in mouse spinal cord, brain, heart, testes, and human brain cerebral cortex. Dnmt3a immunoreactivity was low or undetectable in mouse spleen, liver, kidney, and lung and in human embryonic kidney cells. Western blot for the mitochondrial marker cyclophilin D is used to show protein loading.

conditions. Gels were transferred to a nitrocellulose membrane using the Xcell surelock system (Invitrogen). The membranes were stained with Ponceau S (Sigma) to determine transfer efficiency, destained, blocked with 1% BSA/0.05% Tween 20/TBS and incubated with primary antibodies: rabbit polyclonal anti-cytochrome P450 reductase (Stressgen) at 1:1000; mouse monoclonal anti-actin (Chemicon) at 1:1000; mouse monoclonal anti-porin (Mitosciences) at 1:3000; rabbit polyclonal anti-lamin (Millipore) at 1:500; sheep polyclonal anti-catalase (Biodesign International) at 1:2000; mouse anti-cyclophilin D (Mitosciences) at 1:3000; mouse monoclonal anti-complex V (Molecular Probes Invitrogen) at 1:10,000; rabbit polyclonal and mouse monoclonal anti-Dnmt3a (Cell Signaling, Abgent, and Alexis) at 1:100–1:500, rabbit polyclonal and mouse monoclonal anti-Dnmt1 (Bethyl, Novus, and Alexis) at 1:500–1:5000, and rabbit polyclonal antibody to Dnmt3b (Abcam) at 1:250. The details of the Dnmt antibodies used are shown in **Table 1**. To confirm the efficacy of proteinase K digestion of putative mitochondrial

**Table 1 | Antibodies to Dnmt isoforms screened in this study.**

| Dnmt target | Antibody clonality | Immunogen | Company and product | Comment |
|---|---|---|---|---|
| Dnmt1 | Mouse monoclonal 60B1220.1 | Synthetic peptide corresponding to aa 637–650 (central region) | Alexis (Enzo) ALX-804-369 | Good for mouse skeletal muscle and CNS westerns |
| Dnmt1 | Rabbit polyclonal | Synthetic peptide corresponding to aa 1–125 (N-terminus) | Novus Biologicals NB100-264 | Good for mouse skeletal muscle and CNS westerns |
| Dnmt1 | Rabbit polyclonal | Synthetic peptide corresponding to ∼ aa 574–846 (encoded by exon 25) | Bethyl Laboratories BL961 | Suitable for mouse tissue westerns |
| Dnmt3a | Mouse monoclonal 64B1446 | Mouse recombinant Dnmt3a (C-terminal epitope aa 705–908) | Alexis (Enzo) ALX-804-370 | Good for mouse skeletal muscle and CNS westerns and immunohistochemistry |
| Dnmt3a | Rabbit polyclonal | N-terminus of human Dnmt3a | Cell Signaling Technology 2160 | Suitable for mouse skeletal muscle and CNS westerns and immunohistochemistry |
| Dnmt3a | Rabbit polyclonal | Synthetic peptide corresponding to aa 400–500 of human Dnmt3a | ABGENT AP1034a | Suitable for mouse skeletal muscle and CNS westerns |
| Dnmt3b | Mouse monoclonal 52A1018 | Mouse recombinant Dnmt3b | Abcam Ab13604 | No detection of Dnmt3b in skeletal muscle mitochondria |

surface-tethered proteins, treated skeletal muscle mitochondria were probed with antibodies to tubulin/microtubule associated proteins (1:1000, Sigma) and desmin (1:5000, Sigma). Antibody to LC3A (Cell Signaling) was used as a mitochondrial autophagy marker. Blots were washed, incubated with secondary antibody (1:10,000–1:50,000), and developed using ECL (Pierce Supersignal West Pico). The membranes were imaged using a CCD camera and BioRad Quantity One software or were developed using X-ray film. Immunoreactivity was quantified using ImageJ.

### IMMUNOFLUORESCENCE

Age-matched non-tg and hSOD1$^{mus}$-G37R, -G93A, and -wildtype tg mice (12–15 months of age) received an anesthetic overdose and were perfused transcardially with 4% paraformaldehyde. The group sizes were 3–4 mice per genotype. Spinal cord, skeletal muscle, and testes were removed and cryoprotected in 20% glycerol and then frozen and cut (40 μm) on a sliding microtome. Sections were permeabilized in 0.4–1% Triton-X100/PBS, blocked in 10% normal donkey serum or goat serum/0.1% Triton/PBS, and incubated in primary antibody to mitochondrial markers SOD2 (1:100, Assay Design) or complex V (1:500, Invitrogen Molecular Probes), 5mC (1:100, Calbiochem), Dnmt3a (1:100–500, Alexis or Abgent), or the autophagy marker LC3A (1:100, Cell Signaling). The sections were then rinsed, incubated in secondary antibody (1:400–800) conjugated to AlexaFluor-488 or -594, Hoechst dye counterstained (for nuclear visualization), and imaged on a Zeiss LSM 510 Meta confocal microscope or Zeiss Axiophot epifluorescence microscope.

### mtDNA ISOLATION AND mtDNA PYROSEQUENCING

The PM fraction was used to isolate mtDNA using a Qiaprep Spin Miniprep kit (Qiagen) or conventional phenol-chloroform extraction, RNase digestion, and ethanol precipitation. mtDNA concentration and relative purity was determined by measuring A260 and A280 and calculating the A260/A280 ratio.

Mouse mtDNA was sequenced using Pyromark Q24 (Qiagen). DNA (2 μg) was bisulfite treated using an Epitek Bisulfite kit (Qiagen). Purified converted DNA (10 ng) was then PCR amplified, and 25 μl of product sequenced on the Pyromark Q24 (primers and conditions supplied by Qiagen using the Pyromark software): Locus 1 (D-loop), forward primer 5′-GGGTTTATTAAATTTGGGGGTAGTT-3′, biotinylated reverse primer 5′- ATAC CAAATA CATAACACCACAAT-3′, sequencing primer 5′- ATTTGGTTTTTACTTTAGGG T-3′; Locus 2 (16S rRNA gene), forward primer 5′- TGTTGGATTAGGATA TTTTAATGGTGTAG-3′, biotinylated reverse primer 5′- CAC CACCCTAATAACCTTCTCTA-3′, sequencing primer 5′- ATT TTAATGGTGTAGAAGT-3′; run conditions: 95°C 15 min, 45× (95°C 30 s, 58°C 30 s, 72°C 30 s), 72°C 5 min. The data was validated by internal controls and presented as percent 5mC/cytosine ± standard deviation ($n = 3$, in duplicate) with high agreement in duplicate measures.

### EM

Age-matched non-tg and hSOD1$^{mus}$-G37R, -G93A, and -wildtype tg mice (15–17 months of age) received an anesthetic overdose and were perfused transcardially with 2% paraformaldehyde/2% glutaraldehyde. The group sizes were two mice per genotype. Tissue samples of left and right biceps femoris were

acquired from each mouse and process and embedded in plastic for conventional transmission EM as described (Martin et al., 1994). Tissue samples were cut in the transverse plane at 0.5 μm for high-resolution light microscopy and then thin sections were cut and collected on copper grids for EM. These sections were viewed and imaged using a Phillips CM12 electron microscope. Digital electron micrographs from each mouse genotype were used to determine subsarcolemmal mitochondrial layer thickness and intermyofibrillar mitochondrial density in at least five images per mouse.

### HUMAN CNS TISSUE

Rapid autopsy neurologic disease-free control human brain ($n = 4$) cerebral cortical samples were obtained through the Human Brain Resource Center at Johns Hopkins. These fresh samples of frontal cortex were used to isolate mitochondria for western blot studies of Dnmts.

### STATISTICAL ANALYSIS

Western blot densitometry measurements, cytosine methylation pyrosequencing measurements, and myofiber mitochondrial density measurements were used to determine group means and variances and comparisons among groups were analyzed using a one-way analysis of variance and a Newman-Keuls *post-hoc* test or a Student's *t*-test.

## RESULTS

### DNMT3A LOCALIZES TO MITOCHONDRIA

We analyzed several tissue types for the presence of Dnmts in mitochondria. A flow diagram for the mitochondrial purification preparation is shown (**Figure 1**, top), and the method was validated by western blotting (**Figure 1**, lower left). The fraction designated as PM was highly enriched for mitochondria, as determined by the enrichment of porin and cyclophilin D, and was essentially free of contamination from other subcellular organelles such as the endoplasmic reticulum (cytochrome p450), cytosol (actin), nucleus (lamin), and microsomes (catalase) (**Figure 1**, lower left). EM was used to show by direct visualization that the PM fraction had a high content of mitochondria (**Figure 1**, lower right).

We examined adult mouse skeletal muscle mitochondria for Dnmts (**Figure 2A**). The purity of the mitochondrial samples was confirmed by the enrichment of complex V (**Figure 2A**). Dnmt3a had a robust presence in skeletal muscle mitochondrial fractions (**Figure 2A**) and was detected with several different antibodies to Dnmt3a that recognize N-terminal, central, and C-terminal domains of the protein (**Table 1**). Dnmt1 was usually not detected in skeletal muscle PM fractions (**Figure 2A**), even after prolonged exposures and use of 3 different primary antibodies recognizing different domains of the protein (**Table 1**). In some mitochondrial preparations of skeletal muscle, Dnmt1 was detected at very low levels (**Figure 2B**) but was not present in fractions of intact mitochondria digested with agarose-bound proteinase K (**Figures 2A,B**). In contrast, Dnmt1 was highly concentrated in crude nucleus-enriched P1 fractions (**Figure 1**, Top) of the same tissue homogenates (**Figures 2A,B**). Dnmt3b was not detected in skeletal muscle mitochondria (data not shown).

To determine whether Dnmt3a is present within mitochondria or bound to the surface of mitochondria, fresh intact mitochondria were digested with agarose-bound proteinase K (**Figure 2C**). Tubulin, microtubule-associated proteins, and desmin are known to be tethered to the surface of striated muscle mitochondria (Capetanaki et al., 2007), and tubulin is known to specifically dock to VDAC (Carré et al., 2002). Proteinase K digestion removed completely or nearly completely mitochondrial-bound cytoskeletal proteins but did not alter the robust detection of Dnmt3a in PM (**Figure 2C**). This finding demonstrates that Dnmt3a is present within skeletal muscle mitochondria.

To confirm previous observations indicating a mitochondrial presence of Dnmt3a in nervous tissue (Chestnut et al., 2011), we tracked by subcellular fractionation the presence of Dnmt3a in mouse brain and spinal cord tissue (**Figure 2D**). Dnmt3a was present in nucleus-containing fractions (**Figure 2D**, lanes H and P1) and cytosol-containing fractions (**Figure 2D**, lanes H, S1, P2, and S2). Several isoforms of Dnmt3a have been reported (Chen et al., 2002).We detected both the 78 and 100 kDa isoforms of Dnmt3a in the different fractions of brain and spinal cord (**Figure 2D**). Brain and spinal cord PM fractions contained Dnmt3a (**Figure 2D**), as detected with three different antibodies to the N-terminal or central regions of Dnmt3a (**Table 1**). The 100 kDa isoform of Dnmt3a was concentrated in PM relative to total homogenate (**Figure 2D**). We were unable to detect Dnmt1 immunoreactivity in PM of brain and spinal cord using three different antibodies, consistent with previous data (Chestnut et al., 2011).

Western blotting for Dnmt3a in adult mouse skeletal muscle and spinal cord in a side-by-side comparison revealed tissue differences in isoform distribution (**Figure 2E**). In skeletal muscle, the 78 kDa isoform predominates over the 100 kDa isoform (**Figure 2F**), but in spinal cord the 100 kDa isoform predominates (**Figure 2E**) and accounts for nearly 100% of the total amount of this isoform in spinal cord mitochondria (**Figure 2F**).

We next tested the hypothesis that mitochondrial Dnmt3a is tissue specific. We examined by immunoblotting the presence of Dnmt3a in highly PM isolated from several adult mouse tissues, including spleen, liver, kidney, lung, heart, and testes (**Figure 2G**). The mitochondrial fractions of brain, spinal, heart, and testes contained the 100 kDa isoform of Dnmt3a (**Figure 2G**). Mitochondrial Dnmt3a protein levels were very low or undetectable in spleen, liver, kidney, and lung. The faintly detectable mitochondrial Dnmt3a in spleen, kidney, and lung appeared as the 78 kDa isoform (**Figure 2G**). To determine if the mitochondrial presence of Dnmt3a was mouse-specific, we prepared PM from human cerebral cortex (frontal cortex) and cultured human embryonic kidney (HEK) 293 cells (**Figure 2G**). Dnmt3a was detected robustly in mitochondria of the human brain but not in HEK293 cells (**Figure 2G**). Thus, the mitochondrial localization of Dnmt3a occurs primarily in the mitochondria of excitable tissues in human and mouse.

### MITOCHONDRIAL DNMT3A LEVELS ARE REDUCED IN SKELETAL MUSCLE AND SPINAL CORD OF tg MOUSE MODELS OF ALS

Abnormalities in Dnmt protein levels and DNA methylation have been observed in human ALS CNS tissues and in mouse and cell

models of motor neuron degeneration (Chestnut et al., 2011). Moreover, in human ALS skeletal muscle, marked mitochondrial abnormalities are found (Wiedemann et al., 1998; Vielhaber et al., 2000; Krasnianski et al., 2005; Echaniz-Laguna et al., 2006; Corti et al., 2009), and it has been proposed that skeletal muscle disease is a driving part of the disease process in ALS (Dupuis et al., 2006; Dobrowolny et al., 2008; Wong and Martin, 2010). We examined the levels of Dnmt3a in skeletal muscle mitochondria in hSOD1 tg mouse models of ALS at presymptomatic or early symptomatic stages of disease. Compared to non-tg age matched littermates, presymptomatic tg mice expressing mutant hSOD1-G37R non-conditionally in most tissues showed reduced levels of Dnmt3a in skeletal muscle mitochondria (**Figures 3A,B**). Tg early symptomatic mice with skeletal muscle-restricted expression of G37R mutant (hSOD1$^{mus}$-G37R) and G93A mutant (hSOD1$^{mus}$-G93A) also had a severe loss of skeletal muscle mitochondrial Dnmt3a (**Figures 3A,B**). Mitochondrial Dnmt3a protein levels were reduced modestly in hSOD1$^{mus}$-wildtype tg mice but were not changed significantly in tg mice with non-conditional expression hSOD1-wildtype allele (**Figures 3A,B**). Spinal cords of hSOD1$^{mus}$-G37R, -G93A, and -wildtype tg male mice at early stages of disease also had marked reduction in mitochondrial Dnmt3a (**Figures 3C,D**). The loss of Dnmt3a was selective for the mitochondrial compartment because Dnmt3a levels in crude homogenates of skeletal muscle and spinal cord were unchanged in tg mice (**Figure 3E**).

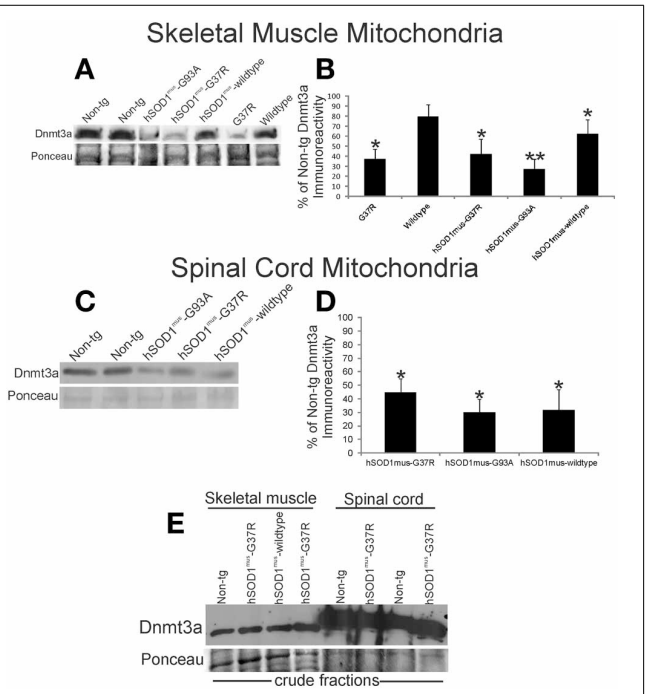


**FIGURE 3 | Dnmt3a levels are reduced in hSOD1 tg mouse models of ALS. (A)** Western blot for Dnmt3a in leg skeletal muscle mitochondria (100 μg/lane) in non-conditional tg mice expressing hSOD1-G37R or hSOD1-wildtype constructs and conditional (muscle-restricted) tg mice expressing hSOD1$^{mus}$-G37R, -G93A, or -wildtype constructs compared to age-matched non-tg littermates. Ponceau S staining of membrane shows protein loading. **(B)** Graph showing the relative levels of Dnmt3a immunoreactivity determined by immunoblotting of pure mitochondria isolated from hSOD1 tg mouse leg skeletal muscle. Values are mean ± SD. Significant differences from non-tg control are indicated by single ($p < 0.05$) or double ($p < 0.01$) asterisks. **(C)** Western blot for Dnmt3a in spinal cord mitochondria fractions (50 μg/lane) in conditional (muscle-restricted) tg mice expressing hSOD1$^{mus}$-G37R, -G93A, or -wildtype constructs compared to age-matched non-tg littermates. Ponceau S staining of membrane shows protein loading. **(D)** Graph showing the relative levels of Dnmt3a immunoreactivity in hSOD1$^{mus}$ tg mouse spinal cord. Values are mean ± SD. Significant differences from non-tg control are indicated by an asterisk ($p < 0.05$). The results have been replicated in at least 3 different experiments. **(E)** Western blot for Dnmt3a in crude fractions of skeletal muscle and spinal cord of conditional (muscle-restricted) tg mice expressing hSOD1$^{mus}$-G37R or -wildtype constructs compared to age-matched non-tg littermates. Ponceau S staining of membrane shows protein loading.

## CELLULAR LOCALIZATION OF DNMT3A AND 5mC IN SPINAL CORD AND SKELETAL MUSCLE AND ABERRANT PATTERNS OF IMMUNOREACTIVITY IN hSOD1$^{mus}$ tg MICE

Our biochemical finding that Dnmt3a is present in excitable tissue mitochondria required direct confirmation of a mitochondrial Dnmt3a localization *in situ* and an assessment of whether Dnmt3a in mitochondria overlaps with a reporter molecule for DNA methylation. We also assessed whether abnormalities in Dnmt3a and 5mC are found in tg mouse models of ALS since abnormalities has been found in human ALS (Chestnut et al., 2011). We used a highly specific monoclonal antibody to detect 5mC (Kang et al., 2006). We have done additional characterization of the 5mC antibody (Chestnut et al., 2011). By immunofluorescence, we visualized Dnmt3a and 5mC localization within spinal cord of 2–4 months old non-tg mice (**Figure 4A**). Extranuclear Dnmt3a and 5mC immunoreactivities colocalized. While Dnmt3a immunoreactivity was present diffusely in the cytoplasm, there were distinct punctate structures positive for Dnmt3a. These cytoplasmic puncta invariably were 5mC-positive (**Figure 4A**, arrows), indicating that these structures contain methylated DNA. Consistent with expectations, 5mC immunoreactivity was also detected in the nucleus of neurons (**Figures 4B,C**, red).

As the only extranuclear cellular structures known to contain DNA are mitochondria, we examined spinal cord for the presence of 5mC within mitochondria. Using immunofluorescence for SOD2, a mitochondrial marker that shows robust punctate labeling in the cytoplasm (Martin et al., 2007), we determined that mitochondria contain 5mC (**Figure 4B**). Not all SOD2-positive structures were also positive for 5mC (**Figure 4B**, arrows). The dual positive structures often appeared larger than the SOD2-only positive mitochondria.

Some of the discreet cytoplasmic puncta that contained 5mC appeared similar to autophagosomes (Martin et al., 1994; Kabeya et al., 2000) or granules based on size and shape (**Figure 4A**). The larger 5mC-positive bodies were not positive for the mitochondrial marker SOD2, while the smaller 5mC-positive bodies did stain for SOD2 (**Figure 4B**), possibly indicating degradation of mitochondria or mitochondrial marker. We next examined the immunolocalization of 5mC and LC3A (**Figure 4C**). LC3A is a marker for autophagosomes (Kabeya et al., 2000) that is known to function in mitophagy (Wang and Klionsky, 2011). 5mC-containing, SOD2-negative bodies were positive for LC3A

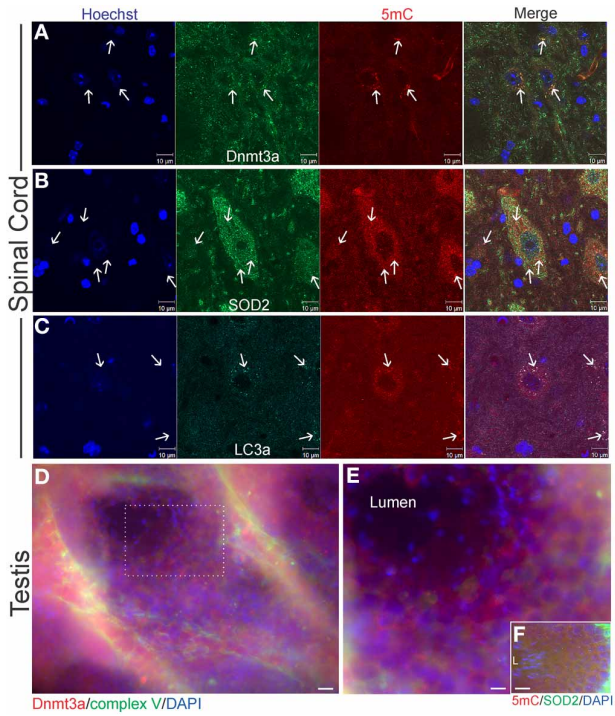


**FIGURE 4 | Cellular localizations of Dnmt3a and 5-methylcytosine (5mC) in wildtype young adult mouse spinal cord and testes. (A)** In 6–8 weeks old non-tg mice, Dnmt3a is present in the nucleus and in the cytoplasm of neurons in spinal cord. Extranuclear cytoplasmic Dnmt3a immunoreactivity (green) is localized diffusely and in discrete bodies (arrows). These discrete bodies also contain 5mC (red, arrows). Hoechst staining was used to identify cell nuclei. **(B)** Cytoplasmic 5mC is localized to mitochondria in neurons. Some mitochondria, identified by SOD2 immunoreactivity (green, arrows), in spinal cord colocalize with 5mC immunoreactivity (red, arrows). Many mitochondria also do not contain 5mC. The SOD2- and 5mC-positive bodies tend to be larger than normal mitochondria (white arrows). **(C)** 5mC is present in autophagosomes. Mouse spinal cord sections were immunostained for an autophagosome marker LC3A (green) and 5mC (red). Extranuclear 5mC immunoreactivity has a high colocalization with LC3A in autophagosomes (arrows). **(D,E)** Immunofluorescent localization of Dnmt3a (red) and complex V (green) in mouse testis. Cell nuclei are blue. Dnmt3a immunoreactivity (red) is enriched in cells in the seminiferous tubules of testes. The boxed area in D is represented at higher magnification in E which shows that Dnmt3a immunoreactivity (red) partly colocalizes (seen as yellow-orange) with mitochondria (green). Scale bars: 30 μm **(D)**; 15 μm **(E)**. **(F)** Immunofluorescence for 5mC (red) and SOD2 (green) in mouse testes. Cell nuclei are blue. 5mC immunoreactivity (red) partly colocalizes (seen as yellow-orange) with mitochondria (green). Mature spermatozoa with scant mitochondria are seen at left in seminiferous tubule lumen (L). Scale bar = 30 μm.

(**Figure 4C**). The prominent colocalization of 5mC and LC3A in the cytoplasm of spinal motor neurons suggests a relationship between cytosine methylation of mtDNA and mitophagy signaling.

In the mouse tissue screening for Dnmt3a protein levels we saw that testes also contained relatively high levels of Dnmt3a (**Figure 2G**). In seminiferous tubules, Dnmt3a and 5mC were discretely localized to mitochondria of spermatocytes (**Figures 4D,E**). Interestingly, immunofluorescent signal for mitochondria, Dnmt3a, and 5mC dissipated with maturation of spermatocytes to spermatozoa within the seminiferous tubule lumen (**Figures 4D,E**) consistent with a predominant pre-fertilization elimination of paternal mitochondria and mtDNA in sperm cells during their maturation, rather than a post-fertilization mechanism (Luo et al., 2013).

Immunofluorescent assessments of Dnmt3a and 5mC were done on skeletal muscle and spinal cord sections of hSOD1$^{mus}$ tg mice to confirm biochemical findings and to put our homogenate-based observations into a cellular localization framework. In non-tg control mice, Dnmt3a colocalized with complex V robustly in subsarcolemmal mitochondria and also in intermyofibrillar mitochondria (**Figure 5A**), while Dnmt3a and complex V immunoreactivity was severely attenuated in hSOD1$^{mus}$-G37R skeletal muscle myofibers (**Figure 5B**). In subsarcolemmal mitochondria adjacent to myonuclei, Dnmt3a was not colocalized with 5mC in non-tg controls (**Figure 5C**), but in hSOD1$^{mus}$ tg mice, Dnmt3a and 5mC were prominently colocalized in apparent subsarcolemmal mitochondria (**Figure 4D**). 5mC immunoreativity was also seen in subsets of myofiber nuclei (**Figures 5C,D**, green). In lumbar spinal cord motor neurons of 15–17 months old non-tg mice, Dnmt3a was normally colocalized with complex V in about a third of the mitochondria in motor neuron cell bodies (**Figure 5E**) and, similarly, about a third of the Dnmt3-positive cytoplasmic particles colocalized with 5mC (**Figure 5G**). In hSOD1$^{mus}$ tg mice, complex V immunoreactivity was enriched in remaining lumbar motor neurons and Dnmt3 immunoreactivity appeared as aggregates associated with some mitochondria (**Figure 5F**). The Dnmt3a-5mC colocalization in remaining mitochondria in motor neurons of hSOD1$^{mus}$ tg mice also revealed large numerous cytoplasmic aggregates containing 5mC (**Figure 5H**) consistent with findings in degenerating motor neurons (Chestnut et al., 2011).

## DIRECT DEMONSTRATION OF 5mC IN mtDNA

We directly identified 5mC in purified mtDNA by DNA pyrosequencing (Marsh, 2007) (**Figures 6A,B**). Two different loci of the mitochondrial genome (D-loop and 16S rRNA gene) were interrogated in bisulfite-treated mtDNA isolated from mouse brain, liver, and testes (**Figure 6A**). Cytosine methylation was determined at five nucleotide sites in locus 1 and at seven nucleotide sites in locus 2 (**Figures 6A,B**). Locus 1 in the D-loop had CpG regions with 5mC content ranging from 9% (brain, position 4) to 2% (liver, position 2) (**Figure 6C**). Most of the mtDNA CpG sites in locus 1 of brain and testes had 5mC content at ∼4–6% (**Figure 6C**). Liver mtDNA was generally at 4% 5mC content at locus 1 (**Figure 6C**). In contrast, locus 2 in the 16S rRNA gene had several CpG regions with 5mC content ranging from 7.5 to 18% in brain and testes mtDNA and testes (**Figure 6D**). Locus 2 CpG position 4 showed the highest 5mC content (**Figures 6B,D**). Liver mtDNA generally had ∼5% 5mC content at locus 2, with the exception of CpG position 4 where it was about 10% (**Figure 6D**).

## ALS MICE HAVE ABERRANT mtDNA 5mC SIGNATURES

Cytosine methylation in mtDNA was assayed in skeletal muscle and spinal cord of different hSOD1 tg mouse models of ALS (**Figures 7**, **8**). Tg mice with non-conditional expression of

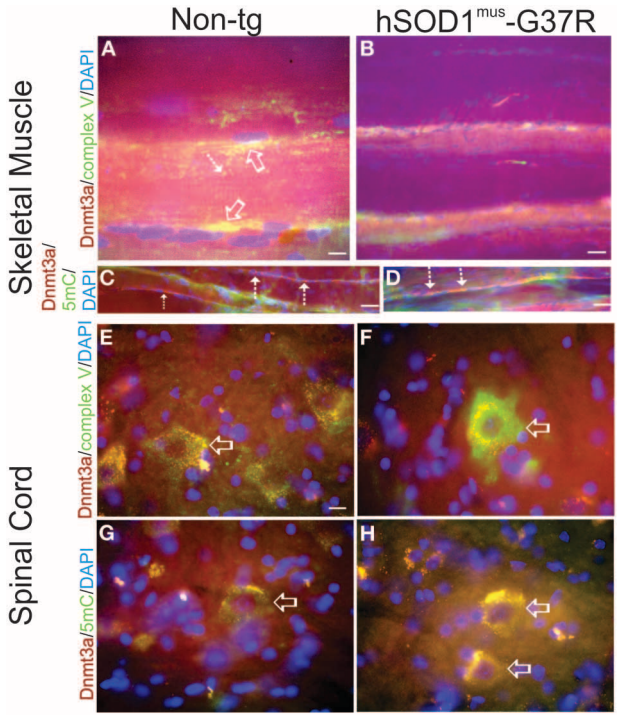


**FIGURE 5 | Immunofluorescent localizations of Dnmt3a, mitochondria, and 5-methylcytosine (5mC) in hSOD1$^{mus}$ tg mouse skeletal muscle and spinal cord. (A,B)** In non-tg mouse skeletal muscle (biceps femoris), Dnmt3a (red) immunoreactivity can be found diffusely in the sarcoplasm and associated with mitochondria (colocalization is seen as yellow) identified by complex V immunoreactivity (green). Dnmt3a/complex V colocalization occur prominently in subsarcolemmal mitochondria in non-tg mice (A, open arrows) and in interfibrillar mitochondria **(A**, hatched arrow**)**. In hSOD1$^{mus}$ mouse hindleg skeletal muscle, Dnmt3a (red) immunoreactivity and mitochondrial complex V immunoreactivity (green) are markedly attenuated. Scale bars = 9 μm **(A)**, 15 μm **(B)**. **(C,D).** Myofiber perinuclear colocalization of Dnmt3a and 5mC is intensified in hSOD1$^{mus}$ tg mice. In non-tg mouse skeletal muscle (biceps femoris), Dnmt3a (red) immunoreactivity can be found clustered around peripheral myonuclei **(C**, hatched arrows**)** and generally has little colocalization with 5mC. In hSOD1$^{mus}$mouse skeletal muscle Dnmt3a (red) and 5mC (green) immunoreactivities have prominent perinuclear colocalizations **(D**, hatched arrows, yellow-orange**)**. Scale bars = 20 μm **(C,D)**. **(E,F)** In ∼17 months old non-tg mice, Dnmt3a immunoreactivity (red) is present in the nucleus and, more prominently, in the cytoplasm of spinal cord motor neurons **(E,G**, open arrows**)**. Cytoplasmic Dnmt3a immunoreactivity (red) is localized in discreet particles in motor neurons **(E**,**G**, arrows**)**. Many of these cytoplasmic particles colocalize (seen as yellow) with complex V immunoreactivity **(E**, green**)**, and thus are mitochondria, and with 5mC **(G**, green, arrow**)** and thus contain methylated mtDNA. Scale bar in **(E)** (same for **F–H)** = 6 μm. In age-matched hSOD1$^{mus}$ tg mice, remaining spinal motor neurons **(F**, open arrow**)** show intensified mitochondrial immunoreactivity for complex V (green) and the Dnmt3a immunoreactivity largely is colocalized with complex V **(F)** and 5mC **(H)** and is aggregated in the cytoplasm.

mutant-G37R and wildtype variants of hSOD1 were analyzed at 8–10 months of age (**Figure 7**). Generally, 5mC content was lower in the D loop (locus 1) than in the 16S rRNA gene (locus 2) in control mice. Within the D-loop, the CpG at site 2 had the lowest % methylation, and sites 3 and 4 had the highest cytosine methylation in skeletal muscle and spinal cord (**Figure 7**). No significant differences in cytosine methylation of the D-loop were seen in tg mice expressing mutant-G37R and wildtype variants of hSOD1 compared to non-tg mice. In the 16S rRNA gene (locus 2), the CpG at site 4 had the highest % methylation of all the sites sequenced (**Figure 7**). Cytosine methylation was significantly increased at sites 1, 3, 4, 5, 6, and 7 in the 16S rRNA gene of spinal cord in tg mice with non-conditional expression of mutant-G37R (**Figure 7**), but no differences were detected in cytosine methylation in the 16S rRNA gene of skeletal muscle (**Figure 7**). Tg mice (16–20 months old) with skeletal muscle-restricted expression of mutant-G37R and -G93A and wildtype variants of hSOD1 (**Figure 8**) showed patterns of mtDNA cytosine methylation that were different compared to non-conditional hSOD1 tg mice. Cytosine methylation in D-loop site 2 of spinal cord was significantly reduced in hSOD1$^{mus}$-G37R (**Figure 8**), but other sites assayed for in the D-loop of spinal cord and skeletal muscle were unchanged (**Figure 8**). In contrast, cytosine methylation in all sites of the 16S rRNA gene of spinal cord was significantly lower in hSOD1$^{mus}$-wildtype mice compared to non-tg mice (**Figure 8**), but G37R$^{mus}$ and G93A$^{mus}$ mice did not differ from control.

### hSOD1$^{mus}$ tg MICE DEVELOP MITOCHONDRIAL ABNORMALITIES IN SKELETAL MUSCLE AND SPINAL CORD

The loss of mitochondrial Dnmt3a seen in tg mouse models of ALS could be related to changes in mitochondrial content. Indeed, skeletal muscle complex V was reduced significantly in hSOD1$^{mus}$-G37R, -G93A, and -wildtype tg mice at middle stages of disease compared to age-matched non-tg mice (**Figure 9A**). The reduced levels of complex V in skeletal muscle were mirrored by increases in the autophagy marker LC3A (**Figure 9B**). In contrast, complex V levels in spinal cord were significantly elevated in hSOD1$^{mus}$ tg mice (**Figure 9C**), consistent with previous histologic observations showing increased numbers of mitochondria in motor neurons of these mice (Wong and Martin, 2010).

Mitochondrial distributions and numbers in skeletal muscle were examined using high-resolution light and transmission electron microscopic evaluation of plastic sections. Non-tg control mice were rich in subsarcolemmal and interfibrillar mitochondria (**Figures 10A,C**). hSOD1$^{mus}$tg mice showed a loss of subsarcolemmal and interfibrillar mitochondria (**Figures 10B,D,E**). In putative type I fibers of non-tg mice the subsarcolemmal mitochondrial layer thickness was ∼4 μm (**Figures 10C,E**), but in hSOD1$^{mus}$-G37R, -G93A, and -wildtype tg mice, the subsarcolemmal mitochondrial layer was significantly attenuated (**Figure 10E**). Putative type II fibers in hSOD1$^{mus}$ tg mouse skeletal muscle displayed a significantly reduced density of interfibrillar mitochondria (**Figure 10F**) compared to age-matched non-tg mice. EM additionally revealed evidence for subsarcolemmal and interfibrillar mitophagy (**Figure 10D**, upper inset), as well as intramitochondrial rod-like inclusions (**Figure 10D**, lower inset) indicative of mitochondrial myopathy (Schlattner et al., 2006).

## DISCUSSION

We demonstrate that the DNA methylating enzyme Dnmt3a is present in mitochondria of mouse CNS, striated muscle, and

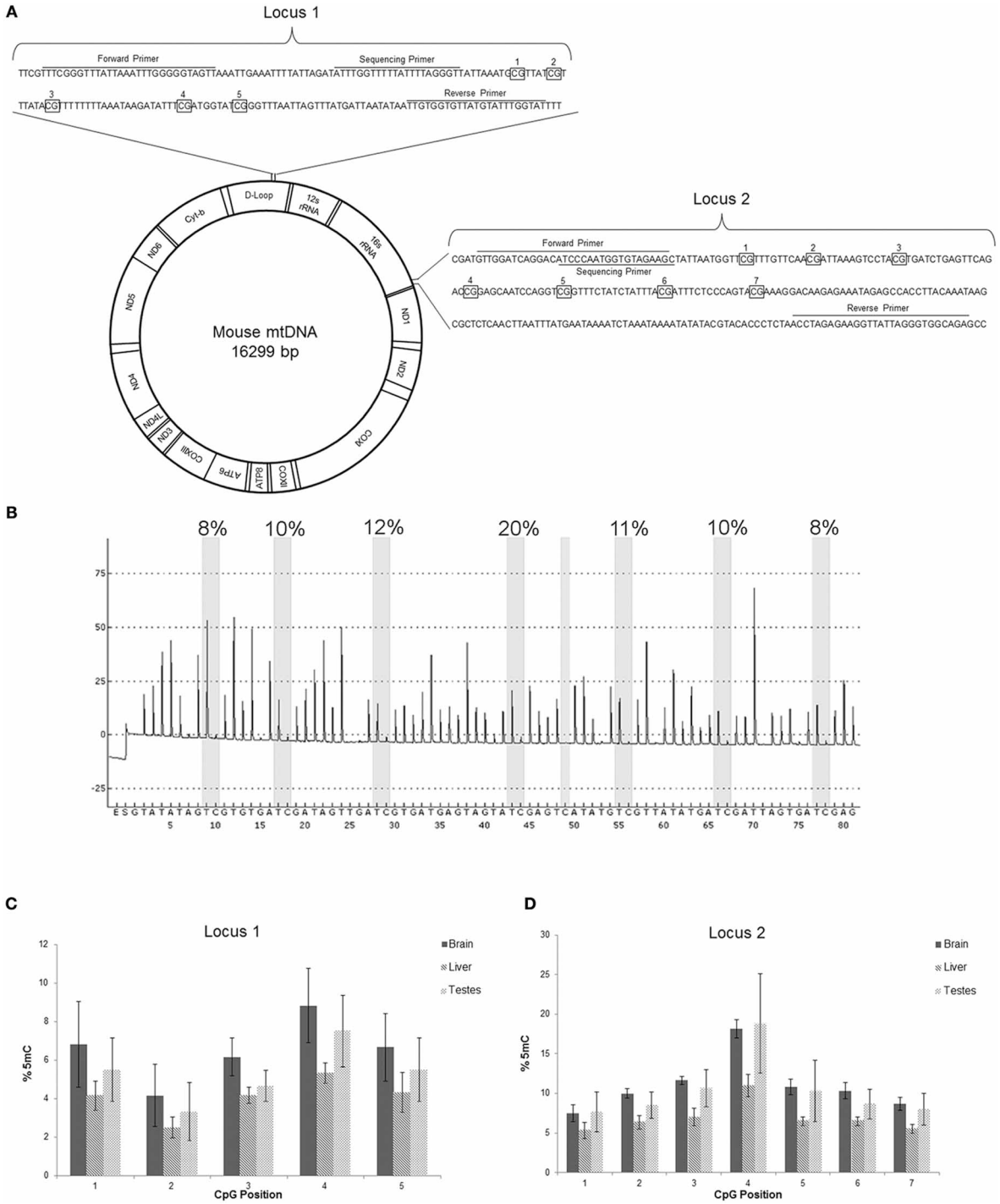


**FIGURE 6 | mtDNA contains 5-methylcytosine (5mC). (A)** Schematic of mouse mtDNA (Bibb et al., 1981) with depictions of the two locations assayed for 5mC. The first locus is in the D-loop, involved in mtDNA replication, and the second locus in the 16s rRNA encoding region. The primer locations are shown in sequences (wildtype or converted shown) with the CpG regions examined that are boxed and numbered. **(B)** Representative pyrogram from pyrosequencing of mouse brain mtDNA locus 2. The Y-axis is relative intensity (in arbitrary units) and the X-axis is the dispensation sequence showing the order of nucleotide addition to the reaction (E, enzyme; S, substrate). The seven thick shaded bars indicate the CpG positions with the degree of methylation (shown at top of bar) calculated from the ratio of the peak heights of C and T. The single narrow bar shows a bisulfite treatment control point where the peak only at the T dispensation and not at the C dispensation confirms full conversion by the bisulfite treatment. **(C,D)** Pyosequencing data of the two regions of mtDNA from mouse brain, liver, and testes. Numbers on the x-axis correspond to the CpG numbering in the sequence in **(A)**, and the y-axis is mtDNA 5mC content. In locus 1 the 5mC content was highest in brain mtDNA. In locus 2 the 5mC content in mtDNA was highest in the brain and testes, especially at position 4. Of the 3 tissues, liver had the lowest mtDNA 5mC content.

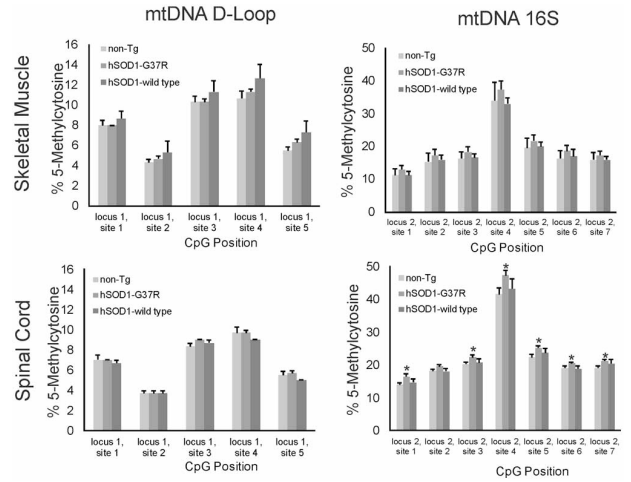


**FIGURE 7 | 5-methylcytosine (5mC) content in two loci of skeletal muscle mtDNA in non-conditional transgenic mouse models of ALS.** Graphs show the %5mC content at 5 CpG sites in the D-loop (**left**) and at 7 CpG sites in the 16S rRNA gene in the skeletal muscle (**top**) and spinal cord (**bottom**) of tg mice with expression of G37R or wildtype hSOD1 variants. Tg and non-tg control mice were 8–10 months of age. Values are mean ± SEM derived from mtDNA isolated from three different mice per genotype. Asterisks denote significant increase in hSOD1-G37R mice compared to non-tg control ($p < 0.05$).

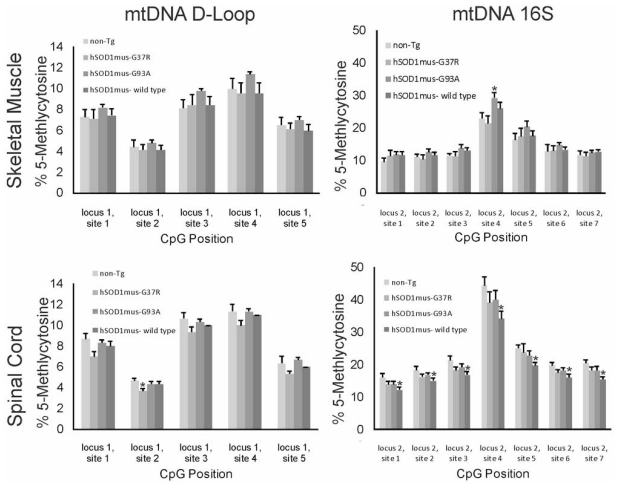


**FIGURE 8 | 5-methylcytosine (5mC) content in two loci of skeletal muscle mtDNA in conditional transgenic mouse models of ALS.** Graphs show the %5mC content at 5 CpG sites in the D-loop (**left**) and at 7 CpG sites in the16S rRNA gene in the skeletal muscle (**top**) and spinal cord (**bottom**) of tg mice with skeletal muscel-restricted expression of G37R, G93A, or wildtype hSOD1 variants. Tg and non-tg control mice were 16–20 months of age. Values are mean ± SEM derived from mtDNA isolated from three different mice per genotype. Asterisks denote significant differences in tg mice compared to non-tg control mice ($p < 0.05$).

testes. We also find Dnmt3a in human cerebral cortex mitochondria. Furthermore, the mtDNA of these tissues contains 5mC as shown directly by DNA pyrosequencing. Dnmt3a protein levels are reduced significantly at early disease in skeletal muscle and spinal cord of tg mouse models of ALS, and these mice also show aberrant patterns in 5mC immunoreactivity in skeletal muscle and spinal motor neurons. Some 5mC-positive structures are sequestered into autophagosomes. The skeletal muscle of presymptomatic tg mouse models of ALS shows accumulation of autophagy marker, loss of subsarcolemmal and intermyofibrillar mitochondria, and ultrastructural evidence for mitophagy. We conclude that mitochondrial localization of Dnmt3a and cytosine methylation of mtDNA are tissue-preferential, mostly confined to excitable tissues, and that regulatory mechanisms for epigenetic modification mtDNA, or non-catalytic functions of mitochondrial Dnmt3a, in skeletal muscle and spinal cord are aberrant in mouse ALS.

DNA methylating enzymes are thought traditionally to localize and function in the cell nucleus where they catalyze cytosine methylation leading to chromatin remodeling (Cheng, 1995; Jones and Takai, 2001; Bird, 2002; Brenner and Fuks, 2006). Here, we show that Dnmt3a is present the mitochondria of adult mouse excitable tissues such as brain, spinal cord, heart, and skeletal muscle. Mitochondrial Dnmt3a was also present in testes. In contrast, Dnmt3a is low or undetectable in spleen, liver, kidney, and lung. We detected mitochondrial Dnmt3a with several different antibodies to Dnmt3a that recognize different epitopes. The finding was not an artifact of subcellular contamination because the mitochondrial preparation was assessed for purity by immunoblotting and EM. Moreover, the Dnmt3a was present within mitochondria rather than being docked to the surface of mitochondria, as was the case for the mitochondrial association of Dnmt1. We also observed that different Dnmt3a isoforms were enriched preferentially in different mouse tissues. Skeletal muscle expressed primarily the 78 kDa Dnmt3a isoform, whereas, nervous tissue expressed primarily the 100 kDa Dnmt3a isoform. Dnmt3a was found also in mitochondria purified from adult human cerebral cortex. Dnmt3b was not detected in mouse skeletal muscle mitochondria (data not shown). We confirmed the mitochondrial expression of Dnmt3a by immunolocalization. Dnmt3a was found to colocalize with mitochondrial markers in spinal cord, skeletal muscle and testes. Another group has shown the presence of a Dnmt isoform associated with mitochondria, but the mitochondria were prepared from cell cultures and only one antibody to Dnmt1 and one antibody putatively to Dnmt3a was used (Shock et al., 2011). Dnmt1, but not Dnmt3a, was found associated with mitochondria from cultured mouse embryonic fibroblasts and human colon carcinoma cells (Shock et al., 2011), but mitochondrial surface-associated Dnmt1 was not ruled out by protease digestion of outer membrane-bound proteins as done here. We did not detect Dnmt1 in mouse skeletal muscle mitochondria using an antibody specifically directed to the N-terminus of Dnmt1. A commercial antibody (Abcam) to amino acids 1–10 of the N-terminus of Dnmt1 was reportedly used in this previous study (Shock et al., 2011), but this antibody was not in inventory for our use. With antibodies to N-terminal and central regions of Dnmt1, we identified here a proteinase K-sensitive Dnmt1 in some mitochondrial preparations, indicating surface-associated Dnmt1. Dnmt1 is associated with imprinting mechanisms involved in maintaining methylation patterns during cell division (Robertson, 2001), likely to be occurring in cultured cycling cells, while Dnmt3a has *de novo* methylating

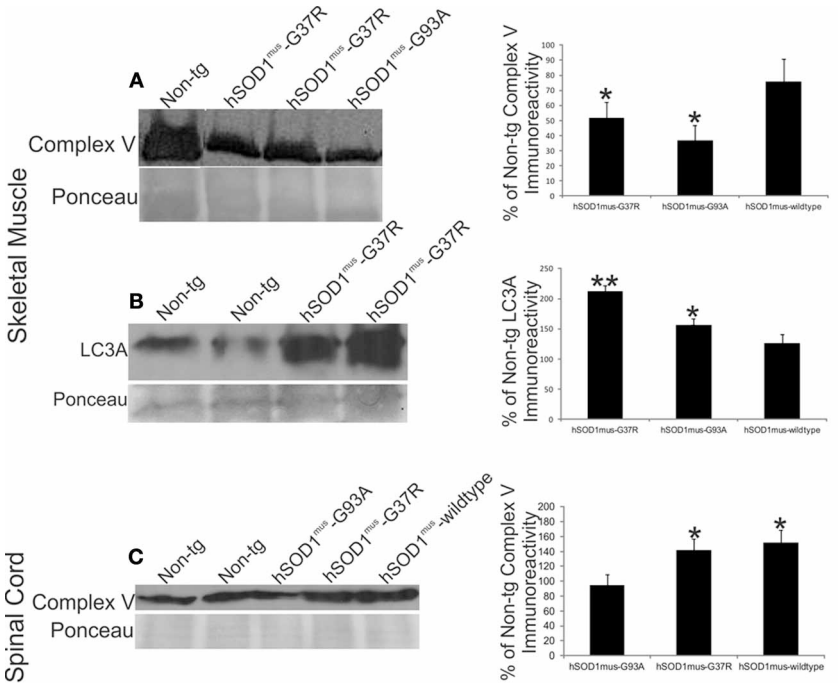


**FIGURE 9 | hSOD1$^{mus}$ tg mouse model of ALS has mitochondrial abnormalities in skeletal muscle and spinal cord. (A)** Western blot for complex V in skeletal muscle crude extracts (100 μg/lane) of early symptomatic tg mice expressing skeletal muscle-restricted hSOD1$^{mus}$-G37R or hSOD1$^{mus}$-wildtype. Ponceau S-stained membrane shows protein loading. Graph (at right) shows the quantification of complex V immunoreactivity in early symptomatic hSOD1$^{mus}$-G37R, hSOD1$^{mus}$-G93A, or hSOD1$^{mus}$-wildtype tg mice. Values (as % of control) are mean ± SD. Asterisk denotes significant differences ($p < 0.01$) from non-tg control. **(B)** Western blot for autophagy marker LC3A in skeletal muscle crude extracts (100 μg/lane) early symptomatic tg mice expressing skeletal muscle-restricted hSOD1$^{mus}$-G37R or hSOD1$^{mus}$-wildtype. Ponceau S-stained membrane shows protein loading. Graph (at right) shows the quantification of LC3A immunoreactivity in early symptomatic hSOD1$^{mus}$-G37R, hSOD1$^{mus}$-G93A, or hSOD1$^{mus}$-wildtype tg mice. Values (as % of control) are mean ± SD. Significant differences from non-tg control are indicated by single ($p < 0.05$) or double ($p < 0.01$) asterisks. **(C)** Western blot for complex V in spinal cord crude extracts (50 μg/lane) of early symptomatic tg mice expressing skeletal muscle-restricted hSOD1$^{mus}$-G37R or hSOD1$^{mus}$-wildtype. Ponceau S-stained membrane shows protein loading. Graph (at right) shows the quantification of complex V immunoreactivity in spinal cord of early symptomatic hSOD1$^{mus}$-G37R, hSOD1$^{mus}$-G93A, or hSOD1$^{mus}$-wildtype tg mice. Values (as % of control) are mean ± SD. Asterisk denotes significant differences ($p < 0.05$) from non-tg control.

activity (Okano et al., 1999). We conclude that the major mitochondrial Dnmt in adult mouse and human excitable tissues *in vivo* is Dnmt3a.

Our finding that Dnmt3a is within mitochondria of excitable tissue led to measurements of mtDNA methylation in these tissues. We demonstrated directly the presence of 5mC in mtDNA by DNA pyrosequencing (Marsh, 2007). Because DNA pyrosequencing cannot distinguish between 5mC and 5-hydroxymethylcytosine, our pryosequencing data represents a measurement of 5mC and 5-hydroxymethylcytosine in mtDNA. A recent study has also demonstrated the presence of 5mC and 5-hydroxymethylcytosine in mtDNA of cultured mouse embryonic fibroblasts and human colon carcinoma cells using qPCR and Gla1 restriction enzyme digestion (Shock et al., 2011). While it is possible the mtDNA methylation is involved in regulating gene expression (Shock et al., 2011), as in the nucleus, the proteins involved in chromatin remodeling, such as histones, are not present in mitochondria (Spelbrink, 2010). This suggests the intriguing possibility that cytosine methylation through Dnmt3a is a major epigenetic mechanism involved in mitochondrial gene regulation without partnership with histone acetylation. Alternatively, the mtDNA methylation may serve some purpose other than gene regulation.

Our immunolocalization experiments on mouse skeletal muscle, spinal cord, and testes revealed that some 5mC-positive structures in the cytoplasm of myofibers, spinal motor neurons, and maturing spermatozoa were mitochondria because the 5mC colocalized with SOD2 or complex V and Dnmt3a. This observation confirms a previous report of mitochondrial 5mC localization using other mitochondrial makers (Chestnut et al., 2011). However, some cytoplasmic 5mC-positive structures were not positive for mitochondrial markers and had a morphology different from mitochondria because they were larger than typical mitochondria and sometimes appeared aggregated. We thus sought to determine possible relationships between methylated mtDNA and mitophagy and found that 5mC colocalized with the autophagosome marker LC3A (Klionsky and Emr, 2000). Our findings in mouse testes suggest that enhanced mtDNA methylation and mitophagy in maturing spermatoza in seminiferous tubules could be a mechanism to reduce the major burden of paternal mtDNA transmission prior to postfertilization autophagy (Al Rawi et al., 2011). Autophagy has also been

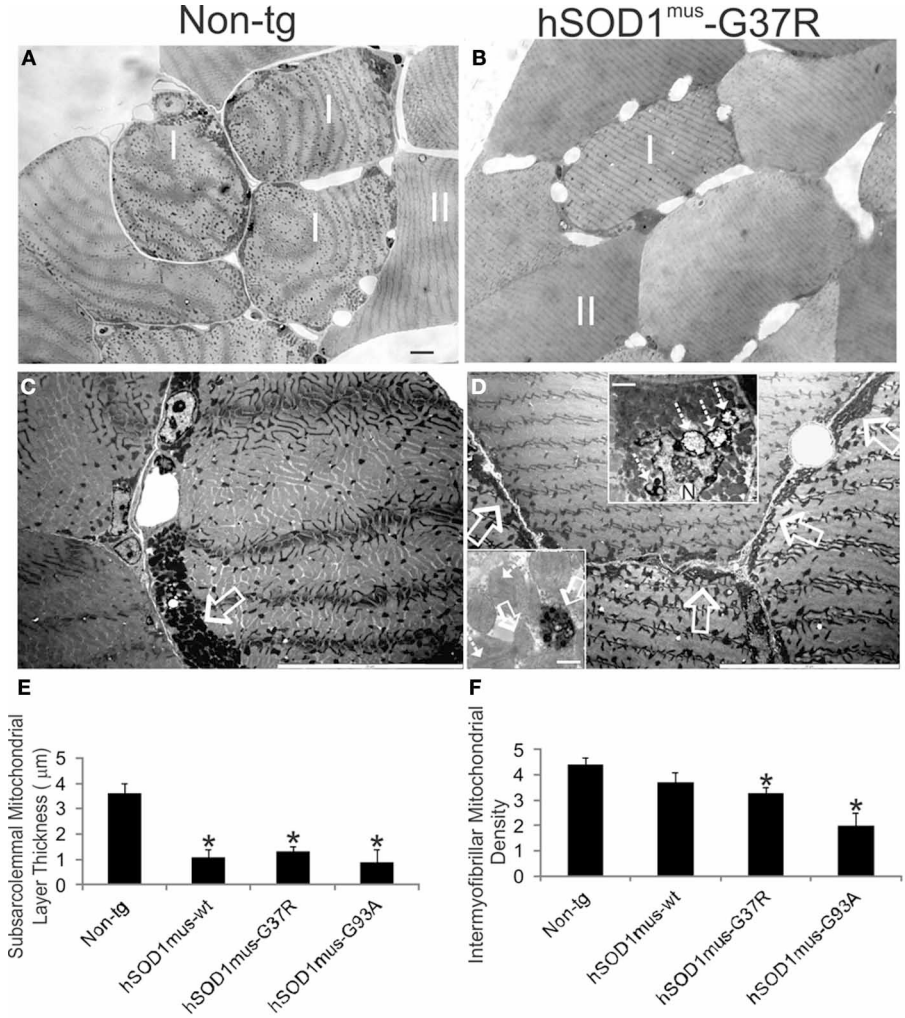


**FIGURE 10 | hSOD1$^{mus}$ tg mouse model of ALS shows loss of mitochondria in skeletal muscle. (A,B)** Toluidine blue-stained plastic sections (0.5 μm thick) of biceps femoris muscle from 15 month-old non-tg and hSOD1$^{mus}$-G37R tg mice. In non-tg mice, type I and type II fibers have distinct appearances as evidenced by the surrounding capillary number and subsarcolemmal and interfibrillar mitochondria density. In tg mice, type I fibers are less prominent with apparent loss of subsarcolemmal and interfibrillar mitochondria. Scale bar (in **A**, same for **B**) = 8 μm. **(C,D)** Electron micrographs showing a high density of subsarcolemmal mitochondria in non-tg mice **(C**, open arrows**)** and prominent attenuation of the subsarcolemmal layer in hSOD1$^{mus}$ tg mice **(D**, open arrows**)**. Upper inset **(D)** shows prominent mitophagy of subsarcolemmal mitochondria (hatched arrows) by a myonucleus (N) in hSOD1$^{mus}$-G37R tg mouse skeletal muscle. Scale bar = 1.3 μm. Lower inset **(D)** shows degenerating mitochondria with round inclusions (open white arrows) or rod inclusions (hatched white arrows) from hSOD1$^{mus}$-G93A tg mouse skeletal muscle. Scale bar = 0.5 μm. **(E)** Graph of the thickness of type I fiber subsarcolemmal mitochondrial layers in age-matched (∼17 months) non-tg and hSOD1$^{mus}$ tg mice. Values are mean ± SD. Asterisk denotes significant differences ($p < 0.01$) from non-tg control. **(F)** Graph of type II fiber interfibrillar mitochondrial densities in age-matched (∼17 months) non-tg and hSOD1$^{mus}$ tg mice. Values are mean ± SD. Asterisk denotes significant differences ($p < 0.01$) from non-tg control.

associated with nervous system and muscle disease (Batlevi and La Spada, 2011). In hSOD1 tg mice with muscle-specific expression, large 5mC-positive aggregates were observed in the cytoplasm of subsets of spinal motor neurons. Similar observations have been shown in adult mouse spinal motor neurons undergoing axotomy-induced apoptosis (Chestnut et al., 2011). The presence of Dnmt3a and 5mC within a subset of mitochondria, many of which are associated with autophagosomes, suggests an upstream epigenetic mechanism for mitophagy in the regulation of normal and pathological mitochondrial dynamics.

We have found that epigenetic mechanisms involving DNA methylation can drive motor neuron apoptosis in cell culture and *in vivo* and that aberrant regulation of DNA methylation is part of the pathobiology of human ALS (Chestnut et al., 2011). Abnormalities in the levels and localizations of Dnmt1, Dnmt3a, and 5mC have been found in human ALS spinal cord and motor cortex (Chestnut et al., 2011), and mitochondrial abnormalities in skeletal muscle, liver, spinal motor neurons, and motor cortex have been reported in human ALS (Sasaki and Iwata, 1999; Menzies et al., 2002). However, causal disease mechanisms are

difficult to pinpoint using human postmortem tissue. We therefore evaluated Dnmt3a and mtDNA methylation in several tg mouse models of ALS expressing mutant and wildtype hSOD1 either non-conditionally with global tissue expression (Gurney et al., 1994; Martin et al., 2007, 2009; Gertz et al., 2012) or conditionally with muscle-restricted expression (Wong and Martin, 2010). In skeletal muscle, mitochondrial Dnmt3a levels were markedly reduced in most hSOD1 tg mouse lines at stages of disease ranging from presymptomatic to mid-symptomatic. The loss of Dnmt3a and mitochondria in skeletal muscle was corroborated microscopically by immunohistochemistry. Interestingly, spinal cord Dnmt3a was reduced in tg mice with muscle-specific expression of hSOD1. This change is interesting because conditional ablation of Dnmt3a specifically in nervous tissue causes an age-related ALS-like phenotype in mice involving neuromuscular junction dismantling, motor neuron loss, and shortened lifespan (Nguyen et al., 2007). The localizations of 5mC immunoreactivity in skeletal muscle and spinal cord were also different in non-conditional and muscle-specific hSOD1 tg mice compared to age-matched control mice. Skeletal muscle disease in ALS mice thus appears to trigger retrograde transynaptic changes in epigenetic DNA methylation machinery in spinal cord mitochondria.

Abnormalities in mtDNA cytosine methylation were detected by pyrosequencing in mouse models of ALS. The cytosines interrogated were in the D-loop and 16S ribosomal RNA gene (**Figure 6A**). The 16S ribosomal RNA gene was more vulnerable than the D-loop to anomalous cytosine methylation. In the 16S ribosomal RNA gene, six cytosine sites showed increased methylation in spinal cord of hSOD1-G37R mice, and one cytosine site showed increased methylation in skeletal muscle of hSOD1$^{mus}$-G93A mice. While five cytosines in the D-loop showed differential levels of methylation, their methylation was similar in control and ALS mice. While the D-loop is generally thought to be a major regulator of mitochondrial genome transcription, it is now known that non-promoter CpG methylation within gene bodies can regulate gene expression in the nuclear genome (Maunakea et al., 2010). We have no direct explanation for the apparent mismatches between mitochondrial Dnmt3a protein levels which decrease and 5mC which increase. Recent studies have found that Dnmt3a has demethylase activity that is stimulated by $Ca^{2+}$ ions (Chen et al., 2013), so the increased cytosine methylation could be construed as being consistent with the loss of Dnmt3a in mitochondria.

A mechanism for the loss of skeletal muscle mitochondria appears to be mitophagy, as evidenced by the ultrastructure of degenerating mitochondrial profiles and the marked upregulation of LC3A seen by western blotting. Because numerous copies of mtDNA reside in single mitochondrion, Dnmt3a protein levels may be more reflective of skeletal muscle mitochondrial numbers, and extensively methylated mtDNA may be more stable than Dnmt3a protein in mitochondria undergoing mitophagy. Recent evidence supports this possibility because some mtDNA can escape autophagy and participate in local inflammatory mechanisms (Oka et al., 2012). Tissue inflammation is a prominent component of the pathobiology of mouse models of ALS (Martin et al., 2007; Chen et al., 2010; Drechsel et al., 2012) and human ALS (McGeer and McGeer, 2002; Corcia et al., 2013).

## AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: Margaret Wong, Barry Gertz, Barry A. Chestnut, Lee J. Martin. Performed experiments: Margaret Wong, Barry Gertz, Lee J. Martin. Analyzed the data: Margaret Wong, Barry Gertz, Lee J. Martin. Wrote paper: Margaret Wong, Barry Gertz, Lee J. Martin.

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# Astrocytes expressing mutant SOD1 and TDP43 trigger motoneuron death that is mediated via sodium channels and nitroxidative stress

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Amyotrophic lateral sclerosis (ALS) is a fatal paralytic disorder caused by dysfunction and degeneration of motor neurons. Multiple disease-causing mutations, including in the genes for SOD1 and TDP-43, have been identified in ALS. Astrocytes expressing mutant SOD1 are strongly implicated in the pathogenesis of ALS: we have shown that media conditioned by astrocytes carrying mutant SOD1$^{G93A}$ contains toxic factor(s) that kill motoneurons by activating voltage-sensitive sodium ($Na_V$) channels. In contrast, a recent study suggests that astrocytes expressing mutated TDP43 contribute to ALS pathology, but do so via cell-autonomous processes and lack non-cell-autonomous toxicity. Here we investigate whether astrocytes that express diverse ALS-causing mutations release toxic factor(s) that induce motoneuron death, and if so, whether they do so via a common pathogenic pathway. We exposed primary cultures of wild-type spinal cord cells to conditioned medium derived from astrocytes (ACM) that express SOD1 (ACM-SOD1$^{G93A}$ and ACM-SOD1$^{G86R}$) or TDP43 (ACM-TDP43$^{A315T}$) mutants; we show that such exposure rapidly (within 30–60 min) increases dichlorofluorescein (DCF) fluorescence (indicative of nitroxidative stress) and leads to extensive motoneuron-specific death within a few days. Co-application of the diverse ACMs with anti-oxidants Trolox or esculetin (but not with resveratrol) strongly improves motoneuron survival. We also find that co-incubation of the cultures in the ACMs with $Na_V$ channel blockers (including mexiletine, spermidine, or riluzole) prevents both intracellular nitroxidative stress and motoneuron death. Together, our data document that two completely unrelated ALS models lead to the death of motoneuron via non-cell-autonomous processes, and show that astrocytes expressing mutations in SOD1 and TDP43 trigger such cell death through a common pathogenic pathway that involves nitroxidative stress, induced at least in part by $Na_V$ channel activity.

**Keywords: ALS, non-cell-autonomous, motor neuron, degeneration, ROS/RNS, anti-oxidants**

## INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a fatal paralytic disorder caused by the progressive degeneration of cranial and spinal motoneurons in adulthood, and leading to death by respiratory failure within 3–5 years of diagnosis. Although the majority of ALS cases are sporadic (SALS), ∼10% are familial (FALS) and are generated by mutations in at least 15 identified ALS-associated gene loci (Bento-Abreu et al., 2010; Ferraiuolo et al., 2011). Dominant mutations in superoxide dismutase 1 (SOD1) and transactive response DNA-binding protein 43 (TARDBP gene, TDP43 protein) are common causes of ALS (Cleveland and Rothstein, 2001; Pasinelli and Brown, 2006; Cozzolino et al., 2012)—to date, more than 150 SOD1 mutations and 40 TARDBP mutations are known to be associated with the ALS phenotype (http://alsod.iop.kcl.ac.uk/; Abel et al., 2012).

Although the molecular underpinnings of motoneuron degeneration in ALS have not yet been elucidated, *in vivo* and *in vitro* studies with use of transgenic mice that carry ALS-causing mutants reveal a large number of pathogenic changes in affected motoneurons: these include mitochondrial dysfunction, hyperexcitability, glutamate excitotoxicity, nitroxidative stress from reactive oxygen species (ROS) or reactive nitrogen species (RNS) (collectively leading to nitroxidative stress), protein aggregation and misfolding, proteasome impairment, cytoskeletal disruption, activation of cell death signals, and dysregulation of transcription and RNA processing (Beckman et al., 2001; Cleveland and Rothstein, 2001; Bruijn et al., 2004; Pasinelli and Brown, 2006; Ferraiuolo et al., 2011; Cozzolino et al., 2012; van Zundert et al., 2012). Despite these advances in identifying these cellular alterations, however, the origin(s) and interplay between multiple pathogenic processes of motoneuron death in ALS remain largely unknown.

A large number of studies highlight the importance of dysregulated crosstalk between motoneurons and non-neuronal cells in ALS (Ilieva et al., 2009). The notion that ALS is at least partially a non-cell-autonomous disease originates in a groundbreaking study from Clement et al. (2003) who generated chimeric mice composed of mixtures of normal and SOD1 mutant-expressing

cells, and showed that wild-type non-neuronal cells extend the survival of motoneurons carrying mutant SOD1. Additional research has since firmly established the contribution of "deadly neighboring cells" (astrocytes, microglia, oligodendrocytes, and Schwann cells) to the degeneration of motoneurons (Boillée et al., 2006; Yamanaka et al., 2008a,b; Lobsiger et al., 2009; Ilieva et al., 2009). Other findings offer compelling evidence that primary mutant SOD1-expressing astrocytes from mouse (Di Giorgio et al., 2007; Nagai et al., 2007; Castillo et al., 2013; Fritz et al., 2013), rat (Vargas et al., 2006; Cassina et al., 2008), and humans (Marchetto et al., 2008) effectively and selectively kill motoneurons, but spare interneurons. Importantly, astrocytes differentiated from neuronal progenitor cells (NPCs) obtained either from post-mortem spinal cord tissue or from skin biopsies from FALS (SOD1 mutations and hexanucleotide expansion in C9orf72) and SALS patients also display non-cell-autonomous toxicity, and selectively kill motoneurons in a co-culture model system (Haidet-Phillips et al., 2011; Meyer et al., 2014). Moreover, astrocytes that express mutants in SOD1 contribute to the pathogenesis of ALS by releasing into the media a toxic factor(s) that kills motoneurons (Nagai et al., 2007; Cassina et al., 2008; Castillo et al., 2013; Fritz et al., 2013). Little is known regarding the non-cell- autonomous toxicity mediated by mutants other than in SOD1, but a recent study suggests that astrocytes expressing mutated TDP43 ($TDP43^{M337V}$) lack non-cell-autonomous toxicity and contribute to ALS pathology only through cell-autonomous processes (Serio et al., 2013).

Here we show that conditioned medium derived from astrocytes that were harvested from transgenic mice carrying ALS-causing mutations in SOD1 ($SOD1^{G93A}$ and $SOD1^{G86R}$) or TDP43 ($TDP43^{A315T}$) contain toxic factors that trigger motoneuron death. Based on earlier studies which document the involvement of $Na_v$ channel-mediated excitability and nitroxidative stress in the pathogenesis of ALS (Ferraiuolo et al., 2011; Cozzolino et al., 2012; van Zundert et al., 2012), we tested here whether these pathogenic changes are induced in motoneurons via non-cell-autonomous processes. We demonstrate that they do so, and our results indicate that nitroxidative stress within the neurons is mediated by $Na_v$ channel activity.

## MATERIALS AND METHODS

### ANIMALS

Care and use of rodents was in accordance with the US National Institute of Health guidelines, and was approved by the Institutional Animal Care and Use Committee of Andres Bello University. Hemizygous transgenic mice carrying mutant human $SOD1^{G93A}$ (high copy number; B6SJL; Cat. No. 002726), wild-type human $SOD1^{WT}$ (B6SJL; Cat. No. 002297), mutant mouse $SOD1^{G86R}$ (FVB crossed on B6SJL background; Cat. No. 005110), or mutant mouse $TDP43^{A315T}$ (B6.Cg crossed on C57BL/6J; Cat. No. 010700) were originally obtained from Jackson Laboratories (Bar Harbor, USA). Non-transgenic littermates and transgenic mice over-expressing the gene for human $SOD1^{WT}$ were used as controls. Transgenes were identified by polymerase chain reaction (Wegorzewska et al., 2009; Castillo et al., 2013; Fritz et al., 2013). The $SOD1^{G93A}$ mice, but not the $hSOD1^{WT}$ mice, develop signs of neuromuscular deficits (tremor of the legs and loss of extension reflex of the hind paws) starting at 3 months of age and have an average lifespan of 19–21 weeks (Gurney et al., 1994). Mice carrying $SOD1^{G86R}$ (Ripps et al., 1995) or $TDP43^{A315T}$ (Wegorzewska et al., 2009) develop similar loss of motor function between 3 and 4 months and do not survive to the age of 4 months.

### CONDITIONED MEDIA PREPARATION

ACM was prepared as described (Nagai et al., 2007; Castillo et al., 2013; Fritz et al., 2013). Briefly, cultures of astrocytes were prepared from P1-2 wild-type mice and from transgenic mice expressing human $SOD1^{G93A}$, mouse $hSOD1^{G86R}$, human $SOD1^{WT}$, or mouse $TDP43^{A315T}$. Cultures were maintained in DMEM (Hyclone, Cat. No. SH30081.02) containing 10% FBS (Hyclone, Cat. No. SH30071.03; lot ATC31648) and 1% penicillin-streptomycin (Gibco, Cat. No. 15070-063) at 37°C 5% $CO_2$. Cultures reached confluence after 2–3 weeks and contained >95% $GFAP^+$ astrocytes. Residual microglia were removed by shaking cultures in an orbital shaker (200 r.p.m. in the incubator) overnight (7 h), at which point media was replaced by spinal culture media (see below). After 7 days, ACM was collected, centrifuged (500 g for 10 min) and stored at −80°C; before use, it was supplemented with 4.5 mg/ml D-glucose (final concentration) and penicillin/streptomycin, and filtered. A chick hindlimb muscle extract was also added to the ACM before use (Sepulveda et al., 2010).

For all experiments the ACM was diluted 8–10-fold. The exact dilution was determined for each new batch of ACM by comparing the motoneuron toxicity of the ACM from transgenic animals carrying the ALS-causing mutants (ACM-$SOD1^{G93A}$, ACM-$SOD1^{G86R}$, and ACM-$TDP43^{A315T}$) to that of ACM generated from mice carrying the wild-type human SOD1 gene (ACM-$SOD1^{WT}$) or from non-transgenic littermates (ACM-NT-Control); at the selected dilutions the conditioned media derived from the astrocytes expressing the ALS-causing genes robustly killed motoneurons, whereas the ACM-NT-Control and ACM-$SOD1^{WT}$ did not affect motoneuron survival. The ACM was applied to ventral spinal cord cultures derived from rats because better quality motoneurons are obtained from rats than from mice; a number of studies have shown that such mixed species co-cultures (from rat, mice, human) do not appear to induce any side effects (e.g., Pehar et al., 2004; Di Giorgio et al., 2007; Nagai et al., 2007; Castillo et al., 2013; Fritz et al., 2013).

### PRIMARY SPINAL CORD NEURONAL CULTURES

Pregnant Sprague–Dawley rats were deeply anesthetized with $CO_2$, and primary spinal cultures were prepared from E14 pups (Sepulveda et al., 2010; Castillo et al., 2013; Fritz et al., 2013). Briefly, whole spinal cords were excised and placed into ice-cold HBSS (Gibco, Cat. No. 14185-052) containing 50 μg/ml penicillin/streptomycin (Gibco, Cat. No. 15070-063). The dorsal part of the spinal cord was removed using a small razor blade, and the ventral cord was minced and enzymatically treated by incubating in pre-warmed HBSS containing 0.25% trypsin (Gibco, Cat. No. 15090-046) for 20 min at 37°C. Cells were transferred to a 15 ml tube containing neuronal growth media containing 70% MEM (Gibco, Cat. No. 11090-073), 25% Neurobasal media (Gibco, Cat. No. 21103-049), 1% N2 supplement (Gibco, Cat.

No. 17502-048), 1% L-glutamine (Gibco, Cat. No. 25030-081), 1% penicillin-streptomycin (Gibco, Cat. No. 15070-063), 2% horse serum (Hyclone, Cat. No. SH30074.03; lot AQH24495) and 100 mM sodium pyruvate (Gibco, Cat. No. 11360-070); they were precipitated, transferred to a new 15-ml-tube containing 2 ml of growth media, re-suspended by mechanical agitation through fire-polished glass Pasteur pipettes of different tip diameters, and counted; $1 \times 10^6$ cells were plated on freshly prepared poly-L-lysine-coated 24-well plates (1 mg/ml; 30.000–70.000 mW; Sigma, Cat. No. P2636). Cells were cultured for 7 days at 37°C under 5% $CO_2$, and supplemented with 45 μg/ml chick hindlimb muscle extract (Sepulveda et al., 2010); the media was refreshed every 3 days.

#### PHARMACOLOGICAL TREATMENTS IN CULTURE

Mexiletine (Tocris, Cat. No. 2596) was dissolved in water to 100 mM and used at final concentration of 25 nM. Riluzole (Sigma, Cat. No. R116) was dissolved in distilled water (plus 10% Tween20) at 100 μM, and added to cultures to final concentration of 100 nM. Spermidine (Sigma, Cat. No. S2626) was dissolved in water at 100 mg/ml and added to cultures to a final concentration of 10 μM. Trolox (Sigma, Cat. No. 238813) was dissolved in distilled water at 100 mM and added to cultures to final concentration of 1 μM. Esculetin (Sigma, Cat. No. 17795) was dissolved in dimethyl sulfoxide (DMSO), and added to cultures to final concentration 25 μM. Resveratrol (Tocris, Cat. No. 1418) was dissolved in DMSO (Sigma) at 100 mM and added to cultures to final concentration of 1 μM. All stock solutions were stored at −20°C.

#### CELL LABELING AND COUNTING

Motoneurons and interneurons were immunolabeled and counted as previously described (Sepulveda et al., 2010; Castillo et al., 2013; Fritz et al., 2013). Briefly, primary spinal cultures were fixed at 7 DIV with 4% paraformaldehyde, and immunostained with an antibody against MAP2 (1:400; Santa Cruz Biotechnology) to label all neurons (interneurons plus motoneurons) and with the SMI-32 antibody (1:1,000, Sternberger Monoclonals) to reveal the presence of unphosphorylated neurofilament-H, which is expressed specifically in motoneurons in spinal cord cultures (Urushitani et al., 2006; Nagai et al., 2007); antibody binding was visualized with the appropriate fluorescent secondary antibodies. Our wild-type primary spinal cord cultures typically contain at least 6–10% motoneurons until 12 DIV (Sepulveda et al., 2010). Immunolabeled neurons were documented on an inverted Nikon Eclipse Ti-U microscope equipped with a SPOT Pursuit™ USB CameraCCD (14-bit), Epi-fl Illuminator, mercury lamp, and Sutter Smart-Shutter with a lambda SC controller. Cells were photographed using a 20× objective; MAP2- and SMI-32-positive neurons were counted offline within 20 randomly chosen fields, and the percentage of SMI-32-positive motoneurons within the total number of MAP2-positive cells was calculated. Each condition was replicated in at least 3 independent cultures, and in duplicate.

#### NITROXIDATIVE STRESS MEASUREMENTS WITH CM-$H_2$DCF-DA

The intracellular levels of ROS/RNS were measured with CM-$H_2$DCF-DA (Invitrogen, Cat. No. C6827). $H_2$DCF-DA is not a specific probe for a particular oxidant and has been used to monitor certain ROS/RNS (see Discussion). The CM-$H_2$DCF-DA stock solution (5 mM) was prepared in DMSO and was diluted in the culture medium to a final concentration of 1 μM just before addition to the cells. After application of the diverse ACMs to the spinal cord cultures for different time (minutes-hours-days), cells were washed (PBS 1×) to remove the ACMs and exposed to CM-$H_2$DCF-DA for 30 min at 37°C in dark, to label both motoneurons and interneurons. To facilitate the CM-$H_2$DCF-DA membrane penetration, 0.004% Pluronic acid F-127 (Invitrogen, Cat. No. P-3000MP) was added to the culture medium to facilitate dye entry, eliminating possible hydrolysis of dyes by external esterases and maintain better cell integrity (Appaix et al., 2012). After the incubation time, the CM-$H_2$DCF-DA-cointaing culture medium was removed and cultures were washed twice with PBS 1× and suspended in culture medium (500 μl final volume). Next, cells were immediately imaged using an inverted Nikon Eclipse Ti-U microscope equipped with a SPOT Pursuit™ USB CameraCCD (14-bit), Epi-fl Illuminator, mercury lamp, and Sutter Smart-Shutter with a lambda SC controller. Cells were photographed using a 20× objective. As CM-$H_2$DCF-DA is a non-fluorescent dye it passively diffuses into cells and is hydrolyzed intracellularly to the DCFH carboxylate anion that is trapped inside; oxidation of DCFH results in the formation of the fluorescent product DCF, with excitation and emission wavelengths $\lambda_{ex}/\lambda_{em}$ = 492–495/517–527 nm. The exposure time was kept below 4 s in order to avoid photo-oxidation of the ROS/RNS sensitive dye and for all given treatments fields were exposed for exactly the same amount of time. At least three independent fields were acquired for each condition and at least 10 cells per field were used for quantification of the fluorescence signal. Cells were marked by drawing a region of interest around the cell body, and mean fluorescence intensity was calculated for each cell after subtraction of the background signal using the image analysis module in ImageJ software. Those cells with a relative intensity unit (RIU) of ≥1.5 were counted as positive. Cultures were also incubated with $H_2O_2$ (200 μM for 20 min) to serve as a positive control and to normalize the number of DCF-positive cells after ACM application.

#### DATA ANALYSIS

ANOVA, followed by *post-hoc* Tukey tests, was used to detect significant changes. Student's *t*-tests were used to compare the response of two cell populations to individual treatments. Unless otherwise stated, error bars represent the mean ± s.e.m.; $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. control.

## RESULTS

#### ACM-SOD1$^{G93A}$, ACM-SOD1$^{G86R}$, OR ACM-TDP43$^{A315T}$ TRIGGERS DEATH OF CULTURED PRIMARY MOTONEURONS

Here we investigated whether astrocytes expressing diverse ALS causing mutants release toxic factor(s) that induce motoneuron death, and if so, whether a common pathogenic pathway is involved. Astrocyte conditioned media (ACM) was derived from astrocytes that were harvested from mice carrying mutant SOD1 (ACM-SOD1$^{G93A}$ and ACM-SOD1$^{G86R}$) or TDP43 (ACM-TDP43$^{A315T}$). These media were added at 8–10-fold dilutions (see Materials and Methods) to wild-type primary rat spinal cultures at 4 DIV for 3 days; effects on neuron survival were assessed at 7 DIV (**Figure 1A**). To define the presence of all

neurons, an antibody against microtubule-associated protein 2 (MAP2) was used (**Figure 1B**; arrowhead); motoneurons were specifically identified with use of the SMI-32 antibody, which recognizes unphosphorylated neurofilament-H (**Figure 1B**; arrow), as previously described (Urushitani et al., 2006; Nagai et al., 2007; Sepulveda et al., 2010; Castillo et al., 2013; Fritz et al., 2013). Chronic (3 days) exposure (from 4 to 7 DIV) of spinal cultures to ACM-SOD1$^{G93A}$, ACM-SOD1$^{G86R}$, or ACM-TDP43$^{A315T}$ induced ∼50% motoneuron death (**Figures 1C,D**). The number of interneurons was unchanged after application of ACM-SOD1$^{G93A}$ ($91 \pm 1\%$ vs. control, $p > 0.05$ by $t$-test), ACM-SOD1$^{G86R}$ ($96 \pm 19\%$ vs. control, $p > 0.05$ by $t$-test), or ACM-TDP43$^{A315T}$ ($103 \pm 23\%$ vs. control, $p > 0.05$ by $t$-test). Our findings that ACM-SOD1$^{G93A}$ and ACM-SOD1$^{G86R}$ robustly reduces motoneuron cell survival, while sparing interneurons, are consistent with previous studies (Nagai et al., 2007; Castillo et al., 2013; Fritz et al., 2013). Our results with ACM-TDP43$^{A315T}$ also show for the first time that astrocytes carrying a TDP43 mutant kill motoneurons through non-cell-autonomous processes.

Three types of control media were used throughout this work. (1) "Control" media that was not conditioned by astrocytes. (2) "ACM-NT-Control" media derived from astrocytes that were harvested from littermate mice that were negative for the SOD1 and TDP43 gene. (3) "ACM-SOD1$^{WT}$" media derived from astrocytes that were harvested from transgenic mice carrying the non-pathological human wild-type SOD1 gene. None of these media caused motoneuron death (**Figure 1E**). In particular, the finding that ACM-SOD1$^{WT}$ was not toxic indicates that the factor inducing motoneuron death is specifically due to the SOD1$^{G93A}$ or SOD1$^{G86R}$ mutation, rather than to overexpression of the human SOD1 protein. By contrast, we can not exclude the possibility that motoneuron death in our spinal cultures is attributable, at least in part, by increased levels of the TDP43 protein itself. In fact, accumulating data with transgenic models have established that excessive levels of even human wild-type TDP43 result in neurodegeneration, likely as a result in the disruption of RNA metabolism (Wegorzewska et al., 2009; Wils et al., 2010; Igaz et al., 2011; Ling et al., 2013).

### ACM-SOD1$^{G93A}$, ACM-SOD1$^{G86R}$, OR ACM-TDP43$^{A315T}$ LEADS TO INCREASES IN INTRACELLULAR ROS/RNS LEVELS

Increased intracellular levels of nitroxidative stress are widely and consistently observed in ALS patients, and in *in vitro* and *in vivo* mouse models that express SOD1 mutants (Barber and Shaw, 2010). To investigate whether soluble toxic factors released by astrocytes that carry SOD1 and TDP43 mutants induce an oxidative burden in primary neurons, we exposed 4 DIV cultures to the diverse ACMs for 30–120 min, washed cells to remove the ACMs, and subsequently loaded the cells with CM-$H_2$DCF-DA for 30 min (**Figure 2A**). CM-$H_2$DCF-DA is a non-fluorescent dye that passively diffuses into cells and is hydrolyzed intracellularly to the DCFH carboxylate anion that is trapped inside; oxidation of DCFH results in the formation of the fluorescent product DCF. Increased intensity in fluorescent DCF could thus reflect detection of certain reactive oxygen and nitrogen species, inducing nitroxidative stress. Combined real-time fluorescence and phase-contrast imaging

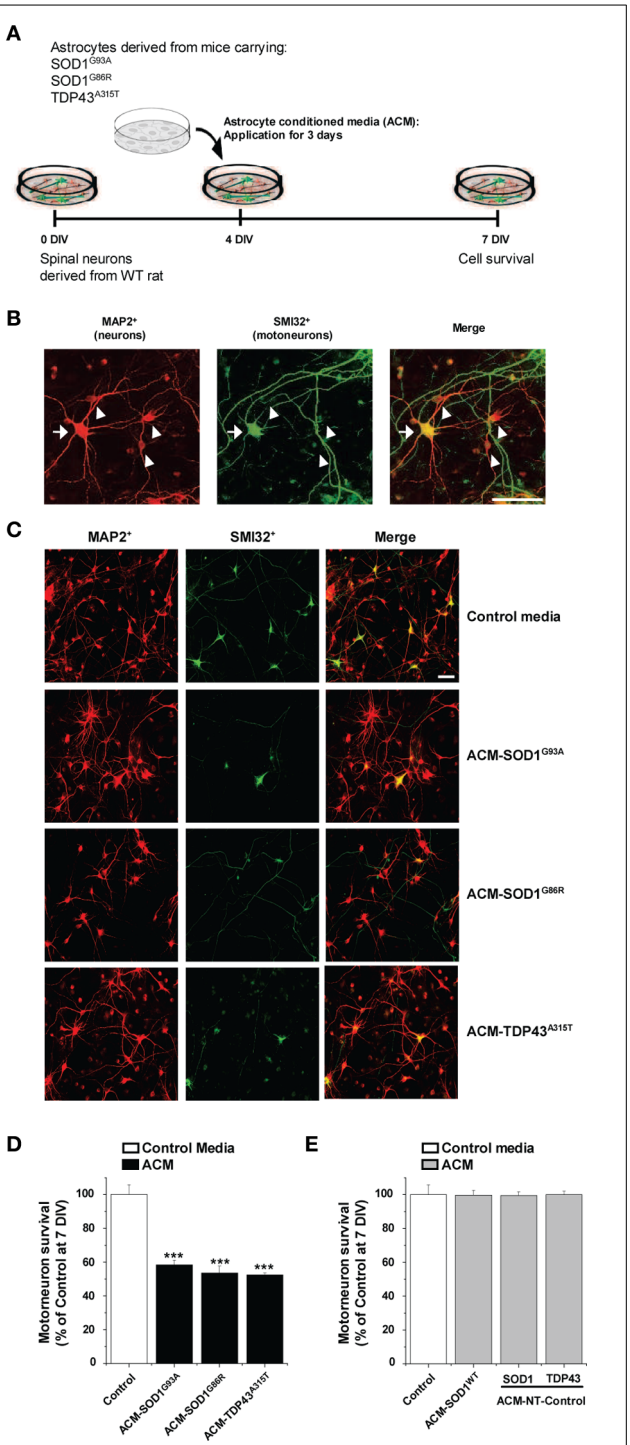


**FIGURE 1 | Exposure of primary spinal cord cultures to astrocyte conditioned media (ACM) derived from SOD1$^{G93A}$, SOD1$^{G86R}$, and TDP43$^{A315T}$ expressing mice triggers death of motoneurons. (A)** Flow diagram of experiment. Primary wild-type (WT) rat spinal cord cultures (4 DIV) were exposed for 3 days to ACM derived from transgenic mice overexpressing SOD1$^{G93A}$ (ACM-SOD1$^{G93A}$), SOD1$^{G86R}$ (ACM-SOD1$^{G86R}$), or TDP43$^{A315T}$ (ACM-TDP43$^{A315T}$). Cells were fixed at 7 DIV, and cell survival was assayed with immunocytochemistry. **(B)** Fixed 7 DIV spinal cord cultures were double-labeled with anti-microtubule-associated

*(Continued)*

**FIGURE 1 | Continued**
protein 2 (MAP2) antibody (red) to visualize interneurons (arrowhead) and with the SMI-32 antibody (green) to identify motoneurons (arrow). Scale bar, 25 μm. **(C)** Representative images of $MAP2^+$/$SMI32^+$-labeled neurons in spinal cultures under control conditions (top image) or treated with the three different ACMs, as indicated in the figure. Scale bar, 200 μm. **(D)** Graph showing the percentage of motoneurons that survived after treatment with ACMs derived from $SOD1^{G93A}$, $SOD1^{G86R}$, and $TDP43^{A315T}$ astrocytes, relative to motoneurons from sister cultures treated with control medium. **(E)** Graph showing the percentage of motoneurons that survived after treatment with media derived from mouse littermates that were negative for the mutated forms of SOD1 and TDP43 (ACM-NT-Control), or with media derived from transgenic mice carrying the non-pathological human wild-type SOD1 gene (ACM-$SOD1^{WT}$). Survival is shown relative to cultures treated with control media. Values represent mean ± s.e.m. from at least 3 independent experiments performed in duplicate, analyzed by One-Way ANOVA followed by a Tukey *post-hoc* test. $^{***}P < 0.001$ relative to control medium at 7 DIV.

showed that DCF levels were very low under basal culture conditions, while application of ACM-$SOD1^{G93A}$ induced both a gradual increase in the intensity and the number of neurons displaying intracellular DCF fluorescence in spinal cord cultures; the fluorescence reached a peak at 30 min (**Figure 2B**). This increase was mimicked by $H_2O_2$ (**Figure 2B**; 200 μM for 20 min) but was blocked by use of diverse general anti-oxidants (see **Figure 4**). In control experiments, application of ACM-$SOD1^{WT}$ did not change DCF fluorescence over the same exposure times (**Figure 2B**$_2$). Exposure of spinal cord cultures to either ACM-$SOD1^{G86R}$ (**Figure 2C**) or ACM-$TDP43^{A315T}$ (**Figure 2D**) also triggered a gradual increase of intracellular DCF fluorescence in neurons; this fluorescence peaked at 60 and 30 min, respectively. Treatment of neuronal cultures with either ACM-NT-Control (**Figures 2C**$_2$**–D**$_2$) or control media did not induce significant differences (see **Supplementary Figure 1** for images of all controls). Together, these results indicate that toxic factors released by astrocytes that carry diverse ALS-causing mutants results in increased nitroxidative stress in spinal cord neurons. The increased nitroxidative stress, however, is unable to induce immediate cell death as the number of motoneurons only starts to significantly reduce after 24 h of ACM application (**Supplementary Figure 2**).

### ANTI-OXIDANTS PREVENT MOTONEURON DEATH INDUCED BY ACM-$SOD1^{G93A}$, ACM-$SOD1^{G86R}$, OR ACM-$TDP43^{A315T}$

To determine whether the increased nitroxidative stress induced by the diverse ACMs contributes to motoneuron cell death, 4 DIV spinal cord neurons were chronically incubated in a combination of the toxic media plus one of the following anti-oxidants: Trolox, esculetin or resveratrol (**Figure 3A**). These anti-oxidants are reported to reduce intracellular levels of ROS/RNS (also documented in the present study—see **Figure 4** for effects on DCF fluorescence; also see reference citations below). Multiple doses (ranging from 100 nM to 100 μM) of the anti-oxidants were used to assess the survival of motoneurons under control conditions, and after co-application with ACM-$SOD1^{G93A}$ (see **Supplementary Table 1**). For each anti-oxidant drug, the maximum effect in preventing motoneuron death induced by ACM-$SOD1^{G93A}$ is displayed in **Figure 3B**; the same concentrations of anti-oxidants were also used to test whether they could rescue motoneuron death induced by ACM-$SOD1^{G86R}$ (**Figure 3C**) or ACM-$TDP43^{A315T}$ (**Figure 3D**) (see below for details on each anti-oxidant). We also applied these doses of anti-oxidants to cultures incubated with ACM-$SOD1^{WT}$ and found that none of the anti-oxidants were successful in significantly increasing the number of motoneurons (**Figure 3E**); similar results were obtained when anti-oxidants were co-applied to spinal cord culture with control media or ACM-NT-Control (not shown), indicating that the beneficial effects of these compounds are specifically attributable to counterbalancing increased nitroxidative stress induced by the diverse toxic ACMs, rather than to simply increasing overall motoneuron cell survival in the cultures.

We first analyzed use of vitamin E, the most potent naturally occurring scavenger of reactive oxygen and nitrogen species known (Tucker and Townsend, 2005). Extensive studies in ALS patients and mice models have shown, however, that vitamin E application *in vivo* is not capable of significantly prolonging survival (Gurney et al., 1996; Desnuelle et al., 2001; Ascherio et al., 2005; Graf et al., 2005) These disappointing results are likely related to the findings that vitamin E poorly penetrates the blood-brain barrier, leading to insufficient doses of this anti-oxidant in the central nervous system; $IC_{50}$ of vitamin E is between 1.5 and 59 μM while ventricular CSF concentration of this vitamin was found at 0.114 μM (reviewed in Barber and Shaw, 2010). Here we opted to use Trolox, a water-soluble vitamin E analog that neutralizes ROS (Ghiselli et al., 1995; Khaldy et al., 2000; Barber et al., 2009; Distelmaier et al., 2012). Chronic co-application of ACM-$SOD1^{G93A}$ (**Figure 3B**) or ACM-$TDP43^{A315T}$ (**Figure 3D**) with 1 μM of Trolox significantly improved motoneuron survival. In the spinal cord cultures treated with ACM-$SOD1^{G86R}$ plus Trolox improvement of motoneurons survival was even better and comparable with motoneuron survival obtained under control conditions (**Figure 3C**).

Next we analyzed esculetin and resveratrol, two molecules that have anti-oxidant activities (Lin et al., 2000; Kaneko et al., 2003; Baur and Sinclair, 2006; Barber et al., 2009). Esculetin and resveratrol were also identified from a screen of the Spectrum Collection library (consisting of 2000 small compounds) as two of the best-hit molecules, based on their ability to function as effective anti-oxidants by reducing DCF fluorescence and to increase the viability of a mutant $SOD1^{G93A}$-expressing cell line; moreover, *in silico* analysis predicted that these two compounds have specific biochemical properties that allow efficient blood-brain barrier penetration (Barber et al., 2009). We found that chronic co-application of ACM-$SOD1^{G93A}$ (**Figure 3B**) or ACM-$TDP43^{A315T}$ (**Figure 3D**) with 25 μM esculetin significantly improved the survival of motoneurons and, in fact, treatment of spinal cord neurons with esculetin plus ACM-$SOD1^{G86R}$ enhanced motoneuron survival to the level obtained under control conditions (**Figure 3C**).

By contrast, chronic application of resveratrol at 1 μM (**Figure 3B**)—as well as at a wide range of concentrations (100 nM to 10 μM) (**Supplementary Table 1**)—failed to prevent motoneuron death induced by ACM-$SOD1^{G93A}$. Co-application

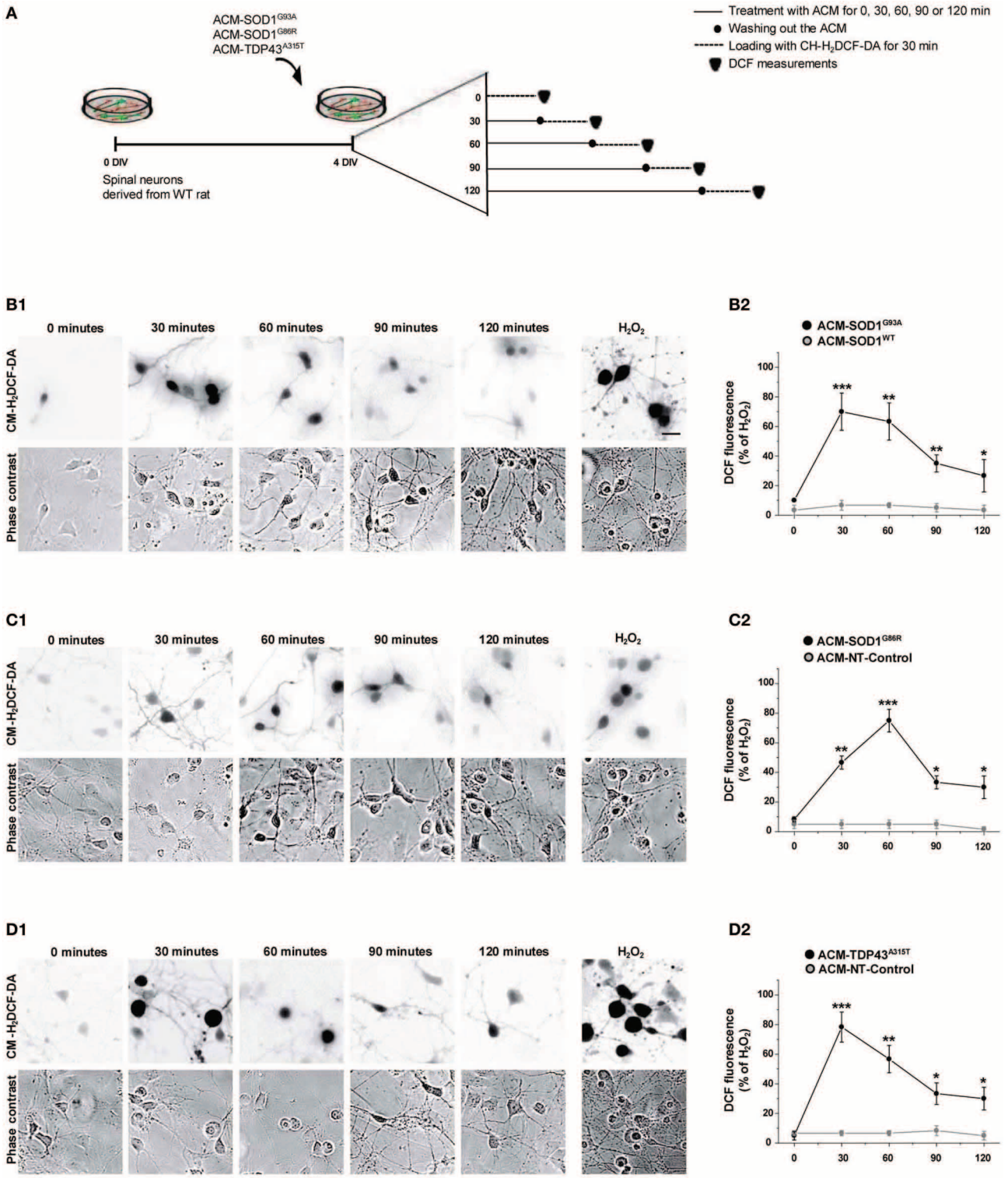


**FIGURE 2 | Exposure of primary spinal cord cultures to ACM-SOD1$^{G93A}$, ACM-SOD1$^{G86R}$, and ACM-TDP43$^{A315T}$ induces rapid increases in intracellular dichlorofluorescein (DCF) fluorescence. (A)** Flow diagram of experiment. Primary wild-type spinal cultures (4 DIV) were exposed for 0–120 min with the different ACMs (solid lines), washed to remove the ACMs (filled circles), and loaded for 30 min with the fluorescent membrane permeable ROS/RNS probe CM-$H_2$DCF-DA (dotted lines). Next cultures were washed and DCF measurements were immediately performed (filled triangles). **(B–D)** Cultures exposed to ACM-SOD1$^{G93A}$ **(B)**, ACM-SOD1$^{G86R}$ **(C),** or ACM-TDP43$^{A315T}$ **(D)**. **($B_1$–$D_1$)** The negatives of representative DCF fluorescent images (in which both motoneurons and interneurons are marked) and corresponding phase contrast images of spinal cord cultures photographed at 0, 30, 90 and 120 min after application of ACM-SOD1$^{G93A}$

*(Continued)*

**FIGURE 2 | Continued**
**($B_1$)**, ACM-SOD1$^{G86R}$ **($C_1$)**, or ACM-TDP43$^{A315T}$ **($D_1$)** are shown. In all experiment, $H_2O_2$ (200 μM for 20 min) served as positive control and to normalize the number of DCF-positive cells after ACM application. Scale bar, 200 μm. **($B_2$–$D_2$)** Graphs showing the percentage of DCF fluorescent cells (including both motoneurons and interneurons) under the conditions indicated. Results obtained with different controls are included in the graphs: control media, ACM-NT-Control, and ACM-SOD1$^{WT}$. DCF fluorescence is relative to cultures treated with $H_2O_2$ (100%). Note that DCF fluorescence peaked after 30 min of incubation of spinal cultures with ACM-SOD1$^{G93A}$ and ACM-TDP43$^{A315T}$ **($B_2$,$D_2$)**, and after 60 min for incubation with ACM-SOD1$^{G86R}$ **($C_2$)**. Values represent mean ± s.e.m. from at least 3 independent experiments performed in duplicate, analyzed by *t*-test. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. control.

of 1 μM resveratrol also did not significantly improve survival of motoneurons incubated with either ACM-SOD1$^{G86R}$ (**Figure 3C**) or ACM-TDP43$^{A315T}$ (**Figure 3D**). This negative effect was not related to toxicity of this anti-oxidant, as overall motoneuron cell survival in cultures treated with resveratrol was similar to that achieved under control conditions or cultures treated with ACM-SOD1$^{WT}$ (see **Figure 3E** and **Supplementary Table 1**).

As discussed before, the application of the diverse ACMs to spinal cord cultures resulted in a strong DCF signals at 30–60 min which then gradually reduced (**Figure 2**). However, this is only a transient reduction and cells start to steadily increase DCF fluorescence from 24 h after ACM application; 7 DIV neurons treated chronically for 3 days with the diverse ACMs resulted in robust levels of ROS/RNS (∼70–80% DCF fluorescence relative to $H_2O_2$) (**Supplementary Figure 3**). We next analyzed whether the doses of anti-oxidants that effectively increased cell survival (as shown in **Figure 3**) also prevented the increase in ROS/RNS levels induced by the diverse ACMs (**Figure 4A**). Co-application of ACM-SOD1$^{G93A}$ plus Trolox (1 μM) or eculetin (25 μM) to spinal cord cultures decreased intracellular DCF fluorescence to a degree similar to that achieved in control cultures (**Figure 4B**). By contrast, resveratrol (1 μM) slightly, but not significantly, reduced nitroxidative stress induced by ACM-SOD1$^{G93A}$. We also observed that co-application of Trolox and esculetin with ACM-SOD1$^{G86R}$ (**Figure 4C**) or ACM-TDP43$^{A315T}$ (**Figure 4D**) had similar beneficial effects in preventing nitroxidative stress; however, again, resveratrol was not effective in significantly reducing the DCF signal. This negative effect of resveratrol was not due to the absence of its anti-oxidant capacity; resveratrol reduced DCF fluorescence induced by $H_2O_2$ (200 μM for 20 min) by ∼50% (**Supplementary Figure 4**).

Application of these anti-oxidants to control cultures (not shown), or to spinal cord cultures treated with either ACM-NT-Control (not shown) or with ACM-SOD1$^{WT}$ (**Figure 4E**) revealed that none of these compounds led to significant decreases in basal DCF fluorescence intensity. Together, our data indicate that the favorable effects of Trolox and esculetin on motoneuron survival principally result from counterbalancing the increases in intracellular levels of ROS induced in neurons by the toxic actions of ACM-SOD1$^{G93A}$, ACM-SOD1$^{G86R}$, or ACM-TDP43$^{A315T}$.

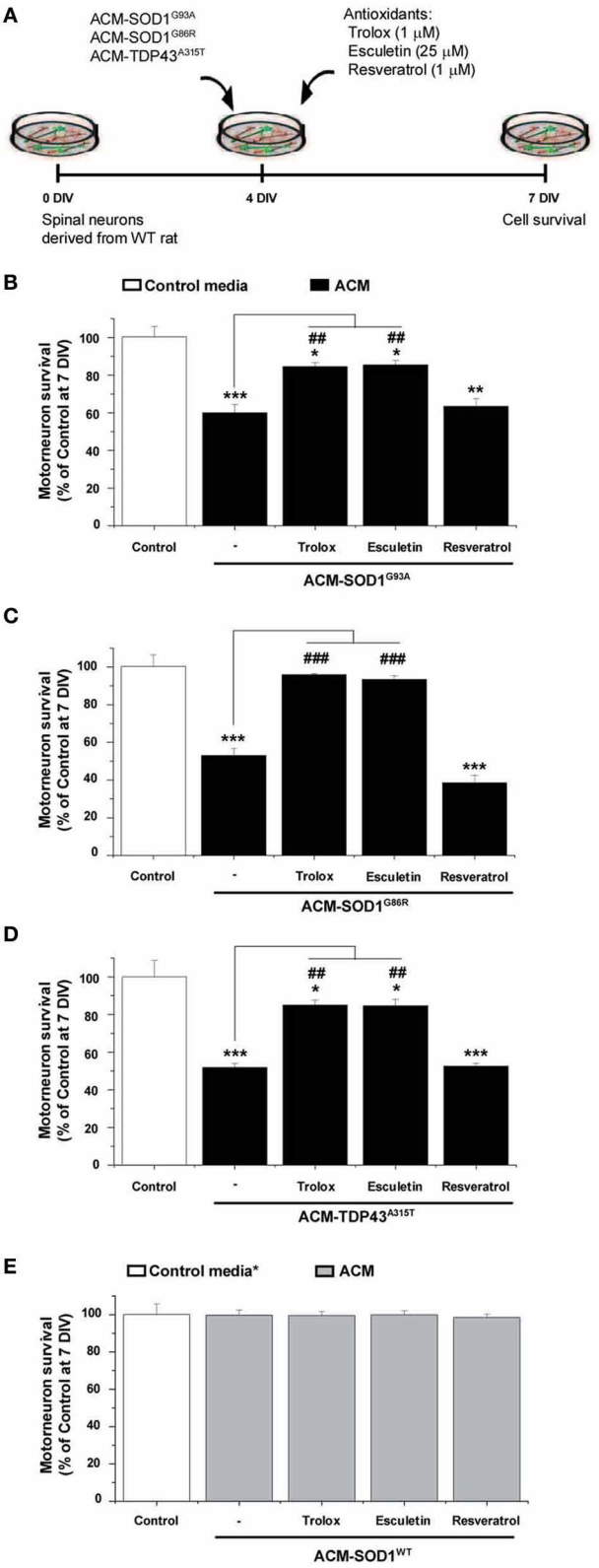


**FIGURE 3 | Anti-oxidants Trolox and esculetin prevent motoneurons death induced by ACM-SOD1$^{G93A}$, ACM-SOD1$^{G86R}$, and ACM-TDP43$^{A315T}$. (A)** Flow diagram of experiment. ACMs were applied
*(Continued)*

**FIGURE 3 | Continued**
chronically starting at 4 DIV alone, or together with the anti-oxidants Trolox (1 μM), esculetin (25 μM), or resveratrol (1 μM). Cell survival was assayed at 7 DIV. **(B–E)** Graphs showing the relative percentage of motoneurons that survived at 7 DIV, after being treated with the diverse anti-oxidants and ACM-SOD1$^{G93A}$ **(B)**, ACM-SOD1$^{G86R}$ **(C)**, ACM-TDP43$^{A315T}$ **(D)**, or ACM-SOD$^{WT}$ **(E)**, relative to motoneurons from sister cultures treated with control medium (indicated with*) or with only the ACM (indicated with$^{\#}$). Note that Trolox and esculetin prevented motoneuron death induced by the diverse ALS-causing ACMs, while resveratrol was ineffective. Note also that none of the compounds improved the survival of control neurons. Values represent means ± s.e.m. from at least 3 independent experiments, analyzed by One-Way ANOVA followed by a Tukey *post-hoc* test. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ relative to survival with control media at 7 DIV; $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$ compared to survival with ALS-causing ACM at 7 DIV. See **Supplementary Table 1**. for the effects of a wide range of concentrations of anti-oxidants on motoneuron survival.

### $Na_V$ CHANNEL BLOCKERS RESCUE MOTONEURON DEATH INDUCED BY ACM-SOD1$^{G93A}$, OR ACM-TDP43$^{A315T}$

To gain insights into the mechanism whereby astrocytes expressing diverse ALS-causing mutant proteins increase intracellular ROS/RNS levels and kill motoneurons, we argued that if the conditioned media from the SOD1 and TDP43 mutant astrocytes share a toxic factor(s), then this toxicity must converge to a common target. We recently reported that ACM-SOD1$^{G93A}$ rapidly (within 30 min) increases neuronal $Na_v$ channel mediated excitability; moreover, the application of several blockers of the $Na_v$ channel activity (including use of mexiletine, spermidine, and riluzole) reduced the hyperexcitability and prevented motoneuron death induced by ACM-SOD1$^{G93A}$ (Fritz et al., 2013). Hence we tested whether the $Na_v$ channel blocker mexiletine (an orally active lidocaine analog that is a local anesthetic and an antiarrhytmic drug that targets the "local anesthetic receptor site" of $Na_v$ channels (Ragsdale et al., 1994; Catterall et al., 2005; Olschewski et al., 2009), spermidine (a polyamine that affects the gating of varies ion channels and serves as an endogenous, activity-dependent $Na_v$ channel blocker (Williams, 1997; Fleidervish et al., 2008) and riluzole which has multiple effects, but at low concentrations (e.g., 100 nM in spinal cord cultures) suppresses neuronal excitability by affecting $Na_v$ channels (Kuo et al., 2005; Theiss et al., 2007; Bellingham, 2011; Fritz et al., 2013) can also rescue motoneuron cell death induced by ACM-SOD1$^{G86R}$ or ACM-TDP43$^{A315T}$ (**Figure 5A**). We used the same concentrations of these $Na_v$ channel blockers as described previously: 25 nM mexiletine, 10 μM spermidine and 100 nM riluzole (Fritz et al., 2013)—these doses were chosen based on earlier determinations that at these concentrations the $Na_v$ channel blockers reduced excitability and also effectively prevented motoneuron cell death induced by ACM-SOD1$^{G93A}$, without affecting overall motoneuron cell survival in control cultures (Fritz et al., 2013; and see also **Figures 5B,E**).

To directly compare the beneficial effect of co-application of mexiletine, spermidine or riluzole on the diverse toxic ACMs, we first analyzed motoneuronal survival when these sodium channel blockers were co-applied with ACM-SOD1$^{G93A}$ (**Figure 5B**). As expected, chronic application (from 4 to 7 DIV) of mexiletine together with ACM-SOD1$^{G93A}$ to spinal cord cultures prevented motoneuron death in those cultures (**Figure 5B**),

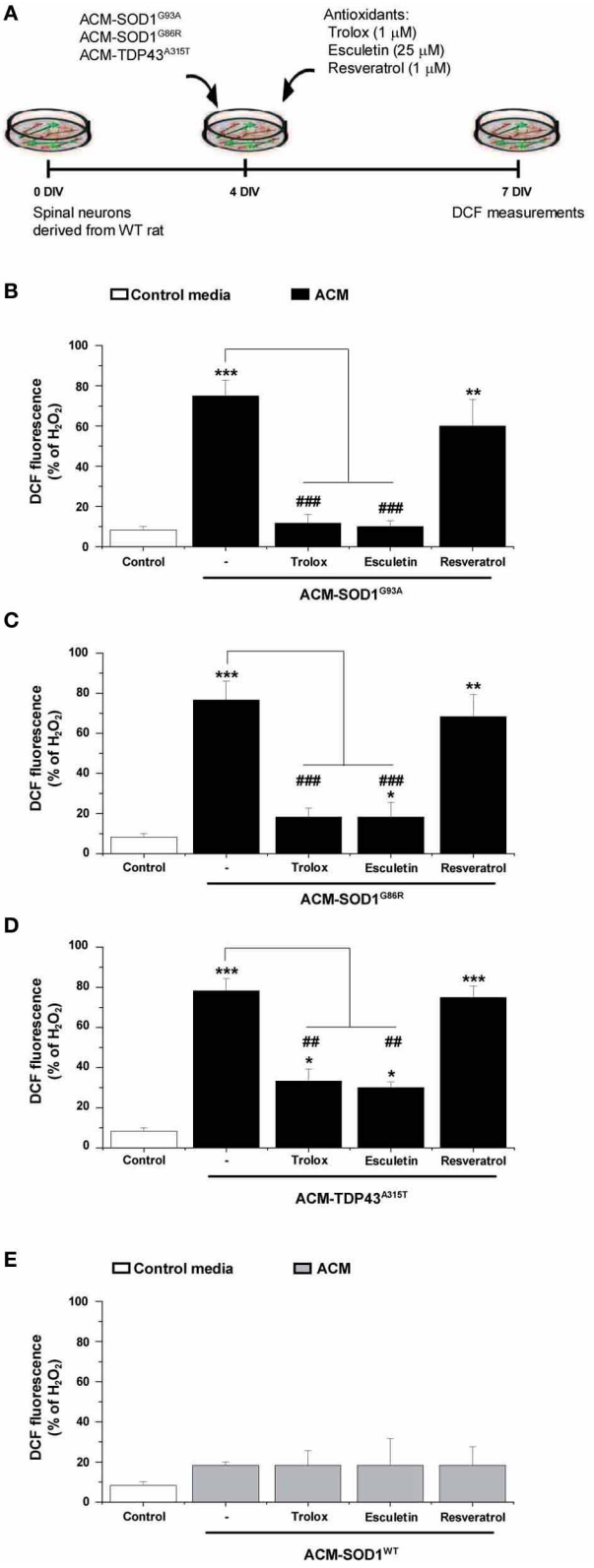


**FIGURE 4 | The effect of anti-oxidants on DCF fluorescence in spinal cord cultures exposed to the diverse ACMs. (A)** Flow diagram of experiment. ACMs were applied chronically starting at 4 DIV alone, or *(Continued)*

**FIGURE 4 | Continued**
together with the anti-oxidants Trolox (1 μM), esculetin (25 μM), or resveratrol (1 μM). At 7 DIV, cultures were incubated with the membrane permeable ROS/RNS probe CM-$H_2$DCF-DA and DCF fluorescence was measured 30 min later. **(B–E)** Graphs showing the percentage of DCF fluorescent cells after being treated with the diverse anti-oxidants and ACM-SOD1$^{G93A}$ **(B)**, ACM-SOD1$^{G86R}$ **(C)**, ACM-TDP43$^{A315T}$ **(D)**, or ACM-SOD$^{WT}$ **(E)**, In all experiment, $H_2O_2$ (200 μM for 20 min) served as positive control and to normalize the number of DCF-positive cells after ACM application. The graphs indicate statistics relative to motoneurons from sister cultures treated with control medium (indicated with*) or with only the ACM (indicated with$^{\#}$). Note that co-application of the diverse ACMs with Trolox or esculetin resulted in significant lower DCF fluorescent intensities, whereas resveratrol was not effective. Values represent means ± s.e.m. from at least 3 independent experiments, analyzed by One-Way ANOVA followed by a Tukey *post-hoc* test. $^{**}P < 0.01$, $^{***}P < 0.001$ relative to DCF fluorescence with control media at 7 DIV; $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$ compared to DCF fluorescence with ALS-causing ACM at 7 DIV.

as we have shown earlier (Fritz et al., 2013). Addition of mexiletine to spinal cord cultures exposed to ACM-SOD1$^{G86R}$ (**Figure 5C**) and ACM-TDP43$^{A315T}$ (**Figure 5D**) was also very beneficial and resulted in partially or complete prevention of motoneuron cell death, respectively. Spermidine (10 μM) also completely rescued motoneuron from death induced by ACM-SOD1$^{G93A}$ (**Figure 5B**), ACM-SOD1$^{G86R}$ (**Figure 5C**), or ACM-TDP43$^{A315T}$ (**Figure 5D**). And furthermore chronic co-incubation of spinal cord cultures with 100 nM riluzole and ACM-SOD1$^{G93A}$ (**Figure 5B**) or ACM-SOD1$^{G86R}$ (**Figure 5C**) completely prevented motoneuron death. The beneficial effects of this drug were less apparent on motoneurons incubated with ACM-TDP43$^{A315T}$ (**Figure 5D**).

### $Na_V$ CHANNEL BLOCKERS PREVENT INCREASES IN NITROXIDATIVE STRESS INDUCED BY ACM-SOD1$^{G93A}$, ACM-SOD1$^{G86R}$, OR ACM-TDP43$^{A315T}$

To determine whether the $Na_v$ channel-mediated hyperexcitability occurs upstream or downstream of the nitroxidative stress detected by DCF, we co-applied the diverse ACMs with $Na_v$ channel blockers to 4 DIV cultures and measured the intensity of DCF fluorescence (**Figure 6A**). The incubation time of the $Na_v$ channel blockers with the ACMs was chosen based on the maximum peak in the established time-course of DCF fluorescence for each conditioned media (see **Figure 2**): ACM-SOD1$^{G93A}$ and ACM-TDP43$^{A315T}$ were tested at 30 min, whereas ACM-SOD1$^{G86R}$ was tested at 60 min. We found that all three $Na_v$ channel blockers significantly reduced DCF fluorescence induced by the diverse ACMs, but to different degrees: mexiletine and spermidine reduced the DCF fluorescent signal induced by ACMs-SOD1$^{G93A}$ to a level similar to that achieved in untreated cultures, while riluzole decreased DCF fluorescence to below basal levels (**Figure 6B**). Similar effects were observed when these $Na_v$ channel blockers were co-applied with ACM-SOD1$^{G86R}$ (**Figure 6C**), ACM-TDP43$^{A315T}$ (**Figure 6D**), or ACM-SOD1$^{WT}$ (**Figure 6E**). Collectively, these data indicate that astrocytes expressing diverse ALS-causing mutant genes, including in the alleles SOD1 and TDP43, secrete soluble factors that kill wild-type motoneurons

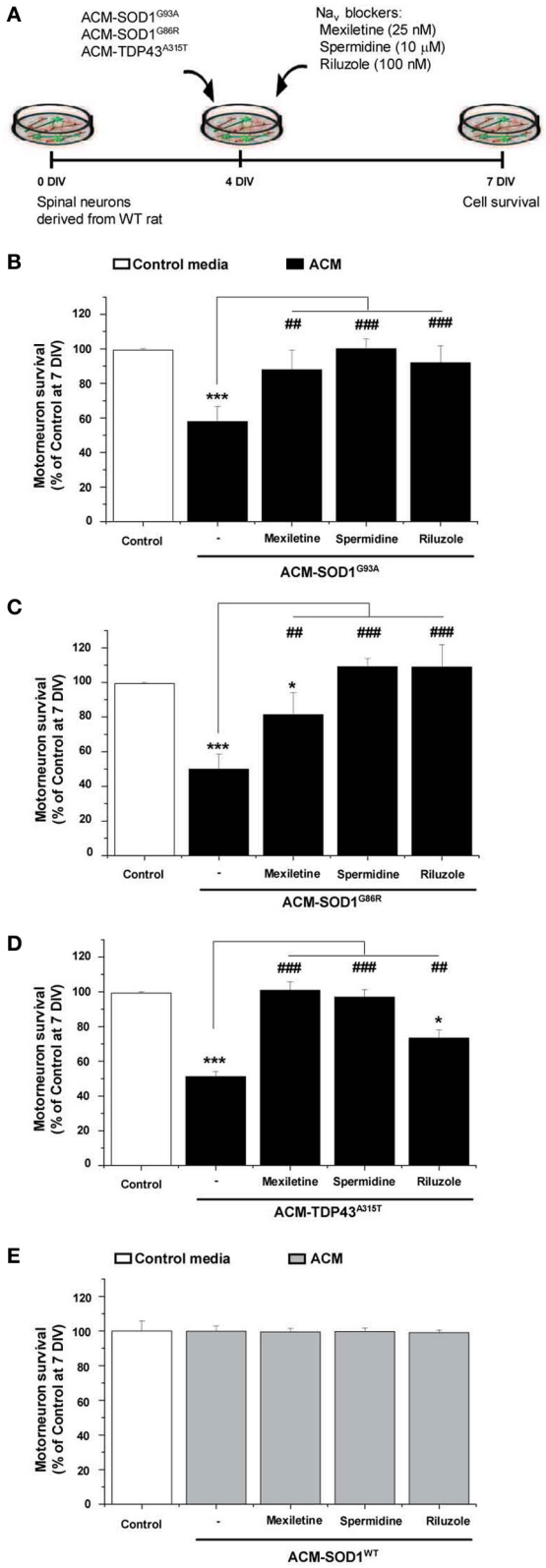


**FIGURE 5 | $Na_v$ channel blockers prevent motoneuron death induced by ACM-SOD1$^{G93A}$, ACM-SOD1$^{G86R}$, and ACM-TDP43$^{A315T}$. (A)** Flow diagram of experiment. ACMs were applied chronically starting at 4 DIV *(Continued)*

**FIGURE 5 | Continued**
alone, or together with the $Na_v$ channel blockers mexiletine (25 nM), spermidine (10 μM), or riluzole (100 nM). Cell survival was assayed at 7 DIV. **(B–E)** Graphs showing the relative percentage of motoneurons that survived at 7 DIV, after being treated with the diverse $Na_v$ channel blockers and ACM-SOD1$^{G93A}$ **(B)**, ACM-SOD1$^{G86R}$ **(C)**, ACM-TDP43$^{A315T}$ **(D)**, or ACM-SOD1$^{WT}$ **(E)**, relative to motoneurons from sister cultures treated with control medium (indicated with*) or with only the ACM (indicated with$^{\#}$). Values represent means ± s.e.m. from at least 3 independent experiments, analyzed by One-Way ANOVA followed by a Tukey *post-hoc* test. $^{*}P < 0.05$, $^{***}P < 0.001$ relative to survival with control media at 7 DIV; $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$ compared to survival with ALS-causing ACM at 7 DIV.

through a common pathway that involves increased nitroxidative stress, mediated at least in part by $Na_v$ channel activity.

## DISCUSSION

We provide evidence to demonstrate that astrocytes expressing mutations in SOD1 and TDP43 genes trigger motoneuron pathology and death through non-cell-autonomous processes mediated by the release of soluble toxic factor(s). We show that conditioned media derived from astrocytes that express ALS-causing mutations in SOD1 (SOD1$^{G93A}$ and SOD1$^{G86R}$) and TDP43 (TDP43$^{A315T}$) enhance ROS/RNS levels in neurons, and reduce motoneuron survival. We also document that application of anti-oxidants to spinal cord cultures prevents the increases in intracellular nitroxidative stress induced by diverse ACMs, and counteracts motoneuron death induced by the toxins in these media. Our finding that addition of $Na_v$ channel blockers to spinal cord cultures also strongly diminish ACM-induced enhancement of DCF fluorescence and motoneuron death further indicates that $Na_v$ channel-mediated excitability occurs upstream of nitroxidative stress and is required for the generation of a certain type of such stress. Collectively, these results indicate that astrocytes that express ALS-causing mutants in SOD1 and TDP43 contribute to ALS pathogenesis by activating a common molecular pathway that involves nitroxidative stress mediated, at least in part, by $Na_v$ channel activity.

### MUTATIONS IN SOD1 AND TDP43 CAUSE MOTONEURON PATHOLOGY AND DEATH BY NON-CELL-AUTONOMOUS PROCESSES

Ample evidence reveals that astrocytes expressing SOD1 mutants contribute to the pathogenesis of ALS: we and others have also demonstrated that such astrocytes release neurotoxic factors that kill primary motoneurons in culture (Nagai et al., 2007; Cassina et al., 2008; Castillo et al., 2013; Fritz et al., 2013). In agreement with these studies, we show here that ACM-SOD1$^{G93A}$ and ACM-SOD1$^{G86R}$ also extensively and selectively kill primary motoneurons. Additionally, we also examined whether mutants in TDP43 contribute to disease pathogenesis by non-cell-autonomous processes. As for ACM-SOD1$^{G93A}$ and ACM-SOD1$^{G86R}$, we document that exposure of wild-type spinal cord cultures to conditioned media generated by astrocytes derived from transgenic mice that express mutant TDP43$^{A315T}$ suffices to trigger robust death of motoneurons. These data indicate that astrocytes harboring mutant TDP43$^{A315T}$ release soluble toxic factor(s) into the media and thereby contribute to disease pathogenesis via non-cell-autonomous processes.

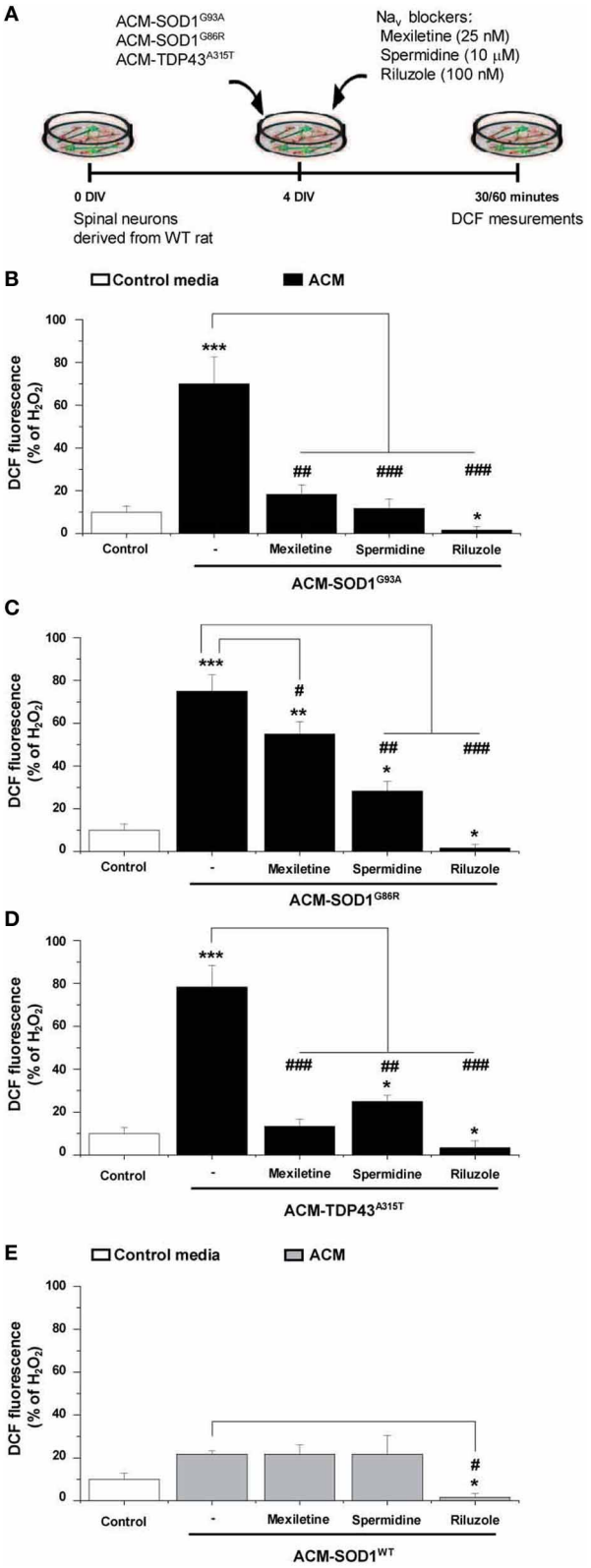


**FIGURE 6 | $Na_v$ channel blockers reduce DCF fluorescence induced by ACM-SOD1$^{G93A}$, ACM-SOD1$^{G86R}$, and ACM-TDP43$^{A315T}$. (A)** Flow diagram of experiment. Spinal cultures (4 DIV) were exposed for 30

*(Continued)*

**FIGURE 6 | Continued**
(ACM-SOD1$^{G93A}$ or ACM-TDP43$^{A315T}$) or 60 (ACM-SOD1$^{G86R}$) minutes (see **Figure 2** for peak of DCF fluorescence), alone or together with $Na_v$ channel blockers mexiletine (25 nM), spermidine (10 μM), or riluzole (100 nM). Next, cultures were incubated with the membrane permeable ROS/RNS probe CM-$H_2$DCF-DA and DCF fluorescence was measured 30 min later. **(B–E)** Graphs showing the percentage of DCF fluorescent cells after being treated with the diverse $Na_v$ channel blockers and $Na_v$ channel blockers and ACM-SOD1$^{G93A}$ **(B)**, ACM-SOD1$^{G86R}$ **(C)**, ACM-SOD$^{WT}$ **(D)**, ACM-TDP43$^{A315T}$ **(E)**. In all experiment, $H_2O_2$ (200 μM for 20 min) served as positive control and to normalize the number of DCF-positive cells after ACM application. The graphs indicate statistics relative to motoneurons from sister cultures treated with control medium (indicated with*) or with only the ACM (indicated with#). Values represent means ± s.e.m. from at least 3 independent experiments, analyzed by One-Way ANOVA followed by a Tukey *post-hoc* test. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ relative to DCF fluorescence with control media at 7 DIV; $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$ compared to DCF fluorescence with ALS-causing ACM at 7 DIV.

Our findings are in contrast to a recent publication by Serio et al. (2013), who showed that astrocytes expressing mutant TDP43$^{M337V}$ contribute to ALS pathology exclusively via cell-autonomous processes, and do not via non-cell-autonomous toxicity. These authors generated astrocytes from induced pluripotent stem cells (IPSCs) derived from a human ALS patient carrying the TDP43$^{M337V}$ mutation. They report that expression of the TDP43 mutant reduced survival of the human astrocytes, but that co-culturing layers of these TDP43$^{M337V}$ mutant containing astrocytes with wild-type motoneurons does not lead to motoneuron death. The reason(s) underlying the difference between their results and ours may be related to technical issues associated with the generation of astrocyte populations from human IPSCs, or to the particular TDP43 mutant involved. Additional experiments on the effects that diverse TDP43 mutants expressed in primary rodent astrocytes, as well as in human IPSCs cell lines, have on motoneurons are needed to resolve this discrepancy.

### EXPOSURE OF MOTONEURONS TO ACM FROM ASTROCYTES CARRYING MUTATIONS IN SOD1 AND TDP43 ACTIVATES A COMMON PATHOGENIC PATHWAY

Several hypotheses, involving the influence of nitroxidative stress, glutamate excitotoxicity, hyperexcitability, formation of high-molecular-weight aggregates, mitochondrial dysfunction, cytoskeletal disruption, and activation of cell death signals, have been proposed to explain the toxic effect of mutated SOD1 (Beckman et al., 2001; Cleveland and Rothstein, 2001; Bruijn et al., 2004; Pasinelli and Brown, 2006; Cozzolino et al., 2012; van Zundert et al., 2012). Our results point to critical roles for nitroxidatve stress as well as $Na_v$ channel activity in inducing motoneuron death. We and others have previously used cell cultures and slice preparations obtained from transgenic mice expressing mutations in SOD1 to report that $Na_v$ channel activity and/or excitability is increased in motoneurons (Kuo et al., 2005; van Zundert et al., 2008; Pambo-Pambo et al., 2009; Pieri et al., 2009; Schuster et al., 2011; Quinlan et al., 2011 and reviewed in ElBasiouny et al., 2010 and van Zundert et al., 2012). Moreover, using the ACM-SOD1$^{G93A}$ model system, we recently found that neuronal hyperexcitability, mediated at least in part through elevated $Na_v$ channel activity, is essential for inducing motoneuron death. And furthermore, the data presented here indicate that soluble factor(s) secreted by astrocytes carrying other ALS-causing mutations in SOD1 (SOD$^{G86R}$), and moreover in TDP43 (TDP43$^{A315T}$), kill motoneurons via activation of $Na_v$ channels.

Soluble mediator(s) secreted by astrocytes with mutations in SOD1 and TDP43 rapidly enhance neuronal nitroxidative stress, and lead to extensive motoneuron death within a matter of days. The rapid elevation in ROS/RNS levels observed in neurons exposed to the toxic ACMs could be due to diffusion of nitroxidative stress generated outside the cell into the motoneurons, or via intracellular *de novo* generation of ROS/RNS. We favor the second option, as the robust increase in the intensity of DCF fluorescence in motoneurons upon application of toxic ACMs is abolished when $Na_v$ channel blockers are applied to the spinal neuron cultures. Note, however, that it has been documented that DCF detects only particular types of reactive species, including hydrogen peroxide ($H_2O_2$; in combination with cellular peroxidases), hydroxyl radicals ($^{\bullet}OH$), and peroxynitrite ($ONOO^-$) (Estévez et al., 1999; Myhre et al., 2003; Gomes et al., 2005; Martin et al., 2007; Kalyanaraman et al., 2012), while it is seems insensitive to nitric oxide (NO) and superoxide ($O_2^{\bullet -}$) (Myhre et al., 2003). It is thus possible that neurons are targets of certain ROS/RNS, including external NO or $O_2^{\bullet -}$, that contribute to neuronal nitroxidative stress without being detected by the DCF probe.

With regard to this idea, amply evidence indicates that NO and $O_2^{\bullet -}$ are produced by ALS glial cells—as a result of either mitochondrial dysfunction, increased NADPH oxidase activity, or inflammation—and play a pivotal role in motoneuron pathology and death (Carter et al., 2009; Drechsel et al., 2012). For example, Barbeito and collaborators (Vargas et al., 2006; Cassina et al., 2008) report that 40% of motoneurons are lost when they are co-cultured on astrocytes carrying mutated SOD1$^{G93A}$ and that these astrocytes were found to produce excessive levels of NO and mitochondrial $O_2^{\bullet -}$. Moreover, cell death is abrogated when these astrocytes are pre-treated with either anti-oxidants capable of reducing $O_2^{\bullet -}$ production, or with inhibitors of NO synthase (NOS) (Vargas et al., 2006; Cassina et al., 2008). Additional studies also indicate that astrocytes and microglia that express mutated SOD1 can generate NO and NADPH oxidase (Nox)-derived ROS (Harraz et al., 2008; Marchetto et al., 2008). Also, *in vivo* production of harmful Nox-derived $O_2^{\bullet -}$ is reported in human SALS patients as well as in the SOD1$^{G93A}$ transgenic mouse model (Wu et al., 2006; Marden et al., 2007; Harraz et al., 2008). And finally, recent studies indicate that even extracellular mutant SOD1 and oxidized/misfolded SOD1 protein (both of which are likely to be secreted by cells) can activate microglia and induce nitroxidative stress (Urushitani et al., 2006; Ezzi et al., 2007; Zhao et al., 2010). Based on these studies, it is plausible that wild-type neurons are targets of external NO or $O_2^{\bullet -}$ that (1) accumulates in media conditioned by astrocytes carrying ALS mutants, or (2) is generated by surrounding wild-type glial cells (astrocytes and/or microglia) within the spinal cord culture via the action of factors present in the ACM (such as mutated or oxidized/misfolded SOD1 that is secreted by the ALS astrocytes). The presence of these RNS/ROS not necessarily affects externally positioned molecules located in or on the cell membranes of neurons: Thus, NO freely diffuses across cell membranes, and

whereas membranes are relatively impermeable to $O_2^{\bullet -}$, recent studies indicate that this oxidative specie could permeate across redoxosomal membranes through undefined chloride channels (Mumbengegwi et al., 2008; Carter et al., 2009). On the basis of our findings presented here and in previous studies, we support the view that induction of $Na_v$ channel activation by toxic ACMs is a central factor in initiating motoneuron death in ALS (van Zundert et al., 2012; Fritz et al., 2013). $Na_v$ channel activity can be increased directly by different oxidative species (Hammarström and Gage, 2000; Kassmann et al., 2008; Nani et al., 2010), but also indirectly by other factors such kinases (e.g., PKC) and the $Na_v$ channel β-subunits (Franceschetti et al., 2000; Goldin, 2003; Aman et al., 2009). It would be interesting to define by mutagenesis whether oxidation of particular amino acids residues (especially methionines and cysteines) influence $Na_v$ channel activation; these types of experiments, however, are beyond the scope of this study.

Here we present the following hypothesis to reconcile the studies by others and the data we have present here and previously (Fritz et al., 2013) showing that ACM-$SOD1^{G93A}$ rapidly (30 min) increases the frequency of calcium transients in cultured spinal cord neurons. Conditioned media derived from astrocytes carrying SOD1 and TDP43 mutants contains NO and/or leads to the generation of NO in spinal cord cultures. This NO in turn induces the activation of $Na_v$ channels, leading to excessive calcium influxes through activated $Ca_v$ channels; the calcium-mediated activation of mitochondrial respiratory chain complexes and/or Nox complexes will produce intracellular $O_2^{\bullet -}$ that can interact with NO to spontaneously generate $ONOO^-$ that hence can be detected by DCF and promotes intracellular damage, including protein nitration (Beckman et al., 2001; Cleveland and Rothstein, 2001; Martin et al., 2007). This hypothesis is supported by the fact that limiting the levels of $O_2^{\bullet -}$ by either reducing Nox activity (Wu et al., 2006; Harraz et al., 2007) or by application of compounds such as Trolox and esculetin (in this study), which have antioxidant and free radical scavenger capacities (Barber et al., 2009), prevented motoneuron death in diverse ALS models. The finding that the antioxidant resveratrol, unlike Trolox and esculetin, was unable to significantly prevent ACM-induced motoneuron death can be explained by the fact that this compound is a poor free radical scavenger compared to the other two compounds (Barber et al., 2009).

### DO SOLUBLE TOXIC FACTOR(S) RELEASED BY ALS ASTROCYTES SPECIFICALLY TARGET MOTONEURONS?

Data presented here and previously (Vargas et al., 2006; Di Giorgio et al., 2007; Nagai et al., 2007; Cassina et al., 2008; Marchetto et al., 2008; Castillo et al., 2013; Fritz et al., 2013) show compelling evidence that whereas astrocytes expressing ALS-linked mutations kill motoneurons in spinal cord cultures, they do not affect the survival of spinal cord interneurons. Data shown here and previously (Castillo et al., 2013; Fritz et al., 2013) indicate that independent of neuronal degeneration exposure of spinal cord cultures to toxic ACMs causes pathophysiological changes—including increases in nitroxidative stress, $Na_v$ channel activity, neuronal excitability, and intracellular calcium transients—in both motoneurons and interneurons. Moreover, detailed analyses in SOD1-ALS mice also indicate that several pathological changes can be detected in interneurons, and importantly, much before the onset of disease symptoms (Martin et al., 2007; van Zundert et al., 2008, 2012; Ramírez-Jarquín et al., 2013; Wootz et al., 2013). If both types of neurons are affected in ALS, why then are motoneurons killed and interneurons spared? Using electrophysiological recordings, we recently showed (Fritz et al., 2013) that application of ACM-$SOD1^{G93A}$ significantly increased the persistent sodium inward current ($PC_{Na}$) of neurons; this $PC_{Na}$ is mediated by $Na_v$ channels ($PC_{Na}$ is greater for $Nav_{1.1}$ and $Nav_{1.6}$ as compared to $Nav_{1.2}$ and $Nav_{1.3}$), and small increases can have important effects on neuronal excitability, leading to excessive influxes of calcium and sodium (ElBasiouny et al., 2010; van Zundert et al., 2012). Interestingly, we found that neurons with a large soma (>20 μm diameter) and expressing ≥5 primary dendrites (typical for motoneurons) displayed a larger $PC_{Na}$ compared to neurons with a smaller soma (<20 μm diameter) and expressing ≤4 primary dendrites (typical for interneurons). Moreover, application of ACM-$SOD1^{G93A}$ further increased the $PC_{Na}$ of neurons, especially of motoneurons. The expression of specific $Na_v$ channel subtypes, and subsequent activation of these channels by soluble toxic factor(s) released by ALS astrocytes, will thus induce larger $PC_{Na}$ and in turn cause more sustained calcium influxes in motoneurons compared to interneurons. In addition, because motoneurons have a limited cytosolic calcium-buffering capacity, excessive uptake of calcium by mitochondria would be an initial step in a cascade of events that impair mitochondrial function, thereby likely producing excessive $O_2^{\bullet -}$ that ultimately leads to motoneuron death (von Lewinski and Keller, 2005; Bento-Abreu et al., 2010; van Zundert et al., 2012).

In summary, we have elucidated critical roles for both nitroxidative stress and $Na_v$ channel activation in the death of motoneuron that is induced in diverse ALS models. Finally, because patients with SALS and FALS display a similar pathology, have comparable clinical symptoms that include hyperexcitability, and experience a beneficial effect of riluzole, it is possible that diverse $Na_v$ channel blockers will show benefit in both forms of ALS.

## AUTHOR CONTRIBUTIONS

Fabiola Rojas, Nicole Cortes, and Sebastian Abarzua performed MN survival experiments. Nicole Cortes and Fabiola Rojas performed DCF measurement. All (Fabiola Rojas, Nicole Cortes, Sebastian Abarzua, Agnieszka Dyrda, and Brigitte van Zundert) analyzed the data and wrote the manuscript.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fncel.2014.00024/abstract

**Supplementary Figure 1 | Exposure of primary spinal cord cultures to Control media or ACM-NT-Control media does not induce increases in intracellular DCF fluorescence. (A)** Flow diagram of experiment. Primary wild-type spinal cultures (4 DIV) were exposed for 0–120 min with the

different medias (solid lines), washed to remove the ACMs (filled circles), and loaded for 30 min with the fluorescent membrane permeable ROS/RNS probe CM-$H_2$DCF-DA (dotted lines). Next, cultures were washed and DCF measurements were immediately performed (filled triangles). **(B–D)** Cultures were exposed to three types of control media: "Control" media that was not conditioned by astrocytes **(B)**, "ACM-SOD1$^{WT}$" media derived from astrocytes that were harvested from transgenic mice carrying the non-pathological human wild-type SOD1 gene **(C)**, and "ACM-NT-Control" media derived from astrocytes that were harvested from littermate mice that were negative for the mutant SOD1 or TDP43 gene; here we show media from TDP43$^{A315T-/-}$ astrocytes. Similar results were obtained after application of media from SOD1$^{G93A-/-}$ or SOD1$^{G86R-/-}$ astrocytes **(D)**. The negatives of representative DCF fluorescent images (in which both motoneurons and interneurons are marked) and corresponding phase contrast images of spinal cord cultures photographed at the different indicated time points of media application are shown. Note that unlike $H_2O_2$ (200 μM for 20 min), which served as a positive control (shown in **B** in the right), none of the control media induced increases in intracellular DCF fluorescence. Scale bar, 200 μm.

**Supplementary Figure 2 | Time-course of motoneuron survival in primary spinal cord cultures exposed to ACM-SOD1$^{G93A}$.** Primary wild-type (WT) rat spinal cord cultures (4 DIV) were exposed for 2, 8, 24, and 72 h to ACM derived from transgenic mice overexpressing SOD1$^{G93A}$ (ACM-SOD1$^{G93A}$) and fixed immediately afterwards. Cell survival was assayed with immunocytochemistry. Graph showing the percentage of motoneurons that survived after treatment with ACMSOD1$^{G93A}$, relative to control medium and with media derived from transgenic mice carrying the non-pathological human wild-type SOD1 gene (ACM-SOD1$^{WT}$). Survival is shown relative to cultures treated with control media. Values represent mean ± s.e.m. from at least 3 independent experiments performed in duplicate, analyzed by One-Way ANOVA followed by a Tukey *post-hoc* test. $^{***}P < 0.001$ relative to control medium at 7 DIV.

**Supplementary Figure 3 | Time-course of DCF fluorescence in spinal cord cultures exposed to ACM-SOD1$^{G93A}$, ACM-SOD1$^{G86R}$, or ACM-TDP43$^{A315T}$.** **(A)** Flow diagram of experiment. Primary wild-type spinal cultures (4 DIV) were exposed for 0, 30, 60, 90, and 120 min and 6, 24, 48, and 72 h with the different ACMs (solid lines), washed to remove the ACMs (filled circles), and loaded for 30 min with the fluorescent membrane permeable ROS/RNS probe CM-$H_2$DCF-DA (dotted lines). Next, cultures were washed and DCF measurements were immediately performed (filled triangles). **(B–D)** Graphs showing the percentage of DCF fluorescent cells (including both motoneurons and interneurons) in cultures exposed to ACM-SOD1$^{G93A}$ **(B)**, ACM-SOD1$^{G86R}$ **(C)**, or ACM-TDP43$^{A315T}$ **(D)**. Results obtained with different controls are included in the graphs: ACMSOD1$^{WT}$ **(B)**, ACM-NT-Control from SOD1$^{G86R-/-}$astrocytes **(C),** and ACM-NT-Control from TDP43$^{A314T-/-}$ astrocytes **(D)**. DCF fluorescence is relative to cultures treated with $H_2O_2$, 200 μM for 20 min (100%). Note that application of toxic ACMs resulted in an initial peak of DCF fluorescence at 30–60 min that was followed by a decline in the DCF signal which then started to steadily increase from 24 h on. Values represent mean ± s.e.m. from at least 3 independent experiments performed in duplicate, analyzed by *t*-test. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. control.

**Supplementary Figure 4 | Anti-oxidant resveratrol is as effective as Trolox and esculetin in reducing DCF fluorescence induced by $H_2O_2$.** **(A)** Flow diagram of experiment. Primary spinal cord cultures (4 DIV) were exposed to 200 μM $H_2O_2$ for 20 min in the presence or absence of anti-oxidants. Cells were washed and then loaded with the membrane permeable ROS/RNS probe CM-$H_2$DCF-DA for 30 min. After washing, DCF fluorescence was measured immediately. **(B)** Graphs showing the percentage of DCF fluorescent cells (including motoneurons and interneurons) in cultures exposed to $H_2O_2$ and Trolox (1 μM), esculetin (25 μM), or resveratrol (1 μM). Values represent means ± s.e.m. from at least 3 independent experiments, analyzed by One-Way ANOVA followed by a Tukey *post-hoc* test. $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ relative to DCF fluorescence in $H_2O_2$ treated cultures.

**Supplementary Table 1 | Effects of a wide range of concentrations of antioxidants on the survival of control motoneurons and those exposed to ACM-SOD1$^{G93A}$.** **(A)** Analysis of survival of motoneurons at 7 DIV in primary cultures after incubation for 4 days with multiple doses of the anti-oxidants Trolox, esculetin, and resveratrol. **(B)** Analysis of surviving motoneurons at 7 DIV in primary cultures after incubation for 4 days with ACM-hSOD1$^{G93A}$ alone (no drug), or with ACM plus different doses of the anti-oxidants. In bold are indicated concentrations at which concentration the anti-oxidant was selected for further analysis on preventing the toxicity induced by the diverse ACMs; those results are displayed in **Figure 3**. Values represent mean ± s.e.m. from at least 3 independent experiments performed in duplicate, analyzed by *t*-test vs. control.

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# Phenotypic transition of microglia into astrocyte-like cells associated with disease onset in a model of inherited ALS

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Microglia and reactive astrocytes accumulate in the spinal cord of rats expressing the Amyotrophic lateral sclerosis (ALS)-linked SOD1$^{G93A}$ mutation. We previously reported that the rapid progression of paralysis in ALS rats is associated with the appearance of proliferative astrocyte-like cells that surround motor neurons. These cells, designated as Aberrant Astrocytes (AbA cells) because of their atypical astrocytic phenotype, exhibit high toxicity to motor neurons. However, the cellular origin of AbA cells remains unknown. Because AbA cells are labeled with the proliferation marker Ki67, we analyzed the phenotypic makers of proliferating glial cells that surround motor neurons by immunohistochemistry. The number of Ki67$^{+}$AbA cells sharply increased in symptomatic rats, displaying large cell bodies with processes embracing motor neurons. Most were co-labeled with astrocytic marker GFAP concurrently with the microglial markers Iba1 and CD163. Cultures of spinal cord prepared from symptomatic SOD1$^{G93A}$ rats yielded large numbers of microglia expressing Iba1, CD11b, and CD68. Cells sorted for CD11b expression by flow cytometry transformed into AbA cells within two weeks. During these two weeks, the expression of microglial markers largely disappeared, while GFAP and S100β expression increased. The phenotypic transition to AbA cells was stimulated by forskolin. These findings provide evidence for a subpopulation of proliferating microglial cells in SOD1$^{G93A}$ rats that undergo a phenotypic transition into AbA cells after onset of paralysis that may promote the fulminant disease progression. These cells could be a therapeutic target for slowing paralysis progression in ALS.

**Keywords: microglia, astrocytes, AbA cells, ALS, phenotypic transformation, neurodegeneration**

## INTRODUCTION

Amyotrophic lateral sclerosis (ALS) may be considered as a paradigm of neurodegeneration involving the progressive death of upper and lower motor neurons (Cleveland and Rothstein, 2001; Boillee et al., 2006a). A consistent neuropathological feature of ALS is the extensive inflammation around motor neurons and axonal degeneration, evidenced by the accumulation of reactive astrocytes, activated microglia and lymphocytes (Engelhardt et al., 1993; Barbeito et al., 2004; Ilieva et al., 2009; Graber et al., 2010). Neuroinflammation is evident in rodent models of inherited ALS overexpressing mutant Cu/Zn superoxide dismutase (SOD1) and in ALS human patients (Gurney et al., 1994; Bruijn et al., 1997; Howland et al., 2002; McGeer and McGeer, 2002; Appel, 2009). Several studies suggest that glial cells, including astrocytes and microglia, play a pathogenic role in ALS through promoting motor neuron death and spreading paralysis after disease onset (Hall et al., 1998; Barbeito et al., 2004; Sargsyan et al., 2005; Boillee et al., 2006b; Papadeas et al., 2011). These observations suggest that therapeutics targeting the inflammatory response of glial cells could slow ALS progression.

We have recently reported the isolation of astrocytes-like glial cells with an aberrant phenotype (AbA cells) from primary spinal cord cultures of symptomatic transgenic rats expressing the SOD1$^{G93A}$ mutation (Diaz-Amarilla et al., 2011). Isolation was based on AbA cell's marked proliferative capacity and lack of replicative senescence. These cells secrete soluble factors that induce motor neuron death with a higher potency than neonatal transgenic astrocytes. Aberrant astrocytes only appear after disease onset in SOD1$^{G93A}$ rats and are localized adjacent to motor neurons, suggesting a link between generation of AbA cells and the progression of paralysis. However, the origin of AbA cells remains unknown. Because AbA cells actively proliferate in the degenerating spinal cord, we hypothesized they could originate from glial progenitors with a high proliferative potential. Previous studies have identified phagocytic microglia as well as NG2$^{+}$ glial progenitors that proliferate during the active phase of motor neuron degeneration in ALS mice and rats (Magnus et al., 2008; Kang et al., 2010; Sanagi et al., 2010). Because we have previously shown that AbA cells do not express NG2 (Diaz-Amarilla et al., 2011), we examined whether AbA cells might be derived from

microglia proliferating adjacent to motoneurons. In this study, we have characterized both *in vivo* and *ex vivo* the phenotype of proliferating glial cells in symptomatic ALS rats and found evidence that neurotoxic AbA cells result from a phenotypic transition from activated microglial cells.

## MATERIALS AND METHODS

### ANIMALS

All procedures using laboratory animals were performed in accordance with the international guidelines for the use of live animals and were approved by the Institutional Animal Committee. Male hemizygous NTac:SD-TgN(SOD1$^{G93A}$)L26H rats (Taconic), originally developed by Howland et al. (2002), were bred locally by crossing with wild-type Sprague–Dawley female rats. Male SOD1$^{G93A}$ progenies were used for further breeding to maintain the line. Rats were housed in a centralized animal facility with a 12-h light-dark cycle with ad libitum access to food and water. Symptomatic disease onset was determined by periodic clinical examination for abnormal gait, typically expressed as subtle limping or dragging of one hind limb. Rats were killed when they reached the end stage of the disease. Both the onset of symptomatic disease (160–170 d) and lifespan (180–195 d) in our colony were delayed considerably compared with earlier reports (Howland et al., 2002). This study was carried out in strict accordance with the IIBCE Bioethics Committee's requirements and under the current ethical regulations of the Uruguayan Law N° 18.611 for animal experimentation that follows the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (USA). All surgery was performed under 90% ketamine – 10% xylazine anesthesia, and all efforts were made to minimize suffering, discomfort or stress.

### CELL CULTURE FROM END-STAGE SYMPTOMATIC SOD1$^{G93A}$ RATS

Microglia cells were obtained from adult spinal cord of symptomatic SOD1$^{G93A}$ rats (175 d) according to the procedures described by Diaz-Amarilla et al. (2011) with minor modifications. Adult age-matched non-Tg rats were used as controls. Briefly, animals were killed by deeply anesthesia, and spinal cord was dissected on ice. After the meninges were removed carefully, spinal cord was chopped finely and dissociated with 0.25% trypsin in calcium-free buffer for 5 min at 37°C. Trypsin treatment was stopped by adding DMEM/10% (vol/vol) FBS in the presence of 50 μg/mL DNaseI and mechanical disaggregation by repeated pipetting. The resulting extract was passed through an 80-μm mesh to eliminate tissue debris and then was spun. The pellet was resuspended in culture medium [DMEM/10% (vol/vol) FBS, Hepes (3.6 g/L), penicillin (100 IU/mL), and streptomycin (100 μg/mL)] and then was plated in a 25-cm$^2$ tissue culture flask. Because large amounts of fat hindered cell counting, the cells isolated from individual spinal cords were plated in individual culture flasks. Culture medium was removed after 24 h and then was replaced every 48 h.

### LEUCINE-METHYL ESTER TREATMENT

Leucine-Methyl Ester (Leu-OMe, Sigma) was prepared in DMEM, pH adjusted to 7.4. Cultures from transgenic symptomatic rats were treated 3 days after plated with 25 mM of Leu-OMe during 1 h. Then, the cells were washed three times with PBS and fixed with cold methanol during 5 min (n = 3).

### IMMUNOCYTOCHEMICAL STAINING OF CULTURED CELLS

Cultured cells were fixed with absolute methanol at -20°C for 5 min on ice and then were washed three times with 10 mM PBS (pH 7.4). Non-specific binding was blocked by incubating fixed cells with 5% BSA in PBS for 1 h at room temperature. Corresponding primary antibodies were diluted in blocking solution and incubated overnight at 4°C in a wet closed chamber. The primary antibodies for microglia recognition were rabbit anti-Iba1 (1:200, Abcam), rabbit anti-CD11b (1:200, Abcam), and mouse anti-CD68 (1:300, Abcam). The antibodies used for astrocyte recognition were mouse anti-GFAP (1:500, Sigma), rabbit anti-GFAP (1:500, Sigma), mouse anti-S100β(1:400, Sigma). After washing, sections were incubated in a 1,000-fold dilution of secondary antibodies conjugated to Alexa Fluor 488 and/or Alexa Fluor 546 (1:1000, Invitrogen). Antibodies were detected by confocal microscopy using a confocal Olympus FV300 microscope.

### ANALYSIS OF MICROGLIAL MARKERS EXPRESSION

After isolation of the symptomatic spinal cord, cells were plated in 35-mm dishes at 1.2 × 10$^4$ cells/cm$^2$. 7 days after plating, cells were fixed and stained with microglia specific markers as described above. The analysis was performed manually in using the cell counter tool of the Image J software. Values were expressed as a percentage ( ± SD) of the total number of DAPI$^+$ nuclei. Only healthy nuclei with clearly defined limiting membranes were counted. Cell counts were performed in duplicate.

### FORSKOLIN TREATMENT

After 20 days *in vitro*, 35 mm dishes were treated with 10 μM of forskolin (FSK; Sigma) during 3 h. Then, the cells were fixed using cold methanol and stained as described above.

### FLOW CYTOMETRIC ISOLATION

After 7 days *in vitro* the cells were incubated at 37°C with 0.25% trypsin without calcium. After 5 min, the cells were harvested in DMEM/10% (vol/vol) FBS and spun at 250 × *g* for 10 min. The resultant pellet was washed three times in PBS at 37°C. After that, the cells were re-suspended in blocking solution (PBS; 5% FBS, and 1% BSA). The cells were labeled at 4°C for 15 min with mouse anti-CD11b-FITC (1:100, Abcam) and sorted using the MoFlo$^{TM}$ XDP–Beckman Coulter. After sorting, the cells were re-plated in a 25 cm$^2$ bottle with DMEM/10% FBS.

### IMMUNOHISTOCHEMICAL STAINING OF RAT SPINAL CORDS

Animals were deeply anesthetized and transcardial perfusion was performed with 0.9% saline and 4% paraformaldehyde in 0.1 M PBS (pH 7.2–7.4) at a constant flow of 1 mL/min. Fixed spinal cord was removed, post-fixed by immersion for 24 h, and then transverse sectioned serially (30–50 μm) on a vibrating microtome. Serial sections were collected in 100 mM PBS for immunohistochemistry. After citrate antigen retrieval, free-floating sections were permeabilized for 15 min at room temperature with 0.1% Triton X-100 in PBS, passed through washing buffered solutions, blocked with 5% BSA:PBS for 1 h at room temperature, and incubated overnight at 4°C in a solution of 0.1% Triton X-100

and PBS containing the primary antibodies, mouse anti-Iba1 (1:200, Abcam), mouse anti CD163 (1:100, Serotec) for microglia recognition, and rabbit anti-GFAP (1:500, Sigma) for astrocyte recognition. A rabbit anti-Ki67 (1:400, Abcam) was used as a proliferation marker. The expression of nitrotyrosine was recognized with a mouse anti-$NO_2$-Tyr (1:300, Millipore) antibody. The immunoreactivity was completely blocked by pre-incubation of the primary antibody with free nitrotyrosine (10 mM). No antigen retrieval was needed to detect nitrotyrosine. After washing, sections were incubated in 1:1,000-diluted secondary antibodies conjugated to Alexa Fluor 488 and/or Alexa Fluor 546 (Invitrogen). Antibodies were detected by confocal microscopy using a confocal Olympus FV300 microscope.

### QUANTITATIVE ANALYSIS OF ABA CELLS IN THE DEGENERATING SPINAL CORD

The number of proliferating cells labeled with Ki67 and also stained for the astrocytic marker GFAP or microglial marker Iba1 was assessed by counting the respective double-positive cells in the gray matter of the lumbar cord of symptomatic or asymptomatic $SOD1^{G93A}$ rats. Quantification was performed only in the ventral horn, comparing the cell numbers in Rexed laminae VII and IX, which display low and high density of large motor neurons, respectively. Double-positive cells were counted in a perimeter of 100 μm, surrounding motor neurons. The analysis was performed manually in 10 histological sections per animal (two different rats for each condition) using the cell counter tool of the Image J software. Values were expressed as a ratio of double-positive cells per motor neuron. The number of double-positive cells labeled with Iba1 or CD163 and GFAP assessed by counting the respective double-positive cells in the gray matter of the lumbar cord of asymptomatic and symptomatic $SOD1^{G93A}$ rats. Quantification was performed only in the ventral horn, comparing the cell numbers in Rexed laminae VII and IX, which display low and high density of large motor neurons, respectively. Values were expressed as the number of double-positive cells per $mm^2$.

## RESULTS

### GLIAL PROLIFERATION ADJACENT TO DEGENERATING MOTOR NEURONS

The number of proliferating glia as identified by $Ki67^+$ nuclei sharply increased in the ventral horn of $SOD1^{G93A}$ symptomatic rats and accumulated near surviving motor neurons as well as at sites of apparent motor neuron loss (**Figure 1**). This population of $Ki67^+$ cells had large cell bodies (30–50 μm) with processes embracing motor neurons and expressed GFAP and Iba1 (**Figure 1A**, upper panels). Both Ki67/GFAP and Ki67/Iba1-positive cells displayed morphological features of AbA cells in culture (Diaz-Amarilla et al., 2011) and could be easily differentiated from astrocytes and microglia from non-transgenic or Tg asymptomatic rat's spinal cord. These large GFAP/Ki67 or Iba1/Ki67 cells were rarely observed in asymptomatic or non-transgenic rats (**Figure 1A**, lower panels). Due to the antigen retrieval procedure, motor neuron cell bodies were non-specifically labeled with Ki67 in all experimental conditions. The ratio of GFAP/Ki67 cells and Iba1/Ki67 cells to motor neurons in symptomatic rats was 2.7 and 2.9 respectively, whereas the ratio was $< 0.3$ in asymptomatic animals for both markers (**Figure 1B**).

### CO-EXPRESSION OF MICROGLIAL AND ASTROCYTIC MARKERS IN ABA CELLS

While astrocytes and microglial cells constituted two separate cell populations in asymptomatic rats, being typically detected by GFAP and Iba1 respectively, most AbA cells surrounding the motor neurons in symptomatic rats were surprisingly co-labeled with both markers as well as CD163 (**Figure 2A**). 70% of the GFAP-positive AbA cells in the ventral horn exhibited microglial markers, as compared as <1% in asymptomatic rats. The number of cells labeled with Iba1/GFAP or CD163/GFAP was similar (∼100 cells per $mm^2$) in symptomatic rats, suggesting the same cells expressed both microglial markers (**Figure 2B**). Detection of the microglial markers required the use of strong antigen retrieval methods, which made motor neuron to artifactually stained with CD163.

Both peri-neuronal AbA cells as well as motor neurons were strongly stained for nitrotyrosine in symptomatic rats, consistent with the production of peroxynitrite (**Figure 2C**). This immunoreactivity was completely blocked by preincubation of the primary antibody with free nitrotyrosine (data not shown).

### CHARACTERIZATION OF FIRST STAGES OF ABA CELLS *IN VITRO*

Because AbA cells can be cultured from symptomatic rats (Diaz-Amarilla et al., 2011), we determined the time course of microglial and astrocytic marker expression. Soon after primary cultures of spinal cord were established (DIV2–DIV7), most of the cells displayed the morphology of phagocytic microglia and were fluorescently labeled with the microglia markers CD11b, CD68, and Iba1 (**Figure 3A**). The detailed morphology of cultured cells closely corresponded to the typical features of microglial cells previously reported (Kreutzberg, 1996; **Figure 3B**). No immunoreactivity for GFAP or S100β was detected in cultures since the establishment of cultures and until 10–12 DIV (data not shown). Microglial cells were also analyzed by flow cytometry (FACS) using FITC-labeled CD11b antibodies. FACS analysis showed that $> 99\%$ of cells of the primary spinal cord culture of symptomatic rats belonged to the microglia lineage. The purity of this culture was also determined by counting $CD68^+$ and $Iba1^+$ cells and found to be $> 98\%$ (**Figure 3C**).

### PHENOTYPIC TRANSFORMATION OF MICROGLIA INTO ABA CELLS *IN VITRO*

To confirm that AbA cells were derived from microglia cell progenitors, $CD11b^+$ expressing cells were FACS-sorted and re-established in culture until DIV15. Sorted cells displayed the typical microglia morphology and phenotypic marker until DIV10. This population of cells progressively transitioned into astrocyte-like cells forming monolayers between DIV10 and DIV15, while losing the morphology and phenotypic markers of microglia (**Figure 4A**). At this time, transition zones in the border separating the microglial and the astrocyte-like cells expressed both microglial (Iba1 and CD11b) and astrocytic (GFAP) markers (**Figure 4B**). These transition zones were transient because most cells expressed S100β without microglial markers by DIV15. The treatment of

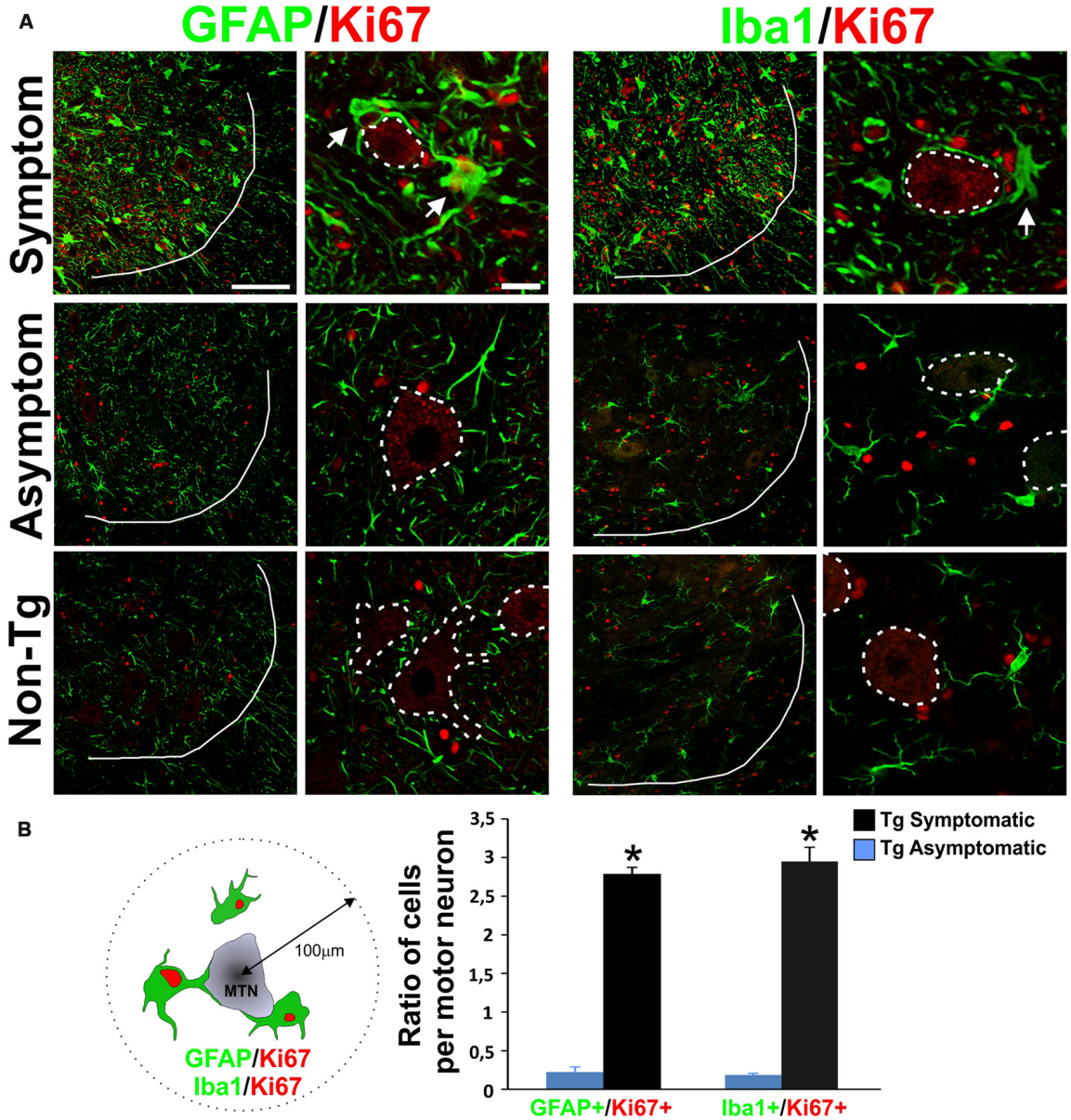


**FIGURE 1 | Characterization of proliferative astrocytes and microglia in the degenerating lumbar spinal cord**. **(A)** Photomicrographs showing GFAP/Ki67 and Iba1/Ki67 stained lumbar spinal cord sections measured in symptomatic (upper panels), asymptomatic (middle panels) $SOD1^{G93A}$ rats, and non-transgenic rats (lower panels). Low magnification panels show the notorious increase in the number of $Ki67^{+}$ red nuclei and the appearance of large $GFAP^{+}$ and $Iba1^{+}$ cells (green) in the symptomatic rats, as compared to low cell proliferation in asymptomatic or non-Tg rats. A white line indicates the border between white and grey matter. The high magnification images show that GFAP/Ki67 and Iba1/Ki67 cells are typically located around the motor neurons (indicated as dotted lines) and their processes closely embrace the neuronal cell body. The arrows indicate double-labeled cells. Note the unspecific binding of Ki67 antibody to motor neuron cell bodies. **(B)** Ratio of GFAP/Ki67 and Iba1/Ki67-positive cells per motor neuron in symptomatic and asymptomatic rats. The scheme at the left shows the methods used to count the cells in a perimeter of 100 μm around motor neurons. The counting was performed in 10 histological sections per animal. Two different rats were used for each condition. Data are shown as mean ± SD; $*P < 0.05$. Scale bars: 100 μm for low magnification panels; 20 μm for high magnification panels.

the cultures with 10 μM FSK, which is known to induce astrocytic processes growth and differentiation (Abe and Saito, 1997), accelerated the transition from microglia to AbA cells, while down-regulating the expression of Iba1 and promoting the growth of processes stained with GFAP (**Figure 4C**). To further confirm the microglia to astrocyte phenotypic switch, we treated the cultures with Leu-OMe, a compound used to selectively deplete microglia from primary cell cultures (Uliasz et al., 2012). **Figure 4D** shows that microglia from ALS rats were completely killed by 25 mM of Leu-OMe at DIV3, whereas there was no toxicity at DIV20 after the cells had undergone the phenotypic transition to an AbA morphology.

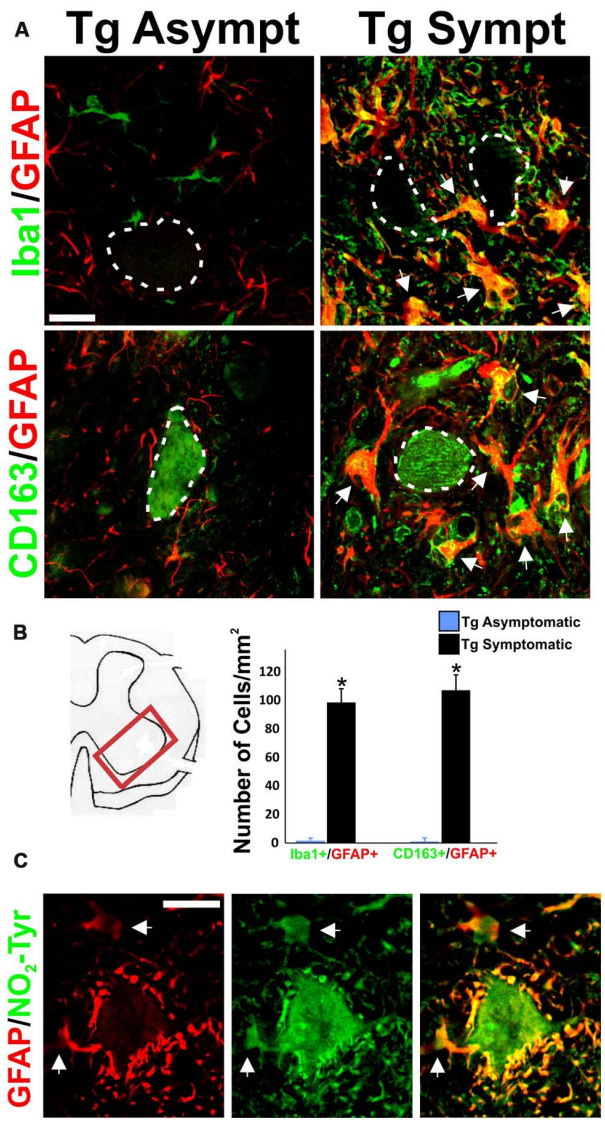


**FIGURE 2 | Concurrent expression of microglia and astrocytic markers in AbA cells.(A)** Representative image of GFAP (red), Iba1 (green), and CD163 (green) in lumbar spinal cord sections from transgenic asymptomatic (Tg-asymp) and symptomatic (Tg-sympt) $SOD1^{G93A}$ rats. Dotted lines indicate motor neuron cells bodies, which artifactually stained for CD163. Note that microglia and astrocytes in asymptomatic rat expressed GFAP and Iba1 in well-segregated cell populations. In comparison, most of the $Iba1^{+}$ and $CD163^{+}$ cells also expressed GFAP in symptomatic animals (arrows). **(B)** Quantification of cells expressing Iba1/GFAP and CD163/GFAP in the lumbar spinal cord. The counting was performed in 10 histological sections per animal. Two different rats were used for each condition. Data are shown as mean ± SD; $*P < 0.05$. **(C)** Representative confocal immunostaining against GFAP (red) and nitrotyrosine ($NO_2$–Tyr, green) in the ventral horn of a symptomatic rat showing the intense nitration of Perineuronal AbA cells (arrows). Scale bars: 20 μm.

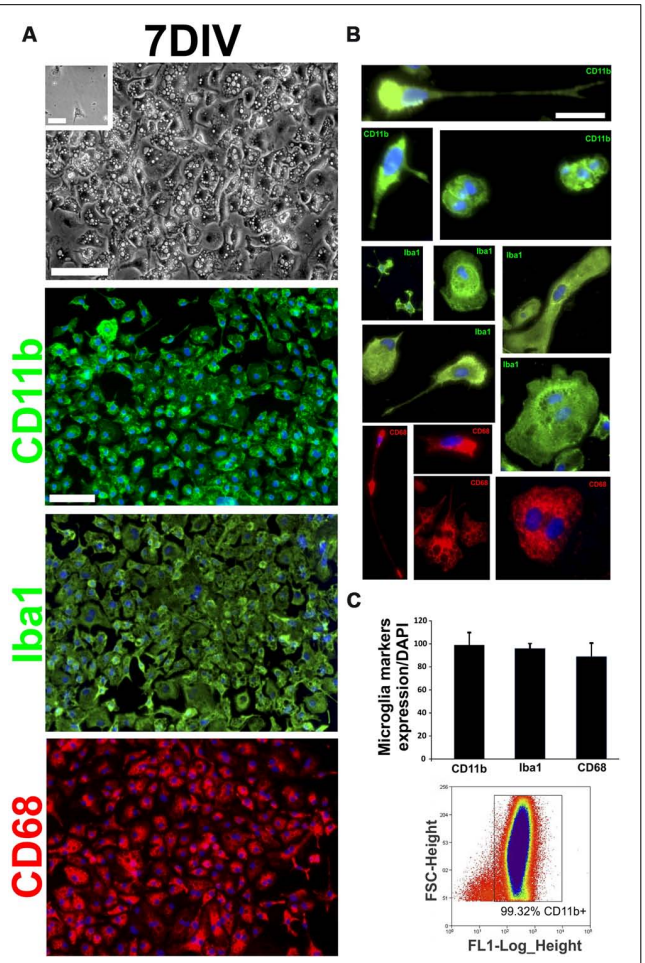


**FIGURE 3 | Characterization of first stages of AbA cells *in vitro*.** Primary cultures of the spinal cord of symptomatic rats were established as in (Diaz-Amarilla et al., 2011) and the cell phenotypes were analyzed on DIV7. **(A)** The upper panel shows a representative phase-contrast image of the culture at DIV7. The inset shows the same culture obtained from non-Tg littermates where no cells could be grown. The lower panels show confocal images of cells immunostained for typical microglia markers such as CD11b, CD68, and Iba1 at 7 DIV. Scale bars: 50 μm. **(B)** Characterization of different typical microglia phenotypes present at 7 DIV, cultured from Tg symptomatic rats. Scale bar: 25 μm. **(C)** Quantification of the microglia markers expression (CD11b, CD68, and Iba1) in culture at 7 DIV, counting cell positive for microglia marker respect to the total number of nuclei labeled with DAPI (upper panel). Typical data from flow cytometry showing that 99% of cells were $CD11b^{+}$. The data are representative of three independent experiments.

## DISCUSSION

Glial cells expressing mutant SOD1 are now well established to be toxic to motor neurons in rodent models as well as in ALS patients (Barbeito et al., 2004; Nagai et al., 2007; Yamanaka et al., 2008; Haidet-Phillips et al., 2011). AbA cells are the most toxic cells yet identified to motor neurons (Diaz-Amarilla et al., 2011). These distinctive glial cells are directly associated with motor neuron disease, because they actively proliferate after the onset of progressive paralysis and make intimate contact with degenerating motor neurons. By analyzing the population of proliferating $Ki67^{+}$ glial cells in the ventral horn of symptomatic SOD1 rats, we found that AbA cells most likely originate from microglia. Notably, purified microglia isolated from the spinal cord of symptomatic rats spontaneously transformed into AbA cells.

The extremely rapid progression of paralysis in $SOD1^{G93A}$ rats is characterized by prominent neuroinflammation associated with

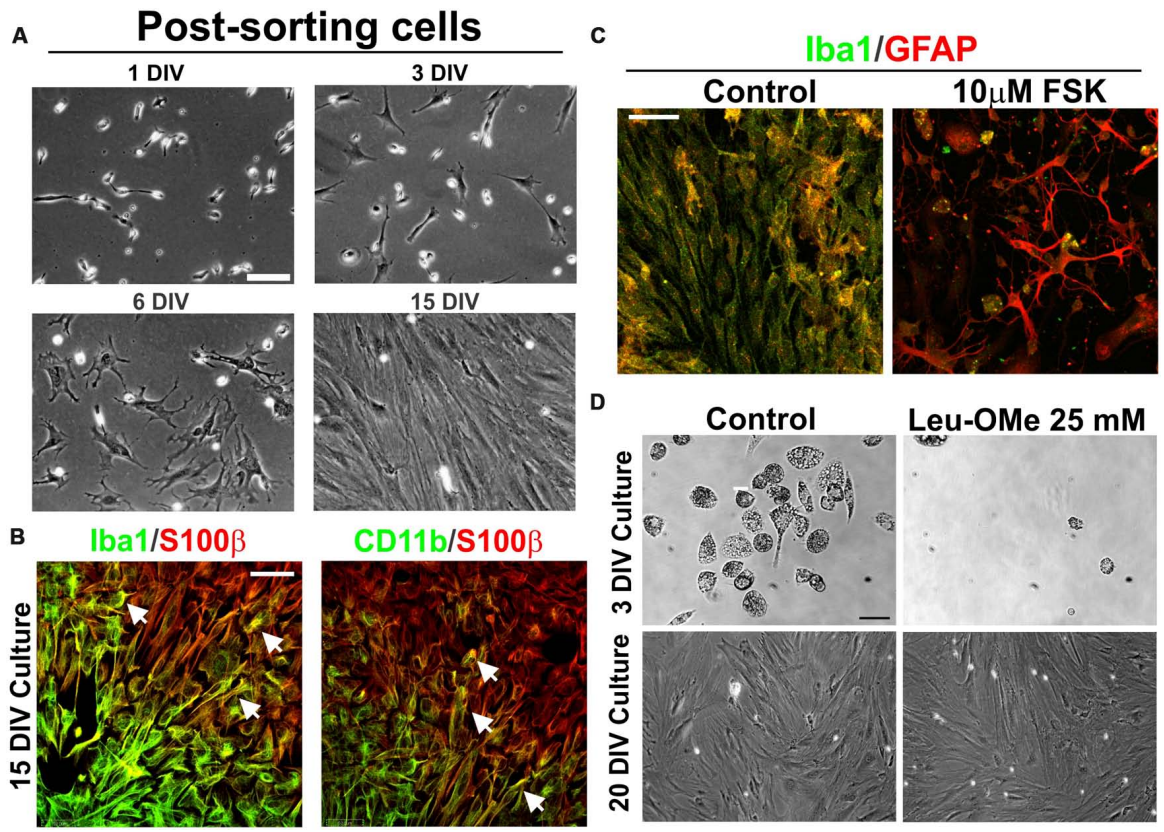


**FIGURE 4 | Phenotypic transition of microglia into AbA cells in culture.** **(A)** Microglia cell cultures (7 DIV) from symptomatic spinal cord were dissociated and stained with FITC-labeled CD11b. After FACS sorting, cells were re-plated and analyzed for phenotypic transition at DIV15. Scale bar: 50 μm. **(B)** Confocal image showing transition zones observed at 15 DIV. Note the segregation of astrocytic S100β staining with the microglia Iba1 or CD11b markers, which coincides with the morphological change of cells. The co-localization of astrocytic and microglia markers was found in a few cells in the border between these zones. Scale bar: 50 μm. **(C)** Treatment of cultures at DIV15 with foskolin (10 μM, 3 h) down regulated Iba1 expression and stimulated GFAP expression and growth of processes. Scale bars: 50 μm. **(D)** Differential effect of 25 μM of Leucine-Methyl Ester (Leu-OMe) before and after the phenotypic transition. Treatment with Leu-OMe was applied to cultures at DIV3 and DIV20 to assess the toxicity. Toxicity of Leu-OMe was restricted to the microglia phenotype. This experiment was repeated with three independent isolations of AbA cells. Scale bars: 20 μm

microglia activation (Sanagi et al., 2010; Philips and Robberecht, 2011). This inflammatory response is consistent with the sharp increase in cell proliferation others and we have observed with Ki67-staining in symptomatic animals (Schaefer et al., 2005; Pun et al., 2006). Previously, BrdU incorporation was used to demonstrate increased cell proliferation in ALS rats of NG2-positive glial progenitor cells (Magnus et al., 2008), which can potentially differentiate into astrocytes. Our results indicate that microglia more likely gave rise to AbA cells expressing astrocytic markers in regions adjacent to motor neurons.

The finding that spinal AbA cells co-express astrocytic GFAP with two different microglia markers Iba1 or CD163 was surprising because such a mixed phenotype is rarely observed. Co-expression of both markers has been observed in neoplastic glioblastoma multiforme cells (Huysentruyt et al., 2011; Persson and Englund, 2012). These human astroglial tumor cells seem to acquire phagocytic properties as a consequence of the dramatic inflammatory conditions occurring in tumors (Persson and Englund, 2012). Similarly, spinal AbA cells may originate from a phenotypic transition of inflammatory microglia into astrocytes-like cells in the degenerating cellular environment of the ventral horn. Other reports have shown aberrant features of microglial cells in symptomatic $SOD1^{G93A}$ rats, including formation of microglia clusters (Howland et al., 2002) and multi-nucleated giant cells (Fendrick et al., 2007). Based on the morphology, localization, high proliferation rate, and other phenotypic features, spinal AbA cells are distinct from previously described M1 or M2 microglia (Kigerl et al., 2009; Durafourt et al., 2012; Liao et al., 2012). It is uncertain whether the phenotypic transition is specific for mutant SOD1 microglia or might also be observed in other CNS insults where phagocytic microglia accumulate around dying neurons (Beyer et al., 2000; Sanagi et al., 2010; Neher et al., 2012). For example, ameboid microglia-like cells expressing markers of oligodendrocyte and monocyte lineages have been described in hippocampus following acute neuronal damage (Fiedorowicz et al., 2008). AbA cells represent a novel pathological phenotype of microglia/macrophages derived from their prominent plasticity following activation (Schwartz et al., 2006; Luo and Chen, 2012). This phenotypic transformation of microglia may explain why the ablation of dividing

astrocytes did not alter astrogliosis in SOD1 mice (Lepore et al., 2008).

Further evidence that AbA cells derive from activated microglia was provided in cell culture experiments showing that purified endogenous microglia can transition to astrocyte-like cells. Microglia expressing CD11b, Iba1, and CD68 represented more than 98% of cells isolated from the spinal cord of symptomatic $SOD1^{G93A}$ rats. Moreover, FACS sorting of these cells using CD11b antibodies resulted in typical microglia cultures that also transitioned to AbA cells after 2 weeks, showing not only that AbA cells are originated from microglia but also the phenotypic change occurs *in vitro*.

Because the phenotypic switch is associated to sustained cell proliferation and a critical cell density, we suggest that inflammatory mediators secreted by the activated microglia induced the transformation. Previous reports have shown the ability of microglia to be progenitors for different neural cell types, including astrocytes *in vitro* (Yokoyama et al., 2004). This property in turn may be related to their hematopoietic origin (Yokoyama et al., 2006). Compared to AbA cells growing in the degenerating spinal cord, the concurrent expression of astrocytic and microglia markers by cultured AbA cells is only transiently in restricted to the borders of the transition zones. Therefore, it appears that activated microglial cells in degenerating spinal cord are prone to transition to astrocyte-like phenotype both in culture conditions as well as *in vivo*, in the cellular niche surrounding the motor neurons. These cells may play a role in the killing and subsequent phagocytosis of motor neurons.

Spinal AbA cells also show a number of aberrant features including high levels of S100β and Cx43 expression (Diaz-Amarilla et al., 2011) that may be relevant for neuronal toxicity through secreted S100 proteins as well as extracellular ATP released through connexin hemichannels. Activation of the extracellular ATP receptor/channel P2X7 has recently been shown to induce motor neuron death and to induce the neurotoxic phenotypes of astrocytes in culture (Gandelman et al., 2010, 2013). Furthermore, endogenous nitration of tyrosine near the ATP binding pocket of HSP90 activates P2X7, which induces motor neuron apoptosis (Franco et al., 2013).

We also showed that spinal AbA cells are strongly stained for nitrotyrosine especially in the distal perineuronal processes, consistent with the production of peroxynitrite. Microglia bearing mutant SOD1 has been shown to damage motor neurons through the production of peroxynitrite (Thonhoff et al., 2012).Because their microglia origin, spinal AbA cells may have primed to generate superoxide and hence peroxynitrite on the exterior face of the plasma membrane in close proximity to motor neurons (Beckman et al., 2001).

## CONCLUSION

Taken together, the present work supports the concept that aberrant astrocyte-like cells in the degenerating spinal cord are derived from activated microglia that proliferate around damaged motor neurons. The present study provides evidence that microglia isolated from the spinal cord of ALS–SOD rats developing paralysis have the potential to transition into an astrocyte-like phenotype. The proliferating spinal AbA cells concurrently express markers of both microglia and astrocytes lineages. Because the appearance of AbA cells is closely associated to the progression of paralysis in $SOD1^{G93A}$ rats, a better understanding of the mediators inducing the phenotypic transition may provide another avenue of intervention to slow the progressive spread of disease in ALS patients.

## AUTHOR CONTRIBUTIONS

Emiliano Trias, Pablo Díaz-Amarilla, Silvia Olivera-Bravo, Joseph S. Beckman, and Luis Barbeito designed research; Emiliano Trias, Pablo Díaz-Amarilla, Silvia Olivera-Bravo, Eugenia Isasi, Derek A. Drechsel, Nathan Lopez, C. Samuel Bradford, and Kyle E. Ireton performed research; Emiliano Trias, Pablo Díaz-Amarilla, C. Samuel Bradford, Joseph S. Beckman, and Luis Barbeito analyzed data; and Emiliano Trias, Joseph S. Beckman, and Luis Barbeito wrote the paper.

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# Beta-2 microglobulin is important for disease progression in a murine model for amyotrophic lateral sclerosis

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Beta-2 microglobulin ($\beta 2m$) is an essential component of the major histocompatibility complex (MHC) class I proteins and in the nervous system $\beta 2m$ is predominantly expressed in motor neurons. As $\beta 2m$ can promote nerve regeneration, we investigated its potential role in amyotrophic lateral sclerosis (ALS) by investigating its expression level as well as the effect of genetically removing $\beta 2m$ on the disease process in mutant superoxide dismutase 1 ($SOD1^{G93A}$) mice, a model of ALS. We observed a strong upregulation of $\beta 2m$ in motor neurons during the disease process and ubiquitous removal of $\beta 2m$ dramatically shortens the disease duration indicating that $\beta 2m$ plays an essential and positive role during the disease process. We hypothesize that $\beta 2m$ contributes to plasticity that is essential for muscle reinnervation. Absence of this plasticity will lead to faster muscle denervation and counteracting this process could be a relevant therapeutic target.

**Keywords: beta-2 microglobulin, amyotrophic lateral sclerosis, motor neuron, neurodegeneration, motor neuron disease**

## INTRODUCTION

Major histocompatibility complex (MHC) class I proteins were originally discovered based on their critical role in the immune system, however immune-independent functions in the nervous system have recently been identified (Huh et al., 2000; Elmer and Mcallister, 2012). Beta-2 microglobulin ($\beta 2m$) is an essential component of MHC class I molecules, being required for expression of all MHC class I on the cell surface. Within the central nervous system $\beta 2m$ has a predominantly motor neuronal expression pattern (Linda et al., 1998, 1999; Thams et al., 2009). This protein is therefore a candidate to contribute to the selective vulnerability of such motor neurons during amyotrophic lateral sclerosis (ALS).

ALS is a progressive neurodegenerative disease, characterized by the selective loss of motor neurons and the denervation of muscle fibers, resulting in muscle weakness and paralysis. In Europe, the disease has an annual incidence of 2.7 cases per 100,000 people (Logroscino et al., 2010) and the disease duration post-diagnosis is 3–5 years. In 10% of patients, ALS is a familial disease and 20% of these familial ALS patients contain mutations in the gene encoding superoxide dismutase 1 (*SOD1*). Based on these mutations, ALS rodent models have been generated that predictably mimic the patient disease process (Julien and Kriz, 2006). As the disease progression is indistinguishable between familial and sporadic ALS, common disease mechanisms are predicted. One of these mechanisms is decreased (peripheral) neuronal plasticity that can influence the ability of neuronal networks to compensate for a loss of (motor) neurons in the network. $\beta 2m$ is expressed in motor neurons in the lumbar spinal cord (Linda et al., 1999) as well as in motor axons (Thams et al., 2009). Additionally, $\beta 2m$ promotes recovery after axotomy (Linda et al., 1998; Oliveira et al., 2004) and sciatic nerve crush (Oliveira et al., 2004), which implies that it may be of importance in ALS too.

In this study, we investigated the role of $\beta 2m$ in ALS mice. To this end, we assessed the gene expression of $\beta 2m$ and interbred mice genetically lacking $\beta 2m$ with $SOD1^{G93A}$ mice and assessed survival and disease pathology.

## MATERIALS AND METHODS

### ANIMAL EXPERIMENTS

Mice overexpressing human wild-type *SOD1* ($SOD1^{WT}$) or human $SOD1^{G93A}$ and $\beta 2m$ knockout mice were purchased from The Jackson Laboratories (Bar Harbor, USA) and maintained on a C57BL/6 background. The $SOD1^{G93A}$ and $\beta 2m$ knockout were interbred allowing for approx. 50% of the mice to be littermate controlled in this study. Chow and water were provided *ad libitum*

and mice were housed in the specific pathogen free animal facility of the KU Leuven under standard conditions according to the guidelines of the KU Leuven. End stage was defined as the age at which mice could no longer right themselves within 30 s when placed on their back. End stage is used as a measurement of survival and is the condition at which mice are euthanized to prevent further suffering. Disease onset was defined as the age at which mouse weight dropped below 90% of the average day 90–105 weight. The animal caretakers and scientists were blinded to the genotypes of the mice when assessing "end stage". All animal experiments were performed with the approval of the Animal Ethical Committee of KU Leuven (020/2010).

#### LASER DISSECTION MICROSCOPY

Murine spinal cords were snap-frozen in Tissue-Tec (Sakura Finetek Europe, Alphen aan de Rijn, The Netherlands) to make cryostat sections of 20-μm thickness. Then, cresyl violet–stained motor neurons, located in the ventral horn of the lumbar spinal cord, were collected on membrane slides 1.0 PEN (Carl Zeiss AG, Oberkochen, Germany), using dissection by a laser-dissection microscope (Carl Zeiss AG) and capturing in Adhesive Cap 500 opaque (Carl Zeiss AG). Only motor neurons in which the nucleus was visible and with soma area > 250 $\mu m^2$, were collected. At least 1,500 motor neurons were dissected for each animal.

#### QUANTITATIVE PCR

Isolation of mRNA was performed using the TriPure (Roche, Basel, Switzerland) method and the RNeasy kit (Qiagen, Venlo, The Netherlands). Reverse transcriptase polymerase chain reaction (PCR) used random hexamers (Life Technologies, Carlsbad, USA) and Moloney Murine Leukemia Virus Reverse Transcriptase (MMLV RT; Invitrogen, Carlsbad, USA). Quantitative PCR (qPCR) was performed with the StepOnePlus (Life Technologies) and TaqMan Universal PCR Master Mix (Life Technologies). Gene expression assays were purchased from Life Technologies and IDT DNA (Coralville, USA): *gapdh* (Mm.PT.39a.1), *β2m* (Mm00437762_m1) and *cd8b1* (Mm.PT49a.10182911). For this analysis, presymptomatic tissue was collected at 90 days of age and symptomatic at 120 days of age. The scientist performing the qPCR was blinded to the genotypes of the samples.

#### NISSL STAINING

To visualize neurons, Nissl staining was performed on 4% formaldehyde fixed spinal cords sections. Sections were briefly immersed in a cresyl violet solution and subsequently in a 70% ethanol with 10% acetic acid. Slides were dehydrated by an increased ethanol concentration series and mounted with PerTex® (Histolab AB, Goteborg, Sweden). Images were collected by Zeiss Axio Imager M1 microscope (Carl Zeiss AG) with AxioCam Mrc5 camera (Carl Zeiss AG). The number of (motor) neurons was quantified by measurement of the soma area as visualized by cresyl violet staining in ImageJ (National Institute of Health) on multiple 40 μm thick sections in the ventral horn of the lumbar spinal cord. Characterisation of motor neurons occurred as previously (Fischer et al., 2004) of at least 5–10 ventral horns of the lumbar spinal cord of 2–4 mice per group. The scientist performing the Nissl staining and neuron quantification was blinded to the genotypes of the samples.

#### IMMUNOHISTOCHEMISTRY

Mice were transcardially perfused with phosphate buffered saline (PBS) and subsequently with 4% formaldehyde. Spinal cords were post-fixed with 4% formaldehyde overnight at 4°C and transferred to 30% sucrose for an additional night. After snap freezing, tissue was sectioned by cryostat at 40 μm thickness and stained with a polyclonal antibody directed against ubiquitin (Dako, Glostrup, Denmark). Images were collected by Zeiss Axio Imager M1 microscope (Carl Zeiss AG) with AxioCam Mrc5 camera (Carl Zeiss AG). Ubiquitin immunopositive aggregates were counted per ventral horn using ImageJ of 2–6 ventral horns of the lumbar spinal cord of 2–4 mice per group and presented as the average of the number of aggregates per ventral horn. The scientist performing the immunohistochemistry and aggregate quantification was blinded to the genotypes of the samples.

#### STATISTICAL ANALYSIS

Analysis was performed with the statistical software package Prism Origin (GraphPad Software, La Jolla, USA). Survival was analyzed by Log-Rank testing. Differences between two groups were analyzed using a Student's *t*-test. Differences between more than two groups were analyzed by ANOVA with Bonferroni correction for multiple testing. Significance was assumed at $p < 0.05$. Error bars represent the standard deviation.

## RESULTS

To assess the potential for *β2m* to have a functional role in ALS pathogenesis, we assessed gene expression in the spinal cords of non-transgenic, $SOD1^{WT}$ and $SOD1^{G93A}$ mice. We observed a strong increase in *β2m* gene expression during disease progression in $SOD1^{G93A}$ mice (**Figure 1A**). This increase is at least partly due to the increased neuron-specific gene expression of *β2m*, as a greater level of upregulation (10-fold) was identified by qPCR on neurons from $SOD1^{G93A}$ mice compared to neurons from $SOD1^{WT}$ mice isolated by laser dissection microscopy (**Figure 1B**). As we do not observe an increase of $CD8^+$ T cells in the spinal cord of end stage $SOD1^{G93A}$ mice, as assessed by the gene expression analysis of *CD8b1* (**Figure 1C**), these data indicate potential for a neuronal role for *β2m* in ALS.

To determine whether *β2m* has an effect in ALS, we interbred $\beta2m^{-/-}$ mice with $SOD1^{G93A}$ mice and assessed disease progression in $\beta2m^{-/-}$ $SOD1^{G93A}$, $\beta2m^{+/-}$ $SOD1^{G93A}$ and $\beta2m^{+/+}$ $SOD1^{G93A}$ mice. The survival of $\beta2m^{+/-}$ $SOD1^{G93A}$ mice did not differ from $\beta2m^{+/+}$ $SOD1^{G93A}$ littermates (data not shown). The complete genetic ablation of *β2m* did not affect onset of disease (data not shown), but significantly decreased average survival of $SOD1^{G93A}$ mice by 8.9 days (**Figure 2A**) and reduced disease duration by approximately 50% (**Figure 2B**). The decrease of survival in $\beta2m^{-/-}$ $SOD1^{G93A}$ mice demonstrated a protective role for *β2m* in ALS mice.

To assess whether genetic ablation of *β2m* alters pathology of $SOD1^{G93A}$ mice, we analyzed pathology in the spinal cords of end stage mice. Decreased numbers of motor neurons were

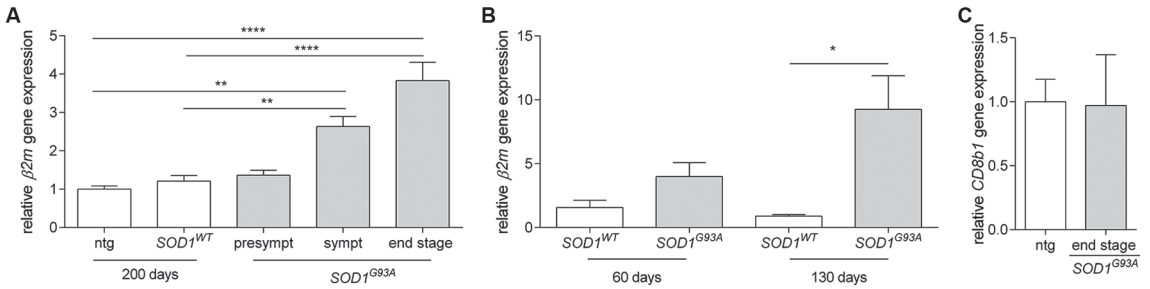


**FIGURE 1 | Increased *β2m* gene expression in ALS mice**. **(A)** Relative *β2m* gene expression in spinal cord of non-transgenic (ntg, $n = 6$) and $SOD1^{WT}$ controls ($n = 6$) compared to presymtomatic (presympt, $n = 6$), symptomatic (sympt, $n = 6$) and end stage ($n = 6$) $SOD1^{G93A}$ mice (ANOVA, Bonferroni post hoc). **(B)** Relative *β2m* gene expression in neurons isolated by laser dissection microscopy from the spinal cord of $SOD1^{WT}$ controls at 60 days ($n = 2$) and 130 days of age ($n = 3$) compared to neurons from $SOD1^{G93A}$ mice at 60 days ($n = 3$) and 130 days of age ($n = 3$; Student's *t*-test). **(C)** Relative *CD8b1* gene expression in the spinal cord of 150 day old non-transgenic mice (ntg, $n = 5$) and end stage ($n = 5$) $SOD1^{G93A}$ mice. $*p < 0.05$, $**p < 0.01$, $****p < 0.0001$.

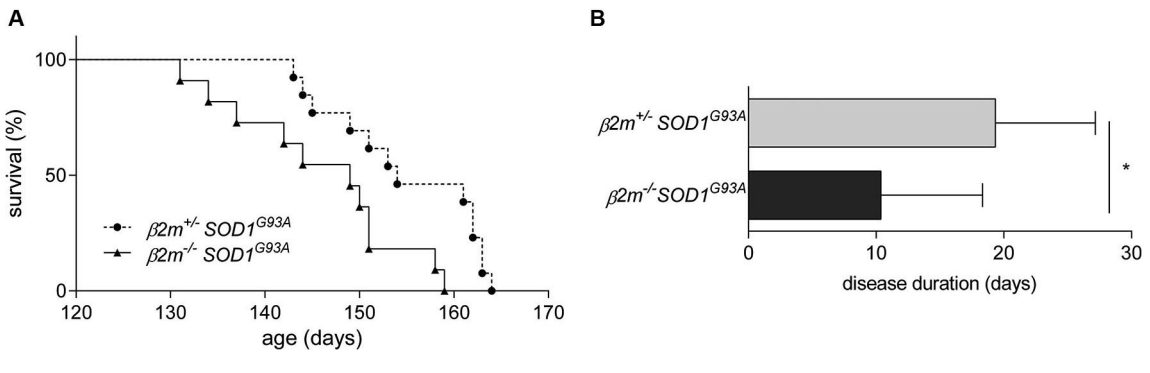


**FIGURE 2 | Decreased survival in *β2m* knockout ALS mice**. **(A)** Survival analysis of $\beta2m^{+/-}$ $SOD1^{G93A}$ ($n = 13$, 154.9 ± 7.7 days) and $\beta2m^{-/-}$ $SOD1^{G93A}$ mice ($n = 11$, 146.0 ± 8.8 days; Log-Rank $p = 0.009$). **(B)** Disease duration of $\beta2m^{+/-}$ $SOD1^{G93A}$ ($n = 9$) and $\beta2m^{-/-}$ $SOD1^{G93A}$ mice ($n = 8$). $*p < 0.05$.

observed in the end stage spinal cord of $SOD1^{G93A}$ and $\beta2m^{-/-}$ $SOD1^{G93A}$ mice (**Figures 3A–C**, quantified in **Figure 3D**), as were increased ubiquitin-positive aggregates (**Figures 3E–G**, quantified in **Figure 3H**). No differences were observed between the end stage pathology of $SOD1^{G93A}$ and $\beta2m^{-/-}$ $SOD1^{G93A}$ mice for motor neurons (**Figures 3B, C**) or ubiquitin immunoreactivity (**Figures 3F, G**). This shows that end stage $\beta2m^{-/-}$ $SOD1^{G93A}$ mice show the same extent of motor neuron loss and aggregate formation as end stage $SOD1^{G93A}$ mice, although disease progression is faster.

## DISCUSSION

Here we show that *β2m* is important in ALS mouse survival and that it is upregulated during disease in the spinal cord and by motor neurons. Upregulation of *β2m* in neuronal tissues has been reported previously when comparing spinal cord (Edstrom et al., 2004) and brain (VanGuilder Starkey et al., 2012) expression of aged rats to adult controls and in the spinal cord of axotomised rats (Maehlen et al., 1988; Olsson et al., 1989; Linda et al., 1998), which may suggest that stressed neurons increase *β2m* gene expression to increase plasticity. This concept fits well with reports of the role of *β2m* in neurons during development and plasticity (Huh et al., 2000; Bilousova et al., 2012), of the hippocampus and visual system (Huh et al., 2000) but not of the cerebellum (Letellier et al., 2008), and the delayed or impaired recovery of *β2m* knockout mice post axotomy (Linda et al., 1998; Oliveira et al., 2004) and sciatic nerve crush (Oliveira et al., 2004).

Impaired (peripheral) plasticity by *β2m* knockout may explain the decrease in survival detected in ALS mice in this study, as increased plasticity is protective in ALS mice and rats (Van Hoecke et al., 2012). A number of plasticity-promoting genetic or pharmacological strategies have proven successful in the past in ALS models, such as EphA4 knockdown and inhibition (Van Hoecke et al., 2012), and vascular endothelial growth factor (VEGF) administration in ALS rodents (Storkebaum et al., 2005).

With the use of a ubiquitous *β2m* knockout mouse we cannot exclude that the decrease of survival of ALS mice lacking *β2m* may be due to the effect of removing *β2m* in the immune system. *β2m* is necessary for the differentiation of $CD8^+$ T cells and natural killer T (NKT) cells (Koller et al., 1990). The role of these cell types is not yet fully understood in ALS, as varying results are obtained for ALS mouse survival when mature lymphocytes are not present (Beers et al., 2008; Tada et al., 2011). Additionally, NKT cells may be associated to ALS disease pathology as impairments in NKT cells are reported in ALS mice (Finkelstein et al., 2011). That being said, qPCR analysis of $CD8^+$ T cells does not suggest a role for

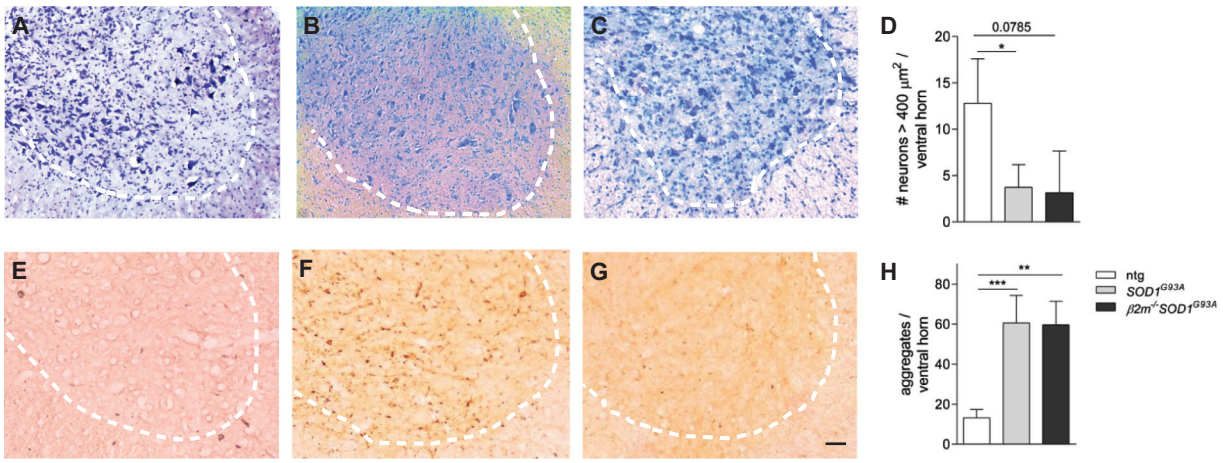


**FIGURE 3 | Unaltered end stage pathology in *β2m*-deficient *SOD1*$^{G93A}$ mice**. Ventral horns of spinal cord sections of non-transgenic (ntg, $n$ = 4; **A** and **E**) and *SOD1*$^{G93A}$ ($n$ = 4; **B** and **F**) and *β2m*$^{-/-}$ *SOD1*$^{G93A}$ ($n$ = 2; **C** and **G**) *SOD1*$^{G93A}$ mice. Nissl staining was used to visualize the (motor) neurons **(A–C)**. The number of > 250 µm$^2$ neurons and > 400 µm$^2$ motor neurons per ventral horn are quantified in **(D)** Ubiquitin immunoreactivity in spinal cord sections of non-transgenic, *SOD1*$^{G93A}$ and *β2m*$^{-/-}$ *SOD1*$^{G93A}$ mice are quantified in **(H)** Dashed lines delineate the ventral horn. Scale bar = 100 µm. *$p$ < 0.05, **$p$ < 0.01, ***$p$ < 0.001.

these cells as *CD8b1* gene expression is not increased in ALS spinal cords. Additionally, a role for CD8$^+$ T cells may be predicted to be detrimental in contrast to our data demonstrating a detrimental role for *β2m* upon removal in ALS mice. Interestingly, *β2m* has been assessed previously as a biomarker in cerebrospinal fluid or venous blood samples from ALS patients, but with variable results (Brettschneider et al., 2008; Mitchell et al., 2009; Baciu et al., 2012).

The mechanism to which *β2m* contributes to neuronal plasticity is not fully understood, though it is proposed that it may be through paired-immunoglobulin like receptor-B (PirB) (VanGuilder Starkey et al., 2012). This receptor is located on axons, dendrites and neuronal somata (VanGuilder Starkey et al., 2012) and could thus easily facilitate plasticity. Additionally, *β2m* is localized at synapses and in synaptosomes (Shatz, 2009). Alternatively, *β2m* and PirB are associated with decreased plasticity and recovery in other neurodegenerative conditions such as stroke (Adelson et al., 2012), experimental autoimmune encephalomyelitis (EAE; Denic et al., 2012) and ischemia (Wang et al., 2012). These paradigms are largely affected by the immune system and the beneficial effect of *β2m* or PirB in these models may be due to the role of the immune system. This notion is supported by work by Linker et al. that show that reconstitution of CD8$^+$ cells in *β2m* knockout mice delays the effect of EAE compared to *β2m* knockout littermates (Linker et al., 2005).

In conclusion, this work shows the detrimental effect of *β2m* knockout in ALS and identifies *β2m* signaling as a potential new direction for the development of therapeutic strategies counteracting ALS.

## AUTHOR CONTRIBUTIONS

Kim A. Staats and Susann Schönefeldt performed the murine behavioral analyses. Kim A. Staats and Marike Van Rillaer conducted the staining experiments. Kim A. Staats and Annelies Van Hoecke analyzed gene expression of motor neurons excised by laser dissection microscopy. Philip Van Damme, Wim Robberecht, Adrian Liston and Ludo Van Den Bosch supervised and designed the experiments. Kim A. Staats, Adrian Liston and Ludo Van Den Bosch wrote the manuscript. All authors approved the final version of the manuscript.

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# Neuroimmunity dynamics and the development of therapeutic strategies for amyotrophic lateral sclerosis

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Amyotrophic lateral sclerosis (ALS) is a fatal paralytic disorder characterized by the progressive and selective loss of both upper and lower motoneurons. The neurodegenerative process is accompanied by a sustained inflammation in the brain and spinal cord. The neuron-immune interaction, implicating resident microglia of the central nervous system and blood-derived immune cells, is highly dynamic over the course of the disease. Here, we discuss the timely controlled neuroprotective and neurotoxic cues that are provided by the immune environment of motoneurons and their potential therapeutic applications for ALS.

**Keywords: inflammation, microglia, lymphocytes, astrocytes, hyperexcitability, cytokine, therapy**

## INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease characterized by the selective and progressive loss of upper and lower motoneurons, with both genetic and sporadic events contributing to the development of the pathological process (reviewed in Bento-Abreu et al., 2010). The term "neuroinflammation" has been attributed to the inflammatory response that occurs within the central nervous system (CNS) concomitantly to neurodegeneration (reviewed in Glass et al., 2010). Astrocytes, microglia, and immune cells are the key cellular modulators of neuroinflammation and have all been shown to actively participate in ALS pathogenesis (Glass et al., 2010; McCombe and Henderson, 2011; Philips and Robberecht, 2011). Importantly, recent reports have highlighted the presence of both neuroprotective and neurotoxic inflammatory cells in ALS animal models and patients that appear to be mainly dependent on the stage of disease progression. Seeing as reviews on the relationship between astrocytic activation and ALS are numerous, we will focus herein on the dynamic functional changes of microglia and immune cells that take place during ALS pathogenesis. A better understanding of these time-dependent modifications is of utmost importance for the development of ALS therapeutic strategies aimed at targeting the neuroinflammatory process.

## A ROLE FOR MICROGLIA IN NEUROINFLAMMATION

### ACTIVATION PROFILE IN HUMAN AND ANIMAL MODELS OF ALS

Microglia, the resident immune cells of the CNS, constantly survey the environment and become activated upon alterations resulting from disease or injury eliciting a strong pro-inflammatory response (reviewed in Hanisch and Kettenmann, 2007). In ALS patients, reactive microglia are observed in the motor cortex, motor nuclei of the brainstem, the entire corticospinal tract, the spinal cord, and within the cerebrospinal fluid (CSF; Engelhardt and Appel, 1990; Kawamata et al., 1992; Banati et al., 1995). Given the relationship between astrocytes and microglia and the importance of astrocytosis in ALS (Davalos et al., 2005; Yamanaka et al., 2008), it has been hypothesized that microgliosis may also participate in ALS pathogenesis.

In rodent ALS models, microgliosis occurs in pre-symptomatic and symptomatic $SOD1^{G93A}$ mice (Hall et al., 1998; Alexianu et al., 2001; Petrik et al., 2007; Gerber et al., 2012) and at both onset and early-stage of the disease in $SOD1^{G37R}$ mice (Boillee et al., 2006). An in-depth *in vivo* characterization of microgliosis in $SOD1^{G93A}$ mice shows that microglia are highly reactive in pre-symptomatic stages while they lose their ability to monitor the environment as the disease progresses (Dibaj et al., 2011). Indeed, microglia isolated from either neonatal or early onset $SOD1^{G93A}$ mice display an activated M2 phenotype and enhance motoneuron survival while microglia isolated from either adult or end stage mice have a classically activated M1 phenotype and induce motoneuron death (Weydt et al., 2004; Liao et al., 2012). In the pre-symptomatic and symptomatic $SOD1^{G93A}$ rat model, microglia aggregates are detected in both the spinal cord and brainstem and display a degenerative and apoptotic phenotype at end stage (Fendrick et al., 2007; Graber et al., 2010). Moreover, microglia of pre-symptomatic $SOD1^{H46R}$ rats express the proliferating marker Ki67 and the phagocytic markers ED1 and major histocompatibility complex (MHC) class II (Sanagi et al., 2010; Bataveljic et al., 2011). These data suggest that microgliosis not only typifies ALS

but that microglia function changes during disease progression, thus exerting differential effects on motoneurons.

## A ROLE FOR MICROGLIA IN ALS PATHOGENESIS

A key finding supporting the contribution of microglia in ALS pathogenesis is the significant extension in lifespan and delay in disease progression when the mutant protein is specifically deleted from macrophages and microglial lineages in both *SOD1*$^{G37R}$ and *SOD1*$^{G85R}$ mice (Boillee et al., 2006; Wang et al., 2009). Similarly, bone marrow transplantation (resulting in donor-derived microglia) of *SOD1*$^{G93A}$ microglia into *PU.1*$^{-/-}$ mice (that lack CNS microglia at birth) did not induce neurodegeneration whereas wild-type donor-derived microglia transplantation into *SOD1*$^{G93A}$; *PU.1*$^{-/-}$ mice improved survival (Beers et al., 2006).

However, phenotypical analysis of microglia in different regions of *SOD1*$^{G93A}$ spinal cord suggests that both neuroprotective and neurotoxic population of microglial cells may co-exist during the disease and that depletion of proliferative microglia does not prevent motoneuron degeneration (Gowing et al., 2008; Beers et al., 2011b). Together, these studies thus suggest that microglia participates, through a complex balance between neuroprotective and neurotoxic signals, to ALS disease progression.

## PROPOSED MECHANISMS OF MICROGLIAL-DERIVED NEUROTOXICITY

Various misregulated pathways within ALS microglia have been identified that may influence motoneuron survival. Endoplasmic reticulum (ER) stress is a characteristic of ALS pathogenesis (reviewed in Lautenschlaeger et al., 2012). In microglia of both sporadic ALS patients and symptomatic *SOD1*$^{G93A}$ mice, there is an increased expression of C/EBP homologous protein (CHOP; Ito et al., 2009), a member of the apoptotic ER stress pathway (reviewed in Oyadomari and Mori, 2004). It remains unclear if it directly participates in microglial neurotoxicity but exposure of microglia to interferon gamma (IFN$\gamma$), which levels are increased in the spinal cord of ALS mice and patients (Aebischer et al., 2011; Aebischer et al., 2012), elicits inducible nitric oxide (NO) synthase (iNOS) expression. The subsequent production of NO can cause an ER stress response that involves CHOP (Kawahara et al., 2001). Interestingly, several SOD1 mouse models show initiation of a specific ER stress response accompanied by microglial activation (Saxena et al., 2009).

Activation of the ligand-dependent CD14 lipopolysaccharide (LPS) receptor located at the microglial surface (Lacroix et al., 1998) initiates a pro-inflammatory Toll-like receptors (TLRs) dependent cascade (Laflamme and Rivest, 2001; Laflamme et al., 2001). Importantly, neurotoxic microglia activation by extracellular SOD1$^{G93A}$ is mediated by the CD14-TLR2 pathway and induces a subsequent release of pro-inflammatory cytokines, including tumor necrosis factor alpha (TNF$\alpha$) and interleukin (IL)-1$\beta$ (Liu et al., 2009; Zhao et al., 2010). Moreover, microglia from sporadic ALS patients show an enhanced TLR2 immunoreactivity (Casula et al., 2011). Microglia may thus participate in motoneuron loss following the specific activation of the CD14-TLR pathway by secreted SOD1 mutant, therefore propagating pro-inflammatory stimuli.

The release of extracellular nucleoside di- and tri-phosphates, in particular ATP, by degenerating neurons can elicit microglia activation through the ionotropic P2X and metabotropic P2Y purinergic receptors which can subsequently elicit a pro-inflammatory response, chemotaxis, and phagocytosis (reviewed in Inoue, 2006; Bours et al., 2011). Notably, P2X is increased within spinal cord microglia of ALS patients (Yiangou et al., 2006). Embryonic microglia and neonatal primary microglial cultures from mutant SOD1 mice display an upregulation of $P2X_4$, $P2X_7$, and $P2Y_6$ receptors (D'Ambrosi et al., 2009). Further, activation of $P2X_7$ in *SOD1*$^{G93A}$ microglia leads to the production of significantly higher levels of TNF$\alpha$, which has a neurotoxic effect on motoneuron cultures (Ugolini et al., 2003), and of cyclooxygenase-2 (COX-2), which produces the potent inflammatory mediators prostaglandins (D'Ambrosi et al., 2009).

Moreover, a reduced ATP hydrolysis activity in mutant SOD1 microglia, suggests a potentiation of a purinergic-mediated inflammation that can participate to the neuroinflammatory state of microglial cells. Since ATP induces an astrocytic neurotoxic phenotype through $P2X_7$ receptor signaling (Gandelman et al., 2010), one can hypothesize that increased extracellular ATP in ALS, whether exacerbated by motoneurons and/or microglia contributes to the pathogenic microgliosis.

## THE POTENTIAL INFLUENCE OF MICROGLIA ON NEURONAL EXCITABILITY

There is presently few assessment of the influence of microglia on motoneuron electrophysiology. However, studies on peripheral nerve or spinal cord injuries show that microglia activation has prominent effects on neuronal inhibitory control and loss of inhibitory control is a contributing mechanism to the motoneuron hyperexcitability that typifies ALS pathogenesis in humans (Bae et al., 2013).

Loss of neuronal inhibitory control occurs by several means including decrease in gamma-aminobutyric acid (GABA)ergic interneurons combined with changes in the expression of the $GABA_A$ receptor messenger RNA subunit (Petri et al., 2003; Maekawa et al., 2004). $GABA_A$ and glycine receptors are chloride ($Cl^-$) channels and the expression of cation-chloride co-transporter contributes to inhibitory effects of these $Cl^-$ currents (Blaesse et al., 2009). Indeed, the entry of $Cl^-$ following the opening of $GABA_A$ and glycine receptor-gated $Cl^-$ channels inhibits neuron excitability by hyperpolarizing membrane potential. Under physiological condition, low intracellular $Cl^-$ concentration $[Cl^-]_i$ is maintained by the potassium ($K^+$)-chloride co-transporter KCC2 that extrudes $Cl^-$ from mature neurons (Rivera et al., 1999). Stimulation of spinal microglia following peripheral nerve injury induces a decrease in KCC2 expression among dorsal horn nociceptive neurons (Coull et al., 2003). KCC2 decrease is induced by the brain-derived neurotrophic factor (BDNF) and this is consistent with the previous observation that BDNF can be produced by non-neuronal cells involved in immune responses, including T and B lymphocytes, monocytes, and microglia (Kerschensteiner et al., 1999; Coull et al., 2005). BDNF produces a depolarizing shift in the anion reversal potential of dorsal horn lamina I neurons due to an increase in $[Cl^-]_i$. This shift prompts an inversion of inhibitory GABA currents that

contributes to neuropathic pain following nerve injury (Coull et al., 2005). Decrease in KCC2 expression is thus responsible for the excitatory effects of GABA on neurons. Microglia activation and BDNF secretion are mediated through ATP activation of microglial P2X receptors. As discussed earlier, P2X receptors might be involved in ALS pathology since a higher density of $P2X_7$-immunoreactive microglial cells/macrophages are found in affected regions of spinal cords from ALS patients (Yiangou et al., 2006). Furthermore, levels of BDNF have been found to be increased in microglial cells isolated from ALS mice at the onset of disease and KCC2 is decreased in vulnerable motoneurons in *SOD1*$^{G93A}$ mice (Fuchs et al., 2010; Liao et al., 2012). Additionally, BDNF might play a role in the influence of microglia on motoneuron electric activity as suggested by work on spasticity. Spasticity is characterized by a velocity-dependent increase in muscle tone resulting from hyperexcitable stretch reflexes, spasms and hypersensitivity to normally innocuous sensory stimulations. Spasticity develops following spinal cord injury and is also regarded as an ALS clinical symptom (Rowland and Shneider, 2001). The main mechanism hypothesized to be responsible for spasticity is increased motoneuron excitability and increased synaptic inputs in response to muscle stretch due to reduced inhibitory mechanisms. Recently, it has been demonstrated that, following spinal cord injury, increased levels of BDNF mediated spasticity, due to post-transcriptional downregulation of KCC2 (Boulenguez et al., 2010). Together, these studies suggest that reactive microglia in ALS may exert an aberrant effect on the electrical activity of motoneurons and highlight the importance of furthering our understanding of this functional interaction.

Lastly, a hypothetical scenario relates to the defect in astrocytic glutamate transporter and the neurotoxic accumulation of the excitatory amino acid. It has been demonstrated that TNFα promotes glutamate release by activated microglia through the cystine/glutamate exchanger (Xc; Piani and Fontana, 1994). Though the implication of the Xc system in ALS has not yet been investigated, it may represent a potential mechanism of microglia-mediated excitotoxicity that warrants further study (Qin et al., 2006).

## THE DUAL ROLE OF NEUROIMMUNITY IN MOTONEURON DISEASE

### PATHOLOGICAL PHENOTYPE OF THE IMMUNE SYSTEM IN ALS

In addition to astrocytes and microglia, blood-derived immune cells may also play synergistic and critical functions during disease progression. Presence of a systemic immune activation is suggested by abnormalities observed in the blood and the CSF of ALS patients such as increased numbers of circulating lymphocytes ($CD4^+$ helper T cells, $CD8^+$ cytotoxic T lymphocytes, CTL, and natural killer, NK cells), increased expression of MHC class II molecules on monocytes as well as higher levels of inflammatory chemokines and cytokines (regulated on activation normal T cell expressed and secreted, RANTES, monocyte chemotactic protein, MCP-1, IL-12, IL-15, IL-17, and IL-23; Zhang et al., 2005; Rentzos et al., 2007, 2010, 2012; McCombe and Henderson, 2011). Further, post-mortem studies of brain and spinal cord lesions from ALS patients show that the activation and proliferation of microglia is associated with an infiltration of activated macrophages, mast cells and T lymphocytes which are found in close proximity to degenerating tissues (Engelhardt et al., 1993; Graves et al., 2004; Lewis et al., 2012). An in-depth autopsy of six ALS patients reveals an enrichment of T-cell receptor Vβ2-positive T cells in the spinal cord and CSF, suggesting an antigen-driven T cell selection (Panzara et al., 1999). Finally, ALS patients with a more rapidly progressing pathology show decreased numbers of regulatory T lymphocytes (Tregs), suggesting that the number of Tregs is inversely correlated with disease progression (Beers et al., 2011a; Rentzos et al., 2012). Tregs secrete anti-inflammatory cytokines such as IL-4, IL-10 and transforming growth factor beta (TGF-β) and has been show to induce the production of the neurotrophic factors glial-derived neurotrophic factor (GDNF) and BDNF by astrocytes (Reynolds et al., 2007). Tregs are also able to dampen a T helper (Th)1 pro-inflammatory response and attenuate toxic microglial responses. Contribution of the innate immune system is also suggested by the presence of immunoglobulins and complement deposition as well as a significant increase of NK cells in the blood of ALS patients (Donnenfeld et al., 1984; Engelhardt and Appel, 1990; Rentzos et al., 2012). While these investigations of ALS samples and tissues do not assess the contributory role of the immune system to disease pathogenesis, they do highlight its active presence.

In support of what is observed in humans, ALS rodent models also display a particular immunological phenotype. Indeed, *SOD1*$^{G93A}$ mice have allowed the demonstration that the inflammatory cellular subtypes are phenotypicaly and functionally different depending upon the disease stage (Liao et al., 2012). During the initial stages, infiltrating $CD4^+$ T cells are mainly Th2 ($IL\text{-}4^+$) while there is a skew toward Th1 ($IFN\gamma^+$) cells and $CD8^+$ T cells (both IL-17A positive and negative) as the disease progresses (Fiala et al., 2010; Beers et al., 2011b). Alteration in inflammatory cell subtypes is associated with, and maybe driven by, differences in Tregs. Interestingly, early symptomatic *SOD1*$^{G93A}$ mice have an increased number of Tregs and a decreased proliferation of effectors T lymphocytes (Teffs), whereas a decreased numbers of Tregs and an increased proliferation of Teffs is found in end stage animals (Beers et al., 2011a; Zhao et al., 2012). The innate immune system is also affected in ALS rodents, displayed by the substantial increase of NKT cells firstly in the liver and then in the spinal cord of *SOD1*$^{G93A}$ mice (Chiu et al., 2008; Finkelstein et al., 2011).

Whether neuroinflammation is a cause or a consequence of motoneuron dysfunction is still debated. It is interesting to note that inflammation is not limited to the CNS but systemic with a correlation between disease evolution and levels of plasma LPS as well as the numbers of activated circulating monocytes and T lymphocytes (Zhang et al., 2005, 2009). A thymic dysfunction also parallels the neurodegenerative process in mutant *SOD1* mice and ALS patients (Seksenyan et al., 2010). In the CNS of ALS patients, TAR DNA-binding protein 43 (TDP-43) displays an increased expression and interacts with nuclear factor kappa B (NF-κB) in glial and neuronal cells. LPS-activation of NF-κB in microglial cells expressing the TDP-43 mutant is associated with the production of pro-inflammatory cytokines, including TNFα, IL-1β, IL-6, and IFNγ (Swarup et al., 2011). NF-κB, is also an important intermediate of the TLR signaling pathway that contribute to the initiation of inflammatory responses (O'Connell et al., 2012). The

central role of inflammation and NF-κB in ALS was recently confirmed by the description in familial ALS of mutations in the gene encoding optineurin, a negative regulator of TNF-induced NF-κB activation (Maruyama et al., 2010).

Additional regulators of the neuroinflammatory response are the microRNAs (miRNA), an abundant class of small, non-coding RNA that regulate gene expression in a wide range of biological processes (O'Connell et al., 2012). Recently, a dominantly inherited mutation in the heterogeneous nuclear ribonucleoprotein (hnRNP) A1 has been associated with familial ALS (Kim et al., 2013). hnRNPA1 is a RNA-binding protein involved in RNA metabolism, including the regulation of alternative pre-mRNA splicing, mRNA export, and stability as well as the processing of miRNA (Guil and Caceres, 2007). Interestingly, hnRNPA1 can directly interact with TDP-43 (Buratti et al., 2005), and TDP-43 was proposed to contribute to the post-translational processing of miRNA through interaction with the endonucleases, Drosha and Dicer (Kawahara and Mieda-Sato, 2012). The activity of Dicer, which processes miRNA precursors at the RNA-induced silencing complex (Wilson and Doudna, 2013), is required to maintain motoneuron functional integrity. Indeed, the conditional deletion of *Dicer* in vesicular acetylcholine transporter-expressing cells leads to motoneuron degeneration and denervation atrophy in mice (Haramati et al., 2010). Another intriguing link with the miRNA pathway in the neuro-immune interaction has been recently revealed by the demonstration that the neurotransmitter acetylcholine can inhibit the production of pro-inflammatory cytokines, TNFα and IL-6, through induction of miRNA-124 in macrophages (Sun et al., 2013). In addition, a subset of $CD4^+$ T cells has been described to produce acetylcholine to modulate the inflammatory response taking part of the autonomic homeostatic reflexes (Rosas-Ballina et al., 2011). Regarding ALS pathogenesis, a dysfunction of the cholinergic circuit has been reported in the spinal cord of SOD1 mutant mice, early in the disease course (Casas et al., 2013). Moreover, the choline acetyltransferase mRNA is a target of TDP-43 (Polymenidou et al., 2011), and the decrease in cholinergic input in the neuroinflammatory context of Alzheimer's disease was also shown to lead to the down regulation of hnRNPA1 (Berson et al., 2012). Despite the sequential events implicating miRNAs and the cholinergic signaling needs to be further explored, this evidence concurs toward the contribution of the neuro-immune interaction in the degenerative process.

The information from pre-clinical models and ALS patients suggests that systemic immune activation (innate and adaptive) might play a key role in ALS pathogenesis and may represent an interesting target for the development of novel treatments. However, a better understanding of the specific roles played by the different subtypes of immune cells is of utmost necessity. Indeed, accumulative evidence suggests that inflammatory cells mediate both protective and deleterious effects on motoneuron survival and that these functions vary during disease progression.

## THE PROTECTIVE FUNCTION OF THE IMMUNE RESPONSE IN ALS

Protective immunity, a crucial homeostatic phenomenon in the repair of damaged tissues, results from both the clearance of debris and the effects of cytokines and growth factors delivered by inflammatory cells to the site of injury (Hohlfeld et al., 2000; Schwartz and Moalem, 2001). The neuroprotective ability of immune cells is also evident in ALS. Indeed, when $SOD1^{G93A}$ mice are bred with mice lacking functional T cells or $CD4^+$ T cells, microglia skew toward an M1 inflammatory phenotype and disease progression accelerates, suggesting that $CD4^+$ T cells provide neuroprotection by suppressing the activation of cytotoxic microglia. Accordingly, reconstitution of T cells following bone marrow transplantation of $SOD1^{G93A}$ mice lacking functional T and B cells prolonged their survival and suppressed the activation of M1 microglia (Beers et al., 2008). Further analysis showed that neuroprotection is mainly supported by $CD4^+CD25^+Foxp3^+$ Tregs that secrete IL-4, thus promoting M2 protective microglia and IL-4 secreting Th2 cells, while inhibiting the neurotoxic Th1 response and IFNγ secretion. The passive transfer of Tregs into ALS mice lacking functional T cells results in lengthened disease duration and prolonged survival (Beers et al., 2011a). Accordingly, these neuroprotective Tregs are increased in the peripheral blood of ALS patients during early stages but their numbers decrease as the disease progression accelerates and are thus inversely correlated with disease progression rates (Beers et al., 2011a; Rentzos et al., 2012; Henkel et al., 2013). Furthermore, Foxp3 and CD25 expression is reduced in Tregs from rapidly progressing patients and are also inversely correlated with disease progression rates (Henkel et al., 2013). Co-culture experiments showed that Tregs suppress the expression of cytotoxic factors Nox2 and iNOS from $SOD1^{G93A}$ microglia through IL-4 secretion and inhibit the proliferation of $SOD1^{G93A}$ Teffs via the combined secretion of IL-4, IL-10, and TGF-β (Zhao et al., 2012). Hence, Tregs enhance the neuroprotective properties of the immune system during the stable disease phase while a switch from a neuroprotective Tregs/M2 to a deleterious Th1/M1 response characterizes disease progression. The key role of this balance between protective and deleterious immune responses in modulating clinical outcome is confirmed by the temporal and regional association between neuroinflammation and motoneuron injury in ALS mice (Beers et al., 2011b). Indeed, initial weakness in the hindlimbs is associated with a Th1 proinflammatory infiltrate in the lumbar spinal cord, while a protective Th2 immune response is observed in the cervical cord and may explain the delayed motor weakness in the forelimbs (Beers et al., 2011b). Therefore, the inflammatory infiltrate observed in ALS lesions appears not simply as a consequence of motoneuron degeneration but is actively involved in the neurodegenerative process. Tregs and Th2 lymphocytes assume the majority of the neuroprotective functions of the immune system and targeting their signaling pathways may be an attractive therapeutic strategy in ALS.

## THE NEUROTOXIC FUNCTION OF THE IMMUNE RESPONSE IN ALS

Cytotoxic T lymphocytes and NK cells are important effector cells of the immune system that eliminate aberrant cells, classically virus-infected cells, or tumorigenic cells (Zhang and Bevan, 2011; Kaur et al., 2012). Interestingly, at symptomatic stage, an increased number of $CD8^+$ T and NK cells is observed in the blood and spinal cord of ALS patients (Calvo et al., 2010; Rentzos et al., 2012). Neurotoxic effects might be associated with a Th1-driven CTL pro-inflammatory immune response. Accordingly, mutant *SOD1* Th1 lymphocytes proliferate to a greater extend and produce more

IFNγ during the rapidly progressing phase than Th1 lymphocytes isolated during the slowly progressing phase.

Different death pathways induced by $CD8^+$ CTL lymphocytes could potentially lead to motoneuron death in ALS. CTL are antigen-specific effector cells that express the ligand for Fas (FasL) and most potential CTL targets express Fas at their surface. The activation of Fas (CD95) by its cognate ligand FasL commits cells to a death program through a caspase cascade (Peter et al., 2007). Interestingly, the activation of Fas triggers a death pathway in motoneurons that appeared restricted to this cell type (Raoul et al., 1999, 2002, 2006; Bernard-Marissal et al., 2012; Aebischer et al., 2013). Motoneurons expressing ALS-linked SOD1 mutations showed an increased susceptibility to Fas-mediated death through activation of a Fas/NO amplification loop (Raoul et al., 2002, 2006). Accordingly, mutant *SOD1* mice with homozygous loss-of-function FasL mutation present a reduced loss of motoneurons and a prolonged life expectancy (Petri et al., 2006). It remains to be determined whether CTL contribute to Fas-induced motoneuron loss. Another cytotoxic mechanism of CTL-mediated killing of target cells is the perforin-granzyme system. Upon recognition of a target cell by CTL, cytotoxic granules containing perforin and granzyme are released in the extracellular space. Perforin is a pore forming protein allowing the entry in the target cells of granzyme serine proteases that subsequently induce caspase activation and cell death (van Domselaar and Bovenschen, 2011). It is noteworthy that increased levels of granzyme A and B isoforms are increased in the serum of ALS patients (Ilzecka, 2011). However, the functional significance of such an increase remains to be determined. IFNγ, which is produced by CTL cells, can exert both immunostimulatory and immunomodulatory effects during an immune response. IFNγ produced by mutant astrocytes and motoneurons can elicit a death program in motoneurons through the activation of the lymphotoxin beta receptor (LT-βR) by its ligand LIGHT (Aebischer et al., 2011, 2012). The genetic deletion of *Light* in $SOD1^{G93A}$ mice suggests that the LIGHT pathway contributes to the progression phase of the disease. Recently, the intracerebroventicular infusion of neutralizing anti-IFNγ antibody has been shown to delay the motor function decline in $SOD1^{G93A}$ mice, suggesting that IFNγ contributes to ALS pathogenesis (Otsmane et al., 2013). However, The precise contribution of IFNγ in the neuroinflammatory response remains to be investigated.

An infiltration of NK cells has been reported in the spinal cord of symptomatic ALS mice (Chiu et al., 2008). While the role of NK cells in ALS remains unknown, several hypothetical mechanisms can be raised about their pathogenic contribution. Indeed, activated NK cells inhibit neurite outgrowth of cerebellar neurons in a cell contact-dependent manner *in vitro* (Pool et al., 2012). In sensory neurons, IL-2-activated NK cells have a killing activity that requires the perforin-granzyme system (Backstrom et al., 2000). Further, the production of IFNγ by activated NK cells might directly trigger motoneuron death through the LIGHT/LT-βR pathway or potentiate a cytotoxic Th1/CTL response via the combined action of other NK-related cytokines such as IL-17 or IL-22 (Cella et al., 2010). NK cells thus represent an interesting branch of the immunopathology that should be further considered.

Several studies suggest that humoral immunity and immunoglobulins could also contribute to the disease. Autoantibodies to voltage-gated $Ca^{2+}$ or $K^+$ channels have been described in ALS patients, which induce specific motoneuron alterations both *in vitro* and *in vivo* after passive transfer in mice (Appel et al., 1991; Engelhardt et al., 1995; Demestre et al., 2005; Pagani et al., 2006; Nwosu et al., 2010). Abnormal levels of anti-Fas antibodies, able to induce neuronal apoptosis *in vitro*, have been detected in the serum of patients with ALS (Yi et al., 2000; Sengun and Appel, 2003). C5a and other complement activation products released after activation of the classical complement pathway by antibodies are elevated in the CSF and spinal cord of ALS mice and patients and specific inhibition of C5a receptor ameliorates disease in $SOD1^{G93A}$ mice (Woodruff et al., 2008; Heurich et al., 2011). Thus, both the innate and adaptive immune system appears to have deleterious consequences on the survival and maintenance of motoneurons in ALS (**Figure 1**).

## EXPLOITING THE NEUROPROTECTIVE AND NEUROTOXIC PROPERTIES OF NEUROIMMUNITY FOR THE DEVELOPMENT OF THERAPEUTIC STRATEGIES

In light of the dynamic functional changes of microglia and immune cells discussed above, attempts to develop therapeutic strategies targeting neuroinflammation have only emphasized the importance of understanding the temporal neuroinflammatory events in ALS.

In pre-clinical mouse models, genetic deletion of the $P2X_7$ receptor, which was previously described as being upregulated in ALS microglia (D'Ambrosi et al., 2009), resulted in increased motoneuron loss, increased microgliosis, and accelerated disease progression, thus suggesting an unanticipated protective role for the $P2X_7$ receptor (Apolloni et al., 2013). Similarly, as mentioned earlier, genetic depletion of functional T cells or $CD4^+$ cells in $SOD1^{G93A}$ mice lead to increased disease progression, decreased survival as well as promoted production of pro-inflammatory effectors (Beers et al., 2008). Finally, eliminating the expression of galectin-3, a multifunctional immunomodulator that is increased in ALS microglia (Norling et al., 2009), in $SOD1^{G93A}$ mice, also results in aberrant microgliosis and increased disease progression (Lerman et al., 2012). These alterations ($P2X_7$, immune cells and galectin-3) were embryonically and permanently induced, implying that at a certain time-point during the development of the animal and the progression of the disease, these molecular and cellular components are necessary for alleviating certain ALS symptoms and pathological features.

At the clinical level, the failure of certain trials assessing the influence of drugs that directly or indirectly impact neuroinflammation may be due to inappropriate knowledge of the dynamic changes that occur within microglia and immune cells. Indeed, drastic immunosuppressive strategies such as cyclosporine, cyclophosphamide, intravenous immunoglobulin G treatment, and total lymphoid irradiation did not provide any significant benefits to ALS patients (Brown et al., 1986; Drachman et al., 1994; Gourie-Devi et al., 1997). Similarly, drugs used to target specific neuroinflammatory effectors that showed promising

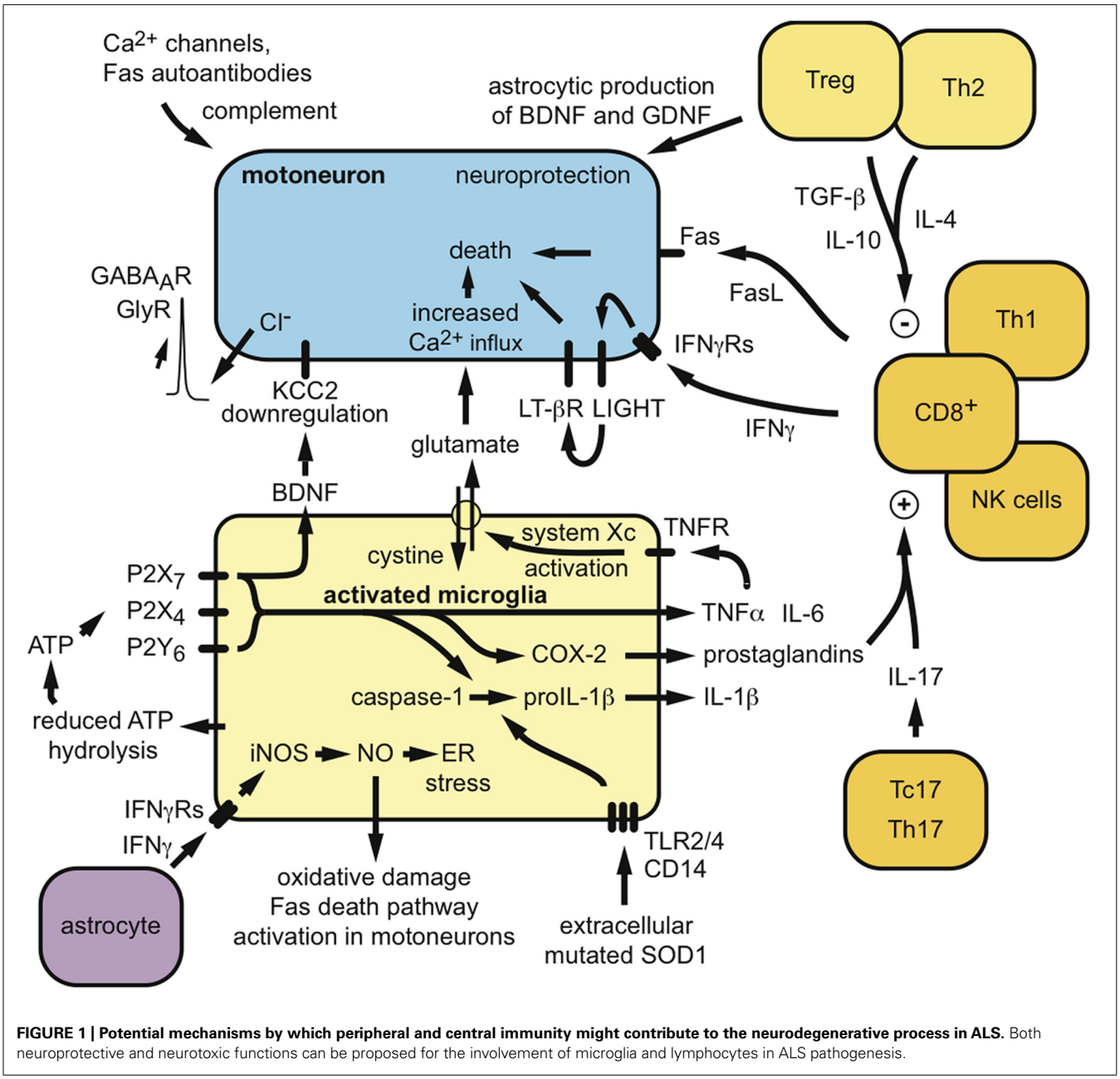


**FIGURE 1 | Potential mechanisms by which peripheral and central immunity might contribute to the neurodegenerative process in ALS.** Both neuroprotective and neurotoxic functions can be proposed for the involvement of microglia and lymphocytes in ALS pathogenesis.

results in pre-clinical models such as celecoxib and pioglitazone (Drachman et al., 2002; Schutz et al., 2005), proved to be ineffective in improving motor functions and survival in ALS patients (Cudkowicz et al., 2006; Dupuis et al., 2012).

The progressive spreading, extension and diffusion of the neurodegenerative process that typically occurs in ALS patients may result from the concurrent progressive invasion of the CNS by glial cells and most importantly, the functional changes that take place within these cells. Importantly, an incomplete understanding of said changes could lead to undesired and unexpected results. Indeed, both minocycline and thalidomide (an analog of lenalidomide) revealed serious harmful effects in patients during a randomized placebo-controlled phase III trial and a single arm, open label phase II study, respectively (Gordon et al., 2007; Stommel et al., 2009).

As translational therapy targeting neuroinflammatory and immunomodulatory effectors is rapidly progressing, it has become clear that a step backward is presently required to better assess the temporal functional changes that occur within glial and immune cells in ALS pathogenesis. The cellular environment being composed of both neuroprotective and neurotoxic functions, specific therapeutic windows may dictate the choice of drugs and their pathogenic targets. Alternatively, a combinatory therapeutic approach may be more efficient at modulating the contributions of non-neuronal cells to ALS pathology. Thus, while neuroinflammation undoubtedly plays a role in ALS pathogenesis, therapeutic

success will be reached in limiting the activation and amplification of toxic glial and immune cells whilst preserving the cellular subtypes that are beneficial to motoneuron survival.

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# Redox environment is an intracellular factor to operate distinct pathways for aggregation of Cu,Zn-superoxide dismutase in amyotrophic lateral sclerosis

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Dominant mutations in Cu,Zn-superoxide dismutase (SOD1) cause a familial form of amyotrophic lateral sclerosis (fALS). Misfolding and aggregation of mutant SOD1 proteins are a pathological hallmark of SOD1-related fALS cases; however, the molecular mechanism of SOD1 aggregation remains controversial. Here, I have used *E. coli* as a model organism and shown multiple distinct pathways of SOD1 aggregation that are dependent upon its thiol-disulfide status. Overexpression of fALS-mutant SOD1s in the cytoplasm of *E. coli* BL21 and SHuffle$^{TM}$, where redox environment is reducing and oxidizing, respectively, resulted in the formation of insoluble aggregates with notable differences; a disulfide bond of SOD1 was completely reduced in BL21 or abnormally formed between SOD1 molecules in SHuffle$^{TM}$. Depending upon intracellular redox environment, therefore, mutant SOD1 is considered to misfold/aggregate through distinct pathways, which would be relevant in description of the pathological heterogeneity of SOD1-related fALS cases.

**Keywords: SOD1, ALS, aggregation, disulfide bond**

## INTRODUCTION

Thiol-disulfide status is critical for functioning of many proteins (Sevier and Kaiser, 2002), and Cu,Zn-superoxide dismutase (SOD1) is one of such proteins in which formation of an intramolecular disulfide bond is required for folding into its enzymatically active conformation (Furukawa et al., 2004). An enzymatic function of SOD1 is to catalyze the removal of a toxic reactive oxygen species, superoxide anion (McCord and Fridovich, 1969), and activation steps of SOD1 *in vivo* include binding of a catalytic copper ion and a structural zinc ion and also formation of an intramolecular disulfide bond. Given that SOD1 isolated from *Bacillus subtilis* (Banci et al., 2005) and *Mycobacterium tuberculosis* (Spagnolo et al., 2004) lacks a copper and zinc binding site, respectively, metal binding seems to be dispensable for SOD1. In contrast, an intramolecular disulfide bond is conserved among all SOD1 proteins identified so far, implying its essential roles in physiological functions of SOD1.

Indeed, abnormalities in a thiol-disulfide status of SOD1 have been proposed as a pathological change in a familial form of amyotrophic lateral sclerosis (fALS) that is caused by dominant mutations in SOD1 (Rosen et al., 1993). For example, in transgenic mice expressing human SOD1 with a fALS mutation (G85R), two Cys residues (Cys57 and Cys146) of SOD1, which normally form an intramolecular disulfide bond, remained reduced (Jonsson et al., 2006). Aggregation of mutant SOD1 is a major pathological change in SOD1-related fALS cases (Bruijn et al., 1998), and inclusions reproduced in diseased mice have been shown to contain disulfide-reduced SOD1 proteins (Jonsson et al., 2006; Karch et al., 2009). *In vitro* studies have also shown increased susceptibility of a disulfide bond in several fALS-mutant SOD1 proteins toward a reducing agent (Tiwari and Hayward, 2003). Furthermore, reduction of a disulfide bond significantly decreased the thermostability and thus facilitated misfolding and aggregation of SOD1 *in vitro*, supporting important roles of a conserved disulfide bond in maintaining an aggregation-resistant structure of SOD1 (Furukawa and O'Halloran, 2005; Furukawa et al., 2008).

In contrast, increased oxidative stress has been reported in fALS patients (Barber and Shaw, 2010), and SOD1 appears to be one of intracellular targets susceptible to oxidative modifications (Guareschi et al., 2012). In transgenic mice expressing human SOD1 with fALS mutations, mutant SOD1 has been shown to form insoluble oligomers cross-linked *via* intermolecular disulfide bonds (Furukawa et al., 2006). *In vitro* experiments have also revealed that aggregation of mutant SOD1 is triggered by abnormal oxidation of histidine and tryptophan residues (Rakhit et al., 2002; Zhang et al., 2003). Under such oxidative conditions, a disulfide-reduced form of SOD1 would not stably exist. Furthermore, structural destabilization of SOD1 by pathogenic mutations has been recently reported to facilitate isomerization of a conserved intramolecular disulfide bond (Cys57–Cys146) into an intermolecular disulfide crosslink (Toichi et al., 2013). Taken together, reduction of a disulfide bond may not be a prerequisite for aggregation of SOD1 *in vivo*; rather, a redox environment surrounding SOD1 would determine how mutant SOD1 is misfolded and aggregated. Indeed, most (>70%) of intracellular SOD1 exist in the reducing environment of cytoplasm (Chang et al., 1988), but a small fraction (∼3%) of SOD1 is also detected in the intermembrane space (IMS) of mitochondria (Okado-Matsumoto and Fridovich, 2001), which is considerably more oxidizing than cytoplasm (Hu et al., 2008). Intracellular SOD1 is thus considered to experience a broad range

of redox environment, which would affect its folding/misfolding processes.

In this study, effects of intracellular redox environment on SOD1 aggregation have been examined in *Escherichia coli* as a model organism. Overexpression of heterologous proteins in bacteria such as *E. coli* often leads to the formation of insoluble aggregates called inclusion bodies, and inclusion bodies of several proteins have been shown to possess amyloid-like properties (Carrio et al., 2005; de Groot et al., 2009; Villar-Pique and Ventura, 2012). Cytoplasm of *E. coli* has been well known as strongly reducing environment (Hwang et al., 1992), while genetically modified *E. coli*, SHuffle$^{TM}$, provides considerably oxidizing cytoplasm (Lobstein et al., 2012). FALS-mutant human SOD1 proteins in *E. coli* BL21 were found to exist as a disulfide-reduced state and form insoluble fibrillar aggregates. In contrast, expression of mutant SOD1 in the oxidizing cytoplasm of SHuffle$^{TM}$ resulted in the formation of insoluble oligomers crosslinked *via* intermolecular disulfide bonds albeit with fibrillar morphologies. Depending upon the intracellular redox environment, therefore, mutant SOD1 proteins form insoluble aggregates with distinct properties, suggesting roles of organelle-specific misfolding pathways of mutant SOD1 in fALS pathomechanism.

## RESULTS AND DISCUSSION

### SOD1 AGGREGATES UNDER REDUCING ENVIRONMENT OF *E. coli* CYTOPLASM

Introduction of an intramolecular disulfide bond between Cys57 and Cys146 in SOD1 has been known to increase the electrophoretic mobility of SOD1 (Furukawa et al., 2004); therefore, thiol-disulfide status of SOD1 can be determined by non-reducing SDS-PAGE analysis. When overexpressed in *E. coli* BL21, wild-type human SOD1 (SOD1(WT)) was found to be in a disulfide-reduced form (SOD1$^{SH}$) with a small fraction of a disulfide-form (SOD1$^{S-S}$; **Figure 1A**). Also notably, most of SOD1$^{SH}$ was insoluble, while SOD1$^{S-S}$ remained soluble (**Figure 1A**). These results are consistent with a previous finding that SOD1$^{SH}$ is highly prone to insoluble aggregation *in vitro* (Furukawa et al., 2008). Given that cytoplasm of *E. coli* provides a highly reducing environment (Hwang et al., 1992), formation of a disulfide bond in proteins will be an unfavorable process in the cytoplasm. Furthermore, metal-chelating capacity of bacterial cytoplasm has been reported to be extremely high (Outten and O'Halloran, 2001; Changela et al., 2003), where SOD1 is supposed to be in a metal-deficient apo state. Indeed, exogenous supplementation of $ZnSO_4$ in a growth media increased the soluble fraction of overexpressed SOD1$^{SH/S-S}$ (**Figure 1A**), supporting previous reports that binding of a zinc ion protects SOD1 from aggregation (Furukawa et al., 2008). In the reducing environment of cytoplasm, where metal-chelating capacity is also significant, SOD1 remain in a disulfide-reduced apo state and is prone to insoluble aggregation.

### OXIDIZING ENVIRONMENT PROTECTS SOD1 FROM AGGREGATION BY DISULFIDE FORMATION

To examine effects of disulfide formation on SOD1 aggregation *in vivo*, SOD1(WT) was overexpressed in *E. coli* SHuffle$^{TM}$, where cytoplasmic reductive pathways are genetically diminished (Lobstein et al., 2012). More specifically, thioredoxin reductase (*trxB*) and glutathione reductase (*gor*) have been removed in *E. coli*

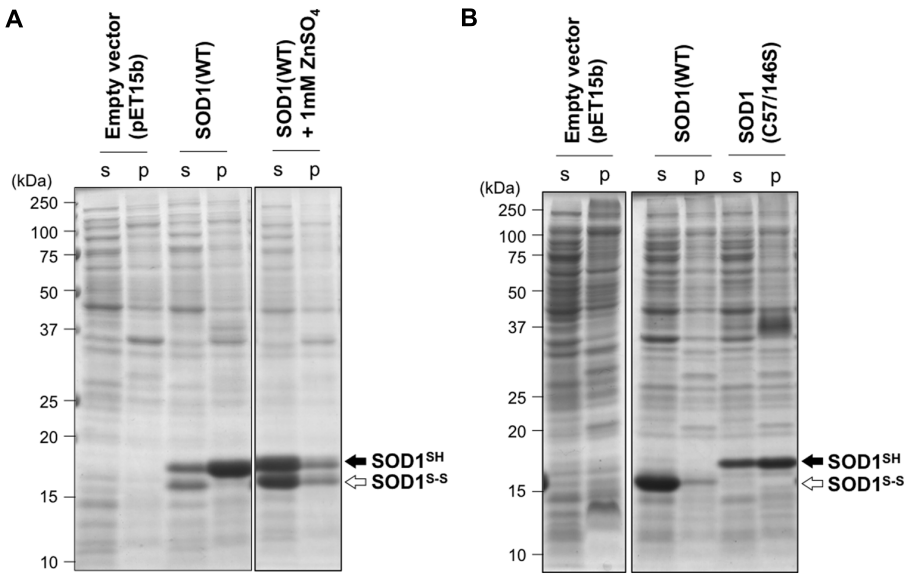


**FIGURE 1 | Redox environment of *E. coli* cytoplasm influences the aggregation propensities of SOD1 by modulating its thiol-disulfide status. (A)** *E. coli* BL21(DE3) or **(B)** SHuffle$^{TM}$ was transformed with pET15b (an empty vector) or pET15b harboring human SOD1(WT) cDNA, and the protein expression was induced with IPTG (see Materials and Methods). Results obtained by addition of 1 mM $ZnSO_4$ at the induction of protein expression were also included in **(A)** (SOD1(WT) + 1 mM $ZnSO_4$). Also, expression of SOD1 with C57/146S mutations, in which an intramolecular disulfide bond cannot form, was examined in *E. coli* SHuffle$^{TM}$ and shown in **(B)**. Cell lysates were fractionated into soluble supernatant (s) and insoluble pellets (p), treated with iodoacetamide, and then analyzed with non-reducing SDS-PAGE by using a 15% polyacrylamide gel. White (SOD1$^{S-S}$) and black (SOD1$^{SH}$) arrows at the right side of the gel image indicate positions of bands corresponding to SOD1 with and without a disulfide bond, respectively.

SHuffle$^{TM}$, which provides oxidizing cytoplasm and thus enables to introduce disulfide bonds in cytoplasmically expressed proteins. As shown in **Figure 1B**, SOD1(WT) overexpressed in *E. coli* SHuffle$^{TM}$ was found to form a disulfide bond and remain soluble, showing that correct introduction of a disulfide bond in SOD1 can prevent its insoluble aggregation *in vivo*. When SOD1 with C57S/C146S mutations, in which a conserved disulfide bond (Cys57–Cys146) cannot form, was overexpressed in *E. coli* SHuffle$^{TM}$, significant amounts of mutant SOD1 was again found in the insoluble fraction. This result hence emphasizes a protective role of the disulfide bond against aggregation of SOD1. In eukaryotes, a copper chaperone for SOD1 (CCS) has been shown to introduce the intramolecular disulfide bond in SOD1 (Furukawa et al., 2004), while CCS-independent pathway(s) for disulfide formation in SOD1 also appears to exist (Subramaniam et al., 2002; Leitch et al., 2009). Given no CCS homologues in bacteria, the results obtained by using SHuffle$^{TM}$ (**Figure 1B**) implies that oxidizing environment is sufficient for introducing a correct disulfide bond into wild-type SOD1 even without CCS and thereby protecting the protein from being misfolded/aggregated.

## ALS MUTATIONS COMMONLY AGGRAVATE THE AGGREGATION PHENOTYPE OF SOD1 UNDER REDUCING ENVIRONMENT

To test effects of fALS-causing mutations on SOD1 aggregation *in vivo*, several types of mutant SOD1 were overexpressed in *E. coli* BL21. SOD1 with A4V mutation has been reported to be expressed in *E. coli* BL21 as insoluble pellets (Leinweber et al., 2004), and all of the other mutant SOD1s tested here were found as a disulfide-reduced form in insoluble pellets regardless of the absence (data not shown) or presence (**Figure 2**) of 1 mM $ZnSO_4$ in a growth media. This is in sharp contrast to wild-type SOD1, which was expressed as a soluble form when cultured in a growth media supplemented with zinc ions (**Figure 1A**). It has been suggested that the decreased affinity of zinc ion is a common pathogenic denominator of fALS-mutant SOD1 (Goto et al., 2000; Hayward et al., 2002); therefore, supplementation of 1 mM $ZnSO_4$ to a growth media is considered to be insufficient for metallation of mutant SOD1 in the *E. coli* cytoplasm. Also notably, significant amounts of SOD1$^{S-S}$ were observed in a soluble fraction when the wild-type protein was expressed in *E. coli* BL21 (**Figure 1A**); however, in fALS-mutant SOD1s, formation of the disulfide bond was hardly observed (**Figure 2**). These results are consistent with previous reports showing increased susceptibility of the disulfide bond to reducing agents by pathogenic mutations (Tiwari and Hayward, 2003). Under the reducing environment with high metal-chelating capacity of *E. coli* cytoplasm, therefore, SOD1 is found to exhibit increased propensities for aggregation with fALS-causing mutations.

## FALS-MUTANT SOD1 TEND TO FORM DISULFIDE-LINKED OLIGOMERS UNDER OXIDIZING ENVIRONMENT

In wild-type SOD1, the intramolecular disulfide bond was efficiently introduced under the oxidizing environment, which then protected the protein from aggregation (**Figure 1B**). In contrast, when several fALS-mutant SOD1s were expressed in the oxidizing cytoplasm of *E. coli* SHuffle$^{TM}$, significant amounts of SOD1 were found to remain in insoluble pellets (**Figure 3**). More specifically, a monomer and higher-order oligomers of mutant SOD1 (G37R, G41S, G85R, G93R, C111Y, and L126X) were evident in an insoluble fraction, while SOD1s with H46R and H80R mutations were obtained as soluble proteins with an intramolecular disulfide bond (**Figure 3A**). When analyzed in reducing SDS-PAGE, furthermore, those higher-order oligomer bands were collapsed and merged to the monomer band (**Figure 3B**), indicating that the oligomers were formed *via* disulfide-crosslinks. Formation of intra- or inter-molecular disulfide bond is considered to depend

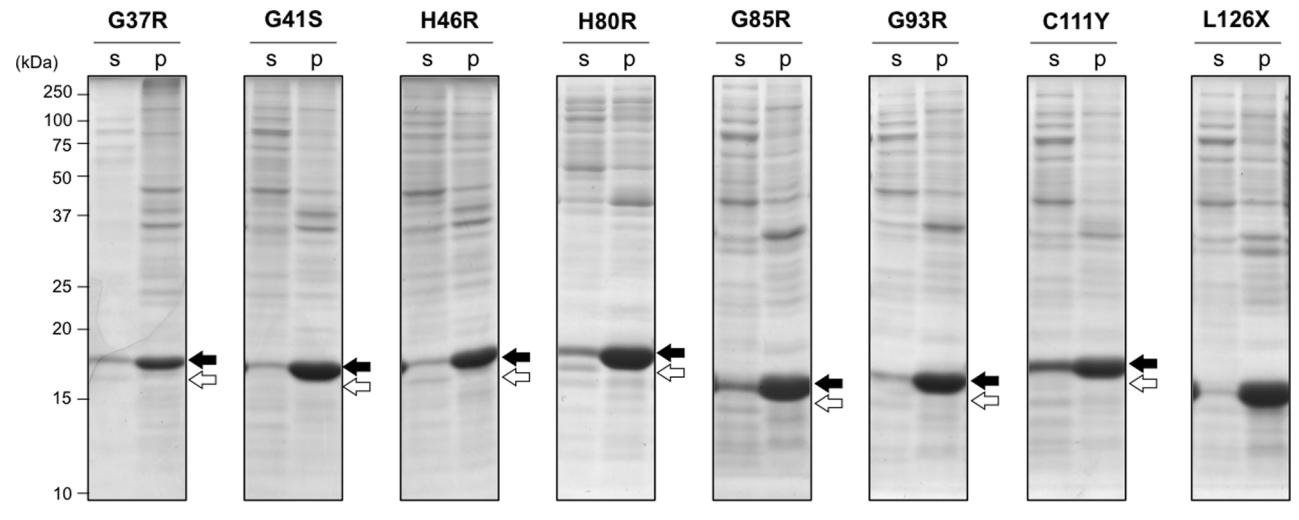


**FIGURE 2 | Aggregation of disulfide-reduced SOD1 with fALS mutations in *E. coli* BL21 was not rescued by addition of $ZnSO_4$.** *E. coli* BL21(DE3) was transformed with pET15b harboring human SOD1 cDNA with indicated fALS mutations, and the protein expression was induced by IPTG in the presence of 1 mM $ZnSO_4$. Cell lysates were fractionated into soluble supernatant (s) and insoluble pellets (p), treated with iodoacetamide, and then analyzed with non-reducing SDS-PAGE by using a 15% polyacrylamide gel. White (SOD1$^{S-S}$) and black (SOD1$^{SH}$) arrows at the right side of the gel image indicate positions of bands corresponding to SOD1 with and without a disulfide bond, respectively.

upon intracellular concentration of SOD1 proteins, but **Figure 3B** shows similar expression levels of SOD1 proteins in *E. coli* examined here. In those mutant SOD1s forming disulfide-linked oligomers (G37R, G41S, G85R, G93R, C111Y, but not L126X), small amounts of proteins were also detected in a soluble fraction with an intramolecular disulfide bond. Notably, SOD1 with H46R and H80R mutations have been shown to exhibit thermostability comparable to that of the wild-type protein (Rodriguez et al., 2005; Vassall et al., 2011); therefore, fALS-causing mutations that significantly destabilize a native structure of SOD1 favor the formation of disulfide-crosslinked oligomers under oxidizing environment.

### MUTANT SOD1 FORMS AMYLOID-LIKE FIBRILLAR AGGREGATES IN *E. coli*

To further characterize the SOD1 aggregates formed in *E. coli*, a protocol to purify those insoluble aggregates were first established, in which insoluble fractions of *E. coli* lysates were extensively washed with 1 M NaCl, 1% Sarkosyl, and then acetone (see Materials and Methods). When purified aggregates of SOD1(G37R) were reacted with iodoacetamide to protect free thiol groups and then analyzed in non-reducing SDS-PAGE, disulfide-linked oligomers were found to be successfully isolated from *E. coli* SHuffle$^{TM}$ (**Figure 4A**, +IA). In contrast, purified aggregates of SOD1(G37R)

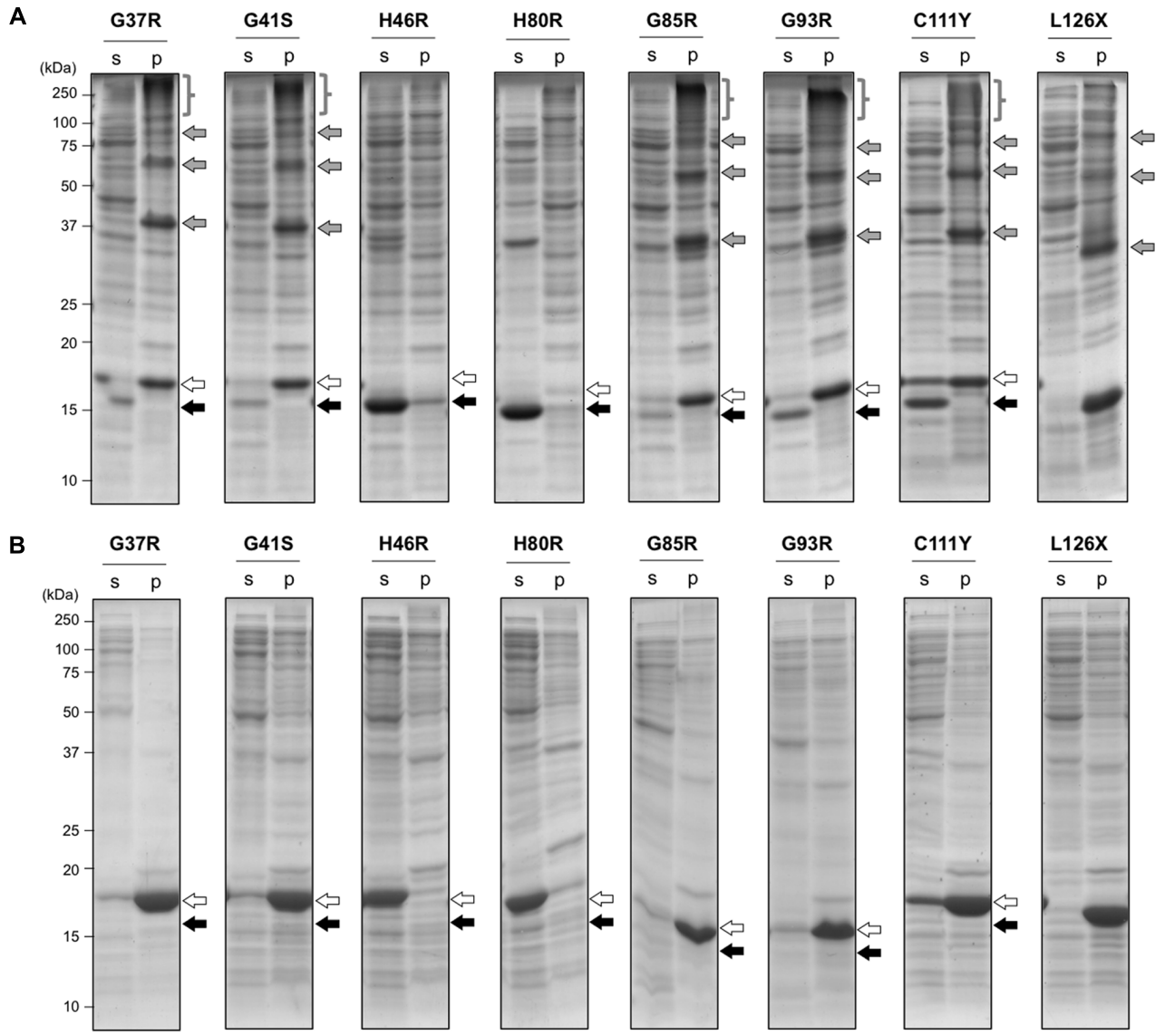


**FIGURE 3 | Aggregation of mutant SOD1 in *E. coli* SHuffle$^{TM}$ associates with the formation of inter-molecular disulfide crosslinks.** *E. coli* SHuffle$^{TM}$ was transformed with pET15b harboring human SOD1 cDNA with indicated fALS mutations, and the protein expression was induced by IPTG. Cell lysates were fractionated into soluble supernatant (s) and insoluble pellets (p), treated with iodoacetamide, and then analyzed with **(A)** non-reducing or **(B)** reducing SDS-PAGE by using a 15% polyacrylamide gel. White (SOD1$^{S-S}$) and black (SOD1$^{SH}$) arrows at the right side of the gel image indicate positions of bands corresponding to SOD1 with and without a disulfide bond, respectively. In addition, gray arrows and braces in **(A)** represent the disulfide-linked SOD1 oligomers, which disappeared in reducing SDS-PAGE **(B)**.

from *E. coli* BL21 were mainly composed of disulfide-reduced monomers with slight contamination of dimers (**Figure 4A**, +IA). Furthermore, after extensive washes, both SOD1(G37R) aggregates from BL21 and SHuffle$^{TM}$ produced a single band in reducing SDS-PAGE (+β-ME), supporting successful purification of the SOD1 aggregates.

Aggregates of SOD1 *in vitro* have been shown to have structural features similar to those of amyloid, which are characterized by fibrillar morphologies with β-sheet-rich structures (Furukawa et al., 2008). As shown in **Figure 4B**, both SOD1(G37R) aggregates purified from *E. coli* BL21 and SHuffle$^{TM}$ were found to increase the thioflavin T fluorescence but with distinct intensity. In addition, those SOD1(G37R) aggregates were found to red-shift an electronic absorption spectrum of Congo red (**Figure 4C**). These tinctorial changes of thioflavin T and Congo red have been typically observed in protein aggregates with amyloid-like properties (Klunk et al., 1999; LeVine, 1999). It is, therefore, possible that SOD1(G37R) forms amyloid-like aggregates both in reducing (BL21) and oxidizing (SHuffle$^{TM}$) environment, but its molecular structure might be dependent upon the redox environment where the aggregates form.

While amyloid-like aggregates generally exhibit fibrillar morphologies, SOD1(G37R) aggregates purified from insoluble inclusions in *E. coli* displayed not fibrils but amorphous, large lump-like structures under an electron microscope (**Figures 4D,E**, left panels). These structures are considered to be attained with aggregation of monomeric and disulfide-linked multimeric SOD1 proteins through SDS-sensitive interactions. When these inclusions were briefly treated with a non-specific protease, Proteinase K, however, a protease-resistant core of inclusions became exposed and was found to exhibit fibrillar morphologies with *approx.* 5.5 nm of the diameter (**Figures 4D,E**, right panels). While morphological differences of SOD1(G37R) aggregates were not clear between *E. coli* BL21 and SHuffle$^{TM}$, exposure of fibrillar structures by treatment with proteases have been previously reported in *E. coli* inclusions of Aβ peptide (Morell et al., 2008) and HET-s (Sabate et al., 2009), which are known to be fibrillogenic. Taken together, therefore, this study successfully reproduces fibrillar aggregation of mutant SOD1 proteins in the cytoplasm of *E. coli* and further implies that intracellular redox environment could modulate the properties of SOD1 aggregates by changing its thiol-disulfide status.

## IMPLICATIONS TO PATHOLOGIES OF SOD1-RELATED fALS CASES

More than 70% of intracellular SOD1 has been detected in the cytoplasm (Chang et al., 1988), which is normally kept as reducing environment by maintaining high concentrations of reduced glutathione (Hwang et al., 1992). Indeed, mutant SOD1 proteins are abnormally accumulated in the cytoplasm as Lewy body-like hyaline inclusions (Shibata et al., 1996), and those insoluble forms of mutant SOD1 have been characterized as a disulfide-reduced state in SOD1-fALS model mice (**Figure 5**, left; Jonsson et al., 2006; Karch et al., 2009). These observations are consistent with the *in vitro* findings that inability to form the disulfide

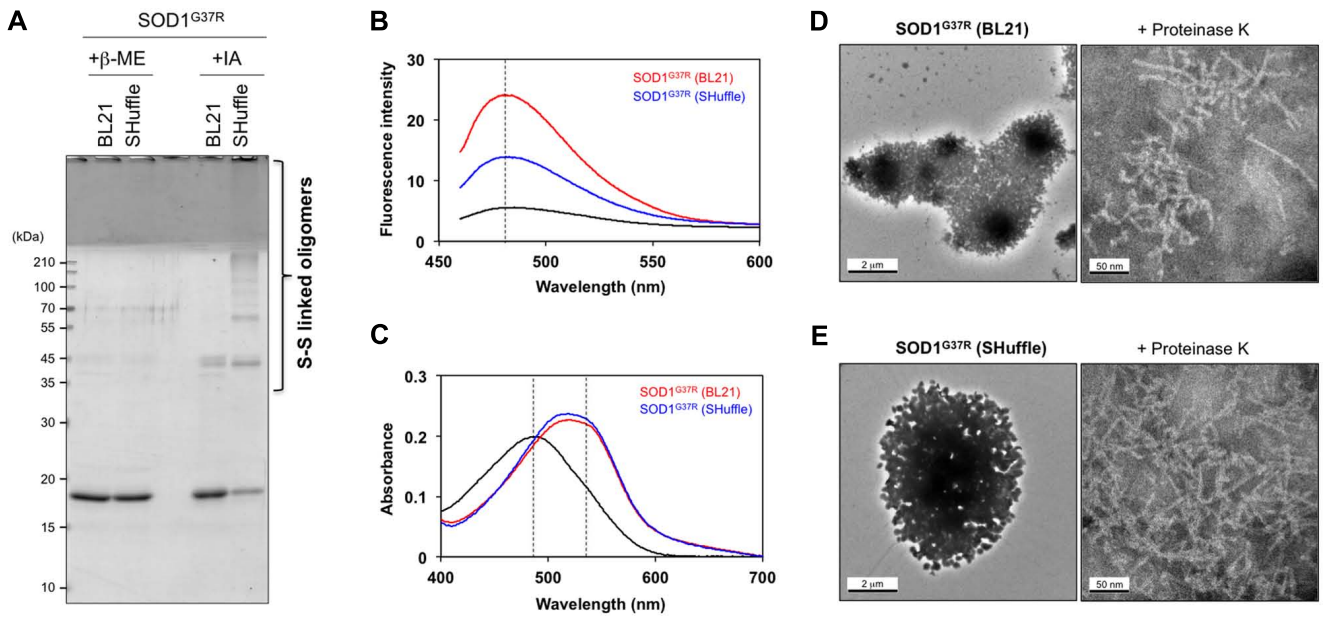


**FIGURE 4 | Amyloid-like characters of SOD1 aggregates purified from insoluble inclusions in *E. coli*. (A)** SOD1(G37R) aggregates purified from insoluble inclusions in *E. coli* were reacted with iodoacetamide for protection of free thiol groups and analyzed with reducing (+β-ME) and non-reducing (+IA) SDS-PAGE. Disulfide-crosslinked oligomers were identified in SOD1(G37R) aggregates purified from insoluble inclusions in *E. coli* SHuffle$^{TM}$ but not in BL21(DE3). **(B, C)** Tinctorial properties of SOD1(G37R) aggregates purified from *E. coli* BL21 and SHuffle$^{TM}$ (red and blue curves, respectively) were examined by **(B)** fluorescence of thioflavin T and **(C)** absorption of Congo red. Black curves represent **(B)** fluorescence spectrum of thioflavin T and **(C)** absorption spectrum of Congo red without addition of SOD1 aggregates. **(D, E)** Electron micrograms of SOD1(G37R) aggregates purified from *E. coli* **(D)** BL21 and **(E)** SHuffle$^{TM}$. SOD1(G37R) aggregates exhibit large, amorphous morphologies (left panels), while fibrillar structures become evident after brief treatment of aggregates with Proteinase K (right panels). A bar represents 2 μm (left panels) or 50 nm (right panels).

bond significantly increases the propensities of SOD1 for fibrillar aggregation (Furukawa et al., 2008). In reducing environment of the cytoplasm, the disulfide bond in SOD1 is introduced by a copper chaperone protein, CCS (Furukawa et al., 2004), and appears to be protected from the reductive cleavage by its burial at the dimer interface. Some fALS mutations would hence disturb the interaction with CCS and/or facilitates the monomerization of SOD1; thereby, stability of the disulfide bond becomes decreased, which then triggers the fibrillar aggregation of disulfide-reduced SOD1 in the cytoplasm (the disulfide-reduction model; Toichi et al., 2013).

In contrast to such disulfide-reduction model of SOD1 aggregation, little accumulation of cytoplasmic inclusions has been observed in some fALS model mice expressing mutant SOD1, albeit with significant mitochondrial pathologies (Nagai et al., 2001; Watanabe et al., 2001). *Approximately* 3% of total SOD1 is known to localize at the IMS of mitochondria (Okado-Matsumoto and Fridovich, 2001), where the redox potential is significantly more oxidizing than that of the cytoplasm (Hu et al., 2008). Furthermore, mitochondria of a motor neuron, which is the most damaged cell in fALS cases (Bruijn et al., 2004), have been reported to provide more oxidizing environment than those of the other types of cells (Ferri et al., 2006). Given that reduction of a disulfide bond in SOD1 is required for its transport into mitochondrial IMS (Field et al., 2003), disulfide-reduced SOD1 just after transported is considered to experience significantly oxidizing environment of motorneuronal IMS (**Figure 5**, right). Transported SOD1 polypeptide then folds into the native three-dimensional conformation, and the intramolecular disulfide bond is introduced by mitochondrial CCS. The folding process of SOD1 is, however, deterred by fALS-causing mutations (Nordlund and Oliveberg, 2006; Bruns and Kopito, 2007), which would increase the chance to form aberrant disulfide crosslinks under oxidizing environment of the mitochondrial IMS (**Figure 5**, right). Indeed, SOD1 oligomers crosslinked *via* disulfide bonds have been observed in mitochondria isolated from spinal cords of fALS-model mice expressing mutant SOD1 (Deng et al., 2006). Also in cultured motorneuronal cells, disulfide-crosslinked oligomers of mutant SOD1 have been reproduced in mitochondria but not in cytoplasm (Ferri et al., 2006). In the oxidizing environment of organelles, therefore, disulfide-reduced mutant SOD1 is highly prone to crosslinking *via* abnormal disulfide bonds, which will not occur under reducing environment of the cytosol.

So far, it has been well known that misfolding/aggregation of mutant SOD1 occurs both in cytoplasm and mitochondrial IMS; however, any possible differences in the molecular properties of SOD1 aggregates between those two cellular compartments have been hardly noticed. An intracellular folding process of protein molecules into their native conformations has been well known to be significantly affected or even controlled by chaperone proteins, but motorneurons are equipped with different sets of chaperones from those in *E. coli*; therefore, a pathological process of SOD1 aggregation in SOD1-related fALS patients, which in general gradually proceed over several decades, would not be precisely reproduced in the *E. coli* overexpression system examined here. Nonetheless, distinct forms of misfolded/aggregated SOD1, especially in terms of their thiol-disulfide status, appear to be possible that depend upon the redox environment surrounding SOD1 proteins (**Figure 5**). While it is further required to test if

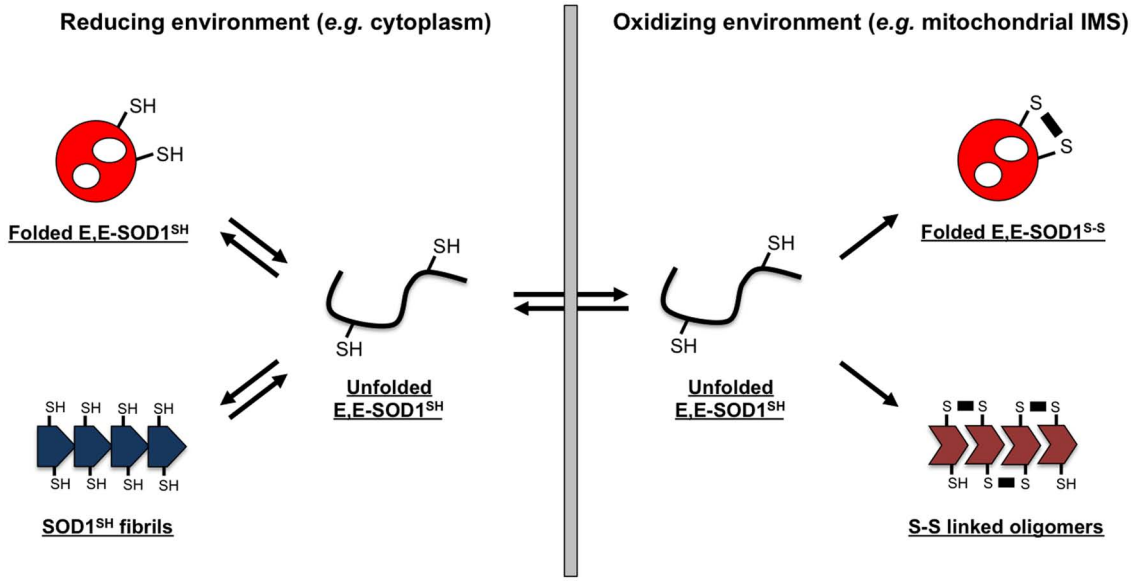


**FIGURE 5 | Schematic representation of a redox-dependent SOD1 folding/misfolding model.** SOD1 exists as an apo and disulfide-reduced state (E,E-SOD1$^{SH}$) and tends to form fibrillar aggregates (SOD1$^{SH}$ fibrils) in reducing environment of the cytosol; in contrast, oxidizing environment stabilizes a soluble, folded state of SOD1 by introduction of an intramolecular disulfide bond (E,E-SOD1$^{S-S}$). Many fALS mutations are considered to destabilize the structure of E,E-SOD1$^{SH}$, increasing the fraction of misfolded state. In such a situation, oxidizing environment favors the formation of SOD1 oligomers cross-linked via abnormal disulfide bonds. Depending upon redox environment where SOD1 exists, several distinct forms in SOD1 aggregates are hence possible.

distinct properties of those SOD1 aggregates include toxicities to motorneurons, the redox-dependent aggregation of mutant SOD1 proteins would be one of the molecular mechanisms describing pathological heterogeneity observed in SOD1-related fALS cases.

## MATERIALS AND METHODS

### PLASMIDS, *E. coli* STRAINS AND PROTEIN EXPRESSION

A vector, pET15b (Novagen), was used for the construction of plasmids expressing human SOD1 without any tags; a SalI site was first introduced between BamHI and Bpu1102I sites of pET15b, and cDNA of human SOD1 was then cloned between NcoI and SalI site. Mutations were introduced by an inverse PCR method using KOD-FX-neo DNA polymerase (TOYOBO), and all constructs used in this study were confirmed by DNA sequencing. Competent cells of *E. coli*, BL21(DE3; New England Biolabs) and SHuffle$^{TM}$ T7 Express lysY (New England Biolabs), were transformed with a plasmid. *E. coli* cells harboring a plasmid were cultured in Luria-Broth media containing 50 mg/L ampicillin (LB/Amp) by shaking at 200 rpm, and the expression of SOD1 proteins was induced with 1 mM isopropyl 1-thio-β-D-galactopyranoside at 37°C for 6 h. To test effects of $Zn^{2+}$ ions on SOD1 aggregation, 1 mM $ZnSO_4$ was further added at the induction of protein expression.

### ELECTROPHORETIC ANALYSIS OF SOD1 PROTEINS EXPRESSED IN *E. coli*

*E. coli* cells cultured in 5 mL LB/Amp media were collected by centrifugation (2,000 × *g*, 10 min) and lysed by ultrasonication in 100 μL of PBS with 2% Triton X-100 and 100 mM iodoacetamide. Soluble supernatant and insoluble pellets were obtained with centrifugation of cell lysates (20,000 × *g*, 10 min), and insoluble pellets were further solubilized by ultrasonication in 100 μL of PBS with 2% SDS and 100 mM iodoacetamide. The soluble supernatant and the re-solubilized insoluble pellets were incubated at 37°C for 30 min, which ensures the protection of thiol groups by modification with iodoacetamide. 0.8 (BL21) or 1.5 (SHuffle$^{TM}$) μL of the samples were then mixed with an SDS-PAGE sample buffer, and 10% β-mercaptoethanol was further added for reducing SDS-PAGE. The samples were boiled at 100°C for 5 min, electrophoresed on a 15% SDS-PAGE gel, and then stained with Coomassie brilliant blue.

### PURIFICATION OF SOD1 AGGREGATES FROM *E. coli*

Insoluble pellets obtained by cell lysis in PBS/2% Triton X-100 were re-suspended in 1 M NaCl/$H_2O$ with ultrasonication and centrifuged at 20,000 × *g* for 10 min to collect insoluble pellets. After washed again by 1 M NaCl, the insoluble pellets were washed three times with PBS/1% Sarkosyl and then washed with cold acetone. After dried up with SpeedVac (Savant), the pellets were re-suspended in 100 mM Na-Pi/100 mM NaCl/5 mM EDTA, pH 7.4 with ultrasonication. Purified pellets were analyzed by SDS-PAGE after being re-dissolved in PBS/2% SDS/100 mM iodoacetamide and loaded on a 15% polyacrylamide gel in the presence and absence of 10% β-mercaptoethanol (**Figure 4A**). Monomer-based concentration of SOD1 aggregates purified from inclusions was spectroscopically determined from the absorbance at 280 nm in 6 M guanidine hydrochloride using 5,500 $cm^{-1}M^{-1}$ as an extinction coefficient.

### CHARACTERIZATION OF SOD1 AGGREGATES FROM *E. coli*

To test if SOD1 aggregates exhibit amyloid-like tinctorial properties, thioflavin T assay was performed. 10 μM (monomer-based) SOD1 aggregates purified from *E. coli* inclusions were mixed with 25 μM thioflavin T in 100 mM Na-Pi/100 mM NaCl/5 mM EDTA, pH 7.4, and fluorescence spectra excited at 442 nm were measured from 460 to 600 nm by using F-4500 (Hitachi). Congo red assay was also performed to test amyloid-like properties in SOD1 aggregates. 3 μM (monomer-based) SOD1 aggregates purified from *E. coli* inclusions were mixed with 5 μM Congo red in 100 mM Na-Pi/100 mM NaCl/5 mM EDTA, pH 7.4, and absorption spectra were measured by UV-2400PC (Shimadzu).

Morphologies of SOD1 aggregates formed in *E. coli* were examined by electron microscopy. Insoluble inclusions purified from *E. coli* were adsorbed on STEM100Cu grids coated by elastic carbon (Okenshoji), washed with water, and then negatively stained with 2% phosphotungstic acid. Images were obtained using an electron microscope (Tecnai$^{TM}$ Spirit, FEI). 500 μL of *approximately* 100 μM (monomer-based) SOD1 aggregates purified from insoluble *E. coli* inclusions were mixed with 2 μL of 5 mg/mL Proteinase K and incubated at 37°C for 10 min. After ultracentrifuged at 110,000 × *g* for 15 min, insoluble pellets were washed with 500 μL of water and again ultracentrifuged. Resultant pellets were re-suspended in 100 μL of water and observed by an electron microscope as mentioned above.

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# An emerging role for misfolded wild-type SOD1 in sporadic ALS pathogenesis

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Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disorder that targets motor neurons, leading to paralysis and death within a few years of disease onset. While several genes have been linked to the inheritable, or familial, form of ALS, much less is known about the cause(s) of sporadic ALS, which accounts for ∼90% of ALS cases. Due to the clinical similarities between familial and sporadic ALS, it is plausible that both forms of the disease converge on a common pathway and, therefore, involve common factors. Recent evidence suggests the Cu,Zn-superoxide dismutase (SOD1) protein to be one such factor that is common to both sporadic and familial ALS. In 1993, mutations were uncovered in SOD1 that represent the first known genetic cause of familial ALS. While the exact mechanism of mutant-SOD1 toxicity is still not known today, most evidence points to a gain of toxic function that stems, at least in part, from the propensity of this protein to misfold. In the wild-type SOD1 protein, non-genetic perturbations such as metal depletion, disruption of the quaternary structure, and oxidation, can also induce SOD1 to misfold. In fact, these aforementioned post-translational modifications cause wild-type SOD1 to adopt a "toxic conformation" that is similar to familial ALS-linked SOD1 variants. These observations, together with the detection of misfolded wild-type SOD1 within human post-mortem sporadic ALS samples, have been used to support the controversial hypothesis that misfolded forms of wild-type SOD1 contribute to sporadic ALS pathogenesis. In this review, we present data from the literature that both support and contradict this hypothesis. We also discuss SOD1 as a potential therapeutic target for both familial and sporadic ALS.

**Keywords: amyotrophic lateral sclerosis (ALS), sporadic amyotrophic lateral sclerosis, SOD1, protein misfolding, immunotherapy**

## INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is the most common motor neuron disease and is clinically characterized by the degeneration of motor neurons in the brain and spinal cord, culminating in paralysis and death within 2–5 years. The only available treatment is riluzole, which in the best cases extends survival by only a few months (Glicksman, 2011). In 1993, mutations in the *SOD1* gene encoding Cu,Zn superoxide dismutase-1 were reported as the first genetic link to familial, or inherited, forms of ALS (FALS) (Rosen et al., 1993). Because of the high incidence of *SOD1* mutations, which account for 20–25% of FALS cases, *SOD1* has been one of the most intensely studied genes in the ALS field and continues to be a primary therapeutic target (Bosco and Landers, 2010).

Much of what we understand about the pathomechanisms of ALS is based on *in vivo* studies with transgenic rodent models expressing FALS-linked SOD1 variants. These FALS-SOD1 animal models recapitulate many key features of the human disease, including motor neuron degeneration, paralysis and shortened life-span (Turner and Talbot, 2008). Moreover, FALS-SOD1 animal models reveal the complex nature of this disease, which involves oxidative stress, a loss of proteostasis (i.e., protein aggregation with defective protein clearance), mitochondrial dysfunction, impaired axonal transport, and glutamate excitotoxicity (Rothstein, 2009). While motor neurons are the primary target in ALS, ALS may actually represent a non-cell autonomous disorder for which glia play an active role (Ilieva et al., 2009). Despite decades of research on FALS-SOD1 *in vitro* and *in vivo*, the exact mechanism of SOD1 in ALS pathogenesis remains unknown (Pasinelli and Brown, 2006; Ling et al., 2013). However, as will be discussed throughout this review, a substantial body of literature points to a gain of toxic function for FALS-SOD1 that stems, at least in part, from SOD1 misfolding.

In contrast to FALS, much less is known about the etiology of sporadic ALS (SALS), which accounts for 90% of ALS cases. Pathological aggregates composed of the TAR DNA-binding protein 43 (TDP-43) are detected in CNS tissues for a majority of SALS cases (Neumann et al., 2006), providing strong evidence for an association of misfolded TDP-43 with ALS pathogenesis (Xu, 2012). Mutations in TDP-43 have also been linked to SALS and FALS (Sreedharan et al., 2008), further establishing a role for this protein in disease. Another RNA-binding protein called fused in sarcoma/translocated in liposarcoma (FUS/TLS) has also been linked to FALS and SALS (Kwiatkowski et al., 2009; Vance et al., 2009). Although the association of FUS/TLS with pathological aggregates in SALS has been reported (Deng et al., 2010), this association is not as common as for TDP-43.

The inheritable nature of FALS facilitates the identification of causal genes, because many FALS-linked genes, such as *SOD1*, *TDP-43* and *FUS/TLS*, are autosomal dominant and segregate according to Mendelian genetics within an ALS family. In an effort to identify genetic susceptibility factors associated with SALS, several genome-wide association studies (GWAS) have been performed. However, by and large these studies have failed to generate confirmed SALS-susceptibility genes (Bosco and Landers, 2010). More recently, repeat expansions within the genome that are associated with different forms of ALS have been identified. Hexanucleotide repeat expansions in the *C9ORF72* gene were linked to familial frontotemporal lobar degeneration (FTLD)/ALS, FALS as well as to SALS (Dejesus-Hernandez et al., 2011; Renton et al., 2011), making this gene the most common factor in all of ALS. Moreover, repeat expansions in ataxin-2 (*ATXN2*) represent a susceptibility factor in SALS (Elden et al., 2010). Despite these advances in the genetics of SALS, the etiology remains unknown for a majority of SALS cases, likely a reflection of the complex nature of SALS. In fact, SALS may arise from genetic as well as environmental and behavioral factors. Smoking, diet, excessive exercise, injury and exposure to environmental toxins have all been implicated in SALS (D'Amico et al., 2013), although none have been shown to unequivocally cause disease. Although FALS is inheritable and SALS is not, the fact that FALS and SALS are clinically indistinguishable raises the possibility that they do in fact emerge from a common source and/or involve similar toxicity factors.

Recent evidence supports SOD1 as a toxic factor that is common to a subset of both FALS and SALS. This evidence is largely based on the observation that aberrant conformations of WT SOD1, induced by oxidation, demetallation and other altered post-translational modifications, cause WT SOD1 to acquire the same toxic functions that are observed for FALS-associated SOD1 variants (Ezzi et al., 2007; Bosco et al., 2010; Guareschi et al., 2012). Moreover, by employing conformation specific antibodies that are selective for SOD1 only when it is mutated or has altered post-translational modifications, misfolded "mutant-like" WT SOD1 has been detected in human post-mortem tissues from SALS individuals (Bosco et al., 2010; Forsberg et al., 2010, 2011; Pokrishevsky et al., 2012), suggesting that such species are in fact pathogenic. The concept that critical proteins can become pathogenic via both germline mutations and non-Mendelian post-translational modifications is not novel, but rather has strong precedence in neurodegeneration with examples including the α-synuclein (Beyer and Ariza, 2013), tau (Mandelkow et al., 1996) and TDP-43 proteins (Arai et al., 2010). The concept that WT SOD1 could play a role in SALS is controversial, since not all conformation specific antibodies employed to date have detected aberrant WT SOD1 species in SALS (Furukawa, 2012; Ling et al., 2013). However, such a role should be strongly considered and fully explored as it has important therapeutic implications for treating both familial and sporadic forms of ALS.

## NORMAL PROPERTIES AND CELLULAR FUNCTIONS OF SOD1

Long before SOD1 was identified as a causative factor in FALS (Rosen et al., 1993), the anti-oxidizing and catalytic properties of native SOD1 were being investigated (McCord and Fridovich, 1969). While the role of SOD1 as an anti-oxidizing enzyme is well known and accepted, the role of SOD1 as a signaling molecule has been relatively underappreciated. Herein we discuss what is known regarding the normal functions of SOD1 in the context of both anti-oxidation and signaling. The normal physiological properties of SOD1 are discussed in order to establish a foundation for the following sections that describe how both FALS-linked mutations and post-translational modifications alter the structure and function of the protein.

### SOD1: LOCATION, ACTIVITY, AND STRUCTURE

Copper,zinc- superoxide dismutase-1 (SOD1) is a member of the human SOD family of proteins, which also includes SOD2 and SOD3. While all three proteins function as anti-oxidizing enzymes that catalyze the dismutation of superoxide radicals ($O_2\bullet^-$) to hydrogen peroxide ($H_2O_2$), they are distinct proteins with unique characteristics (Zelko et al., 2002). SOD1 is highly abundant, comprising ∼1% of total protein in the cell (Pardo et al., 1995), and resides mainly in the cytosol with some degree of localization in the mitochondrial inner membrane space (Fukai and Ushio-Fukai, 2011). The mitochondrion is also home to SOD2, which is localized to the mitochondrial matrix. In contrast to both SOD1 and SOD2, SOD3 is predominately located outside the cell in the extracellular matrix. Other key differences amongst the SOD proteins include their quaternary structures and mechanism of superoxide dismutation: SOD1 is a homodimer while SOD2 and SOD3 are homotetrameric proteins; SOD1 and SOD3 catalyze the dismutation of $O_2\bullet^-$ through the alternate reduction and reoxidation of $Cu^{2+}$, whereas SOD2 utilizes manganese (Mn) as a redox active transition metal for this purpose. The role of SOD in FALS, which will be discussed in detail below, is specific to the SOD1 isoform as there is no compelling evidence supporting the involvement of either SOD2 or SOD3 in FALS pathogenesis (Tomkins et al., 2001).

While coordination of copper to SOD1 is required for dismutation of $O_2\bullet^-$, other post-translational modifications, such as $Zn^{2+}$ coordination (Kayatekin et al., 2008) and disulfide oxidation, help create a mature and structurally stable protein. The 32 kDa homodimeric SOD1 protein adopts an eight-stranded Greek key beta-barrel structural motif (**Figure 1A**). Two functional loops are present in SOD1: the electrostatic loop that guides superoxide into the redox active site where $Cu^{2+}$ is located and the zinc-binding loop. All tolled, each SOD1 molecule coordinates two copper and two zinc atoms, one of each per subunit. A unique functional feature of SOD1 is the presence of an intra-subunit disulfide bond between Cys57 and Cys146 (C57–C146), which is unusual for proteins that reside in the highly reducing environment of the cytosol. Both copper coordination and formation of C57-C146 is facilitated by the cytosolic copper carrier protein CCS (copper chaperone for SOD1) (Furukawa et al., 2004; Seetharaman et al., 2009). A recent study utilizing both electrospray ionization mass spectrometry (ESI-MS) and nuclear magnetic resonance (NMR) spectroscopy support a step-wise model for SOD1 maturation: (i) SOD1 is loaded with Zn, (ii) heterodimerization between SOD1 and CCS, (iii) Cu is transferred from CCS to SOD1, (iv) C57–C146 is formed, and (v) SOD1 homodimerization (Banci et al., 2012). Together these

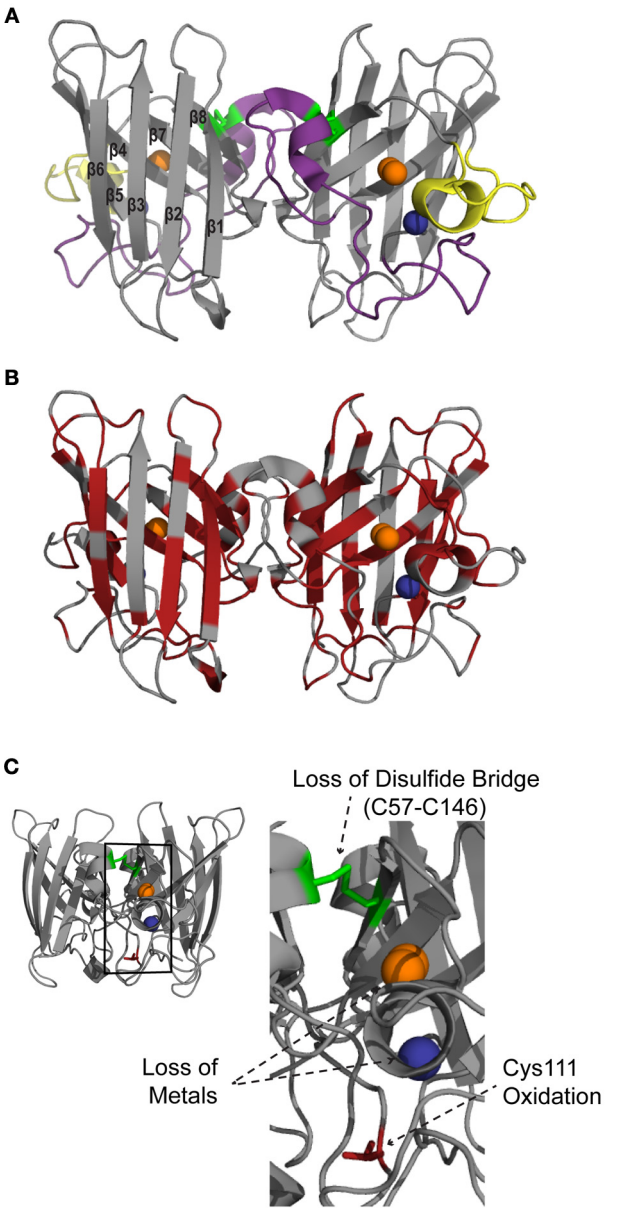


**FIGURE 1 | Structural features of SOD1.** SOD1 is a homodimer with one copper (orange) and one zinc (blue) per subunit (PDB:2C9V). **(A)** SOD1 consists of eight beta sheets that form the beta barrel core. The major functional loops are the zinc binding loop (purple; residues 49–81) and electrostatic loop (yellow; residues 124–139). An intramolecular disulfide bond (C57–C146; green) stabilizes the protein structure. **(B)** Mapping the ALS-linked mutations (red) onto the structure of SOD1 illustrates that these mutations are present throughout the entire protein. **(C)** Alterations to normal post-translational modifications, such as loss of the disulfide bridge (C57–C146; green), demetallation and monomerization (not shown), as well as aberrant modifications such as oxidation of amino acid side chains (shown for Cys 111; red), cause WT SOD1 to misfold.

post-translational modifications produce a highly stable protein, as evidenced by a high melting temperature ($T_m$) of ~92°C and resistance to denaturation in both 6 M GdmCl and 4% SDS (Forman and Fridovich, 1973; Bartnikas and Gitlin, 2003). Demetalation of SOD1 and/or reduction of C57–C146 destabilizes the protein and drastically decreases the melting temperature (Forman and Fridovich, 1973; Furukawa and O'Halloran, 2005). As will be discussed below, these post-translational modifications are compromised by both FALS-linked mutations and oxidation, which in turn destabilize SOD1 in the context of disease.

## SOD1 IN SIGNAL TRANSDUCTION

The physiological relevance of SOD1 catalysis extends beyond oxidative stress protection. In fact, SOD1 catalysis plays a key role in signal transduction, a function that is largely underappreciated compared to its role as an anti-oxidizing enzyme (**Figure 2**). For instance, $H_2O_2$ generated by SOD1 can reversibly and specifically react with proteins, generally by oxidizing Cys residues. Cys oxidation in turn alters the biochemical and functional properties of those proteins in a redox dependent manner (Georgiou, 2002). A variety of signal transduction pathways are modulated by $H_2O_2$, including but not limited to gene expression, cell proliferation, differentiation and death (Rhee, 2006; Brown and Griendling, 2009). NADPH oxygenases (Nox) function as upstream regulators of these signal transduction pathways through the production of $O_2\bullet^-$, which is either converted to $H_2O_2$ spontaneously or catalytically by SOD1. SOD1 comes into close proximity with Nox2-derived $O_2\bullet^-$ at the surface of endosomes in response to proinflammatory cytokines (Harraz et al., 2008). A report by Harraz et al. (2008) demonstrated that SOD1 not only acts downstream of Nox2 but can also modulate Nox function through an interaction with Rac1. SOD1 directly binds and stabilizes the active form of Rac1 in its GTP-bound state, leading to Nox2 activation and $O_2\bullet^-$ production. Interestingly, $H_2O_2$ generated by SOD1 serves as a negative feedback of Nox2 activity: $H_2O_2$ induces the dissociation of the SOD1/Rac1 complex, thereby inactivating Rac1 and Nox2 (Harraz et al., 2008). The mechanism for how $H_2O_2$ disrupts the interaction between SOD1 and Rac1 has not been elucidated. One possibility is that the $H_2O_2$ generated by SOD1, which is in close proximity to Rac1, oxidatively modifies Cys residues within Rac1 in such a way that disrupts the SOD1/Rac1 binding interaction.

Another example of SOD1 redox-sensing and signaling activity is in the context of respiratory repression, which occurs during aerobic fermentation in proliferating cells, including in the context of some cancers. In *Saccharomyces cerevisiae*, a loss of SOD1 activity impairs respiratory repression (Sehati et al., 2011). SOD1 was shown to modulate respiratory repression through binding and stabilizing Yck1p and Yck2p, two casein kinase 1-gamma homologs in yeast that inhibit respiration (Reddi and Culotta, 2013). The authors speculate that SOD1 stabilizes Yck1p/Yck2p through the action of the reaction product $H_2O_2$, where oxidative modification of lysine residues within Yck1p/Yck2p by $H_2O_2$ prevents their ubiquitination and degradation by the ubiquitin-proteasome system. Therefore, both the Rac1/Nox2 and Yck1p/Yck2p interactions with SOD1 demonstrate that the catalytic action of SOD1 can direct the modification and activity of specific protein substrates. In this manner, SOD1 catalyzes a "molecular redox switch" that ultimately controls protein function and signaling, much like phosphorylation.

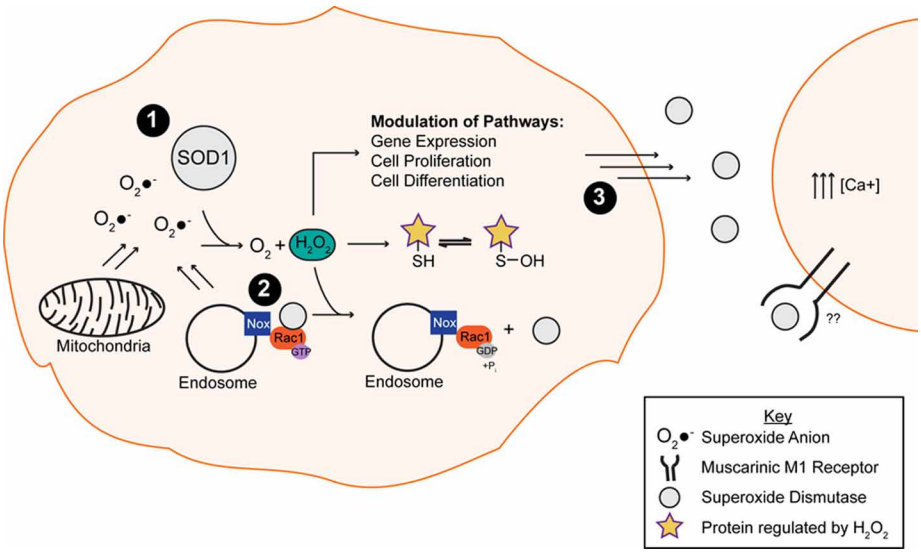


**FIGURE 2 | The putative normal function of the native WT SOD1 protein.** (**1**) SOD1 is responsible for converting the toxic superoxide anion ($O_2^{\bullet-}$) into oxygen ($O_2$) and hydrogen peroxide ($H_2O_2$), the latter of which is involved in the modulation of multiple pathways through oxidation of exposed thiols. (**2**) SOD1 binds and stabilizes Rac1 in its active, GTP bound state, resulting in Nox2 (Nox) activation and superoxide production. The hydrogen peroxide by-product of the dismutase reaction of SOD1 and superoxide anion promotes the disassociation of SOD1 from the Rac1 complex, resulting in Nox inactivation. **(3)** Secreted SOD1 plays a role in extracellular signaling processes. The presence of extracellular SOD1 leads to an increase in intracellular calcium via a mechanism involving the phospholipase C/protein kinase C pathway.

While the catalytic activity of SOD1 is required for these interactions, it is not simply to remove $O_2^{\bullet-}$ from circulation, but rather to modulate signaling pathways in a redox sensitive manner. One can imagine that redox signaling needs to be regulated, as an excess of either $O_2^{\bullet-}$ or $H_2O_2$ would have deleterious effects on the cell. The levels of $H_2O_2$ in the cell are further controlled by the antioxidant enzymes catalase, peroxiredoxins and glutathione peroxidases (Fukai and Ushio-Fukai, 2011), which convert $H_2O_2$ into water and oxygen.

Extracellular SOD1 has also been shown to play a role in signaling. Although SOD1 is predominately localized to the cytoplasm, multiple reports have demonstrated that SOD1 is secreted (Mondola et al., 1996, 1998, 2003; Cimini et al., 2002; Turner et al., 2005). The presence of extracellular SOD1 can in turn increase intracellular calcium levels (Mondola et al., 2004), a phenomena shown to have neuroprotective effects on cerebellar granular neurons exposed to a dopaminergic toxin (Polazzi et al., 2012). This increase in intracellular calcium results from SOD1 activating the phospholipase C/protein kinase C pathway, a pathway implicated in calcium homeostasis (Mondola et al., 2004) through a mechanism involving signal transduction of the muscarinic acetylcholine M1 receptor (M1) (Damiano et al., 2013). Moreover, M1 activation in response to extracellular SOD1 jumpstarts downstream pathways such as the extracellular regulated protein kinase (ERK 1/2) and the Akt signaling cascades (Damiano et al., 2013). Unlike the previous SOD1 signaling pathways discussed above, the activation of the M1 receptor does not appear to be dependent upon the production of $O_2^{\bullet-}$, as the ROS scavenger N-acetylcysteine did not alter the signaling effect of SOD1 in this context.

## SOD1 MISFOLDING: LESSONS FROM FAMILIAL ALS

For the past 20 years, mutant-SOD1 has been the most intensely studied molecule in the ALS field. Studies in animal and cell culture models, as well as extensive biochemical and biophysical analyses of recombinant mutant-SOD1 proteins have collectively revealed a gain-of-toxic mechanism for mutant-SOD1 in ALS that is linked to its propensity to misfold. Aberrantly modified WT SOD1 adapts a conformation and toxic nature much like FALS-linked SOD1 mutants, and is therefore proposed as a pathogenic factor in SALS. Below we introduce what is known about the structure and aberrant properties of FALS-SOD1.

### FALS-LINKED SOD1 MUTATIONS: EFFECT ON STRUCTURE AND CONFORMATION

Currently, 171 mutations have been identified within SOD1 that are linked to ALS (http://alsod.iop.kcl.ac.uk/) (Abel et al., 2012). Approximately 20–25% of FALS cases and 6% of all ALS cases are caused by mutations in *SOD1* (Pasinelli and Brown, 2006). The majority of these mutations (>80%) result in amino acid substitutions while the remaining lesions are a combination of insertions, polymorphisms, and deletions. FALS-linked mutations are not localized to one portion of SOD1, but rather span the entire protein (**Figure 1B**). Moreover, relatively conservative amino acid substitutions within SOD1 can cause ALS, suggesting that even minor alterations severely affect SOD1 structure and/or function. Much effort has been focused on determining the common "toxic" feature within SOD1 that is induced by all of these ALS-linked mutations. Except for the mutations that directly interfere with metal coordination, as copper coordination is required for catalytic activity, many ALS-linked mutations

have no effect on SOD1 dismutase activity (Borchelt et al., 1994; Bowling et al., 1995; Hayward et al., 2002; Potter and Valentine, 2003). The effect of ALS-linked mutations on SOD1 signaling activity remains largely unexplored. However, in the context of Nox2 signaling, FALS-linked SOD1 mutations were shown to alter the SOD1/Rac1 interaction, resulting in persistent activation of Nox2 and abnormally high levels of ROS (Harraz et al., 2008).

A general feature of FALS-linked SOD1 mutants is that they are destabilized and exhibit reductions in $T_m$ relative to WT SOD1 (Stathopulos et al., 2006; Vassall et al., 2006). In fact, FALS-linked SOD1 mutants are further destabilized when normal post-translational modifications such as Cu/Zn coordination and C57–C146 oxidation are impaired (Furukawa and O'Halloran, 2005; Rodriguez et al., 2005; Kayatekin et al., 2010; Svensson et al., 2010), demonstrating interconnection between normal post-translational modifications and the stability of SOD1. Additionally, mutant SOD1 exhibits an altered tertiary structure, evidenced by enhanced hydrophobicity compared to WT SOD1 (Tiwari et al., 2005; Munch and Bertolotti, 2010). While X-ray crystallography has failed to reveal significant structural differences between WT- and mutant-SOD1 proteins, (Hart et al., 1998; Elam et al., 2003; Hough et al., 2004; Cao et al., 2008; Galaleldeen et al., 2009), solution-based structural studies indicate that FALS-linked mutations induce some degree of SOD1 unfolding, or "misfolding" (**Figure 3**), within the electrostatic and/or zinc loops (**Figure 1**). For example, NMR relaxation experiments reveal an overall increase in protein dynamics for SOD1 G93A compared to WT SOD1 (Shipp et al., 2003). The region proximal to the G93A mutation, termed the "β-barrel plug," as well as residues within the zinc-binding loop exhibited the greatest increase in mobility, whereas the dynamics within the electrostatic loop were comparable between SOD1 G93A and WT (Shipp et al., 2003). Similarly, β-strands 3 and 4, which contain the β-barrel plug, displayed high deuterium exchange rates as assessed by mass spectrometry for several demetallated mutant forms of SOD1 (Durazo et al., 2009). In a separate study using similar methodology but with metallated forms of SOD1, only 1 out of the 13 mutants exhibited relatively high deuterium exchange rates in the β-barrel plug (Molnar et al., 2009). However, this study detected a 10–31% increase in deuterium exchange within the electrostatic loop for 7 out of 8 "WT-like" ALS-linked SOD1 mutants (i.e., mutant-SOD1 proteins that coordinate Cu and Zn), including A4V. Deuterium exchange increased within both the electrostatic and zinc binding loops for those SOD1 mutants with impaired metal binding (Molnar et al., 2009).

The generation of conformation specific antibodies has also provided important insights into the misfolded nature of FALS-linked mutant-SOD1 proteins. Most conformation specific antibodies reported to date are selectively reactive for mutant SOD1 over WT SOD1, consistent with the notion that mutations induce some degree of misfolding that exposes linear sequences or conformational epitopes that are otherwise buried in the intact, native protein (Rakhit et al., 2007; Urushitani et al., 2007; Liu et al., 2009; Bosco et al., 2010; Forsberg et al., 2010; Gros-Louis et al., 2010; Grad et al., 2011; Brotherton et al., 2012; Fujisawa et al., 2012; Broering et al., 2013) (**Figure 4**, **Table 1**). For example, the conformation specific antibodies B8H10, D3H5, and A5C3 that were generated against apo-SOD1 G93A do not immunoprecipitate WT SOD1, but rather exhibit differential reactivity for various mutant-SOD1 proteins (G93A, G37R, G85R, G127X, and D90A) from spinal cord lysates derived from the respective ALS mouse models. That B8H10, D3H5, and A5C3

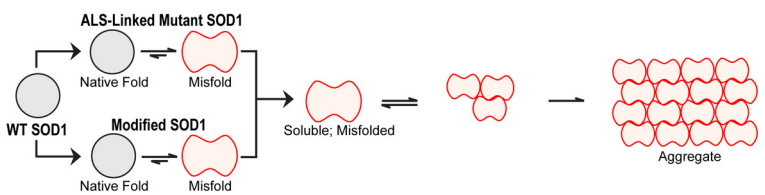


**FIGURE 3 | Misfolded vs. aggregated SOD1.** Misfolded SOD1 results from both ALS-linked mutations and aberrant post-translational modifications. Throughout the review, the term "misfolding" refers to the structural loosening of SOD1 due to a mutation and/or altered post-translational modification within a soluble form of the protein. Misfolded, soluble SOD1 can engage in aberrant protein-interactions and acquire new toxic functions (illustrated in **Figure 5**). Misfolded SOD1 can also assemble into aggregates. In this review, "aggregates" refer to those insoluble species characteristic of end-stage pathological inclusions present in human post-mortem CNS tissues.

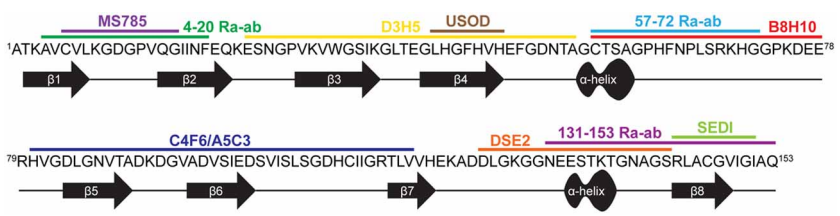


**FIGURE 4 | Conformation specific antibodies highlight regions exposed in SOD1 upon misfolding.** The primary sequence for WT SOD1 is shown with the secondary structure displayed below. The binding regions of 11 antibodies specific for misfolded SOD1 are depicted above the sequence (see **Table 1** for antibody details).

**Table 1 | Conformation specific antibodies recognize regions of SOD1 exposed upon misfolding.**

| Antibody | SOD1 epitope (aa) | Reactive for FALS SOD1 | Reactive for SALS SOD1 | | Therapeutic benefits | Reference |
|---|---|---|---|---|---|---|
| | | | *Patient tissue* | *Modified SOD1* | | |
| B8H10[e] | 57–78[a] | + | nd[b] | nd | nd | Gros-Louis et al., 2010; Pickles et al., 2013 |
| C4F6[e] | 80–118 | + | + | + (ox) | nd | Urushitani et al., 2006; Bosco et al., 2010; Prudencio and Borchelt, 2011; Brotherton et al., 2012; Pickles et al., 2013 |
| MS785 | 6–16 | + | nd | +[c] | nd | Fujisawa et al., 2012 |
| D3H5 | 24–55[a] | + | nd | + (apo) | + | Gros-Louis et al., 2010 |
| A5C3[e] | 80–118[a] | + | nd | nd | − | Gros-Louis et al., 2010 |
| SEDI | 143–151 | + | − | + (ox) | +[d] | Rakhit et al., 2007; Liu et al., 2009, 2012; Kerman et al., 2010; Prudencio and Borchelt, 2011; Mulligan et al., 2012 |
| USOD | 42–48 | + | − | − (ox) | nd | Kerman et al., 2010; Mulligan et al., 2012 |
| DSE2 (3H1) | 125–142 | + | + | nd | nd | Vande Velde et al., 2008; Grad et al., 2011; Pokrishevsky et al., 2012 |
| 4–20Ra-ab | 4–20 | + | + | nd | nd | Jonsson et al., 2004; Forsberg et al., 2010 |
| 57–72Ra-ab | 57–72 | + | + | nd | nd | Stewart et al., 2006; Forsberg et al., 2010, 2011 |
| 131–153Ra-ab | 131–153 | + | + | nd | nd | Jonsson et al., 2004; Forsberg et al., 2010, 2011 |

*Misfolded SOD1-specific antibodies highlight regions in SOD1 that become exposed as a result of misfolding.*

[a] *Determined using similar methodology as in C4F6 epitope mapping (Bosco et al., 2010) (unpublished data, Bosco DA).*

[b] *nd, Not determined.*

[c] *Serum starvation in cultured cells results in SOD1 reactivity with antibody.*

[d] *Benefits observed from active immunization with SOD1 peptide recognized by SEDI.*

[e] *Commercially Available.*

become reactive for denatured WT SOD1 is consistent with a linear epitope within natively folded WT SOD1 that is exposed only upon SOD1 unfolding (Gros-Louis et al., 2010). The C4F6 antibody is also selective for FALS-linked SOD1 mutants G37R, G85R, G93A (Urushitani et al., 2007) A4V (Brotherton et al., 2012) over WT SOD1 when these proteins are in their native state but exhibits weak reactivity for denatured SOD1 proteins, suggesting that this antibody recognizes a conformational epitope (Bosco et al., 2010). Interestingly, both D3H5 (Gros-Louis et al., 2010) and C4F6 (Brotherton et al., 2012) immunoreactivity for SOD1 G93A directly correlates with disease progression in the transgenic SOD1 G93A ALS mouse model, indicating that these antibodies report on the presence of "toxic," misfolded forms of SOD1 G93A.

In contrast to the aforementioned antibodies, for which the epitope within SOD1 remains unknown, several conformation specific antibodies have been generated against sequences that are predicted to become exposed only upon SOD1 misfolding (Rakhit et al., 2007; Grad et al., 2011; Fujisawa et al., 2012). One such antibody, MS785, targets amino acids 6-16 at the N-terminus of SOD1. This portion of mutant SOD1 is implicated in binding Derlin-1, a protein involved with ER associated degradation, and is thus referred to as the Derlin-1 binding region (DBR). Fujisawa et al., demonstrated through immunoprecipitation experiments that mutant-SOD1 associates with Derlin-1 when coexpressed in HEK cells. Their comprehensive immunoprecipitation strategy assessed the interaction between Derlin-1 and 132 ALS-linked SOD1 mutants, of which 124 were found to associate with Derlin-1. Further, MS785 selectively immunoprecipitated SOD1 from B-lymphocytes derived from all 14 SOD1 positive ALS cases examined but not from 11 healthy controls (Fujisawa et al., 2012). An alternative antibody called SEDI was created against an epitope at the opposite end of the SOD1 molecule. SEDI stands for SOD1 Exposed Dimer Interface and was raised against the amino acids 143–151 located at the dimer interface in SOD1 (Rakhit et al., 2007). SEDI reactivity is specific for mutant SOD1 over native WT SOD1 in the context of transgenic mouse tissue [G37R, G93A, and G85R (Rakhit et al., 2007)] and human post-mortem tissues harboring SOD1 mutations [A4V (Rakhit et al., 2007), A4T, V14M, ΔG27/P28, and I113T (Liu et al., 2009)]. Therefore, MS785 and SEDI antibodies report on misfolding events within the N- and C-termini, respectively, that are induced by ALS-linked mutations.

A direct consequence of mutation-induced misfolding of SOD1 is aggregation, which refers to the irreversible assembly of misfolded SOD1 species into an insoluble structure (**Figure 3**). SOD1 aggregation has been extensively investigated *in vivo*, both in ALS human post-mortem tissues and in mutant-SOD1 transgenic mice. The enhanced aggregation propensities of FALS-linked SOD1 mutants have also been comprehensively examined in cell culture and in other *in vitro* assays. We refer the reader to many excellent reviews and original works that detail current models of mutant-SOD1 aggregation (Durham et al., 1997; Bruijn et al., 1998; Johnston et al., 2000; Stathopulos et al., 2003;

Furukawa et al., 2006; Wang et al., 2008; Prudencio et al., 2009) Review: (Chattopadhyay and Valentine, 2009; Turner and Talbot, 2008).

Although it remains unclear whether SOD1 aggregation is a causative or protective factor in disease progression, several recent reports demonstrate that misfolded SOD1 species can spread from cell to cell in a prion-like fashion (Grad et al., 2011; Munch et al., 2011; Sundaramoorthy et al., 2013). Munch et al demonstrated that the uptake of aggregated ALS-linked SOD1 mutants in cultured neuronal cells seeded aggregation of endogenous mutant-SOD1. These endogenous SOD1 aggregates persisted well after (>30 days) the original aggregates dissipated from cell division, consistent with a prion-like propagation of aggregated SOD1 (Munch et al., 2011). More recently, uptake of both misfolded mutant-SOD1 as well as aggregated mutant-SOD1 was shown to induce aggregation of the native WT SOD1 protein (Sundaramoorthy et al., 2013). This latter report demonstrates how misfolded SOD1 can alter the conformation of otherwise normally folded SOD1, however, it remains to be determined whether this spreading of misfolded and aggregated SOD1 directly impacts ALS pathogenesis *in vivo* (Guest et al., 2011).

## MISFOLDED FALS-LINKED SOD1 EXERTS A GAIN OF TOXIC FUNCTION IN ALS

Studies using transgenic rodent models have generated the majority of data pointing to a gain of toxic mechanism for mutant-SOD1 in FALS (Turner and Talbot, 2008). Several transgenic mouse models have been engineered to overexpress ALS-linked SOD1 mutants. Mutant-SOD1 transgenic mice develop an ALS-like phenotype that includes motor neuron degeneration, neuroinflammation, severe paralysis and premature death (Gurney et al., 1994; Dal Canto and Gurney, 1995; Wong et al., 1995; Bruijn et al., 1997; Dal Canto and Gurney, 1997). In addition, cytosolic SOD1-containing ubiquitinated aggregates are detected within CNS tissues from these mice, recapitulating the pathological features of the human disease (Gurney et al., 1994; Dal Canto and Gurney, 1995; Bruijn et al., 1997; Dal Canto and Gurney, 1997; Watanabe et al., 2001). These mice express endogenous murine SOD1, and yet develop motor neuron disease upon expression of exogenous human mutant-SOD1, providing evidence that SOD1 mutations lead to a gain of toxic function. Further, SOD1-deficient mice develop normally with no overt signs of neurodegeneration (Reaume et al., 1996). However, it is noted that SOD1 null mice are more susceptible to axonal (Reaume et al., 1996) and ischemic brain (Kondo et al., 1997) injuries, and therefore a complete loss of SOD1 may be disadvantageous, especially in the context of disease (van Blitterswijk et al., 2011).

Because the aforementioned SOD1 pathological aggregates are a downstream consequence of SOD1 misfolding, the presence of such aggregates in human post-mortem tissues and ALS-mice argues for a role of misfolded SOD1 in disease. This notion is supported by immunization strategies that both target misfolded SOD1 species and have therapeutic outcome in ALS mice. A passive immunization strategy with the D3H5 antibody that specifically reacts with misfolded SOD1 extended survival in SOD1 G93A transgenic mice (Gros-Louis et al., 2010)(**Figure 4**, **Table 1**). A greater therapeutic impact was shown in active immunization trials with the SOD1 G37R transgenic mouse model, using both recombinant apo-SOD1 G93A (Urushitani et al., 2007) and the SEDI (SOD1 exposed dimer interface) peptide (Liu et al., 2012) as immunogens. Use of SEDI increased survival and delayed disease onset to a greater extent than the full-length SOD1 immunogen, likely due to specific targeting of a misfolded toxic epitope within mutant-SOD1 (Liu et al., 2009, 2012). While these studies provide a direct correlation between misfolded SOD1 species and disease in mice, a survival benefit was not realized using humanized SOD1 antibodies in SOD1 transgenic mice (Broering et al., 2013). Going forward, immunotherapeutic strategies in humans may require the specific targeting of regions within SOD1 that are only exposed upon misfolding. Moreover, these regions should mediate some toxic effect *in vivo*, so that antibodies have the potential to neutralize or otherwise block that "toxic epitope." The latter criterion is important in light of the fact that not all mutant-specific antibodies have produced a therapeutic outcome in SOD1 transgenic mice (Gros-Louis et al., 2010).

The immunization studies described above demonstrate that misfolded SOD1 can impact the disease course in ALS mice. What evidence supports a toxic role of misfolded and/or aggregated SOD1 in the human disease? Wang et al., found an inverse correlation between SOD1 aggregation propensity and disease duration in human ALS cases (i.e., mutants that are more aggregation prone are associated with cases that exhibit relatively short survival) using the Chiti-Dobson equation (Wang et al., 2008). A complementary study determined the relative aggregation propensities of 30 SOD1 mutants in a cell culture assay and also reported an indirect correlation between SOD1 aggregation propensity and disease duration (Prudencio et al., 2009). However, this correlation was not statistically significant, possibly due to the limited number of SOD1 mutants that could be included in this type of analysis that utilizes patient data (Prudencio et al., 2009). Nonetheless, these studies implicate misfolded SOD1 as a factor in human ALS pathogenesis.

## TOXIC, SOLUBLE MISFOLDED FALS-SOD1 SPECIES IN DISEASE

While end-stage pathological aggregates composed of insoluble mutant-SOD1 are detected in ALS-mouse models and in the human disease, emerging evidence suggests that the toxic SOD1 species is in fact a misfolded, soluble form of the protein (**Figure 3**). One can imagine that an aggregated, insoluble form of SOD1, much like what is found at end stage of disease, is isolated and unable to diffuse within the cell. Conversely, a non-aggregated misfolded form of SOD1 is soluble, accessible, and able to engage in aberrant interactions, thereby enabling a gain of toxic function. To enumerate all of the aberrant functions and interactions that have been observed for mutant-SOD1 is beyond the scope of this review and therefore we refer the reader to excellent reviews that cover this topic (Cleveland and Rothstein, 2001; Bruijn et al., 2004; Pasinelli and Brown, 2006; Joyce et al., 2011). Herein, we will focus on those gain-of-toxic functions for FALS-linked mutants that are thought to involve misfolded, soluble SOD1 and that may also have relevance to sporadic ALS (**Figure 5**).

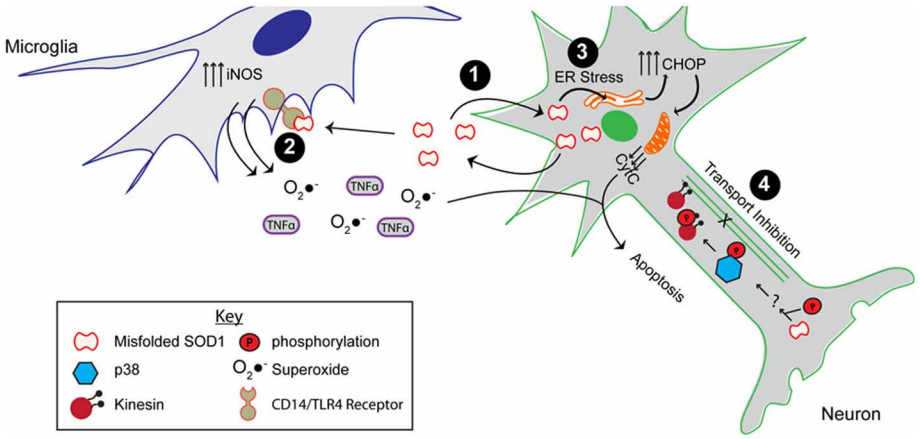


**FIGURE 5 | The toxic properties shared by ALS-linked mutant SOD1 and modified WT SOD1.** As shown, misfolded SOD1, resulting from mutations or aberrant post-translational modifications, causes the protein to engage in aberrant interactions. **(1)** Misfolded SOD1 is both secreted, possibly through chromogranins, and taken up from the extracellular environment. **(2)** Extracellular misfolded SOD1 activates microglia by binding to the CD14/TLR4 receptor, thereby elevating nitric oxide synthase (iNOS) activity as well as secretion of superoxide anion ($O_2\bullet^-$) and proinflammatory cytokines (e.g., TNFα). **(3)** Expression and uptake of misfolded SOD1 leads to ER stress, which elevates the pro-apoptotic CHOP protein and induces mitochondrial damage. **(4)** The presence of misfolded SOD1 in the axon results in axonal transport inhibition through a mechanism involving the phosphorylation of p38 MAPK and the kinesin motor. All of these aberrant functions compromise the integrity of the motor neuron, and potentially contribute to both FALS and SALS pathogenesis.

Support for a soluble misfolded form of SOD1 comes from studies in both SOD1 animal models and cell culture. Zetterstrom et al., identified an accumulation of soluble misfolded SOD1 that was enriched in the spinal cord of SOD1 G93A transgenic mice. The fraction of soluble misfolded SOD1 was quantified with hydrophobic interaction chromatography (HIC) (Zetterstrom et al., 2007). Interestingly, not only is the solubility of FALS-linked SOD1 variants enhanced upon heterodimerization with WT SOD1, but the toxicity of these variants is also enhanced, consistent with the toxic SOD1 species being soluble-misfolded rather than insoluble-aggregated (Witan et al., 2008, 2009). In CHO cells, the fraction of soluble SOD1 was found to correlate with cellular toxicity (Brotherton et al., 2013). This cellular toxicity was attenuated upon expression of exogenous hsp70 (Brotherton et al., 2013), a chaperone that refolds misfolded proteins, although it is not clear whether this is due solely to a reduced load of misfolded SOD1 or whether the anti-apoptotic function of hsp70 also contributed to this effect (Beckmann et al., 1990; Beere et al., 2000; Luders et al., 2000).

Misfolded mutant SOD1 can have downstream effects both outside and inside the cell. Although WT SOD1 is known to be secreted normally (Mondola et al., 1996, 1998, 2003; Cimini et al., 2002; Turner et al., 2005; Urushitani et al., 2006; Santillo et al., 2007), Urushitani et al. demonstrated that mutant-SOD1 can be secreted by an alternate pathway that involves the secretory chromogranin proteins (Urushitani et al., 2006). In contrast, Turner et al., demonstrated an impaired secretion of mutant SOD1 compared to WT SOD1 (Turner et al., 2005). The discrepancy between these studies may de due to the different cell types employed. Nonetheless, both studies are consistent with a dysregulation of mutant-SOD1 secretion compared to native WT SOD1. Once secreted into the extracellular space, mutant-SOD1 activates microglia though a mechanism that involves binding to the CD14/TLR receptor (Zhao et al., 2010), resulting in a typical proinflammatory response (i.e., increased levels of TNFα and IL-1β). This mode of microglia activation leads to motor neuron death (Urushitani et al., 2006; Zhao et al., 2010).

Inside the cell, mutant SOD1 can induce stress in the context of various pathways. For example, primary neurons derived from SOD1 G93A transgenic mice, as well as cell culture models of mutant-SOD1, exhibit signs of ER stress including spliced XBP1 and induction of CHOP (Nishitoh et al., 2008; Prell et al., 2012). ER stress has been shown to be a major player in ALS pathogenesis [reviewed in Kanekura et al. (2009), Matus et al. (2011)]. It should be noted that endogenous expression of mutant-SOD1 is not required for activation of the ER stress response pathway. Uptake of extracellular SOD1 G93A is sufficient to induce ER stress and neuronal toxicity (Sundaramoorthy et al., 2013). Thus, the combination of mutant-SOD1 uptake and intracellular expression in neurons could exacerbate ER stress, and tilt the scale from the UPR coping mechanism toward apoptosis *in vivo*. Mitochondria represent an additional intracellular compartment that is affected by mutant-SOD1 (Pickles et al., 2013). Recent evidence indicates that mutant-SOD1 directly interacts with Bcl-2, leading to exposure of the toxic BH3 domain, which in turn causes mitochondrial damage (Pasinelli et al., 2004; Pedrini et al., 2010). Interestingly, enhanced exposure of the BH3 domain has been detected in FALS SOD1 A4V patient spinal cord homogenates, and increases as a function of disease in spinal cords of SOD1 G93A transgenic mice (Pedrini et al., 2010). Finally, much evidence suggests that mutant-SOD1 impairs the process of axonal transport in different model systems (Morfini et al., 2009). In studies of fast axonal transport (FAT) performed in squid axoplasm, there is compelling evidence that it is a soluble

misfolded form of mutant-SOD1 that inhibits transport in the anterograde direction (Morfini et al., 2009, 2013; Song et al., 2013). Moreover, these studies were extended to explore the role of WT SOD1 in SALS, which is the topic of the following section.

## MISFOLDED WILD-TYPE SOD1: IMPLICATIONS FOR SALS

Since the discovery of FALS-linked mutations in SOD1 (Rosen et al., 1993), there has been speculation that the wild-type version of the protein could play a role in sporadic ALS (Selverstone Valentine et al., 2005; Kabashi et al., 2007). It has become increasingly clear that alterations of the normal post-translational modifications of SOD1 and/or introduction of aberrant modifications in WT SOD1 cause this otherwise stable protein to misfold and adapt properties similar to FALS-linked SOD1 mutants (**Figure 1C**). Until recently, however, there was a lack of direct evidence for aberrant forms of WT SOD1 in SALS. This is probably because the field lacked the appropriate tools and methodologies to detect such species. Below we describe modifications of WT SOD1 that cause this protein to become misfolded and "toxic." We also present evidence, largely from the use of conformation specific antibodies that recognize misfolded SOD1 species, that support or contradict the hypothesis that WT SOD1 plays a role in sporadic ALS.

### LOSS OF NATIVE SOD1 POST-TRANSLATIONAL MODIFICATIONS LEADS TO WT SOD1 MISFOLDING

As discussed above, coordination of Zn, oxidation of the C57–C146 intrasubunit disulfide bond and homodimerization of SOD1 contribute to the structural stability of the molecule. A loss in integrity of any one of these normal post-translational modifications compromises the stability of WT SOD1 and contributes to its misfolding and aggregation (**Figures 1C**, **3**). For example, a reduction of WT SOD1 stability upon demetallation is a widely documented observation (Stathopulos et al., 2003; Lynch et al., 2004; Furukawa and O'Halloran, 2005; Ding and Dokholyan, 2008). Interestingly, demetallation of WT SOD1 induces similar conformational perturbations within the zinc binding and the electrostatic loops (loops IV and VII, respectively, **Figure 1**) as do FALS-linked SOD1 mutations (Strange et al., 2003, 2007; Ding and Dokholyan, 2008; Durazo et al., 2009; Molnar et al., 2009). A direct comparison of NMR backbone chemical shifts for apo dimeric WT SOD1 vs. holo dimeric WT SOD1 revealed the largest structural variations within the electrostatic loop (Banci et al., 2009). These structural alterations were accompanied by pronounced changes in backbone dynamics that further support demetallation-induced misfolding (Banci et al., 2009). Molecular dynamics (Ding and Dokholyan, 2008) and H/D exchange by mass spectrometry (Durazo et al., 2009) also report enhanced flexibility within the aforementioned loops of apo WT SOD1 compared to holo SOD1, but in addition these studies also detect misfolding within the beta barrel of SOD1 upon demetallation (Ding and Dokholyan, 2008; Durazo et al., 2009). Furthermore, the misfolding of WT SOD1 induced by demetallation leads to aggregation of the protein (Banci et al., 2007), which may be driven by the exposure of hydrophobic regions within misfolded SOD1 that are otherwise buried in the native protein (Tiwari et al., 2009).

In addition to demetallation, reduction of the C57–C146 disulfide bond also has a destabilizing effect on WT SOD1 that can lead to protein misfolding and aggregation in a manner similar to FALS-linked mutant SOD1 (**Figure 3**) (Furukawa et al., 2008; Chan et al., 2013). Several studies have demonstrated that a reduction of C57–C146 in apo-SOD1 shifts the monomer-dimer equilibrium toward the monomeric state. Addition of zinc or oxidation of C57–C146 shifts the equilibrium back to the dimeric state, demonstrating interdependence between Zn coordination, C57–C146 bond integrity, and dimerization on the structural stability of SOD1 (Arnesano et al., 2004; Lindberg et al., 2004; Hornberg et al., 2007).

### INTRODUCTION OF ABERRANT POST-TRANSLATIONAL MODIFICATIONS INDUCE WT SOD1 MISFOLDING

In addition to alterations of the normal post-translational modifications of SOD1, WT SOD1 misfolding and aggregation can be induced by the formation of aberrant modifications. Oxidation of SOD1 side chains represents one such modification that has been postulated to play a significant causal role in both FALS and SALS (Kabashi et al., 2007). In the context of WT SOD1, which has relevance to SALS, metal catalyzed oxidation with $CuCl_2$ and ascorbic acid leads to oxidative modification of SOD1 histidine residues and subsequent SOD1 aggregation (Rakhit et al., 2002, 2004). This mode of oxidation-induced SOD1 aggregation proceeds by way of SOD1 dimer dissociation/monomer formation, demonstrating interdependence between aberrant and normal SOD1 post-translational modifications (Rakhit et al., 2004). Cys111 within SOD1 is particularly susceptible to $H_2O_2$ induced oxidation (**Figure 1C**). Prolonged exposure of SOD1 to $H_2O_2$ results in the irreversible conversion of the Cys111 sulfhydryl group to sulfonic acid (Fujiwara et al., 2007; Bosco et al., 2010; Auclair et al., 2013), which may be detrimental since WT SOD1 oxidized by $H_2O_2$ (hereafter referred to as SOD1ox) exhibits an enhanced propensity to misfold and aggregate (Ezzi et al., 2007; Fujiwara et al., 2007; Bosco et al., 2010; Chen et al., 2012). Interestingly, modifications such as β-mercaptoethanol (Fujiwara et al., 2007), persulfide (de Beus et al., 2004) and cysteinylation (Auclair et al., 2013) on Cys111 protect SOD1 against oxidation and possibly against subsequent misfolding.

### ABERRANTLY MODIFIED WT SOD1 PROTEINS ARE TOXIC AND MIMIC FALS-LINKED SOD1 MUTANTS

The observation that zinc depleted WT SOD1 exerts a toxic effect onto motor neurons by a mechanism involving nitric oxide provided one of the first clues that modified WT SOD1 species are toxic and may contribute to SALS pathogenesis (Estevez et al., 1999; Beckman et al., 2001). More recently, zinc-deficient SOD1 was shown to exhibit a toxic effect related to mitochondrial dysfunction in *Drosophila* (Bahadorani et al., 2013). That metal deficient WT SOD1 can be induced to misfold and exhibit a toxic nature analogous to FALS-linked SOD1 is supported by the immunization trial in ALS mice reported by Takeuchi et al. Low copy SOD1 G93A transgenic mice vaccinated with the apo WT SOD1 immunogen exhibited delayed disease onset and prolonged survival compared to control mice injected with saline/adjuvant, and importantly, to a similar degree as mice vaccinated with apo

SOD1 G93A (Takeuchi et al., 2010). This study also implicates WT SOD1 as a viable therapeutic target for SALS.

In recent years, several reports have demonstrated a toxic effect of SOD1ox in the context of ALS relevant pathways and processes (**Figure 5**). For example, SOD1ox acquires aberrant protein-interactions that are also observed for FALS-linked SOD1 mutants. SOD1ox interacts with the heat shock protein Hsc70 that plays a role in refolding misfolded proteins (Ezzi et al., 2007), the secretory protein chromagranin B that actively secretes misfolded forms of SOD1 (Urushitani et al., 2006; Ezzi et al., 2007), and the anti-apoptotic protein Bcl-2 in a manner that induces mitochondrial damage (Pasinelli et al., 2004; Guareschi et al., 2012). SOD1ox was also shown to mimic FALS-linked SOD1 mutants in the inhibition of anterograde FAT in squid axoplasm. These studies demonstrated that the inhibitory effect of SOD1ox and mutant SOD1 was mediated by activated p38 MAPK, indicating that misfolded SOD1 can trigger kinase-dependent signaling cascades (Morfini et al., 2009; Bosco et al., 2010; Morfini et al., 2013; Song et al., 2013). Extracellular derived WT SOD1 can also induce a toxic effect onto cells. SOD1ox applied to cell culture media activates immortalized microglia (Ezzi et al., 2007), which in turn may cause motor neuron death (Ezzi et al., 2007; Zhao et al., 2010). Furthermore, uptake of aggregated WT SOD1 species by macropinocytosis in neuronal cells caused ER stress and seeded the aggregation of intracellular, endogenous SOD1 (Sundaramoorthy et al., 2013).

Studies in various SOD1 transgenic ALS mouse models have provided direct evidence for WT SOD1 mediated toxicity *in vivo*. Intriguingly, an ALS-like phenotype was only observed in transgenic mice expressing the human SOD1 A4V variant, a mutation that corresponds to an aggressive ALS phenotype in humans, when these mice expressed the human WT version of SOD1 (Deng et al., 2006). This paradoxical result may be explained by heterodimerization of WT and A4V subunits, affording a "stabilized" and thus more toxic version of SOD1 A4V that would otherwise be degraded (Witan et al., 2008, 2009). A similar phenomenon was observed in double-transgenic mice expressing human SOD1 WT and G85R, where disease onset was hastened relative to single-transgenic SOD1 G85R mice (Wang et al., 2009). Recently, a transgenic mouse model was developed that over-expresses human WT SOD1 at similar levels to the established high-copy SOD1 G93A mouse model (Gurney et al., 1994; Graffmo et al., 2013). Compared to transgenic mice expressing fewer copies of the human WT SOD1 gene, the mice generated by Graffmo et al exhibit an ALS-like phenotype that includes significant weight loss, SOD1 aggregation, neurodegeneration, gliosis, and a shortened life-span of ∼360 days (Graffmo et al., 2013). The authors posit that the ALS-like phenotype is not a general effect of SOD1 overexpression *per se*, but rather a direct consequence of a substoichiometric population of misfolded SOD1. Although the exact mechanism for WT SOD1 mediated toxicity is not well understood in these models, and the extent to which WT SOD1 is post-translationally modified has not been addressed, these studies clearly demonstrate a link between WT SOD1 and motor neuron degeneration characteristic of ALS.

## EVIDENCE FOR MISFOLDED, TOXIC WT SOD1 SPECIES IN SALS

There is accumulating evidence that WT SOD1 can misfold *in vitro* and exert a toxic effect *in vivo*. That genetic mutations in SOD1 are sufficient to cause ALS raises the possibility that modified forms of WT SOD1 may cause SALS, especially because modified WT SOD1 closely mimics the toxic behavior of FALS-linked SOD1 mutants in the context of numerous assays described above. To date, there is no animal model for SALS. Therefore, investigations into the relevance of WT SOD1 in SALS are focused on biological samples from individuals with this disease.

Early immunohistochemistry (IHC) studies using pan-SOD1 antibodies detected SOD1 within Lewy body-like inclusions in spinal cord sections from individuals with SALS (Shibata et al., 1994, 1996), implicating WT SOD1 in SALS pathology. However, pan-SOD1 antibodies failed to detect SOD1 containing aggregates in every IHC study that included SALS cases (Watanabe et al., 2001). In recent years, conformation specific antibodies have been developed that discriminate between native WT SOD1 and mutant and/or misfolded SOD1. These antibodies are being employed using IHC to address whether misfolded WT SOD1 is in fact present in post-mortem SALS tissues [**Figure 4**, **Table 1**, reviewed in Bosco et al. (2011), Furukawa (2012)]. Forsberg et al generated multiple polyclonal antibodies against small peptide sequences spanning the entire SOD1 protein. Two antibodies targeted to amino acids 4–20 within β-strands 1 and 2, and amino acids 131–153 that includes both the electrostatic loop and the SOD1 dimer interface [i.e., the same sequence used to develop the SEDI antibody Rakhit et al. (2007)] were shown to detect SOD1-containing aggregates within the motor neurons of all 29 sporadic cases examined and punctate staining in only 2 out of 19 non-neurological controls (Forsberg et al., 2010). The C4F6 conformation specific antibody introduced earlier was shown to react with misfolded WT SOD1 in a subset (4 out of 9) of SALS spinal cord sections (Bosco et al., 2010), but produced a diffuse staining pattern as opposed to a punctate pattern that would be consistent with insoluble aggregates (Bosco et al., 2010; Brotherton et al., 2012). The DSE2 antibody, which recognizes a "Disease-Specific Epitope" located within the electrostatic loop that is only exposed when SOD1 is misfolded (Vande Velde et al., 2008), was shown to detect misfolded SOD1 within SALS cases that also exhibit TDP-43 pathology (Pokrishevsky et al., 2012). Although these conformation specific antibodies appear to recognize different types of WT SOD1 species (i.e., soluble misfolded vs. aggregated), these reports collectively implicate SOD1 in SALS.

However, not all SOD1 conformation specific antibodies have detected misfolded or aggregated SOD1 in SALS cases. The USOD antibody, which was generated against residues 42–48 within "Unfolded SOD1" failed to detect misfolded SOD1 in SALS cases as did the SEDI antibody, whereas both antibodies detected aggregated SOD1 in FALS (Liu et al., 2009; Kerman et al., 2010). Furthermore, some antibody studies have produced conflicting results. While Bosco et al did not detect C4F6 reactivity in 17 control spinal cord sections, Brotherton et al., reported C4F6 reactivity in SALS tissues as well as tissues from controls (Brotherton et al., 2012). The source of these discrepancies is not clear, and may stem from technical differences in the experimental procedures, inherent variations in the nature of these antibodies (i.e.,

different epitopes) or inconsistancies amongst the SALS cases utilized across studies.

Biochemistry-based methods offer an alternative approach to IHC for the investigation of misfolded WT SOD1 in SALS. One study reported that SOD1 present in FALS and SALS spinal cord extracts is more susceptible to forming a 32 kDa cross-linked species upon treatment with a biotinylation reagent, suggestive of a misfolded SOD1 molecule that is common to FALS and SALS (Gruzman et al., 2007). This 32 kDa species, however, was later identified as carbonic anhydrase and not misfolded SOD1 (Liu et al., 2010). Subsequently, SOD1 immunopurified from SALS spinal cord tissues was shown to inhibit FAT in squid axoplasm to the same extent as recombinant forms of both oxidized WT and FALS-linked mutant SOD1 (Bosco et al., 2010). Although misfolded SOD1 has been detected in SALS tissues with different antibodies by IHC, this study demonstrated that SOD1 derived from SALS tissues could in fact exert a "toxic" effect in an ALS-relevant assay (Bosco et al., 2010; Yates, 2010). Importantly, this effect was blocked by C4F6 (Bosco et al., 2010), indicating that C4F6 reports on the toxic region within SOD1 and therefore may be useful for designing immunotherapeutic strategies for humans with ALS.

Haidet-Phillips et al. further demonstrated the toxic nature of SOD1 in the context of SALS, as astrocytes derived from human SALS spinal cords exert a toxic effect onto motor neurons only when they expressed near-endogenous levels of SOD1 (Haidet-Phillips et al., 2011). This is consistent with the observation of misfolded SOD1 in glia (Forsberg et al., 2011). Moreover, an ELISA designed for the detection of auto-SOD1 antibodies in human sera demonstrated that these antibodies can influence the SALS disease course. Elevated auto-SOD1 antibodies reactive for the misfolded oxidized form of WT SOD1 conferred a survival benefit within the SALS cohort examined, whereas those cases with elevated auto-SOD1 antibodies against the native WT SOD1 exhibited shorter survival (van Blitterswijk et al., 2011). These data are consistent with a toxic effect of misfolded SOD1 in human SALS, but also indicate a disadvantage to lowering endogenous levels of normal WT SOD1 in SALS. Therefore, it may be necessary to avoid immunotherapeutic and anti-sense oligonucleotide strategies that reduce levels of the normal, native WT SOD1 protein.

The actual molecular nature of "toxic" WT SOD1 species within the aforementioned human SALS studies was not addressed, and therefore it is not clear whether WT SOD1 in SALS exhibits aberrant post-translational modifications. It is intriguing that the $H_2O_2$ product of the dismutation reaction that is catalyzed by SOD1 (**Figure 2**) can also induce the conversion of this otherwise normal protein into a misfolded, toxic species (Ezzi et al., 2007; Bosco et al., 2010). A report by Guareschi et al., may shed some light onto the nature of the SOD1 modifications *in vivo*, as elevated levels of carbonylated SOD1 species were detected in lymphoblast cell lines from a subset of SALS patients with bulbar onset (Guareschi et al., 2012). An over-oxidized form of WT SOD1 that could explain both SOD1 misfolding and toxicity in SALS is reasonable, considering that oxidative stress is a pathological hallmark of SALS (D'Amico et al., 2013).

While there is compelling data supporting that misfolded and/or aggregated WT SOD1 is *associated* with SALS, it is still unclear whether WT SOD1 can *cause* SALS. The existing data cannot exclude the possibility that misfolded SOD1 is simply a downstream effect of disease. In fact, oxidized SOD1 has been detected in the brains of individuals with Alzheimer's and Parkinson's disease (Choi et al., 2005). Moreover, the conformation specific antibodies that detect misfolded and aggregated SOD1 in SALS also detect misfolded SOD1 in SOD1-negative FALS cases as well as in the context of other neurodegenerative disorders (Forsberg et al., 2010, 2011; Brotherton et al., 2012; Pokrishevsky et al., 2012). Therefore, misfolded SOD1 may represent a general consequence of aging and disease. Nonetheless, one would still expect the presence of misfolded SOD1, whether it be the mutant or WT form, to exacerbate disease based on all of the data demonstrating the toxic effects associated with these proteins. Several therapies are under development, with some in clinical trials, that target SOD1 (Glicksman, 2011). It will be important to determine whether any SOD1-based therapies can confer a therapeutic benefit to those individuals with SALS as well as FALS. Such an outcome would provide unequivocal evidence that SOD1 is indeed a pro-active factor is SALS pathogenesis.

## FUTURE OUTLOOK

There is an indisputable role for mutant-SOD1 in FALS; however, whether there is an analogous role for WT SOD1 in the context of SALS is unclear and controversial. The evidence that misfolded WT SOD1 is present in human post-mortem SALS samples, together with two decades worth of evidence that misfolded SOD1 can exert a toxic effect onto cells, supports the hypothesis that WT SOD1 is a causal factor in SALS. However, the presence of misfolded WT SOD1 may simply represent a downstream, non-specific consequence of aging and disease. Because SALS accounts for 90% of ALS cases, and the field is lacking an effective therapy for this devastating disease, a role for misfolded WT SOD1 in the pathogenesis of SALS should be considered. Such a role may include an upstream trigger of the disease, and/or a factor that promotes disease progression. As therapies become available to treat SOD1 in the context of FALS, it will be important to assess whether these therapies can also be applied to all or at least a subset of SALS.

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# A seeded propagation of Cu, Zn-superoxide dismutase aggregates in amyotrophic lateral sclerosis

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Abnormal accumulation of protein inclusions in motor neurons has been known as a major pathological change in amyotrophic lateral sclerosis (ALS). Increasing numbers of proteins including mutant Cu, Zn-superoxide dismutase (SOD1) have been identified as constituents of pathological inclusions in a form of insoluble fibrillar aggregates. Notably, protein fibrillar aggregates exhibit a self-perpetuating property, which can convert a soluble native protein into insoluble fibrillar aggregates. Such "seeding reaction" of protein fibrils can accelerate the aggregation significantly and would contribute to the spread of inclusion pathologies from an affected cell to its neighboring cells in neurodegenerative diseases. In ALS, a pathological change first occurs at the site of disease onset and then propagates throughout the affected tissues in a time-dependent manner; therefore, it can be assumed that seeded aggregation may be the key factor of disease progression in ALS. In this mini review, we will briefly summarize recent studies on possible roles of a seeded aggregation of SOD1 in pathomechanism of ALS.

**Keywords: SOD1, aggregation, seeding reaction, protein misfolding, amyloid**

## INTRODUCTION

Many proteins gain physiological functions by folding into their own unique three-dimensional structures, and any disturbance during this folding process potentially disrupts protein functions, which is considered to cause a variety of diseases (Morimoto, 2008). Among those, neurodegenerative diseases have been well characterized by abnormal accumulation of "mis"-folded proteins in brains and spinal cords of patients (Soto, 2003; Ross and Poirier, 2004). More specifically, certain misfolded proteins form insoluble, fibrillar aggregates that are rich in β-sheet structures, widely known as amyloid (Nelson et al., 2005).

In many neurodegenerative diseases, neurological symptoms appear in middle age (50 years ∼), suggesting that it is a rare event for proteins to become misfolded/aggregated. In fact, protein fibrillar aggregation *in vitro* requires a significant conformational conversion of proteins to form oligomers (also known as "nucleus"), which is a rate-limiting step of the overall aggregation reaction (Harper and Lansbury, 1997). Once the nucleus forms, however, it functions as a structural template (or so called "seed") to convert native proteins into β-sheet-rich structures and then elongate the protein fibril. This mechanism, which accelerates and even triggers protein aggregation, is called the seeding reaction. While it remains controversial whether protein aggregation is the direct cause or a mere result of neurodegeneration (Winklhofer et al., 2008; Treusch et al., 2009), this seeding mechanism may explain why many neurodegenerative diseases progress rapidly after the symptoms first appear.

One notable example for a seeding reaction is the infectivity of Prion diseases, in which the spread of fibrillar aggregates of prion proteins is considered to be the main cause of neurodegeneration (Prusiner, 1982; Aguzzi and Rajendran, 2009). Fibrils of prion proteins are considered to work as infectious agents that can be transmitted between individuals. As exemplified in kuru (Gibbs et al., 1980), eating affected tissues of the disease could introduce fibrillar prion aggregates into a brain of a healthy control as seeds and thereby trigger fibrillation of prion proteins and cause neurodegeneration. While no infectivity between individuals has been reported in neurodegenerative diseases besides prion diseases so far, a seeding phenomenon appears to be common to protein fibrillar aggregates (Dobson, 1999); therefore, increasing numbers of researchers have pursued possible roles of seeding reactions in pathologies of neurodegenerative diseases (Aguzzi and Rajendran, 2009; Polymenidou and Cleveland, 2011; Soto, 2012). For example, Alzheimer's disease (AD) is characterized by fibrillar aggregation of Aβ peptides in brains (Hardy and Selkoe, 2002), and accelerated accumulation of Aβ fibrils has been confirmed in primate and rodent models that are injected with brain homogenates of an AD patient (Meyer-Luehmann et al., 2006; Ridley et al., 2006). Several other pathogenic proteins also forms fibrillar aggregates *in vitro*, which have been tested for their *in vivo* seeding activity by being transduced into cultured cells and brains of transgenic mouse model (Aguzzi and Rajendran, 2009; Polymenidou and Cleveland, 2011; Soto, 2012). A seeding reaction of protein fibrils is thus considered to play important roles in pathological progression of neurodegenerative diseases, and in this mini review, we will focus upon roles of seeded aggregation of proteins in pathologies of amyotrophic lateral sclerosis (ALS).

## A SEEDED FIBRILLATION OF SUPEROXIDE DISMUTASE (SOD1) AS A PATHOLOGICAL PROPAGATION OF AMYOTROPHIC LATERAL SCLEROSIS (ALS)

ALS is a devastating motor neuron disease, mainly caused by abnormal accumulation of inclusions in the spinal cord (Bruijn et al., 2004). Notably, ALS has been known to occur as a focal process, which spreads contiguously throughout upper and lower motor neurons (Ravits and La Spada, 2009; Holmes and Diamond, 2012; Kanouchi et al., 2012). In other words, motor neuron degeneration in ALS is an orderly and actively propagating process, which appears to share characteristics of a seeded aggregation of proteins seen in Prion diseases.

Most ALS cases (∼90%) are sporadic with no known genetic factors (sporadic ALS, sALS), while the remaining cases have been known to exhibit a family history (familial ALS, fALS; Robberecht and Philips, 2013). In 1993, dominant mutations in the gene encoding Cu, Zn-superoxide dismutase (SOD1) were identified as one of major genetic causes of fALS (Rosen et al., 1993), and mutant SOD1 proteins have been known to accumulate abnormally in the form of insoluble inclusions within affected spinal motor neurons of SOD1-related fALS patients (Bruijn et al., 1998). Ultrastructural analysis of SOD1-positive inclusions in fALS cases has identified their fibrillar morphologies (Kato et al., 2000); however, those inclusions were not stained by amyloid-diagnostic dye, Thioflavin S, which has made it controversial whether fibrillar aggregates of mutant SOD1 *in vivo* are rich in β-sheets (Kerman et al., 2010). Moreover, SOD1-positive inclusions have never been isolated from fALS cases, so further biochemical tests will be required to characterize pathological SOD1 aggregates.

In contrast, SOD1-positive inclusions with ALS-like symptoms were reproduced in a fALS-model mouse expressing human SOD1 with a pathogenic mutation (Turner and Talbot, 2008) and were found to be stained by Thioflavin S, supporting the formation of amyloid-like, β-sheet-rich fibrils in mouse (Wang et al., 2002; Furukawa et al., 2008). Insoluble SOD1 aggregates were also successfully isolated from the spinal cords of affected fALS-model mice, and quite notably, those SOD1 aggregates exhibited seeding activity toward fibrillation of purified SOD1 proteins *in vitro*. Chia et al. have prepared homogenates of spinal cords of transgenic mice expressing human SOD1 with G93A mutation and shown that the homogenates triggered fibrillation of wild-type as well as G93A-mutant human SOD1 proteins under *in vitro* conditions with acidic pH of solution in the presence of a chaotropic reagent, guanidine hydrochloride (Chia et al., 2010). While destabilization of SOD1 proteins under artificial conditions appears to be required for a seeded acceleration of fibrillar aggregation, inclusions containing mutant SOD1 would function as seeds and thereby contribute to propagation of pathological changes among contiguous motor neurons and then disease progression of SOD1-related fALS cases.

Fibrillogenic propensities of SOD1 proteins have been well characterized in *in vitro* studies using purified recombinant proteins. SOD1 is a cytoplasmic enzyme (Chang et al., 1988) that catalyzes the conversion of superoxide radicals to hydrogen peroxide and oxygen (McCord and Fridovich, 1969) and is activated by binding of a catalytic copper and a structural zinc ion and also by forming an intramolecular disulfide bond (Furukawa et al., 2004). Wild-type holo-SOD1 with a disulfide bond exhibits high thermostability ($T_m$ ∼ 90°C), conferring significant resistance to structural changes and aggregation (Forman and Fridovich, 1973). In contrast, when SOD1 lacks both metal ions and a disulfide bond (apo-SOD1$^{SH}$), its melting temperature decreases down to 43°C and become more prone to misfolding and aggregation at physiological temperature (Furukawa and O'Halloran, 2005). *In vitro* aggregates of human SOD1 polypeptide without any modifications possess amyloid-like characters with fibrillar morphologies and show a seeding activity to accelerate fibrillation of native human SOD1 proteins (Furukawa et al., 2008). More importantly, amyloid-like fibrils of human apo-SOD1$^{SH}$ retain their seeding activity in the intracellular environment; transduction of those human SOD1 fibrils into cultured cells (mouse neuroblastoma, *Neuro2a*) has been shown to trigger the aggregation of stably-transfected human SOD1 (Furukawa et al., 2013).

fALS-causing mutations have been shown to decrease affinity for copper/zinc ions and/or stability of a disulfide bond (Hayward et al., 2002; Tiwari and Hayward, 2003; Furukawa et al., 2008). Therefore, in a reducing environment of the cytoplasm with high metal-chelating capacity, pathogenic mutations are supposed to increase intracellular fractions of fibrillation-prone apo-SOD1$^{SH}$ (Furukawa et al., 2008). Nonetheless, it remains unclear how mutant SOD1 forms aggregates under pathological conditions. To elucidate how SOD1 aggregates form, several pathways for aggregation have been proposed in SOD1 proteins *in vitro* (Furukawa, 2012b). As reported by Munch et al. mutant SOD1 was found to form fibrillo-granular aggregates by addition of trifluoroethanol (TFE; Münch and Bertolotti, 2010), which penetrated inside neuronal cells through macropinocytosis and then acted as seeds to trigger intracellular aggregation of endogenously expressed SOD1 variants (Münch et al., 2011). Once SOD1 aggregation occurs in a cell, the aggregates can be released to the extracellular space and then transferred from cell to cell (*vide infra*). Intracellular aggregation of mutant SOD1 is thus considered to be persistent and heritable after passages, supporting prion-like propagation of aggregation phenotypes.

As mentioned above, two distinct types of SOD1 aggregates, i.e., apo-SOD1$^{SH}$ amyloids and TFE-induced aggregates, have been found to function as seeds to trigger SOD1 aggregation intracellularly, but their structural and biochemical properties depend on how aggregation was induced (Furukawa et al., 2008; Münch and Bertolotti, 2010). Based upon previous *in vitro* studies, several distinct pathways for aggregation are possible in SOD1 (Toichi et al., 2013) and are expected to produce SOD1 aggregates with a varying degree of a seeding activity. This might describe heterologous progression and severity of diseases among SOD1-related fALS patients. Indeed, disease phenotypes of fALS cases have been known to be variable among different mutations in SOD1 (Wang et al., 2008), and furthermore, mutation-dependent structures of SOD1 fibrils closely correlate with their distinct biochemical properties (Furukawa et al., 2010). Therefore, it will be interesting to test if SOD1 fibrils with different mutations exhibit distinct activity as seeds *in vitro* and *in vivo*.

## PROPAGATION OF PROTEIN MISFOLDING IN AMYOTROPHIC LATERAL SCLEROSIS (ALS)

In a seeding reaction, sheared pieces of insoluble fibrils can act as structural templates for a "phase-like transition" from soluble native conformers to generally insoluble fibrillar state, but this view now appears to be necessary for revision. Grad et al. utilized antibodies (3H1 and 10C12) that exclusively recognize misfolded SOD1 with disease-specific epitopes, which are not available in the natively folded state, and showed that misfolding of endogenous wild-type SOD1 in human cells (e.g., human embryonic kidney 293 cells (HEK293)) is induced by co-expression of a soluble misfolded form of human SOD1 with pathogenic mutations (Grad et al., 2011). In other words, soluble misfolded conformers of SOD1 are also transmissible without adopting classical, insoluble fibrillar states. Furthermore, transient expression of mutant human SOD1 in murine cells (e.g., *Neuro2a*) did not induce misfolding of endogenous mouse wild-type SOD1 (Grad et al., 2011). The difference lies in the amino acid sequence of murine and human SOD1, where the only tryptophan in human SOD1 (Trp32) is replaced by serine in murine counterpart. Indeed, misfolding of wild-type human SOD1 was observed by human SOD1 with a pathogenic (G127X) mutation but was significantly mitigated when G127X human SOD1 with W32S mutation was used (Grad et al., 2011). Trp32 in human SOD1 is highly solvent-exposed and distant from the native dimer interface, which might provide an alternative site for abnormal intermolecular interactions through hydrophobic interactions. It is interesting to note that expression of mutant TAR DNA binding protein 43 (TDP-43) and Fused in Sarcoma (FUS), pathogenic proteins also known to be found in ALS patients (Arai et al., 2006; Neumann et al., 2006; Kwiatkowski et al., 2009; Vance et al., 2009), can increase the immunoreactivity for misfolded SOD1 using a disease-specific antibody (3H1), both in patients and cultured human cells (SH-SY5Y; Pokrishevsky et al., 2012). While pathological involvement of wild-type SOD1 in ALS remains to be established, aberrant conformers of wild-type SOD1 have been reported in sporadic ALS with no genetic background (Furukawa, 2012a). Accordingly, toxic conformers of SOD1 might be produced by abnormal interactions of folded SOD1 with misfolded SOD1 or other proteins (such as TDP-43/FUS) at the site surrounding Trp32. In other words, as proposed in the template-assisted misfolding of prion proteins (Horwich and Weissman, 1997), soluble but misfolded conformers of protein molecules can be propagated through abnormal interactions among homologous proteins even without the formation of classical amyloid-like fibrils.

## A CELL-TO-CELL TRANSFER OF INTRACELLULAR SUPEROXIDE DISMUTASE (SOD1)

SOD1 is known as one of major intracellular proteins, and most of SOD1 (∼70%) exist in the cytoplasm (Chang et al., 1988). To confirm that seeded aggregation or misfolding of SOD1 is the key molecular mechanism of pathological propagation of SOD1-fALS, it is required to understand how intracellular misfolded/aggregated SOD1 is transferred from the cytoplasm to extracellular environment. As a relatively simple process, misfolded/aggregated SOD1 would be released to extracellular environment by death of an affected cell and then phagocytosed by the other cell. Recent studies have nonetheless suggested more sophisticated processes for a cell-to-cell transfer of SOD1 proteins (Grad et al., 2011; Münch et al., 2011). In fact, active secretion of SOD1 to extracellular space has been suggested in several different types of cultured cells (Mondola et al., 1996, 1998), and both wild-type and mutant SOD1 can be also detected in the cerebrospinal fluid of healthy controls as well as fALS patients (Zetterström et al., 2011).

In conditioned media of mouse motor neuron-like hybrid (NSC-34) cell line, impaired secretion of mutant SOD1 was associated with intracellular formation of inclusions and toxicity, suggesting secretion of mutant SOD1 as a beneficial process for cell survival (Turner et al., 2005). In contrast, Urushitani et al. have found that mutant SOD1 proteins are secreted in association with chromogranins and cause microgliosis and neuron death (Urushitani et al., 2006), leading to the idea that suppression of extracellular mutant SOD1 is a promising strategy for therapeutics of SOD1-related fALS cases. Indeed, passive as well as active immunizations targeting extracellular mutant SOD1 proteins have successfully prolonged lifespan of transgenic mice expressing mutant human SOD1 (Urushitani et al., 2007). Toxic roles of secreted SOD1 are further supported by the findings that motor neurons are killed by being co-cultured with astrocytes derived from adult neural progenitor cells isolated from post-mortem lumber spinal cord tissues from sporadic ALS as well as SOD1-related fALS (Haidet-Phillips et al., 2011). Also importantly, suppression of SOD1 in both fALS and sporadic ALS astrocytes was found to negate such toxicity of astrocytes toward motor neurons. Recently, furthermore, Basso et al. have shown the increased release of exosomes from astrocytes overexpressing fALS-causing mutant SOD1 and found that astrocyte-derived exosomes contained mutant SOD1 proteins and were transferred to the cytoplasm of spinal neurons (Basso et al., 2013).

Based upon these results, secretion of SOD1 is considered to occur through several distinct pathways and appears to be a normal physiological process. Experimental evidences are further required to show that secretory vesicles act as a messenger to generate seeding activity of SOD1. More specifically, conformational analysis of SOD1 (folded, misfolded, or fibrillized) included in those vesicles will reveal the molecular mechanism of pathological propagation in ALS through a seeding reaction.

## SUMMARY

As briefly summarized above, increasing numbers of recent studies have supported the idea that misfolding/aggregation of mutant SOD1 is transmissible through a seeding mechanism inside the cell and among cells. In that sense, it is interesting to test pathological roles, if any, of SOD3, which resides at the extracellular matrix and possesses a structural domain almost homologous to SOD1 (Folz and Crapo, 1994). A SOD1-like domain of SOD3 has been shown to exhibit propensities for aggregation (Son et al., 2003), implying its involvement in the formation of seeds that can be taken up by cells. In summary, a seeded aggregation of SOD1 proteins including wild-type SOD1 will be a key event to understand progression/propagation of pathological changes in SOD1-related fALS and even sALS cases without mutations in

SOD1, and extracellular SOD1 with aberrant conformations is a promising target for therapeutics of those devastating diseases.

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# Differential effects on KCC2 expression and spasticity of ALS and traumatic injuries to motoneurons

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Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease manifested by progressive muscle atrophy and paralysis due to the loss of upper and lower motoneurons (MN). Spasticity appears in ALS patients leading to further disabling consequences. Loss of the inhibitory tone induced by downregulation of the potassium chloride cotransporter 2 (KCC2) in MN has been proposed to importantly contribute to the spastic behavior after spinal cord injury (SCI). The aim of the present study was to test whether the alterations in the expression of KCC2 are linked to the appearance of spasticity in the $SOD^{G93A}$ ALS murine model. We compared $SOD^{G93A}$ mice to wild type mice subjected to SCI to mimic the spinal MN disconnection from motor descending pathways, and to sciatic nerve lesion to mimic the loss of MN connectivity to muscle. Electrophysiological results show that loss of motor function is observed at presymptomatic stage (8 weeks) in $SOD^{G93A}$ mice but hyperreflexia and spasticity do not appear until a late stage (16 weeks). However, KCC2 was not downregulated despite MN suffered disconnection both from muscles and upper MNs. Further experiments revealed decreased gephyrin expression, as a general marker of inhibitory systems, accompanied by a reduction in the number of Renshaw interneurons. Moreover, 5-HT fibers were increased in the ventral horn of the lumbar spinal cord at late stage of disease progression in $SOD1^{G93A}$ mice. Taken together, the present results indicate that spasticity appears late in the ALS model, and may be mediated by a decrease in inhibitory interneurons and an increase of 5-HT transmission, while the absence of down-regulation of KCC2 could rather indicate an inability of MNs to respond to insults.

**Keywords: motoneuron disease, spasticity, hypereflexia, KCC2 transporter, $SOD1^{G93A}$ mice**

## INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is an adult onset neurodegenerative disorder that clinically manifests by progressive muscle atrophy and paralysis (Wijesekera and Leigh, 2009) due to the loss of upper and lower motoneurons (MN). The 90% of ALS cases are sporadic with unknown etiology whereas the remaining 10% are inherited forms, caused by genetic mutations. Among these, mutations in the gene encoding for the enzyme Cu/Zn superoxide dismutase 1 (SOD1) have been reported in about 20% of the patients (Rosen, 1993). Several transgenic animal models of ALS have been developed during the last decades. The most widely used is a transgenic mouse that over-expresses the human mutated form of the *sod1* gene with a glycine to alanine conversion at the 93rd codon (Ripps et al., 1995). This model recapitulates most relevant clinical and histopathological features of both familial and sporadic forms of the human disease (Ripps et al., 1995). Moreover, it has been recently reported that alterations of SOD1 protein are also present in sporadic ALS cases, increasing the interest of this model (Bosco et al., 2010).

Spasticity is a secondary complication of different upper MN syndromes characterized by a velocity-dependent increase in muscle tone resulting from hyperexcitability of the stretch reflex (Lance, 1980). This phenomenon is present in ALS patients and leads to important disabling complications that compromise their manual dexterity and gait (Wijesekera and Leigh, 2009; Kiernan et al., 2011). It has been hypothesized that spasticity may occur due to the loss of upper MN and/or alterations of intraspinal motor circuitry (Schütz, 2005; Chang and Martin, 2009, 2011; Dentel et al., 2013). The appearance of spasticity has also been described in the SOD1 ALS murine model (Dentel et al., 2013). In fact, despite the remaining hindlimb muscle innervation at the end stage of the disease could be enough to allow movement, the animals appear paralyzed due to spastic paresis (Mancuso et al., 2011).

Recent studies have demonstrated the relevance of MN increased excitability for the appearance of spasticity after traumatic injuries to the spinal cord (Lu et al., 2008; Boulenguez et al., 2010; Kakinohana et al., 2012; Bos et al., 2013). Rather than be mediated by an increase in excitatory transmission, these studies postulated that the loss of the inhibitory tone below the lesion is mediated by downregulation of the potassium chloride cotransporter 2 (KCC2) in spinal MNs (Boulenguez et al., 2010; Bos et al., 2013). Modulation of the inhibitory amino acids (GABA and glycine) response is determined by changes in the intracellular chloride concentration $[Cl_i^-]$. KCC2 is the main chloride extruder expressed in adult neurons, being responsible for the maintenance of the low $[Cl_i^-]$ (Ganguly et al., 2001; Rivera et al., 2002; Wang et al., 2002; Payne et al., 2003; Stein et al., 2004;

Bray and Mynlieff, 2009). At birth, when GABA and glycine responses are excitatory (Ben-Ari et al., 2007), KCC2 is barely detectable but increases progressively during the early post-natal days of the murine life (Rivera et al., 1999; Wang et al., 2002; Payne et al., 2003). Although excitatory actions of GABA and glycine during early development are relevant for the establishment of circuitry in the spinal cord and the development of motor functional patterns (Stil et al., 2011), the increased excitability induced by KCC2 down regulation in the adult spinal cord after trauma has also been linked to alterations of locomotor pattern, chronic pain and spasticity (Boulenguez et al., 2010; Bos et al., 2013).

In the present experiment, we tested whether changes in the expression of KCC2 in the SOD$^{G93A}$ ALS model are of relevance for the appearance of spasticity at late stages of the disease process. For assessing the potential contributing mechanisms we compared the changes in KCC2 induced by SCI and by peripheral nerve lesions in wild type mice.

## MATERIAL AND METHODS

### TRANSGENIC SOD1$^{G93A}$ MICE

Transgenic mice with the G93A human SOD1 mutation [B6SJL-Tg(SOD1-G93A)1Gur] were obtained from the Jackson Laboratory (Bar Harbor, ME, USA), and maintained at the Animal Service of the Universidad de Zaragoza. Hemizygotes B6SJL SOD1$^{G93A}$ males were obtained by crossing with B6SJL females from the CBATEG (Bellaterra, Spain). The offspring was identified by PCR amplification of DNA extracted from the tail tissue. All experimental procedures were approved by the Ethics Committee of the Universitat Autònoma de Barcelona, where the animal experiments were performed, and followed the guidelines of the European Commission on Animal Care and the Canadian Council on Animal Care.

### SURGICAL PROCEDURES

Adult (8–10 weeks old) female wild type C57BL/6 mice (Charles River) were anesthetized by i.p. injection of ketamine (10 mg/kg; Imalgene) and xylazine (1 mg/kg; Rompun). For the nerve crush, the sciatic nerve was exposed at the mid-thigh and subjected to a crush during 30 s for three times in succession with a Dumont no. 5 forceps. For SCI a laminectomy at the 11th thoracic vertebra was performed. The exposed spinal cord was contused using the Infinite Horizon Impactor device (Precision Scientific Instrumentation), using a force of 50 kdynes and with tissue displacement ranging between 500 and 700 μm. After injury, the skin was sutured and animals were left to recover on a hot pad and returned to their home cages with free access to food and water.

### FUNCTIONAL ASSESSMENT

Locomotors recovery was evaluated in an open-field test using the nine-point Basso Mouse Scale (BMS) (Basso et al., 2006; Klopstein et al., 2012), which was specifically developed for locomotors testing after spinal cord contusion injuries in mice. The BMS analysis of hindlimb movements and coordination was performed by two independent researchers and the consensus score was taken. The final score is presented as mean ± s.e.m.

### NERVE CONDUCTION TESTS

The sciatic nerve was stimulated percutaneously by means of single pulses of 0.02 ms duration (Grass S88) delivered through a pair of needle electrodes placed at the sciatic notch. The compound muscle action potential (CMAP, M wave) and the reflex H wave were recorded from the tibial anterior (TA) and the plantar (interpose) muscles with microneedle electrodes (Valero-Cabré and Navarro, 2001; Mancuso et al., 2011). For evaluation of the motor central pathways, motor evoked potentials (MEP) were recorded from the same muscles in response to transcranial electrical stimulation of the motor cortex by single rectangular pulses of 0.1 ms duration, delivered through needle electrodes inserted subcutaneously, the cathode over the skull overlaying the sensorimotor cortex and the anode at the nose (García-Alías et al., 2003; Mancuso et al., 2011). All potentials were amplified and displayed on a digital oscilloscope (Tektronix 450S) at settings appropriate to measure the amplitude from baseline to the maximal negative peak. To ensure reproducibility, the recording needles were placed under microscope to secure the same placement on all animals guided by anatomical landmarks. During the tests, the mice body temperature was kept constant by means of a thermostated heating pad.

### HISTOLOGY

SOD$^{G93A}$ mice at 8, 12 and 16 weeks of age, and sciatic nerve crushed (at 7 days post injury, dpi) and spinal cord injured (at 28 dpi) wild type mice were included in the histological analysis (4–5 mice per group). Animals were transcardially perused with 4% paraformaldehyde in PBS and the lumbar segment of the spinal cord was harvested, post-fixed overnight, and cryopreserved in 30% sucrose. Transverse 40-μm thick sections were serially cut with a cry tome (Lexica) between L2 and L5 segmental levels. Spinal cord slices were sequentially collected free-floating in Olmos medium.

For immunohistochemistry, sections were blocked with PBS-Triton 0.3%-normal donkey serum 5% and incubated overnight at 4°C with primary antibodies: rabbit anti-KCC2 (KCC2, 1:500, Millipore), mouse anti-neurofilament non-phosphorylated heavy chain (SMI-32, 1:1000, Covance), mouse anti-activating transcription factor 3 (ATF3, 1:500, Abeam), rabbit anti-ionized calcium binding adaptor molecule 1 (Iba1, 1:1000, Wako), rabbit anti-serotonin (5-HT, 1:5000, Sigma) or rabbit anti-calbindin (1:200, Chemicon). After washes, sections were incubated for 1 h at room temperature with Alexi 488 or Alexi 594 conjugated secondary antibody (1:200; Life Science). For co-localization, spinal NMS were labeled with NeuroTrace 500/525 Green Fluorescent Nissl (1:200, Life Science).

To quantify microglial immunoreactivity, microphotographs of the ventral horn gray matter were taken at ×400 and, after defining the threshold for background correction, the integrated density of Iba1 labeling was measured using ImageJ software (Mancuso et al., 2012). The integrated density is the area above the threshold for the mean density minus the background.

### PROTEIN EXTRACTION AND WESTERN BLOT

For protein extraction, another subset of mice ($n = 3$–4) of the same experimental groups, i.e., SOD$^{G93A}$ mice at 8, 12 or 16

weeks of age, mice with sciatic nerve crush and mice with SCI, were anesthetized and decapitated. The lumbar spinal cord was removed and divided into quarters to isolate the ventral quadrants. In animals that received a sciatic nerve crush, only the ventral horn of the lesioned side was used for protein extraction. Samples were prepared for protein extraction and homogenized in modified RIPA buffer (50 mom Tris–HCl pH 7.5, 1% Triton X-100, 0.5% sodium deoxycholate, 0.2% SDS, 100 mM NaCl, 1 mM EDTA) adding 10 μl/ml of Protease Inhibitor cocktail (Sigma) and PhosphoSTOP phosphatase inhibitor cocktail (Roche). After clearance, protein concentration was measured by Lowry assay (Bio-Rad, Dc protein assay).

Western blots were performed by loading 20 μg of protein of each sample in SDS-poliacrylamide gels. The transfer buffer was 25 mM trizma-base, 192 mM glycine, 20% (v/v) methanol, pH 8.4. The membranes were blocked with 5% BSA in PBS plus 0.1% Tween-20 for 1 h, and then incubated with primary antibodies at 4°C overnight. The primary antibodies used were: mouse anti-GAPDH (1:20000, Millipore), rabbit anti-phospho-$^{Ser940}$ KCC2 (1:1000, Phosphosolutions), rabbit anti-KCC2 (1:500, Millipore), mouse anti-gephyrin (1:1000, BD Bioscience) and anti-GAD65/67 (1:1000, Abcam). Horseradish peroxidase–coupled secondary antibody (1:5000, Vector) incubation was performed for 1 h at room temperature. The membranes were visualized using enhanced chemiluminiscence method and the images were collected and analyzed with a Gene Genome apparatus and Gene Snap and Gene Tools software (Signee), respectively.

### STATISTICAL ANALYSIS

Data are expressed as mean ± s.e.m. Electrophysiological test results were statistically analyzed using repeated measurements and One-Way ANOVA, applying Turkey *post-hoc* test when necessary. For immunobloting and histological data we used Mann-Whitney (for two groups comparison) or Kruskal-Wallis tests (for multiple groups comparison) followed by Dunn's *post-hoc* test (Prism 6 software; Graphpad). The level of significance was set at $p < 0.05$.

## RESULTS

### SOD1$^{G93A}$ ANIMALS SHOW HYPERREFLEXIA AND SPASTICITY AT LATE STAGES OF DISEASE PROGRESSION

We first evaluated peripheral motor nerve conduction to assess the progressive muscle denervation by stimulating the sciatic nerve and recording in TA and plantar muscles (Mancuso et al., 2011). Results revealed a different pattern of muscle denervation in the two tested muscles; the plantar muscle CMAP showed a fast drop in amplitude at 12 weeks of age, whereas the TA CMAP progressively decreased in amplitude from 8 weeks of age (**Figure 1A**). The monosynaptic spinal reflex activity was assessed by the H/M ratio (Mancuso et al., 2011). Results evidenced a significant increase of the H/M ratio in both tested muscles ($p < 0.01$) at the end stage of the disease (16 weeks), coincident with the spastic condition (**Figure 1A**). **Figure 1B** shows representative recordings to illustrate the increased H/M ratio in the plantar muscle of 16 weeks aged SOD1$^{G93A}$ mice. Finally, we assessed central motor conduction by means of MEPs to evaluate the state of the spinal motor descending pathways. Results showed a significant reduction of MEPs amplitude from 12 weeks of age both in TA and plantar muscles (**Figure 1A**).

### SPINAL CORD INJURY CAUSES LOCOMOTOR IMPAIRMENT AND HYPERREFLEXIA

We evaluated the locomotor function of wild type mice after SCI by means of the BMS score (Basso et al., 2006; Klopstein et al., 2012). Results revealed that injured mice achieved less than 3 over the 9 points scale at 28 days, evidencing the inability to support their own weight (**Figure 1C**). Then, we assessed central motor conduction preservation and hyperreflexia of the animals at 28 dpi. Results showed a significant reduction of MEPs and an increased H/M ratio, coincident with the spastic behavior in the animals' hindlimbs (**Figure 1C**).

### KCC2 EXPRESSION IS NOT ALTERED IN LUMBAR SPINAL MNs OF SOD1$^{G93A}$ ANIMALS

We did not observe changes in the KCC2 oligomer/monomer ratio (**Figures 2A,B**) in SOD1$^{G93A}$ mice at 8, 12, or 16 weeks of age compared to wild type mice. Accordingly, no significant changes in the phosphorylated form were observed in SOD1$^{G93A}$ mice (**Figures 2A,B**). We then characterized the localization of KCC2 by immunohistochemistry in lumbar MNs of SOD1$^{G93A}$ mice along disease progression. Confocal images showed no changes in subcellular localization of KCC2 at 8, 12 and 16 weeks of age (**Figure 2C**). In fact, co-labeling of KCC2 and SMI-32 confirmed that KCC2 remained in the MNs plasma membrane at late stages of the disease, as no co-localization of both markers was observed in the samples of SOD1$^{G93A}$ mice, even in degenerating MNs (**Figure 3**). Taken together, these findings indicate that KCC2 expression and localization in the MN plasma membrane remains unchanged during ALS disease progression.

### REDUCED ACTIVE FORM OF KCC2 IN LUMBAR SPINAL MNs AFTER SCI

We first examined KCC2 expression by western blotting in the lumbar ventral spinal cord of animals with SCI at 28 dpi. Compared to naïve animals, we observed a slight reduction of total KCC2 after SCI (around 20%), although the difference did not reach statistical significance ($p = 0.06$, data not shown). This tendency is in agreement with previously reported results by. Boulenguez et al. (2010) after SCI in the rat. To check the activation state of the KCC2, we also performed western blotting for the phosphorylated form of the KCC2 (pKCC2) with a specific antibody against phopho-Ser$^{940}$. In contrast to total KCC2 expression, the phosphorylated form was decreased after SCI compared to control samples ($p < 0.05$; **Figures 4A,B**). Since it has been previously described that KCC2 oligomer/monomer ratio is increased at maturity and correlates with KCC2 activation (Blaesse et al., 2006; Boulenguez et al., 2010; Bos et al., 2013), we also analyzed the ratio between the oligomeric and monomeric states of the KCC2. The results showed a significant reduction of the oligomer/monomer ratio (**Figures 4A,B**; $p < 0.05$), confirming the above results on the reduction of the pKCC2 expression.

Confocal immunohistochemical images confirmed KCC2 staining into the MNs cytoplasm. This labeling pattern contrasted

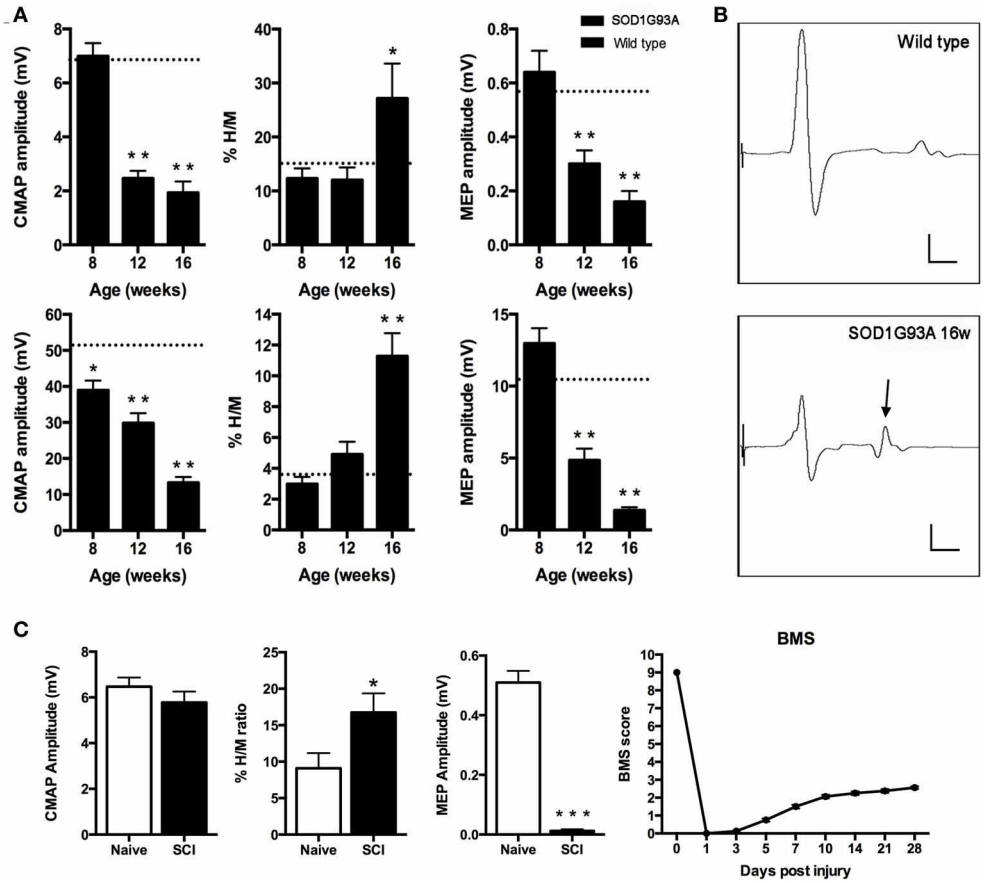


**FIGURE 1 | (A,B)** Peripheral and central motor conduction along disease progression in SOD1$^{G93A}$ mice. **(A)** Plantar (upper) and tibialis anterior (lower) compound muscle action potential (CMAP) amplitude, %H/M ratio and motor evoked potentials (MEP) amplitudes of SOD1$^{G93A}$ animals at 8, 12, and 16 weeks of age. Values are represented as mean ± s.e.m. Dashed line represents wild type mean value for each parameter. $^{*}p < 0.05$; $^{**}p < 0.01$ vs. wild type littermates. **(B)** Representative recordings of plantar muscle CMAPs. Note the relative increase of the H wave (arrow) reflecting hyperreflexia. Scale bars: 2 mV; 2 ms. **(C)** Functional assessment of SCI animals. Compound muscle action potential (CMAP) amplitude of the plantar muscle. Hyperreflexia evaluated by means of the %H/M ratio recorded in the plantar muscle after sciatic nerve stimulation. $^{*}p < 0.05$ vs. naïve mice. The lack of differences indicates that the increased %H/M ratio is not due to CMAP alterations. Motor evoked potentials (MEP) amplitude. BMS score as a measure of the locomotor capacity of the animals in the open field walking test. Note that at 28 dpi the score remains below 3, indicating that animals cannot support their own weight.

to the uniform band surrounding MNs in naïve animals (**Figure 4C**). Together, these results indicate that the translocation of KCC2 to the plasma membrane of MN is reduced after SCI.

### REDUCED ACTIVE FORM OF KCC2 IS OBSERVED IN LUMBAR SPINAL MNs AFTER SCIATIC NERVE CRUSH

In order to mimic the muscle denervation that occurs early in the ALS progression, we performed a sciatic nerve crush to analyze early changes of KCC2 after MN axotomy. Results of western blotting showed a significant decrease of the KCC2 oligomer/monomer ratio and of the phosphorylated active form of KCC2 in the injured side of the spinal cord in contrast to naïve animals (**Figures 4A,B**, $p < 0.01$). Immunohistochemical labeling confirmed that after sciatic nerve crush KCC2 was internalized into the MNs cytoplasm in the injured side forming intracellular clusters. In contrast, KCC2 localization in intact animals, showed a well-defined line surrounding MNs indicating a preferably membrane location of KCC2 in non-injured conditions (**Figure 4C**).

### ABSENCE OF DOWN-REGULATION KCC2 RESPONSE TO AXONAL INSULT IN ALS MNs

We then studied the KCC2 response in injured MNs, identified by labeling the activation transcription factor 3 (ATF3) as a marked of axonal damage (Tsujino et al., 2000), to assess whether KCC2 behave similarly between ALS and axotomized MNs. After sciatic nerve crush, KCC2 cytoplasmic inclusions were found into injured ATF3-positive MNs. On the contrary, the same analysis performed on SOD1$^{G93A}$ ventral spinal cord revealed that KCC2 localization remained normal even if MNs expressed ATF3 (**Figure 5**). These results suggest an altered response regarding KCC2 expression of ALS MNs after muscle denervation.

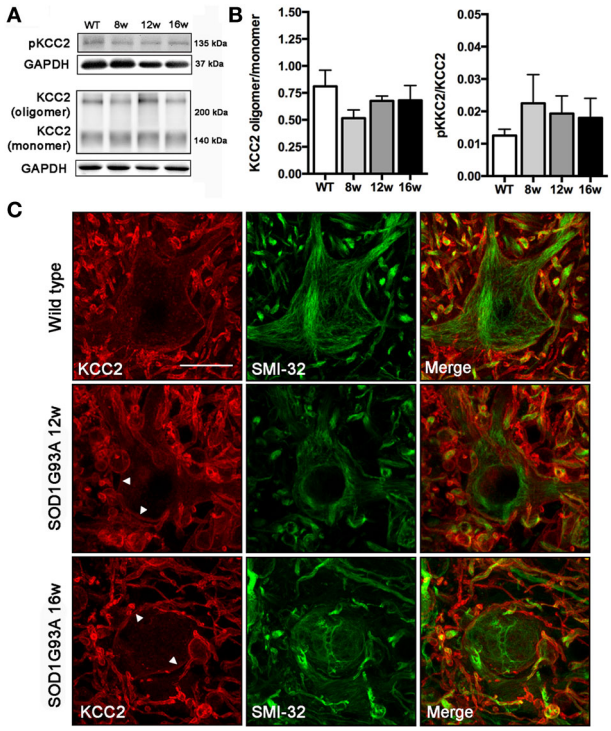


**FIGURE 2 | Analysis of KCC2 and phopho-KCC2 expression in the ventral spinal cord of SOD1$^{G93A}$ mice at 8, 12, and 16 weeks of age. (A)** Representative blots of phopho-KCC2 and total KCC2 (on both monomeric and oligomeric states). **(B)** KCC2 western blots quantifications. Graphs represent the ratio between the oligomeric KCC2 (functional state) vs. monomeric KCC2 and the phopho-KCC2 (active form) vs. monomeric KCC2. Note the lack of significant differences between SOD1$^{G93A}$ at any age and wild type littermates. Data are represented as mean ± s.e.m. **(C)** Confocal images of wild type and SOD1$^{G93A}$ L4 spinal MNs confirmed the maintenance of membrane-bound (active) KCC2 in transgenic animals along disease progression, even in abnormal MNs at 16 weeks of age. Arrowheads show the membrane bound KCC2 in SOD1$^{G93A}$ L4 spinal MNs. Scale bar 20 μm.

### MICROGLIAL REACTIVITY IS INCREASED IN SOD1$^{G93A}$ AND AFTER SCIATIC NERVE CRUSH AND SCI

It has been reported that microglial cells play a central role in the pathway that leads to KCC2 dephosphorylation (Coull et al., 2005; Ferrini et al., 2013). Thus, we evaluated the microglial activation by immunohistochemistry in SOD1$^{G93A}$ , sciatic nerve crush and SCI animals. We focused on the L4-L5 lamina IX in order to analyze the reaction of the microglial cells adjacent to MNs. Results revealed a progressive increase of microglial immunoreactivity in SOD1$^{G93A}$ mice from 8 to 16 weeks of age. On the other hand, SCI and sciatic nerve crush mice also showed an increase in Iba1 reactivity after the lesion at 28 and 7 dpi, respectively. SOD1$^{G93A}$ mice at 16 weeks of age showed similar levels of Iba1 immunoreactivity to those observed in nerve crush and SCI mice, when compared to their respective controls (wild type and naïve) (**Figure 6**).

### INCREASED SEROTONIN PROJECTIONS IN SOD1$^{G93A}$ LUMBAR SPINAL CORD

Serotonin (5-hydroxytryptamine, 5-HT) has been postulated as an important factor that contributes to MN excitability by promoting slight depolarization of their membrane potential through increased persistent inward currents (Heckman et al., 2003). 5-HT has been also related to KCC2 phosphorylation and binding to the cell membrane and the consequent decrease in spasticity after SCI (Bos et al., 2013). For this reason, we assessed the 5-HT projections that arrive to L4-L5 spinal MNs. Immunohistochemical analysis revealed an important increase of 5-HT projections in 16 weeks old SOD1$^{G93A}$ lumbar spinal cord (**Figure 7**, $p < 0.05$).

### REDUCED INHIBITION AND RENSHAW CELLS DEGENERATION IN SOD1$^{G93A}$ LUMBAR SPINAL CORD

Once revealed that KCC2 expression and localization is not altered along ALS progression, we studied the inhibitory circuits in the lumbar spinal cord of 16 weeks aged SOD1 animals to evaluate its potential involvement on hyperreflexia. We analyzed gephyrin as a general marker of inhibitory glycine and GABA systems. Results showed a significant decrease of gephyrin expression in SOD1$^{G93A}$ mice compared to non-transgenic littermates (**Figures 8A,B**). We also labeled the Renshaw cells in lamina VII of the L4 spinal cord, since these cells are glycinergic and importantly contribute to the inhibition of MNs. As described previously (Chang et al., 2009), we found a significant decrease in the number of calbindin positive cells in 16 weeks aged SOD1$^{G93A}$ when compared to WT littermates (**Figure 8C**, $p < 0.05$). The findings suggest that inhibition is reduced in SOD1$^{G93A}$ lumbar spinal cord due to alterations of the glycinergic system.

## DISCUSSION

The results of the present work demonstrate that KCC2 is downregulated after peripheral and central nerve injuries. However, although KCC2 downregulation has been demonstrated to be a key factor in the appearance of hyperreflexia and spasticity after such injuries, we did not found changes in the KCC2 dephosphorylation that could explain the appearance of spastic behavior at the late stage (16 weeks of age) of the ALS murine model. On the other hand, our results suggest that the increased spinal excitability and the appearance of spasticity in ALS may be a consequence of two abnormalities: the loss of inhibitory tone due to loss of Renshaw glycinergic interneurons, and the increased 5-HT projections present in the ventral horn that would directly contribute to increasing MN excitability.

### FACTORS CONTRIBUTING TO SPASTICITY IN SOD1$^{G93A}$ MICE

Spasticity is present in ALS patients and leads to disabling complications in hand function and gait (Wijesekera and Leigh, 2009; Kiernan et al., 2011). Several works have investigated the mechanisms underlying spasticity after traumatic SCI. A relevant finding of these studies is that KCC2 loss of function is an important hallmark of MN increased excitability and thus, of spasticity after SCI (Boulenguez et al., 2010; Bos et al., 2013). The KCC2 is a potassium-chloride cotransporter, responsible for the low intracellular chloride concentration that allows GABA and glycine inhibitory synaptic responses in the adulthood (Rivera et al., 1999; Ganguly et al., 2001; Wang et al., 2002; Payne et al., 2003; Stein et al., 2004; Bray and Mynlieff, 2009). Phosphorylation of S940 in the intracellular C-terminal domain of the KCC2 has

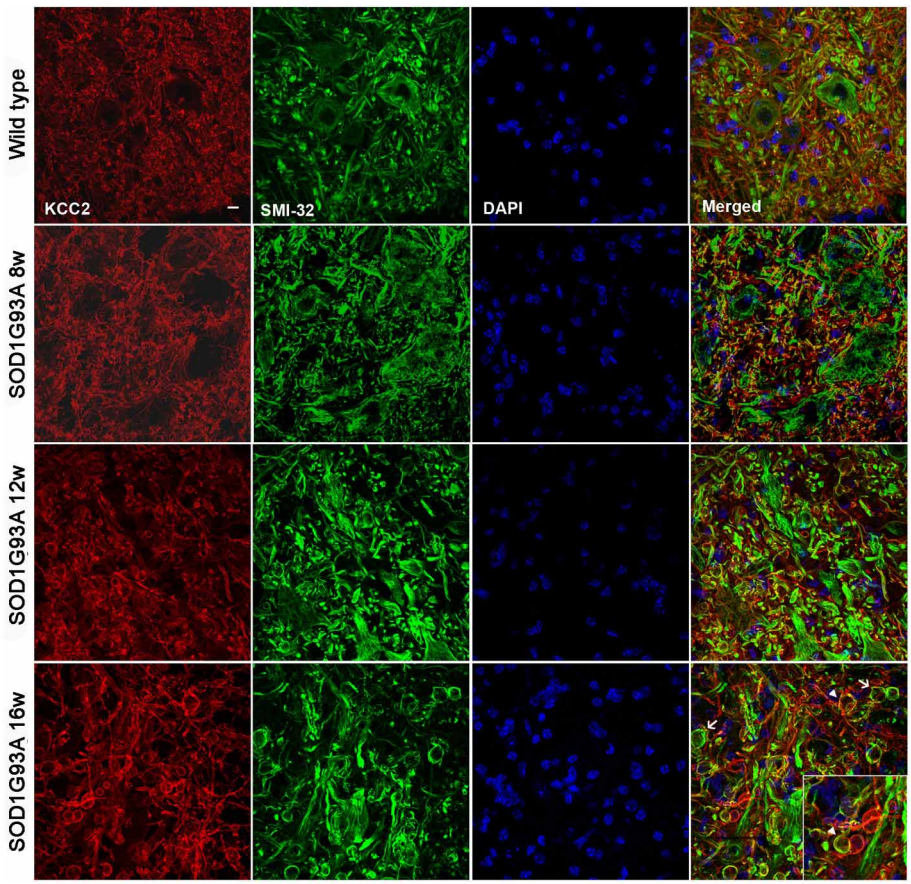


**FIGURE 3 | Confocal images of L4 ventral spinal cord of wild type and SOD1$^{G93A}$ mice at 8, 12, and 16 weeks of age.** Note the progressive increase in number and volume of abnormal swollen structures (arrow) along the disease progression in SOD1$^{G93A}$ animals. The KCC2 remained localized in the cell membrane even in these swallows (arrowheads). Scale bar 10 μm.

been demonstrated to be responsible for the stabilization of KCC2 on the neuronal cell surface, increasing its functional expression (Li et al., 2007; Lee et al., 2007, 2010). The expression and function of KCC2 is reduced after neural injuries, participating in the lowered strength of inhibitory transmission (Coull et al., 2003). The most prevalent mechanism underlying KCC2 regulation has been postulated to be mediated by brain-derived neurotrophic factor (BDNF) released through microglial signaling (Ulmann et al., 2008; Ferrini and De Koninck, 2013). Indeed, microglia react to alterations of the extracellular milieu with a protective and defensive role secreting specific messengers (including BDNF), that in turn sculpt neuronal circuit excitability (Ferrini and De Koninck, 2013). Although, this mechanism has been commonly described in the dorsal horn of the spinal cord, other studies also described the microglia-BDNF-TrkB-KCC2 signaling in the spinal motor system (Ferrini et al., 2013). In agreement, we found that the decrease of KCC2 phosphorylation after central or peripheral nerve injuries in the ventral horn of the spinal cord was also accompanied by increased microglial reactivity. Although this phenomenon has been reported to contribute to spasticity after SCI (Boulenguez et al., 2010; Bos et al., 2013), here we demonstrate that KCC2 is not altered in SOD1$^{G93A}$ mice MN, and unlikely contributing to the hyperreflexia and spasticity that occurs in SOD1$^{G93A}$ animals. Our results revealed that, opposite to what occurs after SCI, KCC2 remained localized in the cell membrane of MN, even in animals of 16 weeks of age when hyperreflexia and spasticity are clearly present. Fuchs et al. (2010) previously reported a down regulation of KCC2 mRNA in spinal MNs of SOD1$^{G93A}$ mice. They found a slight reduction of mRNA signal in only a few large MNs of 80 days old animals (11 weeks of age), despite that almost 50% of lumbar MNs are not functionally connected to muscle at this time (as evidenced by the CMAP reduction). When they analyzed KCC2 mRNA at 120 days (17 weeks of age) they found a significant reduction of mRNA signal area per neuron, although some of the MNs could be in a degenerative state. KCC2 immunoreactivity was reduced in the neuropil surrounding MNs, similar to what we observed (see **Figure 3**), whereas some MNs bodies had increased KCC2 labeling in the cytoplasm (Fuchs et al., 2010). These findings suggest that KCC2 disregulation is slight and does not occur until very advanced stages in SOD1$^{G93A}$ mice, quite later than the muscle denervation process.

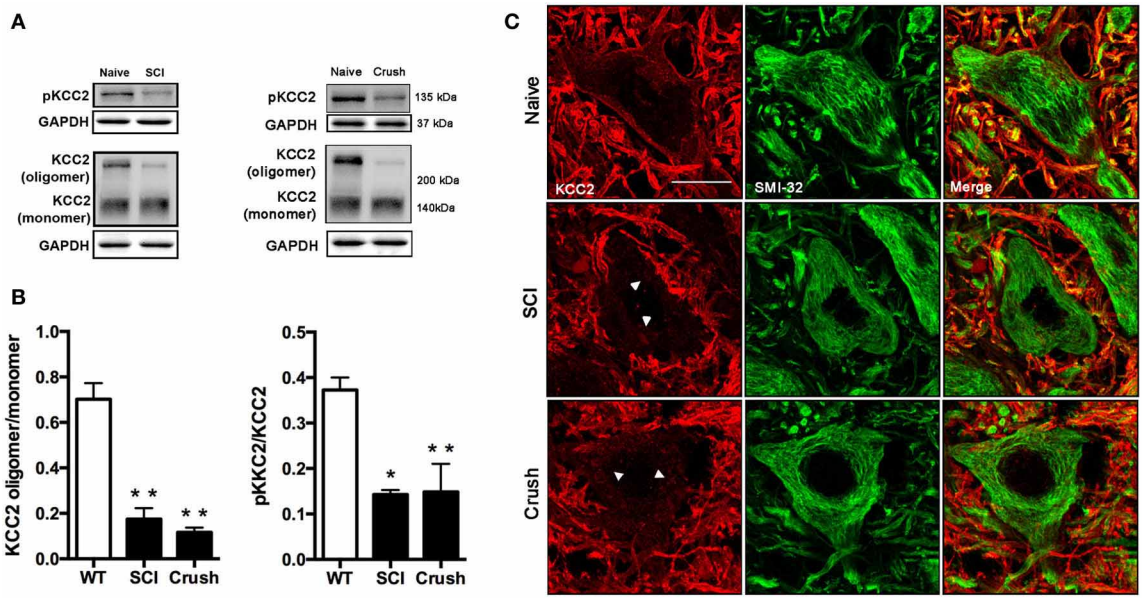


**FIGURE 4 | Analysis of KCC2 and phopho-KCC2 expression in the ventral spinal cord of SCI and sciatic nerve injured mice. (A)** Representative blots of phopho-KCC2 and total KCC2 (on both monomeric and oligomeric states) in SCI and crush mice. **(B)** KCC2 western blots quantifications. Graphs represent the ratio between the oligomeric KCC2 (functional state) vs. monomeric KCC2 and the phopho-KCC2 (active form) vs. monomeric KCC2. Both analyses revealed a decrease in the active form of the KCC2 in SCI (28 days after injury) and in sciatic nerve crush (7 days after injury). Data are represented as mean ± s.e.m.; $*p < 0.05$, $**p < 0.01$ vs. naïve animals. **(C)** Confocal images of L4 spinal MNs revealed the presence of membrane-bound (active) KCC2 in naïve but not in SCI and sciatic nerve injured animals. Arrowheads point internalized KCC2 aggregates. Scale bar 20 μm.

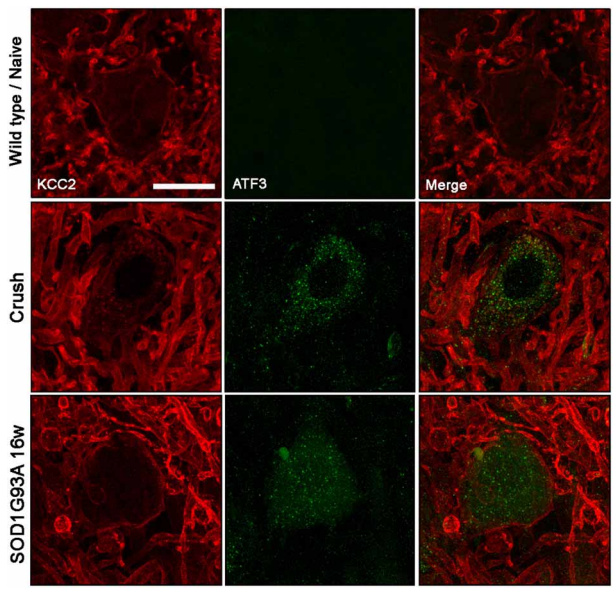


**FIGURE 5 | KCC2 expression and localization in ATF3 labeled MN in wild type/naïve, sciatic nerve injured and 12 weeks old SOD1$^{G93A}$ mice.** Note that membrane-bound KCC2 is reduced in ATF3-positive injured neurons after nerve crush, but it remains in the membrane in ATF3-positive SOD1$^{G93A}$ MNs. Scale bar 20 μm.

Mechanisms underlying spasticity have been mostly studied in experimental models of SCI. It is considered that SCI associated spasticity arises from several mechanisms, one of the most important being alterations of 5-HT inputs to spinal MNs. 5-HT descending axons from brainstem nuclei densely innervate spinal MNs, maintaining their excitability through increased persistent calcium current (Heckman et al., 2003). Although damage of serotonergic axons caused by a SCI leads to a transient hypoexcitability of spinal MNs, after a few weeks, MNs compensate for the loss of serotonin inputs through the overexpression of 5-HT receptors. These synaptic modifications promote hyperexcitability and consequent spasticity (Murray et al., 2010, 2011). In accordance, a recent study showed that the heterogeneity of 5-HT receptors is an important feature of hyperexcitability in MNs after SCI. Bos et al. (2013) reported that activation of 5-HT2B and 5-HT2C receptors induced a depolarizing shift in MNs. However, activation of 5-HT2A participated in the activation and restoration of KCC2 expression. Our results reveal that MNs of SOD1$^{G93A}$ mice receive an increased amount of 5-HT projections at 16 weeks of age. Moreover, in contrast to the localized presence of 5-HT projections around MNs in WT animals, in SOD1$^{G93A}$ mice they became spread over lamina IX in the ventral horn of the spinal cord. As a result, 5-HT labeling was found increased and could partially explain the hyperreflexia and spasticity observed at the late stage of the disease, but also the maintenance of KCC2 in the MN membrane. Further studies assessing the differential

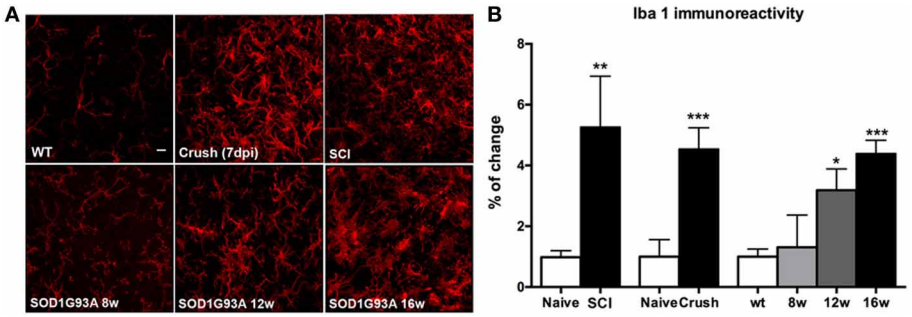


**FIGURE 6 | Comparison of microglial (Iba-1 labeled) immunoreactivity in SOD1$^{G93A}$, SCI and sciatic nerve injured mice. (A)** Representative confocal images of the ventral part of the lumbar spinal cord of wild type/naïve, SOD1$^{G93A}$ at 8, 12, and 16 weeks of age, SCI and sciatic nerve crush injured animals. Scale bar 10 μm. **(B)** Iba-1 immunoreactivity quantification revealed a progressive increase of microglial reactivity during disease progression in SOD1$^{G93A}$ mice. At late stages (16 weeks of age), Iba-1 immunoreactivity level is similar to that observed in SCI and sciatic nerve injured animals. Values are mean ± s.e.m. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. respective wild type/naïve animals.

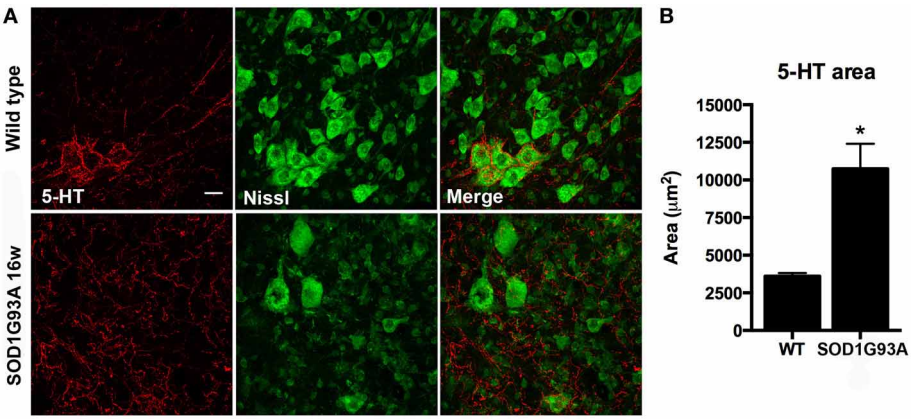


**FIGURE 7 | Serotonin (5-HT) projections to MN pools in the lumbar spinal cord of 16 weeks old SOD1$^{G93A}$ mice and wild type littermates. (A)** Respresentative confocal images show increased 5-HT projections in SOD1$^{G93A}$ compared to wild type mice. Scale bar 20 μm. **(B)** Quantification of the immunolabeling shows a 2 fold increase of 5-HT labeled area in SOD1$^{G93A}$ animals at 16 weeks of age. Values are mean ± s.e.m. $^{*}p < 0.05$ vs. wild type.

expression of 5-HT receptors in SOD1$^{G93A}$ mice would be of interest for understanding their interaction with the KCC2 role in ALS.

We further assessed the inhibitory state of the lumbar spinal cord by measuring gephyrin expression. Gephyrin is a structural component of the postsynaptic protein network of both glycine and GABA inhibitory synapses in the spinal cord (Bohlhalter et al., 1994). Our results show a significant reduction of gephyrin expression in 16 weeks old SOD1$^{G93A}$ animals, evidencing decreased inhibition in the ventral spinal cord. Since gephyrin participates in glycine receptor clustering but not in GABAergic synapses formation (Lévi et al., 2004), we further investigated the glycinergic Renshaw cells at late stages in SOD1 mice. Renshaw cells are spinal interneurons located in the ventral horn gray matter that mediate recurrent inhibition to spinal MNs (Katz and Pierrot-Deseilligny, 1999; Alvarez and Fyffe, 2007). These cells have been related to spasticity (Mazzocchio and Rossi, 1997) and, recently, postulated as contributors to spasticity in ALS (Mazzocchio and Rossi, 2010). In fact, our results revealed a significant decrease in the number of calbindin labeled Renshaw cells in the lumbar spinal cord of SOD1 mice at 16 weeks of age. The loss of inhibitory interneurons could partially explain the abnormally increased spinal excitability found in SOD1$^{G93A}$ animals at late stages. Indeed, Chang and Martin have reported an early presymptomatic loss of glycinergic synaptic buttons onto MNs (Chang and Martin, 2009) and the abnormal properties of glycinergic channels in dissociated G93A MNs (Chang and Martin, 2011).

Taken together, our results suggest two distinct mechanisms that may be contributing to hyperreflexia and increased spinal excitability in the SOD1 mouse model. On the one hand, increased lumbar 5-HT may enhance MN excitability through the activation of 5-HT2B and 5-HT2C receptors and, on the other hand, the loss of Renshaw cell leads to a reduction in inhibitory inputs onto MNs. Both phenomena may explain the disabling spastic paresis observed in SOD1$^{G93A}$ animals at the late stage. These exogenous influences may add to the mild MN

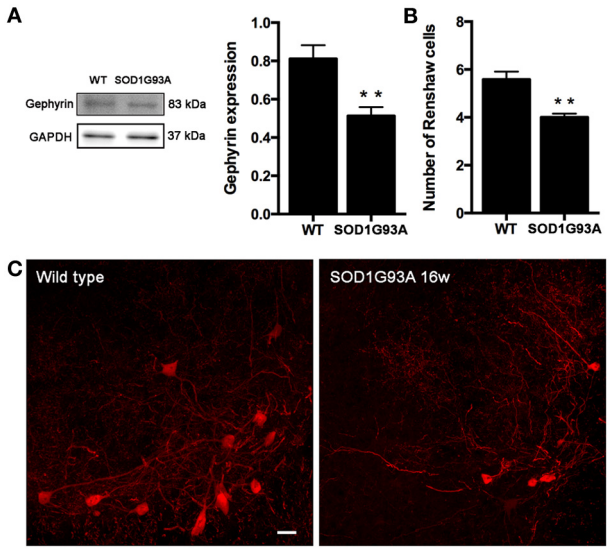


**FIGURE 8 | Evaluation of inhibitory intraspinal circuits in SOD1$^{G93A}$ mice at 16 weeks of age. (A)** Representative blots and quantification of gephyrin expression in the ventral part of the lumbar spinal cord. Results revealed that gephyrin expression in 16 weeks old SOD1 mice was reduced when compared to wild type mice. **(B)** Number of Renshaw cells in the ventral horn of L4 spinal cord of wild type and SOD1$^{G93A}$ animals at 16 weeks of age. Values are mean ± s.e.m. $^{**}p < 0.01$ vs. wild type animals. **(C)** Representative confocal images of Renshaw cells in the L4 spinal cord of wild type and 16 weeks old SOD1$^{G93A}$ animals. Scale bar 10 μm.

depolarization state described in this ALS murine model (Boërio et al., 2010).

## DIFFERENTIAL MN RESPONSE BETWEEN SOD1$^{G93A}$ AND NERVE CRUSHED MNs

Once demonstrated the lack of KCC2 downregulation in SOD1$^{G93A}$ MNs when compared to SCI, we explored whether the KCC2 response could be an intrinsic feature of ALS MNs. To test this hypothesis, we compared the differences in KCC2 expression between ALS and axotomized wild type MNs. Our results demonstrate for the first time that KCC2 down regulation also occurs in spinal MNs after peripheral nerve injury, as showed by the decrease in the active pKCC2 after sciatic nerve crush. Immunohistochemical evaluation also revealed that MNs labeled for ATF3, a typical marker of axonal injury (Tsujino et al., 2000; Navarro et al., 2007), presented an evident translocation of KCC2 from the plasma membrane to cytoplasmic aggregates. This fact indicates that injured MNs were actively down regulating KCC2 from their cell membrane. After lesion of peripheral axons, differential regulation of protein expression occurs and plays a role in transitioning the neuron from a transmission mode to a regenerative, growth mode (Fu and Gordon, 1997; Pieraut et al., 2007, 2011). One example of the plastic changes that occur after axotomy, are the excitatory responses to GABA and glycine induced by the loss of KCC2 in the neuronal cell membrane. This shift in the balance between excitatory and inhibitory influences that renders injured networks hyperexcitable has been implicated in the pathogenesis of neuropathic pain in dorsal horn neurons (Coull et al., 2003; Cramer et al., 2008; Hasbargen et al., 2010; Janssen et al., 2011, 2012).

Our results in SOD1$^{G93A}$ mice revealed that KCC2 remained in its active phosphorylated form and located in the cell membrane even in MNs that highly expressed ATF3, evidencing a recent process of target muscle disconnection (Vlug et al., 2005; Saxena et al., 2009). After axotomy, KCC2 loss of function would contribute to an increase of spinal synaptic excitability and hyperreflexia (Valero-Cabré and Navarro, 2001), as a feature of the plastic changes that may play a role on nerve regeneration and functional recovery. The lack of such changes in ALS MNs could suggest an inability of these cells to initiate some cellular events in response to muscle disconnection that would allow for axonal regeneration and re-establishment of new neuromuscular junctions. The abnormal response to insults would be also manifested by a progressive increase of neurofilament aggregates (Julien, 1997) surrounded by active and phosphorylated KCC2 with age in SOD1$^{G93A}$ MNs. This observation could explain the fact that we did not observe any changes in the KCC2 activity by analyzing the WB result when comparing with wild type animals. Nevertheless, despite the active KCC2 downregulation could be likely due to muscle disconnection *per se*, it cannot be discarded that such changes are produced by transynaptic effects of the injured primary sensory afferents, explaining the differences we observed between ALS and nerve crushed mice. Thus, further experiments are needed to understand which mechanisms underline the reduction of KCC2 activity.

In summary, the present results demonstrate that there is not downregulation of KCC2 expression from the plasma membrane of ALS MNs, even at advanced stage of the disease, when they have suffered deafferentation from upper MNs and axonal damage and muscle disconnection. This is in contrast to what we found after either SCI or peripheral nerve injury, which induced a rapid decrease of KCC2 phosphorylation in MNs. Such a KCC2 change has been previously described in spinal MNs linked to the development of spasticity (Boulenguez et al., 2010), and in dorsal horn neurons related to the appearance of neuropathic pain (Janssen et al., 2012). The fact that KCC2 is not downregulated along the lifespan of SOD1 mice could indicate that ALS MNs do not react as axotomized normal MNs after muscle denervation.

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# Gacyclidine improves the survival and reduces motor deficits in a mouse model of amyotrophic lateral sclerosis

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Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disorder typified by a massive loss of motor neurons with few therapeutic options. The exact cause of neuronal degeneration is unknown but it is now admitted that ALS is a multifactorial disease with several mechanisms involved including glutamate excitotoxicity. More specifically, *N*-methyl-D-aspartate (NMDA)-mediated cell death and impairment of the glutamate-transport has been suggested to play a key role in ALS pathophysiology. Thus, evaluating NMDAR antagonists is of high therapeutic interest. Gacyclidine, also named GK11, is a high affinity non-competitive NMDAR antagonist that may protect against motor neuron death in an ALS context. Moreover, GK11 presents a low intrinsic neurotoxicity and has already been used in two clinical trials for CNS lesions. In the present study, we investigated the influence of chronic administration of two doses of GK11 (0.1 and 1 mg/kg) on the survival and the functional motor activity of hSOD1$^{G93A}$ mice, an animal model of ALS. Treatment started at early symptomatic age (60 days) and was applied bi-weekly until the end stage of the disease. We first confirmed that functional alteration of locomotor activity was evident in the hSOD1$^{G93A}$ transgenic female mice by 60 days of age. A low dose of GK11 improved the survival of the mice by 4.3% and partially preserved body weight. Improved life span was associated with a delay in locomotor function impairment. Conversely, the high dose treatment worsened motor functions. These findings suggest that chronic administration of GK11 beginning at early symptomatic stage may be beneficial for patients with ALS.

**Keywords: GK11, NMDA receptor antagonist, ALS, survival, locomotion**

## INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a chronic neurodegenerative disease characterized by neuronal death of both lower and upper motoneurons in the spinal cord, the brain stem and the motor cortex. This chronic motor neuronopathy leads to progressive atrophy of skeletal muscles, paralysis and ultimately to death of the patients mainly due to respiratory failure [for review see (Turner et al., 2013)].

Pathogenesis and mechanisms of selective vulnerability of motoneurons in ALS are still largely unknown although, within the past two decades, it has been demonstrated that ALS is a complex multifactorial disease. Indeed many factors including protein misfolding, glutamate-mediated excitotoxicity, oxidative stress and impaired axonal transport may contribute to motoneuron death in ALS [for review see Robberecht and Philips (2013)]. An excessive stimulation of glutamate receptors induces excitotoxic processes; this phenomenon being largely implicated in both acute and chronic neurodegenerative diseases (Olney, 1989; Plaitakis and Constantakakis, 1993; Mehta et al., 2013), and in particular in ALS (Heath and Shaw, 2002; Bogaert et al., 2010). In the objective of translation to clinics, excitotoxicity is one of the key pharmacological targets as attested by the only FDA-approved drug, riluzole (2-amino-6-trifluoromethoxy benzothiazole) which is a modulator of excitatory neurotransmitters activity (including glutamate) (Bogaert et al., 2010). Glutamate interacts with a large range of specific transporters and receptors such as N-methyl-D-aspartate receptors (NMDARs) (Mehta et al., 2013) and a promising, but so far overlooked therapeutic strategy, is to reduce excitotoxicity using NMDA receptor antagonist [for review see Spalloni et al. (2013)]. The low-affinity non-competitive NMDAR antagonist, memantine displayed encouraging results in hSOD1$^{G93A}$ mice (Wang and Zhang, 2005; Joo et al., 2007), an ALS animal model. Clinical trials have shown that memantine is safe and well tolerated by ALS patients (de Carvalho et al., 2010; Levine et al., 2010) but no evidence of its efficacy had been reported yet.

Gacyclidine (GK11), a phencyclidine, is a non-competitive NMDA receptor antagonist, with a selective affinity for NR2B receptors, that had been shown to prevent glutamate-induced neuronal death *in vitro* and is less neurotoxic than other NMDA receptor antagonists (Hirbec et al., 2001; Vandame et al., 2007). Moreover, *in vivo* GK11 exhibits neuroprotective effects following organophosphorous nerve agents-induced convulsions (Bhagat et al., 2005) and following spinal cord injury (Feldblum et al.,

2000; Gaviria et al., 2000a,b; Kouyoumdjian et al., 2009; Lonjon et al., 2010).

In this study we have evaluated the effect of chronic treatment of two doses of GK11 on the survival and the locomotor function of hSOD1$^{G93A}$ mice. We demonstrate a dose effect of GK11 on the survival of the mice, treatment with a low GK11 dose induced an increase in life span conversely to a high dose that reduced it. Moreover, treatment-induced increase in survival was associated with a reduction of locomotor function impairment whereas high dose treatment worsened the motor phenotype.

## MATERIALS AND METHODS

### ANIMALS

Transgenic mice carrying the G93A human SOD1 mutation, B6SJL-Tg (SOD1-G93A) 1Gur/J (ALS mice, high copy number) were purchased from The Jackson Laboratory (Bar Harbor, ME, USA) and bred on a B6SJL background. Transgenic mice were identified by PCR and housed in controlled conditions (hygrometry, temperature and 12h light/dark cycle); the environment was not modified over the course of the protocol. Only females were used and litter-matching between groups were done as much as possible. We carried out all animal experiments in accordance with the guidelines approved by the French Ministry of Agriculture and following the European Council directive (2010/63/UE). Every effort was made to minimize the number and suffering of animals. Age of death was defined as functional paralysis of both hindlimbs and a righting reflex >20 s. These criteria follow the commonly accepted guide lines for working on ALS mice (Leitner and Lutz, 2009; Solomon et al., 2011).

### GK11 TREATMENTS

Twice a week, mice (transgenic and control) were intraperitoneally injected with either gacyclidine (two different concentrations; 1 mg/kg or 0.1 mg/kg) (Neuréva, Montpellier, France) or NaCl. The treatment doses were determined according to the following criteria: the dose of 1 mg/kg corresponds to the acute therapeutic dose in rat (Feldblum et al., 2000; Gaviria et al., 2000b) and we wanted to test the possible toxic effect of a chronic administration. Repeated administration of low dose of non-competitive NMDA receptor antagonists induces hyperlocomotion in rat (Wolf and Khansa, 1991; Loscher and Honack, 1992; Matsuoka et al., 2005). Injections were thus not done daily not only to reduce the risk of peritonitis but also to prevent possible interference of injections with behavioral tests. Treatment started at 60 days of age and was carried until the death of the animal. Number of mice: controls injected with GK11 (0.1 mg/Kg, $n = 10$; 1 mg/Kg, $n = 5$) or NaCl ($n = 14$); transgenic mice injected with GK11 (0.1 mg/Kg, $n = 15$; 1 mg/Kg, $n = 5$) or NaCl ($n = 21$).

### BEHAVIORAL ANALYSIS

#### *Catwalk*

We used the CatWalk™ (Noldus, Wageningen, The Netherlands) to study dynamic and voluntarily walking patterns of the mice. As previously described (Gerber et al., 2012a) we selected amongst locomotor patterns, the "relative position" that corresponds to the distance between the placement of front and hind paws over one walking step. For data collection, six runs per animal were performed on a weekly basis from day 60 (just prior the first injection) and until animals were not able to correctly cross the walkway due to hindlimb paralysis. For each mouse, a minimum of three runs crossed at the same speed with 3-full step sequence patterns per run were recorded. To accustom the animals to the environment and thus to avoid bias due to stress, we placed transgenic and control littermates mice on the CatWalk 7 and 3 days prior to the first recording session. Catwalk analyses started before the first injection, the next behavioral session was done at least 72 h after treatment. Recordings were performed until the failure to obtain satisfactory paw patterns with the CatWalk analysis system due to hindlimb paralysis and thus absence of paw detection in the transgenic groups. Data analysis was done in collaboration with InnovationNet (Tiranges, France).

#### *Open field activity*

Spontaneous locomotor activity of mice was monitored in an open field test. Animals were placed in an empty test arena (45 × 45 cm box) and movements automatically recorded. We analyzed the total distance (cm) (Bioseb, Open field, Actitrack software, Vitrolles, France). Recording sessions started at P53 and until the end of life of the transgenic mice; analysis correspond to a weekly 8 min' sessions preceded by 2 min without recordings to avoid any bias due to stress. Open field test were performed on a weekly basis, 48 h after the injection. Recording sessions were performed until mice were unable to move in the test arena

### STATISTICAL ANALYSIS

Kaplan Meier analysis and log-rank test were applied for survival curves (**Figure 1A**). Two ways ANOVA followed by Tukey-Kramer test was used for all analysis that consist on a combination of a treatment (at three different doses, NaCl, GK11 0.1 mg/kg and GK11 1 mg/kg) on two distinct genotypes over time (**Figures 1B**, **2B**, **3A–C**, **4**). From 116 days onward transgenic and control mice could not be directly compared due to speed differences, moreover due to death GK11 1 mg/kg-treated transgenic mice were not included from P123 onwards. We thus used one way ANOVA followed by Tukey-Kramer test for **Figure 3D** and one way ANOVA followed by Mann-Whitney test for **Figures 3E,F**. For all analysis at P60 i.e., before the treatment started *t*-test was done (**Figures 1A**, **A1**). CatWalk data consist on replicates of a minimum of 3 and a maximum of 6 runs (same speed with 3 full step sequence) per animal in all cases these values were averaged. Experiments were designed to reach a 95% power to detect a 10% or greater difference between groups. We used GraphPad Prism version 5.03 (GraphPad software, CA, USA, and Minitab 15, Minitab Inc, USA).

## RESULTS

### DOSE EFFECT OF GACYCLIDINE ON THE SURVIVAL OF THE MICE

We first assessed the influence of a chronic treatment with GK11 (0.1 mg/kg), a non-competitive NMDA receptor antagonist, on the survival of hSOD$^{G93A}$ female mice. Animals treated with GK11 display an increased survival as compared to animals

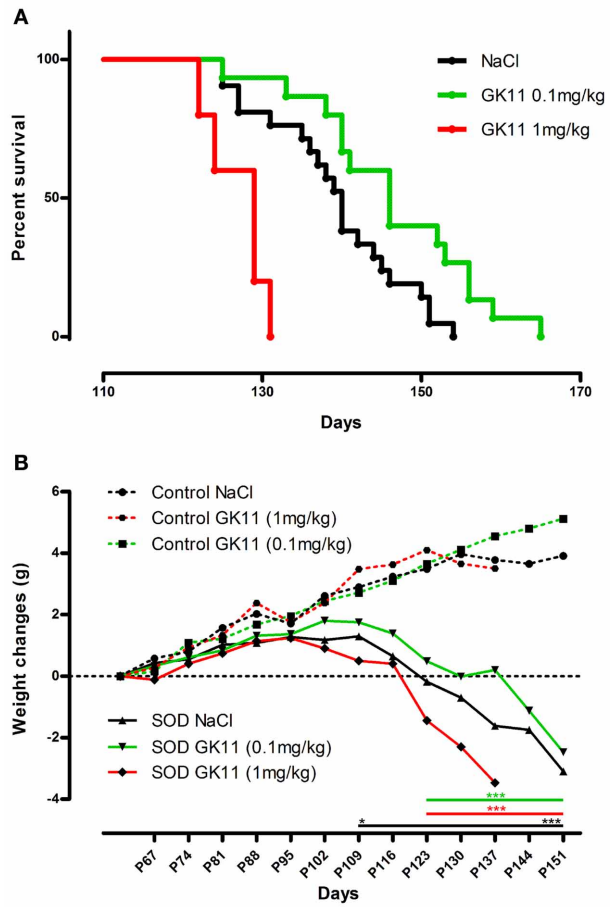


**FIGURE 1 | Dose effect of Gacyclidine on hSOD1$^{G93A}$ mice survival and weight. (A)**—Kaplan-Meier curves of hSOD1$^{G93A}$ mice injected i.p with NaCl ($n = 21$), GK11 0.1 mg/kg ($n = 15$) or GK11 1 mg/kg ($n = 5$). **(B)**—Weight changes (g) in control (dash line) and transgenic mice (continuous line) injected with NaCl, GK11 0.1 mg/kg or GK11.1 mg/kg. Two-ways ANOVA followed by Tukey-Kramer test [$F_{(10,\ 695)=96.18}$]. Weight of NaCl treated controls and transgenics significantly differs from P109 whereas weight of GK11 (both doses) treated controls and transgenics significantly differ from P123. $^{*}p < 0.05$ and $^{***}p < 0.001$.

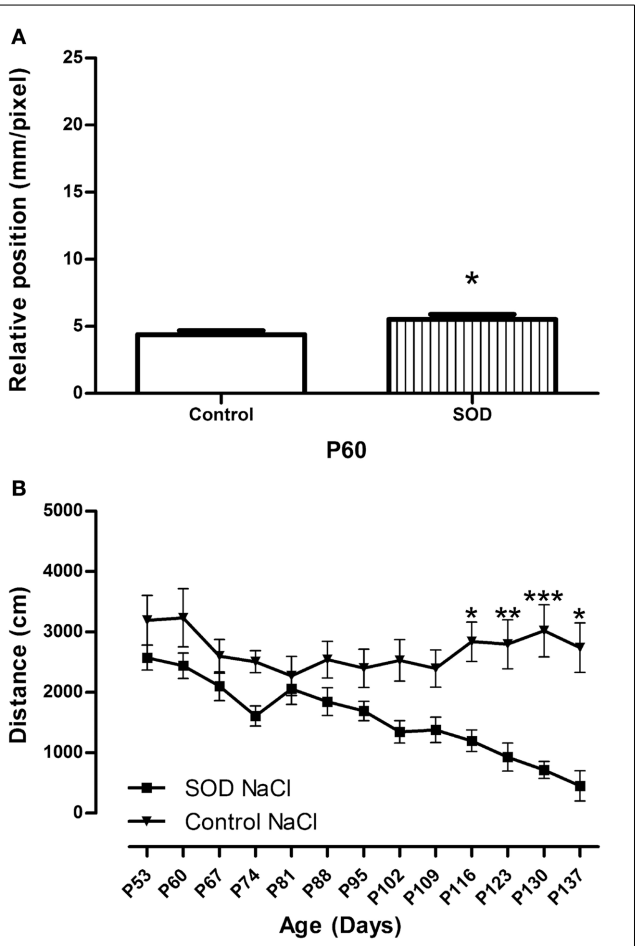


**FIGURE 2 | Locomotor function analysis of hSOD1$^{G93A}$ female mice. (A)**—CatWalk gait analysis. Graph represent the distance between the front and hind paws on a step sequence ("relative position") of control females ($n = 32$) and hSOD1$^{G93A}$animals ($n = 41$) before treatment. Statistics: *t*-test: $^{*}p < 0.05$ **(B)**—Open field test. Spontaneous locomotor activity represented by the mean distance covered by control female and hSOD1$^{G93A}$ mice in a weekly 8 min recording test over disease progression. For Open field test a minimum of 10 animals were used from day 53 to 130, then due to the death of hSOD1$^{G93A}$ mice, a minimum of 5 animals were analyzed (P137). Statistics: two ways ANOVA followed by Tukey Kramer test. $F_{(12,\ 262)=95.95}$. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$.

injected with NaCl. Median survivals are indeed of 146 and 140 for mice injected with GK11 and NaCl respectively; this represent a significant 4.3% increase in life span (Kaplan Meier, *P*-value = 0.0341) (**Figure 1A**).

To evaluate the possible toxicity margin of the molecule, we injected mice with a 1 log higher GK11 concentration (1 mg/kg). Mice chronically injected with this high dose show a significant decrease in lifespan as compared to the littermates injected with NaCl. Median survivals are indeed of 129 and 140 for mice injected with GK11 and NaCl respectively; this represents a significant 7.9% decrease in life span (Kaplan Meier, *P*-value = 0.006) (**Figure 1A**).

Over the progression of the disease, transgenic mice lost weight due to muscular atrophy. We monitored body weight changes from P60 until death. NaCl injected transgenic mice weight differs significantly from those of the NaCl injected control group from P109 whereas weight of GK11 treated transgenic mice (both doses) is significantly different from GK11 treated control mice from P123 (**Figure 1B**). There is no effect of GK11 treatment on the body weight of control mice.

We thus evidence a beneficial effect of a chronic low dose of GK11 on the survival of hSOD1$^{G93A}$ female mice whereas a high dose of GK11 has a detrimental effect.

## LOCOMOTOR FUNCTION IS ALTERED AT 60 DAYS OF AGE IN FEMALE hSOD1$^{G93A}$ MICE

In the perspective of translational studies, it is important to apply the therapeutic strategy at early symptomatic period. Experiments were done on female mice, it was thus mandatory to thoroughly characterize disease onset and progression in female

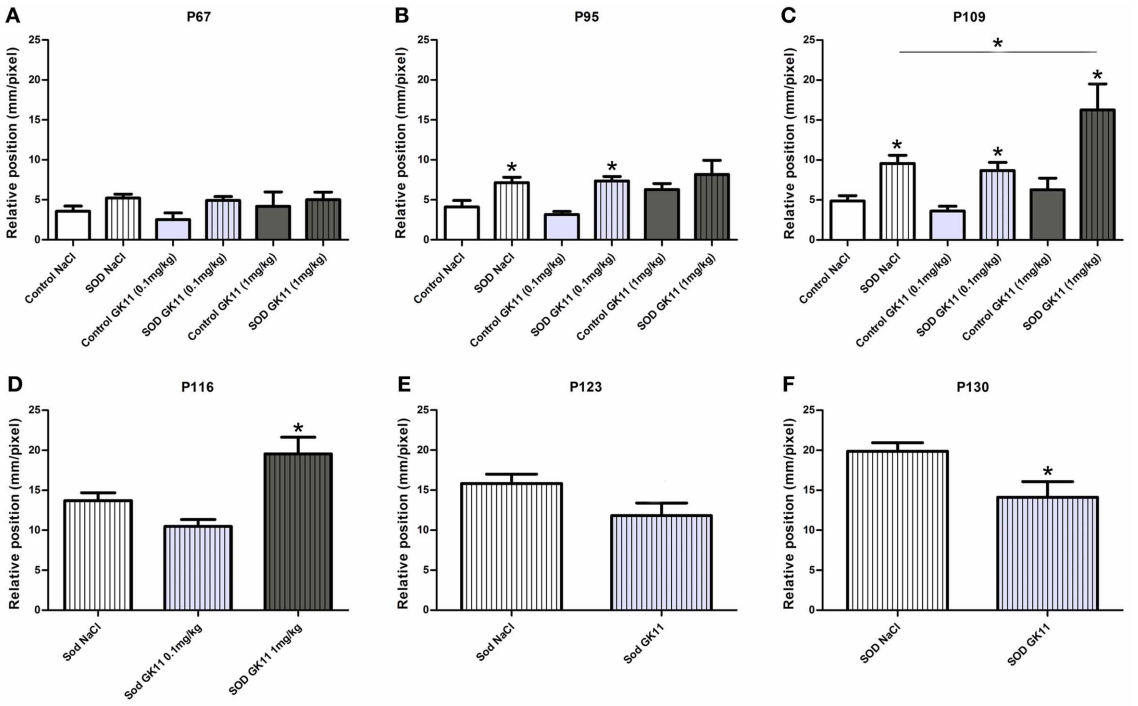


**FIGURE 3 | Evaluation of Gacyclidine effects on hSOD1$^{G93A}$ mice motor pattern: CatWalk gait analysis.** Graphs represent the distance between the front and hind paws on a step sequence ("relative position"). **(A—C)**—control and hSOD1$^{G93A}$ animals (NaCl, GK11 0.1 mg/kg or GK11 1 mg/kg treated)—Respectively at 67, 95, and, 109 days of age. **(D)**—hSOD1$^{G93A}$ animals (NaCl, GK11 0.1 mg/kg or GK11 1 mg/kg injected) at 116 days. **(E, F)** hSOD1$^{G93A}$ animals (NaCl or GK11 0.1 mg/kg injected) at 123 and 130 days. A minimum of 4 and up to 24 animals per time point were analyzed from 67 to 116 days of age; recording were stopped for GK 11 1 mg/kg after P116 due to hindlimb paralysis. Then due to progression of the paralysis a minimum of 8 and up to 15 animals per time point were analyzed from 123 to 130 days of age. Statistics: (**A–C**): two ways ANOVA followed by Tukey Kramer test. **D**: one way ANOVA followed by Tukey Kramer test. (**E**,**F**): one way ANOVA followed by Mann-Whitney test. $^*p < 0.05$.

hSOD1$^{G93A}$ mice. We have used the previously described combination of CatWalk and open field analysis (Gerber et al., 2012a) to evaluate locomotor alterations. To compare with our former motor analysis on male hSOD1$^{G93A}$ mice, we tested the female mice on a weekly basis from 60 days of age and selected the same parameters. We quantified the spontaneous motor activity using the open field system and the "relative position" of the paws (distance between front and hind footprints over a step cycle) using the CatWalk system.

As a prerequisite for accurate CatWalk investigation, we verified if the B6SJL strain is suitable for gait analysis. In that aim, we analyzed the stride of control females from P60 to P130. It had indeed been shown that stride must remain stable in control mice all along the study protocol to avoid any misleading conclusions (Clarke and Still, 1999). The stride remains identical throughout our study (**Table A1**).

At 60 days of age hSOD1$^{G93A}$ female mice present an alteration in gait pattern appearing as the inability to draw their hind limbs up to the previous position of their front limbs (**Figure 2A**) This alteration increased further up to 109 days of age (**Figures 3A–C**, SOD NaCl vs. control NaCl). To evaluate the spontaneous motor activity of hSOD1$^{G93A}$ and control mice, we used the open field system. We evidence a decreased in the mean distance covered by transgenic animals as compared to their control littermate from P116 (**Figure 2B**).

Combination of CatWalk and open field analysis allows an accurate description of motor alterations and permit to identify gait pattern alteration, and thus clinical onset of the disease, at 60 days of age. This is in agreement with our previous results in male hSOD1$^{G93A}$ mice (Gerber et al., 2012a) (**Figure A1**).

## INCREASED LIFE SPAN CORRELATED WITH DELAYED MOTOR DEFICITS

We then used the CatWalk system to tentatively correlate the effects of the drug on the lifespan with modification of disease onset and/or progression of the disease; we compared locomotor functions of transgenic hSOD1$^{G93A}$ females injected with either GK11 (0.1 mg/kg or 1 mg/kg) or NaCl. In parallel, we evaluated whether GK11 had an effect on non-transgenic animal by comparing treated versus NaCl injected control mice.

From 67 days to 109 days, the relative position is identical in transgenic groups injected with GK11 0.1 mg/kg or 1 mg/kg compared to transgenic mice injected with NaCl. Controls animals of all groups (GK11 0.1 mg/kg, 1 mg/kg or NaCl) remain stable over the same period. However, transgenic mice (0.1 mg/kg and NaCl) show an alteration of the motor pattern when compared to their respective controls that increased from 95 to 109 days of age. At 109 days, transgenic mice injected with GK11 at high concentration show a critically altered phenotype compared not only to controls but also to the transgenic group injected with NaCl (**Figures 3A–C**). From 116 days onward, due to hindlimb

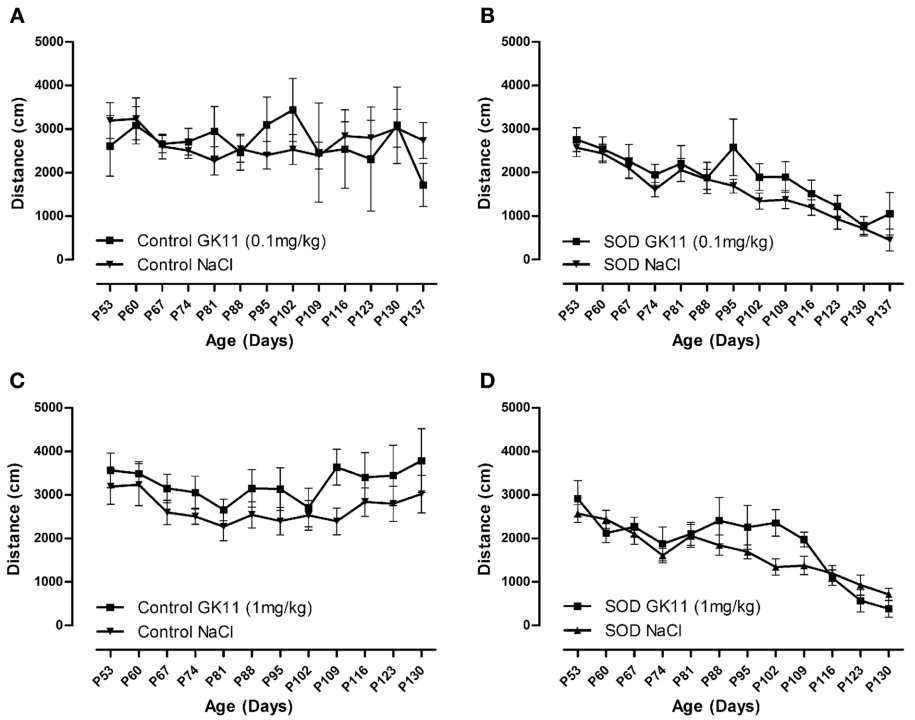


**FIGURE 4 | Evaluation of Gacyclidine effects on the spontaneous motor activity of hSOD1$^{G93A}$ mice: Open Field analysis.** Spontaneous locomotor activity represented by the mean distance covered by female controls and hSOD1$^{G93A}$ mice in a weekly 8 min recording test over disease progression included. **(A,B)**—Control and hSOD1$^{G93A}$ mice injected with NaCl or GK11 0.1 mg/kg. **(C,D)**—Control and hSOD1$^{G93A}$ mice injected with NaCl or GK11 1 mg/kg. A minimum of 5 animals were analyzed per time point. Statistics: two ways ANOVA followed by Tukey Kramer test.

paralysis, transgenic and control mice do not achieve the CatWalk test at the same speed and thus, referring to Clarke's et al. study (Clarke and Still, 1999), cannot be compared. We thus analyzed separately hSOD1$^{G93A}$ and control mice. At 116 days, mice injected with GK11 1 mg/kg show an altered phenotype compared to the NaCl injected mice (**Figure 3D**). No satisfactory recording sessions were obtained for the GK11 1 mg/kg from P123 onwards (**Figures 3E,F**). GK11 0.1 mg/kg treated mice exhibit a reduction of locomotor function impairment compared to the transgenic NaCl group at P130 (**Figure 3F**). No difference between control groups were seen over the same period (data not shown). These results suggest that treatment with a low dose of GK11 induces a slight but significant delay in hindlimb paralysis progression. Oppositely, treatment with a high dose of GK11 worsened the motor pattern.

We used an open field system to measure the spontaneous locomotor activity of control and transgenic mice. No difference has been noted in the mean crossed distances when we compared both GK11 doses (0.1 and 1 mg/kg) and NaCl injected mice for both control and transgenic groups (**Figures 4A–D**).

## DISCUSSION

Glutamate mediated excitotoxicity is involved in ALS pathogenesis (Foran and Trotti, 2009) and several studies have evaluated the pharmacological profile of a number of molecules acting on glutamate release and transport [for review (Corona et al., 2007; Bogaert et al., 2010)]. Gacyclidine (GK11), a non-competitive NMDA receptor antagonist, has already been used in the clinics (Hirbec et al., 2001; Tadie et al., 2003; Lepeintre et al., 2004) and a single GK11 dose of 1 mg/kg has demonstrated neuroprotective effects on a model of photochemical spinal cord injury (Gaviria et al., 2000b). Chronic GK11 administration has never been reported. In view of these data, we decided to analyze the impact of a chronic treatment of GK11 on the survival and locomotor activity of hSOD1$^{G93A}$ mice.

Our study clearly shows a dose response effect; indeed although a high GK11 dose is detrimental by shortening the life span of the mice by 7.9%, a low dose induces a 4.3% increase in survival vs. controls. Moreover, this increased life span is associated with a reduction in walking pattern deficits whereas high dose treatment worsened the motor phenotype.

### GENDER EFFECTS ON SURVIVAL AND FUNCTIONAL DEFICITS IN THE SOD1$^{G93A}$ MOUSE MODEL OF ALS

We have recently carried out a thorough analysis analyses of survival and motor function in hSOD1$^{G93A}$ male mice (Gerber et al., 2012a,b). For the present study we have used females. Transgenic females survive significantly longer than males; median survivals were of 129 and 140 days for males and females respectively. Comparison with a previous study (Heiman-Patterson et al., 2005) attests for the stability of this mouse model; indeed

reported life span was of 128 and 133 days for males and females respectively.

We have evaluated locomotor function of female hSOD1$^{G93A}$ mice using the same combination of dynamic walking patterns and spontaneous motor activity analysis as previously done with males (Gerber et al., 2012a). As for males, we detect early functional deficits that confirm symptoms onset at 60 days of age, i.e., 20 days earlier than previously described (**Figure A1**). Indeed, dynamic gait analysis using the CatWalk system demonstrates alteration in gait pattern for both males and females from 60 days onwards. Spontaneous motor activity evaluated with an open field highlighted a decrease in the mean distance covered by transgenic animals as compared to their control littermate from 116 days onwards for females as compared to 102 days of age onwards for males (Gerber et al., 2012a). This difference may reflect gender-specific mechanism of synaptic impairment since a recent study demonstrated sexual dimorphism in the decline of the presynaptic machinery at an early symptomatic stage (Naumenko et al., 2011). Mean survival of hSOD1$^{G93A}$ mice is of 140 days for female as compared to 129 days for males (Gerber et al., 2012a), however, even if female mice survive longer than male counterparts, early motor symptoms impairment are detected at 60 days of age in both gender.

### ANTIGLUTAMATE DRUGS

One of the popular hypothesis regarding mechanisms involved in ALS is that an excessive activation of glutamate receptors may be responsible for motoneuron death (Corona et al., 2007). NMDA receptors are known for their permeability to cations and in particular to calcium; their over activation leading to excitoxic phenomenon. Recent findings have shown that subtypes of AMPA receptors, that either do not express the GluR2 subunit or express an edited form of this subunit, present a similar permeability to calcium than NMDA receptors (Van Den Bosch et al., 2000; Kawahara et al., 2006; Corona et al., 2007). These AMPA receptor subtypes are widely expressed in motoneurons and are therefore involved in excitotoxic processes (Carriedo et al., 1996; Gerardo-Nava et al., 2013). However, modulating AMPA excitoxicity mediated pathway is mostly ineffective in clinical trials and induce various side effect (such as dizziness and Ataxia) [for review see (Chang et al., 2012)]. Nevertheless, perampanel, a non-competitive AMPA receptor antagonist to AMPAR is very promising in the treatment of epilepsy (Rogawski, 2011). Several antiglutamate drugs have been evaluated in ALS [for review see (Gibson and Bromberg, 2012)]. Riluzole is the only approved treatment and prolongs median survival by about 2 to 3 months in ALS patients (Miller et al., 2012) and by 10.5% in mice (Gurney et al., 1996). Riluzole induces an inhibition of glutamate release and modulation of both alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and N-methyl-d-aspartate (NMDA) receptors (Jin et al., 2010; Cifra et al., 2013). However, Riluzole displays other diverse and multiple non-specific pharmacological properties (Cheah et al., 2010) that may cause adverse effects such as nausea, asthenia and raised alanine transferase (Miller et al., 2012). As recently pinpointed (Spalloni et al., 2013) the role of NMDA receptors activation in ALS has not received much attention, even if a low affinity non-competitive NMDAR antagonist, memantine, has shown promising results in an ALS animal model. In this context, evaluations of new therapeutic agents are of utmost importance. GK11, a high affinity uncompetitive NMDAR antagonist, presents a low intrinsic neurotoxicity, paucity of side effects attributed to its selectivity for NR2 B containing receptors (Vandame et al., 2007). No interaction was found with glutamatergic AMPA, kainate, or $\sigma$1 and $\sigma$2 receptors (Hirbec et al., 2001).

In our study, early symptomatic (starting at P60) bi-weekly chronic administration of a low dose of GK11 (0.1 mg/kg) yields a 4.3% increase in the life span of the female hSOD1$^{G93A}$ mice. These results are similar to the effects of memantine (Wang and Zhang, 2005; Joo et al., 2007). Conversely to memantine (Joo et al., 2007), increase in survival induced by GK11 is associated with a significant protective effect on locomotor deterioration at the end stage of the disease. Of particular interest is the fact that, to-date, GK11 is the most specific NMDA receptor antagonist efficient in an animal model of ALS, thus definitely pinpointing the involvement of this receptor in the pathogenesis of the disease. High dose GK11 treatment leads to a decrease in the life span of female hSOD1$^{G93A}$ associated to a more abrupt development of motor symptoms and hindlimb paralysis. Importantly, high dose drug treatment has no effect on weight loss, CatWalk or open field performances in the control group as reported in a recent study in rats undergoing an acute treatment (Vandame et al., 2013). This toxic side effect might reflect an induction of specific cascade events in the SOD context.

In conclusion, we demonstrate here that a chronic administration (starting at early symptomatic stage) of a non-competitive NMDA receptors antagonist significantly increases the survival of a mouse model of ALS. Indeed, a chronic low dose of GK11 prolongs the life span and reduces weight loss and motor deficits in hSOD1$^{G93A}$ female mice. These results, combined with those from previous studies on CNS injury, demonstrating in patients a relative efficacy and the absence of side effects (Tadie et al., 2003; Lepeintre et al., 2004), suggest that low dose of GK11 could be used as a new and effective NMDA receptor antagonist for ALS patients.

## AUTHOR CONTRIBUTIONS

Yannick N. Gerber: study conception and design, acquisition of data, analysis and interpretation of data and drafting of manuscript. Alain Privat: study conception and design and critical revision. Florence E. Perrin: study conception and design, analysis and interpretation of data and critical revision.

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## APPENDIX

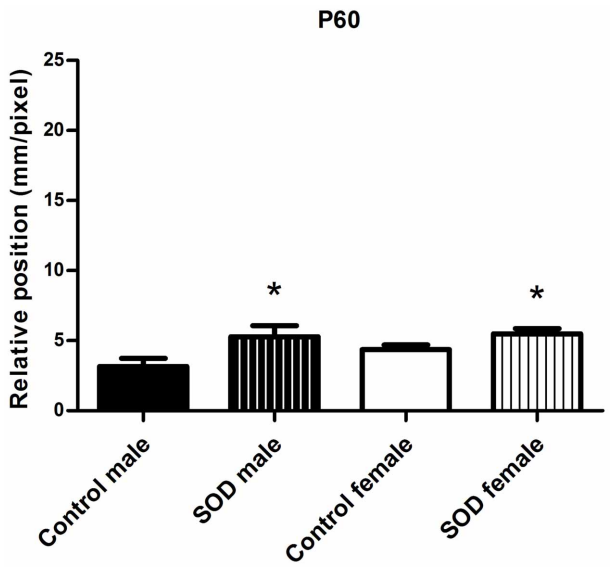


**FIGURE A1 | Locomotor function analysis of hSOD1$^{G93A}$ female and male mice.** CatWalk gait analysis. Graph represent the distance between the front and hind paws on a step sequence ("relative position") of control males ($n = 16$), hSOD1$^{G93A}$ males ($n = 11$), control females ($n = 32$) and hSOD1$^{G93A}$ animals ($n = 41$) at 60 days of age. Statistics: $t$-test: $^*p < 0.05$.

**Table A1 | Frontlimb and hindlimb stride (mm) of control mice at P60 ($n = 22$), Control NaCl at P81 ($n = 12$), P109 ($n = 13$) and P130 ($n = 11$).**

| Colonne1 | P60 | P81 | P109 | P130 |
|---|---|---|---|---|
| Frontlimb (mm) | 58.81 ± 1.275 | 59.00 ± 1.317 | 59.14 ± 1.549 | 61.48 ± 2.053 |
| Hindlimb (mm) | 59.28 ± 1.393 | 59.27 ± 1.372 | 59.14 ± 1.507 | 61.20 ± 1.945 |

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# Trophic factors as modulators of motor neuron physiology and survival: implications for ALS therapy

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Motor neuron physiology and development depend on a continuous and tightly regulated trophic support from a variety of cellular sources. Trophic factors guide the generation and positioning of motor neurons during every stage of the developmental process. As well, they are involved in axon guidance and synapse formation. Even in the adult spinal cord an uninterrupted trophic input is required to maintain neuronal functioning and protection from noxious stimuli. Among the trophic factors that have been demonstrated to participate in motor neuron physiology are vascular endothelial growth factor (VEGF), glial-derived neurotrophic factor (GDNF), ciliary neurotrophic factor (CNTF) and insulin-like growth factor 1 (IGF-1). Upon binding to membrane receptors expressed in motor neurons or neighboring glia, these trophic factors activate intracellular signaling pathways that promote cell survival and have protective action on motor neurons, in both *in vivo* and *in vitro* models of neuronal degeneration. For these reasons these factors have been considered a promising therapeutic method for amyotrophic lateral sclerosis (ALS) and other neurodegenerative diseases, although their efficacy in human clinical trials have not yet shown the expected protection. In this minireview we summarize experimental data on the role of these trophic factors in motor neuron function and survival, as well as their mechanisms of action. We also briefly discuss the potential therapeutic use of the trophic factors and why these therapies may have not been yet successful in the clinical use.

**Keywords: amyotrophic lateral sclerosis, spinal cord neurodegeneration, motor neurons, neurotrophic factors, VEGF**

## INTRODUCTION

Neuronal development and survival depend on a balanced and tightly regulated support from trophic factors. Such factors are capable of regulating several important physiological processes, such as neuronal differentiation, maintenance of synapses, neuronal survival through the inhibition of apoptosis, neurogenesis and axonal outgrowth (Korsching, 1993; Boonman and Isacson, 1999; Hou et al., 2008). In addition, they provide an environmental niche suitable for neuronal survival (Mudò et al., 2009). Trophic support is essential for neurons in the spinal cord and is conferred from many different cellular sources including astrocytes, microglia, neurons and endothelial cells (Ikeda et al., 2001; Béchade et al., 2002; Dugas et al., 2008; Su et al., 2009; Hawryluk et al., 2012). Therefore, trophic support is considered a promising therapeutic strategy for neurodegenerative diseases (Kotzbauer and Holtzman, 2006), and it plays an important role in cellular therapy aimed at the reinnervation of lost neuromuscular synapses (Casella et al., 2010).

Amyotrophic lateral sclerosis (ALS) is caused by the selective and progressive loss of spinal, bulbar and cortical motor neurons that lead to irreversible paralysis, speech, swallowing and respiratory malfunctions and eventually death of the affected individuals in a rapid disease course. ALS is mostly sporadic with 90% of the cases occurring without a family history of the disease. However, in the recent years it has become evident that many sporadic cases carry alterations in proteins that have been found mutated in familial cases that might, at least, increase the probability for developing ALS (Deng et al., 2010). Many of these mutations involve alterations in the TAR DNA-binding protein 43 (TDP43) and Fused in sarcoma (FUS) genes that bind RNA molecules (Gordon, 2013; Sreedharan and Brown, 2013), whereas most familial cases with a dominant autosomal inheritance pattern are caused by mutations in superoxide dismutase 1 (SOD1; Rosen et al., 1993). Transgenic mice expressing a mutant form of the human SOD1 are the most widely used model for *in vivo* studies of ALS (Gurney et al., 1994). Trophic factors have been thought as therapeutic targets for ALS, aiming at restoring lost neuromuscular synapses and rescuing motor neurons from toxicity.

There is a series of well characterized trophic factors for the CNS, such as brain-derived neurotrophic factor (BDNF), insulin-like growth factor 1 (IGF-1), ciliary neurotrophic factor (CNTF), glial-derived neurotrophic factor (GDNF), nerve growth factor (NGF), growth hormone and vascular endothelial growth factor (VEGF). Many of these have been tested for neuroprotective potential in different experimental models of ALS. In fact, viral vectors encoding growth factors are among the most effective ways to delay the progression of degenerative processes and prolong survival in ALS mice (Wang et al.,

2002; Kaspar et al., 2003; Azzouz et al., 2004; Dodge et al., 2008).

## TROPHIC FACTORS DURING MOTOR NEURON DEVELOPMENT

Motor neuron development is differentially affected by specific trophic factor shortage, and loss of particular trophic signaling alters the development of different subpopulations of motor neurons in heterogeneous ways. The absence of GDNF alters the location of developing motor neurons that innervate the limbs in the spinal cord (Haase et al., 2002; Kramer et al., 2006) and selectively affects the innervation of intrafusal muscle spindles (Gould et al., 2008). Interestingly, the overexpression of this factor in muscle during development causes a hyperinnervation of neuromuscular junctions (Nguyen et al., 1998). In contrast, BDNF may not be as important for motor neurons, because although the lack of this trophic factor severely affects the normal development of sensory neurons, motor neurons are able to develop without major alterations (Ernfors et al., 1994a; Jones et al., 1994). Furthermore, distinct motor neuron subpopulations show different sensitivities to the lack of neurotrophins. For example, the absence of neurotrophin-3 produces a complete loss of spinal motor neurons while facial motor neurons are spared (Ernfors et al., 1994b; Gould et al., 2008), and the absence of CNTF produces no alterations for motor neuron development at the spinal or cranial levels (DeChiara et al., 1995), although the loss of its receptor CNTFRα generates severe motor neuron deficits and mice lacking this receptor die perinatally (DeChiara et al., 1995). A possible alternate ligand for this receptor is the dimer formed by cardiotrophin-like cytokine/cytokine-like factor 1, whose deletions have been shown to cause a significant reduction in the number of motor neurons (Forger et al., 2003). The absence of other factors such as cardiotrophin-1 has also been reported to produce a significant loss of motor neurons (Oppenheim et al., 2001; Forger et al., 2003), and the loss of IGF-1 causes significant reduction in the number of trigeminal and facial motor neurons (Vicario-Abejón et al., 2004). Finally, while the lack of VEGF is lethal, a deletion of the hypoxia response element in the promoter region of the VEGF gene causes a decrease in the expression of this factor that leads to an adult-onset progressive loss of motor neurons in mice (Oosthuyse et al., 2001). After this fortuitous discovery, it was reported that certain VEGF haploytpes (-2578C/A, -1154G/A and -634G/C) conferred an increased susceptibility to ALS in humans, but later on in a meta-analysis conducted with more than 7000 subjects from at least eight different populations no association between these haplotypes and ALS was found (Lambrechts et al., 2009). Moreover, no mutations in the hypoxia response element of the VEGF promoter (Gros-Louis et al., 2003), or in the VEGF receptor 2 (Brockington et al., 2007) were found in ALS patients.

Neurotrophic factors are not only important during development, but they also regulate motor neuron maintenance and survival even long after neurons have become fully differentiated. As well, they might be able to trigger the activation of endogenous regenerative processes. Aside from the synthesis of trophic factors in the local spinal microenvironment, synaptic targets of motor neurons also play important roles in the trophic feedback. As a matter of fact, this is an essential event for the development of the CNS during which originating neurons receive trophic input from their target tissues that enables them to surpass an endogenous-codified programmed cell death (Oppenheim, 1991). In the case of motor neurons these effects are mostly mediated by skeletal muscle-derived factors (Oppenheim et al., 1988; Grieshammer et al., 1998; Kablar and Rudnicki, 1999).

## TROPHIC FACTOR EFFECTS ON MOTOR NEURON SURVIVAL

Among all the trophic factors tested in experimental ALS models, VEGF has been shown to be one of the most potent motor neuron protectors. VEGF remarkably retards the progression of the disease and the loss of motor neurons in familial (Azzouz et al., 2004; Zheng et al., 2004; Storkebaum et al., 2005; Wang et al., 2007), as well as in sporadic (Tovar-Y-Romo et al., 2007; Tovar-Y-Romo and Tapia, 2010, 2012) experimental models of motor neurodegeneration.

Activation of VEGF receptor 2 triggers the phosphorylation of intracellular pathways driven by phosphatidyl-inositol-3-kinase (PI3-K), phospholipase C-γ, and mitogen-activated protein kinase (MEK) that promote the inhibition of pro-apoptotic factors like Bad (Yu et al., 2005) and caspases 9 (Cardone et al., 1998) and 3 (Góra-Kupilas and Joško, 2005; Kilic et al., 2006). The activation of these intracellular signaling pathways has been extensively studied in the CNS (Zachary, 2005). VEGF-dependent activation of PI3-K/Akt is sufficient to prevent motor neuronal death in familial models of ALS *in vitro* (Li et al., 2003; Koh et al., 2005; Tolosa et al., 2008) and in experimental *in vitro* models of excitotoxic neuronal death (Matsuzaki et al., 2001). Furthermore, the activation of PI3-K/Akt is required for motor neuron survival and axonal regeneration after spinal cord injury (Namikawa et al., 2000). We have demonstrated that the signaling mediated by PI3-K is critically involved in the protective effect of VEGF against AMPA-induced excitotoxic spinal neurodegeneration *in vivo* (Tovar-Y-Romo and Tapia, 2010).

VEGF also mediates neuroprotection through the inhibition of stress activated protein kinases like p38 mitogen-activated protein kinase. Increased levels of phosphorylated p38 have been found in motor neurons and glia in the familial mouse model of ALS (Tortarolo et al., 2003; Holasek et al., 2005; Veglianese et al., 2006; Dewil et al., 2007), even at the pre-symptomatic stage (Tortarolo et al., 2003), and p38 is also an important factor in a cell death pathway specific for motor neurons (Raoul et al., 2006). Interestingly, the inhibition of p38 prevents motor neuron death in an *in vitro* familial model of ALS (Dewil et al., 2007), and we and others have proven that VEGF can suppress p38 activation in both familial (Tolosa et al., 2009) and excitotoxic (Tovar-Y-Romo and Tapia, 2010) models of spinal cord neurodegeneration.

An increased expression of the VEGF-inducing factor Hypoxia induced factor 1 (HIF-1α) in the spinal cord may occur due to relative hypoxic conditions that exist in the spinal microenvironment, although motor neurons seem to be unable to fully respond to increased downstream effectors such as VEGF (Sato et al., 2012). One possible explanation for this and for the decrease of VEGF levels found in human patients (Devos et al., 2004) might be that inducing factors such as HIF-1α are prevented from translocating to the nucleus even though their concentrations are

increased in the cytoplasm (Nagara et al., 2013). This failure to mount the complete response of VEGF synthesis during hypoxia is not cell type specific and it has been demonstrated to occur in monocytes from ALS patients (Moreau et al., 2011).

In contrast to the good protection potential of VEGF, other factors like BDNF failed to protect in different experimental paradigms. BDNF is synthesized by activated microglia in the first stages of the disease when the glial response mainly exerts anti-inflammatory and protective effects, but its production is lost when microglia turns toxic at later stages (Liao et al., 2012). In addition, BDNF does not protect motor neurons from excitotoxicity in experimental models *in vitro* (Fryer et al., 2000) and *in vivo* (Tovar-Y-Romo and Tapia, 2012). This could be possibly due to the sequestration of the ligand by a truncated isoform of the high affinity receptor that is known to be expressed in motor neurons, because removing this truncated receptor significantly delays the disease onset in the mouse familial model (Yanpallewar et al., 2012). In spite of this, BDNF may be a risk factor for neurons by increasing their sensitivity to excitotoxicity (Fryer et al., 2000), or through the activation of NADPH oxidase (Kim et al., 2002), an enzyme involved in motor neuron pathology by damaging the survival pathways activated by trophic factors (Wu et al., 2006). Other growth factors have also been shown to be beneficial although to a lesser extent.

The expression of GDNF by astrocytes is up-regulated after spinal cord ischemia and this might be a mechanism of protection for motor neurons against excitotoxic death (Tokumine et al., 2003). GDNF exerts its neuroprotective effects preferentially on neuronal somas rather than on nerve endings at the neuromuscular synapse when it is administered directly in the spinal cord (Suzuki et al., 2007). Conversely, when it is administered directly in the muscle, GDNF preserves the muscle-nerve synapse and promotes motor neuron function and survival in a familial model of ALS (Suzuki et al., 2008), implying that the protective effects exerted by GDNF are rather limited by the proximity to the trophic source. Nonetheless, GDNF can be retrogradely transported along motor neuronal axons (Leitner et al., 1999), which allows the opportunity to explore a delivery route that will impact both somas and nerve endings. Interestingly, human ALS patients show an up-regulation of GDNF in muscle (Grundström et al., 1999), and the overexpression of GDNF in muscle but not in astrocytes extends lifespan in ALS mice (Mohajeri et al., 1999). Combined growth factor therapy might be an alternative that is worth exploring, as suggested by a recent report in the rat transgenic ALS model showing that VEGF and GDNF administered through an implant of human mesenchymal stem cells exert a synergistic protection in preserving nerve muscular synapses (Krakora et al., 2013).

In the case of CNTF, although the blockade of its expression has been reported to result in the loss of motor neurons and the development of motor symptoms (Masu et al., 1993), these effects are relatively mild when compared to those induced by the loss of other factors like VEGF. Interestingly, ALS patients have a selective decrease of CNTF expression in the CNS regions affected by the disease (Anand et al., 1995). Conversely, serum levels of CNTF are generally elevated in ALS patients, especially among those with the lumbar-onset form of the disease (Laaksovirta et al., 2008).

## TROPHIC FACTORS AS THERAPY FOR AMYOTROPHIC LATERAL SCLEROSIS (ALS)

Clinical trials administering trophic factors to ALS patients have not been successful yet. Subcutaneous injections of CNTF, which was effective in the mutant mice models of motor neuron disease *pmn/pmn* (Sendtner et al., 1992) and wobbler (Mitsumoto et al., 1994), did not affect the progression of disease in humans, but caused minor adverse side effects (ALS CNTF Treatment Study Group, 1996). Similarly, disease progression was not modified in ALS patients treated with subcutaneous administration of BDNF (The BDNF Study Group, 1999). Two randomized double-blind placebo-controlled clinical trials administering recombinant human IGF showed little (Lai et al., 1997) or no effect (Borasio et al., 1998) on disease progression, even when IGF-1 was found to be protective in the transgenic rodent model of ALS (Kaspar et al., 2003; Dodge et al., 2008). A combined meta-analysis of both trials showed slight retardation in the disease progression in the group treated with IGF-1, although the results are not conclusive (Beauverd et al., 2012). Interestingly, it has been recently reported that skeletal muscle fiber production of IGF-1 is impaired in ALS patients (Lunetta et al., 2012), so that the modest effects found in some of the patients enrolled in the clinical trials might have been due to a compensation of impaired IGF-1 production by the exogenous administration of the factor. Finally, even when according to one report (Morselli et al., 2006) the majority of ALS patients showed deficiencies in growth hormone secretion, in a recent clinical trial the administration of this hormone to ALS patients did not produce any benefit as compared to patients that received placebo (Saccà et al., 2012).

The time of administration after symptom onset in a trophic factor-based therapy is critical. Trophic factors have a short time frame for protection of motor neurons once the noxious process is triggered and this is probably due to the rate at which motor neurons die during the time course of the disease. Histological studies of human spinal cord showed a large variability between the degree of motor neuron loss and muscle weakness (Stephens et al., 2006), and transgenic familial amyotrophic lateral sclerosis (FALS) mice bearing human (Dal Canto and Gurney, 1995; Bruijn et al., 1997) or murine (Morrison et al., 1998) mutant SOD1 do not present a significant loss of motor neurons prior to the onset of symptoms, and the neuronal loss occurs at a very fast rate over a period of 10 days. In our model of chronic spinal cord excitotoxicity we found that the onset of motor deficits, characterized by limping of the rear limbs, occurs before the loss of motor neurons, suggesting that the time at which the cellular death process starts but prior to clear neuronal degeneration constitutes a therapeutic frame within which growth factor administration could result effective (Tovar-Y-Romo et al., 2007; Tovar-Y-Romo and Tapia, 2012). In fact, in the FALS murine models the administration of VEGF (Azzouz et al., 2004; Storkebaum et al., 2005) or IGF-1 (Kaspar et al., 2003; Dodge et al., 2008) well before the beginning of symptoms confers a significantly better protection, observed by a delay in the progression of symptoms and increased lifespan, as compared to that produced when administered at the symptoms onset. A similar result was obtained in rats subjected to spinal AMPA-induced excitotoxicity, in which a delayed administration of VEGF clearly protected but only when administered before

the beginning of motor deficit symptoms (Tovar-Y-Romo and Tapia, 2012). This difference possibly means that growth factors are helpful at preventing the accumulating toxicity that arises from neurodegenerative processes that begin before motor neuron death or symptoms onset (Dal Canto and Gurney, 1995; Bendotti et al., 2001). Unfortunately, obtaining a correct diagnosis of ALS is a complicated and slow process due to the many parameters needed to meet diagnosis criteria (Shook and Pioro, 2009; Bedlack, 2010), so that the earliest intervention with trophic factors once a patient is diagnosed may be already too late.

Administration routes for trophic factor therapy are also important. This is of special interest when considering that in the actual human disease cellular alterations take place along the entire spinal cord, which might be a target particularly difficult to reach. Therefore, assessing different ways to deliver trophic factors is worth trying. Intracerebroventricular (ICV) administration of VEGF has been proven efficient in the rat transgenic model of FALS (Storkebaum et al., 2005) and in our acute model of spinal cord excitotoxicity (Tovar-Y-Romo and Tapia, 2012). ICV administration has the capability to cover the entire spinal cord although it most probably creates a concentration gradient (Storkebaum et al., 2005). The continuous perfusion of trophic factors in the spinal cord by intrathecal infusions or into the brain by ICV injections overcome the blockade that the blood brain barrier represents for the delivery of these molecules. In fact, intrathecal injections have been tried in ALS patients for the delivery of IGF-1, with modest results (Nagano et al., 2005). Clinical trials for VEGF are now underway to assess the safety and tolerability of VEGF (Siciliano et al., 2010).

Other important aspects to consider in growth factor therapies are the stability of the molecule, the half-life of the proteins, the need for sustained delivery and exposure, the dose, their ability to cross the blood brain barrier, and the unwanted side effects on non-targeted cells (Suzuki and Svendsen, 2008). Nonetheless, the neuroprotective potential that growth factor represent overweighs the obstacles that need to be overcome in order to achieve a successful therapy.

## CONCLUSIONS

Because trophic support is an essential component for neuronal maintenance and survival, supplying motor neurons subjected to stressful or noxious stimuli with molecular factors that help them counteract cellular death processes, growth factors represent a therapeutic tool that is undoubtedly worth exploring for ALS. However, we still need to understand a great deal of the molecular pathways that cause growth factor shortage during the course of disease and the cellular and molecular mechanisms that limit the responses elicited by these factors when they are supplied exogenously. As well, we still need to identify proper therapeutic regimens and treatment approaches to be able to translate the findings we have made in experimental models into useful therapeutic procedures.

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# Cellular and molecular mechanisms involved in the neuroprotective effects of VEGF on motoneurons

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Vascular endothelial growth factor (VEGF), originally described as a factor with a regulatory role in vascular growth and development, it is also known for its direct effects on neuronal cells. The discovery in the past decade that transgenic mice expressing reduced levels of VEGF developed late-onset motoneuron pathology, reminiscent of amyotrophic lateral sclerosis (ALS), opened a new field of research on this disease. VEGF has been shown to protect motoneurons from excitotoxic death, which is a relevant mechanism involved in motoneuron degeneration in ALS. Thus, VEGF delays motoneuron degeneration and increases survival in animal models of ALS. VEGF exerts its anti-excitotoxic effects on motoneurons through molecular mechanisms involving the VEGF receptor-2 resulting in the activation of the PI3-K/Akt signaling pathway, upregulation of GluR2 subunit of AMPA receptors, inhibition of p38MAPK, and induction of the anti-apoptotic molecule Bcl-2. In addition, VEGF acts on astrocytes to reduce astroglial activation and to induce the release of growth factors. The potential use of VEGF as a therapeutic tool in ALS is counteracted by its vascular effects and by its short effective time frame. More studies are needed to assess the optimal isoform, route of administration, and time frame for using VEGF in the treatment of ALS.

**Keywords: VEGF, motoneuron, ALS, AMPA receptors, excitotoxicity, Akt**

## INTRODUCTION

Vascular endothelial growth factor (VEGF) was originally described as a factor with a regulatory role in vascular growth and development (reviewed by Carmeliet, 2003; Ferrara, 2004); currently, it is also known for its direct effects on a variety of neuronal cells, modulating neuronal migration, neuritic outgrowth, axon guidance and neuronal survival (reviewed by Ruiz de Almodovar et al., 2009; Mackenzie and Ruhrberg, 2012; Rosenstein et al., 2012).

The VEGFs form a family of growth factors that includes VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, and placental growth factor (Takahashi and Shibuya, 2005). The biological activity of the VEGF family is mediated through the binding to two classes of receptors. The tyrosine kinase receptors include the VEGF receptor-1 (VEGFR1, Flt-1), VEGF receptor-2 (VEGFR2, KDR, Flk-1), and VEGF receptor-3 (VEGFR3, Flt-4). The second class, the non-tyrosine kinase receptors, are the neuropilin-1 (NP-1) and neuropilin-2 (NP-2), which are also receptors for semaphorins, and function as co-receptors for the VEGFRs (reviewed by Carmeliet and Ruiz de Almodovar, 2013).

Vascular endothelial growth factor-A (hereafter referred as VEGF) is expressed in different isoforms in humans, which differ in molecular mass, solubility, receptor affinity, and most likely, in its biological function. $VEGF_{165}$ is the predominant isoform and is secreted as a 45-kDa covalently linked homodimer (reviewed by Bogaert et al., 2006). VEGF is widely expressed throughout the central nervous system. Its expression has been reported in neurons (Ogunshola et al., 2002; Schiera et al., 2007), astroglia (Ijichi et al., 1995), and microglia (Bartholdi et al., 1997). VEGF expression is low in the normal adult spinal cord (Fu et al., 2005); however, it increases in response to injury (Choi et al., 2007). VEGF binds to VEGFR1, VEGFR2, NP-1, and NP-2. VEGFR2 is expressed in many populations of neurons and some glial cells; whereas VEGFR1 is predominantly expressed by activated astrocytes and microglia following acute injury (Ogunshola et al., 2002; Choi et al., 2007; Krum et al., 2008; Ruiz de Almodovar et al., 2009). In addition, direct effects of VEGF on Schwann cells have been described (Sondell et al., 1999). NP-1 and NP-2 are expressed in different types of neurons (Kolodkin et al., 1997; Giger et al., 1998), and also in spinal cord motoneurons (Oosthuyse et al., 2001).

Vascular endothelial growth factor has pro-survival effects on some neuronal cells, protects against experimentally induced cell death (Jin et al., 2000), stimulates axonal growth, and guidance (Sondell et al., 2000; Erskine et al., 2011; Ruiz de Almodovar et al., 2011), stimulates neurogenesis (Jin et al., 2002), regulates neuronal migration (Schwarz et al., 2004; Ruiz de Almodovar et al., 2010), and promotes dendrite patterning and synaptic plasticity (Licht et al., 2010, 2011). In addition to the vascular effects of VEGF protecting motoneurons by ensuring optimal blood supply to brain and spinal cord, it functions as a neurotrophic factor for motoneurons (Oosthuyse et al., 2001; Van Den Bosch et al., 2004). VEGF protects motoneurons from insults such as oxidative stress (Li et al., 2003), hypoxia/hypoglycemia (Van Den Bosch et al., 2004), and glutamate-excitotoxicity (Tovar-Y-Romo et al., 2007; Tolosa et al., 2008; Tovar-Y-Romo and Tapia, 2010).

## ROLE OF VEGF IN AMYOTROPHIC LATERAL SCLEROSIS PATHOGENESIS

The discovery in the past decade that transgenic mice with a homozygous deletion in the hypoxia response element site in the VEGF promoter ($VEGF^{\delta/\delta}$ mice) expressed reduced levels of VEGF (25–40% less) and developed late-onset motoneuron pathology reminiscent of amyotrophic lateral sclerosis (ALS), opened a new field of research on this dramatic disease. Interestingly, all the classic features of ALS including misaccumulation of neurofilaments in brainstem and spinal cord motoneurons, degeneration of motor axons, and denervation-induced muscle atrophy can be observed in these mice (Oosthuyse et al., 2001). As expected, mice engineered to overexpress VEGF had a delayed motoneuron degeneration and an increased survival when crossed to the superoxide dismutase-1 (SOD1) mouse model of ALS (Wang et al., 2007). In addition, the reduction in the levels of VEGF in the SOD1 mutant mice by crossbreeding the SOD1 mouse model of ALS with $VEGF^{\delta/\delta}$ mice worsened the disease, resulting in a decrease in survival due to more severe motoneuron degeneration and earlier onset of muscle weakness (Lambrechts et al., 2003). Interestingly, in the SOD1 mutant mice model of ALS, mutant SOD1 can disrupt the post-transcriptional regulation of VEGF, leading to decreased production of this neurotrophic factor. This effect seems to be restricted to spinal cord, and the decline in VEGF mRNA levels is apparent before onset of weakness, and is more pronounced at middle and end-stages of the disease (Lu et al., 2007). Together, these results suggest a clear relationship between VEGF expression and the familial forms of ALS linked to SOD1 mutations. It still remains unknown the role that VEGF could play in sporadic ALS. In this sense, genetic studies in humans have indicated that VEGF is a modifier of motoneuron degeneration, as a low-VEGF genotype was associated to an increased susceptibility to ALS (Lambrechts et al., 2009).

It is accepted that the major mediator of the trophic effects on spinal cord motoneurons is VEGFR2 (Tolosa et al., 2008; Tovar-Y-Romo and Tapia, 2010), and the concurrent expression of VEGF and VEGFR2 may suggest autocrine/paracrine effects on these cells (Oosthuyse et al., 2001; Ogunshola et al., 2002; Brockington et al., 2006). Interestingly, both VEGF and VEGFR2 expression is reduced in motoneurons and spinal cord of ALS patients (Brockington et al., 2006). Furthermore, the importance of VEGFR2 has been reinforced by experiments showing increased survival of SOD1 mutant mice after overexpression of VEGFR2 (Storkebaum et al., 2005). These findings support the hypothesis that reduced VEGF signaling may play a role in the pathogenesis of ALS (reviewed by Sathasivam, 2008).

Excitotoxicity is a fundamental mechanism involved in motoneuron degeneration in ALS (reviewed by Van Den Bosch et al., 2006). Defective glutamate transport, causing an abnormally increased extracellular concentration of glutamate and over activation of glutamate receptors, has been proposed as an important mechanism in the excitotoxic process in ALS (Rothstein, 2009). In this regard, a decreased expression of the GLT-1 astroglial transporter has been found in the SOD1 animal models around spinal cord motoneurons (Bendotti et al., 2001; Howland et al., 2002). Excessive calcium influx through α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) glutamate receptors is the final effector of motoneuron death in the excitotoxic process. Motoneurons are especially vulnerable to AMPA receptor-mediated excitotoxicity both *in vitro* and *in vivo* as they express a high number of $Ca^{2+}$-permeable AMPA receptors (Carriedo et al., 1996; Van Den Bosch et al., 2000). The permeability of the AMPA receptor depends upon the GluR2 subunit, which regulates the permeability to calcium: only AMPA receptors lacking GluR2 are permeable to calcium. In this regard, motoneurons express low levels of GluR2 and this renders them vulnerable to AMPA receptor-mediated excitotoxicity (Van Damme et al., 2002). Thus, selective loss of motoneurons can be induced experimentally by intrathecal or intraspinal administration of AMPA receptor agonists (Corona and Tapia, 2004; Sun et al., 2006).

In our laboratory, we used spinal cord organotypic cultures to create a model of chronic glutamate excitotoxicity in which glutamate transporters were inhibited by threohydroxyaspartate (THA) to induce motoneuron death. The exposure of these cultures to THA in the presence of VEGF significantly increased motoneuron survival (Tolosa et al., 2008). Similar results were previously obtained *in vivo* after AMPA-induced chronic excitotoxicity in rat spinal cord (Tovar-Y-Romo et al., 2007). Thus, VEGF protects motoneurons from excitotoxic death; however, it has been recently demonstrated *in vivo* that the therapeutic potential of VEGF against excitotoxicity has a short effective time frame, i.e., VEGF was effective only when administered before the onset of motor symptoms (Tovar-y-Romo and Tapia, 2012).

## MECHANISMS OF VEGF PROTECTION AGAINST EXCITOTOXICITY IN ALS

Matsuzaki et al. (2001) initially identified VEGFR2 as the receptor responsible for the neuroprotective effects of VEGF against excitotoxicity in hippocampal neurons. VEGFR2 is expressed by motoneurons in humans (Brockington et al., 2006), mouse (Oosthuyse et al., 2001), and neonatal (Tolosa et al., 2008) and adult rats (Tovar-Y-Romo and Tapia, 2010), and the anti-excitotoxic effects of VEGF in these cells have also been attributed to this receptor (Bogaert et al., 2006; Tolosa et al., 2008; Tovar-Y-Romo and Tapia, 2010).

The signal transduction pathways activated by VEGF are well-characterized in endothelial cells; however, the knowledge of the signaling pathways involved in the anti-excitotoxic effects of VEGF is still incomplete. Upon ligand binding, VEGFR2 undergoes phosphorylation (Meyer et al., 1999), activating intracellular signaling pathways including phosphatidylinositol 3-kinase (PI3-K)/Akt and mitogen-activated protein kinase/extracellular signal-regulated kinase (MEK)/extracellular signal-regulated kinase (ERK). The relevance of the PI3-K/Akt pathway in the neuroprotective effects of VEGF was first proven on the motoneuron-like NSC34 cell line (Li et al., 2003) and also in SOD1 mutant rats where it was shown to counteract the loss of Akt activity preceding motoneuron degeneration (Dewil et al., 2007b). We demonstrated for the first time in spinal cord organotypic cultures that inhibition of the PI3-K/Akt pathway abolishes the anti-excitotoxic effects of VEGF on motoneurons exposed to a glutamate transporter inhibitor (Tolosa et al., 2008). These results were further confirmed *in vivo* in rats exposed to AMPA (Tovar-Y-Romo and

Tapia, 2010). These studies also suggested that the MEK/ERK was less relevant than the PI3-K/Akt signaling pathway, as MEK inhibition had a limited effect on the VEGF-mediated neuroprotection against AMPA-induced excitotoxicity (Tovar-Y-Romo and Tapia, 2010).

Activation of PI3-K by VEGF has additional neuroprotective implications as Akt phosphorylates and activates the cyclic AMP-response element binding protein (CREB), involved in the transcription of the Bcl-2 gene (Pugazhenthi et al., 2000). We demonstrated that excitotoxic conditions are associated to a decreased expression of Bcl-2 in spinal cord cultures, and that VEGF-induced neuroprotection in motoneurons could be related to the restoration, via PI3-K, of Bcl-2 levels in these cultures, and specifically in motoneurons (Tolosa et al., 2008). Bcl-2, besides its ability to block cytochrome *c* release, has been shown to increase calcium uptake and buffering capacity in mitochondria (Zhong et al., 1993), thus protecting against excitotoxicity. Additionally, it has been shown that Bcl-2 overexpression attenuates motoneuron degeneration in the SOD1 animal model (Azzouz et al., 2000).

Interestingly, it has been suggested that the PI3-K/Akt signaling pathway could be involved in GluR2 subunit assembly into AMPA receptors (Rainey-Smith et al., 2010). In this sense, VEGF has been shown, both *in vitro* and *in vivo*, to increase the expression of GluR2 subunit, thus reducing the permeability of AMPA receptors to calcium, and minimizing the vulnerability of motoneurons to AMPA-mediated excitotoxicity (Bogaert et al., 2010). Thus, a potential mechanism for VEGF protection against excitotoxicity would be through a PI3-K/Akt-mediated insertion of the GluR2 subunit of the AMPA receptor in motoneurons. Astrocytes are able to protect against excitotoxicity by inducing GluR2 expression in motoneurons. Interestingly, mutant SOD1 abolishes the ability of astrocytes to regulate GluR2 and thus, increase the susceptibility of motoneurons to excitotoxicity (Van Damme et al., 2007). It remains unknown if the VEGF-induced insertion of GluR2 could be astrocyte-mediated.

p38 mitogen-activated protein kinase (p38MAPK) belongs to a family of protein kinases activated by a range of stimuli including proinflammatory cytokines and oxidative stress (Mielke and Herdegen, 2000). As increased phosphorylation of p38MAPK has been reported in the spinal cord of SOD1 mutant mice, in motoneurons and glial cells, this kinase has been suggested to play a role in the pathogenesis of ALS (Tortarolo et al., 2003; Bendotti et al., 2004). In addition, a motoneuron specific death pathway, involving Fas, p38MAPK, and neuronal nitric oxide synthase activation has been described. Motoneurons from SOD1 mutant mice displayed increased susceptibility to activation of this pathway (Raoul et al., 2002).

Rho-mediated calcium-dependent activation of p38αMAPK has been described as a trigger of excitotoxic cell-death (Semenova et al., 2007). In this regard, it has been shown that VEGF is able to block the AMPA-induced phosphorylation of p38MAPK (Tovar-Y-Romo and Tapia, 2010), thus identifying another molecular mechanism for the anti-excitotoxic effects of VEGF. However, the sole inhibition of p38MAPK activity is not sufficient to protect motoneurons against excitotoxicity as the anti-excitotoxic effects of VEGF are also dependent on the activation of the PI3-K/Akt pathway (Tovar-Y-Romo and Tapia, 2010). In this regard, PI3-K/Akt has been reported to inhibit the phosphorylation of p38MAPK in an apoptosis signal-regulating kinase 1 (ASK1)-dependent manner (Ichijo et al., 1997; Kim et al., 2001). In agreement with that, our group has demonstrated that VEGF protects motoneurons from serum deprivation-induced cell death through PI3-K-mediated inhibition of p38MAPK phosphorylation (Tolosa et al., 2009). Moreover, the inhibition by VEGF of p38MAPK might protect motoneurons in ALS tissue exerting a dual role both through an indirect effect on glial cells (Tortarolo et al., 2003), and a direct anti-apoptotic effect on motoneurons (Dewil et al., 2007a).

## ROLE OF NON-NEURONAL CELLS IN THE NEUROPROTECTIVE EFFECTS OF VEGF

Astroglia (Oosthuyse et al., 2001) and microglia (Bartholdi et al., 1997) are sources of VEGF in the spinal cord and a role for non-neuronal cells has been described in the onset and progression of the pathology in ALS (Clement et al., 2003; Barbeito et al., 2004; Sargsyan et al., 2005). It has been hypothesized that VEGF may also affect motoneurons through an indirect effect on glial cells, as both astrocytes (Krum et al., 2002) and microglia (Ryu et al., 2009) respond to VEGF stimulation. On the one hand, VEGF may affect the glial release of trophic factors, and thus, indirectly, protect motoneurons (reviewed by Bogaert et al., 2006). On the other hand, VEGF decreases the astroglial activation observed in the SOD1 mouse model of ALS, and also enhances neuromuscular junction formation (Zheng et al., 2007). Moreover, the neuroprotective effects observed with lithium in animal models of ALS could be due, in part, to an upregulation of VEGF in non-neuronal cells, as an increase in VEGF has been observed after lithium exposure in brain astrocytes and endothelial cells (Guo et al., 2009). In spite of these potential neuroprotective effects of VEGF involving non-neuronal cells, recently, it has been demonstrated that, under inflammatory conditions, astrocytic expression of VEGF is a key driver of blood–brain barrier disruption, leading to edema, excitotoxicity, and entry of inflammatory cells (Argaw et al., 2012).

Several *in vivo* and *in vitro* studies have indicated that VEGF induces adult neurogenesis (Jin et al., 2002; Cao et al., 2004). It still remains unknown if VEGF *in vivo* induces neurogenesis directly in neural stem cells or indirectly through effects on endothelial cells or other cell types (reviewed by Carmeliet and Ruiz de Almodovar, 2013). The potential of VEGF generating new neurons, together with its ability to induce axon growth could be relevant in its neuroprotective effects on ALS.

## POTENTIAL USE OF VEGF AS A THERAPEUTIC TOOL IN ALS

Vascular endothelial growth factor clearly ameliorates the illness in the mutant SOD1 mice and rats (Azzouz et al., 2004; Storkebaum et al., 2005; Wang et al., 2007), supporting the hypothesis of a role for VEGF in ALS. VEGF has been administered to animals using different strategies. VEGF was administered using lentiviral vectors (intramuscularly delivered and then retrogradely transported) increasing the life expectancy of ALS mice. The treatment was more effective when initiated before disease onset (Azzouz et al., 2004).

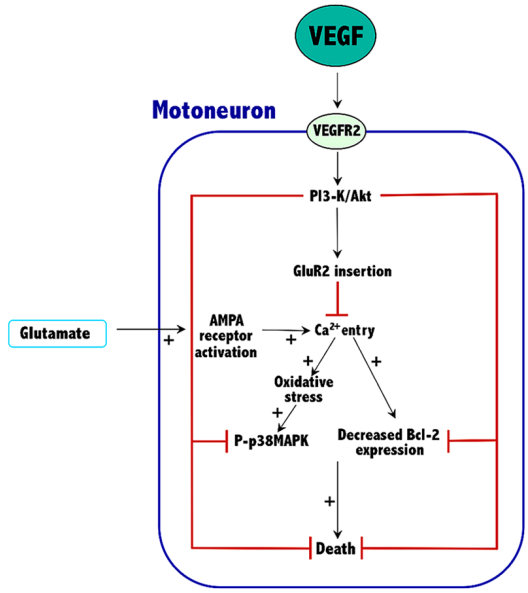


**FIGURE 1 | Mechanisms involved in the neuroprotective effects of VEGF against excitotoxicity in spinal cord motoneurons.** The plus symbols (+) indicate the signaling pathways that are potentiated by excitotoxicity. See Section "Conclusion" for further details.

Intravenous administration of VEGF induces vascular effects: blood vessel growth or blood–brain barrier alterations (Young et al., 2004). To avoid these problematic side-effects, continuous intracerebroventricular (i.c.v.) administration of VEGF in ALS rats was performed. VEGF at doses between 0.2 and 2 $\mu g \cdot kg^{-1} \cdot day$ was safe as it did not induce angiogenesis or inflammation. Besides, it was demonstrated that VEGF diffused from the cerebrospinal fluid to the spinal cord parenchyma, reaching motoneurons, and thus, improving motor performance and prolonging survival of SOD1 rats (Storkebaum et al., 2005). Thus, either retrograde (Azzouz et al., 2004) or paracrine (Storkebaum et al., 2005) delivery of VEGF is effective in the animal models of ALS.

Poesen et al. (2008) have demonstrated that the VEGF-$B_{186}$ isoform is also expressed in the nervous system, has less vascular effects, and also functions as a neuroprotective factor for motoneurons. Interestingly, in contrast to VEGF-A, the presence of VEGF-B is not critical for survival or for motoneuron development in physiological conditions; however, crossing VEGF-$B^{-/-}$ mice with SOD1 mice aggravated motoneuron degeneration. The effect of VEGF-$B_{186}$ seems to be mediated by VEGFR-1, which is also expressed by spinal cord motoneurons, indicating that they can respond to this VEGF-B isoform. In addition, as VEGFR1 is also expressed on astrocytes, an indirect effect on glia could not be ruled out. Finally, the authors demonstrated that i.c.v. delivery of VEGF-B ameliorated the disease in SOD1 rats without exhibiting side vascular effects (Poesen et al., 2008).

Taking advantage of these previous studies on animal models of ALS, ongoing clinical trials are essaying direct i.c.v. administration of VEGF in humans. Clinical trials on phase I/II investigate safety parameters in ALS patients and those on phase II/III are intended to evaluate the efficacy to increase lifespan (http://www.neuronova.com/index.php?option=com_content&task=view&id=40&Itemid=71).

## CONCLUSION

Current knowledge indicates that VEGF can prevent excitotoxic motoneuron death, thus prolonging survival in an animal model of ALS. These effects are VEGFR2-mediated and involve the activation of the PI3-K/Akt signaling pathway, which results in an increased expression of both Bcl-2 and the GluR2 subunit of AMPA receptors. The overall effect of these proteins would be to reduce the excessive entry of calcium characteristic of the excitotoxic process. Thus, Bcl-2 increases the calcium uptake and the buffering capacity of mitochondria, and GluR2 assembly into AMPA receptors reduces their permeability to calcium. By reducing calcium levels into motoneurons of ALS tissue, VEGF reduces oxidative stress and p38MAPK activity, thus improving survival (**Figure 1**).

Although many of the experimental evidences of the benefits of VEGF in ALS are taken from *in vitro* or *ex vivo* experiments, the promising results obtained in animal models of familial ALS substantiate a potential use of VEGF as a therapeutic tool. However, its effectiveness may be counteracted by its vascular effects and by its expected short effective time frame (Tovar-y-Romo and Tapia, 2012). Clearly, more studies are needed to assess the optimal family member/isoform, the route of administration and the time frame for using VEGF in the treatment of ALS. In addition, a better understanding of the cellular and molecular mechanisms involved in the neuroprotective effects of VEGF will be crucial for its therapeutic development.

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# Histone deacetylases and their role in motor neuron degeneration

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Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease, characterized by the progressive loss of motor neurons. The cause of this selective neuronal death is unknown, but transcriptional dysregulation is recently emerging as an important factor. The physical substrate for the regulation of the transcriptional process is chromatin, a complex assembly of histones and DNA. Histones are subject to several post-translational modifications, like acetylation, that are a component of the transcriptional regulation process. Histone acetylation and deacetylation is performed by a group of enzymes (histone acetyltransferases (HATs) and deacetylases, respectively) whose modulation can alter the transcriptional state of many regions of the genome, and thus may be an important target in diseases that share this pathogenic process, as is the case for ALS. This review will discuss the present evidence of transcriptional dysregulation in ALS, the role of histone deacetylases (HDACs) in disease pathogenesis, and the novel pharmacologic strategies that are being comprehensively studied to prevent motor neuron death, with focus on sirtuins (SIRT) and their effectors.

**Keywords: amyotrophic lateral sclerosis, neurodegeneration, neuroprotection, transcription dysregulation, histone deacetylases, sirtuins**

## INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease characterized by the death of upper and lower motor neurons. There are two type of ALS based on the presence of an identified mutation as a cause of the disease. The familial type (fALS) is most commonly caused by mutations in the superoxide dismutase type 1 (SOD1) gene, for which there is a widely studied transgenic mouse model; however fALS represents only about 10% of cases. The most common type of ALS is called sporadic (sALS), but because there are no identified etiologic factors, validated experimental models lack for this type of the disease. Nevertheless, disease progression and pathologic characteristics in both, fALS and sALS, are similar. Also, in both types of the disease, motor neuron degeneration has been associated with the same mechanisms, such as glutamate-mediated excitotoxicity, inflammatory events, axonal transport deficits, oxidative stress and mitochondrial dysfunction (for recent reviews see Corona et al., 2007; Santa-Cruz et al., 2012).

Besides these factors, a novel mechanism that may be involved in motor neuron death and other neurodegenerative diseases is transcriptional dysfunction, which consists of aberrations of the molecular machinery that regulates gene expression, especially at the transcriptional levels through the manipulation of epigenetic marks (Robberecht and Philips, 2013). These marks are covalent modifications of the chromatin components, DNA and histone proteins, carried out by several key enzymes that finely modulate the status of these marks. Among these marks, histone acetylation has been characterized as an important mechanism in the regulation of the opening of chromatin configuration leading to increased transcription (Kouzarides, 2007). The status of histone acetylation strongly depends on the activity of histone deacetylases (HDACs), a widely conserved family of enzymes that catalyze the removal of acetyl groups from histones and from other proteins. This family, which includes sirtuins (SIRT), has been shown to be important for several cellular processes, such as cell death and stress responses, which makes them attractive for the study of their pathogenic role and as potential therapeutic targets (Haigis and Sinclair, 2010).

This paper will review, first the evidence of transcriptional dysregulation in ALS, then the role of the different members of the HDACs family in disease pathogeny and as therapeutic candidates, and finally the role of SIRT and their effectors in ALS.

## RELEVANCE OF HISTONE ACETYLATION IN GENE EXPRESSION

Protein acetylation is an important postranslational modification that regulates numerous cellular functions, such as microtubule dynamics and intracellular transport (Hubbert et al., 2002), development (Bhaumik et al., 2007), metabolism (Cantó et al., 2009), and transcriptional regulation through chromatin remodeling (Kouzarides, 2007).

In eukaryotic cells, including neurons, the genome is organized through the association of DNA with protein complexes, an assembly called chromatin. The most abundant proteins are histones, and these proteins assemble themselves into octamers, composed of two copies of the histones types H2A, H2B, H3 and H4. Around the octamer, ∼147 bp of DNA is wrapped,

forming the basic unit of chromatin, the nucleosome. Nucleosomes further organize themselves in increasingly complex structures, all of which are subject to modulation, by changing the accessibility of the regulatory regions of DNA (such as promoters, enhancers, silencers or insulators) to the transcriptional machinery, ultimately affecting gene expression (Zhou et al., 2011). This regulation can be accomplished through covalent modifications of the chromatin components: in the DNA it occurs through cytosine methylation and hydroxymethylation, and in the histones through methylation, ubiquitinylation, sumoylation, phosphorylation and acetylation of selected aminoacid residues (Kouzarides, 2007). In general terms, gene expression is directly proportional the level of histone acetylation; because of this, the regulation of this postranslational modification is essential for gene homeostasis. Such regulation depends on two groups of enzymes: histone acetyltransferases (HATs) and HDACs, and drastic alterations to this control of gene expression can be deleterious, as has been proven in various tumor cell models (Frew et al., 2009). Therefore, the upregulation of the expression of a given gene can be accomplished by stimulating the activity of HATs or by inhibiting the activity of HDACs (Kazantsev and Thompson, 2008). Currently, research on HATs and on their possible protective effects on neurodegenerative processes is scarce and no specific activators are known, whereas information on HDACs has become the subject of many studies (Selvi et al., 2010). Although histones are not the only target of these enzymes, and in some instances they are not a target at all, the name HDACs is preserved for historical reasons. They constitute a family integrated in a complex network of intracellular signaling participating through acetylation/deacetylation reactions, with numerous protein substrates and multiple physiological consequences depending on the protein involved (de Ruijter et al., 2003; Kazantsev and Thompson, 2008).

## EVIDENCE OF TRANSCRIPTIONAL DYSREGULATION IN ALS

There is evidence that transcriptional dysregulation could contribute to, and possibly be the cause of, some neurodegenerative disorders. This was first proposed for Huntington's disease (Cha, 2000) and relevant findings have been encountered in experimental in vitro and in vivo models of Alzheimer's (Robakis, 2003) and Parkinson's diseases (Yacoubian et al., 2008). In the case of ALS, there is also evidence of transcriptional dysregulation and alterations in the transcriptome in both the sporadic (Figueroa-Romero et al., 2012) and the familial (Kirby et al., 2005) types of the disease. Although the mechanisms and causal relationships have yet to be elucidated, it has been proposed that neuronal protein inclusions, such as those formed by mutated SOD1 or TDP43 (43-kDa transactivator response region (TAR) DNA-binding domain protein) which have been found in ALS and in frontotemporal dementia, could cause their toxicity by acting as surface attractants that sequester vital components of the transcriptional machinery, as was first proved for huntingtin (Cha, 2000). For example, in a screening study to identify targets that disrupt SOD1 aggresome in cultured cells, HDAC inhibition was shown to prevent aggresome formation (Corcoran et al., 2004).

Transcription might also be altered indirectly by modifications of other regulatory components of the transcriptional process, such as HDAC activity. For example, in a *Drosophila* model of polyglutamine neurodegeneration, it was shown that the upregulation of HDACs can ameliorate neuronal death due to a selective transcriptional repression of the CGG repeat-containing gene (Todd et al., 2010). Also, HDAC inhibition is protective for cultured motor neurons against excitotoxicity, a mechanism known to be involved in ALS pathophysiology, due to their ability to modulate gene expression (Kanai et al., 2004). Hence, although the role of transcriptional dysfunction in neurodegenerative diseases is still under study, there is increasing evidence for a role of HDACs in the neurodegenerative processes through the modulation of transcriptional machinery.

## HISTONE DEACETYLASES: ROLE OF CLASSES I AND II IN MOTOR NEURON DEGENERATION

Based on structural, localization and functional criteria, HDAC superfamily is composed of five classes. First, in the next paragraphs, we will focus on the "typical" HDACs (11 enzymes, classified in classes I, IIa, IIb and IV), their distribution and the evidence of their role in ALS. The role of SIRT (seven enzymes that constitute class III or "atypical" HDACs) will be addressed later.

Class I and II HDACs are $Zn^{2+}$-dependent enzymes. Class I include HDAC1, HDAC2, HDAC3 and HDAC8, which are localized in the nucleus and ubiquitously expressed in mammalian tissues (except HDAC8, which is muscle-specific). Broadly speaking, these enzymes are involved in the regulation of gene-specific transcription through the formation of stable transcriptional complexes (de Ruijter et al., 2003). Of these, HDAC2 and HDAC3 seem to have a more important role in the physiology of the central nervous system. Broide et al. (2007) described the distribution of 11 HDACs mRNAs in 50 screened areas of the rat brain, using high-resolution in situ hybridization; HDAC2 and HDAC3 were widely expressed in all areas, especially those of the limbic system (amygdala, piriform cortex, olfactory bulb and hippocampus) and in the granule cell layer of cerebellum, and at the cellular level were found in neurons and oligodendrocytes. In a recent report, a similar distribution of HDAC2 was found in the mouse brain, as well as a moderate expression in primary and secondary motor cortices, and Rexed's laminae 4–9 of the cervical spinal cord (where motor neurons reside). Again, HDAC2 was noted only in neurons and oligodendrocytes, within the nucleus (Yao et al., 2013). Due to their ubiquitous expression in brain and spinal cord, these HDACs have also been implicated in ALS. Janssen et al. (2010) found in ALS patients that HDAC2 expression was upregulated in motor cortex (in layers III–V, where upper motor neuron are located) and in spinal cord grey matter, particularly in the nuclei of motor neurons; they interpreted this result as a protective role of HDAC2 in ALS pathogenesis, although the mechanisms of this effect were not detailed. Regarding this issue, in an in vitro study Kernochan et al. (2005) reported that HDAC inhibition increased promoter activity of the survival motor neuron 2 gene, and this was associated with HDAC2 levels.

Class IIa –HDACs 4, 5, 7 and 9– shuffle between the nucleus and the cytoplasm, and their substrates have not been defined; they have histone deacetylase activity only by interacting with HDAC3 (de Ruijter et al., 2003). HDAC4 and 5 expressions were high in all screened areas, and their locations mimic that of class I HDACs (Broide et al., 2007).

Class IIb is composed of HDAC6 and HDAC10. Again, HDAC10 substrates have not been defined, but it is known that it associates with HDAC3 (de Ruijter et al., 2003). HDAC6 is an unusual enzyme, in the sense that it has two catalytic domains and functions in the cytoplasm where it deacetylates $\alpha$-tubulin and alters microtubule stability (Hubbert et al., 2002). Furthermore, inhibition of this enzyme stimulates autophagy and the proteasome system in a *Drosophila* model of spinal muscular atrophy (a neurodegenerative disease of spinal motor neurons) resulting in increased survival (Pandey et al., 2007). In the rat brain, its expression is low (Broide et al., 2007) and no changes were noted in ALS patients (Janssen et al., 2010). Nevertheless, evidence of the role of HDAC6 in ALS is emerging. In a recent work it was found that TDP43 and fused in sarcoma/translated in liposarcoma (FUS/TLS), which are proteins that regulate RNA processing and have been found to be mutated in some cases of ALS, interact with each other forming a ribonucleoprotein complex that regulates the expression of HDAC6 through its mRNA stability (Kim et al., 2010).

Class IV HDAC only member, HDAC11, is localized in the cell nucleus and is structurally different from the other classes (Kazantsev and Thompson, 2008). Although its functions are poorly understood, recent reports indicate that this HDAC is abundantly and almost exclusively expressed in the mammalian nervous system, within oligodendrocytes (Liu et al., 2008), and also plays an important role in the maturation of this cell type (Liu et al., 2009b). In other study in the rat brain, HDAC11 expression was found to be ubiquitously distributed, in both neurons and oligodendrocytes; in fact, this HDAC displayed the highest levels of expression of all HDACs (Broide et al., 2007). In nervous tissue of ALS patients, including the ventral horn of the spinal cord, the nucleus and the cytoplasm of motor neurons showed decreased HDAC11 mRNA levels (Janssen et al., 2010).

## PROTECTIVE EFFECTS OF HDAC INHIBITION

Chen et al. (2012) addressed the role of class I and II HDACs in an experimental model of acute stroke in rodents. There was progressive decline of mRNA levels of HDACs 1, 2, 5 and 9 in cerebral cortex, while HDACs 3, 6 and 11 mRNA levels were transiently increased. In addition, in an in vitro model of glucose-oxygen deprivation, they reported that selective inhibition of HDAC3 and HDAC6 promoted neuronal survival.

Currently there is an ample battery of small-molecule HDACs inhibitors, that were initially developed to halt cell proliferation in cancer experimental models; HDACs inhibitors are classified as hydroxamate-based (vorinostat, valproic acid (VPA), sodium butyrate, trichostatin A, 4-phenyl butyrate, MC1568 –class II selective–, and tubucin –HDAC6 specific–), and benzamide-based (MS275, compound 106). Their potencies and selectivities vary, but most of them can be considered pan-HDACs inhibitors (Kazantsev and Thompson, 2008).

Several of these compounds have been studied in the transgenic SOD1 mouse model. Trichostatin A induced a modest improvement in motor function and survival as well as protection against motor neuron death, axonal degeneration, muscle atrophy and neuromuscular junction denervation; these effects were attributed to reduced gliosis and upregulation of the glutamate transporter (GLT-1) in the spinal cord (Yoo and Ko, 2011).

VPA is a drug currently in clinical use for other neurological disorders (such as epilepsy and bipolar disorder) due to its numerous mechanisms of action, including HDAC inhibition (Monti et al., 2009). VPA was tested in vivo in the transgenic ALS SOD1 mice, where it did not improve survival or motor performance, but it did improve the acetylation status in the spinal cord through the restoration of the cAMP response element binding protein (CREB) levels in motor neurons and slightly prevented motor neuron death (Rouaux et al., 2007). VPA has already been tested in ALS patients in one clinical trial, showing no benefits in survival or in disease progression in doses commonly used to treat epilepsy (Piepers et al., 2009).

Sodium phenylbutyrate (SPB), another pan-HDAC inhibitor, was shown to extend survival and motor performance in the transgenic ALS SOD1 animal model, and these effects were attributed to an upregulation in the expression of nuclear factor $\kappa$B (NF-$\kappa$B), the active form of the inhibitory subunit of NF-$\kappa$B (i-$\kappa$B) and of beta cell lymphoma 2 (bcl-2) proteins, all involved in survival and stress responses (Ryu et al., 2005). SPB efficacy has been tested also in combinations with other agents with different mechanisms of action, in the same experimental model. For example, the combination of SPB with riluzole potentiated the beneficial effects of SPB on survival and motor performance, although the authors did not address the possible mechanisms (Del Signore et al., 2009). SPB has also been tested in combination with an antioxidant agent (AEOL 10150), where it was found that this combination extended survival more than either treatment alone (Petri et al., 2006). Finally, it is worth to emphasize that SPB has already been tested in a phase 2 clinical trial, where its effects on histone acetylation status, safety and tolerability were addressed; no toxic effects were noted, and significant increases were observed in the blood histone acetylation status, but the therapeutic efficacy of such treatment was not studied (Cudkowicz et al., 2009).

## HISTONE DEACETYLASES: ROLE OF SIRTUINS IN MOTOR NEURON DEGENERATION

Class III HDACs, also called SIRT, are the most divergent class from the rest of the family. This class includes seven members (SIRT1 to SIRT7) with various cellular localizations and preferred enzymatic targets, but what makes unique this class is the dependence of nicotine adenine dinucleotide ($NAD^+$) to perform their catalytic deacetylation and mono-ADP-ribosyl transferase activities (Michan and Sinclair, 2007). SIRT1, SIRT2, SIRT3 and SIRT5 have predominant histone deacetylase activity (Haigis and Sinclair, 2010). SIRT1, SIRT6 and SIRT7 are located in the nucleus. SIRT1 has a preference for euchromatin and can be shuttled to the cytoplasm, depending on the cell type and the developmental stage (Tanno et al., 2007). SIRT6 tends to associate with heterochromatin and SIRT7 is located in the nucleolus.

SIRT2 resides mostly in the cytoplasm, having an important role in regulation cytoskeletal dynamics. SIRT3, SIRT4 and SIRT5 are mitochondrial (Michan and Sinclair, 2007).

SIRT are named after the silent information regulator 2 (sir2) gene in yeast. When overexpressed, the product of this gene extended the lifespan of budding yeast by repressing genomic instability (Kaeberlein et al., 1999). After that discovery, it was learned that SIR2-like genes or SIRT are found in most organisms, where they regulate basic vital functions. In mammals, sirtuin activation or overexpression do not extend lifespan, but appear to be involved in the benefits of calorie restriction, are responsive to environmental stimuli (e.g., daylight and cell stress), and play important roles in numerous human diseases, such as cancer, diabetes, cardiovascular disease and neurodegeneration (Haigis and Sinclair, 2010).

The catalytic deacetylase activity of SIRT is performed on histones and on other proteins, and this ability place SIRT in a privileged position to exert their actions on the genome through direct and indirect pathways. Among the non-histone protein substrates, the peroxisome proliferator-activated receptor gamma coactivator 1 alpha (PGC1-$\alpha$; Cantó and Auwerx, 2009) and the forkhead box O3a (FoxO3a) transcription factor (Zhao et al., 2011b) are two of the most studied, and their relation to ALS will be discussed below. Also, SIRT play an important role as cellular energy sensors (Smith et al., 2000), due to their dependence on $NAD^+$ as a substrate (Imai et al., 2000); in humans, SIRT1-3 and 5 have shown in vitro $NAD^+$-dependent deacetylating activity (Smith et al., 2000). Therefore, SIRT provide a connection between cellular energy states and transcriptional control (Li, 2013).

SIRT have a widespread distribution in the mammalian tissues, including the nervous system. In the mouse, rat and human CNS, SIRT1 mRNA localization was found to be ubiquitous and prominent in various tissues (such as all regions of spinal cord, hippocampus, basal ganglia, brain stem and cerebellum); SIRT1 expression is confined to the neuronal nucleus in parvalbumin (GABAergic) and tyrosine hydroxylase (dopaminergic) neurons (Zakhary et al., 2010). SIRT1 expression was increased in cerebral cortex (especially in the pyramidal cell layer), hippocampus, thalamus and spinal cord in symptomatic SOD1 G93A mice, but spinal motor neurons were not studied in detail; these changes were interpreted as a stress response to the neurotoxic form of SOD1 (Lee et al., 2012).

## PROTECTIVE EFFECTS OF SIRTUINS ACTIVATION

In contrast to other HDACs, the activity of SIRT in the CNS results in neuroprotection. Among the agents with protective action are the polyphenols (vegetal compounds that serve hormonal and protective function in these organisms), particularly resveratrol, which is present in grapes and in red wine, and SRT1720, a synthetic compound derived from resveratrol. These compounds activate selectively both SIRT1 and SIRT2, whereas activators of the remaining SIRT have not yet been described (Kazantsev and Thompson, 2008).

There is a paucity of studies that address SIRT role in ALS pathology. In primary cortical cultures of transgenic SOD1 G93A mice, resveratrol protected against neuron death, and this protection was due to the activation of SIRT1 (Kim et al., 2007). In agreement with these results, in another in vitro study of SOD1 G93A murine motor neurons, it was found that SIRT1 expression was downregulated, and resveratrol prevented neuronal death (Wang et al., 2011). However, the neuroprotection exerted by resveratrol may be due to the stimulation of the activities of other enzymes such as AMP protein kinase (Dasgupta and Milbrandt, 2007). In our laboratory we have recently found that the administration of resveratrol directly in the lumbar spinal cord in rats delays the progress of motor deficits induced by chronic AMPA ($\alpha$-amino-3-hydroxy-5-methyl-4-isoxazol-propionate) infusion in the lumbar spinal cord (Lazo-Gómez and Tapia, in preparation, an in vivo model of excitotoxic motor neuron death (Tovar-y-Romo et al., 2007).

The downstream effectors of SIRT that confer protection in neurodegenerative settings are beginning to be elucidated. For example, in a transgenic mouse model of Huntington's disease, SIRT1 overexpression exerted its beneficial effects through the deacetylation of FoxO3a and p53 (Jiang et al., 2012). This relation has not been explicitly addressed in ALS experimental models, but there is evidence of the role of SIRT1 effectors in motor neuron death. FoxO3a is a transcriptional factor involved in determining cell fate (survival or apoptosis) in stressful situations, such as starvation and oxidative stress. It has been shown that SIRT1 modulates FoxO3a activities through the deacetylation of specific lysine residues, resulting in activation or inhibition, and this depends on the deacetylated lysine position or on FoxO3a cellular location (nucleus or cytoplasm) (Eijkelenboom and Burgering, 2013). In an in vitro study of motor neuron death induced by several insults (excitotoxicity, the overexpression of mutant SOD1, of mutant $p150^{glued}$ or of poly-glutamin expanded androgen receptor), targeted expression of FoxO3a to the nucleus (where it can modulate the transcription of its target genes) by genetic or pharmacologic means prevented neuron death (Mojsilovic-Petrovic et al., 2009). This is in contrast with other in vitro studies, where motor neuron cultures deprived of trophic support were found to overexpress Fas ligand (FasL), due to FoxO3a translocation to the nucleus, thus resulting in cell death (Barthélémy et al., 2004). The reason for this discrepancy is not clear, but it could be due to differences in the death process triggered by the various insults used, which might be regulated in different ways by FoxO3a.

Another well studied SIRT1 effector is PGC1-$\alpha$, a transcriptional coactivator involved in the modulation of the responses necessary to overcome cellular energetic deficiencies, such as the stimulation of mitochondrial biogenesis and the respiratory rate, and the increase of the uptake and metabolism of energy substrates. SIRT1 physically interacts with, deacetylates and activates PGC1-$\alpha$ (Cantó and Auwerx, 2009). It has been shown that PGC1-$\alpha$ plays a role in ALS pathology, because decreased mRNA and protein levels of this coactivator were found in both transgenic mice and in patients with the sporadic form of the disease, in muscle and in spinal cord tissues (Thau et al., 2012). Moreover, PGC1-$\alpha$ may play a therapeutic role, as shown by two studies in the transgenic mouse model. Zhao et al. (2011a) used a targeted overexpression of PGC1-$\alpha$ in neurons of SOD1 transgenic mice, which modestly increased survival and significantly improved

motor performance; these changes were due to the restoration of mitochondrial activities. This is in contrast to the findings of other study in a double transgenic mouse overexpressing PGC1-$\alpha$ and mutant SOD1 in all tissues, showing that survival was not increased albeit motor performance improved and motor neuron loss was ameliorated. An augmentation of the excitatory amino acid transporter protein 2 (EAAT2) in astrocytes was noted in the double transgenic, suggesting that PGC1-$\alpha$ could exert its beneficial effects through other mechanisms (Liang et al., 2011). Regarding other sirtuin isoforms, such as SIRT3, a mitochondrial sirtuin, Song et al. (2013) showed, in primary spinal motor neuronal cell cultures of transgenic SOD1 G93A mice, that the overexpression of SIRT3 and of PGC1-$\alpha$ protected against mitochondrial fragmentation and neuronal cell death, although the authors did not demonstrate the relation between SIRT3 and PGC1-$\alpha$.

These findings indicate that sirtuin activation protects against motor neuron degeneration, although some findings in different experimental models suggest that activators may protect through other mechanisms (Tang, 2010) or even that sirtuin inhibition may be protective. For example, in a model of brain ischemia sirtuin inhibition through nicotinamide administration conferred neuroprotection by preserving $NAD^+$ cellular levels (Liu et al., 2009a).

## CONCLUSION

The opportunities for a really effective therapy for ALS are scarce, and therefore HDAC inhibition and sirtuin activation merit further investigation. Studies on the modification of the activity of these enzymes in in vitro and in vivo experimental models of neurodegeneration, including ALS, have given valuable information suggesting potential therapeutic opportunities for this disease. Nevertheless, the mechanisms underlying such effects are poorly understood and their clarification is necessary for designing more specific and potent treatment strategies. Accumulated evidence on sirtuin activation has provided valuable information about the molecular pathways that could be relevant to halt motor neuron degeneration, such as those related to cellular energetic state and PGC1-$\alpha$. Not all types of HDACs have been evaluated in ALS models and the effects, toxicity, dosage, timing, and mode of administration of specific drugs that inhibit HDAC or activate SIRT have not been established. Of note is the fact that the most widely used model of ALS in which these strategies have been studied is the transgenic rodent with mutations in the human SOD1 gene, but this represent only about 2% of all ALS cases. Novel experimental models of motor neuron degeneration in vivo are clearly needed before safely moving these drugs to clinical trials for a disorder that has proved recalcitrant to all, past and current, therapeutic procedures.

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# A plural role for lipids in motor neuron diseases: energy, signaling and structure

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Motor neuron diseases (MNDs) are characterized by selective death of motor neurons and include mainly adult-onset amyotrophic lateral sclerosis (ALS) and spinal muscular atrophy (SMA). Neurodegeneration is not the single pathogenic event occurring during disease progression. There are multiple lines of evidence for the existence of defects in lipid metabolism at peripheral level. For instance, hypermetabolism is well characterized in ALS, and dyslipidemia correlates with better prognosis in patients. Lipid metabolism plays also a role in other MNDs. In SMA, misuse of lipids as energetic nutrients is described in patients and in related animal models. The composition of structural lipids in the central nervous system is modified, with repercussion on membrane fluidity and on cell signaling mediated by bioactive lipids. Here, we review the main epidemiologic and mechanistic findings that link alterations of lipid metabolism and motor neuron degeneration, and we discuss the rationale of targeting these modifications for therapeutic management of MNDs.

**Keywords: ALS, motor neuron, lipid, metabolism, SMA, SBMA**

## INTRODUCTION

Motor neuron diseases (MNDs) are a group of incurable neurological disorders caused by the selective degeneration of motor neurons. Amyotrophic lateral sclerosis (ALS) is the most representative MNDs among adults with a incidence rate of 2–3 per 100,000 (Brooks et al., 2000). It is characterized by progressive muscle weakness and atrophy, loss of upper and lower motor neurons and death ensuing 3–5 years after diagnosis. Majority of ALS patients are of sporadic origin with unclear ethiopathology. Several mutations are associated with ALS, in particular in genes encoding superoxide dismutase 1 (*SOD-1*), TAR DNA binding protein of 43-kDa (*TDP-43*), fused in sarcoma *(FUS*) and chromosome 9 open reading frame 72 (*C9ORF72*) (Rosen et al., 1993; Mackenzie et al., 2007; Deng et al., 2010; Laaksovirta et al., 2010; Shatunov et al., 2010). Thus, several transgenic mouse models overexpressing various mutant genes have been developed, and the SOD1 model, overexpressing a mutated form of SOD1 gene, is the most studied in ALS (Ripps et al., 1995). Spinal muscular atrophy (SMA) is a genetic autosomal and recessive neuromuscular disease, caused by loss of functional survival motor neuron (SMN) gene 1. Patients suffer from degeneration of spinal motor neurons, muscle weakness leading to atrophy. Disease severity ranges from severe, with death of patients before the age of 10, to mild with moderate symptoms with no alteration of life expectancy. Spinal bulbar muscular atrophy (SBMA), also known as Kennedy's disease, is an X-linked recessive disease caused by a CAG-repeat expansion in the gene coding for the androgen receptor, leading to a poly-Q repeat expansion in the protein (Brooks and Fischbeck, 1995; Fischbeck et al., 1999). The disease affects mainly males, even though it has been described also in female patients. SBMA is characterized by progressive muscle atrophy and degeneration of lower motor neurons in the brain stem and spinal cord. Similarly to ALS, mouse models have been developed to study SMA and SBMA (Katsuno et al., 2003; Bebee et al., 2012).

Along with neuronal degeneration, several alterations of lipid metabolism are found in these diseases. Here, we review the role of lipids in MNDs, with a special attention on energy homeostasis, cell signaling and structure. We further discuss the rationale of targeting lipid metabolism for therapeutic management of MNDs.

## ENERGETIC ALTERATIONS IN AMYOTROPHIC LATERAL SCLEROSIS

Multiple previously unrecognized phenotypes occur in ALS patients, in particular, the unbalance between food intake and energy (Braun et al., 2012; Muscaritoli et al., 2012). ALS patients often present with dyslipidemia, reduced body mass and increased resting energy expenditure (**Table 1**, Desport et al., 2001; Funalot et al., 2009; Dupuis et al., 2011). Energetic alterations are similarly found in transgenic animal models of ALS, the SOD1 mice. These mice are leaner than controls, hypermetabolic, hypolipidemic and present increased fatty acid uptake in muscles (**Table 2**, Dupuis et al., 2004; Fergani et al., 2007; Kim et al., 2011). Several lines of evidence point to lipid metabolism alterations being crucial for ALS progression. A first incidental event shed some light on the subject. During 2 years, a group of scientists remain in an isolated environment, and were submitted to long term caloric restriction (Walford et al., 2002). From eight members of the group, one died from ALS and another one developed progressive gait impairment and motor

**Table 1 | Altered energetic metabolism in ALS patients.**

| Items | Cohort size (ALS patients) | Outcomes | References |
|---|---|---|---|
| **BASAL METABOLISM IN ALS** | | | |
| | $N = 62$ | ALS patients are hypermetabolic. | Desport et al., 2001 |
| | $N = 44$ | ALS patients are hypermetabolic. | Funalot et al., 2009 |
| **EPIDEMIOLOGIC AND RISKS TO ALS** | | | |
| | $N = 153$ | High carbohydrates and low fat intakes increase ALS risk. | Okamoto et al., 2007 |
| | $N = 222$ | High prediagnostic body fat is associated with a decreased risk of ALS mortality. | Gallo et al., 2013 |
| **LIPIDS AND PROGNOSIS IN ALS** | | | |
| | $N = 369$ | High LDL/HDL ratio correlates to longer survival. | Dupuis et al., 2008 |
| | $N = 658$ | Hyperlipidemia does not correlate to longer survival. | Chio et al., 2009 |
| | $N = 658$ | Low level LDL/HDL ratio correlates to respiratory dysfunction. | Chio et al., 2009 |
| | $N = 285$ | Low BMI correlates to faster decline. | Jawaid et al., 2010 |
| | $N = 92$ | Poor nutritional status is associated with higher mortality. | Marin et al., 2011 |
| | $N = 488$ | High triglycerides or cholesterol correlates to longer survival. | Dorst et al., 2011 |
| | $N = 427$ | High BMI correlates to longer survival. | Paganoni et al., 2011 |
| | $N = 77$ | Fast reduction of BMI predicts faster decline. | Shimizu et al., 2012 |
| | $N = 150$ | High BMI correlate to slower ALSFRS score decline. | Reich-Slotky et al., 2013 |
| | $N = 62$ | High subcutaneous fat positively correlate to survival. | Lindauer et al., 2013 |

**Table 2 | Altered energetic metabolism in mouse models of MNDs.**

| Model | Cohort size | Conclusion | References |
|---|---|---|---|
| **ALS** | | | |
| SOD1 G93A mice | $N = 8$ | Altered composition of lipids in spinal cord. | Cutler et al., 2002 |
| SOD1 G86Rand G93A mice | $N = 7$–$8$ | Dramatic defect in energy homeostasis, hypermetabolism mainly of muscular origin. | Dupuis et al., 2004 |
| | $N = 13$ | High fat diet delays disease onset and extent survival. | Dupuis et al., 2004 |
| SOD G93A mice | $N = 27$ | The ketogenic diet protects against motor neuron death. | Zhao et al., 2006 |
| SOD1 G86Rand G93A mice | $N = 10$–$15$ | Hypolipidemia is found in SOD1 mice. | Fergani et al., 2007 |
| SOD1 G93A mice | $N = 49$ females<br>$N = 31$ males | Caloric restriction shortens lifespan through an increase in lipid peroxidation, inflammation and apoptosis. | Patel et al., 2010 |
| SOD1 G93A mice | $N = 30$ males | Hypolipidemia is present at the presymptomatic stage of disease. | Kim et al., 2011 |
| SOD1 G93A mice | $N =$ males | Medium chain triglycerides protect motoneurons survival but does not extent survival. | Zhao et al., 2012 |
| **SMA** | | | |
| SMN1 deficient mice | $N = 30$ males | Hypolipidemia is present at the pre-symptomatic stage of disease. | Butchbach et al., 2010 |

neuron degeneration. Epidemiological studies went beyond this case study and documented an association between nutrition and the risk of developing ALS. First, a case control survey made in Japan in 2007 was conducted with the aim to study pre-illness nutritional habits of ALS patients. The authors identified that high carbohydrate and low fat intakes are associated with higher ALS risk (**Table 1**, Okamoto et al., 2007). More recently, another epidemiologic study focused on anthropometric characteristics of the general population with a follow up over 10 years. The investigators reached the conclusion that high fat content reduces the risk of developing ALS (Gallo et al., 2013). These clinical data are consistent with the reduced overall survival of SOD1 mice under caloric restriction (**Table 2**) (Hamadeh et al., 2005).

Conversely, increased energy intake is beneficial for SOD1 mice. The first evidence emerged in 2004 from our laboratory, when we fed SOD1 mice with a diet enriched in lipids. The treatment restored normal body mass and adiposity, delayed disease onset and motor neuron degeneration, and life expectancy was extended by 20% (Dupuis et al., 2004). The beneficial effects of high fat diet, or ketogenic diet, for SOD1 mice were confirmed by other groups (**Table 2**, Mattson et al., 2007; Zhao et al., 2006, 2012). In 2008, we aimed to study the link between lipids and disease progression, by quantifying circulating lipids in ALS patients. We found that dyslipidemia, defined by high LDL/HDL ratio, was a characteristic of the ALS group (Dupuis et al., 2008), and this dyslipidemia positively correlated with longer survival, increased by 13 months in the group of ALS patients with higher LDL/HDL ratio. Since then, others reported that either hyperlipidemia or high body mass index is a strong prognostic factor for survival (Dorst et al., 2011; Paganoni et al., 2011; Shimizu et al., 2012; Reich-Slotky et al., 2013). For instance, the median life expectancy was higher than 14 months in patients with high

serum triglyceride levels (Dorst et al., 2011). Last, it was recently observed that subcutaneous fat positively correlated with survival of ALS patients (Lindauer et al., 2013). Conversely, lower LDL/HDL ratio has been linked to respiratory impairments (Chio et al., 2009), fast loss of BMI was associated with faster decline (Jawaid et al., 2010) and poor nutritional status is a negative prognosis factor (Marin et al., 2011).

These reports claim for a positive correlation between high circulating lipid levels of lipids or high fat mass and prognosis of ALS patients. The mechanisms in place deserve further investigation, to understand how peripheral lipids interfere with disease progression and how lipids can preserve motor axis integrity.

## ENERGETIC SUBSTRATES AND MOTOR UNITS

The motor unit is an anatomical structure responsible for the control of muscle contraction and its destruction represents the first detectable event in ALS (Dupuis and Loeffler, 2009). The motor units are composed of different sorts of spinal motor neurons and muscles fibers, depending on their activity and energetic capacities. Fast fatigable motor units are composed of large alpha-motor neurons and glycolytic muscle fibers that burn preferentially glucose to exert heavy force on a short period. Conversely, slow motor units correspond to small alpha-motor neurons and oxidative muscle fibers, which store and use preferentially fatty acids to produce less intense but constant strength. Large alpha motor neurons are the first to degenerate in ALS models (Pun et al., 2006; Hegedus et al., 2007), and fast-twitch motor units are preferentially affected in both ALS patients and mouse models (Schmied et al., 1999; Atkin et al., 2005; Gordon et al., 2010). Stimulation of motor units via regular training was proposed to maintain and strengthen motor function in ALS (**Table 3**). Clinical investigations reported benefit for ALS patients whom followed moderate and mainly aerobic exercise program, which use lipids as energy source (de Almeida et al., 2012). In particular, specific training of the diaphragma preserved respiratory functions in ALS patients (Mahajan et al., 2012). It should be noted that these studies were not randomized and suffer from the small size of the cohorts. Moreover, higher release of reactive oxygen species during exercise was reported (Siciliano et al., 2002) and suggest that design for muscular training in ALS patients should be considered with caution (**Table 3**).

In an animal model of ALS, effects of high frequency and high amplitude training were compared to moderate and endurance training. SOD1 mice were subjected either to swimming-based training, targeting glycolytic motor units, or to running-based training, targeting oxidative motor units (Deforges et al., 2009). After training, the group of "swimmer" mice presented with benefits in terms of counts of motor neurons and survival, when compared to proper control. On the contrary, the "running"-trained mice presented a similar disease course compared to sedentary mice. Effect of training on SOD1 mice is not fully clarified. Others reported claimed that moderate exercise alone can improve outcomes in SOD1 mice (Carreras et al., 2010), and most importantly, Gerber and colleagues recently demonstrated that benefits of training in mice might come from the enrichment of the environment rather than mobilization of muscle fibers (Gerber et al., 2012). Therefore, the benefit after different types of training for SOD1 mice targeting different pool of motor units remains to be clarified. Currently, the effects of aerobic versus anaerobic exercises on vital capacity and muscular strength are under clinical investigations (**Table 4**, NCT01650818, NCT01521728). Additional work on this topic is required to understand the impact of different exercises, using different energetic sources, on motor units in ALS patients, and why glycolytic motor units are preferentially affected.

## ENERGY, METABOLISM, AND MITOCHONDRIA

Taken separately, the two extremities of the motor units, the muscles and the motor neurons, show abnormal lipid metabolism. Indeed, early in the disease course, glycolytic muscles of SOD1 mice switch toward an oxidative phenotype, presumably due to the loss of their connection to large motor neurons and subsequent reinnervation by "slow" motor neurons (Sharp et al., 2005). The selective vulnerability of large motor neurons is potentially due to higher energetic needs that could either be not fulfilled or a source of oxidative stress. In case of higher needs, neurons can use ketone bodies as energetic substrate when glucose level becomes low (Guzman and Blazquez, 2004; LaManna et al., 2009). In this situation, astrocytes will use lipid to provide ketone bodies to neurons, and potentially motorneurons in ALS (Yi et al., 2011). Interestingly, medium chain triglycerides, precursors of ketone bodies, preserve motor functions and promote motorneuron survival in SOD1 mice through the enhancement of oxidative metabolism (**Table 2**, Zhao et al., 2012).

Moreover two recent studies have shown abnormally enhanced levels of ketone bodies, released by the breakdown of fatty acids, in the cerebrospinal fluid of ALS patients (Blasco et al., 2010; Kumar

**Table 3 | Effect of exercise in ALS.**

| Cohort | Outcomes | References |
|---|---|---|
| **SOD1 MICE G93A** | | |
| | High frequency and amplitude training support in motor neuron survival. | Deforges et al., 2009 |
| | Moderate frequency and amplitude training support motor neurons survival. | Carreras et al., 2010 |
| | Benefits of training might come from the enrichment of the environment. | Gerber et al., 2012 |
| **ALS PATIENTS** | | |
| | Regular moderate physical exercise should be recommended. | Drory et al., 2001 |
| | Higher release of ROS during exercise suggests that design of training should be considered with caution. | Siciliano et al., 2002 |
| | Benefits of moderate aerobic exercise. | Bello-Haas et al., 2007 |

**Table 4 | Ongoing clinical trials in ALS.**

| Items | Clinical trial ID | Status | Study type | Intervention | Estimated enrollment | Study |
|---|---|---|---|---|---|---|
| **EXERCISE** | | | | | | |
| | NCT01650818 | Recruiting | Interventional | Endurance training | 40 | Aerobic exercise training in ALS |
| | NCT01521728 | Recruiting | Interventional | Resistance exercise | 60 | Trial of resistance and endurance exercise in ALS |
| **DIETARY SUPPLEMENT** | | | | | | |
| | NCT00983983 | Completed | Interventional | Oxepa | 30 | High fat/high calorie trial in ALS |
| | NCT01016522 | Terminated | Interventional | KetoCal | NA | Safety and tolerability of the ketogenic diet in ALS |
| **BASAL METABOLISM** | | | | | | |
| | NCT00714220 | Recruiting | Observational | / | 150 | Quantitative measurement of nutritional substrate utilization in patients with ALS |
| | NCT01592084 | Completed | Observational | / | 267 | Hyperlipidemia and statin therapy in ALS |
| **PHARMACOLOGY** | | | | | | |
| | NCT00690118 | Terminated | Interventional | Pioglitazone (45 mg/day) | 219 | Study of pioglitazone in patients with Amyotrophic Lateral Sclerosis |
| | NCT00876772 | Unknown | Interventional | Olanzapine (10 mg/day) | 40 | Olanzapine for the treatment of appetite loss in Amyotrophic Lateral Sclerosis (ALS) |

*Clinical trial IDs refer to the current nomenclature used at clinicaltrial.gov.*

et al., 2010) that could account for an altered lipid beta oxidation in the CNS of ALS patients.

Higher energetic needs have been documented in muscle of SOD1 mice (Dupuis et al., 2004). In the animal model, the metabolic shift in muscle fibers (glycolytic to oxidative) could explain the pronounced appetence for fatty acids in SOD1 muscles, as well as the changes in the expression profile of genes involved in lipid metabolism (Fergani et al., 2007; Gonzalez de Aguilar et al., 2008; Thau et al., 2012). Interestingly, the predominance of oxidative metabolism goes along with deficiency in oxidative mitochondrial chain function, in particular in the muscles of ALS patients (Echaniz-Laguna et al., 2002; Dupuis et al., 2003; Crugnola et al., 2010). Mitochondria bioenergetic functions are impaired, as shown by Zhou and colleagues, whom described the presence of defective mitochondria in mass near the neuromuscular junctions that may contribute to the progression of muscle atrophy in ALS (Zhou et al., 2010). The origin of mitochondrial defect is under investigation. Several potential pathways are proposed to explain the shift in metabolism and the alterations of mitochondrial functions, and they concern the tuning of metabolic pathways.

Mitochondrial biogenesis and functions are orchestrated in part by peroxisome proliferator-activated receptor gamma coactivator (PGC)-1 alpha (Lin et al., 2002, 2005; Handschin, 2010). In ALS, the implication of PGC-1 alpha has been recently highlighted, as its expression is diminished in the muscles of patients and SOD1 mice (Thau et al., 2012). The downregulation of PGC-1 alpha triggers modification of lipid metabolism, and impacts the use of fatty acids (Barroso et al., 2011). Interestingly, we have very recently shown that deficiency in PGC1-alpha leads to hasten disease progression in the males of a mouse model of ALS (Eschbach et al., 2013), strengthening the relation between lipid metabolism alterations and disease progression, at least in SOD1 mice. When Da Cruz and collaborators overexpressed PGC1-alpha selectively in the muscles of SOD1 mice, they observed improved locomotor activity and reduced muscle atrophy, but no effects on the overall survival of this mouse line (Da Cruz et al., 2012). Their results suggest that improving muscle activity and reducing atrophy through increased PGC1-alpha could be used as a palliative treatment in ALS. In addition, the overexpression of PGC1-alpha selectively in the central nervous system (CNS) of SOD1 mice restored the activity of mitochondrial complexes in the spinal cord, supported motor functions and enhances survival by 8% (Zhao et al., 2011). Moreover sirtuin 3, a downstream target of PGC-1 alpha, protects neurons *in vitro* against SOD1 G85R toxicity (Song et al., 2013). General mitochondrial activity, including mitochondrial proliferation, is impaired in ALS and represents a promising therapeutic target for ALS as recently discussed (Cozzolino et al., 2013; Dupuis, 2013; Pasinetti et al., 2013).

Stearoyl-Coa desaturase 1 (SCD-1) is a key enzyme for the regulation of fatty acid metabolism, and it can impact fatty acid oxidation taking place in mitochondria. SCD-1 introduce a double bond in the carbon chain of saturated, to generated monounsaturated fatty acids that are more prone to be stored in fat tissues. We have recently reported a downregulation of SCD-1 in the muscle of SOD1 mice (Hussain et al., 2013), and in a subpopulation of ALS patients (Pradat et al., 2011). The function of SCD-1 is associated to regulation of energetic metabolism, and most particularly the management of lipid reserves. Downregulation of SCD-1 is known to trigger increased expression of genes involved in the beta-oxidation of fatty acids, increased energy expenditure and reduced fat storage, a metabolic phenotype exhibited by SOD1 mice (Ntambi et al., 2002; Dupuis et al., 2004). We aimed to study the impact of a low SCD-1 activity for the motor function. We recently described that knock-out mice for SCD-1, and non-transgenic mice treated with a SCD-1 inhibitor, present improved nerve regeneration after peripheral nerve injury (Hussain et al., 2013). Moreover, the products of SCD-1, the mono-unsaturated fatty acids, favor cytotoxic SOD-1 aggregation (Kim et al., 2005), and the accumulation of toxic lipid species such as ceramide

(Dobrzyn et al., 2005), suggesting that loss of SCD-1 activity could lower cytotoxicity in ALS. Further work is needed to understand the link between loss of SCD-1 activity and benefits for the motor units, especially in ALS. Aside from its role in energetic metabolism, SCD-1 is additionally required in the synthesis of more complex lipids, including phospholipids. Alterations in lipid metabolism will have repercussion not only on the energy homeostasis, but also on a wide range of cellular functions, including membrane fluidity and signaling.

## A ROLE FOR LIPIDS BESIDE ENERGETIC METABOLISM

Lipids play a critical role in the structure of the central and peripheral nervous systems in particular at the cell membrane level. They control membrane fluidity, improve transmission of electrical signals and stabilize synapses.

### MEMBRANE FLUIDITY

Basic cellular functions depend on the composition in lipids of plasmatic membranes. Enrichment of sphingolipids and cholesterol, as well as content in polyunsaturated fatty acids (PUFA), directly determines membrane fluidity and movement of membrane proteins in lipid rafts (Xu et al., 2001; Lang, 2007; Lingwood and Simons, 2010). Although the level of these lipids is altered in ALS patients and SOD1 mice, membrane fluidity *per se* has not been extensively investigated. One recent study described loss of membrane fluidity in the SOD1 mice at disease onset (Miana-Mena et al., 2011) presumably due to oxidative stress and lipid peroxidation. Membrane phospholipids in the CNS are rich in PUFA and in particular of docosahexaenoic acid (DHA). Interestingly, the profile of fatty acids in the brain cortex and spinal cord of ALS patients revealed an increase of DHA level with potential consequences on membrane fluidity (Ilieva et al., 2007). Changes in the membrane fluidity could affect wide range of cellular functions such as ligand-receptor signal transduction and membrane trafficking (Simons and Vaz, 2004), with consequences on cell functions and survival.

### A DIRECT ROLE FOR LIPIDS IN MOTORNEURON SURVIVAL?

Back in 2002, the group of Pr. Mattson studied the lipid metabolites present in the spinal cord of both ALS patients and pre-symptomatic SOD1 mice, and reported higher amounts of sphingolipids and cholesterol associated with increased lipid peroxidation (Cutler et al., 2002). These findings are important as these lipid metabolites modulate vital cellular functions in the CNS that may be involved in ALS pathophysiology. Aberrant accumulation of ceramides is commonly seen as being toxic. It mediates neuron death by oxidative stress and apoptosis in animal models and patients of neurodegenerative diseases (Brugg et al., 1996; France-Lanord et al., 1997; Bras et al., 2008; Car et al., 2012; Filippov et al., 2012). Ceramides are precursor molecules at the crossroads of the sphingolipid metabolism and they can be converted into sphingomyelin, ceramide-1-phosphate and gangliosides. Abnormal repartition of gangliosides was described in the CNS of ALS patients and presence of antibodies anti-gangliosides has been described in their serum (Mizutani et al., 2003). Gangliosides are important for axonal function and regeneration, and neuronal survival (Akasako et al., 2011). In the 80's, clinicians initiated half-dozen trials in ALS with injection of gangliosides with the aim to protect the motor units, but lead to no benefit (Bradley, 1984; Hallett et al., 1984). These studies were however underpowered. Sphingomyelin is particularly abundant in the nervous systems and represents another lipid impacting motor neuron survival. Cutlers and colleagues proposed that the increase of sphingomyelin in the spinal cord of ALS patients mediates motor neuron death via oxidative stress (Cutler et al., 2002), and in 2007, another group reported that p75-mediated motor neuron death is stimulated upon sphingomyelin-associated ROS production in an animal model of ALS (Pehar et al., 2007).

### SIGNALING MOLECULES

In addition to their role in the membrane structure, PUFA also have intrinsic functions on cell signaling, in particular on neuroinflammation and regulation of energetic metabolism. First, PUFA are known to bind to transcription factors, such as liver-X receptor and retinoic-X receptor (Yoshikawa et al., 2002), to stimulate the expression of genes involved in energy homeostasis and dysregulation of their levels could account for the altered metabolism in ALS.

PUFA can be also converted to active molecules. Depending on the location of the unsaturations, PUFA present either anti-inflammatory and neuroprotective effects, for the omega 3 fatty acids, or pro-inflammatory for omega 6 fatty acids (Schmitz and Ecker, 2008). For instance, eicosapentaenoic and arachidonic acids can be oxidized to give rise to prostanglandins or leukotrienes, and the oxidation of DHA produces the neuroprotectin D1, a signaling molecule that promotes beneficial effects on cell survival under stress (Bazan et al., 2011). Prostaglandin E2 (PGE2) is synthetized by cyclooxygenase-2 from the arachidonic acid, an omega 6 fatty acid, to promote inflammation after binding to its receptor. In ALS patients, level of PGE2 is increased in the serum and the cerebrospinal fluid (Ilzecka, 2003). Evidence from animal models also suggests a role for PGE2 in ALS physiopathology. Indeed, the pharmacological inhibition of PGE2 receptor or the silencing of the gene coding for cyclooxygenase-2 can lower neuroinflammation in SOD1 mice, preserve motor functions and extend survival (Pompl et al., 2003; Klivenyi et al., 2004; Liang et al., 2008).

The omega 3 fatty acids can be converted into anti-inflammatory and neuroprotective molecules. Many reports have shown that treatments based on omega 3 fatty acids exert beneficial effects in various animal models of neurodegenerative disease, such as Parkinson's or Alzheimer's diseases (Arsenault et al., 2011; Ozsoy et al., 2011). In a recent study, Michael-Titus and colleagues investigated the neuroprotective effect of eicosapentaenoic acid, an omega 3 fatty acid precursor for DHA, in SOD1 mice. Unexpectedly, the treatment resulted in enhancement of neuroinflammation, faster disease progression and hastened death for SOD1 mice (Yip et al., 2013). One explanation proposed by the authors is the greater susceptibility for PUFA to be peroxidized and therefore toxic (Kanner et al., 1987). Therefore, the increase in DHA of spinal cord from ALS patients could favor lipid peroxidability, and be potentially a toxic factor in ALS (Ilieva et al., 2007).

Taken together, all these results clearly argue for a strong relationship between lipid metabolism and motor neuron degeneration. Most of the findings originate from ALS, as it is the most studied MNDs. However, alterations of lipid metabolism are also present in other MNDs.

## LIPID ALTERATIONS IN OTHER MOTOR NEURON DISEASES

Motor neuron degeneration is present in several diseases that complete the spectrum of MNDs with ALS. The role of lipids in those diseases is poorly understood, although some lines of evidence exist.

Progressive muscular atrophy (PMA) is rare subtype of ALS characterized by loss of lower motor neurons. To our knowledge, only one report described alterations of circulating lipids in two patients with a PMA syndrome (Yao et al., 1983). However, these patients suffer from other neurological disorders making difficult to reach a conclusion.

SBMA is characterized by progressive muscle atrophy and degeneration of lower motor neurons in the brain stem and spinal cord. Role and regulation of lipids have not been accessed in SBMA patients, although androgen receptors are known to modulate lipid metabolism (Singh et al., 2006). Indeed, the analysis of the muscle transcriptome of SBMA mice revealed that several differentially regulated genes relate to lipid metabolism, from an energetic and a structural point of view (Mo et al., 2010). For instance, the phospholipase A2, group VII ($PLA_2g7$) is downregulated in the muscles of SBMA mice. $PLA_2g7$ degrades phospholipids to release poly-unsaturated fatty acid and, in muscle, it is involved in the differentiation of myoblasts (Xiao et al., 2012) and reduction of adiposity (Rao et al., 2006). Ddit4l is another example of genes deregulated in SBMA mice. Ddit4l, also known as REDD2, is involved in muscle growth via stimulation of the IGF1/mTOR pathway and muscle fiber switch from oxidative to glycolytic metabolism (Pisani et al., 2005; Miyazaki and Esser, 2009; Mo et al., 2010).

SMA is a progressive, recessively inherited, neuromuscular disease. SMA is characterized by weakness and muscle atrophy due to loss of spinal cord motor neurons. Similarly to ALS, metabolic abnormality is part of pathophysiology of SMA. In SMA, an initial study reported normal body mass index in a mixed population of SMA patients with severe to moderate symptoms. However, in this cohort, the authors described a reduction in fat-free mass but an increase in total fat mass (Sproule et al., 2009). A second study, focusing only on the most severe form of the disease, described a lower calorie intake than the recommended dietary allowance in SMA patients, associated to higher fat mass (Poruk et al., 2012). These alterations potentially participate to failure to thrive described in SMA patients. The reason why SMA patients have high fat mass despite low caloric intake is unclear but strengthen the role of metabolism of lipids in the disease. Indeed, a misuse of lipids has been documented in SMA patients with various symptom severities, with an impaired mitochondrial fatty acid beta-oxidation and a loss of free circulating carnitine, arguing for impairment of fatty acid entry into mitochondria (Tein et al., 1995). Later, the same group of clinicians described fatty acid abnormalities in SMA, such as high dicarboxylic acid to ketone ratio in the plasma when fasting, or low C12:C14 fatty acid ratio, that further decreased within disease duration. These two parameters are strong markers for fatty acid beta oxidation defects (Crawford et al., 1999). The authors concluded that fatty acid abnormality in severe SMA is primary and general defect, directly caused by the loss of the SMN function.

Additionally, in a transgenic animal model of SMA, deficient for the SMN gene, pups survive longer when mothers were fed with a diet enriched in lipid content (**Table 2**). The treatment also corrected motor dysfunction despite lack of effect on motor neuron survival (Butchbach et al., 2010). Recently, a clinical trial investigated the effect of a combined treatment of valproic acid and L-carnitine in SMA patients, however, no to limited positive effects have been reported in ambulatory and non-ambulatory SMA patients (Swoboda et al., 2010; Kissel et al., 2011). However, further work is required to assess the role of lipids in SMA and their therapeutic potential.

## PATHS TO EXPLORE

Clinical studies and basic research undoubtedly demonstrate a particular connection between lipid metabolism and ALS (**Table 4**). Contrary to that observed in other neurological diseases, such as Alzheimer's and Parkinson's diseases, or even ageing (Maswood et al., 2004; Patel et al., 2005), high level of lipids positively correlates to better prognosis in ALS. Preclinical data and clinical studies clearly show that dietary lipid supplementation is a promising strategy to treat ALS, although there is no extensive clinical research at this level.

We are aware of only three clinical trials based on nutritional intervention. In a recently published article, Dorst and colleagues aimed to stop weight loss in ALS patients with high caloric diets, either based on fats or carbohydrates. Both interventions were able to stabilize weight loss although the effect was larger in the high-fat diet group (Dorst et al., 2013). Another trial aims to correct malnutrition with high fat dietary supplement in ALS patients (NCT00983983). The second ongoing trial is a phase III that use high ketogenic supplementation (high fat and low carbohydrate) in ALS patients fed through a gastrostomy tube (NCT01016522). These studies are dedicated to the prevention of malnutrition in ALS patients, and are not designed to identify benefit on prognosis after high fat diet. In parallel, how patients on and off respiratory support use diverse nutritional substrates (e.g., lipids) is under investigation (NCT00714220), as well as the influence of lipid lowering therapy in ALS (NCT01592084). There are two additional trials based on pharmacological modulation of energetic metabolism, using pioglitazone or olanzapine. Pioglitazone is an anti-diabetic drug known to manage energetic metabolism and to lower level of circulating lipids in patients suffering from metabolic disorders. This phase II clinical trial has been stopped after interim analysis that revealed no benefit after treatment on primary and secondary outcomes (Dupuis et al., 2012, NCT00690118). A phase II/III using olanzapine is currently ongoing. Olanzapine is a neuroleptic drug with metabolic side effects, and investigators treated ALS patients with olanzapine, based on the weight-increasing effect of the drug (NCT00876772). Study results have not been released yet. The results of these trials will certainly help to determine whether modulation of lipids, though supplementation or pharmacology,

is a feasible and rational treatment for ALS, and potentially for other MNDs.

As discussed in this review, the role of lipids in ALS and MND pathophysiology goes beyond energetic metabolism, to structure and signaling. These alterations, in particular those in the CNS, could represent an important source of therapeutic options in ALS. Indeed, there is a large spectrum of drugs targeting sphingolipids that are well characterized in terms of safety and bioavailability. They could be suitable for ALS patients, once clear targets are identified. A clinical trial has been very recently registered with the aim to test in ALS patients, the safety and tolerability of fingolimod, an agonist for sphingosine-1-phosphate receptors (NCT01786174), already used in multiple sclerosis to lower neuroinflammation in the CNS. In addition to pharmacological agents, dietary supplementation with neuroprotective and anti-inflammatory fatty acids, or derivatives, is worthy of further characterization. Altogether, alterations of lipid metabolism in MND deserve further work that will undoubtedly lead to new and interesting therapeutic options for patients.

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# Blood–CNS barrier impairment in ALS patients versus an animal model

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Amyotrophic lateral sclerosis (ALS) is a severe neurodegenerative disease with a complicated and poorly understood pathogenesis. Recently, alterations in the blood–Central Nervous System barrier (B-CNS-B) have been recognized as a key factor possibly aggravating motor neuron damage. The majority of findings on ALS microvascular pathology have been determined in mutant superoxide dismutase (SOD1) rodent models, identifying barrier damage during disease development which might similarly occur in familial ALS patients carrying the SOD1 mutation. However, our knowledge of B-CNS-B competence in sporadic ALS (SALS) has been limited. We recently showed structural and functional impairment in postmortem gray and white matter microvessels of medulla and spinal cord tissue from SALS patients, suggesting pervasive barrier damage. Although numerous signs of barrier impairment (endothelial cell degeneration, capillary leakage, perivascular edema, downregulation of tight junction proteins, and microhemorrhages) are indicated in both mutant SOD1 animal models of ALS and SALS patients, other pathogenic barrier alterations have as yet only been identified in SALS patients. Pericyte degeneration, perivascular collagen IV expansion, and white matter capillary abnormalities in SALS patients are significant barrier related pathologies yet to be noted in ALS SOD1 animal models. In the current review, these important differences in blood–CNS barrier damage between ALS patients and animal models, which may signify altered barrier transport mechanisms, are discussed. Understanding discrepancies in barrier condition between ALS patients and animal models may be crucial for developing effective therapies.

**Keywords: amyotrophic lateral sclerosis, blood–CNS barrier, patients, animal models, microvascular pathology**

## INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease affecting upper and lower motor neurons in the brain and spinal cord, damage which leads to progressive muscle atrophy, paralysis and death typically within three to five years from diagnosis (Rowland and Shneider, 2001). Most ALS cases are sporadic amyotrophic lateral sclerosis(SALS) with only 5–10% genetically linked familial amyotrophic lateral sclerosis (FALS); 20% of FALS cases show missense mutations in the Cu/Zn superoxide dismutase (SOD1) gene (Rosen et al., 1993). Clinical presentation and pathology of SALS and FALS, however, are similar. Numerous hypotheses exist regarding ALS pathogenesis (Alexianu et al., 2001; Cleveland and Rothstein, 2001; Bruijn et al., 2004; Strong et al., 2005; Pasinelli and Brown, 2006; Van Den Bosch et al., 2006; Mitchell and Borasio, 2007; Rothstein, 2009; Saleh et al., 2009; Hovden et al., 2013), but the causes of the diffuse motor neuron degeneration are still uncertain.

The blood–Central Nervous System barrier (B-CNS-B) is composed of the blood–brain barrier (BBB), blood–spinal cord barrier (BSCB), and blood–cerebrospinal fluid barrier (BCSFB) and has a crucial role in controlling CNS homeostasis by selective transport of substances to and from the systemic compartment and blocking passive diffusion of harmful blood solutes (Bradbury, 1985; Dermietzel and Krause, 1991; Pardridge, 1991, 1999; Nag, 2003; Vorbrodt and Dobrogowska, 2003; Ballabh et al., 2004). This control is possible due to the unique composition of the microvasculature – capillaries formed by endothelial cells (BBB and BSCB) and epithelial cells of the choroids plexus (BCSFB). Exchange by free diffusion is limited to molecules massing less than 450 Da; more massive substances require specific transport mechanisms. These mechanisms allow influx of required substances and efflux of cell waste (Begley and Brightman, 2003; Begley, 2004; Pardridge, 2005). Endothelial cells and their tight/adherens junctions are the primary components of the BBB and BSCB systems, while other barrier elements (pericytes, astrocytes, perivascular macrophages, and the basal lamina) also have essential roles in the tightly integrated unit maintaining the CNS environment for proper function of neuronal cells. Although major structural and functional properties are shared between the BBB and BSCB, some morphological and physiological differences have been noted in the BSCB (Bartanusz et al., 2011). Glycogen microvessel deposits,

increased capillary permeability for some tracers, and decreased tight/adherence junction protein expressions were described for the BSCB in comparison to the BBB. Therefore, dysfunctional or structural impairment of any B-CNS-B component may lead to an increasingly toxic CNS environment. Microvascular endothelial dysfunction, in particular, might be implicated in the pathogenesis of various neurodegenerative diseases (Grammas et al., 2011).

Early studies in the 1980s reported altered BCSFB permeability as indicated by abnormal serum proteins and complement in the CSF of ALS patients (Leonardi et al., 1984; Annunziata and Volpi, 1985). These observations were followed by detection of blood-borne substances in the CNS tissue of ALS patients (Donnenfeld et al., 1984), suggesting BBB and BSCB leakage. Only relatively recent research has focused on microvascular competence in the brain and spinal cord, indicating impairment of BBB and BSCB integrity in animal models of ALS and in ALS patients. Compelling evidence of this B-CNS-B damage has been demonstrated at structural and functional levels in an animal model of ALS at initial stage of disease and this damage was exacerbated with disease progression (Garbuzova-Davis et al., 2007a,b; Zhong et al., 2008; Nicaise et al., 2009a,b; Miyazaki et al., 2011). Importantly, BSCB breakdown was found in SOD1 mutant mice and rats prior to motor neuron degeneration and neuroinflammation (Zhong et al., 2008; Nicaise et al., 2009a; Miyazaki et al., 2011). Evidence of BSCB impairment has also been observed in postmortem tissue from ALS patients. Loss of endothelium integrity, as shown by significant reductions of tight junction proteins and astrocyte end-feet dissociated from the endothelium, was observed in spinal cords from ALS patients (Henkel et al., 2009; Miyazaki et al., 2011). Recently, we showed structural and functional impairment in postmortem gray and white matter microvessels of medulla and spinal cord tissue from SALS patients, suggesting pervasiveness of the B-CNS-B damage (Garbuzova-Davis et al., 2012). These results strengthen the likelihood that barrier disruption contributes to disease pathogenesis (Garbuzova-Davis et al., 2008). However, B-CNS-B disruption could also trigger, as well as aggravate, motor neuron degeneration in ALS. Vascular impairment has only recently been recognized as a key factor in ALS, identifying ALS as a neurovascular disease (Garbuzova-Davis et al., 2011; Rodrigues et al., 2012). Although numerous similarities in barrier damage have been identified in mutant SOD1 animal models of ALS and ALS patients, some differences in the B-CNS-B alterations have been noted. In the current review, these discrepancies in the BBB and BSCB competence between ALS patients and animal models are discussed with an aim toward developing effective new therapies for ALS.

## BBB AND BSCB IMPAIRMENT IN TRANSGENIC RODENT MODELS OF ALS

Only comparatively recent research has focused on investigations of BBB and BSCB integrity in ALS. Initially, Garbuzova-Davis et al. (2007a) demonstrated ultrastructural capillary alterations in the brainstem and spinal cord (cervical and lumbar) in both early and late stages of disease in G93A SOD1 mice. Electron microscopy analysis showed highly vacuolated and degenerated endothelial cells, mitochondrial degeneration within endothelial cells, extensive perivascular edema, and swelling of astrocyte end-feet adjacent to capillaries. Capillary rupture was also indicated by the presence of erythrocytes in the extracellular space of brainstem microvessels in early symptomatic G93A mice. These findings were later confirmed by a study from the same research group (Garbuzova-Davis et al., 2007b), showing Evans blue leakage in spinal cord capillaries of G93A mice at 13 weeks of age, indicating functional impairment of the BSCB in early stage disease. The study also demonstrated endothelial damage through downregulation of the transporter protein Glut-1 and CD146 expressions, associated with decreased laminin, a component of the basement membrane in capillaries. The alterations were mainly detected in the ventral horns of the spinal cords, areas most affected by ALS. Importantly, motor neurons demonstrated intracellular edema and cytoplasmic vacuolization, in addition to degenerated axons with myelin disruption near capillaries in the brainstem and spinal cords of G93A mice at early disease stage.

The study by Zhong et al. (2008) not only confirmed these observations on microvascular barrier damage in the spinal cord of symptomatic G93A mice, but also showed that BSCB disruption precedes neuroinflammation and might initiate disease symptoms. Western blot analysis evidenced diminished levels of zonula occludens-1 (ZO-1), occludin, and claudin-5 tight junction proteins and Glut-1 prior to disease onset in ALS mice. Although these alterations were observed, markers of endothelial activation (intercellular adhesion molecule-1, ICAM-1) and inflammation (monocyte chemoattractant protein-1, MCP-1) and cycloxygenase-2 (COX-2) were not indicated. Also prior to motor neuron loss and inflammatory changes, the investigators showed 10–15% reductions in total capillary length and 30 to 45% decreases in spinal cord blood flow of SOD1 transgenic mice. Additionally, microhemorrhages and hemosiderin deposits were found in spinal cord parenchyma, demonstrating BSCB functional impairment and disruption.

Miyazaki et al. (2011) also evaluated BSCB integrity in G93A SOD1 mice and observed progressive downregulation of occludin and platelet-endothelium cell adhesion molecule-1 (PECAM-1 or CD31) and vascular collagen IV, associated with increased activity of matrix metalloproteinase-9 (MMP-9), indicating endothelial cell and basement membrane involvement in microvascular pathological changes. All of these observations preceded motor neuron death and were in agreement with Zhong et al. (2008). However, the decline of occludin expression was moderate in the spinal cord tissues from ALS mice from 10 to 15 weeks of age and the expression of this protein significantly decreased in late symptomatic mice at 18 weeks of age. Surprisingly, the quantification of collagen IV in gray matter tissue evidenced protein up-regulation, a finding in opposition to immunohistochemical observations in the perivascular areas. These divergent results were ascribed to increased glial production of collagen IV as a consequence of disease progression and neuroinflammation, possibly reflecting an attempt by neural cells to compensate for endothelium disruption. In support of this suggestion, the authors showed higher collagen IV expression in microglia of G93A mice at 18 weeks of age vs. controls. Hence, it is still unclear whether glial cells,

especially astrocytes, induce or prevent microvascular CNS barrier damage.

Another study, based on the G93A SOD1 rat model of ALS, demonstrated interesting results. Ultrastructural alterations of the capillaries such as perivascular swollen astrocyte end-feet, Evans blue leakage, reduced mRNA expression of ZO-1 and occludin, and of agrin, a basement membrane component, were observed in animals only at symptomatic stage (Nicaise et al., 2009a). Conversely, IgG and hemosiderin deposits, other indicators of capillary leakage, were detected in the brainstem and lumbar spinal cord of pre-symptomatic ALS rats. Additionally, the same group (Nicaise et al., 2009b) showed increased expression of aquaporin-4 (AQP4) mRNA and protein in the spinal cord gray matter of end-stage SOD1 rats. Immunohistochemistry revealed increased AQP4 in areas surrounding vessels and motoneuron perikaria. Electron microscopic analysis confirmed localization of this protein in association with perivascular swollen astrocytic processes. The authors suggested that the aquaporin channels may promote perivascular edema and AQR4 might be a potential marker of barrier disruption in ALS (Nicaise et al., 2010). Since elevated AQP4 was also detected near motor neurons, it is possible that dysfunctional astrocytes contribute to further motor neuron degeneration. A more recent study by Bataveljić et al. (2012) confirmed overexpression of AQP4 in the brainstem (facial and trigeminal nuclei) and motor cortex of G93A SOD1 rats at end-stage of disease. Notably, increased AQP4 immunoreactivity was observed in astrocytic processes around blood vessels in studied brain areas of ALS rats. In parallel, the authors determined decreased expression of potassium channel (Kir4.1) in the brainstem and cortex of rats by immunolabeling and Western blot analyses. The authors concluded that the functional changes in these channels could reduce astrocytes' ability to properly maintain water and potassium CNS homeostasis, not only affecting the BBB but also impeding motor neuron survival in ALS. Moreover, increased vascular permeability in the brain was determined in symptomatic G93A SOD1 rats using Gd-DTPA-enhanced MRI (Andjus et al., 2009). In addition to the BBB leakage, the authors showed marked lateral ventricle dilatation in ALS rats as "the most apparent feature of brain tissue atrophy."

Thus, the compromised BBB and BSCB are evident in the SOD1 animal model of ALS. Endothelial cell degeneration, swollen astrocyte end-feet and dissociation from the endothelium, capillary leakage, perivascular edema, downregulation of tight junction proteins, microhemorrhages, and reduction of basement membrane components are the main hallmarks of the B-CNS-B impairment. This altered vascular barrier, normally preventing entry of various blood-borne harmful substances into the CNS, could contribute to motor neuron death. Although capillary barrier damage in the CNS of both mouse and rat SOD1 models of ALS has been demonstrated prior to motor neuron loss and neuroinflammation, the specific cause of B-CNS-B breakdown has not yet been identified. It is possible that endothelial cells are more susceptible to detrimental involvement of the misfolding mutant SOD1 protein. However, the particular mechanism(s) responsible for the endothelial cell alteration observed in ALS still needs to be determined.

## BBB AND BSCB IMPAIRMENT IN SPORADIC AND FAMILIAL ALS PATIENTS

The transgenic rodent models expressing mutant SOD1 have greatly contributed to the understanding of ALS pathogenesis. Relatively new research on B-CNS-B competence in ALS has largely used mutant SOD1 rodent models, but determination of barrier integrity without involvement of the mutant SOD1 protein is necessary to clarify the pathogenesis of sporadic human ALS cases. ALS is a multifactorial disease with a complexity of underlying intrinsic and extrinsic factors related to motor neuron death. Some of these detrimental factors might be directly associated with B-CNS-B impairment.

Although the B-CNS-B regulates cellular infiltration into the CNS (Engelhardt, 2008), under inflammatory conditions, extensive leukocyte migration into the CNS occurs following cytokine releases from inflammatory/immune cells (de Vries et al., 1997; Sagar et al., 2012; Sallusto et al., 2012). Leukocyte trafficking through the B-CNS-B is a multistep process mediated by adhesion molecules, classified as immunoglobulins, integrins, cadherins, or selectins. These molecules are up-regulated on the surfaces of the endothelial cells, allowing adhesion and migration of cells from the bloodstream to the CNS (Ley et al., 2007) and are often accompanied by an increased influx of serum proteins. In example, Lindsberg et al. (2010) discussed the deleterious role of the mast cell, a potent inflammatory cell, in cerebral ischemia. These cells located within the cerebral microvasculature secrete cytokines, histamine, heparin, and proteases which can degrade the basement membrane and exacerbate barrier damage, promoting edema, prolonged extravasation, and microhemorrhage and attracting new inflammatory cells. The paracellular pathway of cell migration through the capillary wall is most common, but some authors hypothesize that penetration of immune cells into the CNS can also be accomplished through the transcellular pathway with intact tight junctions (Carman and Springer, 2008).

In the CNS tissue of ALS patients, inflammation and immune cell activation have been detected and are associated with motor neuron degeneration (Donnenfeld et al., 1984; Engelhardt and Appel, 1990; Engelhardt et al., 1993, 1995; Henkel et al., 2004; Boillée et al., 2006). Early studies found IgG and C3/C4 complement deposits in the spinal cord and motor cortex tissues from ALS patients (Donnenfeld et al., 1984). Engelhardt and Appel, (1990) also detected active macrophages and IgG within the endoplasmic reticulum of motor neurons in ALS patients. Interestingly, IgG from sera of ALS patients induced death of a motor neuron cell line (VSC 4.1) *in vitro* (Engelhardt et al., 1995). These study results suggest alteration of B-CNS-B permeability and thus recent investigations have begun to focus on potential endothelial barrier damage in ALS patients.

Henkel et al. (2009) demonstrated diminished mRNA expression of occludin and ZO-1 in human lumbar spinal cord tissue from both sporadic and familial forms of ALS. Similarly, decreased immunostaining for occludin was observed in a small cohort of ALS patients (Miyazaki et al., 2011). These results agreed with the experimental findings, confirming loss of endothelial integrity, and indicating BSCB disruption that might contribute to disease pathogenesis.

A study by Garbuzova-Davis et al. (2010) showed a significant reduction in the numbers of circulating endothelial cells in the peripheral blood of ALS patients with moderate or severe disease. Increased circulating endothelial cells is considered a marker for endothelial damage (Blann et al., 2005) and has been noted in several vascular diseases, including acute myocardial infarct and acute ischemic stroke (Nadar et al., 2005; Chong et al., 2006). These unexpected results in ALS may be explained by a lack of endothelial shedding, resulting in the attachment of new endothelial cells over the damaged cells and thus a multilayer endothelium (Garbuzova-Davis et al., 2010). Indeed, electron microscopy images of ALS mouse tissue have revealed multiple layers of endothelial cells in the brain and spinal cord capillaries (Garbuzova-Davis et al., 2007a). Also, a reduction of circulating endothelial cells in peripheral blood of ALS patients with disease progression could be due to impaired re-endothelialization. The structural and functional integrity of the vascular network, normally maintained by continuous renewal of the endothelial cell layer with a low replication rate of 0.1% per day (Hunting et al., 2005), might be weakened in ALS. It is possible that insufficient production of endothelial progenitor cells by the bone marrow might be an issue. Recent reports demonstrated the functional deficiency of bone marrow mesenchymal stromal cell in ALS patients by reductions in pluripotency and secretion of various trophic factors (Koh et al., 2012) as well as by abnormal productions of MMPs and tissue inhibitors of metalloproteinases (TIMPs; Bossolasco et al., 2010).

In our recent study (Garbuzova-Davis et al., 2012), we examined structural and functional integrities of capillaries in the gray and white matter of the brainstem (medulla) and spinal cord (cervical and lumbar) in postmortem tissue from SALS patients. Study results showed capillary ultrastructural abnormalities in CNS tissues from SALS patients, similar to results from our animal studies (Garbuzova-Davis et al., 2007a). Mainly, severe intra- and extracellular edema, endothelial cell impairment as characterized by swelling and cytoplasmic vacuolization, pericyte degeneration, and degeneration of astrocyte end-feet processes surrounding capillaries were determined by electron microscopic analysis of the medulla and spinal cords. Also, separation of the endothelial cells from the basement membrane, allowing plasma to contact the basal lamina, was a significant capillary alteration noted in brain and spinal cord tissues of SALS patients. Observed capillary endothelium damage led to vascular leakage in the brain and spinal cord as determined by immunostaining for endogenous IgG, confirming previous study results on an animal model of ALS (Garbuzova-Davis et al., 2007b; Nicaise et al., 2009a). Microvascular leakage was also determined in CNS tissues from SALS patients by perivascular fibrin deposits in our electron microscopy images and was recently confirmed (Winkler et al., 2013). Winkler et al. (2013) additionally demonstrated parenchymal accumulation of the plasma-derived proteins trombin and IgG as well as erythrocyte-derived hemoglobin and iron-containing hemosiderin in the cervical gray matter from both SALS and FALS patients via immunostaining. The authors noted that these abnormal deposits in the postmortem tissues were detected only in tissues from ALS patients, but not from controls, and there were unusually widespread pathological depositions at a significant distance from capillaries. In our study (Garbuzova-Davis et al., 2012), fibrin filament deposits and IgG leakage, determined by electron microscopic and immunostaining analysis respectively, were predominantly limited to within the capillary basement membrane or were in close proximity to capillaries. Moreover, erythrocyte extravasation was not determined perivascularly or at neuropil locations in our numerous electron microscope images at different magnifications of the medulla, cervical and lumbar spinal cords from ALS patients. Microhemorrhages within CNS tissues in ALS are possible, even though not supported by MRI evaluations of microbleeds in the brain of ALS patients (Verstraete et al., 2010), so the presence of erythrocytes in CNS parenchyma observed by Winkler et al. (2013) needs further confirmation. Erythrocytes are normally restricted from entry into the CNS and these cells typically extravasate into CNS tissue due to capillary rupture as shown in multiple sclerosis (Adams, 1988), post-traumatic epilepsy (Willmore and Triggs, 1984), and cerebral ischemia (Simard et al., 2007). However, capillary rupture was not evident morphologically in the brain or spinal cords from ALS patients, even by electron microscopy imaging.

Importantly, complementary approaches have identified pericyte degeneration by electron microscopy (Garbuzova-Davis et al., 2012) and shown reduction in pericyte numbers via immunostaining (Winkler et al., 2013) in capillaries of ALS patients, deficiencies which might severely compromise the B-CNS-B. Hence, the cause(s) of pericyte deterioration in human ALS tissues should be determined.

Additionally, analysis of tight junction protein expressions in our study (Garbuzova-Davis et al., 2012) using Western immunoblot showed significant decreases of primarily ZO-1 expression in gray and white matter in all examined SALS tissues, similarly to previous studies (Henkel et al., 2009; Miyazaki et al., 2011). For occludin and claudin-5, diminished protein expressions were mostly found in ALS medulla and cervical spinal cord.

Moreover, our new findings (Garbuzova-Davis et al., 2012) showed extensive vascular basement membrane collagen IV accumulation, 2–2.5 times higher than controls, in the majority of brain and spinal cord vessels from SALS patients. Also, collagen fiber calcifications were determined in some CNS capillaries. Although similar results were noted in Alzheimer's patients (Claudio, 1996), some previous reports conflict with our study results. Decreased perivascular collagen IV was noted in postmortem ALS spinal cord tissue (Ono et al., 1998; Miyazaki et al., 2011). This discrepancy needs clarification.

Finally, our study results showed a significant increase of microvascular density in gray matter of the lumbar spinal cord from SALS patients vs. controls (Garbuzova-Davis et al., 2012) suggesting that neovascularization occurred to compensate for vascular insufficiency due to dysfunctional capillaries. Currently, we are investigating possible new vessel formation in both an ALS mouse model and ALS patients. Supporting the likelihood of ALS neovascularization, Biron et al. (2011) similarly (to our results) demonstrated increased microvascular density in brains from Alzheimer's patients, suggesting a relationship between hypervascularity, neoangiogenesis and BBB disruption. Also, ongoing angiogenesis resulted in increased vascular density in postmortem brain tissues, mainly in the hippocampus, in Alzheimer's patients (Desai et al., 2009). Recently, Desai Bradaric et al. (2012) showed

the presence of newly created vessels in postmortem brain tissues such as the substantia nigra pars compacta, locus ceruleus, and putamen from subjects with Parkinson's disease and progressive supranuclear palsy. The authors suggest that these new angiogenic vessels could contribute to disease inflammatory processes by failing to restrict extravasation of immune cells and inflammatory or toxic factors from the peripheral circulation to the CNS due to the new vessels having incompletely developed BBB properties. This possibility might also be an issue for ALS.

Thus, there is compelling evidence of BBB and BSCB impairment in areas of motor neuron degeneration in ALS patients. Importantly, microvascular alterations seen in both gray and white matter of medulla, cervical, and lumbar spinal cord from SALS patients indicate pervasive B-CNS-B damage that might contribute to disease pathogenesis.

## SIMILARITIES AND DIFFERENCES IN THE B-CNS-B IMPAIRMENT BETWEEN ALS PATIENTS AND ANIMAL MODELS OF ALS

Convincing findings indicate B-CNS-B alterations in both ALS patients and the SOD1 animal model of ALS and suggest these alterations as a possible factor aggravating motor neuron damage. Numerous signs of barrier damage, such as endothelial cell degeneration, capillary leakage, perivascular edema, downregulation of tight junction proteins, and microhemorrhages are common in both mutant SOD1 animal models of disease and ALS patients. To date, other pathogenic features linked to barrier alterations have so far only been identified in ALS patients. Mainly, pericyte degeneration, perivascular basement membrane collagen IV expansion, and white matter capillary abnormalities in SALS patients are significant barrier related pathologies yet to be noted in ALS SOD1 animal models. In **Table 1**, current evidence of B-CNS-B impairment in ALS is provided from animal and human studies.

Severe capillary pericyte damage (Garbuzova-Davis et al., 2012; Winkler et al., 2013) is an important finding in ALS patients. At the ultrastructural level, complete pericyte degeneration or cell fragmentation in the adjacent extracellular space was determined in numbers of gray and white matter microvessels in the medulla and cervical/lumbar spinal cord of SALS patients (Garbuzova-Davis et al., 2012). Pericytes play essential roles in maintaining B-CNS-B integrity by regulating capillary permeability, blood flow, vascular tone, and angiogenesis (Hirschi and D'Amore, 1996; Kutcher and Herman, 2009; Armulik et al., 2010; Winkler et al., 2011, 2012; Sá-Pereira et al., 2012). These functions are associated with the cells' anatomical location, in close proximity to endothelial cells and sharing a common basement membrane. Pericytes are also involved in modulation of immunological response by their phagocytic function (Balabanov and Dore-Duffy, 1998; Dalkara et al., 2011). For example, amyloid deposits within degenerating pericytes were detected in the brains of Alzheimer's patients (Dalkara et al., 2011). The authors discussed the role of pericyte dysfunction in cerebral hypoperfusion and suggest that "microvascular dysfunction due to pericyte degeneration initiates secondary neurodegenerative changes" (Dalkara et al., 2011). In ALS, Rule et al. (2010) reported reduced capillary blood flow in

**Table 1 | Evidence of blood–CNS barrier impairment in ALS patients and SOD1 animal models of ALS.**

| | References | |
|---|---|---|
| **Description of evidence** | **Animal model of ALS** | **Human ALS** |
| Endothelial cell degeneration or damage | Garbuzova-Davis et al. (2007a), Nicaise et al. (2009a), Miyazaki et al. (2011) | Garbuzova-Davis et al. (2012) |
| Capillary leakage | Garbuzova-Davis et al. (2007b), Nicaise et al. (2009a), Andjus et al. (2009) | Garbuzova-Davis et al. (2012), Winkler et al. (2013) |
| Pericyte degeneration or damage | | Garbuzova-Davis et al. (2012), Winkler et al. (2013) |
| Perivascular edema | Garbuzova-Davis et al. (2007a) | Garbuzova-Davis et al. (2012) |
| Astrocyte end-feet capillary damage and dissociation | Garbuzova-Davis et al. (2007a,b), Nicaise et al. (2009a), Miyazaki et al. (2011), Bataveljić et al. (2012) | Miyazaki et al. (2011), Garbuzova-Davis et al. (2012) |
| Altered basement membrane components | Garbuzova-Davis et al. (2007b), Nicaise et al. (2009a), Miyazaki et al. (2011) | Ono et al. (1998), Miyazaki et al. (2011), Garbuzova-Davis et al. (2012) |
| Microhemorrhages or perivascular hemosiderin | Garbuzova-Davis et al. (2007a), Zhong et al. (2008), Nicaise et al. (2009a) | Verstraete et al. (2010) (unsupporting), Winkler et al. (2013) |
| Altered blood flow or capillary lengths and diameters | Zhong et al. (2008), Miyazaki et al. (2011) | Rule et al. (2010) |
| Downregulation of junctional complex proteins | Zhong et al. (2008), Nicaise et al. (2009a), Miyazaki et al. (2011) | Henkel et al. (2009), Miyazaki et al. (2011), Garbuzova-Davis et al. (2012) |
| Altered endothelial transporter protein expression | Garbuzova-Davis et al. (2007b), Zhong et al. (2008), Milane et al. (2010), Jablonski et al. (2012) | Jablonski et al. (2012) |

brains of patients correlating with disease severity, a reduction likely associated with pericyte impairment. A similar decrease in blood flow was determined in the spinal cord of G93A SOD1 mice preceding inflammation and motor neuron injury (Zhong et al., 2008). However, there were no obvious abnormalities detected in pericyte morphology in the brainstem or spinal cord capillaries via electron microscope even in late symptomatic G93A mice (Garbuzova-Davis et al., 2007a). This discrepancy between animal data and results from ALS patients should be investigated.

Another difference between human and animal studies in ALS is vascular basement membrane collagen IV abnormalities. As we noted above, our recent study (Garbuzova-Davis et al., 2012) showed extensive basement membrane collagen IV accumulation and even collagen fiber calcifications in numerous capillaries in gray and white matter brain and spinal cords from SALS patients. However, opposing reports demonstrated decreased perivascular collagen IV in postmortem ALS spinal cord tissue (Ono et al., 1998; Miyazaki et al., 2011). In a study by Miyazaki et al. (2011), reduction of immunoexpression for collagen IV was observed in the anterior horns of the spinal cord from ALS patients as well as in G93A mice during disease progression and was accompanied by MMP-9 up-regulation. The authors conclude that this vascular damage is common to humans and this ALS animal model. However, the observed collagen IV reduction might be due to the diminished capillary density described in the same study. In addition, the authors provided conflicting data with double immunofluorescence staining for collagen IV and Iba-1 (microglial marker) in the spinal anterior horn of G93A mice at 18 weeks of age showing higher collagen IV expression vs. controls along with the appearance of collagen IV-positive microglia. Microglial cells are resident cells in the CNS with macrophagic properties upon their activation and likely overexpression of collagen IV by microglia indicates uptake of this protein. Supportive evidence of the ability of microglia to express collagen IV was not provided (Miyazaki et al., 2011). Also, it is unclear if this collagen IV immunostaining was also associated with capillaries or astrocytes. Double immunostaining for collagen IV and astrocytes might be a reasonable confirming procedure. It has been shown that reactive astrocytes expressed type IV collagen after spinal cord injury in promoting glial scar formation (Liesi and Kauppila, 2002). In the same study, the authors reported that IL-1 beta and TGF beta-1 cytokines induced collagen IV expression in astrocytes *in vitro*. Since reactive astrogliosis and microglia activation are major contributors to inflammatory processes in ALS by secretion of various pro-inflammatory cytokines, particularly TNF-alpha and TGF beta-1 (Schiffer et al., 1996; Hall et al., 1998; McGeer and McGeer, 1998; Elliott, 2001; McGeer and McGeer, 2002; Consilvio et al., 2004; Henkel et al., 2004; Xie et al., 2004; Vargas and Johnson, 2010; Appel et al., 2011; Haidet-Phillips et al., 2011; Sica, 2012; Evans et al., 2013; Phatnani et al., 2013) it is possible that reactive astrocytes not only affect motor neurons but also promote a collagenous vascular basement membrane. Moreover, inflammation may initiate barrier damage by impairing endothelial cell function. Mantovani et al. (1992) showed that the inflammatory environment in ALS affected endothelial cell gene expression, altering cell function. An additional discrepancy between our and the above mentioned studies regarding basement membrane collagen deposition was discussed in detail (Garbuzova-Davis et al., 2012) and primarily focused on the potential imbalance between MMPs and TIMPs due to defective regulation of the MMP pathway by damaged endothelial cells. Also, since downregulation of other basement membrane components such as laminin (Garbuzova-Davis et al., 2007b) and argin (Nicaise et al., 2009a) has been shown in symptomatic G93A mice but not yet confirmed in ALS patients, it is possible that buildup of perivascular collagen IV occurs as a compensatory mechanism for maintenance of vascular integrity.

We strongly believe that abnormal perivascular collagen accumulation in SALS patients takes place over a long period of time. Our notion is partially supported by Ono et al. (1998) who showed widely separated and fragmented collagen bundles in the interstitial tissue surrounding capillaries in the posterior half of lateral funiculus and anterior horn of cervical spinal cord from ALS patients by ultrastructural analysis. Yet total collagen content determined for each of these spinal cord regions was lower in ALS patients than in controls with or without neurological diseases. Although these data are interesting, the existence of basement collagen abnormalities needs clarification.

Thus, the complexity of the B-CNS-B alterations in ALS is evident. Commonalities in barrier pathologies between humans and an animal model of ALS are essential for understanding involvement of the B-CNS-B in disease pathogenesis. However, the disparities in barrier competence in humans with ALS vs. animal model should be considered. Despite a growing research effort, more studies are needed to reveal specific mechanisms of barrier breakdown in ALS. The question still remains: is barrier damage an initial disease factor or a secondary element in human ALS?

## CONCLUSION

ALS has been, and remains, a challenge for developing therapeutics. More than 30 drug compounds have already been tested in ALS clinical trials yet the only modestly effective treatment is riluzole (Aggarwal and Cudkowicz, 2008). Some tested substances failed to prove effective and even showed harmful effects in Phase III clinical trials: IGF-1 (insulin-like growth factor type 1; Sorenson et al., 2008), minocycline (Gordon et al., 2007), creatine (Groeneveld et al., 2003), and topiramate (Cudkowicz et al., 2003).

These disappointing results might reflect defective transport systems in damaged BBB and BSCB in ALS. Degeneration of endothelial and pericyte cells, compromising vascular barrier integrity in the brain and spinal cord in ALS patients, could be the main obstacles for effective drug delivery to the CNS. Also, it is hard to imagine proper transport of pharmaco-therapeutics across an extensively expanded vascular collagenous basement membrane. Such abnormal buildup of basement membrane collagen seems likely to alter barrier influx and efflux transport systems and, as result, motor neurons might suffer both from reduced nutritional deliveries and increased metabolite levels. Additionally, increases of P-glycoprotein (P-gp) along with the breast cancer resistance protein (BCRP) were determined in brain and spinal cord microvessels in both SOD1 animal models and ALS patients (Milane et al., 2010; Jablonski et al., 2012). Jablonski et al. (2012) conclude that the impairment of these P-gp and BCRP efflux transporters might induce pharmaco-resistance in ALS.

Numerous comprehensive reviews discuss therapeutic strategies of transport drugs across the blood–CNS barrier (Abbott and Romero, 1996; Pardridge, 2002; Misra et al., 2003; McCarty, 2005; Patel et al., 2009; Pathan et al., 2009; Gabathuler, 2010) including chemical (i.g., lipid-mediated transport), biological (i.g., specific transporters for pharmaceuticals), or particular drug carrier systems. Also, various drug delivery systems (e.g., liposomes, nanoparticles, or microspheres) and routes (e.g., intranasal, intraventricular, or intrathecal) have been proposed. However, all of these various strategies for effective drug delivery to the CNS rely on a normally functioning BBB/BSCB. In ALS, deliveries of therapeutic drugs are likely to be complicated by the pervasiveness of the B-CNS-B damage.

In conclusion, the blood–CNS barrier should be considered as a primary therapeutic target prior to development of any treatment approach for ALS.

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