Catarina de Pais Paiva Santos

Licenciada em Biologia Celular e Molecular

Glia-Motoneuron dialogue in ALS onset and progression in SOD1G93A-mice model

Dissertação para obtenção do Grau de Mestre em
Genética Molecular e Biomedicina

Orientador: Dora Maria Tuna de Oliveira Brites, Investigadora Coordenadora e Professora Catedrática Convidada, Faculdade de Farmácia, Universidade de Lisboa

Co-orientador: Ana Rita Mendonça Vaz, PhD, Faculdade de Farmácia, Universidade de Lisboa

Júri:

Presidente: Doutora Margarida Casal Ribeiro Castro Caldas Braga

Arguente: Doutora Maria Teresa Albuquerque Santos Faria Pais

Vogal: Doutora Dora Maria Tuna de Oliveira Brites

FACULDADE DE CIÊNCIAS E TECNOLOGIA
UNIVERSIDADE NOVA DE LISBOA

Dezembro, 2014

Catarina de Pais Paiva Santos

Licenciada em Biologia Celular e Molecular

Glia-Motoneuron dialogue in ALS onset and progression in SOD1G93A-mice model

Dissertação para obtenção do Grau de Mestre em
Genética Molecular e Biomedicina

Orientador: Dora Maria Tuna de Oliveira Brites, Investigadora Coordenadora e Professora Catedrática Convidada, Faculdade de Farmácia, Universidade de Lisboa

Co-orientador: Ana Rita Mendonça Vaz, PhD, Faculdade de Farmácia, Universidade de Lisboa

Júri:

Presidente: Doutora Margarida Casal Ribeiro Castro Caldas Braga

Arguente: Doutora Maria Teresa Albuquerque Santos Faria Pais

Vogal: Doutora Dora Maria Tuna de Oliveira Brites

FACULDADE DE CIÊNCIAS E TECNOLOGIA
UNIVERSIDADE NOVA DE LISBOA

Dezembro, 2014

Glia-Motoneuron dialogue in ALS onset and progression in SOD1G93A-mice model

Part of the results discussed in this thesis were presented in the following meetings:

Paiva Santos, C., Gomes, C., Fernandes, A., Sebastião, AM., Vaz, AR., Brites, D,. Studying the role of glial cells during amyotrophic lateral sclerosis progression. 6th Postgraduate iMed.ULisboa Students Meeting, Faculty of Pharmacy, University of Lisbon - Lisbon, Portugal, July 2nd, 2014. [Poster] (See annex 1.1)

Paiva Santos, C., Gomes, C., Fernandes, A., Sebastião, AM., Vaz, AR., Brites, D, The microglial-motoneuron dialogue in ALS: studies in the G93ASOD1 mice model. III Jornadas Intercalares das dissertações anuais dos mestrados do Departamento de Química e do Departamento de Ciências da Vida. FCT-UNL. 6-7 Feb 2014 [Oral communication]

Cunha, C., Paiva Santos, C., Gomes, C., Sebastião, AM., Fernandes, A., Vaz, AR., Brites, D. Exploring the pro-inflammatory environment in the spinal cord of mutant SOD1G93A mice: potential biomarkers at pre- and symptomatic phases. XVIII Congress of the Portuguese Biochemical Society, Coimbra, Portugal, December, 17-20, 2014.

Vaz, AR. Motoneuron degeneration and glial reactivity in ALS: insights from cellular to animal models.Neuroscience Seminars at IMM 2012, Instituto de Medicina Molecular, Universidade de Lisboa, Lisbon, Portugal, June 9, 2014. [Oral communication by invitation]

Vaz, AR., Cunha, C., Nascimento, F., Sebastião AM., Brites, D. Profiling microglial reactivity in a mice model of Amyotrophic Lateral Sclerosis. I Luso-Brasilian Glial Network Symposium, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brasil, March 10-12, 2014.

This work was supported by grants PTDC/SAU-FAR/118787/2010 (DB) and PEst-OE/SAU/UI4013/2013-2014, from Fundação para a Ciência e a Tecnologia.

PARA OS MEUS PAIS

AGRADECIMENTOS

Em primeiro lugar queria agradecer à Professora Doutora Dora Brites por me ter recebido no grupo e pela confiança depositada em mim. Estou muito grata pela oportunidade e experiência que adquiri no nosso grupo. Quero também agradecer toda a disponibilidade que demonstrou e por incutir rigor científico e espírito crítico ao longo do trabalho.

Rita, quero agradecer-te toda a disponibilidade e paciência que demonstraste ao longo do trabalho. Pelo conhecimento adquirido, rigor e espírito crítico que sempre incutistes quer no laboratório quer no trabalho escrito muito obrigada. Foi um prazer trabalhar contigo ao longo desta experiência.

Quero também agradecer à orientadora interna da minha tese, à Professora Doutora Margarida Castro Caldas, pela sua disponibilidade no esclarecimento de qualquer dúvida.

À Professora Doutora Adelaide Fernandes agradeço toda a disponibilidade demonstrada e pelos ensinamentos e pela afável recepção no grupo.

Agradeço à Professora Doutora Alexandra Brito, ao Professor Doutor Rui Silva e à Doutora Sofia Falcão pela disponibilidade e amabilidade que sempre demonstraram ao longo do ano.

Cátia, um especial obrigado por todas as chatices que te dei e te estar sempre a importunar com infindáveis dúvidas existências e problemas. Obrigada por tudo o que me ensinaste e pela amabilidade e boa disposição que sempre demonstraste. Sempre com o teu "não há problema" ajudaste-me a enfrentar as adversidades do laboratório e a arranjar sempre uma forma de contornar os problemas. Estiveste sempre lá para me apoiar, excepto quando me gozavas (eu confiei em ti!). Muito obrigada por tudo ☺

Carolina, muito obrigada por tudo em que me ajudaste no laboratório e em todo o trabalho. Quero também agradecer-te todas as lições de geografia e gramática que davas de vez em quando. Mesmo que estivesses sempre à espera que eu dissesse coisas à Catarina para me gozares eu sei que o fazias de bom coração (e até foste aprendendo algumas coisas parvas mas engraçadas). Muito obrigada por tudo!

Gisela, obrigada por tudo. Pelos almoços, pela ajuda, pela amizade… Foste bestial durante este ano que passei no cpm. Obrigada pelo companheirismo e pelos bons momentos (mesmo quando andamos a atazanar a cabeça uma à outra) ☺

À menina Cláudia, muito obrigada por estares sempre de bom humor e fazeres sempre uma pessoa sorrir. Sempre estiveste disponível para me ajudar e és um grande exemplo de dedicação e trabalho. Boa sorte para o futuro e um muito obrigada.

Filipa, o meu primeiro contacto com o grupo foi através do teu trabalho na barreira e ajudaste a promover a neurociência como o meu projecto de tese. Obrigada por toda a disponibilidade e amabilidade que sempre demonstraste (mesmo quando estavas cheia de trabalho). Muito obrigada e boa com essa defesa que se está a aproximar.

Inês Figueira, mesmo estando no cpm de vez em quando trazes sempre boa disposição. Queria agradecer-te toda a amizade e disponibilidade que me demonstraste, e por todos os bons momentos. Obrigada ☺

Maria Inês, companheira de mestrado. Quero agradecer-te todos os bons momentos que partilhámos durante este ano e pelo sangue, suor e lágrimas que derramámos para fazer a tese. Obrigada pelo companheirismo e boa disposição.

Rui, pá és um tipo bestial. Obrigada por tudo, pelas cervejas, jantares e sobretudo ginjinhas :P Especialmente, obrigada pela amizade. Nunca mudes porque quando for grande quero ser como tu!

À menina Vera. Embora já não estejas no cpm tenho que te agradecer também todo o apoio e boa disposição que sempre proporcionaste. Fizeste muita falta quando te foste embora. Um grande beijinho e obrigada.

Cátia Janota. Pá não sei o que te dizer a não ser obrigado. És uma pessoa especial que sempre esteve lá para me apoiar (mesmo quando não estava no cpm). Estiveste lá nos bons e nos maus momentos. Muito obrigada miúda! ☺

Às meninas novas, Carla, Maria e Mafalda, vocês são umas melguinhas muito queridas. Chegaram com muita animação e sei que vão ter muito sucesso neste vosso ano de tese. Um grande beijinho e boa sorte.

Tiago, obrigada por tudo. Por esta amizade de 5 anos e pelo apoio incondicional e por também me meteres no sítio quando é preciso. Obrigada por todos os bons momentos ☺

Aos meninos Ricardo, Joana, João, Mafalda e Micaela queria também agradecer por tudo! Pelo apoio e pelas gargalhadas partilhadas. Um grande beijinho para vocês.

Mais importante que tudo é a minha família. Sem os meus pais a apoiarem-me não estaria aqui. Obrigada por nunca me deixarem desistir e por me fazerem sempre ir à luta. Sei que por mais longe que esteja vocês estão sempre disponíveis para me apoiarem seja no que for. Obrigada por todos os sacrifícios e por me amarem incondicionalmente. Ao meu maninho, aos meus avós e ao resto da minha família um muito obrigado por todo o vosso apoio!

RESUMO

Esclerose lateral amiotrófica (ELA) é uma doença neurodegenerativa fatal caracterizada pela perda progressiva de neurónios motores (NMs). Estudos apontam as células gliais como factores cruciais na origem e progressão da doença. De facto, vias de sinalização NM-microglia envolvendo neuroprotecção ou inflamação, são susceptíveis de estarem alteradas na ELA.

Neste trabalho procurámos estudar moléculas relacionadas com função glial e/ou reactividade pela avaliação de marcadores gliais e hemicanais, maioritariamente expressos nos astrócitos. Adicionalmente, estudámos moléculas envolvidas no diálogo NM-microglia (CXCR3/CCL21; CX3CR1/CX3CL1; MFG-E8), assim como na proliferação (Ki-67), moléculas envolvidas em inflamação (TLR2/4, NLRP3; IL-18) e em sinais de alarme/calmantes (HMGB1/autotaxin). Para tal usámos homogeneizados de medula espinhal lombar de ratinhos que expressam a proteína humana mutada SOD1 (mSOD1) num estadio pré-sintomático e sintomático de ELA. Ratinhos SJL (WT) com as mesmas idades foram usados como controlos.

Observámos expressão reduzida de genes associados a marcadores astrocitários (GFAP e S100B) e microgliais (CD11b) em mSOD1 na fase pré-sintomática, assim como níveis diminuídos de componentes de junções aderentes, panexina1 e conexina43, e expressão de Ki-67 e autotaxina diminuída. Adicionalmente, o diálogo NM-microglia estava reduzido em ratinhos mSOD1 assim como a resposta inflamatória. Curiosamente, observámos reactividade asctrocítica (S100B) e microglial (CD11b) aumento da proliferação (Ki-67) e expressão de autotaxina diminuída em ratinhos mSOD1 sintomáticos. Diálogo NM-microglia (CXCR3/CCL21; CX3CR1/CX3CL1; MFG-E8) e actividade de hemicanais (conexina43 e panexina1) foram observados na fase sintomática, juntamente com resposta inflamatória exacerbada, assim indicada pelos níveis aumentados de HMGB1 e NLRP3.

Os nossos resultados sugerem que a diminuição da expressão de autotaxina constitui uma característica da fase pré-sintomática, e precede a rede de determinantes inflamatórios na fase sintomática, incluindo HMGB1, CCL21, CX3CL1, e NLRP3. Identificação de moléculas e vias de sinalização que estão diferencialmente activadas durante a progressão da ELA vão contribuir para um melhor desenvolvimento de estratégias terapêuticas para a doença.

Termos-chave: esclerose lateral amiotrófica; neurodegeneração; diálogo neurónio motor-microglia; neuroinflamação; modelo ratinho G93ASOD1

ABSTRACT

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease characterized by the progressive loss of motoneurons (MN). Increasing evidence points glial cells as key players for ALS onset and progression. Indeed, MN-glia signalling pathways involving either neuroprotection or inflammation are likely to be altered in ALS.

We aimed to study the molecules related with glial function and/or reactivity by evaluating glial markers and hemichannels, mainly present in astrocytes. We also studied molecules involved in microglia-MN dialogue (CXCR3/CCL21; CX3CR1/CX3CL1; MFG-E8), as well as proliferation (Ki-67) and inflammatory-related molecules (TLR2/4, NLRP3; IL-18) and alarming/calming signals (HMGB1/autotaxin). We used lumbar spinal cord (SC) homogenates from mice expressing a mutant human-SOD1 protein (mSOD1) at presymptomatic and late-symptomatic ALS stages. SJL (WT) mice at same ages were used as controls.

We observed decreased expression of genes associated with astrocytic (GFAP and S100B) and microglial (CD11b) markers in mSOD1 at the presymptomatic phase, as well as diminished levels of gap junction components pannexin1 and connexin43 and expression of Ki-67 and decreased autotaxin. In addition, microglial-MN communication was negatively affected in mSOD1 mice as well as inflammatory response. Interestingly, we observed astrocytic (S100B) and microglial (CD11b) reactivity, increased proliferation (Ki-67) and increased autotaxin expression in symptomatic mSOD1 mice. Increased MN-microglial dialogue (CXCR3/CCL21; CX3CR1/CX3CL1; MFG-E8) and hemichannel activity, namely connexin43 and pannexin1, were also observed in mSOD1 at the symptomatic phase, along with an elevated inflammatory response as indicated by increased levels of HMGB1 and NLRP3.

Our results suggest that decreased autotaxin expression is a feature of the presymptomatic stage, and precede the network of pro-inflammatory-related symptomatic determinants, including HMGB1, CCL21, CX3CL1, and NLRP3. The identification of the molecules and signaling pathways that are differentially activated along ALS progression will contribute for a better design of therapeutic strategies for disease onset and progression.

Keywords: amyotrophic lateral sclerosis; neurodegeneration; glial-motoneuron dialogue; neuroinflammation; G93ASOD1 mouse model

INDEX

INDEX OF FIGURES

INDEX OF TABLES

ABBREVIATIONS

ALS	Amyotrophic lateral sclerosis
ATP	Adenosine-5'-triphosphate
ATX	Autotaxin
BDNF	Brain-derived neurotrophic factor
CCL21	Chemokine (C-C motif) ligand 21
cDNA	Complementary DNA
CNS	Central nervous system
CX3CL1	CX3C chemokine ligand 1
CX3CR1	CX3C chemokine receptor 1
Cx43	Connexin 43
CXCR3	Chemokine (C-X-C motif) receptor 3
DNA	Deoxyribonucleic acid
EAAT	Excitatory amino acid transporter
ER	Endoplasmic reticulum
fALS	Familial amyotrophic lateral sclerosis
FUS/TLS	Fused in sarcoma/translated in liposarcoma
GDNF	Glial derived neurotrophic factor
GFAP	Glial fibrillary protein
HMGB1	High mobility group box 1
INF-γ	Interferon gamma
IGF-1	Insulin growth factor 1
IL	Interleukin
LMN	Lower motoneuron
MFG-E8	Milk fat globule factor E8
miRNA	microRNA
mSOD1	Mutant SOD1
NF-κB	Nuclear factor- κB
NO	Nitric oxide
RAGE	Receptor for advanced glycation end products
RIPA	Radio-immunoprecipotation assay
ROS	Reactive oxygen species

sALS	Sporadic amyotrophic lateral sclerosis
SC	Spinal cord
siRNA	Small interference RNA
SOD1	Superoxide dismutase 1
TDP-43	TAR DNA-binding protein 43
TGF-β	Transforming growth factor-β
TLR	Toll-like receptor
TNF	Tumor necrosis factor
UMN	Upper motoneuron
UPR	Unfolded protein response
PAN1	Pannexin 1
WT	Wild type

I. INTRODUCTION

1. Amyotrophic lateral sclerosis: an overview

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease characterized by selective loss of upper motoneurons (UMN) in the cortex and in the brainstem and lower motoneurons (LMN) in the spinal cord (SC) of the patients, which progressively leads to muscle atrophy and paralysis and ultimately death, usually by respiratory failure (Liao *et al.*, 2012; Pratt *et al.*, 2012; Robberecht and Philips, 2013). This disease was first described in 1869 by the French neurologist Jean-Martin Charcot and can sometimes be referred to as Lou Gehrig's disease (Gonzalez de Aguilar *et al.*, 2007). Etymologically, ALS refers to the lack of muscle nourishment (amyotrophic), lateral meaning the corticospinal tract which descends through the lateral spinal cord and sclerosis meaning hardening or scarring (Bento-Abreu *et al.*, 2010). The onset of ALS is about 50 to 60 years of age, although it may occur earlier in life, with a life expectancy of 3 to 5 years after symptoms onset (Kiernan *et al.*, 2011; Mitchell and Borasio, 2007). This disease is the most common adult-onset motoneuron disorder, affecting approximately 1-2 people per 100,000 individuals worldwide (Factor-Litvak *et al.*, 2013) with men being slightly more affected than women by a factor between 1.2 and 1.5 (Al-Chalabi and Hardiman, 2013), and approximately 5,600 people are diagnosed each year (Gilbert, 2014). Although there are no epidemiological studies of ALS in Portugal, according to Professor Mamede de Carvalho clinical practice (a renowned clinician in the area of ALS in Portugal at Centro Hospitalar Lisboa-Norte – Hospital de Santa Maria) it is estimated that there are 600 Portuguese patients suffering from this disease, with a tendency to increase due to population aging. ALS has an array of clinical manifestations which overlap with other neurodegenerative diseases which makes the diagnosis very difficult and until now there is no definitive diagnosis for the disease (Hardiman *et al.*, 2011; Robberecht and Philips, 2013). The main presentations, according to the location where the disease begins, include: (1) limb-onset ALS, more common, with a combination of UMN and LMN signs in the limbs; (2) bulbar-onset ALS, presenting speech and swallowing difficulties, and with limb features developing later in the course of the disease; (3) the less common primary lateral sclerosis with pure UMN involvement; and (4) progressive muscular atrophy, with pure LMN involvement (Figure I.1.) (Kiernan *et al.*, 2011). Currently, riluzole (Rilutek®) is the only drug approved by the United States Food and Drug Administration (FDA), although it was proven that this drug slows disease progression by extending patient survival only by 2-3 months (Gordon, 2013; McGeer and McGeer, 2005).

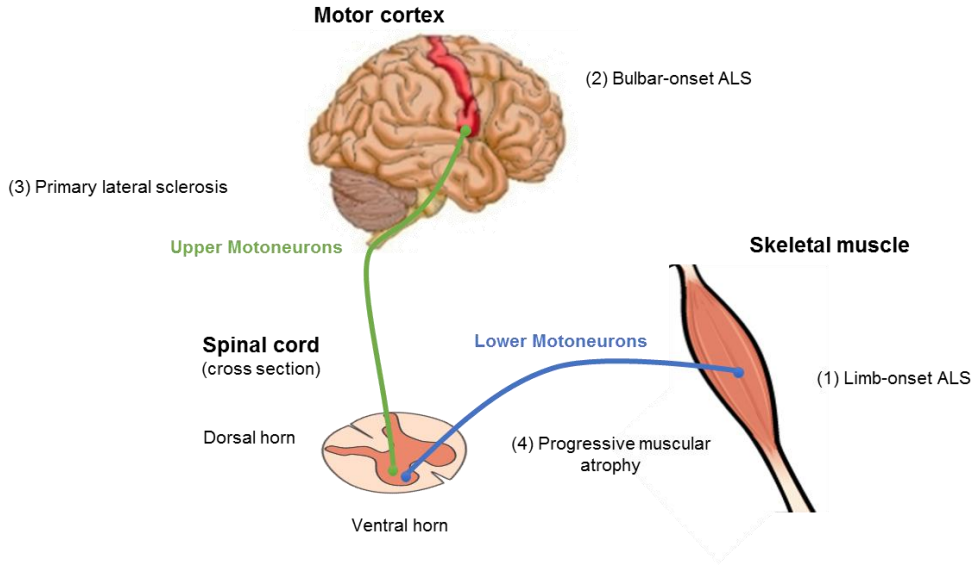


Figure I.1. Amyotrophic lateral sclerosis (ALS) selectively affects lower motor neurons (MN) from the ventral horn of the spinal cord and brainstem and upper MN from the motor cortex. Main presentations of ALS include (1) limb-onset ALS, more common, with a combination of UMN and LMN signs in the limbs; (2) bulbar-onset ALS, presenting speech and swallowing difficulties, and with limb features developing later in the course of the disease; (3) the less common primary lateral sclerosis with pure UMN involvement; and (4) progressive muscular atrophy, with pure LMN involvement. Adapted from Kiernan et al. (2011).

1.1. Genetic features

Approximately 10% of ALS cases have genetic origin (also designated familial ALS, or fALS) (Kurland and Mulder, 1955a, b) following a predominantly autosomal dominant pattern (Chen *et al.*, 2013), while the majority of ALS cases (90%) are non-genetic, also designated as sporadic ALS (sALS), as reviewed in Julien (2001). Currently there are 32 genes reported as being involved in ALS (Abel, 2014) and mutations in some can lead to disease and some might increase susceptibility (Robberecht and Philips, 2013). Both fALS and sALS have common pathological mechanisms with indistinguishable clinical features along disease progression, as well as similar dysfunctional features (Calvo *et al.*, 2012; Li *et al.*, 1988; Phatnani *et al.*, 2013).

Therefore, studies of fALS can be informative either for both sporadic and familial ALS pathology. The copper/zinc superoxide dismutase 1 (SOD1) was the first gene (in 1993) to be identified as being mutated in ALS (Rosen *et al.*, 1993) and accounts for 10-20% of fALS cases (Ticozzi *et al.*, 2011). There are currently 178 different mutations spanning all five exons that have been identified in the SOD1 gene (Abel, 2014). Most of these mutations are missense with a dominant pattern of inheritance (Robberecht and Philips, 2013). Interestingly, the mutation D90A in Scandinavian populations is characterized by a recessive pattern of inheritance and produce a distinctive phenotype (Pasinelli and Brown, 2006). This enzyme is a ubiquitous, predominantly cytosolic protein that catalyzes the dismutation of superoxide anion radicals leading to the formation of hydrogen peroxide (Bendotti *et al.*, 2012;

Shaw, 2005). SOD1 functions as a homodimer, in which each monomer binds one zinc and one copper atom. Copper binding is thought to be important for catalytic activity while zinc binding is believed to be critical for structural stability (Bendotti *et al.*, 2012; Shaw, 2005). The mechanisms through which the expression of mutant SOD1 (mSOD1) results in MN injury and death involves misfolding of mSOD1 and escapes the ubiquitin-proteasome system whilst having an apparently toxic effect on the cell (Robberecht and Philips, 2013). The acquisition of the toxic function might reside in the propensity of mutant enzymes to form insoluble aggregates hindering intracellular functions or in a novel enzymatic activity, prompting a pro-oxidative chemistry in a typical anti-oxidant enzyme, or both (Bendotti and Carri, 2004).

Other genes in which mutations can lead to adult-onset ALS include fused in sarcoma/translated in liposarcoma (FUS/TLS) (Kwiatkowski *et al.*, 2009; Vance *et al.*, 2009) and TAR DNA-binding protein (TDP-43) (Rutherford *et al.*, 2008; Sreedharan *et al.*, 2008). These are multifunctional proteins related to gene expression, RNA splicing, transport and translation. TDP-43 is reported to participate in mRNA transport to synapses followed by local protein synthesis in neurons, thus playing a crucial role in maintenance of the synaptic activity/function (Lattante *et al.*, 2013). They are also responsible for the processing of microRNAs (miRNAs), RNA maturation and splicing (Colombrita *et al.*, 2011). ALS-causing mutations in both genes result in the movement of mutant proteins from their normal location in the nucleus to the cytoplasm, where they form inclusions. In fact, *in vitro* studies show that mutant TDP-43 has an inherent tendency to aggregate as mutant TDP-43, but not wild-type (WT) (Lattante *et al.*, 2013). However, it is still unclear whether the cytoplasmic inclusions are themselves toxic to the cells or act to sequester protein, thereby reducing the pool of functional protein, (Barber and Shaw, 2010; Pratt *et al.*, 2012; Robberecht and Philips, 2013).

Most recently, the discovery of C9ORF72 repeat expansion that causes ALS constituted a breakthrough for ALS development (DeJesus-Hernandez *et al.*, 2011; Renton *et al.*, 2011). C9ORF72 contains a GGGGCC hexanucleotide that is rarely repeated more than two to five times. An abnormal expansion of this sequence is found in about 40% of families with ALS and in about 7% of sALS cases. The normal function of the protein, presumably cytoplasmic, is unknown. The normal transcription of the ORF72 leads to the formation of at least three different RNAs, however, when repetitions are present, incorrect recruitment of splicing factors occurs and leads incorrect splicing that may generate insoluble and potentially toxic dipeptide repeat proteins (Robberecht and Philips, 2013).

Both the genetic and non-genetic forms of ALS are suggested to share similar pathogenic mechanisms such as the presence of abnormal neurofilamentous accumulations in degenerating MNs (Julien, 2001). Additionally, cytoplasmic aggregation of TDP-43 and FUS are common pathological features of both sALS and fALS (Lagier-Tourenne *et al.*, 2010).

In addition, cytoplasmic aggregation of nuclear TDP-43 and FUS in the degenerating neurons and glia of ALS patients were recently reported, as well as the release of the accumulated mutant SOD1 to the extracellular space (Li *et al.*, 2013b; Ogawa and Furukawa, 2014).

1.2. Environmental causes

The fact that sALS represents approximately 90% of ALS cases suggests that specific environmental factors may play a prominent role in the pathogenesis of the disease. It has been proposed that exposure to cyanobacteria, heavy metals (Factor-Litvak *et al.*, 2013), pesticides, intense physical activity, head injury, cigarette smoking, electromagnetic fields and electrical shocks may contribute to ALS onset (Huisman *et al.*, 2013; Kiernan *et al.*, 2011; Redler and Dokholyan, 2012; Sutedja *et al.*, 2009; Trojsi *et al.*, 2013). To add to the relevance of the role of toxic agents, Chamorro indigenous people of Guam have a high incidence rate of ALS among its population as a result of the cumulative consumption of cycad flour and flying foxes (Redler and Dokholyan, 2012; Trojsi *et al.*, 2013). In particular, cycad produces the neurotoxin beta-N-methylamino-L-alanine (BMAA), the seeds are eaten by the flying foxes which then lead to the increase of toxin dosage in its tissues (Redler and Dokholyan, 2012; Trojsi *et al.*, 2013). Additionally, geographically limited populations with dramatically increased ALS incidence such as inhabitants of the Kii peninsula in Japan, the Chamorro people of Guam, Gulf War veterans and Italian football players, certainly lead one to suspect the environment as a potential modifier of disease susceptibility. However, no conclusive statements can be made from these few examples, leaving ALS risk associated with BMAA exposure to be further clarified.

1.3. Pathogenic mechanisms involved in motoneuron susceptibility/vulnerability

ALS is a multifactorial disease in which particular neuronal groups selectively suffer degeneration. Certain motor neuron populations are less vulnerable, specifically sensory and autonomic neurons and cerebellar neurons are almost always either preserved or affected to a very mild and subclinical degree (Barber and Shaw, 2010; Robberecht and Philips, 2013). Also, MNs and frontotemporal neurons are affected to variable degrees (Robberecht and Philips, 2013). The underlying mechanisms that lead to selective death of these MNs are not fully understood, however MNs present some features which may render this cell group vulnerable to such insults. These features include a large cell size with long axonal processes with a very high neurofilament content, which predicts high metabolic demands (Ferraiuolo *et al.*, 2011; Shaw and Eggett, 2000). Hence, MNs rely on optimal mitochondrial function which potentiates the risk of oxidative stress due to the increased generation of reactive oxygen species (ROS) (Ferraiuolo *et al.*, 2011; Shaw and Eggett, 2000). MNs reveal a vulnerability to glutamatergic toxicity along with altered intracellular calcium homeostasis due to low expression of calcium-buffering proteins combined with high expression of α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA)/kainate receptors lacking the GluR2 (Jaiswal and Keller, 2009; Shaw and Eggett, 2000). Moreover, high expression of certain proteins, such as mutant SOD1, that may aggregate (Ogawa and Furukawa, 2014), can also lead to increased vulnerability to toxicity (Ferraiuolo *et al.*, 2011; Shaw and Eggett, 2000). Finally, reduced capacity for heat shock response and chaperone activity represent key features for MN vulnerability in ALS (Ferraiuolo *et al.*, 2011).

Recently, Kaplan and collaborators (2014) have identified that matrix metalloproteinase-9 (MMP-9) is a determinant of the selective neurodegeneration (Kaplan *et al.*, 2014). The authors demonstrated that MMP-9 is expressed selectively by the vulnerable fast MNs whilst triggering ER stress and axonal

die-back. This finding provides a basis for considering MMP-9 as a candidate target for novel therapeutic approaches to ALS. In addition, studies by Vaz et al (2014) have shown increased levels of MMP-9 in response to SOD1 accumulation in NSC-34 mSOD1 model (Vaz *et al.*, 2014).

The major molecular pathways involved in MN degeneration in ALS are depicted in Figure I.2. and the molecular mechanisms that have been described in ALS pathology are further detailed in the next section (1.3.1-1.3.9).

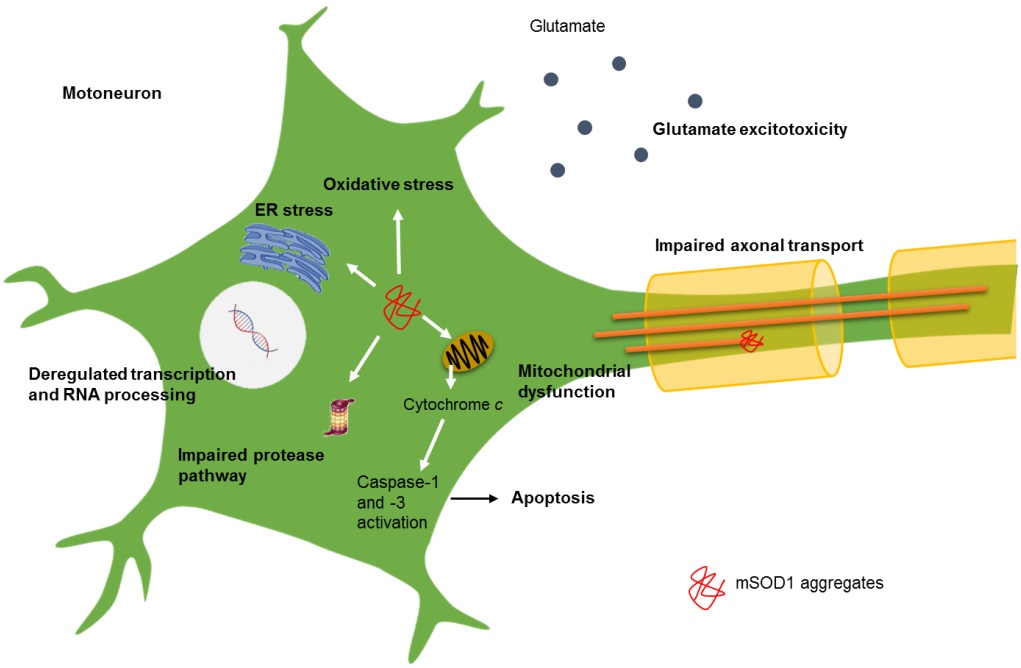


Figure I.2. Molecular pathways involved in motoneuron degeneration in ALS. Mutant superoxide dismutase 1 (mSOD1) accumulation and aggregation promotes the disruption of several mechanisms in motoneurons (MNs). Aggregation of mSOD1 leads to oxidative stress due to increased production of reactive oxygen species (ROS). mSOD1 also elicits endoplasmic reticulum stress (ER) together with impaired proteasome response. Mitochondrial dysfunction is observed ALS due to mSOD1 aggregation inside its intermembrane space leading to dysregulated energy production and cytochrome *c* leading to caspase-1 and -3 activation and the apoptotic signaling pathway. Glutamate accumulation in the extracellular space can induce excitotoxicity with high Ca^{2+} entry which aggravates mitochondrial dysfunction. Impaired axonal transport is also observed with accumulation of neurofilaments which may induce axonal retraction. Although not directly related to mSOD1-mediated motor neuron injury, altered RNA metabolism is observed in different ALS models being a transversal mechanism of toxicity in motor neurons. Adapted from Pasinelli and Brown (2006).

1.3.1. Deregulated transcription and RNA processing

Alterations in RNA processing were first implicated in MN degeneration by Lefebvre and colleagues in 1995 by the identification of mutations in survival MN protein or gemin-1 gene (SMN1) as a cause for SC muscular atrophy (Lefebvre *et al.*, 1995). This protein has a role in the assembly of small nu-

clear ribonucleo-proteins, which are important for pre-mRNA splicing (Ferraiuolo *et al.*, 2011). More recently, microarray analysis of the transcription profiles in a MN-like cell line (NSC-34) has showed alterations in the transcription of genes involved in antioxidant and stress responses, apoptosis, immunity and protein degradation, in late-stage disease (Ferraiuolo *et al.*, 2011; Kirby *et al.*, 2005)

As previously mentioned, TDP-43 and FUS/TLS are nuclear proteins involved in RNA processing events such as splicing and transcriptional regulation. Pathogenic inclusions, possibly originating from the stress granules, may cause mRNA splicing failure which leads to aberrant protein sequences (Colombrita *et al.*, 2011). Also, TDP-43 accumulations may sequester RNAs (and other proteins) (Polymenidou *et al.*, 2012; Robberecht and Philips, 2013). In addition, oxidation of mRNA has been also identified in ALS patients and transgenic (Tg) mice expressing a variety of fALS-linked SOD1 mutations (Barber and Shaw, 2010).

Other genes coding proteins involved in RNA-binding and/or processing protein dysfunctions include TATA-binding protein associated factor 15 (TAF15), Ewing sarcoma breakpoint region 1 (EWSR1), angiogenin (ANG) and senataxin (SETX) (Robberecht and Philips, 2013).

1.3.2. Oxidative stress

Oxidative stress consists in a disruption of the cellular redox signaling that recognizes the occurrence of compartmentalized redox circuits, by which reactive oxygen species (ROS) production is increased (Packer and Cadenas, 2007). MNs are susceptible to ROS damage due to low concentrations of antioxidant enzymes, such as catalase and glutathione peroxidase, high content of easily oxidized substrates and inherent high flux of ROS generated during neurochemical reactions (Carri *et al.*, 2003). Furthermore, in nervous tissue oxidative stress can be detrimental by several interacting mechanisms, including direct damage to crucial molecular species, increase in intracellular free Ca^{2+} and release of excitatory amino acids (Barber *et al.*, 2006). Elevated levels of oxidative stress cause structural damage and changes in redox-sensitive signaling. Also, high levels of free radicals can hinder normal function of several proteins, lipids and DNA, as observed in samples of cerebrospinal fluid (CSF) of ALS patients and *post-mortem* tissue from both sALS and fALS cases (Barber and Shaw, 2010; Ferraiuolo *et al.*, 2011). Chang and colleagues (2008) have also showed that mRNA oxidation is present in both ALS patients and mSOD1 mice (Chang *et al.*, 2008).

The identification of SOD1 mutations as contributors to ALS has revealed a clear association between oxidative stress and disease onset (Rosen *et al.*, 1993). Indeed, mSOD1 plays an important role due to its loss of function in the removal of ROS, however there are studies indicating a gain of toxic function that is responsible for the acquisition of the pathological phenotype, rather than an impairment of its anti-oxidant activity (Carri *et al.*, 2003). In addition, not only in neurons but also in microglia, mSOD1 increases superoxide production by NADPH oxidases (Nox), transmembrane proteins capable of reducing oxygen to superoxide (Marden *et al.*, 2007). The increase in its activity can be pathogenic and was found to be up-regulated in both sALS patients and SOD1G93A mutant mouse model (Marden *et al.*, 2007). Additionally, microarrays studies have shown down-regulation of genes

involved in antioxidant response in MNs expressing mSOD1 (Kirby *et al.*, 2005). Nrf2, a transcription factor involved in regulating antioxidant response, was reported to have decreased mRNA and protein expression in ALS patients (Sarlette *et al.*, 2008), which suggests alterations in signaling cascades that may hinder the cell's ability to fight oxidative stress.

Multiple studies have implicated oxidative stress with other pathological processes that occur in ALS and possibly having a role in their exacerbation. These processes include excitotoxicity, mitochondrial damage, protein aggregation and microglial and astrocyte signaling (Barber and Shaw, 2010; Ferraiuolo *et al.*, 2011).

1.3.3. Excitotoxicity

Glutamate is the main excitatory neurotransmitter in the human CNS, acting on both ionotropic and metabotropic receptors, the main ionotropic receptor classes being NMDA and α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA)/kainate (Dingledine *et al.*, 1999; Heath and Shaw, 2002). Excitotoxicity, which has been reported as an important cause of neuronal death in ALS, is neuronal degeneration induced by overstimulation of glutamate receptors and this mechanism can occur when extracellular glutamate concentration increases or when the postsynaptic neuron becomes vulnerable to normal glutamate levels (Barber and Shaw, 2010; Van Damme *et al.*, 2005). In particular, MNs are susceptible to toxicity through activation of AMPA receptors (Shaw, 2005). Additionally, lethal injury to neurons, astrocytes or microglia may lead to the release of intracellular glutamate which can result in excitotoxicity to the surrounding neurons (Van Damme *et al.*, 2005).

The excitatory amino acid transporters (EAATs) play a crucial role in the regulation of extracellular glutamate concentration, by stimulating the reuptake of glutamate to maintain a physiological concentration in the synaptic cleft. In particular, EAAT2 is the major glutamate transporter and is widely distributed in the human CNS (Musaro, 2010). It has been reported that EAAT2 is diminished in sporadic and familial ALS patients, both in the motor cortex and SC (Rothstein *et al.*, 1992; Van Damme *et al.*, 2005). This will lead to an excessive glutamate content in the synaptic cleft, which is toxic to neurons. This excessive glutamate will result in glutamate-triggered Ca^{2+} entry, which may come from a subset of AMPA/kainate lacking GluR2 AMPA subunit allowing them to become Ca^{2+}-permeable (Rao and Weiss, 2004). This permeability of Ca^{2+} allows its uptake into MN mitochondria increasing ROS generation, therefore contributing to the oxidative stress and excitotoxicity that ultimately leads to MN degeneration (Rao and Weiss, 2004; Van Damme *et al.*, 2007).

1.3.4. Protein aggregation

Abnormal protein folding and aggregation is a common pathological feature of many neurodegenerative diseases, including ALS. Their role in disease pathogenesis, however it is not well defined, as they could represent harmless bystanders, or they could be beneficial to the cell by sequestering potentially toxic intracellular proteins (Barber and Shaw, 2010; Shaw, 2005). A variety of protein aggregates has been described in ALS, the most common of which is the ubiquitinated inclusion. Many cy-

toplasmic aggregates from different proteins were found in fALS cases, such as SOD1, TDP-43 and FUS.

Mutant SOD1 (mSOD1) aggregates are found in fALS Tg mice and in cell culture models (Gurney *et al.*, 1994; Vaz *et al.*, 2014) which will contribute to aberrant protein folding and degradation in disease pathogenesis (Wood *et al.*, 2003). A set of hypotheses for the pathogenesis of ALS is the conformational instability of mSOD1 that induces the formation of harmful aggregates (Pasinelli and Brown, 2006). Several hypotheses have been put forward to explain how mSOD1 aggregates could produce cellular toxicity: (i) there might be by sequestration of other proteins required for normal MN function; (ii) by repeatedly misfolding, the SOD1 aggregates may reduce the availability of chaperone proteins required for the folding and function of other essential intracellular proteins; (iii) the SOD1 mutant protein aggregates may reduce the proteasome activity needed for normal protein turnover; (iv) there could be an inhibition of the function of specific organelles (e.g. mitochondria) by aggregation on or within these organelles (Shaw, 2005).

TDP-43 is present in neuronal inclusions in most ALS patients (Rutherford *et al.*, 2008; Sreedharan *et al.*, 2008). Under normal conditions, TDP-43 is predominantly localized in the nucleus, and loss of nuclear TDP-43 staining is seen in most cells containing TDP-43-positive cytoplasmic inclusions (Neumann *et al.*, 2006). TDP-43 inclusions are not restricted to MNs, and it seems that cytoplasmic redistribution of TDP-43 is an early pathogenic event in ALS. In 2008, mutations in TARDBP, the gene encoding TDP-43, were discovered in several fALS pedigrees (Sreedharan *et al.*, 2008). Similarly, cytoplasmic inclusions containing mutant fused in sarcoma (FUS) protein have been observed in some patients with FUS-related fALS (Hewitt *et al.*, 2010).

1.3.5. Mitochondrial dysfunction

Mitochondria plays an essential role in intracellular energy production, calcium homeostasis and apoptosis control. This organelle is especially important due to MNs inherent high metabolic demand that requires optimal mitochondrial function. These functions, along with other properties of the mitochondria are potentially relevant in the pathogenesis of ALS and in other neurodegenerative diseases. Indeed, mitochondrial degeneration occurs early in disease onset as a result of mSOD1-caused dysfunction and structural damage in both cultured neuronal cells and Tg mice through unknown mechanisms (Higgins *et al.*, 2003). Evidence for mitochondrial dysfunction include: (i) alterations in the morphology of mitochondria in hepatocytes, muscle, and MNs; (ii) increased mitochondrial volume and Ca^{2+} levels within motor axon terminals in muscle biopsies from sALS cases (Siklos *et al.*, 1996); (iii) reduced complex IV activity in spinal motor neurons in sALS (Borthwick *et al.*, 1999); (iv) high frequency of mitochondrial DNA mutations in motor cortex tissue in sALS (Dhaliwal and Grewal, 2000); (v) multiple mutations and decreased mitochondrial DNA in muscle and SC in in sALS (Wiedemann *et al.*, 2002); (vi) ALS-like phenotype in one patient with a deletion in the cytochrome oxidase *c* subunit gene (Comi *et al.*, 1998). In addition, it was proposed that mSOD1 is imported and accumulates inside the mitochondria, which causes direct damage of the organelle and activation of cell death through the activation of caspases-1 and -3 (Pasinelli *et al.*, 2004; Pasinelli *et al.*, 2000). Pasinelli and colleagues

(2004) have also demonstrated that mSOD1 aggregates interact with the anti-apoptotic protein Bcl-2 both *in vitro* and *in vivo* mouse models and human SCs (Pasinelli *et al.*, 2004). This binding can consequently lead to the entrapment of Bcl-2 and rendered nonfunctional as the mSDO1 protein aggregates, thus facilitating triggering of apoptosis (Pasinelli *et al.*, 2004).

Mitochondrial dysfunction has been offered as a converging point for the multiple pathways that cause apoptosis activation and neuronal loss in ALS. The role of disturbance in the mitochondrial function and transport in the pathogenesis of ALS has been revealed using mSOD1 mice as a familial model of ALS (Bendotti and Carri, 2004). The cause of mitochondrial malfunction may be linked to the accumulation of aggregated misfolded proteins such as SOD1 and TDP-43. These aggregates form in the ubiquitin-containing inclusions in cell bodies and axons of MNs. Notably, SOD1 is basically a cytosolic protein but it partly deposits in the intermembrane spaces of mitochondria in mSOD1 mice (Higgins *et al.*, 2003), a fact that can explain its harmful effect on the mitochondrial function.

1.3.6. Endoplasmic reticulum stress

The endoplasmic reticulum (ER) functions as the site of translation, folding and transport of membrane proteins and secreted proteins, along with Ca^{2+} storage and has a role in lipid synthesis. The presence of misfolded proteins or protein aggregates triggers a defense mechanism called unfolded protein degradation (UPR) through ER-resident chaperones (Matus *et al.*, 2013). The presence of these non-functional proteins can lead to translation and ER-association protein degradation suppression, however, if not stopped, these mechanisms can lead to the activation of apoptotic signaling (Ferraiuolo *et al.*, 2011). mSOD1 aggregates were found to accumulate in ER membranes binding to the ER-luminal polypeptide chain binding protein (BiP) in affected tissues in a progressively negative manner (Ilieva *et al.*, 2009; Kikuchi *et al.*, 2006), a chaperone that regulates the activation of ER transducers such as inositol-requiring kinase 1 (IRE1), PKR-like endoplasmic reticulum kinase (PERK) and activating transcription factor 6 (ATF6).

Furthermore, ER has been implicated in the pathology of ALS at early stages of the disease where a marker of protein disulphide isomerase (PDI), a chaperone and a marker of the UPR, was found to co-localize with mSOD1 inclusions in Tg mice, and also in biosamples from patients with sALS. Markers of ER stress were also found to be upregulated in the CSF and SC of sALS patients (Atkin *et al.*, 2006; Atkin *et al.*, 2008).

1.3.7. Deregulated endosomal trafficking

Endocytosis is the process by which cells perform the uptake of extracellular molecules into their cytoplasm through vesicles or vacuoles that will fuse with early endosomes (Cooper and Hausman, 2004). The cargo is conducted to its destination via the endosomal network. This system has been implicated in several subtypes of ALS. Specifically, in the subtype ALS2/ALSIN, a rare autosomal recessive with a juvenile onset, alsin is a guanine nucleotide exchange factor (GEF) acting to promote neurite outgrowth (Hadano *et al.*, 2001). In cultured MNs, alsin protects MNs from mSOD1-mediated

toxicity which infers to a possible neuroprotective role (Kanekura *et al.*, 2004). Mutations in alsin cause disruption of the endolysomal system, leading to an aggregation of immature vesicles and misfolded proteins in neurons (Chen *et al.*, 2013; Ferraiuolo *et al.*, 2011). Other ALS-associated mutations suggest dysregulated endosomal trafficking as pathophysiological mechanism in ALS. These include mutations in the vesicle associated membrane protein-associated protein B (VAPB), optineurin, a component of the endosomal sorting complex required for transport (ESCRTIII), charged multivesicular protein 2B (CHMP2B), VCP which forms a complex with the endocytotic protein clathrin, and Sac domain-containing protein gene family (FIG4) a phosphoinositide 5-phosphatase ($PI(3,5)P_2$ which is involved in helping the retrograde trafficking of endosomal vesicles to the trans-Golgi network (Chen *et al.*, 2013; Ferraiuolo *et al.*, 2011). Recently, Farg and colleagues (2014) have reported C9ORF72 has being implicated in the regulation of endosomal trafficking. This study reveals that the C9ORF72 co-localizes with Rab proteins in neuronal cell lines and human SC, consistent with the suspicion of a RabGEF activity for C9ORF72 (Farg *et al.*, 2014).

1.3.8. Impaired axonal transport

MNs are characterized by having long axonal processes necessary for conduction of electrical impulses and transport of essential constituents (e.g. RNA, organelles and proteins), to and from the synaptic structures to the cell body. This transport involves two main molecular motors: microtubule-dependent kinesin, which mediates transport from the soma to the neuromuscular junction (NMJ) (anterograde transport) and dynein towards the cell body (retrograde transport). This system is set upon a neuronal intermediate filament network, composed mainly by neurofilaments (NFs), that is responsible for cell shape and axonal caliber maintenance. Dysfunction in the axonal transport is characteristic of the pathology of ALS as the incorrect assembly of neurofilaments due to over- or under-expression, mutation or deficient transport of individual subunits causes them to accumulate, both in human and mouse models (Redler and Dokholyan, 2012). mSOD1, contrary to wild-type SOD1, is reported to bind to the 3' un-translated region of neurofilament-light subunits (NF-L) mRNA increasing mRNA instability and degradation (Ge *et al.*, 2005). Axonal transport dysfunction may also be a consequence of other pathogenic mechanisms involved in ALS, such as mitochondrial impairment which can lead to insufficient energy required for the energy-dependent transport mechanism. In mSOD1 mice, tumor necrosis factor (TNF) is elevated which causes disruption of kinesin function via a mechanism involving p38 mitogen-activated protein kinase (p38 MAPK), a stress-activated protein that can phosphorylate NFG/NFM (Ackerley *et al.*, 2004). Hyperphosphorylation of neurofilaments contributes to defective transport by causing their detachment from motor complexes and promoting aberrant self-association (Redler and Dokholyan, 2012). Also, pro-inflammatory cytokines lead to activation of p38, and p38 activity is shown to be elevated in SCs of mSOD1 mice (Ackerley *et al.*, 2004; Hu *et al.*, 2003). Recently, Alami and collaborators (2014), implicate a role for TDP-43 in the cytoplasm of MNs cells supporting anterograde axonal transport of target mRNAs from the cell body to axonal compartments. Therefore, mutations in TDP-43 induce partial loss of function and can cause deficient axonal transport of mRNA targeted by TDP-43 (Alami *et al.*, 2014). Interestingly, ALS has been described as a "dying-back axonopathy" due to the progression of defects from distal to proximal with probable con-

tribution from abnormalities in anterograde transport and mitochondrial dysfunction (Ferraiuolo *et al.*, 2011; Fischer *et al.*, 2004).

1.3.9. Cell death

There are several mechanisms of cell death that may be implicated in MN degeneration in ALS and apoptosis seems to be the most common mechanism by which these cells die (Guegan and Przedborski, 2003). As reviewed by Ghavami *et al.* (2014), apoptosis is a mechanism of programmed cell death in which biochemical events lead to characteristic cellular changes and ingestion by phagocytes. The main molecular components of the apoptotic mechanism include the Bcl-2 family of oncoproteins, caspase family of proteolytic enzymes and the apoptosis inhibitor family of proteins (Guegan and Przedborski, 2003). Caspase activation can be triggered by three main pathways: release of pro-apoptotic factors (e.g. cytochrome *c* released from mitochondria); activation of cell surface ligand receptor systems of the tumor necrosis factor family including Fas-Fas ligand, with subsequent recruitment of cytosolic adaptor proteins; and stress to the endoplasmic reticulum with activation of caspase-12 (Shaw, 2005).

Evidence that MNs die in ALS by programmed cell death include evidence of structural morphology of degenerating MNs compatible with apoptosis (Guegan and Przedborski, 2003). Studies in *in vitro* cultures, rodent models and *post-mortem* human spinal cord demonstrate that mSOD1 aggregates can bind to Bcl-2 proteins in a manner that the anti-apoptotic function of the protein in cancelled, thus promoting an imbalance of the Bcl-2 family of proteins towards apoptosis (Pasinelli *et al.*, 2004). In addition, increased activation of the initiator caspase-9 was reported, as well as an increase in the activity of caspases-1 and -3 in MNs and astrocytes in ALS mice (Li *et al.*, 2000; Pasinelli *et al.*, 2000; Vaz *et al.*, 2014; Yoshihara *et al.*, 2002).

While activation of apoptotic pathways have long been implicated in the demise of MNs in ALS, it is becoming increasingly clearer that apoptosis is not the only cellular mechanism that regulates cell death in this pathology. Recently, a relatively new form of necrosis has been characterized as necroptosis (Wu *et al.*, 2012), which is biochemically regulated and requires the involvement of receptor interaction protein kinase 1 and 3 (RIP1 and RIP3). Interestingly, Re et al. (2014) showed that in the absence of caspases activation, MN demise triggered by sALS as well as fALS astrocytes might involve necroptosis, a specialized form of necrotic cell death that involves the activation of the kinase domain of RIP1 and subsequent recruitment of a mixed lineage kinase domain-like protein (MLKL).

Another cell death mechanism that deserves our attention is autophagy. Autophagy plays a pivotal role in keeping the cell homeostasis by digestion of dysfunctional organelles and proteins (Chen *et al.*, 2012). High levels of aggregated proteins can cause UPS dysfunction and subsequently, compensatory activation of autophagy (Korolchuk *et al.*, 2010). In the recent years, defective autophagy pathway or alterations in autophagy-related genes has been shown in various human pathologies such as neurodegenerative diseases (Ghavami *et al.*, 2014). For instance, *post-mortem* studies, as well as animal models, have demonstrated that the number of autophagosomes is increased in ALS SCs (Kim *et al.*,

2013). In addition, Pizzasegola and collaborators (2009) have reported that treatment with lithium, which activates autophagy, exacerbates disease progression in ALS (Pizzasegola *et al.*, 2009). Another study with rapamycin, which is also an inducer of autophagy, aggravates disease progression in ALS mice (Zhang *et al.*, 2011). However, studies using lithium and rapamycin have shown the opposite effects thus leaving the precise role of autophagy in ALS unsettled (Fornai *et al.*, 2008; Wang *et al.*, 2012a)

2. ALS is a non-cell autonomous disease: the importance of glial cells

Studies of mutant SOD1 mice have shown that cell death in ALS is non-cell autonomous, as astrocytes and microglial cells that surround motor neurons may contribute to disease onset and/or progression (Figure I.3) (Clement *et al.*, 2003; Evans *et al.*, 2013). Indeed, it is described that glial cells, namely astrocytes and microglia, become increasingly activated as the disease progresses in both animal models and patients (Gurney *et al.*, 1994). Expression of the mSOD1 protein in microglia is sufficient to cause cellular toxicity to the neurons, resulting in neuronal degeneration. This has been shown using both cell culture and *in vivo* experiments, and illustrates the importance of both astrocytes and microglia in ALS (Evans *et al.*, 2013). In addition, Clement and colleagues (2003), by using chimeric mice, demonstrated that the toxicity to MNs requires non-neuronal cells expressing mSOD1, as shown by the fact that mSOD1 MNs surrounded by healthy microglia and astrocytes presented delayed degeneration and prolonged survival, whereas the reverse (healthy MNs surrounded by mSOD1-overexpressing microglia) shows the development of the pathology (Clement *et al.*, 2003). In addition, several studies with different approaches have demonstrated that mutant SOD1 expression in MNs determines the initial timing of disease onset and early progression in some cases, but does not have a significant contribution to later disease progression (Ilieva *et al.*, 2009). Consequently, astrocytes and microglia must have decisive implications in disease progression after its onset. It is also important to mention that, mutant SOD1 gene excision from microglia and selective reduction in astrocytes significantly slowed disease progression (Boillee *et al.*, 2006a). In addition to astrocytes and microglia, oligodendrocytes may also have a role in ALS. In fact, it was recently reported the loss of the monocarboxylate transporter 1 (MCT1) in both ALS patients and mSOD1 transgenic mice (Lee *et al.*, 2012b) together with the identification of an apoptotic oligodendrocyte phenotype in the ventral grey matter of the SC from mSOD1 transgenic mice prior to the actual MN loss became evident (Valori *et al.*, 2014).

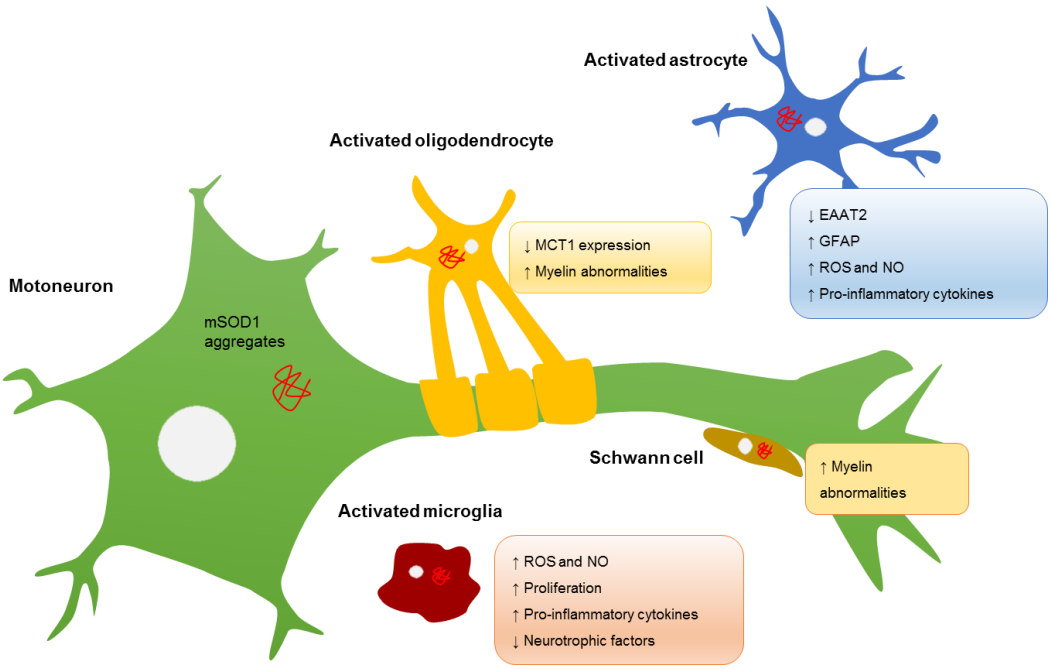


Figure I.3. ALS is a non-autonomous cell disease. Motoneuron (MN) degeneration is dependent on glial cell, microglia has increased proliferative and phagocytic abilities whilst having increased production reactive oxygen species (ROS) and nitric oxide (NO) as well as inflammatory molecules like tumor necrosis factor α (TNF-α) and interleukin 1β (IL-1β). Astrocytes have been shown to have downregulation of excitatory aminoacid transporter 2 (EAAT2) high expression of glial fibrillary acidic protein (GFAP) from activation. Furthermore, astrocytes have increased generation of pro-inflammatory cytokines along with ROS and NO. Oligodendrocytes have been reported having loss of monocarboxylate transporter 1 (MCT1) thus contributing to ALS progression. Schwann cells involvement in ALS is controversial, however some studies show increased myelin abnormalities in presymptomatic mSOD1 mice. Adapted from Ilieva (2009).

2.1. Oligodendrocytes and Schwann cells

Oligodendrocytes are essential cells for the normal function of the CNS. They play a pivotal role in axonal maintenance by providing myelin sheaths for rapid signal conduction (Valori *et al.*, 2014). As previously mentioned, ALS is characterized by axonal loss and neuronal atrophy which may be associated with an important role of oligodendrocytes due to its myelinating function (Lasiene and Yamanaka, 2011). Indeed, recent studies report abnormalities in these cells in *post-mortem* ALS patient's SC and mouse models. Studies in ALS SOD1G93A mice reveal an increase of proliferation rate of oligodendrocyte precursor (NG2) cells (Kang *et al.*, 2013), a class of progenitor cells that have the capacity to differentiate into oligodendrocytes. Oligodendrocytes normally express MCT1 which provides neurons with lactate, and studies in *in vitro* using neuroblastoma 2a cells as well as mSOD1 *in vivo* models have shown that the expression of this transporter is reduced, and this loss is toxic to MNs (Philips *et al.*, 2013). In a study by Phillips and collaborators (2013) they investigated oligodendrocytes and NG2 cells and found that oligodendrocyte lineage cells may be targets of disease in both human patients with ALS and mSOD1 mice. In SOD1G93A mice, oligodendrocytes showed signs of degeneration and death before MN loss together with upregulation of caspase-3 (Philips *et al.*, 2013).

Another evidence for the potential involvement of oligodendrocytes in ALS is the discovery of a disruption of gap junctions, focusing on the study of connexins (Cx) 32 and 47 expressed by these cells. Cui et al. (2014) demonstrated that the levels of oligodendrocytic Cx47 and Cx32 are markedly diminished in the anterior horns of SCs from SOD1G93A mice, suggesting that disruption of the glial syncytium due to alteration of connexin expression might contribute to the progression of MN disease (Cui *et al.*, 2014).

Another evidence for the potential involvement of oligodendrocytes in ALS is the aberrant location of TDP-43 to the cytoplasm rather than the nucleus, which is a morphological hallmark of patients with sALS (as previously addressed in 1.3.4), is not limited to MNs, but also occurs in oligodendrocytes (Seilhean *et al.*, 2009).

In the peripheral nervous system the role of myelination is performed by Schwann cells (Morrison *et al.*, 2013). The involvement of Schwann cells in ALS pathology is still poorly understood. However, studies using G37R mice with mSOD1 knockdown in Schwann cells show aggravated disease progression (Lobsiger *et al.*, 2009). On the other hand, Wang et. al have shown that the selective knockdown of mSOD1 in Schwann cells mitigates disease progression using G85R mice (Wang *et al.*, 2012b). These results suggest that different mutations can have different impacts on cellular toxicity and disease progression.

2.2. Astrocytes

Astrocytes comprise about 85% of all glial cells, and contribute to the maintenance of vascular, ionic, redox and metabolic homeostasis in the brain (Allen and Barres, 2005). They fulfill a structural role along with maintenance of the homeostasis of the extracellular environment, influencing neuronal excitability by regulating the levels of ions and neurotransmitters, more specifically the uptake of glutamate from the synaptic cleft and nourish motoneurons through the release of neurotrophic factors (Evans *et al.*, 2013; Philips and Robberecht, 2011). It has being proposed that a hallmark of the pathophysiology of ALS is the reactive astroglyosis as a response to brain injury, which is accompanied by a characteristic up-regulation of the intermediate filament glial fibrillary acidic protein (GFAP), an astrocytic marker, as well as the marker aldehyde dehydrogenase 1 family, member L1 (ALDH1L1) (Evans *et al.*, 2013; Philips and Robberecht, 2011). More recently, Diaz-Amarilla et al. (2011) have identified a population of phenotypically aberrant astrocytes in which there is a decrease of the GLT-1/EAAT2 transporter, thus impairing glutamate clearance, as previously addressed in point 1.3.3. of this section (Yamanaka *et al.*, 2008), as well as increased expression of S100B and connexin 43 (Cx43). In this model, aberrant astrocytes from transgenic SOD1G93A rats produced soluble factors that were bound to be toxic to co-cultured MNs. One of the pathways by which astrocytes may induce MN toxicity is through the up-regulation of NADPH oxidase 2 (NOX2) to produce superoxide, since the NOX2 inhibitor, apocynin, was demonstrated to prevent motor neuron loss caused by mSOD1 astrocytes (Zhao *et al.*, 2013a). In addition, models of ALS using mSOD1G93A rats showed that astrocytes induce up-regulation of the glutamate receptor subunit GluR2 in neighboring (co-cultured) MNs. The GluR2 subunit produces receptors that are impermeable to Ca^{2+}, thereby protecting the MNs from ex-

citotoxic damage (Ilieva *et al.*, 2009). Indeed, it was shown that mSOD1 expression in astrocytes abrogated their GluR2-regulating capacity, rendering MNs vulnerable to excitotoxicity (Van Damme *et al.*, 2007). Astrocytes are also able to modulate neuronal integrity through the release of neurotrophic factors, of which glial-cell derived neurotrophic factor (GDNF), brain-derived neurotrophic factor (BDNF), insulin-like growth factor 1 (IGF1) and vascular endothelial growth factor (VEGF) are well studied examples (Ekestern, 2004). Interestingly, exogenous administration of these factors increases survival of MNs in the mSOD1 model which appear to be related to a disruption of the release of neurotrophic factors (Philips and Robberecht, 2011). One other molecule that deserves our attention is the nuclear factor erythroid-2-related transcription factor 2 (Nrf2). Activation of Nrf2 in astrocytes is thought to coordinate the up-regulation of antioxidant defenses, thereby conferring protection to neighboring neurons (Vargas *et al.*, 2008). Indeed, astrocyte-selective up-regulation of Nrf2 produced significant delay in disease onset in mouse models overexpressing dismutase-active and –inactive SOD1 mutants (Lasiene and Yamanaka, 2011; Vargas *et al.*, 2008). On the other hand, astrocytes release the glutathione precursor (CysGly), which is used by MNs to synthesize the antioxidant glutathione to provide significant protection against oxidative stress (Zhao *et al.*, 2013a). Direct evidence of altered glutathione or glutathione utilizing enzymes levels in sporadic and familial ALS remain controversial as some studies in *post-mortem* brain samples from ALS patients (Przedborski *et al.*, 1996a), and in blood samples from ALS patients (Przedborski *et al.*, 1996b) observed reduced glutathione peroxidase activity while others do not detect changes in activity (Fujita *et al.*, 1996). Astrocytes can also help suppress cytotoxic microglial activation by releasing Transforming growth factor-β (TGF-β), whilst M1 microglial release of IL-1β can promote astrocytic activation (Liu *et al.*, 2011). Finally, the role of astrocytes in the maintenance of CNS homeostasis may be compromised due to Cx expression dysregulation, namely, Cxs 43 and 30, which were found to be overexpressed in both SOD1G93A mice model and cell cultures obtained from adult spinal cord of symptomatic SOD1G93A rats (Cui *et al.*, 2014; Diaz-Amarilla *et al.*, 2011).

2.3. Microglia

Microglia are cells derived from the hematopoietic cell lineage and are considered the primary immune cells of the CNS. Microglia are the resident macrophages of the CNS, and have two distinct roles: first they have a supporting role to neurons in conjunction with astrocytes and they have an immunological role, which may have cytotoxic properties. Under physiological conditions microglia are in a state called “resting microglia” in which surveys the surrounding environment through its processes in order to sense insults such as abnormal proteins produced in neurodegenerative diseases (Graeber and Streit, 2010).

Microgliosis has been proposed as a main feature of ALS pathology, as well as neuroinflammation, where microglia certainly plays a key role (Moisse and Strong, 2006). In fact, there is a marked activation microglia in the brain of living ALS patients detectable by positron emission tomography (PET) (Turner *et al.*, 2004). The role of microglia in neuroinflammation in ALS will be further detailed in the following chapter.

3. Neuroinflammation in ALS: the role of microglia

Neuroinflammation is a hallmark of ALS pathology and is characterized by gliosis and the accumulation of an increased number of activated microglia and astrocytes, in both ALS patients and mouse models (Evans *et al.*, 2013; Turner *et al.*, 2004). Markers for such glial cells have been found to be altered in ALS patients, namely, the increased expression of astrocytic marker glial fibrillary acidic protein (GFAP) (Kawamata *et al.*, 1992), together with higher expression of leukocyte common antigen (LCA), lymphocyte function associated molecule-1 (LFA-1) and complement receptors CR3 (CD11b) and CR4 (CD11c) suggesting increased microglial immune activity (Phani *et al.*, 2012). These last markers suggest an activation of microglia and macrophages in ALS affected regions such as the motor cortex, brainstem and corticospinal tract (Kawamata *et al.*, 1992; Papadimitriou *et al.*, 2010). Other studies reveal immunological differences between the blood of ALS patients and healthy controls such as increased levels of cluster of differentiation (CD4+) cells and reduced CD8+ T-lymphocytes (Phani *et al.*, 2012). In addition, there is a marked decrease in expression of CD4+CD25+ T-regulatory cells and CD14+ monocytes (Mantovani *et al.*, 2009). Analysis of CSF of ALS patients show a dysregulation in pro- and anti-inflammatory cytokines such as IL-1β, TNF-α, Interferon- γ (INF-γ), IL-6 and IL-10, along with altered levels of growth factors like VEGF (Evans *et al.*, 2013; Ghavami *et al.*, 2014; McGeer and McGeer, 2002).

As previously mentioned, the pathogenesis of ALS is non-cell autonomous disease, which means that the MN degeneration requires the participation of non-neuronal cells. This concept is particularly important for the role of the immune system in the pathological progression of the disease. Microglia play a pivotal role in neuroinflammation as they act as the first line of immune defense in the CNS and respond to “danger signals” released from the damaged tissue.

3.1. Microglia phenotypes: neuroprotection vs. neurotoxicity

In physiological conditions, microglia in the CNS is most of the time in a state called “resting microglia” (Li *et al.*, 2013a) characterized by its thin, highly branched processes emerging from the cell body which survey the environment for potential threats (Luo and Chen, 2012). In response to insults such as microbial infection, serum microhemorrage of blood vessels, immunoglobulin-antigen complexes and abnormal protein produced in neurodegenerative diseases (e.g. mSOD1), microglia contributes to the neuroinflammatory process acquiring rapid morphological and functional activation which includes phagocytosis, antigen presentation, as well as the production and secretion of ROS, cytokines and growth factors (Evans *et al.*, 2013; Lasiene and Yamanaka, 2011). These insults can be exogenous or endogenous, the latter including cytokines and chemokines, including IFN-γ, TNF-α, IL-1β and IL-6 (Dewil *et al.*, 2007). Cumulative studies using diverse animal models of CNS injury have demonstrated that microglia have very distinct and different phenotypic states, as schematically represented by Figure I.4., and, in line with other tissue macrophage populations, they may exert either a neurotoxic or neuroprotective response depending on the physiological conditions they encounter. These states are classified as classically activated microglia (M1), which are cytotoxic due to the secretion of ROS, pro-inflammatory cytokines, and increased levels of NOX2 expression (Liao *et al.*, 2012), and this pheno-

type is influenced by T helper cell type 1 (Th1) that release GM-CSF and INF- γ, thus triggering M1 proliferation (Brites and Vaz, 2014). In contrast, alternatively activated microglia (M2) can be promoted by IL-4 and IL-13 (Olah *et al.*, 2011). This phenotype promotes neuroprotection by blocking the pro-inflammatory response and producing high levels of anti-inflammatory cytokines and neurotrophic factors (Evans *et al.*, 2013; Henkel *et al.*, 2009; Liao *et al.*, 2012). This M2 phenotype can be divided into three sub-phenotypes: the M2a or alternate activation repair/regeneration/remodeling phenotype, the M2b immunoregulatory and the M2c acquired-deactivating as reviewed in Brites (2014). Markers for M2a include arginase (Arg1) and mannose receptor and IGF-1 (Varnum and Ikezu, 2012). M2b is stimulated by immune complexes, TLR agonists and IL-1R ligands and useful markers are interleukin-1 receptor antagonist (IL-1Ra) and suppressor of cytokine signaling 3 (SOCS3) (Chhor *et al.*, 2013). Characterization of M2c stimulated by IL-10, transforming growth factor-β (TGF-β) and glucocorticoids is obtained through the increased levels of anti-inflammatory cytokines (IL-10,TGF-β), low levels of pro-inflammatory cytokines and enhanced IL-4Rα, Arg1, (SOCS3), and CD206 (Varnum and Ikezu, 2012). M1 and M2c phenotypes are proposed as being those capable of expressing and releasing MFG-E8 for phagocytic purposes (Brites and Vaz, 2014).

Recently, Liao and collaborators (2012) have demonstrated that there are both states of activated microglia over the course of disease and indicate that early in the disease process, mSOD1 microglia expressed more M2 related mRNAs, such as Ym1 and CD206, when compared with near end-stage mSOD1 microglia as the near end-stage microglia expressed more M1 markers, namely NOX2. A transition between states of activation suggests that earlier in disease, M2 microglia mainly has a role of neuroprotection and then undergo into a phenotypically transformation to a more neurotoxic M1 microglia. In addition, it was reported in the same study that, at early stages, the neuroprotection from M2 mSOD1 microglia is enhanced by astroglia leading to MN protection, while during disease progression it seems to be a transformation of mSOD1 microglia from a neuroprotective M2 phenotype to a cytotoxic M1 phenotype (Liao *et al.*, 2012).

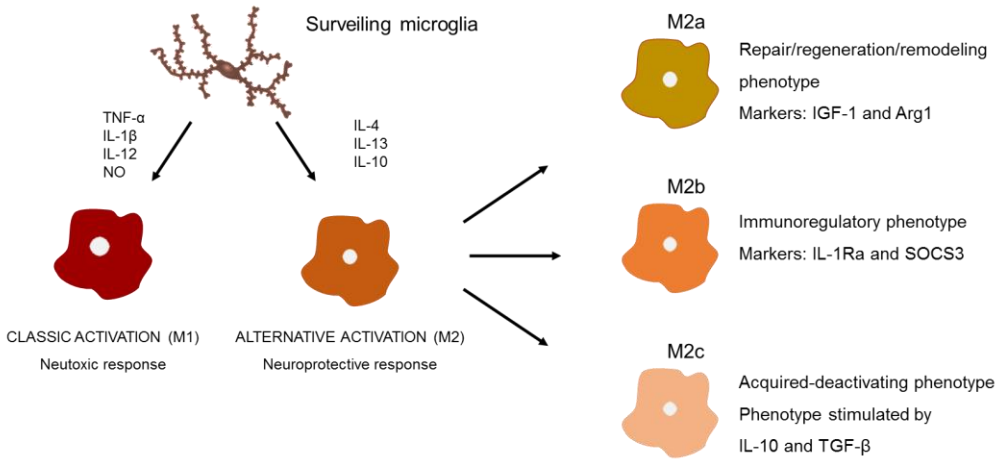


Figure I.4. Microglia phenotype diversity. Surveiling microglia changes phenotype in response to various stimuli. Classical activation phenotype (M1), characterized as having a neurotoxic response, is induced by Tumor Necrosis Factor α (TNF-α), Interleukin (IL) -1β and -12 and Nitric Oxide (NO). Alternative activation (M2), charac-

terized as having a neuroprotective response, is induced by IL-4, IL-13 and IL-10. M2 phenotype can be further divided into three sub-phenotypes: M2a repair/regeneration/remodeling phenotype, expressing Insulin-like growth factor 1 (IGF-1) and arginase (Arg1) markers; M2b immunoregulatory phenotype expressing interleukin-1 receptor antagonist (IL-1Ra) and suppressor of cytokine signaling 3 (SOCS3); M2c acquired-deactivating phenotype which is stimulated by IL-10 and Transforming growth factor-β (TGF- β).

3.2. Neuron-microglia dialogue in ALS

Communication between neurons and glial cells is essential for the maintenance of the CNS. Neurons are highly active and vulnerable cells that require microglial support. Microglia can release factors such as IGF-1 and BDNF (Olah *et al.*, 2011). In addition, peripheral nerve damage specifically activates microglia in the CNS at the corresponding innervation site of the injured nerve, which indicates a directed communication between damaged neurons and microglia (Biber *et al.*, 2007).

Chemokines are also able to induce a chemotactic cellular response. Chemokines are classified into four main subfamilies: CXC, CC, C and CX3C. Most of the members of all these families (except for C chemokines) have been described in neurons and for most of them microglia express the corresponding receptors and respond *in vitro* by having an increased chemotaxis (Biber *et al.*, 2008). Chemokine (C-X3-C motif) ligand 1 (CX3CL1), or fractalkine, was found in neurons to have a role in specific communication with microglia, which are the only cells in the CNS that expresses the correspondent receptor CX3C chemokine receptor 1 (CX3CR1) (Harrison *et al.*, 1998). MNs have been documented to promote microglia-mediated neuroprotection through CX3CL1-CX3CR1 signaling. *In vivo*, CX3CR1 deficiency deregulates microglial response, resulting in neurotoxicity (Cardona *et al.*, 2006). Also, Tg mSOD1/CX3CR1$^{-/-}$ mice exhibited more extensive neuronal cell loss than CX3CR1^{lps} littermate controls. Thus, CX3CL1 release from MNs enhances neuroprotection, and the loss of CX3CR1 on microglia promotes neurotoxicity. Another report indicates that CX3CL1 treatment of cultured microglia increases milk fat globule factor-E8 (MFG-E8) mRNA levels, thus promoting phagocytosis of neurons by microglia (Leonardi-Essmann *et al.*, 2005). CX3CL1 is a membrane-bound protein that is cleaved to its soluble form following cell stress or injury, which then acts as a chemoattractant for T cells, monocytes and microglia (Fuller and Van Eldik, 2008). Another relevant chemokine is CCL21, which was shown to be implicated in signaling neuronal injury to microglia through the receptor CXCR3. This chemokine appears to have be a chemotactic agent release by damaged neurons and is important to drive microglia to the site of lesion (de Jong *et al.*, 2005). These authors ascertain that CCL21 is transported within vesicles that are transported along the neuronal process towards presynaptic structures. Astrocytes also exhibited the receptor CXCR3 but do not reveal significant alterations unless high levels of CCL21 are produced (van Weering *et al.*, 2010). Curiously, the CCL21/CXCR3 signaling axis was never explored in the context of ALS.

As previously mentioned, one of the roles of alternatively activated microglia is phagocytosis. Through this process, MFG-E8 produced by microglia recognizes phosphatidylserine (PS) as a “eat me” signal that is expressed on the surface of apoptotic neurons, thus triggering the phagocytic mechanism (Fuller and Van Eldik, 2008). Leonardi-Essmann and collaborators proved using cultured rat

microglia that the up-regulation of CX3CL1 also leads to an increase in MFG-E8 expression (Leonardi-Essmann *et al.*, 2005) suggesting a combined effort in injury response. Importantly, astrocytes can also produce MFG-E8 in order to provide additional support to microglial phagocytic functions (Kranich *et al.*, 2010).

In the same manner, CD200, a member of the immunoglobulin superfamily, is constitutively expressed at the neuronal membrane surface, and its receptor CD200R is primarily present on microglia (Biber *et al.*, 2007). Mice deficient for CD200, a neuronal glycoprotein whose receptor, CD200R, is expressed by all myeloid cells, show aberrant microglial physiology including morphological activation of microglia in the resting CNS and accelerated response to facial nerve transection (Hoek *et al.*, 2000). In fact, there are studies showing that WT microglia, after LPS activation, is less neurotoxic than mSOD1 microglia due to the enhanced release of neurotrophic factors, such as IGF-1, and attenuated release of free radicals and proinflammatory cytokines (Beers *et al.*, 2006; Xiao *et al.*, 2007). Additionally, in microglia-MN co-cultures, treatment with IL-4 suppressed M1 microglial activation promoting an M2 phenotype, reduced release of ROS, enhanced IGF-1 secretion, and improved MN survival (Zhao *et al.*, 2006).

CD14 is a pattern recognition receptor for misfolded proteins, which is very important in ALS since it is described by Zhao and colleagues (2010) to bind mSOD1, and that the microglial activation mediated by mSOD1 can be attenuated using TLR2, TLR4 and CD14 blocking antibodies in SOD1G93A mice model (Zhao *et al.*, 2010). Furthermore, Appel et al. (2011) also demonstrated that mSOD1G93A binds to CD14 and its blockade leads to diminished proinflammatory cytokines and increased IGF-1 released from mSOD1G93A-treated microglia (Appel *et al.*, 2011). As mentioned above in 1.3.4 of this introduction, there can also be protein inclusions of TDP43 which was demonstrated by Zhao and colleagues (2013) to interact with the CD14 receptor inducing microglial cytotoxic activation and the NOD-like receptor family, pyrin domain containing 3 (NLRP3) inflammasome (Zhao *et al.*, 2013b). TLR2 and TLR4 are co-receptors for CD14 and previous studies suggested that CD14 and TLR contribute to the inflammatory responses initiated by microglia (Olson and Miller, 2004; Zhao *et al.*, 2010). Another microglial receptor involved in triggering inflammatory response is the receptor for advanced glycation end products (RAGE), which can function also as receptor for high mobility group box 1 (HMGB1). HMGB1 is an alarmin that binds to DNA and capable of exerting both extra-nuclear and extra-cellular functions. It can be released by dying neurons (Scaffidi *et al.*, 2002). Within the cell it acts as a transcription factor, whilst outside it behaves as a cytokine which can lead to a positive feedback loop potentiating the secretion of more HMGB1 and can bind to RAGE, TLR2 and TLR4, thus generating an inflammatory response (Casula *et al.*, 2011; Muller *et al.*, 2004). Activation of these pathways can also lead to the activation of NFκB resulting in production of inflammatory cytokines and angiogenic factors (van Beijnum *et al.*, 2008). Casula and colleagues found increased expression of TLR2, TLR4 and RAGE concomitant with higher expression of HMGB1 mRNA levels using *post-mortem* spinal cord samples obtained from ALS patients indicating an increased inflammatory state in late stage disease (Casula *et al.*, 2011).

Autotaxin (ATX) functions as a secreted lysophospholipase D that converts lysophosphatidylcholine (LPC) to lysophosphatidic acid (LPA), which is involved in a wide array of processes namely, neural development and the stimulation of cell migration, proliferation and survival of various cells, namely microglia (Perrakis and Moolenaar, 2014). LPA receptors are expressed by microglia, astrocytes and oligodendrocytes (Hoelzinger *et al.*, 2008). Most importantly, ATX secretion by microglial cells was shown to be stimulated by oxidative stress, and that ATX overexpression reduces microglial ROS-mediated damage and microglial activation (Awada *et al.*, 2012).

Chromogranins, which are components of neurosecretory vesicles, were documented to interact with mutant forms of SOD1 (Urushitani *et al.*, 2006). Mutant SOD1 was also found in immune-isolated trans-Golgi network and in some preparations, suggesting that it could be secreted, furthermore, chromogranins were demonstrated to act as chaperone-like proteins and promote secretion of SOD1 mutant proteins (Appel *et al.*, 2011). In fact, since SOD1 is a cytosolic protein that lacks a signal peptide, there must be an alternative mechanism for the extracellular export of this protein. Hence, neuronal ability to produce and release exosomes has drawn attention. Exosomes are small lipid membrane vesicles that formed by fusion of multivesicular bodies with plasma membrane and the subsequent release of their cargo. They are important for a variety of pathways, and one of the biological functions of exosomal release is the secretion of membrane proteins meant to be discarded or to be passed onto other cells (Fruhbeis *et al.*, 2012). These exosomes are characterized by the presence of nucleic acids, including mRNAs and miRNAs. Microglia can release exosomes containing IL-1β (Bianco *et al.*, 2009) and was shown to internalize oligodendroglial exosomes, with apparent inhibitory function of membrane expansion and myelin formation, by micropinocytosis (Bakhti *et al.*, 2011; Fitzner *et al.*, 2011). Gomes and collaborators (2007) describe SOD1 secretion in NSC-34 cells associated with exosomes (Gomes *et al.*, 2007). This evidence suggests a new cellular mechanism contributing for dissemination of mSOD1 toxicity in ALS pathogenesis. Figure I.5 summarizes major communication pathways between MN and microglia.

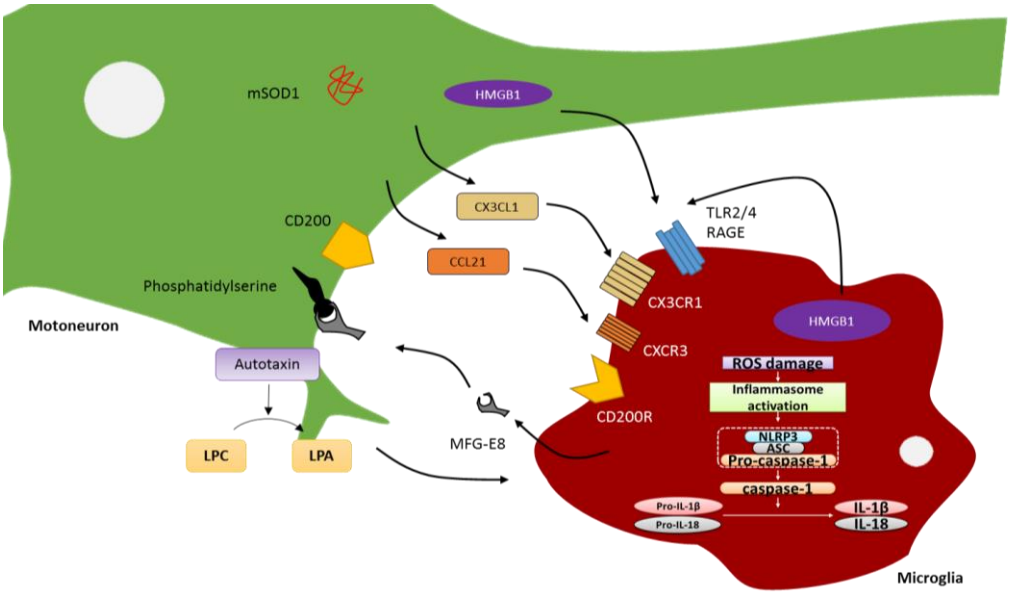


Figure I.5. Dialogue between microglia and motor neurons. Activation of microglia in ALS can be mediated by the release of mutant SOD1 (mSOD1) by motor neurons (MNs) through a chromogranin chaperone-like process. mSOD1 is recognized by toll-like receptors (TLRs), stimulating an inflammatory response. HMGB1 can be released from MNs and is recognized by TLRs and the receptor for advanced glycation end products (RAGE). Reactive oxygen species (ROS) and TLR activation lead to inflammasome activation through the formation of NOD-like receptor family, pyrin domain containing 3 (NLRP3) and Pro-caspase-1 which then converts Pro-IL-1β and Pro-IL-18 into their active forms. Chemokine (C-C motif) ligand 21 (CCL21) can be secreted by MNs and act upon microglial chemokine (C-X-C motif) receptor 3 (CXCR3) triggering microglial activation and proliferation. In the same manner, CX3C chemokine ligand 1 (CX3CL1) is released and is recognized by CX3C chemokine receptor 1 (CX3CR1) inducing a neuroprotective response. CD200, a member of the immunoglobulin superfamily, is constitutively expressed at the neuronal membrane surface, and its receptor CD200R is primarily present on microglia interaction. Milk fat globule factor 8 (MFG-E8) can be released by microglia and recognize externalized phosphatidylserine at the surface of apoptotic neurons. Autotaxin converts lysophosphatidylcholine (LPC) to lysophosphatidic acid (LPA), which can be perceived by microglia.

3.3. The relevance of microglia in ALS progression

As previously stated, microglia plays a key role in ALS progression. This importance was established by studies such as those by Boillee and colleagues in mice showing that diminished levels of mSOD1 specifically in microglia greatly decrease disease progression (Boillee *et al.*, 2006b). It was also proposed that this slowed disease progression is a consequence of the increased neurotoxicity of mSOD1G93A microglia when compared to WT microglia in microglia/MN co-cultures (Xiao *et al.*, 2007), where higher expression of NO and superoxide levels were found, together with a decreased expression of IGF-1.

To further assess the effects of mSOD1 microglia in ALS pathoprogression, Beers et al. (2006) developed a knockout mouse model unable to produce myeloid cells, which was transplanted WT microglia and found that this mice did not develop MN injury nor an ALS-like disease (Beers *et al.*, 2006). Interestingly, replacement of microglia cells using clodronate liposomes, which specifically target monocyte/macrophage system (such as microglia cells), significantly slowed disease progression and prolonged survival of the transgenic ALS mice after bone marrow transplantation (BMT) (Lee *et al.*, 2012a). Nevertheless, influences of BMT may not be efficient enough since microglia are replaced by tissue-resident microglia rather than bone marrow cells (BMCs) (Ohnishi *et al.*, 2009). Altogether, these studies indicate a pivotal role for microglia during the progression of the disease, as well as a putative beneficial role of healthy microglia in rescuing MN degeneration in ALS *in vitro* and *in vivo* animal models.

4. Therapeutic approaches – recent findings

ALS is a devastating MN disease characterized by progressive wasting and weakness leading to respiratory failure and death. Despite the advances in the comprehension of the mechanisms that trigger neurodegeneration in ALS, there is not an effective treatment for this disease. Currently, the strat-

egies that are being developed use pharmacotherapy, physical therapy, gene therapy, stem-cell therapy, RNA therapy and immunotherapy areas.

The only available drug approved by the American Food and Drug Administration (FDA) is the 2-amino-6-trifluoromethoxy-benzothiazole (Riluzole) which has been shown to prolong survival of ALS patients only by a few months. Riluzole aims to perverse neuronal motor function by exerting its neuroprotective effects through blocking both voltage-gated sodium channels (Na+) and NMDA receptors, thereby preventing excessive calcium influx into neurons (Pratt *et al.*, 2012; Venkova-Hristova *et al.*, 2012). Riluzole modulates GABAergic systems and acts as Ca^{2+}, Na^{+} channel blocker with antidepressant and anti-convulsant properties (Pandya *et al.*, 2012). The drug was tolerated well, but survival was extended by only 2-3 months compared with placebo. Other medications targeting glutamate pathways in neurons – talampanel, memantine, topiramate, pamotrigine, gabapentin and ONO2506 – have been studied, but all trials have been negative (Zinman and Cudkowicz, 2011). Inactivation of synaptic glutamate is a key function of the EAAT2 glutamate transporter on astrocytes in the protection of MNs from toxic effects. Cetftriaxone, a beta-lactam antibiotic, increased astrocyte-mediated glutamate transport by stimulating expression of EAAT2 in an *in vitro* blind screening study of 1040 medications approved by the US FDA. In an animal model of ALS, use of this compound was associated with prolonged survival and upregulated transcription of mRNA for EAAT2 (Zinman and Cudkowicz, 2011).

Stem cells have the potential to develop into many different cell types in the body during early life and growth. There are two kinds of stem cells in animals and humans: embryonic stem cells (ESC) and somatic or adult stem cells. The potential of stem cell therapies in neuronal replacement and regeneration, as well of other neural tissues, has become a source of great hope and expectation for ALS patients. Stem cell therapy in ALS comprises two approaches: cell replacement and neural protection (Meamar *et al.*, 2013). Cell replacement involves the substitution of dead neurons of the spinal cord with stem cell-derived spinal MN precursors, while neural protection aims to supply trophic factors to support and preserve endogenous cells (Mazzini *et al.*, 2003; Mitchell and Borasio, 2007). Studies in mSOD1 rats show a delay in disease onset and prolonged survival by providing genetically non-compromised supporting cells (Xu *et al.*, 2009). It has been reported that transplantation of autologous mesenchymal stem cells into the SC of ALS patients is safe and well tolerated (Mazzini *et al.*, 2003). More recently, the use of induced pluripotent stem cells has generated a great deal of attention due to their ability to differentiate into MNs. However, stem-cell treatment requires much work to be done before this treatments can be regarded as an experimental therapeutic for ALS. The potential of induced pluripotent stem cells (iPSCs) has attracted a great deal of attention due to its potential. Namely, Nizzardo et al. (2014) have observed beneficial effects of injecting iPSC-derived NSCs in ALS mice (Nizzardo *et al.*, 2014).

Among other mechanisms, loss of neurotrophic support to MNs has been implicated in the pathogenesis of ALS (Pandya *et al.*, 2012). A deficiency of growth factor support could provoke MN death in patients in ALS (Zinman and Cudkowicz, 2011). Various growth factors including GDNF, brain-derived neurotrophic factor (BDNF), VEGF, and IGF-1 are expressed differently in ALS, and all have enor-

mous neuroprotective influence and promote proliferation among MNs in ALS (Federici and Boulis, 2012). Viral delivery of IGF-1, glial-cell-derived neurotrophic factor (GDNF) and VEGF to MNs in the SOD1G93A mouse models prolongs their survival (Turner and Talbot, 2008; Vincent *et al.*, 2008).

Gene therapy involves the usage of a vector, most commonly viral, to deliver the therapeutic gene of interest to the affected region or tissue. Vaccines targeting SOD1G93A have been developed and shown to be effective in mouse models of ALS (Takeuchi *et al.*, 2010). Also, SOD1 gene-silencing approach may be useful to delay disease onset or progression. Intraventricular infusion of antisense DNA oligonucleotides is one such approach. It reduces SOD1 protein and mRNA in the brain and spinal cord (Smith *et al.*, 2006).

RNA interference (RNAi) is a mechanism of gene silencing in which siRNAs target cellular mRNAs for degradation. Therapies using small interfering RNA (siRNA) have shown down-regulation in human mutant SOD1G93A gene in lumbar SC of ALS mice (Rizvanov *et al.*, 2009). miR-206 is a skeletal muscle–specific micro RNA that is a key regulator of signaling between neurons and skeletal muscle fibers at neuromuscular synapses. Mice that are genetically deficient in miR-206 have accelerated ALS progression. Because miR-206 mediates its action partially through histone deacetylase-4 and fibroblast growth factor signaling pathways, it delays ALS progression and promotes regeneration of neuromuscular synapses in mice. miR-206 also leads to regeneration of neuromuscular synapses (Williams *et al.*, 2009).

Animal models of ALS provide unique opportunities to study this lethal human disease both clinically and pathologically. ALS animal models have allowed controlled testing of various pathogenic mechanisms and arise as a medium clinical research and development.

5. SOD1G93A mouse model for studying degeneration in ALS

The development of experimental of both *in vitro* and *in vivo* models is essential for the study of cellular and molecular pathophysiological mechanisms of neurodegenerative diseases. As previously mentioned, mutations in SOD1 are one of the most frequent in fALS, together with the characteristic inclusions of mSOD1 being also present in sALS conceives the establishment mSOD1 models as one of the most widely studied models. Such models include mice, rats, zebrafish, *Drosophila melanogaster* and *Caenorhabditis elegans* (Joyce *et al.*, 2011). Although none of these models are capable to fully mimicking the pathological and behavioral features of ALS, they still provide a valuable platform for the testing of pathogenic hypotheses.

Approaches for the study of cellular and molecular pathophysiological mechanisms of ALS includes *in vitro* models. There is a wide array of these models, namely primary spinal cord cultures, NSC-35 cell lines expressing mSOD1 as well as organotypic cultures (Tovar *et al.*, 2009). For instance, primary spinal cord cultures allowed for the identification of MN vulnerability to glutamate excitotoxicity through overstimulation of AMPA receptors (Carriedo *et al.*, 1996), while using the NSC-34 cell line Golgi apparatus fragmentation and mitochondrial dysregulation were described (Gomes *et al.*, 2008; Raimondi *et al.*, 2006) and finally resorting to organotypic cultures there was identification of neu-

rotrophic factors acting to protect MNs from excitotoxicity (Tolosa *et al.*, 2008). The knowledge obtained using *in vitro* models can present itself as clues for further studies in vivo models as the latter doesn't present the same limitations and better presents correlations between cellular alterations as well as motor behavior which can only be obtained in the whole animal.

The first animal model was developed by Gurney and collaborators (1994) where they created a Tg mouse that expressed the human SOD1 gene with a substitution of a glycine for an alanine at position 93 (G93A) (Gurney *et al.*, 1994). The construct generally comprises 12-15 kb human genomic fragments encoding SOD1 driven by the endogenous promoter and regulatory sequences (Turner and Talbot, 2008). Currently, there are 177 different SOD1 human ALS mutations expressed in the mouse as well as artificially induced SOD1 mutant transgenes that either prevent copper binding (Turner and Talbot, 2008). Most mutations are missense, however there are C-terminally truncated variants. Different SOD1 mutants reveal a degree of variation of transgene copy number, transcript and protein levels, enzymatic activity and neuropathology, however, a common feature is the induction of fatal symptoms (Julien and Kriz, 2006). These mSOD1 mouse models include G37R, G85R, D90A and G86R that show a similar phenotype as the SOD1G93A model (Van Den Bosch, 2011).

The disease in SOD1G93A mice begins with hind limb tremor, impaired leg extension and reduced stride length around three months. These symptoms develop to hyperreflexia and complete paralysis of the limbs culminating in premature death at around 4 months of age (Dal Canto and Gurney, 1994; Durand *et al.*, 2006; Turner and Talbot, 2008). Neuromuscular junctions degenerate around 47 days, which appears selective for fast-fatigable axons (Fischer *et al.*, 2004), although a report by Gould et al. (2006) places peripheral denervation earlier at 25 days (Gould *et al.*, 2006). Proximal axonal loss is prominent at 80 days and is concomitant with motor impairment and a severe 50% drop-out of lower MNs is evident at 100 days (Fischer *et al.*, 2004). These data support that ALS is a dying back axonopathy (Ferraiuolo *et al.*, 2011). However, there is controversy about where the disease begins with the dying forward hypothesis proposing that the MNs in the cortex are the main part of the disorder, connecting with anterior horn cells thus mediating anterograde degeneration of anterior horn cells via glutamate excitotoxicity (Kiernan *et al.*, 2011). Pathologic features include mitochondrial vacuolization, Golgi apparatus fragmentation, neurofilament-positive inclusions and cytoplasmic immune-reactive mSOD1 aggregates (Dal Canto and Gurney, 1995; Tu *et al.*, 1996). There is also suggested a marked astroglial and microglial activation around symptom onset (Turner and Talbot, 2008; Van Den Bosch, 2011). Degeneration of cranial nuclei such as trigeminal, facial and hypoglossal nerves also occurs in mice (Angenstein *et al.*, 2004; Zang *et al.*, 2004). Phenotypically, the symptom age onset and its severity is directly proportional to the amount of SOD1 expressed in the tissue, supporting the hypothesis of gain of toxic function of mSOD1 (Tovar *et al.*, 2009). Expression of different SOD1 gene mutations leads to different biochemical properties in Tg mice, even in the presence of endogenous mSOD1 gene, triggering the development of a neurodegenerative disease similar to the human illness (Ripps *et al.*, 1995).

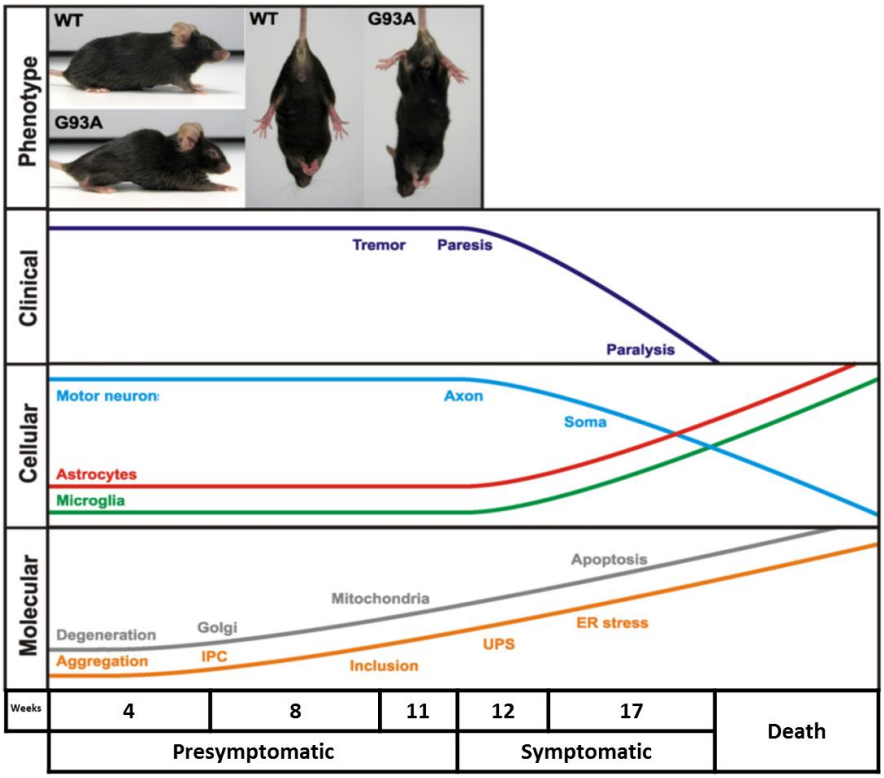


Figure I.6. Schematic time progression of amyotrophic lateral sclerosis (ALS) in mSOD1 mice. Phenotypic differences between control (WT) mice and high copy number transgenic SOD1G93A mice (mSOD1) show muscle atrophy of mSOD1 mice. Clinically, mSOD1 mice exhibit hindlimb tremor, weakness and locomotor deficits at about 30 days old which then evolves into paresis and progressive paralysis culminating in premature death at around 120 days. On a cellular level, Motoneuron degeneration is preceded by distal synaptic and axonal degeneration. Progressive astrocytosis and microgliosis is observed at 90 days old. On a molecular approach, progressive mSOD1 aggregation is observed resulting in insoluble protein complexes (IPC), inclusion bodies modified by the ubiquitin-proteasome system (UPS) resulting in increasing endoplasmic reticulum (ER) stress. Furthermore, increasing Golgi degeneration and mitochondria dysregulation trigger apoptosis. Adapted from Turner *et al.* (2008).

Other mSOD1 models present similar phenotypes as the G93ASOD1 mouse line. For instance the D90ASOD1 mutation shows more stability, however it is not as neurotoxic as other SOD1 mutants (Jonsson *et al.*, 2009; Jonsson *et al.*, 2006). The G86R model also indicate a possible gain of toxic function by the mSOD1 protein as well symptoms onset around 3 months of age with of paralysis leading to premature death around 4 months (Ripps *et al.*, 1995). Watanabe and colleagues (2005) developed a model with a 2-base pair deletion at codon 126 of the SOD1 gene, where the mice start with symptoms of hindlimb paraparesis around 14 months progressing to muscle atrophy and death within 40 days (Watanabe *et al.*, 2005). The homozygous strains, although phenotypically indistinguishable from their heterozygous counterparts, exhibit mean ages of onset of the disease and die earlier.

Further developed models include TDP-43 and FUS mutations. Mouse models overexpressing TDP-43 show toxicity and suffer neuronal loss (D'Alton *et al.*, 2014; Xu *et al.*, 2011). Studies by Veerbeeck and colleagues (2012) show accumulation of FUS in the cytoplasm comparatively to WT, with

the degree of this shift correlating to the severity if the FUS mutation as reflected by disease onset in humans (Verbeeck *et al.*, 2012).

Rodent models, such as mSOD1, have the advantage of a consistent motor phenotype; readily available, low maintenance; homologous basic neuromuscular structures to humans and a short life cycle (Mead *et al.*, 2011). Presenting common pathological feature between different models is progressive accumulation of aggregates containing SOD1 protein and aberrant neurofilament accumulations in degenerating MNs, mSOD1 is still the most used animal model for the study of ALS.

6. Aims

The aims of this thesis are to evaluate molecules involved in cellular dysfunction and reactivity in ALS pathogenesis and to identify motoneuron-glia signaling pathways that are altered along ALS progression. This will be achieved by using samples obtained from lumbar spinal cord of the transgenic mice carrying a human mutant SOD1G93A gene, B6SJL-TgN (SOD1-G93A) 1Gur/J (mSOD1), at two different stages of disease progression: presymptomatic (4-6 weeks) and symptomatic (14-16 weeks). Results will be compared to similar samples obtained from the control B6SJLF1/J (WT) mice.

The specific aims are:

1. To evaluate both mRNA and protein expression levels of specific glial makers involved in ALS onset and progression, namely GFAP and S100B for astrocytes and CD11b for microglia.
2. To evaluate both mRNA and protein expression levels of gap junction components (Connexin 43 and Pannexin 1) involved in cell-cell communication and proliferation and migration markers Ki-67 and Autotaxin.
3. To evaluate both mRNA and protein expression levels of motoneuron-microglia dialogue pathways, namely CXCR3-CCL21, CX3CR1-CX3CL1 and MFG-E8.
4. To evaluate both mRNA and protein expression levels of inflammation-related molecules, namely TLR2, HMGB1, IL-18 and NLRP3.

II. MATERIALS AND METHODS

1. Materials

1.1. Animals

Transgenic B6SJL-TgN (SOD1-G93A)1Gur/J males (Jackson Laboratory, No. 002726) overexpressing the human SOD1 gene carrying a glycine to alanine point mutation at residue 93 (G93A) (Gurney *et al.*, 1994) and wild-type B6SJLF1/J females were purchased from The Jackson Laboratory (Bar Harbor, ME, USA) and were breed at Instituto de Medicina Molecular rodent facilities where a colony was established. Maintenance and handling was performed according to the European Community guidelines (Directives 86/609/EU and 2010/63/EU, Recommendation 2007/526/CE, European Convention for the Protection of Vertebrate Animals used for Experimental or Other Scientific Purposes ETS 123/Appendix A) and Portuguese Laws on Animal Care (Decreto-Lei 129/92, Portaria 1005/92, Portaria 466/95, Decreto-Lei 197/96, Portaria 1131/97). The protocols used in this study were approved by the Portuguese National Authority (General Direction of Veterinary) and by the Ethics Committee of the Institute of Molecular Medicine. Every effort was made to minimize the number of animals used and their suffering.

mSOD1 mice were used to study presymptomatic (4–6 weeks old) and symptomatic (12–14 weeks old) phases of the disease. 4–6 and 12–14 weeks old wild-type (WT) animals served as controls. Both male and female mice were used in a 1:1 proportion as no gender influences over the intrinsic features of neuromuscular transmission have been detected in this model (Rocha *et al.*, 2013). Mice were anesthetized with a lethal dose of Pentobarbital and intracardially perfused. For quantitative real time transcription polymerase chain reaction (qRT-PCR) or Western blot (WB), mice were perfused with 0.1 M phosphate buffer at pH 7.4 and lumbar spinal cord dissected and rapidly frozen at -80ºC.

1.2. Chemicals

Bovine serum albumin (BSA) and β-mercaptoethanol were purchased from Sigma-Aldrich (St. Louis, MO, USA). Nitrocellulose membrane was obtained from Amersham Biosciences (Piscataway, NJ, USA); sodium dodecyl sulfate (SDS) was acquired from Prolabo (Poole, UK); LumiGLO® was from Cell Signalling (Beverly, MA, USA); acrylamide, bis-acrylamide, Tween 20, glycerol, sodium chloride (NaCl) were obtained from Merck (Darmstadt, Germany); Bio-Rad's Protein Assay Reagent was obtained from BioRad Laboratories (Hercules, CA, USA); Complete, EDTA-free Protease inhibitor cocktail tables (Roche). All the other common chemicals were of analytical grade and were purchased either from Sigma-Aldrich or Merck.

1.3 Primers and Antibodies

Primary antibodies: mouse polyclonal anti-CXCR3, rabbit polyclonal anti-MFG-E8, rabbit polyclonal anti-SOD1 were purchased from Santa Cruz Biotechnology® (Santa Cruz, CA, USA). Mouse monoclonal anti-HMGB1 was acquired from BioLegend® (San Diego, CA, USA). Mouse anti-β-actin, rabbit polyclonal anti-GFAP were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Secondary antibodies: Horseradish peroxidase-labelled goat anti-rabbit IgG, horseradish peroxidase-labelled goat anti-mouse IgG and horseradish peroxidase-labelled rabbit anti-goat IgG were purchased from Santa Cruz Biotechnology® (Santa Cruz, CA, USA).

Table II.1. Primer sequences for genes evaluated

Gene	Primer sequence
ATX	Fwd 5'- GAC CCT AAA GCC ATT ATT GCT AA -3'
	Rev 5'- GGG AGG TGC TGT TTC ATG T -3'
CCL21	Fwd 5'- CAG GAC TGC TGC CTT AAG TA -3'
	Rev 5'- GCA CAT AGC TCA GGC TTA GA -3'
CD11b	Fwd 5'- CAG ATC AAC AAT GTG ACC GTA TGG G -3'
	Rev 5'- CAT CAT GTC CTT GTA CTG CCG CTT G -3'
CX43	Fwd 5'- ACA GCG GTT GAG TCA GCT TG -3'
	Rev 5'- GAG AGA TGG GGA AGG ACT TGT -3'
CX3CL1	Fwd 5'-CTC ACG AAT CCC AGT GGC TT-3'
	Rev 5'-TTT CTC CTT CGG GTC AGC AC-3'
CX3CR1	Fwd 5'-TCG TCT TCA CGT TCG GTC TG-3'
	Rev 5'-CTC AAG GCC AGG TTC AGG AG-3'
GFAP	Fwd 5'- CCA AAC TGG CTG ATG TCT ACC -3'
	Rev 5'- GCT TCA TCT GCC TCC TGT CTA -3'
HMGB1	Fwd 5'- CTC AGA GAG GTG GAA GAC CAT GT-3'
	Rev 5'-GGG ATG TAG GTT TTC ATT TCT CTT TC-3'
IL-18	Fwd 5'-TGG TTC CAT GCT TTC TGG ACT CCT-3'
	Rev 5'-TTC CTG GGC CAA GAG GAA GTG-3'
Ki-67	Fwd 5'- CAG TAC TCG GAA TGC AGC AA -3'
	Rev 5'- CAG TCT TCA GGG GCT CTG TC -3'
MFG-E8	Fwd 5'- TGA CTT TGG ACA CAC AGC GT -3'
	Rev 5'- GTG TAG AAC AAC GGG AGG CT -3'
NLRP3	Fwd 5'-TGC TCT TCA CTG CTA TCA AGC CCT-3'
	Rev 5'-ACA AGC CTT TGC TCC AGA CCC TAT-3'
PAN1	Fwd 5'- TGT GGC TGC ACA AGT TCT TC -3'
	Rev 5'- ACA GAC TCT TGC CCC ACA TTC -3'
S100B	Fwd 5'- GAG AGA GGG TGA CAA GCA CAA -3'
	Rev5'- GGC CAT AAA CTC CTG GAA GTC -3'
TLR2	Fwd 5'- TGC TTT CCT GCT GAA GATT T -3'
	Rev 5'- TGT ACC GCA ACA GCT TCA GG -3'

Table II.2. Primary antibodies used and respective information

Primary antibody	Clonality	Host	Brand	Dilution
β-actin	Monoclonal	Mouse	Sigma-Aldrich	1:5000
CXCR3	Polyclonal	Mouse	Santa Cruz Biotechnology®	1:300
GFAP	Polyclonal	Rabbit	Sigma-Aldrich	1:750
HMGB1	Monoclonal	Mouse	Biolegend®	1:200
MFG-E8	Polyclonal	Rabbit	Santa Cruz Biotechnology®	1:125
SOD1	Polyclonal	Rabbit	Santa Cruz Biotechnology®	1:500

1.4. Equipment

Mini-PROTEAN Tetra cell system used for Western Blot from Bio-Rad (Hercules, CA, USA). Micro-plate reader (PR 2100) was also from Bio-Rad and it was used for spectrophotometric measurement of protein. For immunodetection in nitrocellulose membranes was used ChemiDoc™ equipment from Bio-Rad Laboratories. Sonication of samples was performed in the Ultrasonic Processor UP100H (Hielscher-Ultrasound Technology, Teltow, Germany). For RNA quantification Nanodrop ND-100 Spectrophotometer (NanoDrop Technologies) was used. qRT-PCR was performed on a 7300 Real-Time PCR System (Applied Biosystems) using Luminaris Color HiGreen High ROX qPCR Master Mix (Thermo Scientific).

2. Methods

2.1. Quantitative Real-Time PCR assay

Total RNA was extracted from spinal cord by using Trizol Reagent (Invitrogen). Briefly, sample tissue was lysated by adding TRIzol reagent, homogenated and then freezed. Chlorophorm was added in 0.2 per ml then incubated for 2-3 minutes at room temperature. Then samples were centrifuged at 12000 for 15 minutes and aqueous phase was recovered and precipitated with 0.5 ml of isopropyl per ml of TRIzol and incubated for another 10 minutes at room temperature. Samples were centrifuged at 12000g for 10 minutes. Supernatant was removed and RNA pellet was washed with 75% ethanol. RNA sample was further centrifuged at 12000g for 5 minutes and 30 µl of DEPC water was and collect to a sterile tube.

Total RNA was then quantified by using Nanodrop ND-100 Spectrophotometer (NanoDrop Technologies) and equal amounts of RNA were tested for qRT-PCR as usual in our lab (Mariano *et al.*, 2013). Results were normalized to β-actin and quantified using the mathematical model developed by Pfaffl (Pfaffl, 2001).

Primer sequences used are listed in Table II.1.

2.2. Western Blot assay

Homogenates from SC were obtained by adding ice radio-immunoprecipitation assay, a lysis buffer composed of 5% of Tris(hydroxymethyl)aminomethane (Tris) 1M pH=8, 1% of Ethylenediamine Tetraacidic acid (EDTA) 0,5M pH=8, 3% of NaCl 5M, 10% of NP-40, 50% glycerol, 0,1% SDS and protease inhibitor, and homogenized using Pellet pestles (Sigma). Then samples were sonicated during 20 seconds and centrifuged at 14000 g for 10 min, at 4ºC, and the supernatants were collected and stored at -80ºC. Protein concentration was determined using the Bradford method (Bradford, 1976) using Bio-Rad's Protein Assay Reagent. Equal amounts of protein were separated on a 10% or on a 12% sodium dodecyl sulfate-polyacrilamide gel electrophoresis (SDS-PAGE) and then transferred to a nitrocellulose membrane. After transfer, blotted membranes were incubated in blocking buffer [Tween 20-Tris buffered saline (T-TBS) plus 5% (w/v) non-fat dried milk] at room temperature during 1 h. After that, membranes were incubated overnight at 4ºC with the primary antibodies specified in Table II.2., diluted in T-TBS with 5% BSA: After washing with T-TBS, the membranes were incubated at room temperature (RT) during 1 h with the respective secondary antibodies: goat anti-rabbit HRP-linked or goat anti-mouse HRP-linked (for each one 1:5000, Santa Cruz Biotechnology®), diluted in blocking solution. After washing membranes with T-TBS, chemiluminescent detection was performed using LumiGLO® reagent, bands were visualized in Chemidoc equipment and relative intensities of protein bands were analyzed using the Image Lab™ analysis software, both from Bio-Rad Laboratories (Hercules, CA, USA). All the results were normalized to β-actin.

2.3. Statistical analysis

Results of at least four different experiments were expressed as mean ± SEM. Comparisons between the different parameters evaluated in WT and mSOD1 mice were made using two-tailed Student's *t*-test for equal or unequal variance, as appropriate. Comparison of more than two groups (considering analysis between WT and mSOD1 either in presymptomatic and in symptomatic stages) was done by one-way Analysis of Variance (ANOVA) using GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA) followed by multiple comparisons Bonferroni post-hoc correction. $p<0.05$ was considered statistically significant and $p<0.01$ very significant.

III. RESULTS

ALS has been characterized as non-cell autonomous disease where glial cells play a pivotal role for its onset and progression, where neuroinflammation is an important hallmark of the disease, where microglia can acquire neurotoxic or neuroprotective properties (Evans *et al.*, 2013). Astrocytes may also have an important roles related with excitotoxicity observed in ALS as well as inflammation, together with alterations in astrocytic phenotypes and function in SOD1 mouse models (Diaz-Amarilla *et al.*, 2011; Philips and Robberecht, 2011). Therefore, a better understanding of MN-microglia dialogue during ALS onset and progression becomes necessary for discerning its pathoprogression.

The mSOD1 mouse model used in the present work was first developed by Gurney and colleagues (1994). These mice overexpress human SOD1 inducing fatal symptoms strongly indicative of ALS and currently serve as one of the most used models for the study of the disease.

Here, we evaluated either mRNA and/or protein expression levels of markers that could indicate astroglial or microglial reactivity and degeneration, as well as molecules involved in MN-glia signaling pathways in the spinal cord of mSOD1 mice at different stages of disease progression, namely at presymptomatic (4-5 weeks) and at symptomatic (14-15 weeks) stages.

1. Glial cell response and cellular communication are damaged in spinal cord of mSOD1 mice at presymptomatic stage

1.1. SOD1 expression is increased in SC of mSOD1 mice

We first evaluated the expression of SOD1. As shown in Figure III.1 our data reveal the presence of a band with molecular weight around 21 kDa, correspondent to human SOD1 (Kikuchi *et al.*, 2006) only in mSOD1 mice. In addition, when normalized to the amount of mouse SOD1, we observed significantly higher SOD1 expression in mSOD1 mice in comparison to WT (p<0.05), confirming that SOD1 accumulation occurs in our experimental model.

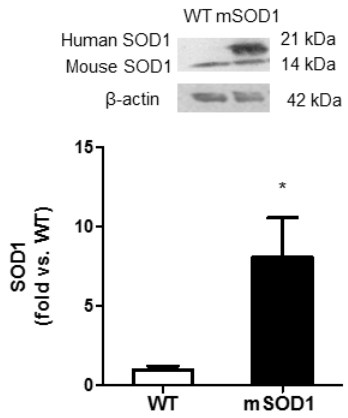


Figure III.1. SOD1 expression is increased in the spinal cord (SC) from presymptomatic mSOD1 mice when compared to aged-matched control (WT). Samples were obtained as indicated in Methods. Results are expressed as mean (± SEM) from six independent experiments *p<0.05 vs. matched WT mice using two-tailed unpaired Student´s *t*-test.

1.2. Glial cellular markers, hemichannels and proliferation indicators are diminished in SC from mSOD1 at PS phase

Since ALS pathology is not exclusively a MN-disease, we then studied markers associated with proliferation and reactivity of glial cells associated to disease progression, namely astrocytes and microglia. First, we observed that astrocytic markers are diminished in the presymptomatic stage at the level of both mRNA (p<0.05) and protein (p<0.05) (Figure III.2 A and B). Also, expression of mRNA S100B, which is a member of a multigenic family of Ca^{2+} binding protein mainly expressed by astrocytes is decreased (Figure III.2 C). The reduction of both cellular markers may indicate an impaired astroglial activation during the presymptomatic stage of the disease. We also evaluated the state of microglial activation through the evaluation of mRNA levels of CD11b, a known indicator of microglia activation (Saijo and Glass, 2011). As shown in Figure III.2 D, we also determined diminished levels of CD11b in mSOD1 mice when compared to control (p<0.05), suggesting lack of microglial activation as well at this phase.

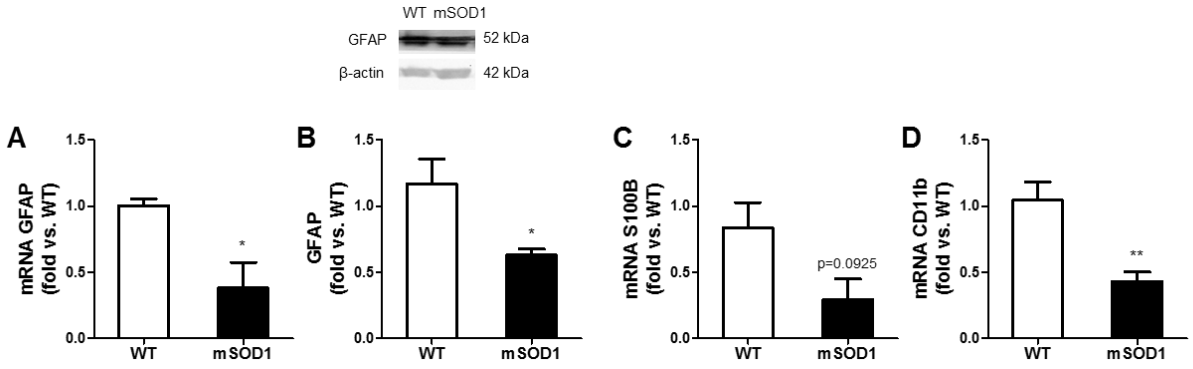


Figure III.2. Expression of markers for glial activation are decreased in the spinal cord (SC) from presymptomatic mSOD1 mice when compared to aged-matched controls (WT). Samples were obtained as indicated in Methods. Results of mRNA GFAP (A), GFAP (B), mRNA S100B (C) and mRNA CD11b (D) are expressed as mean (± SEM) from four (mRNA GFAP), three (GFAP and S100B) and six (CD11b) independent experiments *p<0.05; **p<0.005 vs. matched WT mice using two-tailed unpaired Student´s t-test.

Next, we focused our study on the evaluation of hemichannels activity. Connexins form gap junctions that appose two cells and form channels for direct intercellular communication, through which intercellular second messengers, such as Ca^{2+} ions and small molecules, are exchanged (Barbe *et al.*, 2006). Astrocytes constitutes the cells that mainly express Cx43 (Orellana *et al.*, 2009). At the mSOD1 presymptomatic stage of ALS progression, mRNA expression of either Cx43 is markedly decreased (p<0.05) (Figure III.3 A), suggesting a decrease in hemichannel activity due to an impairment in astroglial communication capacity. PAN1 is expressed by neurons and glia (Penuela *et al.*, 2013) allowing cell-cell communication through channels permeable to ATP and also the propagation of Ca^{2+} waves. In addition, they play a role in the release of “find-me” signals of apoptotic cell clearance (Chekeni *et al.*, 2010). In Figure III.3. we observed that PAN1 mRNA levels are significantly dimin-

ished (p<0.05) suggesting a deregulation in cellular communication and apoptotic pathways. Ki-67 has a role in proliferation (Kreitz *et al.*, 2000). As such, mRNA expression of Ki-67 was visibly decreased (p<0.05) when compared to control at presymptomatic stages, as portrayed in Figure III.3 C, thus proposing lessened cellular capacity for migration.

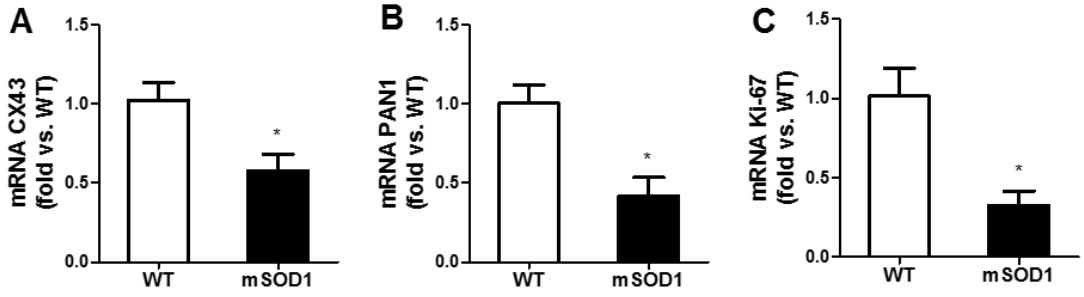


Figure III.3. Hemichannel proteins Cx43 and PAN1 and proliferation marker Ki-67 are decreased in the spinal cord (SC) from presymptomatic mSOD1 mice when compared to aged-matched control (WT). Samples were obtained as indicated in Methods. Results of mRNA Cx43 (A), mRNA PAN1 (B) and mRNA Ki-67 (C) are expressed as mean (± SEM) from five (Cx43), four (PAN1) and three (Ki-67) independent experiments *p<0.05 vs. matched WT mice using two-tailed unpaired Student´s t-test.

1.3. MN-microglia dialogue is impaired in SC from mSOD1 mice at presymptomatic stage

Having observed such alterations at the level of glial function, we further explored MN-microglia communication. We focused on the following axis: CXCR3-CCL21, CX3CR1-CX3CL1 and phagocytosis mediated through MFG-E8.

CXCR3-CCL21 signaling has not been studied in ALS context. CXCR3 is a receptor present in microglia which recognizes CCL21, a chemokine released by neurons that acts a chemokine that acts to drive microglia to the site of lesion and potentiate inflammation in conditions of spinal cord injury, when it's expressed by apoptotic neurons (Biber *et al.*, 2008). Although astrocytes also have CXCR3, they do not respond in terms of proliferation unless high levels of CCL21 occur (van Weering *et al.*, 2010). CXCR3 protein expression is unaffected in mSOD1 when compared to control levels (Figure III.4.A). However, CCL21 mRNA levels are significantly decreased (p<0.05) during the presymptomatic stage (Figure III.4.B). CCL21 decrease suggests decreased microglial mobility and lack of astrocytitc.

On the other hand, CX3CR1-CX3CL1 communication may have a role in enhancing neuroprotection, through the modulation of microglial proliferation (Appel *et al.*, 2011). As demonstrated in Figure III.4 C and D, we observed a marked decrease (p<0.05) in mRNA expression of both these molecules, suggesting decreased neuroprotection capacity, due to its role in microglial proliferation and phagocytic properties and reducing neuronal apoptosis (Deiva *et al.*, 2004).

Interestingly, MFG-E8 MRNA and protein levels were also decreased (p<0.05) in mSOD1 presymptomatic mice (Figure III.4 E and F), suggesting compromised phagocytic capacity at this ALS stage. Since MFG-E8 expression is directly implicated by CX3CL1 expression (Leonardi-Essmann *et al.*, 2005), these results may indicate possible weakening in phagocytic capacity. Indeed, MFG-E8

seems to be essential for microglia engulfment and removal of dying neurons therefore acting as a phagocytic ability marker (Fuller and Van Eldik, 2008). This may however, be lost if the cell is continuously stressed by a neurotoxic stimulus.

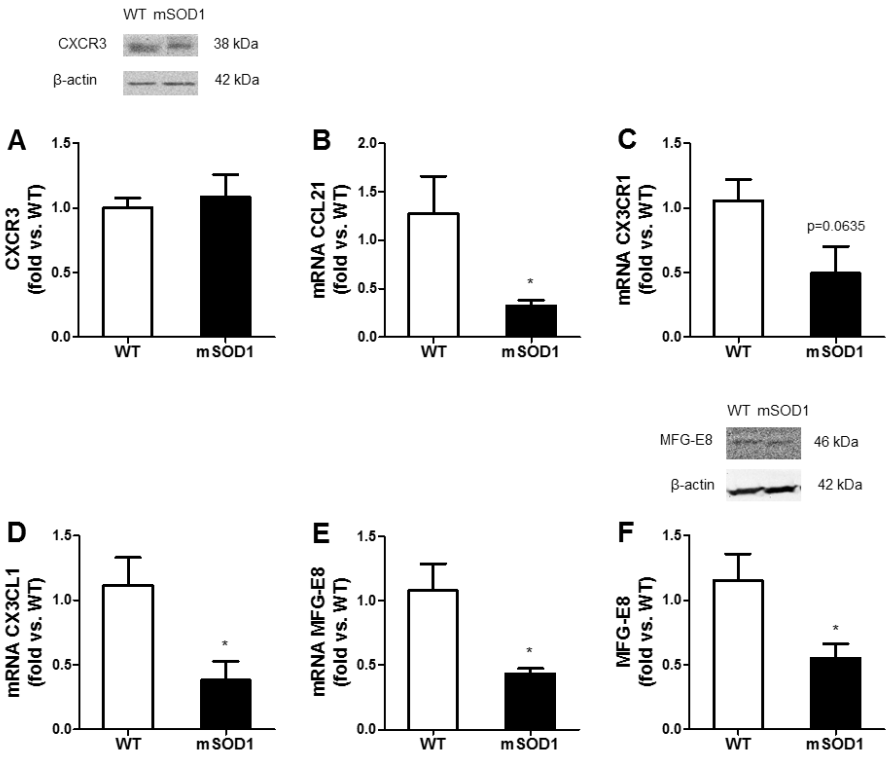


Figure III.4. MN-microglia communication is impaired in the spinal cord (SC) from presymptomatic mSOD1 mice when compared to age-matched control (WT). Samples were obtained as indicated in Methods. Results from CXCR3 (A), mRNA CCL21 (B), mRNA CX3CR1 (C), mRNA CX3CL1 (D), mRNA MFG-E8 (E) and MFG-E8 (F) are expressed as mean (± SEM) from four (CXCR3, CX3CR1 and MFG-E8) and five (CCL21, CX3CL1 and mRNA MFG-E8) independent experiments **p*<0.05 vs. matched WT mice using two-tailed unpaired Student´s *t*-test.

ATX is also involved in microglial migration, alongside proliferation (Perrakis and Moolenaar, 2014). Thus, ATX expression was evaluated. In SC from mSOD1 mice, Figure III.5 shows that ATX levels are decreased compared to control samples. This seems to indicate an impairment in cellular migration and also could provide a potentiation in neuroinflammation as overexpression of ATX inhibits microglial activation and migration (Awada *et al.*, 2012).

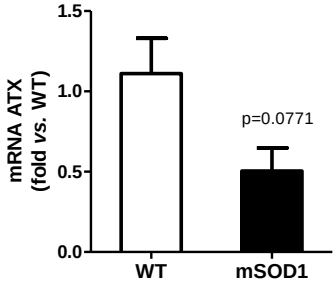


Figure III.5. Autotaxin (ATX) expression showed to be slightly decreased in the spinal cord (SC) from presymptomatic mSOD1 mice when compared to age-matched control (WT). Samples were obtained as indicated in Methods. Results are expressed as mean (± SEM) from at least four independent experiments **p*<0.05 vs. matched WT mice using two-tailed unpaired Student´s *t*-test.

1.4. Inflammation-related markers are altered in SC of mSOD1 mice

Since progressive neuroinflammation is a hallmark of ALS, the study of inflammation-related molecules is imperative. Additionally, our previous results show a lack of neuroprotective capacity which may be related to progressive neuroinflammation. Accordingly, TLR2 a pattern recognition receptor, which can recognize mSOD1 (Zhao *et al.*, 2010), is increased at the presymptomatic stage (Figure III.5.A) possibly indicating an attempt of immune cells, such as microglia, to produce an inflammatory response. Regarding HMGB1, an alarmin that can be released by astrocytes, microglia and neurons (mainly when cells are dying) (Gao *et al.*, 2011; Lo Coco *et al.*, 2007), we found that this alarmin is significantly decreased (p<0.05) in the SC of presymptomatic stages as illustrated by Figure III.6. Protein expression was also evaluated but revealed apparent unaltered levels (0.92 fold, see Annex 1.1). Since HMGB1 can interact with TLR2 due to the decrease of HMGB1, TLR2 could be overcompensating its lack thereof (Casula *et al.*, 2011). Another inflammatory indicator that we have measured is IL-18, which slightly increased at presymptomatic stage (Figure III.6.C). In addition, the inflamasomme component NLRP3 showed a significant decrease (p<0.05) when compared to control values (Figure III.6.D), consequently indicating an impairment of the inflammatory response.

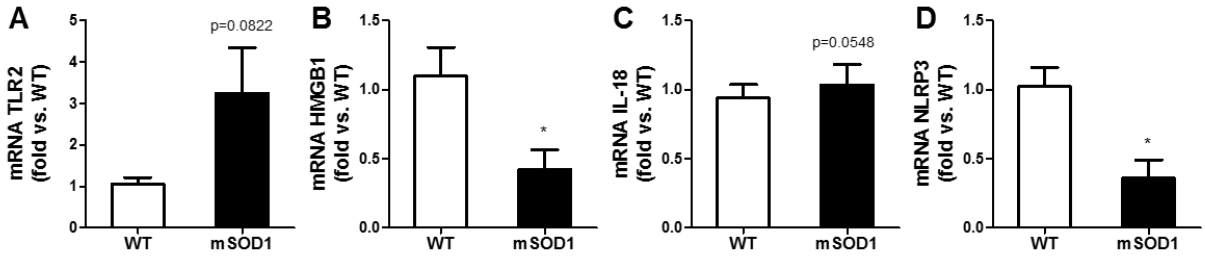


Figure III.6. Inflammatory-related biomarkers are altered in the spinal cord (SC) from mSOD1 mice when compared to age-matched control (WT). Results from mRNA TLR2 (A), mRNA HMGB1 (B), mRNA IL-18 (C) and mRNA NLRP3 (D) are expressed as mean (± SEM) from four independent experiments *p<0.05 vs. matched WT mice using two-tailed unpaired Student´s *t*-test.

Altogether these results indicate lack of glial cell activation and damaged cellular communication with an affected inflammatory response.

2. Spinal cord from mSOD1 mice at symptomatic stage is characterized by an exacerbated inflammatory environment

The transition from the presymptomatic phase to the symptomatic phase is accompanied by a number of alterations both at clinical and molecular levels (Turner and Talbot, 2008). Following the same rationale of the previous section, we investigated possible alterations in the same aspects of the symptomatic stage.

2.1. SOD1 expression is increased in SC of mSOD1 mice

As illustrated in Figure III.7, we observed higher SOD1 protein levels in mSOD1 mice than in those from WT expression (p<0.05).

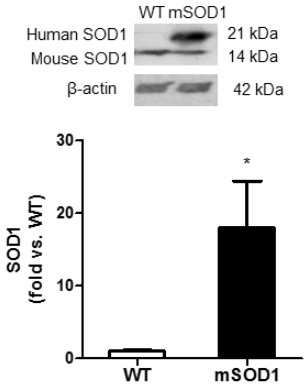


Figure III.7. SOD1 expression is increased in the spinal cord (SC) from symptomatic mSOD1 mice when compared to aged-matched control (WT). Samples were obtained as indicated in Methods. Results are expressed as mean (± SEM) from six independent experiments *p<0.05 vs. matched WT mice using two-tailed unpaired Student´s t-test.

2.2. Glial cell activity, communication and proliferation capacities are increased in SC of mSOD1 mice at symptomatic stage

Regarding cellular markers, namely astrocytic marker GFAP, we observed a significant increase in mRNA levels (Figure III.8.A, p<0.05 *vs.* WT), but a decrease was found for protein expression (Figure III.8.B, p<0.05 vs. WT). This lower protein content may possibly indicate the presence of an astrocyte population with an aberrant phenotype, as if has been suggested by some authors in mSOD1 rats (Diaz-Amarilla *et al.*, 2011). Accordingly, mRNA levels of S100B were slightly increased as compared to control samples (Figure III.8.C).

In addition, as illustrated in Figure III.8.D, mRNA expression of CD11b was also markedly increased (p<0.01 vs. WT), suggesting that microglia is also activated during the symptomatic stage of the disease.

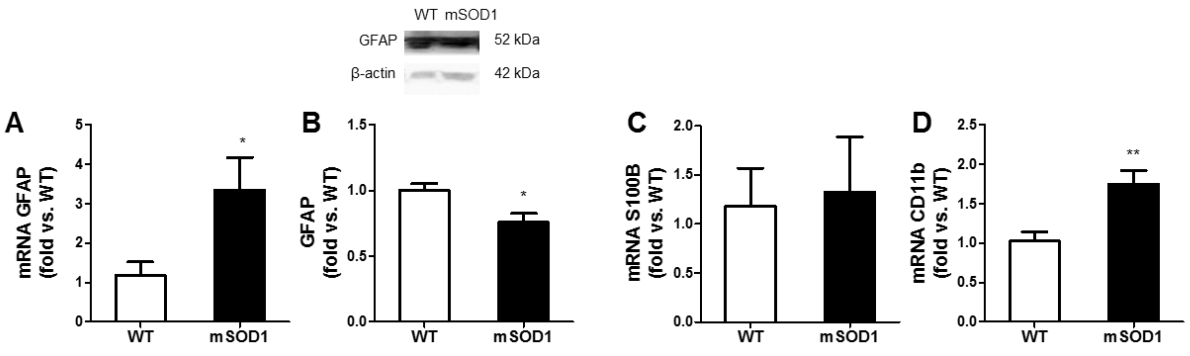


Figure III.8. Expression of markers for glial activation are increased in symptomatic stage of mSOD1 mice when compared to aged-matched controls (WT). Samples were obtained as indicated in Methods. Results of mRNA GFAP (A), GFAP (B), mRNA S100B (C) and mRNA CD11b (D) are expressed as mean (± SEM) from four (GFAP and S100B) and six (CD11b) independent experiments *p<0.05 vs. matched WT mice using two-tailed unpaired Student´s t-test.

Furthermore, we found increased mRNA expression of both Cx43 and PAN1 of symptomatic mSOD1 mice (Figures III.9.A and B), suggesting larger communication between cells at this stage. Expression of mRNA Ki-67 was also elevated (Figure III.9.C), indicating an increase in cellular proliferation.

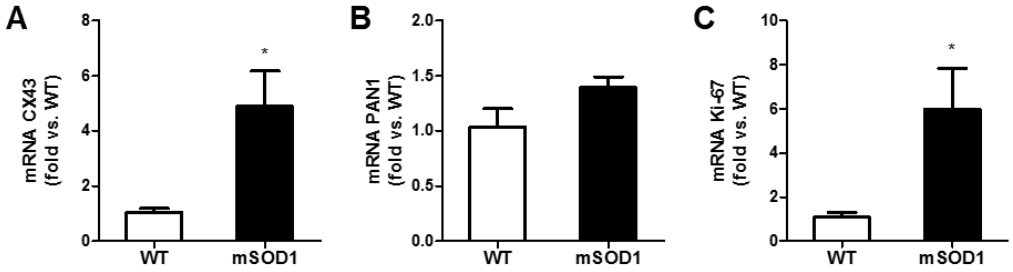


Figure III.9. Connexin 43 (Cx43) and proliferation marker Ki-67 are decreased in the spinal cord (SC) from symptomatic mSOD1 mice compared to control (WT), but no changes were noticed in Pannexin 1 (PAN1). Samples were obtained as indicated in Methods. Results of mRNA Cx43 (A), mRNA PAN1 (B) and mRNA Ki-67 (C) are expressed as mean (± SEM) from five (Cx43), four (PAN1) and three (Ki-67) independent experiments **p*<0.05 vs. matched WT mice using two-tailed unpaired Student´s *t*-test.

In relation to MN-microglia dialogue, the CXCR3-CCL21 axis was positively affected revealing a slight enhancement of CXCR3 (Figure III.10.B) and mRNA CCL21 (*p*<005) (Figure III.10.B) expression levels. This increase suggests higher microglial mobility towards the site of lesion (Biber *et al.*, 2007).

Likewise, the CX3CR1-CX3CL1 axis was also significantly increased in mSOD1 *vs.* WT as depicted in Figures III.10.C and D (*p*<0.05), respectively, suggesting an attempt to modulate the inflammatory microglial response (Briones *et al.*, 2014). Additionally, both mRNA and protein expression levels of MFG-E8 (Figures III.10.E and F, respectively) were also increased in mSOD1 mice, proposing higher microglial phagocytic capacity at symptomatic stage (Appel *et al.*, 2011).

2.3. MN-microglia signaling is increased in SC of mSOD1 at S stage

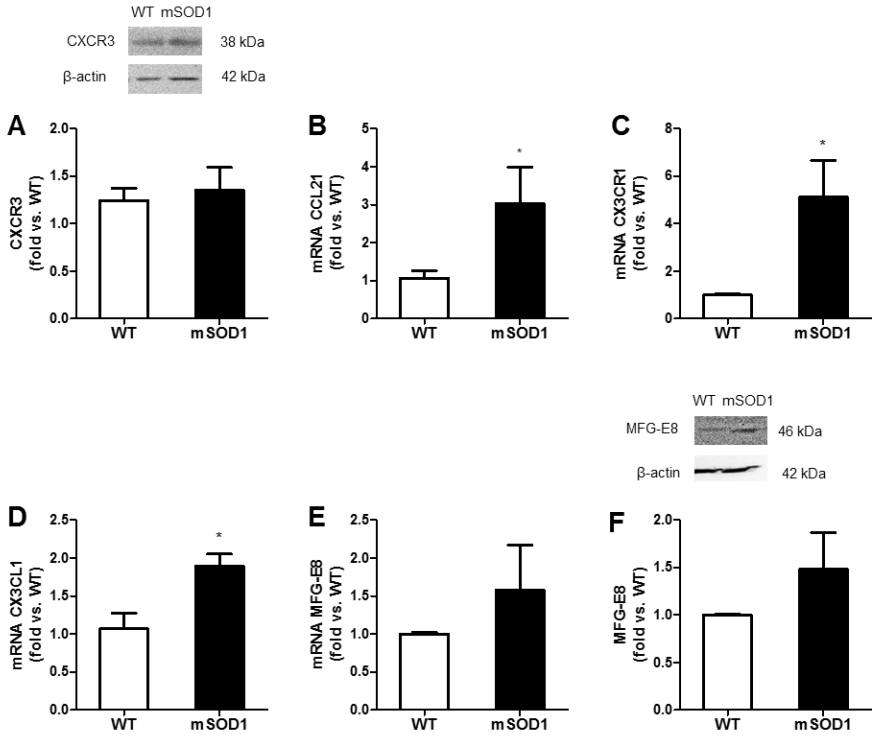


Figure III.10. MN-microglia communication is increased in the spinal cord (SC) from symptomatic mSOD1 mice when compared to aged-matched controls (WT). Samples were obtained as indicated in Methods. Results from CXCR3 (A), mRNA CCL21 (B), mRNA CX3CR1 (C), mRNA CX3CL1 (D), mRNA MFG-E8 (E) and MFG-E8 (F) are expressed as mean (± SEM) from three (CXCR3 and MFG-E8), four (CX3CL1, CCL21 and mRNA MFG-E8) and five (CX3CR1) independent experiments *p<0.05 vs. matched WT mice using two-tailed unpaired Student´s *t*-test.

Regarding the evaluation of cellular migration and proliferation, we observed in Figure III.10 that there was a rise in mRNA expression of ATX. This shift could be an attempt to promote inhibition of microglial activation, as well as to confer higher oxidative stress protection (Awada *et al.*, 2012).

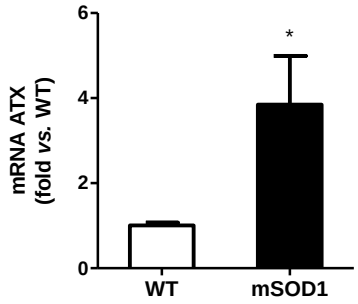


Figure III.11. Autotaxin expression is increased in the spinal cord (SC) from symptomatic mSOD1 mice compared to aged-matched controls (WT). Samples were treated as indicated in Methods. Results are expressed as mean (± SEM) from four independent experiments *p<0.05 vs. matched WT mice using two-tailed unpaired Student´s *t*-test.

2.4. Inflammation-related markers are altered in SC of mSOD1 mice at symptomatic stage

Finally, addressing neuroinflammation-related molecules we show in Figure III.12.A a slight decrease mRNA expression of TLR2 in symptomatic mSOD1, however other inflammatory markers were found be highly increased. Indeed, mRNA HMGB1 levels were significantly higher in symptomatic mSOD1 mice than in WT (p<0.01 vs. WT, Figure III.6.B), as well as HMGB1 protein expression (~1.35 fold, see Annex 1.2). Analysis of IL-18 mRNA levels revealed a slight increase (Figure III.12.C), which was even higher for mRNA expression of NRLP3 (Figure III.12.D) suggesting the inflammasome activation at symptomatic phase of mSOD1 mice.

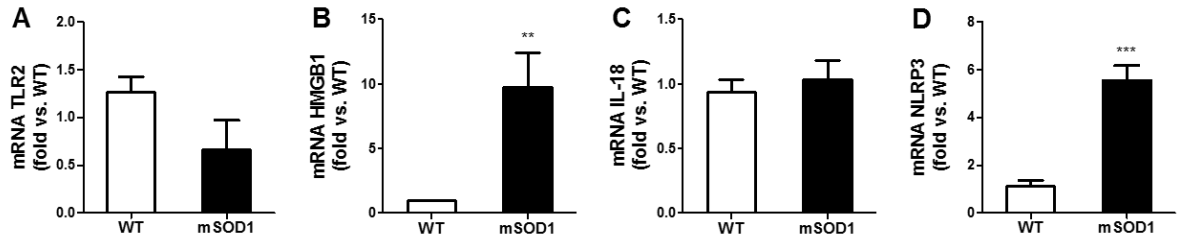


Figure III.12. Inflammatory-related biomarkers are altered in the spinal cord (SC) from symptomatic mSOD1 mice compared to aged-matched controls (WT). Results from mRNA TLR2 (A), mRNA HMGB1 (B), mRNA IL-18 (C) and mRNA NLRP3 (D) are expressed as mean (± SEM) from four independent experiments *p<0.05 vs. matched WT mice using two-tailed unpaired Student´s t-test.

Altogether, analysis of the SC from mSOD1 at symptomatic stage of ALS showed an increased cellular activation and proliferation concomitant with elevated hemichannel activity, MN-microglia dialogue and inflammation-related molecules. These alterations seem to potentiate the pro-inflammatory state characteristic of the symptomatic phase of ALS.

3. Evaluation of molecular markers variation along ALS pathoprogression

After studying each individual phase, we performed an evaluation of the variation of each molecular marker along the progression of the disease. This analysis was accomplished by matching mSOD1 from symptomatic stage against mSOD1 results from the pre-symptomatic stage and using one-way ANOVA followed by Bonferroni's multiple comparison test. These results are summarized in Table III.1.

Table III.1. Variation of different markers between the presymptomatic and symptomatic stages of mSOD1 mice vs. aged-matched controls (WT). Results are expressed as mean (± SEM) from at least three independent experiments *p<0.05, **p<0.005 and ***p<0.001 *vs.* matched mSOD1 mice using one-way ANOVA followed by Bonferroni's multiple comparison test. ↑, ↓ refer to an increase and decrease, respectively, of each molecule vs. respective control, with number of arrows referring to greater or lesser parameter variation.

Molecule	***Presymptomatic***	***Symptomatic***	***Statistical variation between stages***
SOD1 (protein)	↑↑	↑↑↑	
Cellular marker			
GFAP (mRNA/protein)	↓/↓	↑↑/↓	**
S100B (mRNA)	↓	↑	
CD11b (mRNA)	↓	↑	***
Hemichannel activity			
CX43 (mRNA)	↓	↑↑	***
PAN1 (mRNA)	↓	↑	**
Ki-67 (mRNA)	↓	↑↑	*
MN-microglia dialogue			
CXCR3 (protein)	↑	↑	
CCL21 (mRNA)	↓	↑↑	**
CX3CR1 (mRNA)	↓	↑↑	**
CX3CL1 (mRNA)	↓	↑	***
MFG-E8 (mRNA/protein)	↓/↓	↑/↑	*
Proliferation and migration marker			
ATX (mRNA)	↓	↑↑	**
Inflammatory-associated molecules			
TLR2 (mRNA)	↑↑	↓	
HMGB1 (mRNA)	↓	↑↑	***
IL-18 (mRNA)	↑	↑	
NLRP3 (mRNA)	↓	↑↑	***

These results show increasingly SOD1 accumulation in mSOD1 mice during disease progression. During the transition from a presymptomatic stage to the symptomatic stage there is a change in mRNA levels of both astrocytes (GFAP p<0.005) and microglia markers (CD11b p<0.001) indicating a shift from an inactivated stage during presymptomatic stage and activated during symptomatic phase.

In the same manner, hemichannels components (Cx43 $p<0.001$; PAN1 $p<0.005$) and proliferation marker Ki-67 ($p<0.05$) also present elevated expression when compared to control values at symptomatic stage. Accordingly, MN-microglia dialogue also present a turnover where communication is increased at symptomatic stage. Specifically, CCL21 ($p<0.005$), CX3CR1-CX3CL1 ($p<0.005$ and $p<0.001$, respectively) and MFG-E8 ($p<0.05$) are significantly increased. Similarly, ATX changes from decreased levels at presymptomatic stage to an overexpression during the later stages of the disease ($p<0.005$). We also observed an increase of inflammatory factors namely HMGB1 ($p<0.001$) and NLRP3 ($p<0.001$) at symptomatic stage.

Altogether these results distinguish two different roles for glial cells and inflammatory response between stages, with the presymptomatic stage presenting lack of glial cell activation and communication ability, impaired MN-microglia dialogue and inflammation incapacity. The symptomatic stage, however shows increased glial activation and communication together with high proliferation capacity, improved MN-microglia dialogue and strong inflammatory response.

IV. DISCUSSION

Amyotrophic lateral sclerosis is a neurodegenerative disease characterized by its progressive loss of MNs. Causes for ALS comprise both genetic and sporadic components and several studies have identified a role of glial cells for the pathoprogression of the disease, which makes ALS a very complex and multifactorial disease. The study model of SOD1G93A (mSOD1) mice have provided great insights into the mechanisms of disease progression (Gurney *et al.*, 1994; Turner and Talbot, 2008). As such, in the present work, we resorted to this mSOD1 model in order to evaluate mRNA and protein expression levels of several cellular markers with a main emphasis on glial function, as well as known MN-microglial communication pathways and molecular indicators inflammation. Our approach focused on the characterization of both presymptomatic and symptomatic disease stage in mSOD1 mice, based on the study of each individual phase as well as on determination of the evolution of such biomarkers along disease progression.

We first observed a significant increase of SOD1 protein expression in samples from the SC of mSOD1 mice at both phases of the disease, with a progressive accumulation of SOD1 levels with the course of the disease. Previous studies using SOD1G93A1 mice have also shown progressive accumulation of human SOD1 in the spinal cord through Northern Blot and immunocytochemistry analysis (Cheroni *et al.*, 2005). Abnormal protein folding and aggregation of SOD1, along with other proteins previously addressed in the Introduction section of this thesis, are a common pathological feature of ALS (Gurney *et al.*, 1994; Vaz *et al.*, 2014). These aggregates have been suggested as harmful through the sequestration of proteins required for normal MN function; reducing availability of chaperones due to repeatedly misfolding; reducing proteasome activity and lead to inhibition of the function of specific organelles (e.g. mitochondria) by aggregation on or within these organelles (Shaw, 2005). As such, constant accumulation could aggravate these insults. Additionally, identification of extracellular mSOD1 inducing microglial-mediated MN injury through stimulation of TLR2/4 and CD14 triggering microgliosis and pro-inflammatory response in primary cultures of MNs and microglia (Zhao *et al.*, 2010) could be factors potentiating MN injury due to increasing mSOD1 accumulation. mSOD1 can be secreted through chromogranin mediation has also been linked to ALS (Urushitani *et al.*, 2006). Further studies should focus the state of aggregation of SOD1 in our model being a characteristic of ALS pathophysiology (Higgins *et al.*, 2003).

In order to evaluate glial cell response associated with SOD1 accumulation, we focused on cellular markers of astrocytes and microglia. Regarding astrocytes, our results show decreased mRNA and protein expression of the astrocytic marker GFAP, during presymptomatic stage of mSOD1 mice but increased levels of mRNA at symptomatic stage, suggesting that astrocytes are being differently modulated as disease progresses. In fact, astrocytes are known to release trophic factors to protect neurons at the very beginning of insult (Gomez-Nicola *et al.*, 2010) but they also may participate in the inflammatory response at later phases (Evans *et al.*, 2013). The diminished levels of GFAP, together with lessened S100B, a known marker of astroglial activation (Sofroniew and Vinters, 2010), found at presymptomatic stage probably indicate that at this time point, astrocytes lack of response capacity

from astrocytes. However, during the symptomatic phase, we found increased levels of S100B and GFAP mRNA expression, along with decreased protein expression of GFAP. Higher levels of S100B, together with Cx43 and PAN1 at this stage indicate astroglisosis. This phenotypic variation may be due to the previous results obtained by Diaz-Amarilla and colleagues (2011) (Diaz-Amarilla *et al.*, 2011) where they identified in primary SC cultures of symptomatic rats expressing SOD1G93A a phenotypically aberrant population of astrocytes in which they have increased S100B expression and Cx43 and decreased expression of GFAP, in which they lack expression of GLT-1. In addition, it is important to notice that at symptomatic stage, GFAP expression is only up-regulated in mSOD1 mice at the level of gene expression. The fact that it doesn't translate into protein is probably a result of altered mRNA processing, a proposed altered mechanism in ALS pathophysiology. (Ferraiuolo *et al.*, 2011). Another work observed a phenotypic transition of microglia into astrocyte-like cells associated with disease onset in a model of inherited ALS (SOD1G93A rats) (Trias *et al.*, 2013), however it is hard to precisely pinpoint these alterations since we have no way to tell from which cells these GFAP alterations result. Another relevant study was done by Yoshii and collaborators (Yoshii *et al.*, 2011) where they identified that the loss of GFAP marginally accelerates disease progression in SOD1H46R, as a consequence, the loss of GFAP we observed could also be contributing to disease progression, Regarding S100B expression, we found their levels diminished during presymptomatic stage of ALS suggesting lack of astrocytic proliferation at this stage (Brozzi *et al.*, 2009). On the other hand, increased S100B expression observed at symptomatic stage may result in higher astrocytic migration in mSOD1 mice. Also, as previously mentioned, phenotypically aberrant astrocytes overexpress S100B which could contribute to the increased levels we observed in our results, together with high Cx43 expression, and suggest the presence of an immature astrocytic phenotype at the symptomatic phase (Diaz-Amarilla *et al.*, 2011).

Regarding microglial activation, our results show down-regulation of mRNA expression of CD11b during the presymptomatic stage of ALS and a significant shift to higher levels of CD1b in mSOD1 than control at the symptomatic phase. These results are in accordance with microglial activation and microgliosis characterized in ALS and the inflammatory state observed at the symptomatic phase (Liao *et al.*, 2012). Altogether, these results indicate a prevalence of activated glial cells only at symptomatic stage of ALS.

In relation to connexins, our results show a significant decrease of mRNA expression levels of Cx43 during the presymptomatic stage of the disease. This supports the previous results of both GFAP and S100B results suggesting lack of astrocytic response during this phase of the disease. Some studies suggested that upregulation of Cx43 can be correlated with microglia activation (Kielian, 2008), which is also in accordance with our results showing lack of activation on both astrocytes and microglia in mSOD1 at presymptomatic stage. In addition, we were able to detect increased mRNA expression of Cx43 in mSOD1 at symptomatic stage, corroborating our assumption that at this time point there is an increase of glial activation. This is not without precedent since Cx43 is primarily expressed in astrocytes and its up-regulation leads to a reactive astroglisosis (Theodoric *et al.*, 2012). Similarly, levels of PAN1, which form hemichannel allowing for cell-cell communication and has a predominant neuronal origin (Barbe *et al.*, 2006), were significantly decreased in presymptomatic

mSOD1, although slightly increased at symptomatic phase. Since these channels are permeable to ATP, allow for propagation of Ca^{2+} waves and play a role in the release of "find-me" signals of apoptotic cell clearance (Chekeni *et al.*, 2010), we may assume that there is a deregulation of cellular communication that may also lead to diminished phagocytic abilities. In addition, we evaluated the mRNA expression of the proliferation marker, Ki-67 (Kreitz *et al.*, 2000). Similarly to Cx43 and PAN1, Ki-67 mRNA expression was decreased during the presymptomatic phase of ALS. Taken all these markers into account, our results infer lack of proliferating capacity during the earlier stages of the disease as well as lack of intercellular communication. At the symptomatic stage we identify a shift towards up-regulation of mRNA expression compared to control values. This suggests a positive turnover between cell-cell communication from a down-regulation at the presymptomatic stage, to an increase at the symptomatic phase. Namely, higher PAN1 expression levels suggest that there might be an increase of transmission of "find-me" signals for apoptotic clearance (Chekeni *et al.*, 2010). Additionally, the overexpression of Ki-67 and Cx43 seem to have positive effects in microglial activation and proliferation capacity (Homkajorn *et al.*, 2010; Kreitz *et al.*, 2000), hence contributing to a pro-inflammatory response at the symptomatic phase.

Having identified a putative role for microglial activation in the pathophysiology of ALS, we explored known communication pathways between these cells and MNs. As such, we focused in the CXCR3-CCL21 axis, which has never been studied in the context of ALS. We showed for the first time that at presymptomatic phase there is unaltered protein expression of CXCR3 and significant decrease in mRNA levels of CCL21, however at symptomatic stage we observed a huge increase in CCL21 in mSOD1 mice. CXCR3, a microglial receptor for CCL21 whose stimulation by CCL21 induces activation (van Weering *et al.*, 2010) suggests that at the presymptomatic stage this axis of communication is impaired, therefore not producing a microglial response at this stage. Since neurons express CCL21 in response to injury (Biber *et al.*, 2001), these results suggest an alteration between the MN-microglia communication, where MNs are not able to release an injury response through chemokine CCL21. We can also extrapolate that there would be no astrocytic response due to low levels of CCL21 mRNA, since these cells are produce a response only when at higher CCL21 concentrations (van Weering *et al.*, 2010). Due to its chemotactic properties induced by the presence CCL21 (Biber *et al.*, 2008), we can deduce that microglial mobility is compromised through this pathway during the presymptomatic phase of ALS. However, we found significantly increased levels of CCL21 in symptomatic phase, which, accordantly with the results obtained for Cx43, CD11b and Ki-67, indicate higher microglia proliferation that may promote an inflammatory response to MN injury. Additionally, since CCL21 is able to interact with astrocytic receptor CCR7, which is found up-regulated after inflammatory injury inducing astrocytic response (Gomez-Nicola *et al.*, 2010), and associating this knowledge with observed astroglisosis in our studies, CCR7 may also be considered a good candidate to evaluate in order to assess whether CCL21 is affecting more microglial or astrocytic response at the symptomatic phase of ALS.

Another described axis of communication between MNs and microglia is CX3CR1-CX3CL1. Our results show diminished mRNA expression of both CX3CL1 and its receptor during the presymptomatic stage. This indicates that this axis might be severely compromised during this phase as not only

MNs have decreased capacity for releasing chemokines but as well as the capacity for microglia to recognize such signals and produce a neuroprotective response (Harrison *et al.*, 1998). This impairment is concomitant with our previously evaluated microglial response and proliferative factors at this stage. Nonetheless, during the symptomatic phase, we observed increased mRNA expression representing an increase in MN-microglial dialogue. Since higher levels of CX3CL1 could contribute to an increased inflammatory response due to its chemotactic ability and drawing activated microglia towards the site of injury, our results identify microglial response at this stage as the probable cause to the inflammation also observed. Furthermore, MN-microglia communication studies were focused on microglial release of MFG-E8, which recognizes phosphatidylserine as an "eat-me" signal expressed on the surface of apoptotic neurons (Fricker *et al.*, 2012). Our results showed a significant decrease of both mRNA and protein expression of MFG-E8 during the presymptomatic phase of ALS, which may be related to lack of PAN1 hemichannels releasing "eat-me" signals, also required for phagocytosis (Chekeni *et al.*, 2010). This result also proposes a damaged phagocytic response from microglia that will interfere with their communication with MNs and contribute to an overall diminished neuroprotective environment at the presymptomatic stage. However, MFG-E8 levels are increased at symptomatic stage, thus suggesting an increase in microglia phagocytic ability. Also, studies show that CX3CL1 up-regulates MFG-E8 expression (Leonardi-Essmann *et al.*, 2005), which, together with higher levels of PAN1, our results show an increased phagocytic response, for which microglia activation and migration is required. Nevertheless, we may not conclude that the effect of a phagocytic microglia at symptomatic stage contributes to a neuroprotective effect, since microglia activation was also observed at this time, as well as an inflammatory environment, as it will be further detailed in this chapter.

One of the possible intervenients that mediate this lack of microglial neuroprotection is autotaxin (ATX). ATX is involved in cellular processes like migration, proliferation and survival of various cells (Perrakis and Moolenaar, 2014). Since ATX promotes the conversion of LPC to LPA whose receptors are expressed by microglia, astrocytes and oligodendrocytes, this molecule has been pointed to play a key role in glial response to injury (Awada *et al.*, 2012). Our results show decreased mRNA expression of ATX during the presymptomatic phase which suggests lack of cellular migration ability in agreement with our previously obtained results for this stage of ALS. ATX levels also indicate a shift between the presymptomatic and symptomatic phases, where we verify a significant increase in ATX mRNA expression after symptom onset which infer higher migration and proliferation capacity at this stage. These results support the alterations we identified for hemichannel components and MN-microglia communication, where migration and proliferation markers are also up-regulated. Additionally, studies have shown that secretion of ATX by microglial cells is stimulated by oxidative stress and that ATX overexpression reduces microglial ROS-mediated damage and microglial activation (Awada *et al.*, 2012). As such, we might be observing an attempt to preserve microglial viability in detriment to an aggravation of inflammatory response also observed.

Having identified a pattern of glial cell activation in our model, we further explored inflammation markers, since it may be considered a hallmark of ALS pathophysiology (Philips and Robberecht, 2011). Our results show increased mRNA expression of TLR2 during the presymptomatic phase

which, combining with increased levels of SOD1 could be triggering an inflammatory response (Zhao *et al.*, 2010).TLR2 is a pattern recognition receptor capable of recognizing mSOD1 aggregates as well as the alarmin HMGB1 and triggering an inflammatory cascade response. Studies using *post-mortem* human spinal cord cultures from ALS patients have shown increased TLR2, mainly in microglia lineage, and HMGB1 mRNA expression levels at end stage of the disease (Casula *et al.*, 2011). Liu et al. (2009) have shown that the expression of mSOD1 increases the neurotoxic potential of microglia via TLR2 (Liu *et al.*, 2009). However, at the symptomatic stage we observe a slight decrease in TLR2 expression, which may indicate that the potentiation of the inflammatory response does occur through this pathway.

Regarding HMGB1, we found decreased mRNA expression levels together with seemingly unaltered protein levels at presymptomatic stage. This molecule can act as a transcription factor for inflammatory response genes or be released to the extracellular medium and act upon TLR2/4 or RAGE receptors prompting an inflammatory response (Muller *et al.*, 2004). As such, during the presymptomatic phase this lack of mRNA HMGB1 may not translate in lack of activation of inflammation due to maintained levels of protein. On the other hand, during the symptomatic phase these expression levels are increased which may potentiate the inflammatory response observed. Since TLR2 expression levels are decreased during this phase, it's likely that HMGB1 is primarily acting on TLR4 and RAGE. Interestingly, according to Casula *et. al* (2011), TLR4 is primarily expressed in astrocytes (Casula *et al.*, 2011), which suggest that in our model TLR4 is probably more activated than TLR2. Further studies taking into account both receptors will add our assumptions in order to better understand the modulation through HMGB1 in our model.

Our results showed IL-18 mRNA levels to be slightly increased during the presymptomatic phase which could mean a slight attempt at an inflammatory response. IL-18 is an inflammatory cytokine that induces activation of Th1 cells and production of INF-γ. One of the inflammatory markers found in the CNS of ALS mice is active caspase-1, which is activated in response to danger signals by inflammasomes and proteolytically matures IL-1β and IL-18 (Meissner *et al.*, 2010). After the transition to the symptomatic phase IL-18 expression levels only slightly increased, which suggest that the aggravation of the response may not be occurring through this pathway.

Nevertheless, NLRP3 evaluation showed a significant decrease in mRNA expression in presymptomatic mSOD1 mice. NLRP3 inflammasome can be induced by numerous stimuli, namely ROS. NLRP3 inflammasome can induce, through caspase-1, IL-18 and IL-1β maturation, the latter being a potent inflammatory factor (Heneka *et al.*, 2013). Altogether, these results suggest a deficiency of inflammatory response during the presymptomatic stage. However, during the symptomatic stage we observed a significant increase in the expression levels of NLRP3 indicating marked pro-inflammatory response involving the formation of inflammasome components at this phase. Posterior measurement of IL-1β expression levels should be performed in order to better assess NLRP3 effects. Overall, these results suggest a pro-inflammatory environment in symptomatic mSOD1 mice, and that glia reactivity and MN-glial communication pathways may be aggravating this inflammatory response.

Chapter IV. DISCUSSION

In conclusion and as schematically represented in Figure IV.1, our results show a distinct transition from a presymptomatic stage characterized by severe impairment of communication, glial and inflammatory responses to symptomatic phase where there is a pattern of glial activation with marked increase of communication pathways and proliferation factors, together with striking intensification of neuroinflammatory response. Altogether, these results allow for a clear distinction between both stages of the disease, which bring new insights to target specific molecules that lead to neurodegeneration in ALS. Interestingly, we hypothesize that by increasing autotaxin expression we may be able to delay ALS onset, before considering anti-inflammatory compounds to fight disease progression.

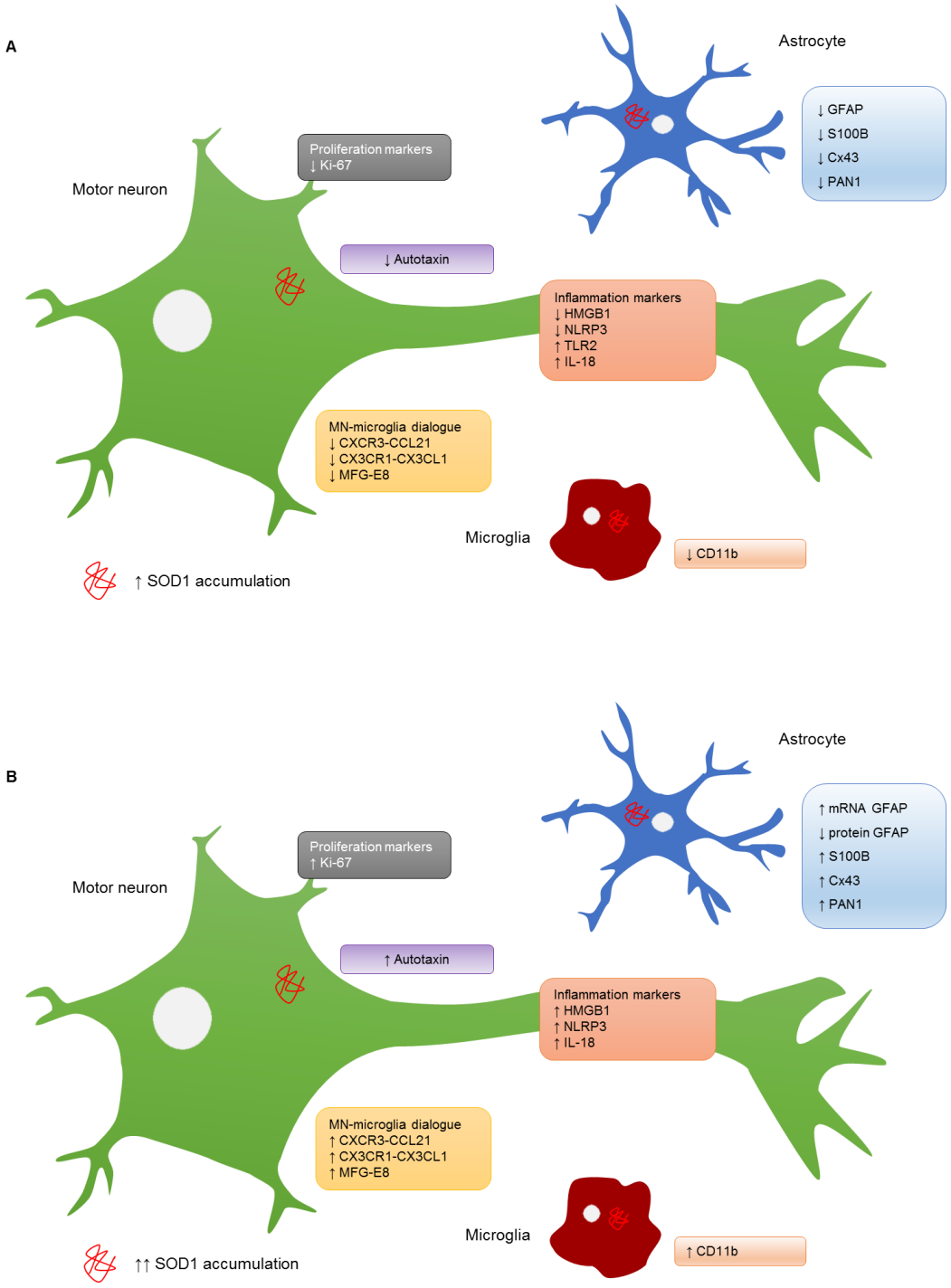


Figure IV.1. Schematic representation of the major findings of this Master Thesis. (A) Presymptomatic stage of amyotrophic lateral sclerosis (ALS) in mSOD1 mice evidence an increase in SOD1 accumulation. Glial cell markers show a decrease in astrocytic markers glial fibrillary protein (GFAP) and S100B and in microglial marker complement receptor CR3 (CD11b). Gap-junction components Connexin 43 (Cx43) and Pannexin 1 (PAN1) are negatively affected. Motoneuron (MN)-microglia dialogue pathways, namely Chemokine (C-C motif) ligand 21 (CCL21) and chemokine (C-X-C motif) receptor 3 (CXCR3), CX3C chemokine receptor 1 (CX3CR1) Chemokine (C-X3-C motif) ligand 1 (CX3CL1) and milk fat globule factor-E8 (MFG-E8) is impaired. Proliferation marker Ki-67 and autotaxin levels are diminished. Inflammation-related molecules high mobility group box 1 (HMGB1) and NOD-like receptor family, pyrin domain containing 3 (NLRP3) are negatively affected, while Toll-like receptor 2 (TLR2) and Interleukin 18 (IL-18) are increased. **(B)** The symptomatic stage of ALS shows high accumulation of SOD1. Astrocytic (GFAP and S100B) and microglial (CD11b) markers are increased. Gap-junction components Cx43 and PAN1 are elevated. MN-microglia dialogue pathways CXCR3-CCL21; CX3CR1-CX3CL1 and MFG-E8 are increased. Proliferation marker Ki-67 and autotaxin levels are elevated. Inflammation-related molecules IL-18, HMGB1 and NLRP3 expression levels are increased.

Future Perspectives

In our study we were able to identify key pathophysiological aspects of the ALS using the mSOD1 study model. Further investigation aims to evaluate new cellular and molecular markers that will provide insight into (de)differentiation state of glial cells along with better understanding of the molecular pathways involved in ALS onset and progression. Additional work should focus on glutamate transporters, due to glutamate excitotoxicity being described an important cause for neuronal death. As well as emphasis in inflammatory-related markers such as IL-1β and NF-κB for better understanding of the major aspects of the inflammatory pathway are altered.

Moreover, studies require the evaluation of these molecules through an immunohistochemistry approach in order to assess proliferation patterns and communication pathways involved as well as phenotypic characterization of the cells studied.

Finally, our approach could be further applied to other known brain regions affected in ALS, namely the motor cortex which could provide a better understanding of the location where the disease begins (Eisen and Weber, 2001) as there is controversy on “dying back” versus “dying forward” mechanisms leading to the disease (Redler and Dokholyan, 2012). As these studies could provide better understanding of the development of cortical and spinal motor neuron populations, their interaction with each other, and how it changes in ALS as well as the identification of the earliest, presymptomatic characteristics of ALS possibly providing new biomarkers for earlier diagnosis.

V. BIBLIOGRAPHY

Abel, O. (2014). The Amyotrophic Lateral Sclerosis Online Genetics Database (ALSoD).

Ackerley, S., Grierson, A.J., Banner, S., Perkinton, M.S., Brownlees, J., Byers, H.L., Ward, M., Thornhill, P., Hussain, K., Waby, J.S.*, et al.* (2004). p38alpha stress-activated protein kinase phosphorylates neurofilaments and is associated with neurofilament pathology in amyotrophic lateral sclerosis. Molecular and cellular neurosciences *26*, 354-364.

Al-Chalabi, A., and Hardiman, O. (2013). The epidemiology of ALS: a conspiracy of genes, environment and time. Nature reviews Neurology *9*, 617-628.

Alami, N.H., Smith, R.B., Carrasco, M.A., Williams, L.A., Winborn, C.S., Han, S.S., Kiskinis, E., Winborn, B., Freibaum, B.D., Kanagaraj, A.*, et al.* (2014). Axonal Transport of TDP-43 mRNA Granules Is Impaired by ALS-Causing Mutations. Neuron *81*, 536-543.

Allen, N.J., and Barres, B.A. (2005). Signaling between glia and neurons: focus on synaptic plasticity. Current opinion in neurobiology *15*, 542-548.

Angenstein, F., Niessen, H.G., Goldschmidt, J., Vielhaber, S., Ludolph, A.C., and Scheich, H. (2004). Age-dependent changes in MRI of motor brain stem nuclei in a mouse model of ALS. Neuroreport *15*, 2271-2274.

Appel, S.H., Zhao, W., Beers, D.R., and Henkel, J.S. (2011). The microglial-motoneuron dialogue in ALS. Acta Myol *30*, 4-8.

Atkin, J.D., Farg, M.A., Turner, B.J., Tomas, D., Lysaght, J.A., Nunan, J., Rembach, A., Nagley, P., Beart, P.M., Cheema, S.S.*, et al.* (2006). Induction of the unfolded protein response in familial amyotrophic lateral sclerosis and association of protein-disulfide isomerase with superoxide dismutase 1. The Journal of biological chemistry *281*, 30152-30165.

Atkin, J.D., Farg, M.A., Walker, A.K., McLean, C., Tomas, D., and Horne, M.K. (2008). Endoplasmic reticulum stress and induction of the unfolded protein response in human sporadic amyotrophic lateral sclerosis. Neurobiology of disease *30*, 400-407.

Awada, R., Rondeau, P., Gres, S., Saulnier-Blache, J.S., Lefebvre d'Hellencourt, C., and Bourdon, E. (2012). Autotaxin protects microglial cells against oxidative stress. Free radical biology & medicine *52*, 516-526.

Bakhti, M., Winter, C., and Simons, M. (2011). Inhibition of myelin membrane sheath formation by oligodendrocyte-derived exosome-like vesicles. The Journal of biological chemistry *286*, 787-796.

Barbe, M.T., Monyer, H., and Bruzzone, R. (2006). Cell-cell communication beyond connexins: the pannexin channels. Physiology (Bethesda, Md) *21*, 103-114.

Barber, S.C., Mead, R.J., and Shaw, P.J. (2006). Oxidative stress in ALS: a mechanism of neurodegeneration and a therapeutic target. Biochimica et biophysica acta *1762*, 1051-1067.

Barber, S.C., and Shaw, P.J. (2010). Oxidative stress in ALS: key role in motor neuron injury and therapeutic target. Free radical biology & medicine *48*, 629-641.

Beers, D.R., Henkel, J.S., Xiao, Q., Zhao, W., Wang, J., Yen, A.A., Siklos, L., McKercher, S.R., and Appel, S.H. (2006). Wild-type microglia extend survival in PU.1 knockout mice with familial

amyotrophic lateral sclerosis. Proceedings of the National Academy of Sciences of the United States of America *103*, 16021-16026.

Bendotti, C., and Carri, M.T. (2004). Lessons from models of SOD1-linked familial ALS. Trends in molecular medicine *10*, 393-400.

Bendotti, C., Marino, M., Cheroni, C., Fontana, E., Crippa, V., Poletti, A., and De Biasi, S. (2012). Dysfunction of constitutive and inducible ubiquitin-proteasome system in amyotrophic lateral sclerosis: implication for protein aggregation and immune response. Progress in neurobiology *97*, 101-126.

Bento-Abreu, A., Van Damme, P., Van Den Bosch, L., and Robberecht, W. (2010). The neurobiology of amyotrophic lateral sclerosis. The European journal of neuroscience *31*, 2247-2265.

Bianco, F., Perrotta, C., Novellino, L., Francolini, M., Riganti, L., Menna, E., Saglietti, L., Schuchman, E.H., Furlan, R., Clementi, E.*, et al.* (2009). Acid sphingomyelinase activity triggers microparticle release from glial cells. The EMBO journal *28*, 1043-1054.

Biber, K., Neumann, H., Inoue, K., and Boddeke, H.W. (2007). Neuronal 'On' and 'Off' signals control microglia. Trends in neurosciences *30*, 596-602.

Biber, K., Sauter, A., Brouwer, N., Copray, S.C., and Boddeke, H.W. (2001). Ischemia-induced neuronal expression of the microglia attracting chemokine Secondary Lymphoid-tissue Chemokine (SLC). Glia *34*, 121-133.

Biber, K., Vinet, J., and Boddeke, H.W. (2008). Neuron-microglia signaling: chemokines as versatile messengers. Journal of neuroimmunology *198*, 69-74.

Boillee, S., Vande Velde, C., and Cleveland, D.W. (2006a). ALS: a disease of motor neurons and their nonneuronal neighbors. Neuron *52*, 39-59.

Boillee, S., Yamanaka, K., Lobsiger, C.S., Copeland, N.G., Jenkins, N.A., Kassiotis, G., Kollias, G., and Cleveland, D.W. (2006b). Onset and progression in inherited ALS determined by motor neurons and microglia. Science (New York, NY) *312*, 1389-1392.

Borthwick, G.M., Johnson, M.A., Ince, P.G., Shaw, P.J., and Turnbull, D.M. (1999). Mitochondrial enzyme activity in amyotrophic lateral sclerosis: implications for the role of mitochondria in neuronal cell death. Annals of neurology *46*, 787-790.

Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical biochemistry *72*, 248-254.

Briones, T.L., Woods, J., and Wadowska, M. (2014). Chronic neuroinflammation and cognitive impairment following transient global cerebral ischemia: role of fractalkine/CX3CR1 signaling. Journal of neuroinflammation *11*, 13.

Brites, D., and Vaz, A.R. (2014). Microglia Centered Pathogenesis in ALS: Insights in Cell Interconnectivity. Frontiers in cellular neuroscience *8*.

Brozzi, F., Arcuri, C., Giambanco, I., and Donato, R. (2009). S100B Protein Regulates Astrocyte Shape and Migration via Interaction with Src Kinase: IMPLICATIONS FOR ASTROCYTE DEVELOPMENT, ACTIVATION, AND TUMOR GROWTH. The Journal of biological chemistry *284*, 8797-8811.

Calvo, A.C., Manzano, R., Atencia-Cibreiro, G., Olivan, S., Munoz, M.J., Zaragoza, P., Cordero-Vazquez, P., Esteban-Perez, J., Garcia-Redondo, A., and Osta, R. (2012). Genetic biomarkers for ALS disease in transgenic SOD1(G93A) mice. PloS one *7*, e32632.

Cardona, A.E., Pioro, E.P., Sasse, M.E., Kostenko, V., Cardona, S.M., Dijkstra, I.M., Huang, D., Kidd, G., Dombrowski, S., Dutta, R.*, et al.* (2006). Control of microglial neurotoxicity by the fractalkine receptor. Nature neuroscience *9*, 917-924.

Carri, M.T., Ferri, A., Cozzolino, M., Calabrese, L., and Rotilio, G. (2003). Neurodegeneration in amyotrophic lateral sclerosis: the role of oxidative stress and altered homeostasis of metals. Brain research bulletin *61*, 365-374.

Carriedo, S.G., Yin, H.Z., and Weiss, J.H. (1996). Motor neurons are selectively vulnerable to AMPA/kainate receptor-mediated injury in vitro. The Journal of neuroscience : the official journal of the Society for Neuroscience *16*, 4069-4079.

Casula, M., Iyer, A.M., Spliet, W.G., Anink, J.J., Steentjes, K., Sta, M., Troost, D., and Aronica, E. (2011). Toll-like receptor signaling in amyotrophic lateral sclerosis spinal cord tissue. Neuroscience *179*, 233-243.

Chang, Y., Kong, Q., Shan, X., Tian, G., Ilieva, H., Cleveland, D.W., Rothstein, J.D., Borchelt, D.R., Wong, P.C., and Lin, C.L. (2008). Messenger RNA oxidation occurs early in disease pathogenesis and promotes motor neuron degeneration in ALS. PloS one *3*, e2849.

Chekeni, F.B., Elliott, M.R., Sandilos, J.K., Walk, S.F., Kinchen, J.M., Lazarowski, E.R., Armstrong, A.J., Penuela, S., Laird, D.W., Salvesen, G.S.*, et al.* (2010). Pannexin 1 channels mediate 'find-me' signal release and membrane permeability during apoptosis. Nature *467*, 863-867.

Chen, S., Sayana, P., Zhang, X., and Le, W. (2013). Genetics of amyotrophic lateral sclerosis: an update. Molecular neurodegeneration *8*, 28.

Chen, S., Zhang, X., Song, L., and Le, W. (2012). Autophagy dysregulation in amyotrophic lateral sclerosis. Brain pathology (Zurich, Switzerland) *22*, 110-116.

Cheroni, C., Peviani, M., Cascio, P., DeBiasi, S., Monti, C., and Bendotti, C. (2005). Accumulation of human SOD1 and ubiquitinated deposits in the spinal cord of SOD1G93A mice during motor neuron disease progression correlates with a decrease of proteasome. Neurobiology of disease *18*, 509-522.

Chhor, V., Le Charpentier, T., Lebon, S., Ore, M.V., Celador, I.L., Josserand, J., Degos, V., Jacotot, E., Hagberg, H., Savman, K.*, et al.* (2013). Characterization of phenotype markers and neuronotoxic potential of polarised primary microglia in vitro. Brain, behavior, and immunity *32*, 70-85.

Clement, A.M., Nguyen, M.D., Roberts, E.A., Garcia, M.L., Boillee, S., Rule, M., McMahon, A.P., Doucette, W., Siwek, D., Ferrante, R.J.*, et al.* (2003). Wild-type nonneuronal cells extend survival of SOD1 mutant motor neurons in ALS mice. Science (New York, NY) *302*, 113-117.

Colombrita, C., Onesto, E., Tiloca, C., Ticozzi, N., Silani, V., and Ratti, A. (2011). RNA-binding proteins and RNA metabolism: a new scenario in the pathogenesis of Amyotrophic lateral sclerosis. Archives italiennes de biologie *149*, 83-99.

Comi, G.P., Bordoni, A., Salani, S., Franceschina, L., Sciacco, M., Prelle, A., Fortunato, F., Zeviani, M., Napoli, L., Bresolin, N.*, et al.* (1998). Cytochrome c oxidase subunit I microdeletion in a patient with motor neuron disease. Annals of neurology *43*, 110-116.

Chapter V. BIBLIOGRAPHY

Cooper, G.M., and Hausman, R.E. (2004). The Cell: A Molecular Approach (ASM Press).

Cui, Y., Masaki, K., Yamasaki, R., Imamura, S., Suzuki, S.O., Hayashi, S., Sato, S., Nagara, Y., Kawamura, M.F., and Kira, J. (2014). Extensive dysregulations of oligodendrocytic and astrocytic connexins are associated with disease progression in an amyotrophic lateral sclerosis mouse model. Journal of neuroinflammation *11*, 42.

D'Alton, S., Altshuler, M., Cannon, A., Dickson, D.W., Petrucelli, L., and Lewis, J. (2014). Divergent phenotypes in mutant TDP-43 transgenic mice highlight potential confounds in TDP-43 transgenic modeling. PloS one *9*, e86513.

Dal Canto, M.C., and Gurney, M.E. (1994). Development of central nervous system pathology in a murine transgenic model of human amyotrophic lateral sclerosis. The American journal of pathology *145*, 1271-1279.

Dal Canto, M.C., and Gurney, M.E. (1995). Neuropathological changes in two lines of mice carrying a transgene for mutant human Cu,Zn SOD, and in mice overexpressing wild type human SOD: a model of familial amyotrophic lateral sclerosis (FALS). Brain research *676*, 25-40.

de Jong, E.K., Dijkstra, I.M., Hensens, M., Brouwer, N., van Amerongen, M., Liem, R.S., Boddeke, H.W., and Biber, K. (2005). Vesicle-mediated transport and release of CCL21 in endangered neurons: a possible explanation for microglia activation remote from a primary lesion. The Journal of neuroscience : the official journal of the Society for Neuroscience *25*, 7548-7557.

Deiva, K., Geeraerts, T., Salim, H., Leclerc, P., Hery, C., Hugel, B., Freyssinet, J.M., and Tardieu, M. (2004). Fractalkine reduces N-methyl-d-aspartate-induced calcium flux and apoptosis in human neurons through extracellular signal-regulated kinase activation. The European journal of neuroscience *20*, 3222-3232.

DeJesus-Hernandez, M., Mackenzie, I.R., Boeve, B.F., Boxer, A.L., Baker, M., Rutherford, N.J., Nicholson, A.M., Finch, N.A., Flynn, H., Adamson, J.*, et al.* (2011). Expanded GGGGCC hexanucleotide repeat in noncoding region of C9ORF72 causes chromosome 9p-linked FTD and ALS. Neuron *72*, 245-256.

Dewil, M., Van Den Bosch, L., and Robberecht, W. (2007). Microglia in amyotrophic lateral sclerosis. Acta Neurol Belg *107*, 63-70.

Dhaliwal, G.K., and Grewal, R.P. (2000). Mitochondrial DNA deletion mutation levels are elevated in ALS brains. Neuroreport *11*, 2507-2509.

Diaz-Amarilla, P., Olivera-Bravo, S., Trias, E., Cragnolini, A., Martinez-Palma, L., Cassina, P., Beckman, J., and Barbeito, L. (2011). Phenotypically aberrant astrocytes that promote motoneuron damage in a model of inherited amyotrophic lateral sclerosis. Proceedings of the National Academy of Sciences of the United States of America *108*, 18126-18131.

Dingledine, R., Borges, K., Bowie, D., and Traynelis, S.F. (1999). The glutamate receptor ion channels. Pharmacological reviews *51*, 7-61.

Durand, J., Amendola, J., Bories, C., and Lamotte d'Incamps, B. (2006). Early abnormalities in transgenic mouse models of amyotrophic lateral sclerosis. Journal of physiology, Paris *99*, 211-220.

Eisen, A., and Weber, M. (2001). The motor cortex and amyotrophic lateral sclerosis. Muscle & nerve *24*, 564-573.

Ekestern, E. (2004). Neurotrophic factors and amyotrophic lateral sclerosis. Neuro-degenerative diseases *1*, 88-100.

Evans, M.C., Couch, Y., Sibson, N., and Turner, M.R. (2013). Inflammation and neurovascular changes in amyotrophic lateral sclerosis. Molecular and cellular neurosciences *53*, 34-41.

Factor-Litvak, P., Al-Chalabi, A., Ascherio, A., Bradley, W., Chio, A., Garruto, R., Hardiman, O., Kamel, F., Kasarskis, E., McKee, A*., et al.* (2013). Current pathways for epidemiological research in amyotrophic lateral sclerosis. Amyotrophic lateral sclerosis & frontotemporal degeneration *14 Suppl 1*, 33-43.

Farg, M.A., Sundaramoorthy, V., Sultana, J.M., Yang, S., Atkinson, R.A., Levina, V., Halloran, M.A., Gleeson, P.A., Blair, I.P., Soo, K.Y*., et al.* (2014). C9ORF72, implicated in amytrophic lateral sclerosis and frontotemporal dementia, regulates endosomal trafficking. Human molecular genetics.

Federici, T., and Boulis, N.M. (2012). Gene therapy for amyotrophic lateral sclerosis. Neurobiology of disease *48*, 236-242.

Ferraiuolo, L., Kirby, J., Grierson, A.J., Sendtner, M., and Shaw, P.J. (2011). Molecular pathways of motor neuron injury in amyotrophic lateral sclerosis. Nature reviews Neurology *7*, 616-630.

Fischer, L.R., Culver, D.G., Tennant, P., Davis, A.A., Wang, M., Castellano-Sanchez, A., Khan, J., Polak, M.A., and Glass, J.D. (2004). Amyotrophic lateral sclerosis is a distal axonopathy: evidence in mice and man. Experimental neurology *185*, 232-240.

Fitzner, D., Schnaars, M., van Rossum, D., Krishnamoorthy, G., Dibaj, P., Bakhti, M., Regen, T., Hanisch, U.K., and Simons, M. (2011). Selective transfer of exosomes from oligodendrocytes to microglia by macropinocytosis. Journal of cell science *124*, 447-458.

Fornai, F., Longone, P., Ferrucci, M., Lenzi, P., Isidoro, C., Ruggieri, S., and Paparelli, A. (2008). Autophagy and amyotrophic lateral sclerosis: The multiple roles of lithium. Autophagy *4*, 527-530.

Fricker, M., Neher, J.J., Zhao, J.W., Thery, C., Tolkovsky, A.M., and Brown, G.C. (2012). MFG-E8 mediates primary phagocytosis of viable neurons during neuroinflammation. The Journal of neuroscience : the official journal of the Society for Neuroscience *32*, 2657-2666.

Fruhbeis, C., Frohlich, D., and Kramer-Albers, E.M. (2012). Emerging roles of exosomes in neuron-glia communication. Frontiers in physiology *3*, 119.

Fujita, K., Yamauchi, M., Shibayama, K., Ando, M., Honda, M., and Nagata, Y. (1996). Decreased cytochrome c oxidase activity but unchanged superoxide dismutase and glutathione peroxidase activities in the spinal cords of patients with amyotrophic lateral sclerosis. Journal of neuroscience research *45*, 276-281.

Fuller, A.D., and Van Eldik, L.J. (2008). MFG-E8 regulates microglial phagocytosis of apoptotic neurons. Journal of neuroimmune pharmacology : the official journal of the Society on NeuroImmune Pharmacology *3*, 246-256.

Gao, H.M., Zhou, H., Zhang, F., Wilson, B.C., Kam, W., and Hong, J.S. (2011). HMGB1 acts on microglia Mac1 to mediate chronic neuroinflammation that drives progressive neurodegeneration. The Journal of neuroscience : the official journal of the Society for Neuroscience *31*, 1081-1092.

Ge, W.W., Wen, W., Strong, W., Leystra-Lantz, C., and Strong, M.J. (2005). Mutant copper-zinc superoxide dismutase binds to and destabilizes human low molecular weight neurofilament mRNA. The Journal of biological chemistry *280*, 118-124.

Ghavami, S., Shojaei, S., Yeganeh, B., Ande, S.R., Jangamreddy, J.R., Mehrpour, M., Christoffersson, J., Chaabane, W., Moghadam, A.R., Kashani, H.H.*, et al.* (2014). Autophagy and apoptosis dysfunction in neurodegenerative disorders. Progress in neurobiology *112*, 24-49.

Gilbert, J.H. (2014). Our Vision: A World without ALS.

Gomes, C., Keller, S., Altevogt, P., and Costa, J. (2007). Evidence for secretion of Cu,Zn superoxide dismutase via exosomes from a cell model of amyotrophic lateral sclerosis. Neuroscience letters *428*, 43-46.

Gomes, C., Palma, A.S., Almeida, R., Regalla, M., McCluskey, L.F., Trojanowski, J.Q., and Costa, J. (2008). Establishment of a cell model of ALS disease: Golgi apparatus disruption occurs independently from apoptosis. Biotechnol Lett *30*, 603-610.

Gomez-Nicola, D., Pallas-Bazarra, N., Valle-Argos, B., and Nieto-Sampedro, M. (2010). CCR7 is expressed in astrocytes and upregulated after an inflammatory injury. Journal of neuroimmunology *227*, 87-92.

Gonzalez de Aguilar, J.L., Echaniz-Laguna, A., Fergani, A., Rene, F., Meininger, V., Loeffler, J.P., and Dupuis, L. (2007). Amyotrophic lateral sclerosis: all roads lead to Rome. Journal of neurochemistry *101*, 1153-1160.

Gordon, P.H. (2013). Amyotrophic Lateral Sclerosis: An update for 2013 Clinical Features, Pathophysiology, Management and Therapeutic Trials. Aging and disease *4*, 295-310.

Gould, T.W., Buss, R.R., Vinsant, S., Prevette, D., Sun, W., Knudson, C.M., Milligan, C.E., and Oppenheim, R.W. (2006). Complete dissociation of motor neuron death from motor dysfunction by Bax deletion in a mouse model of ALS. The Journal of neuroscience : the official journal of the Society for Neuroscience *26*, 8774-8786.

Graeber, M.B., and Streit, W.J. (2010). Microglia: biology and pathology. Acta neuropathologica *119*, 89-105.

Guegan, C., and Przedborski, S. (2003). Programmed cell death in amyotrophic lateral sclerosis. The Journal of clinical investigation *111*, 153-161.

Gurney, M.E., Pu, H., Chiu, A.Y., Dal Canto, M.C., Polchow, C.Y., Alexander, D.D., Caliendo, J., Hentati, A., Kwon, Y.W., Deng, H.X.*, et al.* (1994). Motor neuron degeneration in mice that express a human Cu,Zn superoxide dismutase mutation. Science (New York, NY) *264*, 1772-1775.

Hadano, S., Hand, C.K., Osuga, H., Yanagisawa, Y., Otomo, A., Devon, R.S., Miyamoto, N., Showguchi-Miyata, J., Okada, Y., Singaraja, R.*, et al.* (2001). A gene encoding a putative GTPase regulator is mutated in familial amyotrophic lateral sclerosis 2. Nature genetics *29*, 166-173.

Hardiman, O., van den Berg, L.H., and Kiernan, M.C. (2011). Clinical diagnosis and management of amyotrophic lateral sclerosis. Nature reviews Neurology *7*, 639-649.

Harrison, J.K., Jiang, Y., Chen, S., Xia, Y., Maciejewski, D., McNamara, R.K., Streit, W.J., Salafranca, M.N., Adhikari, S., Thompson, D.A.*, et al.* (1998). Role for neuronally derived fractalkine in mediating

interactions between neurons and CX3CR1-expressing microglia. Proceedings of the National Academy of Sciences of the United States of America *95*, 10896-10901.

Heath, P.R., and Shaw, P.J. (2002). Update on the glutamatergic neurotransmitter system and the role of excitotoxicity in amyotrophic lateral sclerosis. Muscle & nerve *26*, 438-458.

Heneka, M.T., Kummer, M.P., Stutz, A., Delekate, A., Schwartz, S., Vieira-Saecker, A., Griep, A., Axt, D., Remus, A., Tzeng, T.C.*, et al.* (2013). NLRP3 is activated in Alzheimer's disease and contributes to pathology in APP/PS1 mice. Nature *493*, 674-678.

Henkel, J.S., Beers, D.R., Zhao, W., and Appel, S.H. (2009). Microglia in ALS: the good, the bad, and the resting. Journal of neuroimmune pharmacology : the official journal of the Society on NeuroImmune Pharmacology *4*, 389-398.

Hewitt, C., Kirby, J., Highley, J.R., Hartley, J.A., Hibberd, R., Hollinger, H.C., Williams, T.L., Ince, P.G., McDermott, C.J., and Shaw, P.J. (2010). Novel FUS/TLS mutations and pathology in familial and sporadic amyotrophic lateral sclerosis. Archives of neurology *67*, 455-461.

Higgins, C.M., Jung, C., and Xu, Z. (2003). ALS-associated mutant SOD1G93A causes mitochondrial vacuolation by expansion of the intermembrane space and by involvement of SOD1 aggregation and peroxisomes. BMC neuroscience *4*, 16.

Hoek, R.M., Ruuls, S.R., Murphy, C.A., Wright, G.J., Goddard, R., Zurawski, S.M., Blom, B., Homola, M.E., Streit, W.J., Brown, M.H.*, et al.* (2000). Down-regulation of the macrophage lineage through interaction with OX2 (CD200). Science (New York, NY) *290*, 1768-1771.

Hoelzinger, D.B., Nakada, M., Demuth, T., Rosensteel, T., Reavie, L.B., and Berens, M.E. (2008). Autotaxin: a secreted autocrine/paracrine factor that promotes glioma invasion. Journal of neuro-oncology *86*, 297-309.

Homkajorn, B., Sims, N.R., and Muyderman, H. (2010). Connexin 43 regulates astrocytic migration and proliferation in response to injury. Neuroscience letters *486*, 197-201.

Hu, J.H., Chernoff, K., Pelech, S., and Krieger, C. (2003). Protein kinase and protein phosphatase expression in the central nervous system of G93A mSOD over-expressing mice. Journal of neurochemistry *85*, 422-431.

Huisman, M.H., Seelen, M., de Jong, S.W., Dorresteijn, K.R., van Doormaal, P.T., van der Kooi, A.J., de Visser, M., Schelhaas, H.J., van den Berg, L.H., and Veldink, J.H. (2013). Lifetime physical activity and the risk of amyotrophic lateral sclerosis. Journal of neurology, neurosurgery, and psychiatry *84*, 976-981.

Ilieva, H., Polymenidou, M., and Cleveland, D.W. (2009). Non-cell autonomous toxicity in neurodegenerative disorders: ALS and beyond. The Journal of cell biology *187*, 761-772.

Jaiswal, M.K., and Keller, B.U. (2009). Cu/Zn superoxide dismutase typical for familial amyotrophic lateral sclerosis increases the vulnerability of mitochondria and perturbs Ca2+ homeostasis in SOD1G93A mice. Mol Pharmacol *75*, 478-489.

Jonsson, P.A., Graffmo, K.S., Andersen, P.M., Marklund, S.L., and Brannstrom, T. (2009). Superoxide dismutase in amyotrophic lateral sclerosis patients homozygous for the D90A mutation. Neurobiology of disease *36*, 421-424.

Jonsson, P.A., Graffmo, K.S., Brannstrom, T., Nilsson, P., Andersen, P.M., and Marklund, S.L. (2006). Motor neuron disease in mice expressing the wild type-like D90A mutant superoxide dismutase-1. Journal of neuropathology and experimental neurology *65*, 1126-1136.

Joyce, P.I., Fratta, P., Fisher, E.M., and Acevedo-Arozena, A. (2011). SOD1 and TDP-43 animal models of amyotrophic lateral sclerosis: recent advances in understanding disease toward the development of clinical treatments. Mammalian genome : official journal of the International Mammalian Genome Society *22*, 420-448.

Julien, J.P. (2001). Amyotrophic lateral sclerosis. unfolding the toxicity of the misfolded. Cell *104*, 581-591.

Julien, J.P., and Kriz, J. (2006). Transgenic mouse models of amyotrophic lateral sclerosis. Biochimica et biophysica acta *1762*, 1013-1024.

Kanekura, K., Hashimoto, Y., Niikura, T., Aiso, S., Matsuoka, M., and Nishimoto, I. (2004). Alsin, the product of ALS2 gene, suppresses SOD1 mutant neurotoxicity through RhoGEF domain by interacting with SOD1 mutants. The Journal of biological chemistry *279*, 19247-19256.

Kang, S.H., Li, Y., Fukaya, M., Lorenzini, I., Cleveland, D.W., Ostrow, L.W., Rothstein, J.D., and Bergles, D.E. (2013). Degeneration and impaired regeneration of gray matter oligodendrocytes in amyotrophic lateral sclerosis. Nature neuroscience *16*, 571-579.

Kaplan, A., Spiller, K.J., Towne, C., Kanning, K.C., Choe, G.T., Geber, A., Akay, T., Aebischer, P., and Henderson, C.E. (2014). Neuronal matrix metalloproteinase-9 is a determinant of selective neurodegeneration. Neuron *81*, 333-348.

Kawamata, T., Akiyama, H., Yamada, T., and McGeer, P.L. (1992). Immunologic reactions in amyotrophic lateral sclerosis brain and spinal cord tissue. The American journal of pathology *140*, 691-707.

Kielian, T. (2008). Glial connexins and gap junctions in CNS inflammation and disease. Journal of neurochemistry *106*, 1000-1016.

Kiernan, M.C., Vucic, S., Cheah, B.C., Turner, M.R., Eisen, A., Hardiman, O., Burrell, J.R., and Zoing, M.C. (2011). Amyotrophic lateral sclerosis. Lancet *377*, 942-955.

Kikuchi, H., Almer, G., Yamashita, S., Guegan, C., Nagai, M., Xu, Z., Sosunov, A.A., McKhann, G.M., 2nd, and Przedborski, S. (2006). Spinal cord endoplasmic reticulum stress associated with a microsomal accumulation of mutant superoxide dismutase-1 in an ALS model. Proceedings of the National Academy of Sciences of the United States of America *103*, 6025-6030.

Kim, J., Kim, T.-Y., Cho, K.-S., Kim, H.N., and Koh, J.-Y. (2013). Autophagy activation and neuroprotection by progesterone in the G93A-SOD1 transgenic mouse model of amyotrophic lateral sclerosis. Neurobiology of disease *59*, 80-85.

Kirby, J., Halligan, E., Baptista, M.J., Allen, S., Heath, P.R., Holden, H., Barber, S.C., Loynes, C.A., Wood-Allum, C.A., Lunec, J.*, et al.* (2005). Mutant SOD1 alters the motor neuronal transcriptome: implications for familial ALS. Brain : a journal of neurology *128*, 1686-1706.

Korolchuk, V.I., Menzies, F.M., and Rubinsztein, D.C. (2010). Mechanisms of cross-talk between the ubiquitin-proteasome and autophagy-lysosome systems. FEBS letters *584*, 1393-1398.

Kranich, J., Krautler, N.J., Falsig, J., Ballmer, B., Li, S., Hutter, G., Schwarz, P., Moos, R., Julius, C., Miele, G.*, et al.* (2010). Engulfment of cerebral apoptotic bodies controls the course of prion disease in a mouse strain-dependent manner. The Journal of experimental medicine *207*, 2271-2281.

Kreitz, S., Fackelmayer, F.O., Gerdes, J., and Knippers, R. (2000). The proliferation-specific human Ki-67 protein is a constituent of compact chromatin. Experimental cell research *261*, 284-292.

Kurland, L.T., and Mulder, D.W. (1955a). Epidemiologic investigations of amyotrophic lateral sclerosis. 2. Familial aggregations indicative of dominant inheritance. I. Neurology *5*, 182-196.

Kurland, L.T., and Mulder, D.W. (1955b). Epidemiologic investigations of amyotrophic lateral sclerosis. 2. Familial aggregations indicative of dominant inheritance. II. Neurology *5*, 249-268.

Kwiatkowski, T.J., Jr., Bosco, D.A., Leclerc, A.L., Tamrazian, E., Vanderburg, C.R., Russ, C., Davis, A., Gilchrist, J., Kasarskis, E.J., Munsat, T.*, et al.* (2009). Mutations in the FUS/TLS gene on chromosome 16 cause familial amyotrophic lateral sclerosis. Science (New York, NY) *323*, 1205-1208.

Lagier-Tourenne, C., Polymenidou, M., and Cleveland, D.W. (2010). TDP-43 and FUS/TLS: emerging roles in RNA processing and neurodegeneration. Human molecular genetics *19*, R46-64.

Lasiene, J., and Yamanaka, K. (2011). Glial cells in amyotrophic lateral sclerosis. Neurology research international *2011*, 718987.

Lattante, S., Rouleau, G.A., and Kabashi, E. (2013). TARDBP and FUS mutations associated with amyotrophic lateral sclerosis: summary and update. Human mutation *34*, 812-826.

Lee, J.C., Seong, J., Kim, S.H., Lee, S.J., Cho, Y.J., An, J., Nam, D.H., Joo, K.M., and Cha, C.I. (2012a). Replacement of microglial cells using Clodronate liposome and bone marrow transplantation in the central nervous system of SOD1(G93A) transgenic mice as an in vivo model of amyotrophic lateral sclerosis. Biochemical and biophysical research communications *418*, 359-365.

Lee, Y., Morrison, B.M., Li, Y., Lengacher, S., Farah, M.H., Hoffman, P.N., Liu, Y., Tsingalia, A., Jin, L., Zhang, P.W.*, et al.* (2012b). Oligodendroglia metabolically support axons and contribute to neurodegeneration. Nature *487*, 443-448.

Lefebvre, S., Burglen, L., Reboullet, S., Clermont, O., Burlet, P., Viollet, L., Benichou, B., Cruaud, C., Millasseau, P., Zeviani, M.*, et al.* (1995). Identification and characterization of a spinal muscular atrophy-determining gene. Cell *80*, 155-165.

Leonardi-Essmann, F., Emig, M., Kitamura, Y., Spanagel, R., and Gebicke-Haerter, P.J. (2005). Fractalkine-upregulated milk-fat globule EGF factor-8 protein in cultured rat microglia. Journal of neuroimmunology *160*, 92-101.

Li, M., Ona, V.O., Guegan, C., Chen, M., Jackson-Lewis, V., Andrews, L.J., Olszewski, A.J., Stieg, P.E., Lee, J.P., Przedborski, S.*, et al.* (2000). Functional role of caspase-1 and caspase-3 in an ALS transgenic mouse model. Science (New York, NY) *288*, 335-339.

Li, T.M., Alberman, E., and Swash, M. (1988). Comparison of sporadic and familial disease amongst 580 cases of motor neuron disease. Journal of neurology, neurosurgery, and psychiatry *51*, 778-784.

Li, Y., Du, X.F., and Du, J.L. (2013a). Resting microglia respond to and regulate neuronal activity in vivo. Communicative & integrative biology *6*, e24493.

Li, Y.R., King, O.D., Shorter, J., and Gitler, A.D. (2013b). Stress granules as crucibles of ALS pathogenesis. The Journal of cell biology *201*, 361-372.

Liao, B., Zhao, W., Beers, D.R., Henkel, J.S., and Appel, S.H. (2012). Transformation from a neuroprotective to a neurotoxic microglial phenotype in a mouse model of ALS. Experimental neurology *237*, 147-152.

Liu, W., Tang, Y., and Feng, J. (2011). Cross talk between activation of microglia and astrocytes in pathological conditions in the central nervous system. Life sciences *89*, 141-146.

Liu, Y., Hao, W., Dawson, A., Liu, S., and Fassbender, K. (2009). Expression of amyotrophic lateral sclerosis-linked SOD1 mutant increases the neurotoxic potential of microglia via TLR2. The Journal of biological chemistry *284*, 3691-3699.

Lo Coco, D., Veglianese, P., Allievi, E., and Bendotti, C. (2007). Distribution and cellular localization of high mobility group box protein 1 (HMGB1) in the spinal cord of a transgenic mouse model of ALS. Neuroscience letters *412*, 73-77.

Lobsiger, C.S., Boillee, S., McAlonis-Downes, M., Khan, A.M., Feltri, M.L., Yamanaka, K., and Cleveland, D.W. (2009). Schwann cells expressing dismutase active mutant SOD1 unexpectedly slow disease progression in ALS mice. Proceedings of the National Academy of Sciences of the United States of America *106*, 4465-4470.

Luo, X.G., and Chen, S.D. (2012). The changing phenotype of microglia from homeostasis to disease. Transl Neurodegener *1*, 9.

Mantovani, S., Garbelli, S., Pasini, A., Alimonti, D., Perotti, C., Melazzini, M., Bendotti, C., and Mora, G. (2009). Immune system alterations in sporadic amyotrophic lateral sclerosis patients suggest an ongoing neuroinflammatory process. Journal of neuroimmunology *210*, 73-79.

Marden, J.J., Harraz, M.M., Williams, A.J., Nelson, K., Luo, M., Paulson, H., and Engelhardt, J.F. (2007). Redox modifier genes in amyotrophic lateral sclerosis in mice. The Journal of clinical investigation *117*, 2913-2919.

Mariano, C., Palmela, I., Pereira, P., Fernandes, A., Falcao, A.S., Cardoso, F.L., Vaz, A.R., Campos, A.R., Goncalves-Ferreira, A., Kim, K.S.*, et al.* (2013). Tricellulin expression in brain endothelial and neural cells. Cell and tissue research *351*, 397-407.

Matus, S., Valenzuela, V., Medinas, D.B., and Hetz, C. (2013). ER Dysfunction and Protein Folding Stress in ALS. International journal of cell biology *2013*, 674751.

Mazzini, L., Fagioli, F., Boccaletti, R., Mareschi, K., Oliveri, G., Olivieri, C., Pastore, I., Marasso, R., and Madon, E. (2003). Stem cell therapy in amyotrophic lateral sclerosis: a methodological approach in humans. Amyotrophic lateral sclerosis and other motor neuron disorders : official publication of the World Federation of Neurology, Research Group on Motor Neuron Diseases *4*, 158-161.

McGeer, E.G., and McGeer, P.L. (2005). Pharmacologic approaches to the treatment of amyotrophic lateral sclerosis. BioDrugs : clinical immunotherapeutics, biopharmaceuticals and gene therapy *19*, 31-37.

McGeer, P.L., and McGeer, E.G. (2002). Inflammatory processes in amyotrophic lateral sclerosis. Muscle & nerve *26*, 459-470.

Mead, R.J., Bennett, E.J., Kennerley, A.J., Sharp, P., Sunyach, C., Kasher, P., Berwick, J., Pettmann, B., Battaglia, G., Azzouz, M.*, et al.* (2011). Optimised and rapid pre-clinical screening in the SOD1(G93A) transgenic mouse model of amyotrophic lateral sclerosis (ALS). PloS one *6*, e23244.

Meamar, R., Nasr-Esfahani, M.H., Mousavi, S.A., and Basiri, K. (2013). Stem cell therapy in amyotrophic lateral sclerosis. Journal of clinical neuroscience : official journal of the Neurosurgical Society of Australasia *20*, 1659-1663.

Meissner, F., Molawi, K., and Zychlinsky, A. (2010). Mutant superoxide dismutase 1-induced IL-1beta accelerates ALS pathogenesis. Proceedings of the National Academy of Sciences of the United States of America *107*, 13046-13050.

Mitchell, J.D., and Borasio, G.D. (2007). Amyotrophic lateral sclerosis. Lancet *369*, 2031-2041.

Moisse, K., and Strong, M.J. (2006). Innate immunity in amyotrophic lateral sclerosis. Biochimica et biophysica acta *1762*, 1083-1093.

Morrison, B.M., Lee, Y., and Rothstein, J.D. (2013). Oligodendroglia: metabolic supporters of axons. Trends in Cell Biology *23*, 644-651.

Muller, S., Ronfani, L., and Bianchi, M.E. (2004). Regulated expression and subcellular localization of HMGB1, a chromatin protein with a cytokine function. Journal of internal medicine *255*, 332-343.

Musaro, A. (2010). State of the art and the dark side of amyotrophic lateral sclerosis. World journal of biological chemistry *1*, 62-68.

Neumann, M., Sampathu, D.M., Kwong, L.K., Truax, A.C., Micsenyi, M.C., Chou, T.T., Bruce, J., Schuck, T., Grossman, M., Clark, C.M.*, et al.* (2006). Ubiquitinated TDP-43 in frontotemporal lobar degeneration and amyotrophic lateral sclerosis. Science (New York, NY) *314*, 130-133.

Nizzardo, M., Simone, C., Rizzo, F., Ruggieri, M., Salani, S., Riboldi, G., Faravelli, I., Zanetta, C., Bresolin, N., Comi, G.P.*, et al.* (2014). Minimally invasive transplantation of iPSC-derived ALDHhiSSCloVLA4+ neural stem cells effectively improves the phenotype of an amyotrophic lateral sclerosis model. Human molecular genetics *23*, 342-354.

Ogawa, M., and Furukawa, Y. (2014). A seeded propagation of Cu, Zn-superoxide dismutase aggregates in amyotrophic lateral sclerosis. Frontiers in cellular neuroscience *8*, 83.

Ohnishi, S., Ito, H., Suzuki, Y., Adachi, Y., Wate, R., Zhang, J., Nakano, S., Kusaka, H., and Ikehara, S. (2009). Intra-bone marrow-bone marrow transplantation slows disease progression and prolongs survival in G93A mutant SOD1 transgenic mice, an animal model mouse for amyotrophic lateral sclerosis. Brain research *1296*, 216-224.

Olah, M., Biber, K., Vinet, J., and Boddeke, H.W. (2011). Microglia phenotype diversity. CNS & neurological disorders drug targets *10*, 108-118.

Olson, J.K., and Miller, S.D. (2004). Microglia initiate central nervous system innate and adaptive immune responses through multiple TLRs. Journal of immunology (Baltimore, Md : 1950) *173*, 3916-3924.

Orellana, J.A., Saez, P.J., Shoji, K.F., Schalper, K.A., Palacios-Prado, N., Velarde, V., Giaume, C., Bennett, M.V., and Saez, J.C. (2009). Modulation of brain hemichannels and gap junction channels by pro-inflammatory agents and their possible role in neurodegeneration. Antioxidants & redox signaling *11*, 369-399.

Packer, L., and Cadenas, E. (2007). Oxidants and antioxidants revisited. New concepts of oxidative stress. Free radical research *41*, 951-952.

Pandya, R.S., Mao, L.L., Zhou, E.W., Bowser, R., Zhu, Z., Zhu, Y., and Wang, X. (2012). Neuroprotection for amyotrophic lateral sclerosis: role of stem cells, growth factors, and gene therapy. Central nervous system agents in medicinal chemistry *12*, 15-27.

Papadimitriou, D., Le Verche, V., Jacquier, A., Ikiz, B., Przedborski, S., and Re, D.B. (2010). Inflammation in ALS and SMA: sorting out the good from the evil. Neurobiology of disease *37*, 493-502.

Pasinelli, P., Belford, M.E., Lennon, N., Bacskai, B.J., Hyman, B.T., Trotti, D., and Brown, R.H., Jr. (2004). Amyotrophic lateral sclerosis-associated SOD1 mutant proteins bind and aggregate with Bcl-2 in spinal cord mitochondria. Neuron *43*, 19-30.

Pasinelli, P., and Brown, R.H. (2006). Molecular biology of amyotrophic lateral sclerosis: insights from genetics. Nature reviews Neuroscience *7*, 710-723.

Pasinelli, P., Houseweart, M.K., Brown, R.H., Jr., and Cleveland, D.W. (2000). Caspase-1 and -3 are sequentially activated in motor neuron death in Cu,Zn superoxide dismutase-mediated familial amyotrophic lateral sclerosis. Proceedings of the National Academy of Sciences of the United States of America *97*, 13901-13906.

Penuela, S., Gehi, R., and Laird, D.W. (2013). The biochemistry and function of pannexin channels. Biochimica et Biophysica Acta (BBA) - Biomembranes *1828*, 15-22.

Perrakis, A., and Moolenaar, W.H. (2014). Autotaxin: structure-function and signaling. Journal of lipid research *55*, 1010-1018.

Pfaffl, M.W. (2001). A new mathematical model for relative quantification in real-time RT-PCR. Nucleic acids research *29*, e45.

Phani, S., Re, D.B., and Przedborski, S. (2012). The Role of the Innate Immune System in ALS. Frontiers in pharmacology *3*, 150.

Phatnani, H.P., Guarnieri, P., Friedman, B.A., Carrasco, M.A., Muratet, M., O'Keeffe, S., Nwakeze, C., Pauli-Behn, F., Newberry, K.M., Meadows, S.K.*, et al.* (2013). Intricate interplay between astrocytes and motor neurons in ALS. Proceedings of the National Academy of Sciences of the United States of America *110*, E756-765.

Philips, T., Bento-Abreu, A., Nonneman, A., Haeck, W., Staats, K., Geelen, V., Hersmus, N., Kusters, B., Van Den Bosch, L., Van Damme, P.*, et al.* (2013). Oligodendrocyte dysfunction in the pathogenesis of amyotrophic lateral sclerosis. Brain : a journal of neurology *136*, 471-482.

Philips, T., and Robberecht, W. (2011). Neuroinflammation in amyotrophic lateral sclerosis: role of glial activation in motor neuron disease. Lancet neurology *10*, 253-263.

Pizzasegola, C., Caron, I., Daleno, C., Ronchi, A., Minoia, C., Carri, M.T., and Bendotti, C. (2009). Treatment with lithium carbonate does not improve disease progression in two different strains of SOD1 mutant mice. Amyotrophic lateral sclerosis : official publication of the World Federation of Neurology Research Group on Motor Neuron Diseases *10*, 221-228.

Polymenidou, M., Lagier-Tourenne, C., Hutt, K.R., Bennett, C.F., Cleveland, D.W., and Yeo, G.W. (2012). Misregulated RNA processing in amyotrophic lateral sclerosis. Brain research *1462*, 3-15.

Pratt, A.J., Getzoff, E.D., and Perry, J.J. (2012). Amyotrophic lateral sclerosis: update and new developments. Degener Neurol Neuromuscul Dis *2012*, 1-14.

Przedborski, S., Donaldson, D., Jakowec, M., Kish, S.J., Guttman, M., Rosoklija, G., and Hays, A.P. (1996a). Brain superoxide dismutase, catalase, and glutathione peroxidase activities in amyotrophic lateral sclerosis. Annals of neurology *39*, 158-165.

Przedborski, S., Donaldson, D.M., Murphy, P.L., Hirsch, O., Lange, D., Naini, A.B., McKenna-Yasek, D., and Brown, R.H., Jr. (1996b). Blood superoxide dismutase, catalase and glutathione peroxidase activities in familial and sporadic amyotrophic lateral sclerosis. Neurodegeneration : a journal for neurodegenerative disorders, neuroprotection, and neuroregeneration *5*, 57-64.

Raimondi, A., Mangolini, A., Rizzardini, M., Tartari, S., Massari, S., Bendotti, C., Francolini, M., Borgese, N., Cantoni, L., and Pietrini, G. (2006). Cell culture models to investigate the selective vulnerability of motoneuronal mitochondria to familial ALS-linked G93ASOD1. The European journal of neuroscience *24*, 387-399.

Rao, S.D., and Weiss, J.H. (2004). Excitotoxic and oxidative cross-talk between motor neurons and glia in ALS pathogenesis. Trends in neurosciences *27*, 17-23.

Redler, R.L., and Dokholyan, N.V. (2012). The complex molecular biology of amyotrophic lateral sclerosis (ALS). Prog Mol Biol Transl Sci *107*, 215-262.

Renton, A.E., Majounie, E., Waite, A., Simon-Sanchez, J., Rollinson, S., Gibbs, J.R., Schymick, J.C., Laaksovirta, H., van Swieten, J.C., Myllykangas, L.*, et al.* (2011). A hexanucleotide repeat expansion in C9ORF72 is the cause of chromosome 9p21-linked ALS-FTD. Neuron *72*, 257-268.

Ripps, M.E., Huntley, G.W., Hof, P.R., Morrison, J.H., and Gordon, J.W. (1995). Transgenic mice expressing an altered murine superoxide dismutase gene provide an animal model of amyotrophic lateral sclerosis. Proceedings of the National Academy of Sciences of the United States of America *92*, 689-693.

Rizvanov, A.A., Mukhamedyarov, M.A., Palotas, A., and Islamov, R.R. (2009). Retrogradely transported siRNA silences human mutant SOD1 in spinal cord motor neurons. Experimental brain research *195*, 1-4.

Robberecht, W., and Philips, T. (2013). The changing scene of amyotrophic lateral sclerosis. Nature reviews Neuroscience *14*, 248-264.

Rocha, M.C., Pousinha, P.A., Correia, A.M., Sebastiao, A.M., and Ribeiro, J.A. (2013). Early changes of neuromuscular transmission in the SOD1(G93A) mice model of ALS start long before motor symptoms onset. PloS one *8*, e73846.

Rosen, D.R., Siddique, T., Patterson, D., Figlewicz, D.A., Sapp, P., Hentati, A., Donaldson, D., Goto, J., O'Regan, J.P., Deng, H.X.*, et al.* (1993). Mutations in Cu/Zn superoxide dismutase gene are associated with familial amyotrophic lateral sclerosis. Nature *362*, 59-62.

Rothstein, J.D., Martin, L.J., and Kuncl, R.W. (1992). Decreased glutamate transport by the brain and spinal cord in amyotrophic lateral sclerosis. The New England journal of medicine *326*, 1464-1468.

Rutherford, N.J., Zhang, Y.J., Baker, M., Gass, J.M., Finch, N.A., Xu, Y.F., Stewart, H., Kelley, B.J., Kuntz, K., Crook, R.J.*, et al.* (2008). Novel mutations in TARDBP (TDP-43) in patients with familial amyotrophic lateral sclerosis. PLoS genetics *4*, e1000193.

Saijo, K., and Glass, C.K. (2011). Microglial cell origin and phenotypes in health and disease. Nat Rev Immunol *11*, 775-787.

Sarlette, A., Krampfl, K., Grothe, C., Neuhoff, N., Dengler, R., and Petri, S. (2008). Nuclear erythroid 2-related factor 2-antioxidative response element signaling pathway in motor cortex and spinal cord in amyotrophic lateral sclerosis. Journal of neuropathology and experimental neurology *67*, 1055-1062.

Scaffidi, P., Misteli, T., and Bianchi, M.E. (2002). Release of chromatin protein HMGB1 by necrotic cells triggers inflammation. Nature *418*, 191-195.

Seilhean, D., Cazeneuve, C., Thuries, V., Russaouen, O., Millecamps, S., Salachas, F., Meininger, V., Leguern, E., and Duyckaerts, C. (2009). Accumulation of TDP-43 and alpha-actin in an amyotrophic lateral sclerosis patient with the K17I ANG mutation. Acta neuropathologica *118*, 561-573.

Shaw, P.J. (2005). Molecular and cellular pathways of neurodegeneration in motor neurone disease. Journal of neurology, neurosurgery, and psychiatry *76*, 1046-1057.

Shaw, P.J., and Eggett, C.J. (2000). Molecular factors underlying selective vulnerability of motor neurons to neurodegeneration in amyotrophic lateral sclerosis. Journal of neurology *247 Suppl 1*, I17-27.

Siklos, L., Engelhardt, J., Harati, Y., Smith, R.G., Joo, F., and Appel, S.H. (1996). Ultrastructural evidence for altered calcium in motor nerve terminals in amyotropic lateral sclerosis. Annals of neurology *39*, 203-216.

Smith, R.A., Miller, T.M., Yamanaka, K., Monia, B.P., Condon, T.P., Hung, G., Lobsiger, C.S., Ward, C.M., McAlonis-Downes, M., Wei, H.*, et al.* (2006). Antisense oligonucleotide therapy for neurodegenerative disease. The Journal of clinical investigation *116*, 2290-2296.

Sofroniew, M.V., and Vinters, H.V. (2010). Astrocytes: biology and pathology. Acta neuropathologica *119*, 7-35.

Sreedharan, J., Blair, I.P., Tripathi, V.B., Hu, X., Vance, C., Rogelj, B., Ackerley, S., Durnall, J.C., Williams, K.L., Buratti, E.*, et al.* (2008). TDP-43 mutations in familial and sporadic amyotrophic lateral sclerosis. Science (New York, NY) *319*, 1668-1672.

Sutedja, N.A., Veldink, J.H., Fischer, K., Kromhout, H., Heederik, D., Huisman, M.H., Wokke, J.H., and van den Berg, L.H. (2009). Exposure to chemicals and metals and risk of amyotrophic lateral sclerosis: a systematic review. Amyotrophic lateral sclerosis : official publication of the World Federation of Neurology Research Group on Motor Neuron Diseases *10*, 302-309.

Takeuchi, S., Fujiwara, N., Ido, A., Oono, M., Takeuchi, Y., Tateno, M., Suzuki, K., Takahashi, R., Tooyama, I., Taniguchi, N.*, et al.* (2010). Induction of protective immunity by vaccination with wild-type apo superoxide dismutase 1 in mutant SOD1 transgenic mice. Journal of neuropathology and experimental neurology *69*, 1044-1056.

Theodoric, N., Bechberger, J.F., Naus, C.C., and Sin, W.C. (2012). Role of gap junction protein connexin43 in astrogliosis induced by brain injury. PloS one *7*, e47311.

Ticozzi, N., Tiloca, C., Morelli, C., Colombrita, C., Poletti, B., Doretti, A., Maderna, L., Messina, S., Ratti, A., and Silani, V. (2011). Genetics of familial Amyotrophic lateral sclerosis. Archives italiennes de biologie *149*, 65-82.

Tolosa, L., Mir, M., Asensio, V.J., Olmos, G., and Llado, J. (2008). Vascular endothelial growth factor protects spinal cord motoneurons against glutamate-induced excitotoxicity via phosphatidylinositol 3-kinase. Journal of neurochemistry *105*, 1080-1090.

Tovar, Y.R.L.B., Santa-Cruz, L.D., and Tapia, R. (2009). Experimental models for the study of neurodegeneration in amyotrophic lateral sclerosis. Molecular neurodegeneration *4*, 31.

Trias, E., Diaz-Amarilla, P., Olivera-Bravo, S., Isasi, E., Drechsel, D.A., Lopez, N., Bradford, C.S., Ireton, K.E., Beckman, J.S., and Barbeito, L. (2013). Phenotypic transition of microglia into astrocyte-like cells associated with disease onset in a model of inherited ALS. Frontiers in cellular neuroscience *7*, 274.

Trojsi, F., Monsurro, M.R., and Tedeschi, G. (2013). Exposure to environmental toxicants and pathogenesis of amyotrophic lateral sclerosis: state of the art and research perspectives. International journal of molecular sciences *14*, 15286-15311.

Tu, P.H., Raju, P., Robinson, K.A., Gurney, M.E., Trojanowski, J.Q., and Lee, V.M. (1996). Transgenic mice carrying a human mutant superoxide dismutase transgene develop neuronal cytoskeletal pathology resembling human amyotrophic lateral sclerosis lesions. Proceedings of the National Academy of Sciences of the United States of America *93*, 3155-3160.

Turner, B.J., and Talbot, K. (2008). Transgenics, toxicity and therapeutics in rodent models of mutant SOD1-mediated familial ALS. Progress in neurobiology *85*, 94-134.

Turner, M.R., Cagnin, A., Turkheimer, F.E., Miller, C.C., Shaw, C.E., Brooks, D.J., Leigh, P.N., and Banati, R.B. (2004). Evidence of widespread cerebral microglial activation in amyotrophic lateral sclerosis: an [11C](R)-PK11195 positron emission tomography study. Neurobiology of disease *15*, 601-609.

Urushitani, M., Sik, A., Sakurai, T., Nukina, N., Takahashi, R., and Julien, J.P. (2006). Chromogranin-mediated secretion of mutant superoxide dismutase proteins linked to amyotrophic lateral sclerosis. Nature neuroscience *9*, 108-118.

Valori, C.F., Brambilla, L., Martorana, F., and Rossi, D. (2014). The multifaceted role of glial cells in amyotrophic lateral sclerosis. Cellular and molecular life sciences : CMLS *71*, 287-297.

van Beijnum, J.R., Buurman, W.A., and Griffioen, A.W. (2008). Convergence and amplification of toll-like receptor (TLR) and receptor for advanced glycation end products (RAGE) signaling pathways via high mobility group B1 (HMGB1). Angiogenesis *11*, 91-99.

Van Damme, P., Bogaert, E., Dewil, M., Hersmus, N., Kiraly, D., Scheveneels, W., Bockx, I., Braeken, D., Verpoorten, N., Verhoeven, K.*, et al.* (2007). Astrocytes regulate GluR2 expression in motor neurons and their vulnerability to excitotoxicity. Proceedings of the National Academy of Sciences of the United States of America *104*, 14825-14830.

Van Damme, P., Dewil, M., Robberecht, W., and Van Den Bosch, L. (2005). Excitotoxicity and amyotrophic lateral sclerosis. Neuro-degenerative diseases *2*, 147-159.

Van Den Bosch, L. (2011). Genetic rodent models of amyotrophic lateral sclerosis. Journal of biomedicine & biotechnology *2011*, 348765.

van Weering, H.R., de Jong, A.P., de Haas, A.H., Biber, K.P., and Boddeke, H.W. (2010). CCL21-induced calcium transients and proliferation in primary mouse astrocytes: CXCR3-dependent and independent responses. Brain, behavior, and immunity *24*, 768-775.

Vance, C., Rogelj, B., Hortobagyi, T., De Vos, K.J., Nishimura, A.L., Sreedharan, J., Hu, X., Smith, B., Ruddy, D., Wright, P.*, et al.* (2009). Mutations in FUS, an RNA processing protein, cause familial amyotrophic lateral sclerosis type 6. Science (New York, NY) *323*, 1208-1211.

Vargas, M.R., Johnson, D.A., Sirkis, D.W., Messing, A., and Johnson, J.A. (2008). Nrf2 activation in astrocytes protects against neurodegeneration in mouse models of familial amyotrophic lateral sclerosis. The Journal of neuroscience : the official journal of the Society for Neuroscience *28*, 13574-13581.

Varnum, M.M., and Ikezu, T. (2012). The classification of microglial activation phenotypes on neurodegeneration and regeneration in Alzheimer's disease brain. Archivum immunologiae et therapiae experimentalis *60*, 251-266.

Vaz, A.R., Cunha, C., Gomes, C., Schmucki, N., Barbosa, M., and Brites, D. (2014). Glycoursodeoxycholic Acid Reduces Matrix Metalloproteinase-9 and Caspase-9 Activation in a Cellular Model of Superoxide Dismutase-1 Neurodegeneration. Molecular neurobiology.

Venkova-Hristova, K., Christov, A., Kamaluddin, Z., Kobalka, P., and Hensley, K. (2012). Progress in therapy development for amyotrophic lateral sclerosis. Neurology research international *2012*, 187234.

Verbeeck, C., Deng, Q., Dejesus-Hernandez, M., Taylor, G., Ceballos-Diaz, C., Kocerha, J., Golde, T., Das, P., Rademakers, R., Dickson, D.W.*, et al.* (2012). Expression of Fused in sarcoma mutations in mice recapitulates the neuropathology of FUS proteinopathies and provides insight into disease pathogenesis. Molecular neurodegeneration *7*, 53.

Vincent, A.M., Sakowski, S.A., Schuyler, A., and Feldman, E.L. (2008). Strategic approaches to developing drug treatments for ALS. Drug discovery today *13*, 67-72.

Wang, I.F., Guo, B.S., Liu, Y.C., Wu, C.C., Yang, C.H., Tsai, K.J., and Shen, C.K. (2012a). Autophagy activators rescue and alleviate pathogenesis of a mouse model with proteinopathies of the TAR DNA-binding protein 43. Proceedings of the National Academy of Sciences of the United States of America *109*, 15024-15029.

Wang, L., Pytel, P., Feltri, M.L., Wrabetz, L., and Roos, R.P. (2012b). Selective knockdown of mutant SOD1 in Schwann cells ameliorates disease in G85R mutant SOD1 transgenic mice. Neurobiology of disease *48*, 52-57.

Watanabe, Y., Yasui, K., Nakano, T., Doi, K., Fukada, Y., Kitayama, M., Ishimoto, M., Kurihara, S., Kawashima, M., Fukuda, H.*, et al.* (2005). Mouse motor neuron disease caused by truncated SOD1 with or without C-terminal modification. Brain research Molecular brain research *135*, 12-20.

Wiedemann, F.R., Manfredi, G., Mawrin, C., Beal, M.F., and Schon, E.A. (2002). Mitochondrial DNA and respiratory chain function in spinal cords of ALS patients. Journal of neurochemistry *80*, 616-625.

Williams, A.H., Valdez, G., Moresi, V., Qi, X., McAnally, J., Elliott, J.L., Bassel-Duby, R., Sanes, J.R., and Olson, E.N. (2009). MicroRNA-206 delays ALS progression and promotes regeneration of neuromuscular synapses in mice. Science (New York, NY) *326*, 1549-1554.

Wood, J.D., Beaujeux, T.P., and Shaw, P.J. (2003). Protein aggregation in motor neurone disorders. Neuropathology and applied neurobiology *29*, 529-545.

Xiao, Q., Zhao, W., Beers, D.R., Yen, A.A., Xie, W., Henkel, J.S., and Appel, S.H. (2007). Mutant SOD1(G93A) microglia are more neurotoxic relative to wild-type microglia. Journal of neurochemistry *102*, 2008-2019.

Xu, L., Ryugo, D.K., Pongstaporn, T., Johe, K., and Koliatsos, V.E. (2009). Human neural stem cell grafts in the spinal cord of SOD1 transgenic rats: differentiation and structural integration into the segmental motor circuitry. The Journal of comparative neurology *514*, 297-309.

Xu, Y.F., Zhang, Y.J., Lin, W.L., Cao, X., Stetler, C., Dickson, D.W., Lewis, J., and Petrucelli, L. (2011). Expression of mutant TDP-43 induces neuronal dysfunction in transgenic mice. Molecular neurodegeneration *6*, 73.

Yamanaka, K., Boillee, S., Roberts, E.A., Garcia, M.L., McAlonis-Downes, M., Mikse, O.R., Cleveland, D.W., and Goldstein, L.S. (2008). Mutant SOD1 in cell types other than motor neurons and oligodendrocytes accelerates onset of disease in ALS mice. Proceedings of the National Academy of Sciences of the United States of America *105*, 7594-7599.

Yoshihara, T., Ishigaki, S., Yamamoto, M., Liang, Y., Niwa, J., Takeuchi, H., Doyu, M., and Sobue, G. (2002). Differential expression of inflammation- and apoptosis-related genes in spinal cords of a mutant SOD1 transgenic mouse model of familial amyotrophic lateral sclerosis. Journal of neurochemistry *80*, 158-167.

Yoshii, Y., Otomo, A., Pan, L., Ohtsuka, M., and Hadano, S. (2011). Loss of glial fibrillary acidic protein marginally accelerates disease progression in a SOD1(H46R) transgenic mouse model of ALS. Neuroscience research *70*, 321-329.

Zang, D.W., Yang, Q., Wang, H.X., Egan, G., Lopes, E.C., and Cheema, S.S. (2004). Magnetic resonance imaging reveals neuronal degeneration in the brainstem of the superoxide dismutase 1 transgenic mouse model of amyotrophic lateral sclerosis. The European journal of neuroscience *20*, 1745-1751.

Zhang, X., Li, L., Chen, S., Yang, D., Wang, Y., Zhang, X., Wang, Z., and Le, W. (2011). Rapamycin treatment augments motor neuron degeneration in SOD1(G93A) mouse model of amyotrophic lateral sclerosis. Autophagy *7*, 412-425.

Zhao, W., Beers, D.R., and Appel, S.H. (2013a). Immune-mediated Mechanisms in the Pathoprogression of Amyotrophic Lateral Sclerosis. Journal of neuroimmune pharmacology : the official journal of the Society on NeuroImmune Pharmacology *8*, 888-899.

Zhao, W., Beers, D.R., Henkel, J.S., Zhang, W., Urushitani, M., Julien, J.P., and Appel, S.H. (2010). Extracellular mutant SOD1 induces microglial-mediated motoneuron injury. Glia *58*, 231-243.

Zhao, W., Bell, S., Beers, D., Liao, B., Henkel, J., Baloh, R., and Appel, S. (2013b). TDP43-Mediated Microglial Activation through NF-{kappa}B, AP-1 and NLRP3 Inflammasome. Neurology.

Zhao, W., Xie, W., Xiao, Q., Beers, D.R., and Appel, S.H. (2006). Protective effects of an anti-inflammatory cytokine, interleukin-4, on motoneuron toxicity induced by activated microglia. Journal of neurochemistry *99*, 1176-1187.

Zinman, L., and Cudkowicz, M. (2011). Emerging targets and treatments in amyotrophic lateral sclerosis. Lancet neurology *10*, 481-490.

VI. ANNEX

Annex 1.

Annex 1.1.

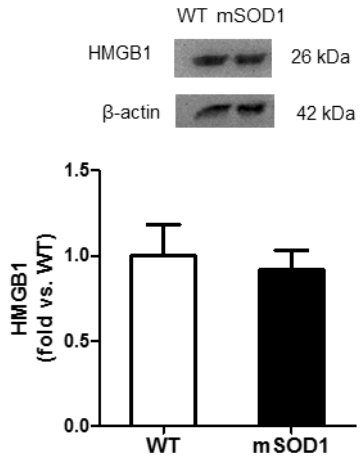


Figure VI.1. HMGB1 protein expression does not change in the spinal cord (SC) of presymptomatic mSOD1 mice when compared to aged-matched controls (WT). Samples were treated as indicated in Methods. Results are expressed as mean (± SEM) from at least three independent experiments **p*<0.05 vs. matched WT mice using two-tailed unpaired Student´s *t*-test.

Annex 1.2.

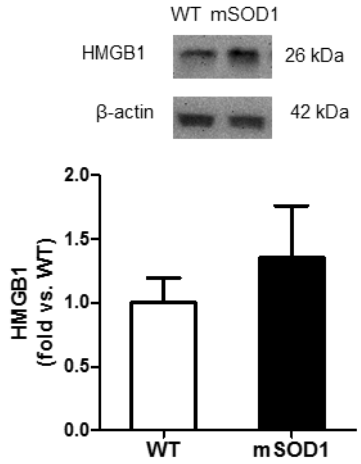
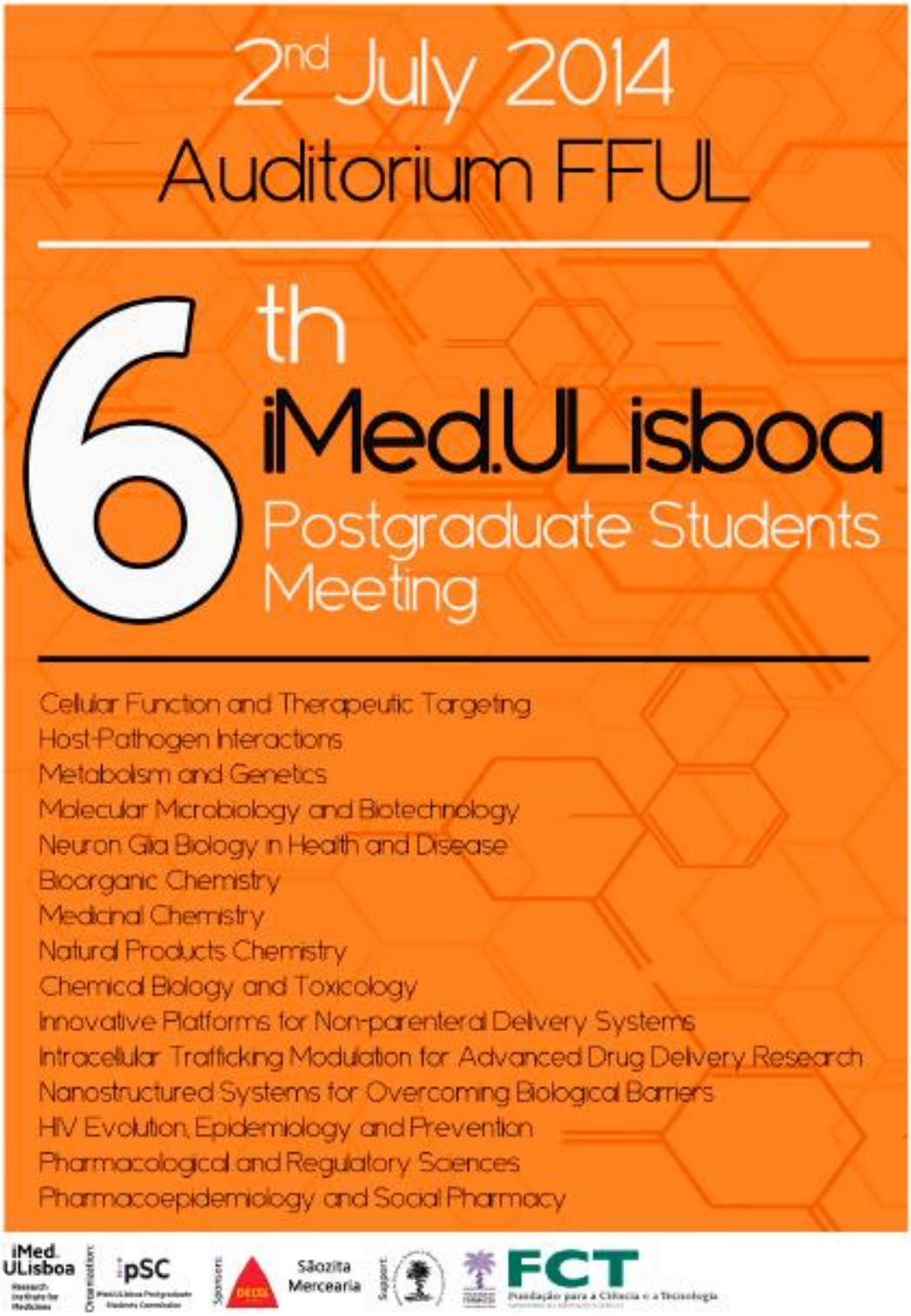


Figure VI.2. HMGB1 protein expression does not change in the spinal cord (SC) of symptomatic mSOD1 mice when compared to aged-matched controls (WT). Samples were treated as indicated in Methods. Results are expressed as mean (± SEM) from at least three independent experiments **p*<0.05 vs. matched WT mice using two-tailed unpaired Student´s *t*-test.

Chapter VI. ANNEX

Annex 2.



Annex 2.1.

6th iMed.ULisboa Postgraduate Students Meeting
July 2nd 2014, Lisbon, Portugal

PC36

Studying the role of glial cells during amyotrophic lateral sclerosis progression

Paiva Santos C[1], Gomes C[1], Fernandes A[1,2], Sebastião AM[3], Vaz AR[1,2], Brites D[1,2]

[1]Research Institute for Medicines (iMed.ULisboa), Faculty of Pharmacy, Universidade de Lisboa, Lisbon, Portugal. [2]Department of Biochemistry and Human Biology, Faculty of Pharmacy, Universidade de Lisboa, Lisbon, Portugal. [3]Institute of Pharmacology and Neurosciences, Faculty of Medicine, Universidade de Lisboa, Lisbon, Portugal; Unit of Neurosciences, Institute of Molecular Medicine, Universidade de Lisboa, Lisbon, Portugal.

catarinapaivasantos@hotmail.com

Neuron Glia Biology in Health and Disease

Amyotrophic lateral sclerosis (ALS) is characterized by selective loss of motoneurons (MN) but glial cells also play a pivotal role for MN injury, thus contributing to ALS progression [1]. Indeed, MN-glia signalling pathways involving either neuroprotection or inflammation are likely to be altered [2]. We aimed to investigate the molecules involved in cellular dysfunction and reactivity along ALS progression in the transgenic mice (TgSOD1-G93A). Thus, we used mRNA and protein extracted from the lumbar Spinal Cord (SC) of TgSOD1-G93A mice collected at 4-6 weeks (pre-symptomatic, PS) or 14-16 weeks (symptomatic, S) stages. Non-tg mice (Wt) at the same age were used as control.

Our results show decreased mRNA expression genes associated with astroglial gap junctions at PS stage, namely connexin 43 and pannexin 1, suggesting astrocyte dysfunction. In addition, we observed increased GFAP and S100B mRNA expression at the S phase, which suggests reactive astrogliosis. Regarding genes involved in neuroinflammation, we found decreased expression of the inflammasome NLRP3 and IL-18 at PS stage but they were increased at S stage. In addtion, we found increased mRNA expression of microglial marker CD11b at S stage, together with higher mRNA levels of the alarmin HMGB1, also suggesting increased neuroinflammation at later stages of ALS progression. Regarding microglia-neuronal communication, we observed increased expression of the chemokine CX3CL1 and its receptor CX3CR1 at S stage, which may have a role in promoting intercellular communication, as well as higher mRNA expression of MFG-E8, CCL21 and Ki-67, which are associated with increased microglial phagocytosis, motility and proliferation, respectively. Autotaxin, an enzime responsible for the production of lysophosphatidic acid which signalling causes cell proliferation and cytokine secretion is upregulated at both PS and S stages. Interestingly, protein analysis of the same molecules show opposite results at PS stage, namely HMGB1, TLR4 and MFG-E8, that may be the consequence of altered transcription and mRNA processing, both phenomenons that have been described in the pathophysiology of the disease [3]. Altogether, these data indicate an increase of both classical and alternative microglial activation phenotypes at S stage, together with increased astroglial response.

Funded by FEDER (COMPETE Programme) and by National funds (FCT-PTDC/SAU-FAR/118787/2010 to DB and PEst-OE/SAU/UI4013/2014 to iMed.ULisboa).

References

[1] Clement AM, *et al. Science*. 2003; 302(5642):113-7.
[2] Brites D, Vaz AR. *Front Cell Neurosci*. 2014; 8:117.
[3] Kirby J, *et al. Brain*. 2005; 128(Pt 7):1686-706.

Chapter VI. ANNEX

Annex 3.

XVIII Congress of the Portuguese Biochemical Society, Coimbra, Portugal, December, 17-20, 2014

Exploring the pro-inflammatory environment in the spinal cord of mutant SOD1G93A mice: potential biomarkers at pre- and symptomatic phases

Carolina Cunha[1], Catarina Santos[1], Cátia Gomes[1], Ana M. Sebastião[3], Adelaide Fernandes[1,2], Ana Rita Vaz[1,2], Dora Brites[1,2]

[1]Faculty of Pharmacy, Universidade de Lisboa, Portugal
[2]Faculty of Pharmacy, Universidade de Lisboa, Department of Biochemistry and Human Biology, Lisbon, Portugal

[3]Unit of Neuroscience, Institute of Molecular Medicine, University of Lisbon, Lisbon, Portugal

Neuroinflammation is a key hallmark of amyotrophic lateral sclerosis (ALS), a severe motoneuron disease. Accumulating evidence suggest the involvement of glial cell activation during disease progression, although the features involved in each specific disease stage are still elusive. Since both diagnosis and treatment of ALS remain scarce, attempts to find novel disease biomarkers and therapies are imperative.

Here we aimed to identify potential inflammatory-related biomarkers that may differentiate ALS stages and constitute targets for therapeutics. We have performed a RT-PCR-based study using lumbar spinal cord (LSC) tissue of the TgSOD1G93A mice (mSOD1), the most used ALS model. Samples were collected at 4-5 weeks-old (presymptomatic stage, PS) and 14-16 weeks-old mice (symptomatic stage, S). Non-tg mice were used as controls.

Analysis of mSOD1 mice LSC indicated that autotaxin and CCL21 were reduced at PS phase (0.8-fold and 0.3-fold, respectively, $p<0.05$) and increased at S stage (4-fold and 3-fold, respectively, $p<0.05$). The alarmin high mobility group box-1 (HMGB1) followed such profile, i.e., low levels at PS (0.4-fold, $p<0.05$) and elevated ones at S (10-fold, $p<0.01$), revealing that a pro-inflammatory environment courses with defensive mechanisms as disease progresses. Regarding CX3CL1-CX3CR1 axis, we observed that the cytokine was down-regulated at PS phase (0.3-fold, $p<0.05$) increasing thereafter (~4-fold, NS), while its receptor was only up-regulated at S stage (5-fold, $p<0.05$). Also increased at S phase were the inflammatory-associated proteins Cx43 (5-fold, $p<0.05$), TLR2 (3-fold, $p<0.05$) and the inflammasome component NLRP3 (6-fold, $p<0.01$). By determining the inflammatory miRNA (miR) profiling, we noticed that miR-155 was already up-regulated at PS phase (1.6, $p<0.05$) while miR-124 and miR-146a were only up-regulated at S stage (1.4-fold and 3.8-fold, $p<0.05$).

Our results suggest that decreased autotaxin expression and increased miR-155 are features of the PS stage, and precede the network of pro-inflammatory-related symptomatic determinants that include HMGB1, CCL21, CX3CL1, miR-124 and miR-146a. We hypothesize that by increasing autotaxin expression and down-regulating miR-155 expression we may be able to delay ALS onset, before considering anti-inflammatory compounds to fight disease progression.

Supported by grants PTDC/SAU-FAR/118787/2010 (DB) and PEst-OE/SAU/UI4013/2013-2014, and by the fellowship SFRH/BD/91316/2012 (CC) from Fundação para a Ciência e a Tecnologia.

Annex 4.

I Luso-Brasilian Glial Network Symposium, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brasil, March 10-12, 2014

Profiling microglial reactivity in a mice model of amyotrophic lateral sclerosis

A. Rita Vaz[1,2*], Carolina Cunha[1], Filipe Nascimento[3], Ana M. Sebastião[3], Dora Brites[1,2]

[1]Instituto de Investigação do Medicamento (iMed.ULisboa), Faculdade de Farmácia, Universidade de Lisboa, Portugal; [2]Departamento de Bioquímica e Biologia Humana, Faculdade de Farmácia, Universidade de Lisboa, Portugal; [3]Unidade de Neurociências, Instituto de Medicina Molecular, Faculdade de Medicina, Universidade de Lisboa, Portugal

Abstract

Amyotrophic lateral sclerosis is a neurodegenerative disease characterized by the selective loss of motor neurons, although glial cells seem to be required for the disease initiation and/or progression. We are investigating how microglia phenotypes and activation stages may influence motor neuron degeneration. For that we use primary cultures of cortical microglia isolated from transgenic mice carrying the mutant human G93A superoxide dismutase 1 gene, as well as spinal cord homogenates from such mice. We observed that microglia reactivity differs from the presymptomatic to the symptomatic stage.