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Dissecting cross-talk between microglia and motoneurons in ALS: signaling events and soluble factors

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ABSTRACT

Convergence of pathways in motoneuron (MN) injury include microglia in the initiation and progression of Amyotrophic Lateral Sclerosis (ALS). Neuroinflammation is a pathological hallmark of ALS and microglia may acquire neurotoxic or neuroprotective properties in response to misfolded superoxide dismutase-1 (SOD1) or other molecules produced by the injured MN.

We assessed: (i) the role of microglia in preventing/restoring MN dysfunction using a mixed culture of NSC-34 MN-like cells (mutated in G93A) and of N9 microglia cells, added at 0 or 2 days-in-vitro (M0, M2) and cultured till 4 and 7 days-in-vitro; (ii) neurodegenerative network in organotypic cultures from lumbar segments of spinal cord (SC) obtained from the ALS mice model TgSOD1-G93A at 7 day-old and aged for 10 days-in-vitro, as well as the response to lipopolysaccharide (LPS, 1 μg/mL) immunostimulation. Western blot assays for SOD1, high-mobility-group-box-protein-1 (HMGB1) and toll-like receptor-4 (TLR-4), and fluorimetric/colorimetric assays for ATP, glutamate and nitric oxide (NO), were used.

Microglia (M0/M2) decreased the accumulation of human/mouse mutated SOD1 ($P<0.01$). In addition, elevation of glutamate efflux ($P<0.01$), and reduction of extracellular ATP ($P<0.01$), MMP-2 ($P<0.05$) and MMP-9 ($P<0.01$) was observed by M2 at 7 days-in-vitro. Reduction of NO ($P<0.05$) and MMP-2 ($P<0.01$) was obtained with M0. HMGB1 increased by M0 and decreased by M2, suggesting HMGB1 release from the cell. Accumulation of SOD1 was verified in SC organotypic cultures, but no changes in ATP or NO were obtained, although a slight decrease in ATP by LPS was verified. Down-regulation of TLR-4 by LPS may indicate the exhaustion of the inflammatory response mechanisms in the aged SC culture.

Together, these results suggest that microglia by inhibiting MMP activation and HMGB1 cytoplasmic translocation in the ALS model are key in modulating MN degeneration and should be considered as therapeutic targets in ALS.

Keywords: Inflammatory mediators**;** Microglia modulatory effects of motoneuron degeneration; microglia-motoneuron communication; neuroinflammation; SOD1 accumulation

RESUMO

A convergência das vias de sinalização envolvidas na lesão dos neurónios motores (NM) inclui a microglia no início e progressão da Esclerose Lateral Amiotrófica (ELA). A neuroinflamação é uma característica da ELA e a microglia pode adquirir propriedades neurotóxicas ou neuroprotectoras em resposta ao *misfolding* da superóxido dismutase 1 (SOD1) ou a outras moléculas produzidas pelos MNs lesados.

Avaliou-se: (i) o papel da microglia na prevenção/reparação da função dos NM usando uma cultura mista de NSC-34 MN-*like cells* (mutada em G93A) e células microgliais N9, adicionadas aos 0 e 2 dias de diferenciação (M0, M2) e cultivadas 4 e 7 dias-in-vitro; (ii) a neurodegenerescência de culturas organotípicas de segmentos lombares da medula espinhal (ME) de murganhos transgénicos TgSOD1-G93A obtidas aos 7 dias de vida, envelhecidas durante 10 dias-in-vitro, e a resposta ao lipopolisacárido (LPS, 1 µg/mL). Utilizou-se o Western blot para a SOD1, *high-mobility-group-box-protein-1* (HMGB1) e *toll-like receptor-4* (TLR-4), bem como ensaios fluorimétricos/colorimétricos para ATP, glutamato e óxido nítrico (NO).

A microglia (M0/M2) diminuiu a acumulação de SOD1 mutada ($P<0.01$). Verificou-se haver aumento da libertação de glutamato ($P<0.01$) e redução de ATP ($P<0.01$), MMP-2 ($P<0.05$) e MMP-9 ($P<0.01$) no meio extracelular pela M2 aos 7 dias-in-vitro. Igualmente se obteve diminuição de NO ($P<0.05$) e MMP-2 ($P<0.01$) com M0. O HMGB1 aumentou pela M0 e diminuiu pela M2, indicando a sua libertação pela célula. Verificou-se a acumulação de SOD1 nas culturas organotípicas, mas sem alteração de ATP ou NO, apesar do LPS ter causado um pequeno decréscimo do ATP. A inibição do TLR-4 pelo LPS sugere o colapso dos mecanismos de resposta inflamatória na cultura.

Os resultados ao evidenciarem a inibição da activação das MMP e da translocação citoplasmática do HMGB1 pela microglia no modelo de ELA apontam-na como alvo terapêutico na modulação da neurodegenerescência dos NM na ALS.

Palavras-chave: Mediadores inflamatórios; efeitos moduladores exercidos pela microglia na degeneração dos neurónios motores, comunicação entre os neurónios motores e a microglia; neuroinflamação; acumulação de SOD1

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ABBREVIATIONS

ALS	Amyotrophic lateral sclerosis
ALS2	Alsin
AMPA	α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANG	Angiogenin
APC	Antigen presenting cell
ARE	Antioxidant-response element
ATP	Adenosine triphosphate
Bcl-2	B-cell lymphoma 2
BDNF	Brain-derived neurotrophic factor
Bip	Binding immunoglobulin protein
BMAA	Toxin β-methyl-amino-L-alanine
BSA	Bovine serum albumin
$CaCl_2$	Calcium chloride
CCL2	C-C motif ligand 2
cDNA	Complementary DNA
CDP	Choline - Cytidine-5- diphosphocholine
CNS	Central nervous system
CNTFR	Ciliary neurotrophic factor
COX-2	Cyclooxygenase 2
CSF	Cerebrospinal fluid
CX_3CL1 or Fractalkine	CX_3C-chemokine ligand 1
CX_3CR1	CX_3C-chemokine receptor 1
DAMP	Damage-associated molecular pattern
DIV	*Days in vitro*
DMEM-Ham´s F-12	Dulbecco's modified Eagle's medium-Ham's F12
DNA	Deoxyribonucleic acid
EAAT	Excitatory amino acid transporter
EDTA	Ethylenediamine tetraacetic acid
EMG	Electromyography
EPO	Erytropoietin
ER	Endoplasmatic reticulum
ERAD	ER-associated protein degradation
fALS	Familiar Amyotrophic Lateral Sclerosis
FBS	Fetal bovine serum
FTD	Frontotemporal dementia
FUS/TLS	Sarcoma/ Translated in liposarcoma
G418	Geneticin sulfate
GDNF	Glial-derived neurotrophic factor

GFAP	Glial fibrillary acidic protein-Cre
GLT1	Glutamate transporter-1
GM-CSF	Granulocyte/macrophage-colony stimulating factor
GUDCA	Glycoursodeoxycholic acid
HBSS	Hank's balanced salt solution
HMGB1	High-mobility group box 1
H_2O_2	Hydrogen peroxide
hSOD1	Human SOD1
IFNγ	Interferon gamma
IGF1	Insulin-like growth factor 1
IL	Interleukin
iNOS	Inducible nitric oxide synthase
iPSC	Induced pluripotent stem cell
KOH	Potassium hydroxide
LC3	Microtubule-associated protein 1A/1B-light chain 3
LMN	Lower motoneurons
LPS	Lipopolysaccharide
M-CSF	Macrophage-colony stimulating factor (M-CSF)
MFG-E8	Milk Fat Globule Factor-E8
MHC	Major histocompatibility complex
miRNAs	microRNAs
MMP(s)	Metalloproteinase(s)
MN(s)	Motoneuron(s)
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MRI	Magnetic resonance imaging
mRNA	Messenger ribonucleic acid
MSC	Mesenchymal stem cell
mSOD1	Mutated SOD1
mTOR	mammalian Target of rapamycin
NaCl	Sodium chloride
NADPH	Nicotinamide adenine dinucleotide phosphate
NEAA	Non-essential aminoacids
NF-H	Neurofilament heavy
NF-κB	Nuclear factor-κB
NF-L	Neurofilament light
NF-M	Neurofilament medium
NGF	Neurotrophic growth factor
NMDA	N-methyl-D-aspartic acid
NMJ	Neuromuscular junction
NO	Nitric oxide

NO_2	Nitrites
NP-40	Nonyl phenoxypolyethoxylethanol
Nrf2	Nuclear erythroid 2 – related factor 2
NSC-34	Neuroblastoma spinal cord 34
PAMP	Pathogen-associated molecular pattern
PDI	Protein disulphide isomerase
PDL	Poly-**D**-Lysine
PET	Positron emission tomography
PGE2	Prostaglandin E2
P38 MAPK	p38 mitogen-activated protein kinase
PMSF	Phenylmethylsulfonyl fluoride
PRR	Pattern-recognition receptor
P2X receptor	Ionotropic receptor
P2Y receptor	Metabotropic receptor
RAGE	Receptor for advanced glycation end-products
RIPA	Ice radio-immunoprecipotation assay
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute
sALS	Sporadic Âmyotrophic Lateral Sclerosis
SC	Spinal Cord
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulfate-polyacrilamide gel electrophoresis
SETX	Senataxin
siRNA	Small interference RNA
SMN1	Gemin-1
SOD1	Superoxide dismutase 1
TARDP	TAR DNA binding protein/TDP-43
TBS	Tris buffered saline
Tg	Transgenic
TGFβ	Transforming growth factor-β
THA	Threo-β-hydroxyaspartate
TIMP(s)	Inhibitor(s) of matrix metalloproteinases
TLR	Toll like receptor
TNF	Tumor necrosis factor
UDP	Uridine diphosphate
UMN	Upper motoneurons
UPR	Unfolded protein response
UPS	Ubiquitin-proteasome system
VABP	Vesicle associated membrane protein-associated protein B
VDAC	Voltage-dependent anion channel

VEGF	Vascular endothelial growth factor
wt	Wild type

I. INTRODUCTION

1. New insights on amyotrophic lateral sclerosis (ALS)

Amyotrophic Lateral Sclerosis (ALS), also known as Charcot's disease or as Lou Gehrig's disease (Naganska and Matyja, 2011) is a neurodegenerative disease characterized by death of lower motoneurons (MN) in the brainstem and spinal cord (SC) and of upper MN in the motor cortex (Ferraiuolo *et al.*, 2011), as showed in Figure I.1. The name "amyotrophic" are related to the atrophy of muscle fibers, weakness and loss of muscle mass; "lateral" refers to the nerve pathways that are localized along both sides of the SC, and "sclerosis" is the scan tissue resulted from the process of nerve degeneration. ALS was first described by Jean- Marie Charcot in 1869, a French neurobiologist and physician who associated most of the symptoms with the lesion of MN that originate in the SC (Musaro, 2010). Some groups of neurons such as the ones in upper brainstem nuclei (that control eye movements) and neurons in Onuf's nucleus within the sacral SC (that control the bladder) are less affected by this disease (Kirby *et al.*, 2005). Despite the cognitive ability, sensation and autonomic nervous functions are usually not affected, 5-10% of patients with ALS also develop fronto-temporal lobar dementia (Redler and Dokholyan, 2012). Clinical presentation includes weakness, wasting, fasciculations and atrophy of muscles involving the bulbar and limb regions. ALS leads to a slurred speech, dysarthria, a progressive paralysis and ultimately death, usually by respiratory failure (Kiernan *et al*., 2011; Musaro, 2010; Shaw, 2005).

If the disease affects only the spinal lower MNs, the condition is called "progressive muscular atrophy"; if only the upper MNs are affected, the condition is called "primary lateral sclerosis"; finally, if only the bulbar musculature is affected, the denomination is "progressive bulbar palsy". Most of the patients with at least one of these initial conditions will develop features of ALS over time (Shaw, 2005). On the other hand, some long-term survivors maintain one of those variants of ALS (Evans *et al.*, 2013). Moreover, these variants differ from ALS in terms of disease progression, survival and clinical presentation; in progressive muscular atrophy the prognosis is better, with a 5-10 year of survival rate, slow progression and absence of upper MN signs (brisk reflexes, spastic catch and Babinski sign) (Kim *et al.*, 2009). Although primary lateral sclerosis has many similarities with ALS, disease progression is slower, survival is longer and patients usually show muscle stiffness or spasticity and, very rarely, limb wasting (Tartaglia *et al.*, 2007). Progressive bulbar palsy tends to have a worse prognosis than ALS and the other variants, and involves death of MN of the lower brainstem with or without involvement of the cortico-bulbar tract (Karam *et al.*, 2010).

The worldwide prevalence is 3-5/100,000 habitants and the lifetime risk of developing ALS is 1-800 (Naganska and Matyja, 2011; Redler and Dokholyan, 2012). Usually, ALS first symptoms occur at middle age (50-60 years), although it may appear in younger people, with an incidence of 1-3/500 000(Ferraiuolo *et al.*, 2011; Kirby *et al.*,2005; Naganska and Matyja, 2011). It generally affects more men than women, with a male to female ratio of approximately 1.6/1. The average survival is 3 years

from symptoms onset but a small proportion of patients have a slower disease course (Wood-Allum and Shaw, 2010).

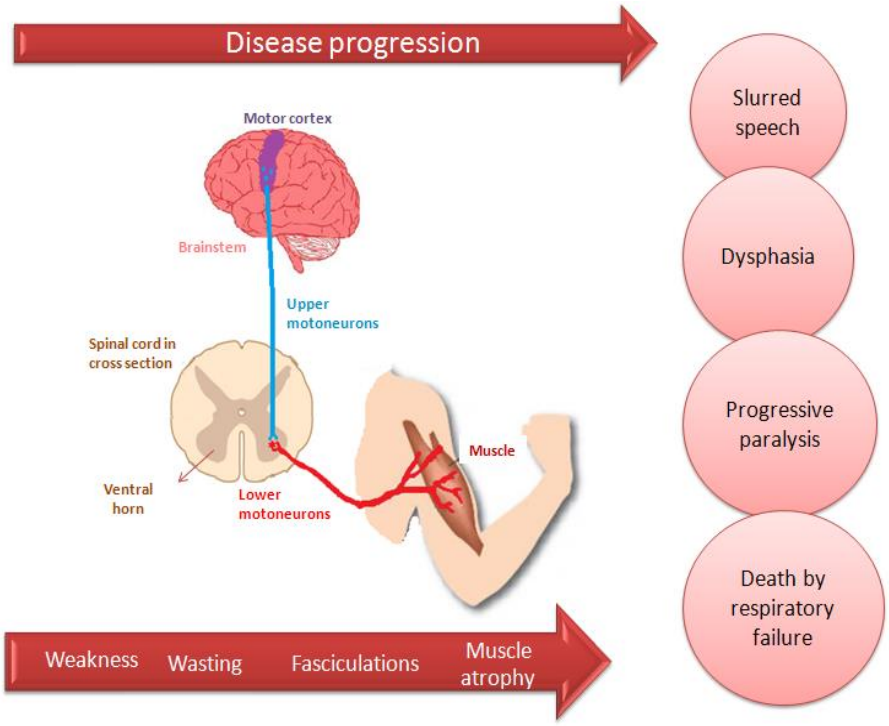

Figure I.1 - Amyotrophic Lateral Sclerosis is a neurodegenerative disease characterized by death of lower motoneurons (LMN) in the brainstem and spinal cord and of upper motoneurons (UMN) in the motor cortex. UMN connect motor cortex and spinal cord, through the extension of their fibers along the brainstem, while LMN connect spinal cord to voluntary muscles. Together, they form the corticospinal tract. A fail in one of these connections originates weakness, wasting, fasciculations and muscle atrophy involving the bulbar and limb regions. Consequently, it leads to a slurred speech, dysarthria, progressive paralysis and ultimately death, usually by respiratory failure.

1.1. Genetic Causes

It has been proposed a large genetic contribution for ALS, once it was observed that Mendelian genes are mutated in individuals with no familial history of this disease (Al-Chalabi and Lewis, 2011). However, it is difficult to identify ALS genes because it is a disease of later life, so it is rare to obtain large pedigrees for linkage studies, and the prognosis is poor, making the point prevalence consequently low (Al-Chalabi *et al.*, 2012). Nevertheless, about 90-95% of ALS cases are classified as sporadic (sporadic ALS or sALS), with unknown causes, and 5-10% of cases are inherited (familiar ALS or fALS). The most common inheritance pattern for fALS is autosomal dominant (Naganska and Matyja, 2011). Several genes have been identified as a cause or risk factor for the development of the disease (Armon, 2005), revealing a high degree of heterogeneity (Sabatelli *et al.*, 2013). That identification has provided important clues about the molecular mechanisms implicated in ALS (Naganska and Matyja, 2011). Among these genes, it stands out the gene encoding superoxide dismutase 1 (*SOD1*), which accounts up to 20% of inherited ALS cases (Andersen, 2006), TAR DNA binding protein/TDP-43 (*TARDP*) (Sreedharan *et al.*, 2008) and mutations in the fused in sarcoma/translated in liposarcoma (*FUS/TLS*) (Kwiatkowski *et al.*, 2009).

1.1.1 Mutations in *SOD1*

The discovery in 1993, by Rosen and collaborators, of the mutations in the gene on chromosome 21q22.1, which encodes the enzyme superoxide dismutase 1 (SOD1), marked the beginning of the research around the causes of ALS (Rosen *et al.*, 1993).

These mutations account for 20% of fALS cases (Rowland and Shneider, 2001), although the frequency varies among populations (Andersen, 2006). The patients with mutant SOD1-related ALS show several phenotypes, but the typical fast progressive ALS phenotype prevails and is clinically not distinguishable from sALS (Ince *et al.*, 2011).

SOD1 is a metalloenzyme that acts as a homodimer and whose function is to convert intracellular superoxide free radicals – a toxic waste product of mitochondrial oxidative phosphorylation – to hydrogen peroxide (H_2O_2). Subsequently, the H_2O_2 is eliminated by the action of other free radical scavenging enzymes (Siegel *et al.*, 2006). The elimination of radicals protects the cell from deoxyribonucleic acid (DNA) and intracellular protein oxidative damage (Naganska and Matyja, 2011). SOD1 contains one cooper and one zinc ion per monomer. The cooper ion has an active scavenging activity (Bendotti *et al.*, 2012), while the zinc ion stabilizes the protein structure (Shaw, 2005). This protein is widely expressed in the mammalian central nervous system (CNS), accounts for about 1% of all brain proteins and is present at a very high content in MN (Siegel *et al.*, 2006).

It has been identified more than 160 mutations so far, spanning all five exons (Sabatelli *et al.*, 2013). Most of the mutations are missense, which most probably alter the active site of the enzyme (Shaw and Eggett, 2000), while non-sense mutations or gene deletions are rare. Most of the mutations are inherited in an autosomal dominant form (Sabatelli *et al.*, 2013); the mutation resulting from substitution of alanine by valine in position 5 of the protein (p.ALA5VAL) is present in 50% of families in USA, and is usually associated with a fast progressive disease (Al-Chalabi *et al.*, 2012).

Initially, there were findings suggesting a loss of SOD1 function as a causative mechanism of ALS, due to the decrease of the enzymatic activity in patients with ALS, and the distribution of ALS-causative mutations spread throughout the SOD1 gene (Deng *et al.*, 1993; Rosen *et al.*, 1993). Further studies evidenced that mutated SOD1 (mSOD1) possesses a neurotoxic property that is responsible for the pathogenic mechanisms of the disease and known as gain of function hypothesis. It began with the analysis of mSOD1 mouse models by Gurney and colleagues (1994). Actually, there are more than 12 different published human mSOD1 transgenic (tg) strains, which contribute to the development of a progressive adult-onset motor phenotype, characterized by the loss of lower MN (Joyce *et al.*, 2011). Although the two hypothesis are still discussed, there is increased evidence that the gain of function hypothesis is most likely because: a) in humans no correlation was found between SOD1 dismutase activity and severity of clinical phenotypes (Ratovitski *et al.*, 1999); b) in mice, no evidence of ALS-like phenotype was found in SOD1 null animals (Reaume *et al.*, 1996); and c) Bruijn and colleagues (1998) found no change in survival time of mice carrying a mSOD1 transgene (SOD1 G85R) on a normal mouse background (i.e. with two copies of the endogenous mouse Sod1 gene) compared with the same transgene on a null SOD1 background.

It has been investigated the correlation between mutations and phenotype due to the large vulnerability of phenotypes in terms of disease progression, extramotor features and age of onset (Milani *et al.*, 2011).The heterogeneity among SOD1 mutation is responsible for a difficult identification of pathways that leads to the cell death of MN (Bruijn *et al.*, 2004).

1.1.2 - Mutations in *TDP-43* and *FUS*

Both TDP-43 and FUS/TLS are multifunctional proteins with functions related to gene expression, transcription, RNA splicing, transport and translation. They are also responsible for the processing of microRNAs (miRNAs), RNA maturation and splicing (Kiernan *et al.*, 2011). Mutations in *TDP-43* and *FUS* account for 4-5% of patients with fALS (Naganska and Matyja, 2011) and 5 % of sALS, in the case of *TDP-43* (Redler and Dokholyan, 2012). It was found an association between ALS with frontotemporal dementia (FTD) and Parkinsonism in patients with chromosome 17-linked disease with mutations in the *TDP-43*. ALS with dementia also occurs in the gene mapped to 9q21-22 (Rowland and Shneider, 2001). The inclusions are present in half patients with FTD (Neumann *et al.*, 2006). In addition, mutations in FUS gene were found in patients with FTD and fALS with Parkinson's disease (Yan *et al.*, 2010).

Regarding TDP-43, mutations in this gene results in a neuronal aggregation of abnormal protein in patients with sALS and non-SOD1 fALS (Tan *et al.*, 2007). This mislocalization within the cell is responsible for the toxicity in MN, but how TPD-43 causes the disease is still unknown (Traub *et al.*, 2011). Besides studies of patients with TARDP mutations, a tg mice expressing the mutated human TDP-43 developed by Wegorzewska and colleagues (2009) causes a clinical syndrome similar to human ALS, but without cytoplasmatic aggregates of TDP-43. Zhou and colleagues (2010a) developed tg rats with mutant TDP-43 that causes nerve degeneration and aggregates of TDP-43. Similar to TDP-43 protein, aggregates of FUS are found in the cytoplasm of MN in patients with no pathological changes in TDP-43 or SOD1, suggesting a novel disease pathway (Kiernan *et al.*, 2011). The discovery of mutations in TDP-43 and FUS/TLS in fALS and the presence of abnormal TDP-43 in sALS opened the development of research on the role of RNA metabolism and processing, which will be further explored in section 1.3.1.

1.2. Environmental causes

It has been proposed that exposure to some chemicals and toxins, viral infection and Prion disease, as well as a lifetime of intensive sport or physical exertion, are some of the environmental causes that may contribute to ALS onset (Kiernan *et al.*, 2011; Redler and Dokholyan, 2012; Rowland and Shneider, 2001). However, most of these reports involve a very small number of cases, which do not allow a rigorous evaluation of these factors as potential risks for ALS development (Redler and Dokholyan, 2012). Nevertheless, regarding exposure to some toxins, there is increased evidence that active smokers have a double risk of developing ALS, compared with people who never smoked (Gallo *et al.*, 2009). The most convincing case related with toxins is the association between β-methyl-amino-L-alanine (BMAA) exposure and the development of an epidemic of ALS-Parkinson's disease on the island of Guam. BMAA is a neurotoxin produced by cyanobacteria and a dietary compound

found in the seed of the cycad Cycas cirinalis. These seeds were used to make flour by the Chamorro during 1950's and are also eaten by flying foxes. Consequently, not the consumption of products derived from flour, but the consumption of flying foxes provoked an increased incidence of ALS (Cox *et al.*, 2005). In addition, some virus, such enterovirus (Berger *et al.*, 2000), immunodeficiency virus and human T-cell lymphotrophic virus type I (Rowland and Shneider, 2001) have been reported in a small number of patients who developed ALS symptoms. Lyme disease, an infectious disease caused by at least three species of bacteria, may occasionally produce a syndrome with both upper and lower MN symptoms, but it did not develop a typical ALS (Berger *et al.*, 2000).

1.3. MN vulnerability

ALS is a multifactorial disease, with pathophysiological mechanisms that show a complex interaction between genetic and molecular pathways, most of them related with the subtype of disease caused by mutations in SOD1 (Ferraiuolo *et al.*, 2011). MNs seem to be the main target of injury in ALS. The mechanisms that lead to the death of MNs are not completely understood, although structural and metabolic specialization could contribute to their vulnerability (Shaw and Eggett, 2000). MN are large cells with a cell body of nearly 50-60 μm and an axon of up to 1 m long (Barber and Shaw, 2010). Consequently, the cell requires high amounts of energy, necessary for the production and transport of cellular components, regulation of messenger ribonucleic acid (mRNA) distribution for protein synthesis, maintenance of the membrane potential along the axon and action potential generation (Ferraiuolo *et al.*, 2011).

Those mechanisms include deregulated transcription and RNA processing, oxidative stress, mitochondrial dysfunction, excitotoxicity, protein aggregation, deregulated endosomal trafficking, endoplasmatic reticulum (ER) stress, cellular death and impaired axonal transport, which will be further dissected in this chapter and illustrated in Figure I.2.

1.3.1. Deregulated transcription and RNA processing

Deregulated transcription and RNA processing was first detected in MN degeneration through the identification of mutations in survival MN protein or gemin-1 gene (*SMN1*) by Lefebvre and colleagues (1995). This gene encodes a protein responsible for the assembly of small nuclear ribonucleoproteins, important for pre-mRNA splicing (Burghes and Beattie, 2009). Gene expression profiling showed a transcriptional repression of mSOD1 in neuroblastoma-spinal cord MN 34 (NSC-34) cells stably expressing SOD1G93A (Kirby *et al.*, 2005) and in isolated MN from SOD1 G93A mice with late-stage disease (Ferraiuolo *et al.*, 2007).

TDP-43 and FUS/TLS are proteins present in the nucleus of healthy cells and are involved in RNA processing events such as splicing and transcriptional regulation (Lagier-Tourenne *et al.*, 2010). The investigations about the mechanisms by which TDP-43 and FUS trigger neurodegeneration are in an initial stage; thus, it is still unknown if the neurodegeneration is due to a loss of function of these proteins, a gain of toxic properties, or a combination of both, associated with nuclear or cytoplasmatic aggregates. The gain of function hypothesis proposes that TDP-43 and FUS/TLS cytoplasmic inclusions drive the disease, through an aggregation mechanism (Polymenidou *et al.*, 2012). TDP-43 and FUS are also involved in the formation of stress granules-cytoplasmic foci containing RNA in

complex with RNA-binding proteins that transiently appear to be under cellular stress (Bosco *et al.*, 2010; Dewey *et al.*, 2011). It may be possible that stress granules transform into pathogenic inclusions during neurodegeneration. Additionally, the reception of specific cellular RNAs within cytoplasmic TDP-43 and FUS inclusions may deplete the cell of essential RNA components, contributing to pathogenesis (Polymenidou *et al.*, 2012). It was reported in ALS patients splicing alterations, some of which may be directly related to TDP-43 misregulation (Xiao *et al.*, 2011). Oxidation of mRNA has been also identified in ALS patients and tg mice expressing a variety of fALS-linked SOD1 mutations (Barber and Shaw, 2010).

Other genes coding proteins involved in RNA transcription, such angiogenin (*ANG*) and senataxin (*SETX*) have similarly been object of studies (Ferraiuolo *et al.*, 2011).

1.3.2. Oxidative stress

Cellular reactive oxidative species (ROS) results mainly from the aerobic metabolism; in fact, during oxidative phosphorylation in the mitochondria, there is a “leakage” of the electrons from the mitochondrial respiratory chain. Posteriorly, these species react with other compounds to produce more potent oxidants (Siegel *et al.*, 2006). These potent oxidants are capable of changing protein conformation, alter cellular membrane dynamics by oxidation of unsaturated fatty acids, and cause damage in DNA and RNA (Barber and Shaw, 2010). Oxidative stress defines a condition that disrupts redox signaling and control, through the damaged compartmentalized cellular redox circuits (Jones, 2006).

ALS-vulnerable MN are particularly susceptible to oxidative stress, since they have low endogenous calcium buffering capacities, which occur due to the low expression of cytosolic calcium-binding proteins (Barber and Shaw, 2010). Consequently, it allows a fast recovery time of activity-related calcium transients at a relatively low energy cost, which is useful in the practice of motor activities like running or breathing (Lips and Keller, 1999). On the other hand, low buffering capacities leads to formation of large volume calcium micro-domains around open channels, mainly near mitochondria (von Lewinski and Keller, 2005). Consequently, mitochondria pick up more free calcium, triggering an increase in ROS production and contributing to oxidative stress (Barber and Shaw, 2010). Moreover, MN have a high threshold for mounting a protective heat shock response, increasing sensitivity to ER stress and mitochondrial features (Ferraiuolo *et al.*, 2011).

It is still debated if oxidative stress is a primary cause of degeneration or results from another toxic insult in the pathogenesis of ALS. In fact, identification of ALS associated SOD1 mutations revealed oxidative stress as a primary driver in ALS pathogenesis (Barber and Shaw, 2010). Moreover, oxidative stress aggravates other pathophysiological processes involved in neurodegeneration, such as excitotoxicity (Rao and Weiss, 2004), mitochondrial impairment (Duffy *et al.*, 2011), protein aggregation (Wood *et al.*, 2003) and ER stress (Kanekura *et al.*, 2009), besides being an underlying cause for alterations in signaling from astrocytes (Blackburn *et al.*, 2009) and microglia (Sargsyan *et al.*, 2005). Indeed, studies of cerebrospinal fluid (CSF) and human postmortem CNS tissue from ALS patients showed biochemical changes that represent the effect of free radical

damage or abnormal free radical metabolism (Ferrante *et al.*, 1997; Shaw *et al.*, 1995; Smith *et al.*, 1998; Tohgi *et al.*, 1999).

SOD1 plays an important role as an antioxidant. So, the accumulation of this enzyme in wild type (wt) or mutated state influences this mechanism. Transgenic mouse models of ALS expressing human mSOD1 showed increased oxidative damage at the level of proteins, lipids and DNA (Casoni *et al.*, 2005; Liu *et al.*,1999; Poon *et al.*, 2005). Moreover, mSOD1 itself was the most severely oxidized protein in the SOD1 G93A mouse (Andrus *et al.*, 1998). As previously mentioned, it is known that mSOD1 is toxic through an unknown gain of function. For example, beyond catalyzing SOD radicals, SOD1 also acts as a peroxidase, producing hydroxyl radicals, using H_2O_2 as a substrate (Yim *et al.*, 1990). Alternatively, mutations in SOD1 may difficult the enzyme to bind zinc (Estevez *et al.*, 1999), which will impede the elimination of ROS by both mutant and wt SOD1. An hypothesis is that this impossibility of SOD1 to bind zinc will favor the reducing agents to react with oxidized calcium at the active site, and consequently more production of superoxide, which reacts with nitric oxide (NO) to produce peroxynitrite, will ultimately cause tyrosine nitration (Barber and Shaw, 2010). Indeed, studies in SOD1 G93A mice with zinc deficiency showed acceleration in the disease progress (Ermilova *et al.*, 2005).

Some studies of MN expressing mSOD1 showed down-regulation of genes involved in the antioxidant response, such as the transcription factor nuclear erythroid 2 – related factor 2 (Nrf2). Activation or Nrf2 determines its translocation from the cytoplasm to the nucleus, where it interacts with the antioxidant-response element (ARE) sequence to increase the expression of proteins with antioxidant function (Nguyen *et al.*, 2009). Down-regulation of Nrf2 expression will reduce the capacity to remove ROS, which has been reported in mSOD1 models of ALS (Kirby *et al.*, 2005) and in the CNS of patients with ALS (Sarlette *et al.*, 2008).

Finally, it was reported the role of microglial mSOD1. It can increase the production of superoxide by nicotinamide adenine dinucleotide phosphate oxidase (NADPH oxidase), through the blockage of Rac1 into its active state in the Nox complex (Harraz *et al.*, 2008). The increasing of Nox2 expression was detected in mSOD1 mice and in the CNS of patients with ALS. Interestingly, knockout of Nox1 or Nox2 revealed to increase the survival rate of SOD1 G93A (Ferraiuolo *et al.*, 2011).

1.3.3. Mitochondrial dysfunction

Mitochondria are responsible for the generation of energy, through the production of adenosine triphosphate (ATP), as well as to maintain calcium homeostasis and regulation of the initiation of apoptotic cell death (Pizzuti and Petrucci, 2011). Besides that, mitochondria generate free radicals, as mentioned in the previous section, which are the main cause for oxidative damage. Moreover, the requirement of high amounts of energy by MN promotes an intense mitochondrial activity and a consequent production of higher quantity of ROS, contributing to a consequent oxidative stress event (Genova *et al.*, 2004).

In fact, mSOD1 provokes dysfunction and structural damage of mitochondria in human patients and mouse models of ALS (Higgins *et al.*, 2003). In SOD1 G93A mice, SOD1 aggregates were observed in vacuoles and mitochondrial intermembrane space, at an early stage of the disease (Higgins *et al.*, 2002). Mutant SOD1 also accumulates in mitochondrial outer membrane, impeding

protein import (Vande Velde *et al.*, 2008), and binds directly to the voltage-dependent anion channel (VDAC), depolarizing the membrane and contributing to the abnormal function of electron transport chain, thus increasing ROS production (Mattiazzi *et al.*, 2002).

The disturbance of mitochondrial function by mSOD1 causes cell death through the activation of the apoptotic cascade. Some of the mechanisms that trigger apoptosis are the release of cytochrome *c* (Kirkinezos *et al.*, 2005) and the binding of mSOD1 to the pro-survival factor B-cell lymphoma 2 (Bcl-2) (Pedrini *et al.*, 2010).

Excitotoxic events trigger a high calcium influx in response to glutamate. Consequently, elevation of cytosolic calcium levels in neurons induce enhanced production of free radicals from mitochondria. Transgenic mouse with mSOD1 G93A also presents a significant decrease in mitochondrial calcium loading capacity in brain and SC (Damiano *et al.*, 2006). In a neuronal model with mSOD1 G93A, it was observed a morphologic swollen mitochondria, impaired activity in electron transport chain, compromised cellular bioenergetic status and alterations in mitochondrial proteome (Fukada *et al.*, 2004; Menzies *et al.*, 2002). In addition, in these cellular models, it was verified a deceleration of fast axonal transport of mitochondria and other membrane bound organelles, caused by energy supply in MN (De Vos *et al.*, 2007; Zhang *et al.*, 2007).

1.3.4. Excitotoxicity

Glutamate is the main excitatory neurotransmitter in the CNS and exerts its effects through an array of ionotropic and metabotropic postsynaptic receptors. G protein-coupled receptor is a metabotropic receptor, which leads to the release of intracellular calcium stores when activated. Glutamate-gated ion channels are ionotropic receptors, which subdivide in N-methyl-D-aspartic acid (NMDA), a calcium permeable channel, and in non-NMDA, a channel whose calcium permeability varies with the subunit composition. α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) is a non-NMDA channel that has a subunit called GluR2, whose activity depends on post-transcriptional editing of GluR2 mRNA. When this subunit is present, the channel is impermeable to calcium; when AMPA lacks GluR2 subunit, it becomes calcium permeable (Siegel *et al.*, 2006).

To remove glutamate from the synaptic cleft, there are the excitatory amino acid transporters (EAATs), predominating the EAAT2, also called glutamate transporter-1 (GLT1), mainly in astrocytes located near post-synaptic neurons (Rowland and Shneider, 2001).

Excitotoxicity results from excessive activation of glutamate receptors and may be caused by an increased sensitivity of the post-synaptic neuron to glutamate or by increased synaptic levels of glutamate (Van Damme *et al.*, 2005). It could disrupt intracellular calcium homeostasis, contributing to the damage of intracellular organelles, perturbation of ATP production and activation of proteolytic and ROS-generating enzyme systems (Arundine and Tymianski, 2003). MN are particularly sensible to glutamatergic toxicity via AMPA receptor activation, since they have a high expression of this receptor lacking the GluR2 subunit (Williams *et al.*, 1997). Consequently, during an excitotoxic event, elevated concentrations of calcium enter in the cell and are captured by mitochondria, contributing to ROS production (Carriedo *et al.*, 2000). Studies demonstrated that the levels of glutamate in CSF of some patients with ALS are elevated (Rothstein *et al.*, 1992). It has also been observed hyperexcitability of

the motor system at early stages of the disease using transcranial magnetic stimulation techniques in conjunction with peripheral nerve excitability studies in humans (Vucic and Kiernan, 2006).

Another via excitotoxicity is the selective loss of EAAT2. This loss can be associated to an aberrant splicing of EAAT2 mRNA in affected regions of the CNS (Lin *et al.*, 1998). Consequently, glutamate remains in the environment and continues to activate their receptors (Musaro, 2010). Indeed, patients with sALS and fALS, as well as mSOD1 rat, have all presented decreased levels of EAAT2 (Fray *et al.*, 1998; Howland *et al.*, 2002).

1.3.5. Protein aggregation

In normal cellular behavior, protein control is maintained by the activation of protein chaperones and ubiquitin-proteasome system (UPS), preventing the toxic effects of mutant proteins. However, under cases of physiological or environmental stress, this system becomes overloaded and impaired (Kabashi and Durham, 2006). One of those cases is the occurrence of extra- or intracellular fibrillar aggregates that contain β-sheet conformation, which constitutes an important feature of ALS (Musaro, 2010). Many cytoplasmic aggregates from different proteins were found in fALS cases, such as SOD1, TDP-43 and FUS (Ferraiuolo *et al.*, 2011).

In SOD1 tg mouse, insoluble inclusion bodies of SOD1 appear in brain stem and in SC, coincident with symptom onset, and accumulate progressively in the terminal stages, causing symptoms only when UPS cannot destroy SOD1 aggregates (Redler and Dokholyan, 2012). Aggregates of SOD1 were also observed in cell culture models and in SOD1-linked fALS patients (Ferraiuolo *et al.*, 2011). Many hypothesis have been proposed to explain how mSOD1 aggregates trigger cell toxicity. One of them is the sequestration by these aggregates of other proteins required for normal MN function (Shaw, 2005). Another option are the continued misfolding of SOD1 aggregates, that maintain chaperone constantly occupied, preventing the access of other proteins necessary for folding or function (Bruening *et al.*, 1999). Additionally, mSOD1 aggregates could reduce proteasome activity needed for normal protein turnover (Allen *et al.*, 2003) and, finally, mSOD1 could inhibit the function of specific organelles by their aggregation on them or even in their inside (Shaw, 2005).

TDP-43 forms aggregates that have an ultrastructural similarity to TDP-43 deposits in degenerating neurons of ALS patients. The C-terminal domain of TDP-43 is crucial for spontaneous aggregation, leading to a distribution of inclusions along the cytoplasm (Johnson *et al.*, 2009). The presence of these aggregates may be a pathogenic marker in sALS and fALS (Sreedharan *et al.*, 2008).

Finally, cytoplasmic inclusions containing mutant FUS have been observed in some patients with FUS-related fALS (Ferraiuolo *et al.*, 2011), providing several important clues about the new disease pathway, once no SOD1 or TDP-43 aggregates are found (Kiernan *et al.*, 2011).

1.3.6. Deregulated endosomal trafficking

Endocytosis is a process for moving a wide variety of molecules from the cell periphery into the cytoplasm of the cell. Specific cargo molecules are transported and delivered to the correct destination within the cells. This transport are mediated by vesicles and/or vacuoles, which then

mature or fuse with early endosomes, before the cargos are delivered to their end destinations (Otomo *et al.*, 2012). Endocytosis are controlled and composed by a variety of molecules, whose mutations have been implicated in several genetic subtypes of ALS. Alsin is a guanine nucleotide exchange factor for the small GTPase protein Rab5 and is involved in endosomal fusion and trafficking, as well as neurite outgrowth (Ferraiuolo *et al.*, 2011). One form of juvenile onset autosomal recessive ALS (ALS2) has been linked to the loss of function of the alsin gene (Yang *et al.*, 2001). Most mutations in this gene lead to premature stop codons, resulting in carboxyl-terminal truncated proteins that are unstable, compared with the wt alsin (Yamanaka *et al.*, 2003). Loss of function of alsin causes the degeneration of MN, but the pathogenic mechanisms of ALS2 remain unknown (Lai *et al.*, 2006).

Vesicle associated membrane protein-associated protein B (VAPB) is a protein resident in endoplasmic reticulum (ER) which is possibly involved in unfolded protein response (UPR). Indeed, studies refer that mutations in this gene constitute a risk factor for MN disease mediated by loss of its function (Kabashi *et al.*, 2013). More rare mutations in genes coding optineurin (Maruyama *et al.*, 2010), charged multivesicular protein 2B (Parkinson *et al.*, 2006) and valosin-containing protein (Johnson *et al.*, 2010) have also been described in ALS.

1.3.7. Endoplasmatic reticulum stress

The ER is responsible for translation, folding and transport of membrane proteins and secreted proteins. It also stores high calcium levels and interacts closely with mitochondria by exchanging calcium in a cyclic manner, which is thought to synchronize energy demand and rates of protein folding, and also to initiate apoptosis. It interacts closely with the nucleus and the Golgi apparatus to direct proteins to axonal transport and exocytosis, and protein degradation pathways. ER can recognize aberrant proteins and refold them through the activation of ER chaperones such as binding immunoglobulin protein (BiP) or drive out them through the ER-associated protein degradation (ERAD) pathway, activating UPR (Siegel *et al.*, 2006). When there are problems in calcium exchanges or the accumulation of misfolded proteins, ER stress can be triggered (Schroder, 2008). Studies showed that markers of ER stress are up-regulated in the CSF and SC of patients with sALS, as early features in ALS (Ferraiuolo *et al.*, 2011). For example, Atkin and colleagues (2008) identified the activation of protein disulphide isomerase (PDI), an ER chaperone and a marker of the UPR derived from the presence of mSOD1 inclusions, both in mSOD1 mice and in autopsies from patients with sALS. PDI and other UPR-induced proteins are up-regulated at the beginning of the disease in mSOD1 rodents, reinforcing the concept that ER stress is involved in the onset of MN injury (Atkin *et al.*, 2008). Nishitoh and colleagues (2008) found that mSOD1 interacts with ER-associated degradation ERAD protein Derlin-1, a transmembranar protein responsible for the translocation of misfolded proteins from the ER lumen, in mouse neuronal tissue, leading to ER stress-mediated cell death through apoptosis and stimulating kinase-1. Vijayalakshm and colleagues (2011) exposed MN-like cells and MN to CSF from patients with ALS and observed an increase in ER stress, which included expression of UPR markers, ER fragmentation and caspase-12 activation. Mutations in VAPB gene are also involved in ER stress, since it forms aggregates through conformational changes (Chen *et al.*, 2010).

1.3.8 – Cellular Death

Apoptosis is a form of programmed cell death, which includes several morphological and biochemical changes that characterize and permit the identification this type of cell death. Interveners in this event include: a) caspase family of proteolytic enzymes responsible for the destruction of structural and regulatory proteins; b) the Bcl-2 family of oncoproteins, which are associated with the mitochondrial membrane and modify its permeability; and c) apoptosis inhibitor family of proteins that prevents proteolytic activation of specific caspases and, therefore, apoptosis (Siegel *et al.*, 2006).

In ALS, it is accepted that MN die by a programmed cell death pathway and biochemical markers of apoptosis were found in the terminal stages of human and in tg mice. Mutated SOD1 is involved in the activation of apoptosis in cultured neuronal cells either transfected or microinjected with mSOD1 complementary DNA (cDNA), evidenced to suffer apoptosis (Durham *et al.*, 1997; Pasinelli *et al.*, 1998). Mitochondria also participate in apoptosis, through the release of pro-apoptotic factors, such as cytochrome *c*. In ALS tg mice, it was observed an impairment in the association of cytochrome *c* with the inner membrane of the mitochondrion, leading to its release to cytoplasm with subsequent activation of the apoptotic cascade (Bacman *et al.*, 2006). Activation of caspases is associated with MN degeneration. In the SOD1 G85R mice, caspase-1 is activated months before caspase-3 activation, MN death and clinical onset (Pasinelli *et al.*, 2000). In SOD1 G93A mice, and accordingly to caspase activation sequence, the cytochrome *c* translocation into the cytosol activate caspase-1, followed by the activation of caspase-9 which, at least, trigger caspase-3 and -7 activation. In this same model, the activation of caspase-7 was shown to match with ALS onset (Guegan *et al.*, 2001).

Some other pathways related with the anti-apoptotic Bcl-2 protein were reported to promote apoptosis. One of them is the entrapment of Bcl-2 into both wt and mSOD1 aggregates, switching the protein to a non-functional stage (Pasinelli *et al.*, 2004). Another hypothesis is related with their conformational modification that results from their binding to mSOD1, leading to the production of a toxic protein (Pedrini *et al.*, 2010). Before these observations were made, Kostic and colleagues (1997) have shown that, in contrast, overexpression of Bcl-2 preserved motor function and extended the life span in SOD1 G93A mice.

Contrary to apoptosis, necrosis is not a developmentally programmed type of cell death. Instead, cells swell, nuclear membrane disrupts, mitochondria and ER lose their structure and become dysfunctional. It is independent of pre-mitochondrial apoptotic proteins and results from a traumatic physical injury or stroke. It affects a large amount of cells, whereas apoptosis typically occurs in individual cells within a population of surviving neighbors. Typically, the cellular contents are released into the extracellular space and damage neighboring cells to evoke an inflammatory response, while in apoptotic events, each cell forms a vesicle (Siegel *et al.*, 2006). Necrotic cell death may also occur in many neurodegenerative diseases, including ALS. However, it is not well studied the mechanisms that trigger this particularly type of event. Studies indicate that necrosis of adult neurons is mediated by increases of intracellular calcium and the major players are cytosolic calpains and spilled lysosomal cathepsins. Cellular energy depletion is a potent trigger of necrosis mediated by excitotoxicity (Artal-Sanz and Tavernarakis, 2005). Acidification, produced by oxygen depletion, also plays an important

role in necrotic neuronal death (Ding *et al.*, 2000). Acidosis activates calcium-permeable acid sensing ion channels, resulting in glutamate receptor-independent neuronal injury due to calcium toxicity (Xiong *et al.*, 2004). Moreover, toxic mutations in several genes can trigger necrotic cell death. Necrosis-like neuronal death was shown to be also determined by gain-of-function mutations in genes that encode the ion channel proteins termed degenerins (Hall *et al.*, 1997).

Autophagy, that constitutes a protein clearing system, is composed by three categories: macroautophagy, microautophagy and chaperone-mediated autophagy. Macroautophagy is characterized by the involvement of organelles with an intracellular membrane to isolate them from cytoplasm, forming a structure called autophagosome. The compartment is then acidified and fuses with lysosome for degradation. Microautophagy is responsible for the turnover of organelles and recycling of biological building blocks. In this case, the organelle fuses directly with lysosome and is degraded. Chaperone-mediated autophagy is a mechanism used to import cytoplasmic proteins into lysosomes for degradation. The proteins targeted for degradation are tagged with a particular peptide motif, which is recognized by a chaperone. Then, the chaperone–protein complex binds to a specific lysosomal membrane receptor for import into the lysosome (Siegel *et al.*, 2006). The macroautophagic pathway has been implicated in several neurodegenerative diseases, including ALS. Kabuta and colleagues (2006) have demonstrated in cellular models that wt and mSOD1 are degraded by macroautophagy. As it reduces the toxicity of mSOD1 proteins, it is proposed that macroautophagy is important for the reduction of mSOD1-mediated neurotoxicity in fALS. However, macroautophagy may disturb the cell homeostasis and lead to cell death when autophagy is over-activated, contributing for the pathogenesis of ALS (Pattingre *et al.*, 2005). Studies in SOD1 G93A mice reported an alteration of autophagy in the beginning of the pre-symptomatic stage of ALS. Compared with the age-matched controls, the number of Microtubule-associated protein 1A/1B-light chain 3 (LC3)-labeled autophagic vacuoles were significant increased in the MN of the SC from SOD1 G93A mice (Li *et al.*, 2008). Increased autophagy was suggested to be partially regulated by a mammalian target of rapamycin (mTOR) signaling pathway (Morimoto *et al.*, 2007). Besides mSOD1, the existence of mutations in other genes may also determine a defective autophagy (Caccamo *et al.*, 2009; Parkinson *et al.*, 2006).

1.3.9 - Impaired axonal transport

MN are high polarized cells with long axons and a cytoskeleton composed by neurofilament (NF) proteins that which ensures the maintenance of cell shape, axonal caliber and delivery of essential components, such as RNA, proteins and organelles to the axonal compartment (Siegel *et al.*, 2006). NF proteins include light (NF-L), medium (NF-M), and heavy (NF-H) subunits, in equal proportion, which are progressively phosphorylated during axoplasmic transport. Axonal transport between the cell body and neuromuscular junction (NMJ) is made by microtubule-dependent kinesin and cytoplasmic dynein/dynactin molecular motor, which mediates anterograde transport (toward NMJ) and retrograde transport (toward cell body), respectively (Duncan and Goldstein, 2006).

Over- or under-expression, mutation or deficient transport of individual subunits determine misassembly of neurofilaments leading to their accumulation and thus determining impairment of axonal transport and resultant MN death (Redler and Dokholyan, 2012). Abnormal accumulation of

neurofilaments is a pathological feature ALS and occurs as an early event (Shaw, 2005), which has been observed in both patients with sALS and fALS (Hirano *et al.*, 1984), as well as in SOD1 G93A mice (Rouleau *et al.*, 1996).

There is not a clear hypothesis about how the aberrant expression of neurofilaments originates the degeneration of MN. The axonal transport of molecules can be disrupted by the disorganization of neurofilaments, process known as “axonal strangulation”, that may derive from mSOD1 toxic effects (Collard *et al.*, 1995). Thus, reduction of the number of axonal neurofilaments, evidence to protect from the mSOD1 toxic effects (Rowland and Shneider, 2001). On the other hand, the accumulation of neurofilaments could exert a protective effect against mSOD1 through buffering calcium, providing more phosphorylation sites for deregulated intracellular kinases, or reducing the burden of axonal transport or diminishing zinc binding (Rowland and Shneider, 2001; Shaw, 2005). The exclusion of the light subunit of neurofilaments (Williamson *et al.*, 1998) or overexpression of the NF-H subunit (Couillard-Despres *et al.*, 1998) was observed to ameliorate ALS in mSOD1 mice.

A reduced expression of NF-L was also observed in different models, such as sALS and fALS cases, tg mice and in a cell culture model of ALS, where an alteration of stoichiometry of neurofilament subunits was observed (Barber and Shaw, 2010). Mutations in the gene coding NF-H subunit were also found in sALS and fALS patients (Al-Chalabi *et al.*, 1999).

The anterograde and retrograde transport are impaired by the presence of mSOD1 and mitochondria trafficking suffer retardation, leading to a defective transport of other cargoes, once the cell lacks energy (De Vos *et al.*, 2007). This may explain the early onset distal axonopathy observed in ALS mouse models and the accumulation of mitochondria in proximal axons in sALS patients (Redler and Dokholyan, 2012). Other factors may account to impaired axonal transport. Indeed, tumor necrosis factor (TNF) may cause kinesin disruption, via a mechanism involving p38 mitogen-activated protein kinase (p38 MAPK), as observed in L929 cell line (De Vos *et al.*, 2000) and glutamate by activating protein kinases, which phosphorylate neurofilament proteins (Ackerley *et al.*, 2000).

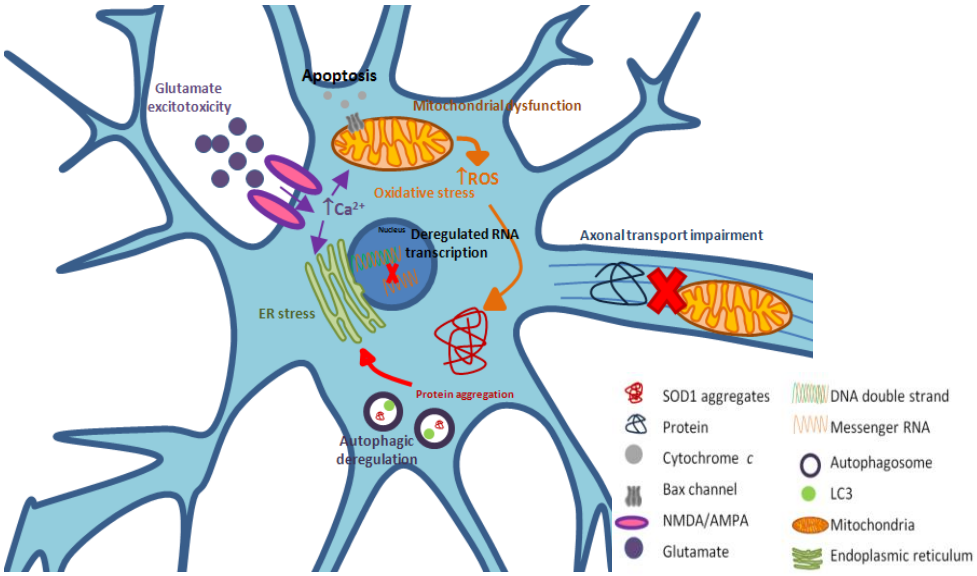

Figure I.2 – Amyotrophic Lateral Sclerosis (ALS) is a multifactorial disease, with pathophysiological mechanisms that show a complex interaction between genetic and molecular pathways, most of them related with the subtype of the disease caused by diverse mutations in superoxide dismutase 1 (SOD1). Mutations in several ALS-related genes, oxidative stress and mitochondrial alterations can be responsible for the formation of protein aggregates, which are harmful for neurons. These aggregates lead to proteasome impairment and endoplasmic reticulum (ER) stress, and ultimately activate autophagy and apoptotic pathways. Autophagy begins with the formation of a phagophore, which expands to cover portions of cytoplasm containing proteins and organelles into the autophagosome. It is assisted by p62, linking ubiquitinated protein aggregates and LC3. After that, autophagosomes are trafficked along microtubules in a dynein-dependent way to lysosomes. Then, they enter in a maturation phase, through the fusion with endosomal vesicles or multivesicular bodies, followed by fusion with lysosomes, forming the autolysomes. In ALS there is an increased expression of the autophagic marker LC3-II, showing the activation of this via in the degradation of SOD1. However when autophagy is overactivated, there is a disturbance of the cell homeostasis, leading to cell death. Mitochondrial dysfunction is characterized by a decrease of the respiratory chain activity, adenosine triphosphate (ATP) levels, together with the release of the cytochrome *c* with consequent activation of the apoptotic cascade. This dysfunction promotes an increase in the production of reactive oxygen species (ROS), leading to oxidative stress. Excitotoxicity mediated by glutamate leads to an overstimulation of N-Methyl-D-aspartate (NMDA) receptors and accumulation of calcium (Ca^{2+}) ions in cellular compartments, which further leads to ER stress and activation of apoptotic pathways. Transcriptional deregulation and abnormal RNA processing, together with overproduction of ROS, also contribute to aberrant protein folding. Accumulation of neurofilament intracellular aggregates disrupts axonal transport processes, thus blocking the energy supply to the distal axon. Adapted from Ferraiuolo *et al.* (2011).

1.4. The role of glial cells and cross-talk with neurons in ALS

Initially, ALS was considered a cell autonomous disease, where MNs were the only cells affected. In fact, many studies in genetically engineered mouse models were made to develop disease from the expression of mSOD1 only in MN, most of them without success (Lino *et al.*, 2002; Pramatarova *et al.*, 2001). Only one that used mSOD1 expression driven by neuron-specific Thy1.2 promoter appeared to cause MN death (Jaarsma *et al.*, 2008). However, more recently, many findings showed the intervention of non-neuronal cells, such as microglia and astrocytes, in the pathogenesis of the disease (Ferraiuolo *et al.*, 2011). Several studies showed a reduction in MN survival when cultured with mSOD1-expressing glial cells. Di Giorgio and colleagues (2007*)* have used pathogenic MN derived from embryonic stem cells carrying G93A mutation and wt MNs to be co-cultured with SOD1G93A glial cells. Mutant glia revealed to induce signs of neurodegeneration in both wt and mSOD1 neurons. By the other side, removal of mSOD1 expression in either astrocytes or microglia using glial fibrillary acidic protein-Cre (GFAP-Cre) or CD11b-Cre transgenes, respectively, slowed the

disease progression and boosted the survival of ALS mice (Boillee *et al.*, 2006; Yamanaka *et al.*, 2008b). However, the individual contribution of each cell is still a matter of debate. Actually, when the expression of mSOD1 was restricted to microglia or astrocytes the disease was not induced (Beers *et al.*, 2006). Nevertheless, other studies point to astrocytes expressing mSOD1 as causing MN degeneration when using an *in vitro* model of ALS (Nagai *et al.*, 2007).

1.4.1. Astrocytes

Astrocytes, the most abundant nerve cell types in the adult CNS, are responsible for the maintenance of the extracellular homeostasis through the modulation of ions and neurotransmitters, nutrients supply and release of growth factors. They also have a structural function, whose membranes cover synaptic contacts and establish contacts with neuronal membranes as well as with blood (Siegel *et al.*, 2006). In case of injury, astrocytes become activated in a process called astrogliosis, characterized by altered cell morphology, hypertrophic nuclei, increase prominence of processes, up-regulation of intermediate filament GFAP (Pekny and Nilsson, 2005) and release of pro-inflammatory factors (Phani *et al.*, 2012), as shown in Figure I.3. These characteristics were observed in both SC and brain motor regions in mSOD1 mice, as well as in human ALS cases (Hall *et al.*, 1998; Kassa *et al.*, 2009; Nagata *et al.*, 1998).

Alterations in astrocytic function, may involve the loss of the EAAT2 transporter and the consequent increase of extracellular glutamate, as observed in SCs of both sALS and fALS (Fray *et al.*, 1998; Rothstein *et al.*, 1995), as shown in Figure I.3. Similar features were noticed in the SCs of mSOD1 tg mice (Bruijn *et al.*, 1997) and rats (Howland *et al.*, 2002), resulting in neuronal excitotoxicity, as previously discussed in section 1.3.4. A recent study performed by Diaz-Amarilla and colleagues (2011), which created primary astrocyte cultures from symptomatic SOD1 rat SCs, identified a high proliferation of a subset of astrocytes, expression of GFAP and other astrocyte markers, a phenotype that they designated as “aberrant astrocytes”, and also include the absence of EAAT2, further confirming previous findings.

Activated astrocytes, if releasing insufficient quantity of neurotrophic factors, may impair MN once they are essential in promoting neuronal survival (Ekestern, 2004). Among such factors are the glial-derived neurotrophic factor (GDNF), brain-derived neurotrophic factor (BDNF), ciliary neurotrophic factor (CNTFR) and vascular endothelial growth factor (VEGF) (Dewil *et al.*, 2007b; Ekestern, 2004), as we can see in Figure I.3. Additionally, mSOD1 astrocytes may release toxic products, such as pro-inflammatory cytokine oxidative stressors (Phani *et al.*, 2012) and neurotrophic growth factor (NGF), which induce the death of MN expressing the receptor p75 through a mechanism involving the synthesis of NO and peroxide (Pehar *et al.*, 2004). Fas ligand and TNF-α produced by reactive astrocytes can also activate death receptors in injured MN (Raoul *et al.*, 2002). Moreover, astrocytes respond to toxic factors in CSF of ALS patients by undergoing morphological transformation from flat to process bearing and by expressing elevated levels of GFAP and S100A6 and S100B proteins (Shobha K, *et al.*, 2010).

1.4.2. Oligodendrocytes and Schwann cells

Oligodendrocytes are the most important glial cells responsible for myelination of upper motor axons and, more recently, to also be involved in axonal energy supply and metabolism (Lee *et al.*, 2012). Recent studies propose a contribution of these cells in the neuropathology of ALS. Seilhean and colleagues (2009) found p62 and TDP-43 positive intracytoplasmic inclusions in oligodendrocytes, while MacKenzie and colleagues (2011) found FUS-positive inclusions in these glial cells. A study of human ALS post mortem tissue showed loss in the number of small fibers, most likely due to intrinsic SC lesions, and diffuse myelin pallor in the anterolateral columns associated with microglial infiltration (Hayashi *et al.*, 2001). Studies in SC myelin from SOD1 G93A rats in fully symptomatic stages of the disease found a disorganization of myelin, and reduced levels of lipids, phospholipids and cholesterol (Niebroj-Dobosz *et al.*, 2007), as shown in Figure I.3. Recently, Philips and colleagues (2013) showed degenerative changes in oligodendrocytes in human patients with ALS and in mSOD1 mouse model. On the contrary, some studies suggested that oligodendrocytes might not be an important element in the disease pathology. Indeed, Yamanaka and colleagues (2008a) by examining chimeric mice, whose all MN and oligodendrocytes express high levels of mSOD1, observed that the disease onset was delayed, supporting that cell types excluding MN and oligodendrocytes must be major contributors to ALS disease onset and progression.

Schwann cells are responsible for the myelination and regeneration of lower motor axons, providing electrical isolation, essential for fast signal conduction (Siegel, 2006). In case of axonal damage, they also participate, together with peripheral macrophages, in clearing debris and participating in the recovery of axons (Ilieva *et al.*, 2009). Many studies showed a limited and unexpected involvement of these cells in ALS disease progression. Turner and colleagues (2010) showed that expression of SOD1 G93A in Schwann cells did not trigger MN degeneration, once no alterations were observed between control and mutated mice; additionally the increase of mSOD1 expression in Schwann cells was shown to not exacerbate the disease phenotype (Figure I.3). Moreover, Lobsiger and colleagues (2009) reported that SOD1 G37R expressed in Schwann cells slowed the ALS progression, while it was accelerated when the mutant was inactivated by Cre-mediated gene excision. In contrast to the effects produced by other mutated glial cells and their influence on disease progression, these features point to the protective effect that mutated Schwann cells may have.

1.4.3. Microglia

Microglia are cells derived from the hematopoietic cell lineage and are considered the CNS tissue-resident macrophage population in the brain parenchyma. They are considered the primary immune cells of the CNS, being responsible for monitoring the environment and respond to infection and injury (Siegel *et al.*, 2006). Under physiological conditions, the cells exhibit a deactivated phenotype, associated with the production of anti-inflammatory and neurotrophic factors (Streit, 2002). In response to a pathogen invasion or tissue damage, microglia switch to an activated phenotype, promoting the inflammatory response through morphology changes, migration to the damaged cells and release of proinflammatory molecules (Lasiene and Yamanaka, 2011), as shown in Figure I.3.

Microgliosis has been known to be a component of ALS pathology in the past 20 years (Engelhardt and Appel, 1990). Evidence of microglial activation in the brain of living ALS patients, using positron emission tomography (PET), was reported by Turner and colleagues (2004). Weydt and colleagues (2004) showed for the first time the role of mSOD1expression in primary cultured microglia from mSOD1 tg mice, in which microglia produced higher levels of TNF-α and released lower levels of interleukin-6 (IL-6), comparatively with wt microglia, when stimulated with lipopolysaccharide (LPS). Later, in microglia/MN co-cultures, SOD1 G93A microglia induced more MN death and decreased neurite number and length as compared with wt microglia, where NO produced by microglia may have had a key role (Xiao *et al.*, 2007).

To evaluate the role of microglia in ALS progression, Boillee and colleagues (2006), by excising mSOD1 from microglia in SOD1 G37R mice using the Cre-Lox system, observed that the intervention resulted in a slower disease progression. Similar results were obtained in the SOD1 G85R mice. (Wang *et al.*, 2009). Other studies, using replacement of microglia via bone marrow transplantation in SOD1 G93A mice, demonstrated that although not affecting the disease onset, microglia were able to slow the disease progression (Beers *et al.*, 2006). It was also observed by Gowing and colleagues (2008) that the disease progression does not require the proliferation of mSOD1 microglia in response to an initial injury, once reduction of proliferating microglia by half did not affect the progression rate.

Thus, the role of microglia is complex, once they are capable of stimulating neuroprotective as well as neurotoxic effects. This dissertation is focused to better dissect the role of microglia in ALS. These properties and influence of microglia in ALS will be further detailed in the section two of this introduction.

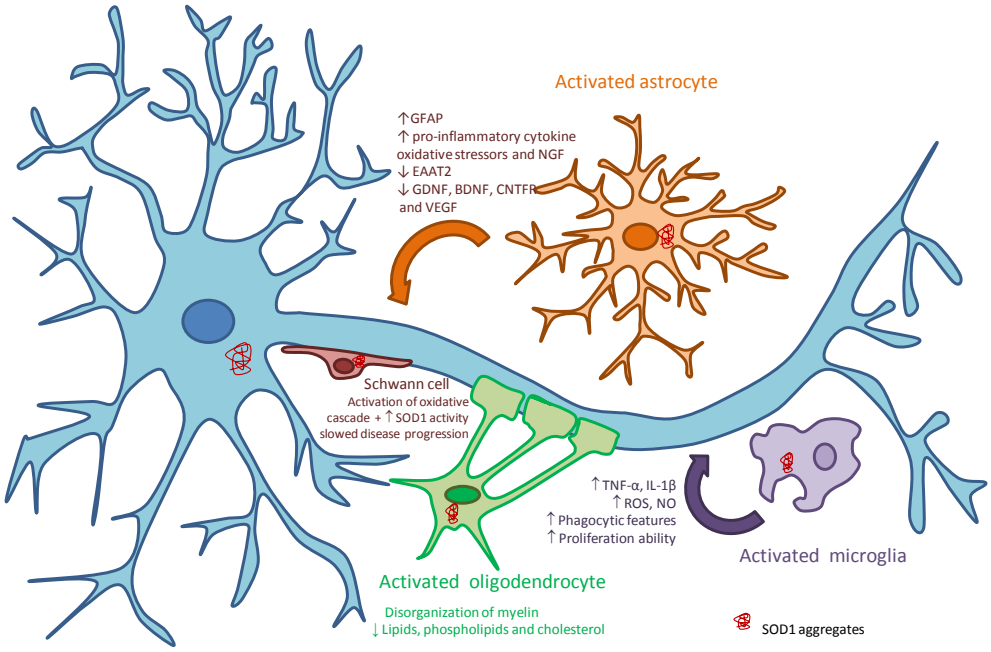

Figure I.3 - Glial cells establish a cross-talk with motoneurons (MNs) in Amyotrophic Lateral Sclerosis. Many studies showed the intervention of mutated superoxide dismutase 1 (mSOD1)-expressing non-neuronal cells in the pathogenesis of the disease. In case of injury, astrocytes become activated in a process called astrogliosis, characterized by the up-regulation of intermediate filament glial fibrillary acidic protein **(**GFAP) and increased levels of toxic products in the extracellular media, such as pro-inflammatory cytokines, oxidative stressors and neurotrophic growth factor (NGF). On the other hand, there is a loss of the excitatory aminoacid transporter-2 (EAAT2), which can cause neuronal excitotoxicity and an insufficient release of neurotrophic factors, essential to promote neuronal survival. These factors include glial-derived neurotrophic factor (GDNF), brain-derived neurotrophic factor (BDNF), ciliary neurotrophic factor (CNTFR), and vascular endothelial growth factor (VEGF). In addition, it was observed disorganization of myelin, a decrease of lipids, phospholipids and cholesterol in activated oligodendrocytes. On the other hand, in Schwann cells, it was verified that an increase of mutant SOD1 expression and activation of oxidative cascade slowed ALS progression. Finally, when mutant SOD1 accumulates within microglia, they increase their proliferation and phagocytic abilities and the production of pro-inflammatory molecules, such as the tumor necrosis factor alpha (TNF-α) or interleukin-1 beta (IL-1β), reactive oxygen species (ROS) and nitric oxide (NO).

1.5. Controversy in ALS—where does the disease begin?

It is not yet established where ALS begins, once it impacts either in UMN as in LMN, or even in both. One hypothesis is that ALS begins in cortico-MN, which connect monosynaptically with anterior horn cells and, via glutamate excitotoxicity, mediates anterograde degeneration of anterior horn cells – dying-forward hypothesis (Kiernan *et al.*, 2011). Miller and colleagues (2006), for instance, suggest that muscle is not a primary target for non-cell autonomous toxicity in fALS. They found that reducing mSOD1 within the muscle, through the use of lentivirus that encodes small interference RNA (siRNA), directed against mSOD1 or the selective excision of mSOD1 gene from muscle, did not impact in the disease. Moreover, the production of chronic muscle hypertrophy through stimulation of myogenesis did not slow the disease (Miller *et al.*, 2006; Shaw and Ince, 1997). Towne and colleagues (2008) showed that a reduction by more than 50% of SOD1 G93A levels in mouse muscles did not alter the course of disease, proving again that SOD1-mediated damage within skeletal muscles does not contribute to death of MN in ALS. Additionally, some clinical observations reinforce this hypothesis: (i) MN lacking a monosynaptic connection with cortico-MN, such as the oculomotor, abducens, and

Onuf's nuclei, are resistant to neurodegeneration; (ii) ALS do not naturally develop in the animal model due to a shortage of cortico-motor neuronal-anterior horn cell connections; (iii) the rare appearance of pure LMN forms of ALS, whereas subclinical UMN involvement is invariably detected with transcranial magnetic stimulation studies (Kiernan *et al.*, 2011).

Another concept supports that ALS begins inside the muscle cells or at the NMJ – dying-back hypothesis. In this case, there is a deficiency in the motor neurotrophic hormone, normally released by postsynaptic cells and transported up from the presynaptic axon to the cell body, through retrograde transport (Kiernan *et al.*, 2011). Studies performed by Dobrowolny and colleagues (2008) demonstrated that selective expression of mSOD1 in the muscle of a tg mouse provoked alterations and induced sings in the pre-symptomatic stage of ALS. Analysis of the SOD1G93A mice showed how the ubiquitous expression causes first muscle atrophy, followed by the destruction of the NMJ, retrograde axonal degeneration and, ultimately, MN death (Dupuis and Loeffler, 2009). In 2010, Wong and Martin created tg mice expressing wt, human SOD1 G37R and human SOD1 G93A gene variants only in skeletal muscle. These tg mice developed age-related neurologic and pathologic phenotypes consistent with ALS, such as limb weakness and paresis with motor deficits.

Muscle selective alterations in mitochondrial function might contribute to initiate NMJ dismantlement, followed by distal axonopathy, astrocytosis in the SC and MN loss in mice (Dupuis *et al.*, 2009). Zhou and colleagues (2010b) reported alterations in mitochondrial inner membrane potential of muscle fibers near NMJ, in young SOD1 G93A mice, prior to disease onset. Finally, studies showed that inhibition of the pro-apoptotic machinery prevented MN loss in mice, but not denervation, neither promoted functional improvement or lifespan extension (Dewil *et al.*, 2007a; Gould *et al.*, 2006).

2. Microglia in ALS: distinguishing between neuroprotective and neurotoxic properties

2.1. Resting Microglia

Microglia are the resident macrophage of the CNS, representing 10% of all cells in nervous system (Heneka *et al.*, 2010). They are part of the innate immune response, participate in events associated with CNS organization, form the neuron-glia network and regulate the brain parenchyma homeostasis (Bessis *et al.*, 2007; Moore and Thanos, 1996). Unlike the neurons and the other glial cells, microglia are not derived from neuroectoderm. Indeed, the original microglial population in the CNS differentiates from cells of the myeloid lineage that originate in the bone marrow and occurs early in embryonic development (Ransohoff and Perry, 2009). After accessing the CNS, microglia disseminate along the parenchyma and transform into the resting state (Heneka *et al.*, 2010), term recently questioned once they are not inactive; in other words, they are continually surveying the surrounding environment by extending and retracting their processes (Davalos *et al.*, 2005; Nimmerjahn *et al.*, 2005). In this state, they acquire a ramified appearance, with thin processes extending from the cell body to the surrounding environment (Lull and Block, 2010), a morphology

seen *in vivo* but absent in cell cultures (Lull and Block, 2010). Each cell occupies a defined territorial domain, in order to avoid the overlap with neighboring microglia (Heneka *et al.*, 2010). One question that remains unclear is if microglia can have a high level of self-renewal to support their population in both resting and activated states. When they are at a resting state the mitotic rate is low, but they can reach a high rate of proliferation when activated, suggesting they have leastwise a partial capacity to cell-turnover (Ajami *et al.*, 2007; Lawson *et al.*, 1992).

Microglia differs from other populations of macrophages relatively to the expression of low levels of CD45 and major histocompatibility complexes (MHCs) and they are poor antigen presenting cells (APCs). One possible reason is the non-existence of serum proteins in the brain, which is responsible for macrophage activation (Lull and Block, 2010). Some *in vitro* experiments showed that resting microglia is responsible for the secretion of neurotrophic factors, such as insulin-like growth factor 1 (IGF1), BDNF, transforming growth factor-β (TGFβ) and NGF, as well as anti-inflammatory factors, such as IL-10 (Bessis *et al.*, 2007; Polazzi and Monti, 2010), as represented in Figure I.4. They also have a phagocytic function, as it has been observed during neurogenesis. An elevated number of neuroblasts generated in subgranular zone of the dentate gyrus undergoes apoptosis, and functional microglia is able to phagocyte these apoptotic cells (Sierra *et al.*, 2010).

There are many mechanisms that maintain the resting state of microglia, most of them influenced by neurons. One of them is the signaling by CX_3C-chemokine ligand (CX_3CL1 or fractalkine) through its cell-surface receptor CX_3CR1 on microglia, which restrains microglial cell activity (Saijo and Glass, 2011). In mice deficient in CX_3CR1, it was observed deregulated microglia response, that further increased in experimental models of ALS (SOD1 G93A mice) and Parkinson's disease induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), where a hyperactive microglial cell phenotype and a high neuronal loss was evidenced (Cardona *et al.*, 2006).

It was also reported by Ransohoff and Cardona (2010) that signaling induced by the microglial cell receptors CD172, CD200R and CD45 following interaction with the neuronal cell-surface proteins CD47, CD200 and CD22, respectively, inhibit microglial cell activity (see Figure I.4).

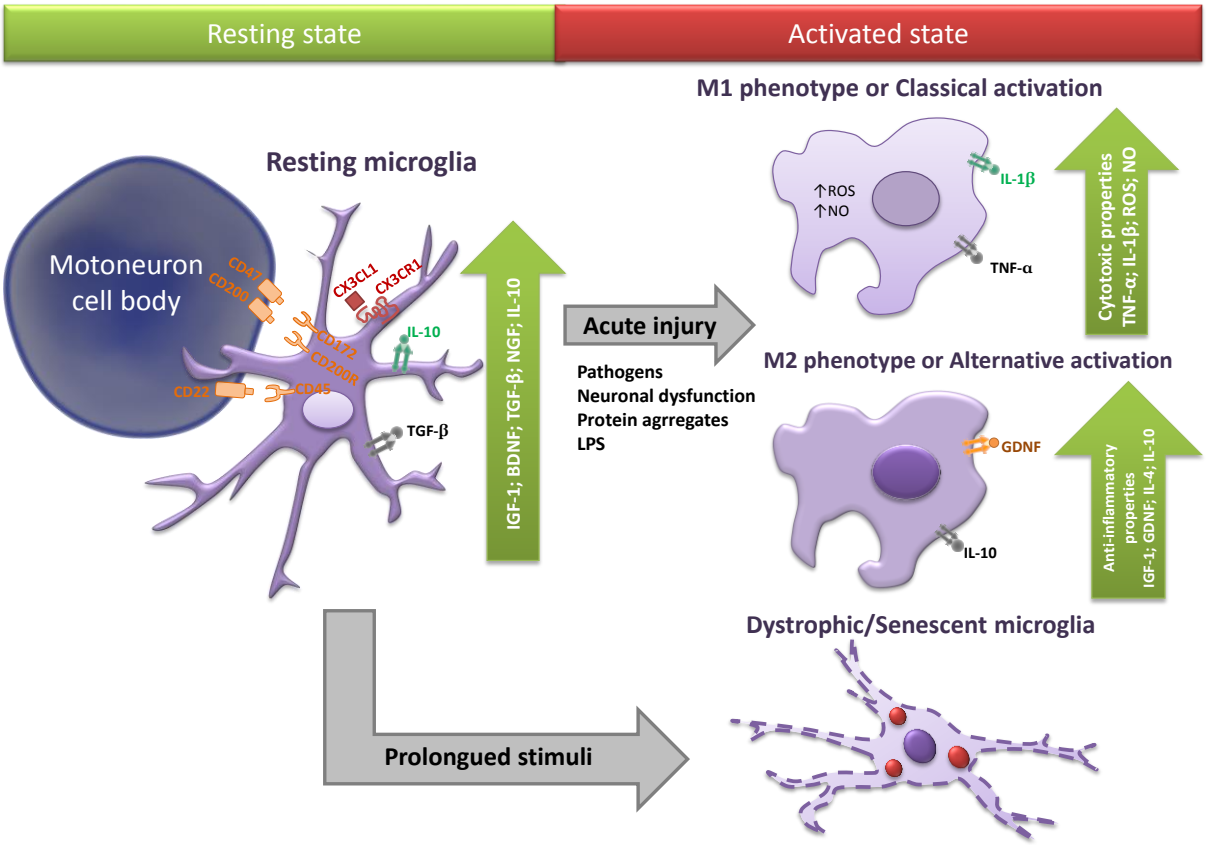

Figure I.4 – **Microglia can change their phenotype in response to a variety of insults**. In the healthy central nervous system microglia are in a resting/vigilant state, characterized by ramified appearance with thin processes extending from the cell body to the surrounding environment. They secrete neurotrophic factors, such as insulin-like growth factor-1 (IGF-1), brain-derived neurotrophic factor (BDNF), transforming growth factor-β (TGF-β) and nerve growth factor (NGF), as well as anti-inflammatory factors, such as interleukin-10 (IL-10). Motoneurons (MNs) influence the resting state of microglia through several mechanisms, such as the signaling by the CX3C-chemokine ligand (CX3CL1 or fractalkine) through its cell-surface receptor CX3CR1. Signaling induced by the microglial cell receptors CD172, CD200R and CD45 following interaction with the neuronal cell-surface proteins CD47, CD200 and CD22, respectively, inhibit microglial cell activity. In response to a wide array of injurious stimuli, microglia undergo activation. They can acquire different activation states and, consequently, they produce substances that can be either beneficial or toxic to MN. M1 phenotype, or "classical activation", which is cytotoxic in nature, describes a pro-inflammatory phenotype characterized by the production of interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α), as well increased release of reactive oxygen species (ROS) and nitric oxide (NO). M2 phenotype, or "alternative activation" refers to a phenotype capable of augmenting a neuroprotective environment, whose treatment with anti-inflammatory cytokines (e.g. IL-4 and IL-10), reduce the inflammation and lead to the release of neurotrophic factors, such as IGF-1 and glial-derived neurotrophic factor (GDNF), which promote neuronal outgrowth and support other glia. Another phenotype, occurring after a prolonged stimuli is called dystrophic or senescent microglia. Changes with ageing include dystrophic cytoplasmic processes characterized by slight enlargement, distinct loss of fine branches, and formation of cytoplasmic spheroids, gnarling, beading and fragmentation.

2.2. Microglial Activation and function in the Healthy CNS

In response to several insults, such as pathogen invasion, ischemia, abnormal protein content or altered neuronal activity, microglia suffer profound changes at the morphological, gene expression and functional levels, and switch into an active phenotype (Kettenmann *et al.*, 2011), as represented in Figure I.4. Many endogenous and exogenous factors are released during insult. Chemokines, cytokines and components of the complement system, such as TNF-α, macrophage-colony stimulating factor (M-CSF), (GM)-CSF, interferon gama (IFNγ), IL-1 and IL-6 are examples of endogenous factors, while LPS constitutes a good example of an exogenous factor (Dewil *et*

al., 2007a). As a consequence, microglia switch from a ramified to an amoeboid shape, with retraction and thickening of processes (Kreutzberg, 1996). They also become motile cells, moving to the damage local through chemotactic gradients and proliferate to increase local densities, ensuring more cells for the defense and restoration of homeostasis (Kettenmann *et al.*, 2011). In this state, they acquire phagocytic features, in order to clean cellular debris resulting from apoptosis and normal cell death (Aloisi, 2001). Upon activation, microglia produce factors that either (i) induce repair and regeneration such as anti-inflammatory cytokines, neurotrophins and growth factors, (ii) worsen neuronal injury such as ROS and pro-inflammatory factors and (iii) recruit and drive immune cell populations, including chemokines, immunogens, and proinflammatory factors, which promote the activation of the immune system (Block and Hong, 2005).

2.3. Neuroinflammation

The inflammatory response occurs in response to environmental factors or subsequent to protein aggregation, enabling the intervention of innate and adaptive immune systems (Siegel *et al.*, 2006). Although inflammation is linked to tissue repair processes and some inflammatory stimuli induce beneficial effects, such as phagocytosis of debris or apoptotic cells, it may determine an uncontrolled state characterized by the production of neurotoxic factors that will then amplify underlying disease mechanisms. Inflammatory response can also establish feed-forward loops, contributing to overwhelm normal resolution mechanisms (Glass *et al.*, 2010).

Neuroinflammation is characterized by the activation and proliferation of microglia, astrocytes and infiltrating T cells at sites of neuron injury, being now established as an important aspect of many neurodegenerative diseases, such as Parkinson's disease, Alzheimer disease, and ALS (Glass *et al.*, 2010; Lewis *et al.*, 2012). In what concerns to ALS, many studies found neuroinflammation in tg mice overexpressing variants of human mSOD1, which was associated with inherited ALS. A study in SOD1 G93A mice showed that astrocytic activation may exert a trophic influence on the MN, but is insufficiently conserved later on the disease course. Concerning microglial activation, it has been documented their contribution to the oxidative stress and damage involved in the disease process (Hall *et al.*, 1998). Using the same model and applying cDNA microarray to monitor gene expression during neurodegeneration, Yoshihara and colleagues (2002) revealed an up-regulation of inflammatory genes related to glial cell activation at 11 weeks of age in the pre-symptomatic stage prior to MN death. In 2003, Hensley and collaborators (2003), using SOD1 G93A mice and a microglial cell line, showed that TNF-α was the principal driver for neuroinflammation, while several co-stimulating cytokines and chemokines act to potentiate TNF-α effects.

Studies with CSF from ALS patients revealed a deregulation of both anti- and pro-inflammatory cytokines, as well as of growth factors, such as IL-6, IL-10 GM-CSF, VEGF and IFN-γ (Mitchell *et al.*, 2009), suggesting the involvement of glial cells. On the contrary, when microglia proliferation was blocked through the ablation of TNF-α in mSOD1 mice, it was shown no effect on the rate of disease progression, placing in doubt that microglia contribute to neurodegeneration in ALS (Gowing *et al.*, 2006). However, recently it was found that early microglia functional deficits precede ALS onset. Indeed, the Authors observed that the number of microglia in the SC of the mSOD1 mice

decrease at the pre-symptomatic age, although no changes on cell morphology were observed. Moreover, two sets of microglia (high and low expression of Iba1 cells) were observed at the early-symptomatic age: increased population of $Iba1^{high}$ cells and decreased number of $Iba1^{low}$ microglia. This finding suggests an early participation of microglia in the initiation of ALS (Gerber *et al.*, 2012).

Many studies focused in T-reg cells, which have been shown to play a significant neuroprotective role through the modulation of neuroinflammation. Huang and Richard (2001) showed an increase of the chemokine (C-C motif) ligand 2 (CCL2), which is responsible for enhancing the trafficking of T cells into CNS. In adition, it was desmontrated that T cells play an endogenous neuroprotective role in ALS through the modulation of the trophic/cytotoxic balance of glia (Beers *et al.*, 2008; Chiu *et al.*, 2008). Others proposed that a reduction of T-reg cells in the blood of sALS patients might result from their recruitment from the periphery into the CNS, in order to activate resident innate immune cells such as microglia, as well as anti-inflammatory cytokines (Kipnis *et al.*, 2004; Mantovani *et al.*, 2009).

2.3.1. Cell communication in response to inflammation

A variety of surface receptors are also up-regulated in the activated microglia, including MHC, complement receptors (Liu and Hong, 2003) and pattern-recognition receptors (PRRs) which include toll like receptors (TLRs). MHC class II expression is important for the activation of naive T cells, suggesting the influence of microglia in activation of the adaptive immune system. Microglia operate as the primary sensors of pathogen-associated molecular patterns (PAMPs) in the CNS and express all TLRs (Saijo and Glass, 2011). For example, TLR4 is responsible for LPS recognition and consequent activation of caspase cascade and nuclear factor-κB (NF-κB) activation, triggering the production of neurotoxic pro-inflammatory mediators and NO (Burguillos *et al.*, 2011), as represented in Figure I.5. Beyond PAMPS, TLRs also recognize endogenous damage-associated molecular patterns (DAMPs) provoked by metabolic products and molecules released by dead cells, as the high-mobility group box 1 (HMGB1) (Chen and Nunez, 2010; Wang *et al.*, 1999). A prolonged microglia activation of TLRs contributes to pathological ways of inflammation, which in turn mediate neurodegenerative diseases (Saijo and Glass, 2011).

Beyond PRRs, other receptors are responsible for the recognition of DAMPs released from damage cells and are important in clearance of debris and in tissue repair. Receptor for advanced glycation end-products (RAGE) is the prototypic DAMP receptor (Sims *et al.*, 2010). Microglia are also known to express both ionotropic receptors, such as the P2X1 to P2X7, and metabotropic receptors, such as P2Y1, P2Y2, P2Y4, P2Y6 and P2Y11 to P2Y14, which bind purines or pyrimidines and activate downstream signaling cascades through G proteins. ATP mainly activates P2X receptors and induces the transcription of pro-inflammatory mediators (Saijo and Glass, 2011), as shown in Figure I.5 Several molecules on the surface of apoptotic cells and corresponding receptors have been shown to be involved in the recognition and ingestion of apoptotic cells by microglia. For example, uridine diphosphate (UDP) released from dying neurons triggers microglial cell P2Y6 receptors and induces phagocytosis of the neurons (Koizumi *et al.*, 2007), as we can see in Figure I.5 Another important molecule secreted by microglia and involved in phagocytosis is Milk Fat Globule Factor-E8 (MFG-E8). It works as a bridge to attach the phagocytic cell to the apoptotic cell, through the recognition of

phosphatidylserine located at the outer membranes of cells suffering apoptosis. After that recognition, a signaling cascade response in microglia is triggered, stimulating the phagocytosis of the dying cell (Liu *et al.*, 2013). Moreover, certain caspases, for example caspase-1, also play a pivotal role in immune-mediated inflammation. Necrotic and apoptotic cell death is known to release nuclear proteins, such as HMGB1 and histones. Both are known to amplify the lethal condition of LPS-mediated sepsis in animals. RAGE, together with TLR2 and TLR4, is triggered by HMGB1 and activates the transcription of pro-inflammatory genes in microglia (Erlandsson Harris and Andersson 2004; Sims *et al.* 2010), as represented in Figure I.5.

Indeed, importance of HMGB1 has risen, in respect to its role in inflammation and immunity. It is a ubiquitous nuclear protein present in almost all cell types. Beyond its function as a nuclear cofactor in transcription regulation, it can be extracellularly released, where it mediates activation of innate immune responses, including chemotaxis and cytokine release. It is passively released by necrotic cells and actively secreted by stimulated monocytes/macrophages and astrocytes, binding to the RAGE and other receptors, including TLR2 and TLR4 (Yang *et al.*, 2013). Evidence suggests a contribution of HMGB1 in many inflammatory diseases, which may be a key point to consider in ALS pathophysiology. In the SC of SOD1 G93A mice a reduction of HMGB1 has been observed in degenerating neurons during the progression of the disease, whereas reactive glial cells displayed HMGB1 in the nucleus, but not in the cytosol (Lo Coco *et al.*, 2007). On the contrary, another study on human tissue (end stage of disease) did not detect a significant change in the number of MN showing nuclear and/or cytoplasmic staining in ALS SC. However, cytoplasmic translocation of HMGB1 in activated microglia and astrocytes were observed, supporting the role of glial cells as major source of extracellular HMGB1 in ALS patients, with both rapid and slow disease progression (Casula *et al.*, 2011).

Matrix metalloproteinases- 9 and 2 (respectively, MMP-9 and MMP-2) are gelatinases responsible for extracellular matrix degradation and are also implicated in inflammation. Secretion by microglia in the context of neuroinflammation is involved in the recruitment of T-lymphocytes to CNS (Nagase *et al.*, 2006). The main cellular sources of MMP-2 in the brain are microglia and vascular endothelium and its increased production may lead to damage in white matter myelin and microvascular beds remodelation (Ihara *et al.*, 2001). MMP-9 has been associated to synaptic plasticity, learning, and memory (Wilczynski *et al.*, 2008). Interestingly abnormally high amount of MMP-9 was found in the motor cortex, as well as thoracic and lumbar cord specimens from ALS patients (Lim *et al.*, 1996).

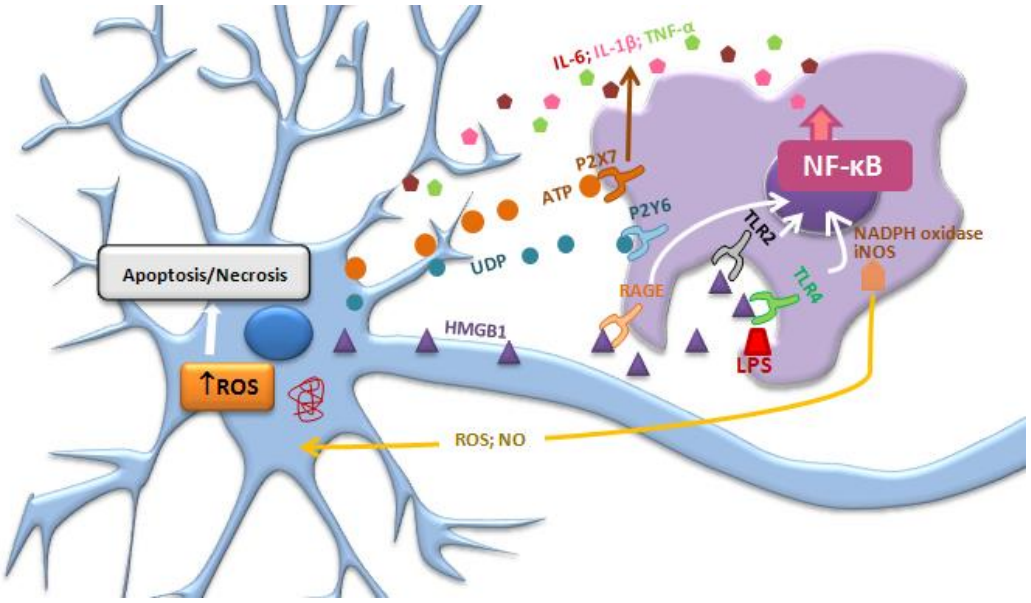

Figure I.5 – Microglia-neuron cross-talk during inflammatory process. Many disease-associated factors are responsible for microglia activation, through pattern-recognition receptors (PRRs), which include toll like receptors (TLR) and receptor for advanced glycation end-products (RAGE), and purinergic receptors (P2X and P2Y), all implicated in M1-activated microglia phenotype. These factors include recognize pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharide (LPS), damage-associated molecular patterns (DAMPs) such as high-mobility group box 1 protein (HMGB1), ATP and uridine diphosphate (UDP), and neurodegenerative disease-specific protein aggregates, such as superoxide dismutase 1 (SOD1) aggregates. The presence of SOD1 aggregates and the increase of reactive oxygen species (ROS) in the injured motoneurons (MNs) induce apoptosis. One of the molecules released by these MN is HMGB1, which binds to TLR-2, TLR-4 and RAGE receptors, all present in microglia. Consequently, NF-κB-mediated gene transcription is activated, leading to the production of interleukin-1 beta (IL-1β), IL-6 and tumor necrosis factor alpha (TNF-α). ATP released by injured MN binds to P2X7 receptor in microglia and also induces the transcription of pro-inflammatory mediators. UDP released from dying neurons triggers microglial cell P2Y6 receptors and induces phagocytosis of the MN. Activated microglial cells release nitric oxide (NO) through the inducible nitric oxide synthase (iNOS) activation, as well as reactive oxygen species (ROS) produced by nicotinamide adenine dinucleotide phosphate-oxidase (NADPH), which contribute to neuroinflammation.

2.4. Defining microglial activation

Activated microglia produce substances that can be either beneficial or toxic to neurons, depending on the phenotypes (Saijo and Glass, 2011). The degree of inflammatory output from microglia is tightly regulated by both intrinsic factors, as well as by cross-talk between neurons and glia (Streit, 2002).

M1 phenotype, or “classical activation”, which is cytotoxic in nature, describes a proinflammatory phenotype characterized by the production of IL-1β and TNF-α, and increased release of ROS and NO through the up-regulation of NADPH oxidase and iNOS (Henkel *et al.*, 2009), as represented in Figure I.4. Their activation and proliferation are influenced by IFN-γ and GM-CSF produced by Th1 lymphocytes and by infiltrating neutrophils (Mosser and Edwards, 2008) and by signaling through TLRs (Glass *et al.*, 2010). *In vitro* co-cultured microglia and neurons, LPS activated TLR4 and provoked increased microglial production of NO and ROS, as well as increased levels of extracellular glutamate, which culminates in the excitotoxic death of neurons (Ajami *et al.*, 2007).

M2 phenotype, or “alternative activation” describes a phenotype capable of augmenting a neuroprotective environment. Treatment with anti-inflammatory cytokines (e.g. IL-4 and IL-10) reduces

the inflammation (Martinez *et al.*, 2006) and leads to the release of neurotrophic factors, such as IGF-1 and GDNF, which promote neuronal outgrowth and support other glia (Isgaard *et al.*, 2007), as shown in Figure I.4. The metabolism of L-arginine also allows the distinction between M1 and M2 activation features: in M1-activated macrophages and microglia, the up-regulation of inducible nitric oxide synthase (iNOS) converts L-arginine to L-citrulline (with consequent activation of NOS), while in M2-activated macrophages and microglia it converts L-arginine to L-ornithine (Mildner *et al.*, 2007).

2.5. Role of microglia activation in ALS

The effects of activation and proliferation of microglia in ALS are reported in various studies and a significant MN loss are seen in specific areas, such as the motor cortex, motor nuclei of the brainstem, the corticospinal tract and the ventral horn of the SC (Ince *et al.*, 1996; Kawamata *et al.*, 1992). Activation of microglia is found even in areas of only mild degeneration (Ince *et al.*, 1996). Studies showed an increase of many factors usually present in microglia activation, such as M-CSF expression, which is up-regulated in ALS pre-central gyrus (Akiyama *et al.*, 1994) and TNF-α levels in ALS serum of ALS patients (Poloni *et al.*, 2000). In general, an increased expression of pro-inflammatory cytokines is found in the CSF from ALS patients (Sekizawa *et al.*, 1998). Furthermore, cyclooxygenase-2 (COX-2) expression is enhanced in mSOD1 mice (Almer *et al.*, 2001). Many of these findings were observed in both the mSOD1 mice and rat in the presymptomatic phase, with a constant microglial response, observed during the active phase of the disease progression (Alexianu *et al.*, 2001; Elliott, 2001; Hensley *et al.*, 2003; Xie *et al.*, 2004). IL-1β, a cytokine released by microglia at the M1 stage was found increased in SOD1 G93A mice (Meissner *et al.*, 2010) and related to a disease acceleration (Nguyen *et al.*, 2001). Indeed, the administration of IL-1 receptor antagonist prevented disease progression in mice. However studies using SOD1 G37R mice in a context of IL-1β knock-out showed no alteration of the disease course, neither any compensatory mechanisms by other cytokines (Nguyen *et al.*, 2001). In another study, it was observed increased COX-2 mRNA and protein levels in SC samples from ALS patients, paralleled by increased prostaglandin E2 (PGE2) content in CSF at regions associated with MN pathology (Maihofner *et al.*, 2003). Excessively activated microglia also seems to provoke MN death, through the release of free radicals, which augment the susceptibility of the MN AMPA/kainate receptor to the toxic effects of glutamate (Zhao *et al.*, 2004). On the other hand, MN death was suppressed by COX-2 inhibition, avoiding chronic glutamate excitotoxicity in organotypic SC system (Drachman and Rothstein, 2000). Furthermore, Turner and colleagues (2004) detected activated microglia in prefrontal cortex, motor cortex, thalamus and pons of ALS patients by histological studies of post-mortem brains and SC tissue using the PET ligand PK1195, which labels the peripheral benzodiazepine receptor that is expressed by activated microglia.

The products of activated microglia could lead to MN death via Fas ligand or NO-induced apoptotic pathways (Raoul *et al.*, 2002), and/or by TNF-α-mediated apoptotic mechanisms (He *et al.*, 2002), whose mechanisms in detail are represented in Figure I.6. Moreover, up-regulation of p38 MAPK pathway that mediates microglial activation (Koistinaho and Koistinaho, 2002) and down-regulation of the anti-apoptotic Akt pathway (Dewil *et al.*, 2007b), have been described in ALS.

About microglial priming, studies using models of MN degeneration, showed that it happens before or concominant with the onset of clinical disease. In fact, it was shown that activation and proliferation of microglia begins during early pre-symptomatic stages of the disease (Alexianu *et al.*, 2001). In chimeric mice the survival of MN injury is influenced by the extension of non-neuronal cells expressing the mutant transgene. Moreover, non-tg MN surrounded by glia expressing mSOD1 evidenced to degenerate, while tg MN surrounded by non-tg glia remained healthy (Clement *et al.*, 2003). To support this study, the genetic knock-down of mSOD1 in cells of the macrophage lineage significantly reduced the progression of ALS (Weydt *et al.*, 2005). In addition, chronic administration of LPS in mSOD1 mice resulted in an exacerbated microglial activation, which consequently contributed to disease progression (Nguyen *et al.*, 2004).

There are several signaling molecules that can also play a role in neuronal-glial cross-talk impairment in ALS. As previously mentioned, fractalkine and CD200 produced by neurons prevent microglia from becoming neurotoxic. Knockout of CX3CR1 showed a deregulated microglia response and an increased cell-autonomous toxicity; however the addition of mSOD1 increased microglial activation and promoted MN death (Cardona *et al.*, 2006; Hoek *et al.*, 2000). MMPs may also have an important role in cell-to-cell communication. MMP-2 and, mostly MMP-9 expression was found to be elevated in the SC of SOD1G93A mice (Fang *et al.*, 2010). Increased expression of NO and ROS was also observed in patients and rodent models, leading to lipid peroxidation and protein carbonylation, which disrupt the integrity and function of neurons and glial cells (Beers *et al.*, 2006; Wu *et al.*, 2006). On the other hand, Kiaei and colleagues (2007) found that the lack of MMP-9 increased the survival of SOD1 G93A mice and the neurotoxicity induced by their presence was suggested to be mediated through the up-regulation of neuronal TNF-α and FasL expression and activation.

All these studies point to the relevance of the activated tg microglia in the spread of the MN neurodegeneration. Interestingly, activated microglia might also have a neuroprotection function, since Chiu and colleagues (2008), demonstrated that the expression of the neurotrophic factor IGF-1 and anti-inflammatory IL-1R antagonist by microglia increased with disease progression. In addition, no changes in TNF-α levels were detected with disease progression, although an increase of IL-1β, TNF-α and NADPH oxidase was obtained in the disease end-stage (Beers *et al.*, 2011). These observations may suggest that, during initial stages of the disease in the mSOD mice, microglia appear to exhibit an M2 phenotype, thus supporting neuronal survival. However, as the disease progress, microglia shift towards an M1 phenotype, although the physiological mechanisms that provoke such activation have not yet been elucidated.

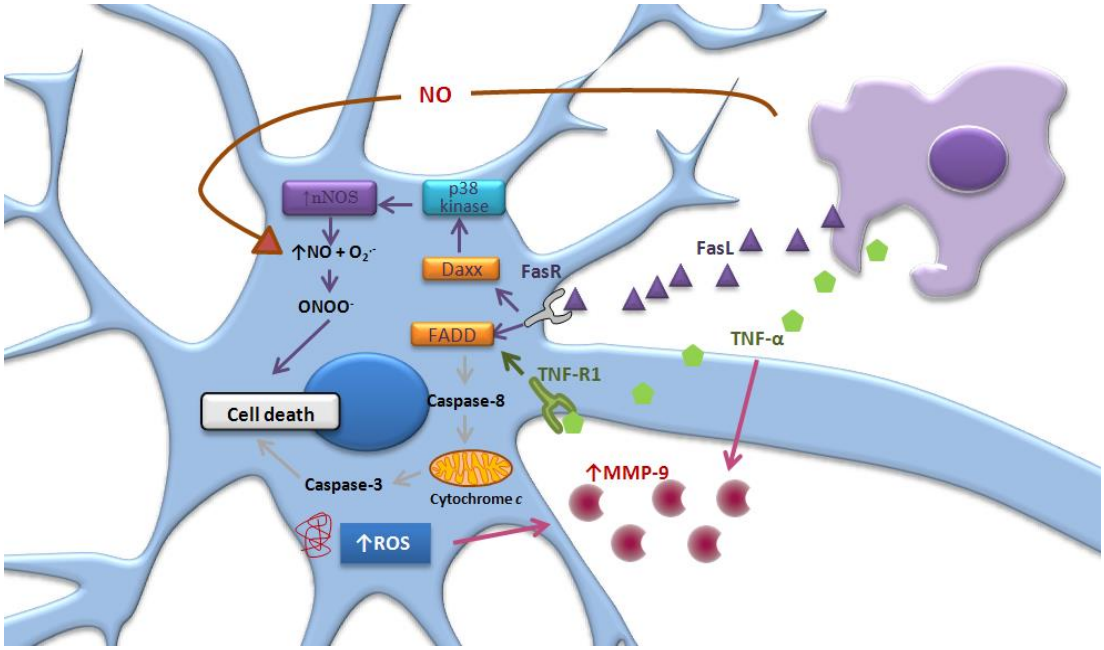

Figure I.6 – Signaling mechanisms involved in neuron-microglia cross-talk impairment in Amyotrophic Lateral Sclerosis. Inflamma-drivers released by activated microglia may lead to motoneurons (MN) death via Fas ligand (FasL), NO-induced apoptotic pathways and tumor necrosis factor-α (TNF-α)-mediated apoptotic mechanisms. FasL also released by microglia in such conditions binds to its receptor (FasR) in the injured MN and recruits the adaptor molecule Fas-Associated protein with Death Domain (FADD). Subsequently, caspase-8 is activated, leading to the activation of caspase cascade, which culminate in apoptosis. Fas-R can also bind to Death-Associated protein (Daxx), which activates mitogen-activated protein (MAP) kinase 38 and neuronal nitric oxide synthase (nNOS). Consequently, NO production increases and their reaction with the anion superoxide ($O_2^{\cdot-}$) contributes to a high production of peroxinitrite ($ONOO^-$), a powerful reactive oxidant that triggers cell death. NO may also be produced by activated microglia, becoming a contributor for NO-induced apoptosis. TNF-α released by microglia binds to its receptor (TNF-R1) in the injured MNs and, like Fas L, triggers cell death through FADD activation. TNF-α released by microglia and reactive oxygen species (ROS) present in injured MN constitute a potent transcriptional activator of metalloproteinase-9 (MMP-9), an enzyme involved in extracellular matrix degradation.

3. Different models for the study of neurodegeneration in ALS

Acquired knowledge about cellular and molecular pathophysiological mechanisms of neurodegenerative diseases is crucial to allow the development of novel therapeutic strategies. For this, creation and use of experimental models is essential to perform studies *in vivo* and *in vitro*. *In vitro* models rely on cell cultures, slices or organotypic cultures bring us information about cellular and molecular pathophysiological mechanisms of the disease, as well as about cellular communication failure. *In vivo* models, usually rodents, allow an approximation to the features involved in human disease (Tovar *et al.*, 2009). The variety of *in vitro* and *in vivo* models for the study of ALS pathogenesis is huge. However, in the next section we will be only focusing in the experimental approaches used in the work developed and here presented.

3.1. *In vitro* models

3.1.1. Primary cultures of MN

Primary SC cultures were established to study morphological, biochemical and electrophysiological characteristics of MN (Ransom *et al.*, 1977). The SCs are obtained from 12-14 days-old rodent embryos because it is easy to access and remove; then, the SC is dissociated by mechanical and enzymatic procedures, and plated on matrix-coated dishes (Tovar *et al.*, 2009). MN,

astrocytes and microglia can be extracted, purified and maintained in culture with high viability yields (Gingras *et al.*, 2007). MN are easy to identify in culture, once they have a large size (up to 20 µm in diameter), dendritic trees and may be stained for the transcription factor Hb9 and choline acetyltransferase, which are specific markers of MN (Gingras *et al.*, 2007; Tradewell *et al.*, 2011). MN are present in very small quantities, so they are dependent of the presence of glial cells to perform trophic support. If absent, the culture only lasts for 2 weeks (Bar, 2000; Lunn *et al.*, 2009).

This type of culture is useful to study effects of long-term cell survival in ALS, once they can be maintained in culture for up to 7 weeks (Tradewell *et al.*, 2011). Purified MN can also be cultured on an astrocyte feeder layer for several weeks to display characteristics similar to adult MN (Bar, 2000). MN can also be induced to express multiple copies of the gene of interest, in this case mSOD1, by microinjection of vectors directly into the cells (Tradewell *et al.*, 2011; Tradewell and Durham, 2010). This type of culture was used to demonstrate that MNs are vulnerable to glutamatergic excitotoxicity through AMPA receptors (Rao *et al.*, 2003).

However, the conditions of the cells in culture cannot mimic *in vivo* environment, once the complex interactions developed in the nervous system are lost and, consequently, some results obtained *in vitro* may not be reproducible *in vivo*. Finally, in these cultures it is not possible to express some features of the adult phenotype, once primary cells are derived from embryos (Park *et al.*, 2004).

3.1.2.NSC-34 and N9 Cell line: assembly of mixed culture

Immortalized and clonally uniform murine NSC hybrid cell lines were developed to dissect MN biology *in vitro*. All NSC hybrids were produced through somatic fusion between the N18TG2 aminopterin-sensitive neuroblastoma and SC MN from enriched primary cultures (Cashman *et al.*, 1992). They are composed by small undifferentiated and proliferative cells and large multinucleate cells. This cell line contains small proliferative and undifferentiated cells and larger multinucleate cells. Characteristics of MN relate to acetylcholine synthesis, storage and release, generation of action potential, expression of neurofilament proteins and association with neuromuscular synapse-specific basal lamina glycoproteins (Tovar *et al.*, 2009). They respond to agents affecting voltage-gated ion channels, organization of cytoskeleton and axonal transport, similarly to primary MN, although they failed in reproducing synaptic connections when at the undifferentiated stage (Durham *et al.*, 1993). This cell line expressing human mSOD1 by transfection with a plasmid is considered a cellular model of ALS and revealed similar features to those of MN degeneration in ALS, like fragmentation of the Golgi apparatus (Gomes *et al.*, 2008), mitochondrial deregulation (Raimondi *et al.*, 2006) and ER stress (Vijayalakshmi *et al.*, 2011), among others. A big problem resides on the characteristics of the neuroblastoma lineage that these cell lines retain, mediated by the oncogene N-myc involved in cell proliferation. Therefore, interference on the mechanisms of neuronal death and its prevention by possible therapeutic agents, cannot be discarded (Tovar *et al.*, 2009).

N9 is a microglia cell line derived from mouse brain that shares many phenotypical features with primary mouse microglia. However, the modified cell has an increased proliferation and adherence capacities (Stansley *et al.*, 2012). N9 microglia cells arose from the immortalizing primary microglia cells with the v-myc or v-mil oncogenes of the avian retrovirus MH2 (Righi *et al.*, 1989). Zhao

and colleagues (2011) demonstrated that LPS increased iROS production and the expression of gp91phox in N9 microglial cells. Consequently, intracellular ROS can activate diverse downstream signaling molecules such as protein kinase C, MAPK and NF-κB to regulate the expression of genes encoding a variety of pro-inflammatory factors (Zhao *et al*., 2011).

Mixed cultures with these two cell types are good candidates to get a suitable model to study the mechanisms involved in neurodegeneration and the specific dialogue of MN with microglia in the pathogenesis of ALS. In fact, it was recently demonstrated that differentiated NSC-34 transfected with human SOD1G93A have increased mitochondrial dysfunction, apoptosis and efflux of neuro-inflammatory markers such as MMP-9 and NO, features of ALS pathogenesis.

3.1.3.Organotypic cultures

Organotypic cultures consist in tissue slices cultivated in air-liquid interface which unlike cell lines, allow to preserve all the cellular content and the complex electrophysiological and biochemical organization of cells in the SC, as a 3D system (Tovar *et al.*, 2009). The slices can be obtained from both embryos and postnatal animals, namely from mutated mice, whose genotype has to be identified after birth (Mazzone and Nistri, 2011). After dissection of the lumbar SC and removal of the meninges, transversal sections with 200-400 μm are separated and transferred into membrane inserts fitting six-well or 12-well culture plates (Caldero *et al.*, 2010), where they can be kept in culture for more than 2 months. These type of culture maintain the choline acetyltransferase and acetylcholinesterase activities (Delfs *et al.*, 1989).

These models allow dynamic studies with many drugs but it cannot reproduce entirely what happens in an *in vivo* system and much less in the ALS patient (Tovar *et al.*, 2009). Here, MN degeneration can be induced by chronic exposure to the glutamate transporter blocker threo-β-hydroxyaspartate (THA) (Rothstein *et al.*, 1993), kainate (Mazzone and Nistri, 2011) or lithium (Caldero *et al.*, 2010).

3.2. *In vivo* models

3.2.1.Transgenic mSOD1 rodents

As previously mentioned, approximately 20% of fALS cases are due to mSOD1. Therefore, Gurney *et al.* created mice that express human mSOD1 (Gurney *et al.*, 1994). The first mutations produced were the substitution of glycine for alanine at position 93 (G93A) and of alanine for valine at position 4 (A4V). Other SOD1 mutations have been subsequently overexpressed in tg mice, such as G37R, G86R, and D90A mSOD1 proteins, all leading to MN degeneration (Borchelt *et al.*, 1994; Jonsson *et al.*, 2006; Ripps *et al.*, 1995) .

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 (Gurney, 2000). 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).

The disease in tg mice begins with hind limb limitation, impaired leg extension and reduced stride length, and continues to complete paralysis of the limbs within few days (Gurney *et al.*, 1994). Cellular alterations include vacuolar deterioration of MN and their processes at early stages, followed by neuronal loss and atrophy of the ventral horns in the SC at a late stage; another regions beyond SC could be affected, such as medulla, pons and midbrain (Dal Canto and Gurney, 1995). Specifically in SOD1 G93A SJL mice, the pre-symptomatic stage of motor dysfunction begins at six week of age and symptomatic stage develops at 13 weeks of age, which culminate with death at 4-5 months of age (Martin *et al.*, 2005). All of these characteristics are also seen in tg rats (Howland *et al.*, 2002; Nagai *et al.*, 2001).

However, it has been difficult to match the findings made in this model with the processes occurring in patients that do not have mutations in SOD1 (Dupuis *et al.*, 2004). This model also fails in the evaluation of drugs that showed therapeutic efficacy, due to the small number of animals usually evaluated, the lack of randomization and the initiation of the treatment before symptoms onset (Benatar, 2007; Scott *et al.*, 2008).

4. Recent findings on diagnosis and therapeutic approaches in ALS

Diagnosis in ALS is based on diagnostic criteria, called El Escorial criteria and more recently the Awaji criteria, which present good sensitivity and specificity. These criteria uses neurophysiological data acquired by electromyography (EMG) together with the clinical information (Kiernan *et al.*, 2011). It can also include other three key principles: evidence of LMN loss, evidence of reinnervation and fibrillation and sharp waves or fasciculation potentials. Using these criteria, it is possible to classify the patient into groups of clinically definite, clinically probable, or clinically possible ALS (Costa *et al.*, 2012). Magnetic resonance imaging (MRI) scan may be also used to rule out any (SC) or brainstem disease. Blood tests are important to detect abnormal proteins or hormone levels, or even the presence of heavy metals such as lead more closely associated with other neurological diseases. The CSF analysis should be done in parallel to evaluate the existence of other abnormalities (e.g., viral, autoimmune, neurotoxic). Recently, metabolomics profile in the CSF was demonstrated to have different signatures between ALS patients with mutations and those without mutations (Wuolikainen *et al.*, 2012). Genetic testing for mSOD1 also exists, but little information can be given to the patient since the majority of families with fALS (80%) do not have alterations in their SOD1 gene (Naganska and Matyja, 2011).

Despite the advances in the comprehension of the mechanisms that trigger neurodegeneration in ALS, there is not an effective therapeutic for this disease. Actual therapeutic strategies being developed use pharmacotherapy, physical therapy, gene therapy, stem-cell therapy, RNA therapy and immunotherapy areas.

By now, Riluzole is the only drug approved by the Food and Drug Administration for the treatment of ALS and its function is to decrease the release of glutamate (Bensimon *et al.*, 1994). It does not reduce MN damage, but two therapeutic trials showed that the drug prolonged survival by 3-6 months (Bensimon *et al.*, 1994; Lacomblez *et al.*, 1996).

Many clinical trials also tried other neuroprotective agents, such as Memantine, Tamoxifen, Ceftriaxone, Creatine, Myotrophin®, Celebrex, Neurodex, Oxandrolone, CoQ10, Topiramate, Xaliproden, Indinavir, Minocycline, Buspiron, Gabapentin, α-tocopherol, among others (Desnuelle *et al.*, 2001; Gordon, 2005; Miller *et al.*, 2001; Phukan and Hardiman, 2009), but none of them has succeeded for treatment in ALS patients.

Citicoline, also called CDP-choline (cytidine-5- diphosphocholine) is an endogenous nucleoside that has neuroprotective properties related to its action on glutamate-mediated cell death. Studies using organotypic cultures of the rat lumbar SC showed a decrease of the extracellular levels of glutamate, by inhibition of neuronal glutamate efflux and an increased astrocytic glutamate uptake (Matyja *et al.*, 2008). Another compound involved in the apoptotic mechanism of cell death is Erytropoietin (EPO). Many studies showed that EPO promote neuronal survival, when exposed with damaging agents through antagonizing glutamate cytotoxic action, enhancing antioxidant enzyme expression, reducing the free radical production rate and affecting neurotransmitter release (Grasso *et al.*, 2007; Liu *et al.*, 2008).

Immunotherapy focuses in the protection and regeneration of the immune system, through appliance of intravenous immunoglobulins and experimental treatment with vaccination, minocycline, neurotrophic factors and antibodies. Several immunosuppressive and immunomodulatory therapies have been tried in ALS but with no success (Calvo *et al.*, 2010). The most studied is minocycline, a second-generation tetracycline with distinct antibiotic and anti-inflammatory properties (Whiteman and Halliwell, 1997). Their neuroprotective effects result from the inhibition of microglial activation (Tikka *et al.*, 2001) and possibly proliferation (Tikka *et al.*, 2002). The studies passed in phase I/II in ALS patients, but no difference between treated and untreated groups was observed (Gordon *et al.*, 2004).

More recently, attention has been given to stem cell transplantation, which consists in restoration of motor function, by introducing human stem cells into the SC to replace degenerating MN (Rowland and Shneider, 2001). The treatment may stop or slow the progression of MN disease, providing growth factors, immunomodulation of injurious lesion environment and anti-inflammatory effects on microglia and astrocytes (Traub *et al.*, 2011). However, in ALS, this treatment is difficult due to the complexity of the pathways involved in motor function (Rowland and Shneider, 2001). Nevertheless, several stem cell sources have been studied in ALS, including bone marrow transplantation (Corti *et al.*, 2010; Ohnishi *et al.*, 2009), mesenchymal stem cell (MSC) transplantation (Mazzini *et al.*, 2010; Vercelli *et al.*, 2008), neural stem cell transplantation (Corti *et al.*, 2007; Xu *et al.*, 2006) and induced pluripotent stem cells (iPSCs) (Ebert *et al.*, 2009), with promising results. Vercelli and colleagues (2008) transplanted human MSCs into the lumbar SC of asymptomatic SOD1G93A mice and proved that these type of stem cells are a good candidate for ALS cell therapy, since they can survive and migrate after transplantation in the lumbar SC, while preventing astrogliosis and microgliosis. Zhou and colleagues (2013) showed that intrathecally transplanted human marrow stromal cells inhibited inflammatory response in mSOD1 mice, evidenced by a reduction in microglial activation, TNF-α secretion and iNOS protein expression.

Gene therapy consists to the usage of a vector, most commonly viral, to deliver the therapeutic gene of interest to the affected region or tissue (O'Connor and Boulis, 2012). Vaccines that

target SOD1G93A have been developed and have shown to be effective in mouse models of ALS (Takeuchi *et al.*, 2010). It was also implemented a SOD1 gene-silencing approach, which may be useful to delay disease onset or progression (Smith *et al.*, 2006).

RNA interference therapy consists in the inhibition and regulation of gene expression through the binding of a noncoding miRNA in mRNA. It is very useful in autosomal-dominant diseases, where the silencing of the dominant mutant allele might cure the patient (Traub *et al.*, 2011). Several studies have demonstrated effective mSOD1 silencing and improved motor outcomes in mice (Ralph *et al.*, 2005; Raoul *et al.*, 2005). miR-155 has shown to be an excellent therapeutic target derived from its abundance and marked elevation in ALS, together with its reproducibility across species and various ALS models, as well as prior work linking miR-155 with immunity and inflammation. Recent data suggest that miR-155 is increased in peripheral monocytes from ALS-model mice and ALS patients, suggesting that modulation of these cells may be a potential therapeutic approach (Butovsky *et al.*, 2012).

For all the reasons mentioned in this chapter, microglia is now seen as a possible therapeutic target for ALS disease and studies involving transplantation of wt microglia seems to provide a promising approach.

AIMS

The main goal of this thesis is to explore the role of microglia in preventing or restoring motoneurons (MNs) function in ALS, through the evaluation of signaling molecules that may be involved in cellular cross-talk, by using two different experimental models: (i) mixed MN-microglial cultures (NSC-34-N9 cell lines); (ii) organotypical slices from mice spinal cord. Parameters to be evaluated will include SOD1 accumulation, oxidative stress markers (glutamate and ATP) and inflammatory mediators (nitric oxide, metalloproteinases, toll-like receptor-4 and high mobility group box 1).

More specifically, the aims are:

i. Optimization of mixed cultures, composed by MN-like cell line (NSC-34) and microglial cell line (N9). For this, we will use a MN-like cell line expressing human-SOD1 with G93A mutation (NSC-34/hSOD1G93A) and the microglial cell line N9. NSC-34 expressing human-SOD1wt will be used as controls. To produce a mixed culture, we will add N9 cells to mutated MNs at 0 and 2 days-of-differentiation (DIV) and cells will be maintained in cultures for 4 and 7 DIV.

ii. To set-up and characterize organotypic cultures obtained from lumbar spinal cord (SC) of ALS-transgenic mice carrying the same human SOD1 mutation (TgSOD1-G93A mice). Response to SOD1 accumulation and to LPS immunostimulatory stimulus will be evaluated to assess the validity of the culture and a more integrative cellular participation in the pathophysiological mechanisms of ALS. Moreover, it will be a suitable ALS model to test the efficacy of novel therapeutic compounds to halt, or at least delay, ALS progression.

The final purpose of this thesis project is to provide insights into microglia and inflammatory mediators as players in the onset and progression of ALS and to establish a SC organotypic culture as resource for future studies on neuroinflammation-neurodegeneration and on drug prevention effects.

II: MATERIALS AND METHODS

1. Materials

1.1 Chemicals

Dulbecco's modified Eagle's medium-Ham's F12 medium (DMEM-Ham´s F-12), DMEM high glucose w/o pyruvate, fetal bovine serum (FBS), Penicillin/Streptomycin, L-glutamine and non-essential aminoacids (NEAA) were purchased from Biochrom AG (Berlin, Germany); Hank's balanced salt solution (HBSS) without phenol red (1x), B-27® Serum-Free Supplement (50X) and Neurobasal medium (1x) were acquired from GIBCO® (Life Technologies, Inc.,Grand Islands, USA); Roswell Park Memorial Institute (RPMI) 1640 medium, trypsin-Ethylenediamine tetraacetic acid (trypsin-EDTA) solution (1X), ATP, β-Nicotinamide adenine dinucleotide 2′-phosphate ($NADP^+$), bovine serum albumin (BSA), Coomassie Brilliant Blue R-250, naphtylethylenediamine ($C_{12}H_{14}N_2$), sulfanilamide ($C_6H_8N_2O_2S$), Tris-base, phenylmethylsulfonyl fluoride (PMSF), Poly-**D**-Lysine (PDL) and β-mercaptoethanol were purchased from Sigma-Aldrich (St. Louis, MO, USA); Geneticin sulfate (G418) and nonyl phenoxypolyethoxylethanol (NP-40) were obtained from Calbiochem (Darmstadt, Germany); **L**-glutamic acid kit, Triton X-100, glucose-6-phosphate dehydrogenase, hexokinase and protease inhibitor cocktail tablets were obtained from Roche Diagnostics (Mannhein, Germany); Nitrocellulose membrane was obtained from Amersham Biosciences (Piscataway, NJ, USA); sodium dodecyl sulphate (SDS) was acquired from VWR-Prolabo; cell lysis buffer® and LumiGLO® were from Cell Signaling (Beverly, MA, USA); acrylamide, bis-acrylamide, glucose anhydre, Tween 20, glycerol, absolute ethanol, acetic acid, sodium chloride (NaCl), potassium hydroxide (KOH), Gelatine, calcium choride ($CaCl_2$) and perchloric acid (70%) were obtained from Merck (Darmstadt, Germany); Bio-Rad's Protein Assay Reagent was obtained from BioRad Laboratories (Hercules, CA, USA). All the other common chemicals were of analytical grade and were purchased either from Sigma-Aldrich or Merck.

1.2 Antibodies

Primary antibodies*:* Rabbit polyclonal anti-superoxide dismutase 1 (SOD1) and rabbit polyclonal anti-toll-like receptor 4 (TLR4) were purchased from Santa Cruz Biotechnology® (Santa Cruz, CA, USA). Mouse monoclonal anti-high mobility group protein B1 (HMGB1) was acquired from BioLegend® (San Diego, CA, USA). Mouse anti-β-actin was obtained from Sigma-Aldrich (St. Louis, MO, USA).

Secondary antibodies: Horseradish peroxidase-labelled goat anti-rabbit IgG was purchased from Santa Cruz Biotechonlogy® (Santa Cruz, CA, USA). Horseradish peroxidase-labelled goat anti-mouse IgG was obtained from Amersham Biosciences (Piscataway, NJ, USA).

1.3 Equipment

Optical microscope with phase-contrast equipment [Olympus, model CK2-TR, purchased from Carl Zeiss, Inc. (North America)] were used for cell morphologic evaluation. Mini-PROTEAN Tetra cell system used for Western Blot and zymography was from Bio-Rad (Hercules, CA, USA). Microplate reader (PR 2100) was also from Bio-Rad and it was used for spectrophotometric measurements of protein, nitrites and glutamate content. GloMax® Multi Detection System (Sunnyvale, CA, USA) was used for ATP quantification. For metalloproteinases gel photographs and immunodetection in nitrocellulose membranes it was used ChemiDoc™ equipment, also from Bio-Rad Laboratories. Sonication of samples was performed in the Ultrasonic Processor UP100H (Hielscher-Ultrasound Technology, Teltow, Germany). To ensure a stable environment to optimal cell growth (37°C and 5% CO_2), cell cultures were maintained in HERAcell 150 incubators (Thermo Scientific, Waltham, MA, USA) and the work performed in sterile conditions in a HoltenLamin Air HVR 2460 (Allerod, Denmark). Eppendorf 580R (Eppendrof, Hamburg, Germany) and a Sigma 3K30 centrifuges were used for different experimental procedures. To slice tissue in organotypic cultures, it was used McIlwain Tissue Chopper (Gomshall, Surrey, UK). In other procedures, were used surgical material and the Stereomicroscope Stemi DV 4 [Carl Zeiss, Inc. (North America)].

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2. Methods

2.1. *In vitro* mixed cultures

For *in vitro* studies, were used two cell lines: NSC-34 and N9. NSC-34 is a murine neuroblastoma and spinal cord hybrid cell line that has many of the unique morphological and physiological characteristics of motoneurons (Cashman *et al.*, 1992), as mentioned in the introduction. N9 cell line was developed by immortalizing primary microglia cells obtained from CD1 mouse cortex (Righi *et al.*, 1989).

2.1.1. *NSC-34* cell line

NSC-34 cell line transfected with human SOD1, either wild type or mutated in G93A (NSC-34/hSOD1wt or NSC-34/hSOD1G93A, respectively), were a gift from Júlia Costa, Instituto de Tecnologia Química e Biológica (ITQB), Universidade Nova de Lisboa, Portugal (Gomes *et al.*, 2008). NSC-34/hSOD1wt were used as control condition. NCS-34 cells were grown in proliferation media (DMEM high glucose, w/o pyruvate, supplemented with 10% of FBS and 1% of Penicillin/Streptomycin) and selection was made with G418 at 0.5 mg/ml. Medium was changed every 2 to 3 days. Culture plates were coated with PDL (50 µM) before plating the cells. Cells were seeded in 12-well culture plates at a concentration of $5x10^4$ cells/ml and maintained at 37ºC in a humidified atmosphere of 5% CO_2.

2.1.2.*N9* cell line

N9 microglia cell line was a gift from Teresa Pais, Instituto de Medicina Molecular (IMM), Lisbon, Portugal. Cells were cultured in RPMI supplemented with FBS (10%), L-glutamine (1%) and Penicillin/Streptomycin (1%), grown to confluency and splitted every 2 to 3 days. No coating was required for the maintenance of these cells. In order to perform mixed cultures, cells were plated with NSC-34 cells in differentiation media at a concentration $2x10^4$ cells/ml. They were maintained at 37ºC in a humidified atmosphere of 5% CO_2.

2.2. *In vitro* treatment of mixed cultures of NSC-34 and N9 cell lines

After 48 h in proliferation media (as described in 2.1.1 section), differentiation of NSC-34 was induced by changing medium for DMEM-F12 plus FBS (1%), NEAA (1%), Penicillin/Streptomycin (1%) and G148 (0.1%) (Cho *et al.*, 2011). To produce a mixed culture, we added N9 microglia cells either to NSC-34/hSOD1wt or NSC-34/hSOD1G93A, at time of differentiation or at two *days in vitro* (0 and 2 DIV, respectively), as represented in Figure II.1. In cultures where microglia were added at 0 DIV, we intended to see if healthy microglia could prevent the development of MN degeneration, since neurons do not appear to be injured at this time. In cultures where microglia were added at 2 DIV, MN had already signs of degeneration, so we planned to see if microglia promote or restore MN degeneration. Mixed cultures were maintained in culture at 37ºC in a humidified atmosphere of 5% CO_2 till 4 DIV, where previous studies in the group revealed high MN degeneration (Vaz *et al.*, 2013), and till 7 DIV to see long term changes in microglia-MN signaling. NSC-34 cells were plated at a concentration of $5x10^4$ cells/ml and N9 cells at a concentration of $2x10^4$ cells/ml, in order to maintain the usual proportion of microglia and neurons in the CNS, as in previous studies with mixed neuron-microglia cultures performed in our group (Silva *et al.*, 2011). All of these procedures were performed in sterile conditions.

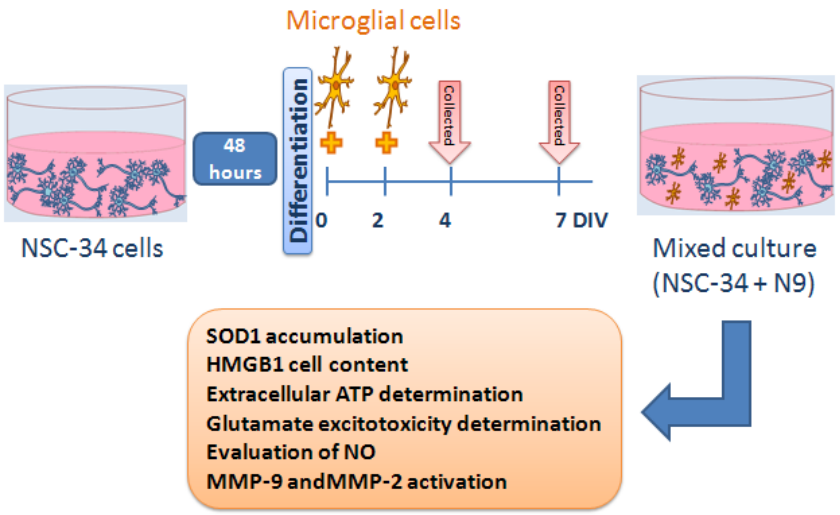

Figure II.1 Experimental procedure used in mixed cultures of transfected NSC-34 cell line and N9 cell line and parameters evaluated. Neuroblastoma Spinal Cord 34 (NSC-34) cells, transfected with human superoxide dismutase 1 (SOD1), wild type or mutated in G93A (NSC-34/hSOD1wt or NSC-34/hSOD1G93A), were grown in proliferation media and selection was made with G418. After 48h in culture, differentiation was induced by

changing medium for Dulbecco's modified Eagle's medium-Ham's F12 medium plus fetal bovine serum (1%) and non-essential amino acids (1%). Microglia (N9) was added at 0 and 2 DIV and cells were maintained at 4 or 7 days after differentiation (DIV). Accumulation of SOD1 and high-mobility group protein B1 (HMGB1) cell content were evaluated by western blot. Extracellular Adenosine Triphosphate (ATP) was quantified by an enzymatic fluorescence assay, glutamate by L-glutamic acid kit, nitric oxide (NO) by Griess reaction and matrix metalloproteinase-2 (MMP-2) and -9 (MMP-9) activity by gelatin zymography assay.

2.3 Organotypic spinal cord culture

Organotypic slice cultures were adapted from the methods previously described (Guzman-Lenis *et al.*, 2009). Briefly, 7-days SJL (Wt) or transgenic mice carrying a human mSOD1 (TgSOD1-G93A mice), previously genotyped, were sacrificed by decapitation and their spinal cords were dissected using surgical equipment and a magnifying glass. After dissection, the lumbar segment of their spinal cord was extracted and cut into 350 µm transverse slices with a McIlwain tissue chopper. Samples were placed in high glucose (6 mg/ml) HBSS with 1.5% Penicillin-Streptomycin, where, they were separated and carefully transferred onto culture plate inserts and placed into a 6-well plate containing Neurobasal medium with 1x B27, 2 mM glutamine, 6 mg/ml glucose and antibiotics, and maintained at the air-liquid interface, at 37ºC in 5% CO_2. The medium was changed the following day and, replaced three times per week until 10 DIV. At 10 DIV, slices were incubated with lipopolysaccharide or LPS (1µg/ml) for 24h, to study their influence on neuroinflammatory response in ALS. Incubations with Neurobasal medium were used as control.

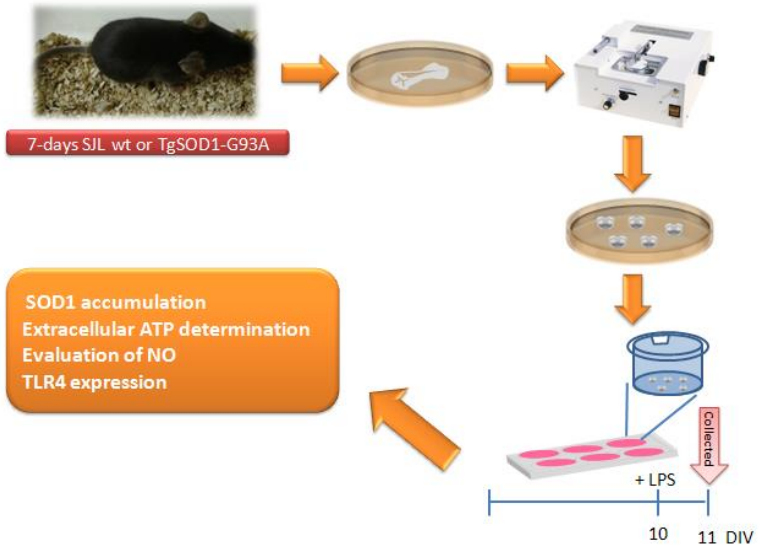

Figure II.2 Experimental scheme of organotypic cultures of 7-days SJL (Wt) and transgenic mice carrying a human mSOD1 (TgSOD1-G93A mice) and parameters evaluated. The lumbar segment of spinal cord was incubated in a 6-well plate and cultured during 10 days. At 10 DIV, slices were incubated with lipopolysaccharide or LPS (1 µg/ml) for 24h, to study their influence on neuroinflammatory response in ALS. After incubation, slices were collected for western blot analysis for superoxide dismutase-1 (SOD1) and Toll-like-receptor-4 (TLR-4) expression and extracellular media was used for quantification of nitric oxide (NO) by Griess reaction and Adenosine Triphosphate (ATP) by an enzymatic fluorimetric assay.

2.4 Western Blot assay

Western Blot was carried out as usual in our lab (Fernandes *et al.*, 2006). Total cell extracts were obtained by lysing cells with 1x Cell Lysis Buffer plus 1 mM PMSF for 5 min, on ice and with shaking,

followed by sonication during 20 seconds. The lysates were centrifuged at 14000 *g* for 10 min, at 4ºC, and the supernatants were collected and stored at -20ºC.

Homogenates from lumbar SC slices were obtained by adding ice radio-immunoprecipitation assay (RIPA), a lysis buffer composed by 5% of Tris(hydroxymethyl)aminomethane (Tris) 1M pH=8, 1% of Ethylenediamine Tetraacetic acid (EDTA) 0,5M pH=8, 3% of NaCl 5M, 10% of NP-40, 50% glycerol, 0,1% SDS and protease inhibitor, and homogeneized using a Pellet pestles (Sigma). Then, samples were treated as described for mixed cell lysates.

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 15% (for SOD1 and HMGB1 evaluation) or on a 12% (for TLR4 evaluation) sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), running with fixed amperage of 30 mA per gel. After running the gel, proteins were transferred to a nitrocellulose membrane with fixed amperage of 300 mA during 1h30, and then blotted membranes were incubated in blocking buffer [Tween 20-Tris buffered saline (T-TBS) plus 0.25% (w/v) non-fat dried milk] at room temperature during 1h. After that, membranes were incubated overnight at 4ºC with the following primary antibodies diluted in T-TBS with 5% BSA: rabbit anti-SOD1 antibody (1:500 for cell lysates and 1:200 for tissue homogenates, Santa Cruz Biotechnology®), rabbit anti-TLR4 (1:100 for homogenates, Santa Cruz Biotechonlogy®), mouse anti-HMGB1 antibody (1:500 for lysates, BioLegend®) and mouse anti-β-actin (1:5000 for cell lysates and 1:2000 for tissue homogenates, Sigma-Aldrich). After washing with T-TBS, the membranes were incubated at room temperature (RT) during 1 h with the respectively 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 by 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 obtained were normalized to β-actin.

2.5 Quantification of extracellular ATP

NSC-34, either alone or in mixed culture with N9 cells, as well as in organotypic culture extracellular media were treated on ice to avoid degradation of ATP. For the determination of extracellular ATP levels, the incubation media was collected and treated with perchloric acid 2M. Then, the pH value was neutralized with 4 M KOH solution. To remove cellular debris, the samples were centrifuged (Eppendorf, 5810R) during 5 min at 10,000 *g* and 4°C, between the different steps. ATP levels were measured indirectly by the production of NADPH, through an enzymatic assay consisting in the addition of a solution containing $NADP^+$, glucose, 6P-glucose dehydrogenase, followed by hexokinase, which is the enzyme that starts the reaction. Fluorescence intensity was quantified using GloMax® Multi Detection System (Promega) at λ_{em}=410-460 nm and λ_{ex}=365 nm. A calibration curve of standard ATP was used for each assay.

2.6 Measurement of extracellular glutamate

Glutamate content was quantified in differentiation media from NSC-34, either alone or in mixed culture with N9 cells by using the L-glutamic acid kit (Roche). The reaction was performed in a 96-well microplate and the absorbance was read in the microplate reader (Bio-Rad Laboratories) at 490 nm. A calibration curve of glutamic acid was used for each assay. All samples and standards were analyzed in duplicate and the mean value was used (Falcão *et al.*, 2005).

2.7 Quantification of extracellular nitric oxide/nitrite levels

NO levels were estimated by measuring the concentration of nitrites (NO_2), the stable end-product from NO metabolism, in the extracellular media of differentiated NSC-34 cells, either alone or in mixed culture with N9 cells, as well as in organotypic culture extracellular media. Cell supernatants free from cellular debris were mixed with Griess reagent [1% (w/v) sulphanilamide in 5% H_3PO_4 and 0.1% (w/v) *N*-1 naphtylethylenediamine, in a proportion of 1:1 (v/v)] in 96-well tissue culture plates for 10 min in the dark at room temperature. The absorbance was determined using a microplate reader (Bio-Rad Laboratories) at 540 nm. A calibration curve of standard nitrites was used for each assay. All samples were measured in duplicate and the mean value was used.

2.8 Gelatin zymography

Metalloproteinases -2 and -9 (respectively MMP-2 and MMP-9) quantification was performed in NSC-34, either alone or in mixed culture with N9 cells, through the gelatin zymography method, which is possible to detect the protease activity in the running gel. Cell supernatants free from cellular debris were used in SDS-PAGE zymography in 0.1 % gelatin-10 % acrylamide gels under non-reducing conditions, at 30 mA/gel. Then, the gels were washed for 1 h at room temperature with 2.5% Triton-X-100 (in 50 mM Tris pH 7.4; 5 mM $CaCl_2$; 1 µM $ZnCl_2$) to remove SDS and to renature the MMP species in the gel. To induce gelatin lysis, the gels were incubated at 37°C in the developing buffer (50 mMTris pH 7.4; 5 mM $CaCl_2$; 1 µM $ZnCl_2$) overnight. For enzyme activity analysis, the gels were stained with 0.5% Coomassie Brilliant Blue R-250 and destained in 30% ethanol/10% acetic acid/H2O. Gelatin activity, detected as a white band on a blue background was photographed in Chemidoc and measured using computerized image analysis (Image Lab) (Silva *et al.*, 2010).

2.9 Statistical analysis

Results of at least three different experiments were expressed as mean ± SEM for NSC-34 cultures either isolated or in mixed culture with N9. Comparisons between the different parameters evaluated in wt and G93A NSC-34 cell line and in organotypic cultures from SC of TgSOD1-G93A or WT mice were made using two-tailed Student's t-test for equal or unequal variance, as appropriate. Comparison of more than two groups in the parameters evaluated in mixed cultures with or without microglia was done by one-way 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

1. Characterization of microglia-motoneurons cross-talk in a model of mixed cultures of NSC-34 and N9 cell lines

Neuroblastoma Spinal Cord 34 (NSC-34) is a hybrid cell line produced through somatic fusion between neuroblastoma and mice spinal cord (SC) motoneurons (MNs) from enriched primary cultures (Cashman *et al*., 1992). In our model, we used NSC-34 that had been transfected either with wild type (wt) human SOD1 (NSC-34/hSOD1wt) or mutated in G93A (NSC-34/hSOD1G93A) (Gomes *et al.*, 2008). The characterization of these cells was recently done in our group and accumulation of mutated SOD1 was shown to occur after 4 days of differentiation (DIV) in NSC-34/hSOD1G93A cells, together with cell dysfunction (Vaz *et al*., 2013), which may represent the progression of MN degeneration in familiar ALS. Therefore, in our model, we considered two different time points after NSC-34 cell differentiation that could mimic two stages of MN degeneration in ALS: (i) 4 DIV – during SOD1 accumulation (symptomatic) and (ii) 7 DIV – after SOD1 accumulation and cell damage.

Since ALS does not exclusively affects MN but also other cell types, and because neuroinflammation is a pathological hallmark of this disease, where microglia can adquire neurotoxic or protective properties, we considered that it would be interesting to develop a model where cellular cross-talk with microglia was taken into account, since increasing evidence point microglia as key players for MN degeneration in ALS (Appel *et al.*, 2011). Therefore we studied the role of microglia in preventing or restoring MN function by using a model of mixed cultures, composed by N9 cell line and NSC-34 cell line. N9 is a microglia cell line derived from mice cortex and shares many phenotypical characteristics with primary cultures of mouse microglia, such as migration, phagocytosis and inflammation-related features (Bruce-Keller *et al.*, 2000; Cui *et al.*, 2002; Fleisher-Berkovich *et al.*, 2010).

N9 cells were added either to NSC-34/hSOD1wt or NSC-34/hSOD1G93A, at time of neuronal differentiation or after two days (0 and 2 DIV, respectively), as described in Methods. In cultures where microglia were added at 0 DIV, we intended to see if microglia can prevent the development of the disease, since NSC-34/hSOD1G93A do not show to be in injured at this time point. In cultures where microglia were added at 2 DIV, NSC-34/hSOD1G93A already presented signs of degeneration, so we planned to see if microglia can rescue MN degeneration.

1.1 Morphological characterization in NSC-34 cell line either expressing human SOD1 wt or mutated in G93A and N9 cell line in mixed culture

On average, the amounts of microglia and MN *in vivo* are near the ratio of 1/3, so NSC-34 cells were cultured at 2.5×10^4 cell/ml and N9 at 1.0×10^4 cell/ml as previously described (Silva *et al.*, 2011). As represented in Figure III.1, we observed differentiated MNs from NSC-34 line with long extension of ramifications and increased number of neurites, as well as microglial cells from N9 cell line, much smaller and with very few or none ramifications, which are typical characteristics from this cell line

(Cashman *et al.*, 1992; Zhao *et al.*, 2011). We can see a higher proportion of MNs compared with microglia, with a ratio 3/1, confirming the proportion *in vivo*.

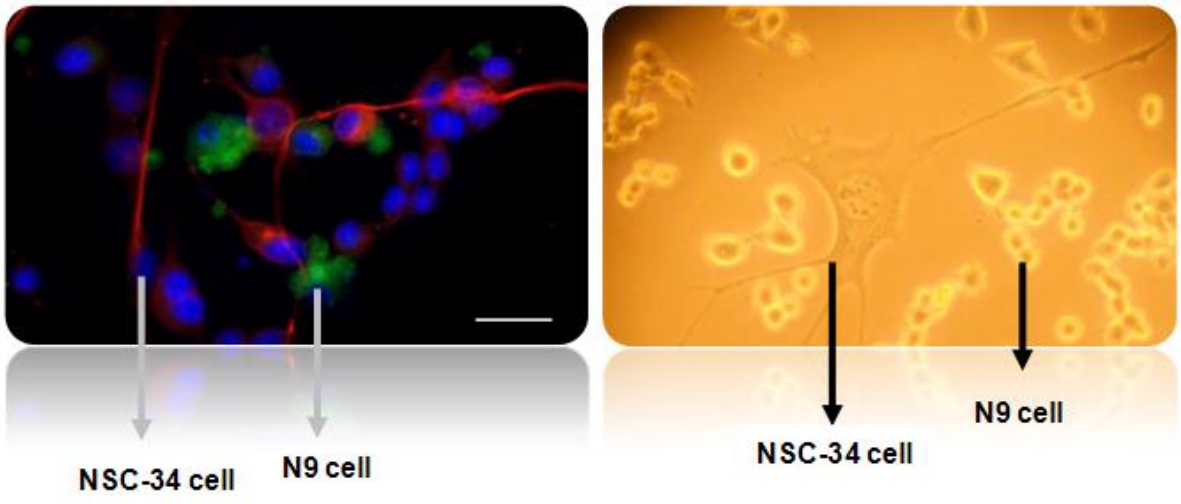

Figure III. 1 - Mixed cultures of NSC-34 cell line and microglial cells from N9 cell line, were successfully implemented and represent the ratio 3/1 as previously described (Silva *et al.*, 2011). Cells were cultured as indicated in Methods. (A) After fixation with paraformaldehyde, cells collected at 4 DIV were double-stained with mouse anti-βIII-tubulin for neurons and with rabbit anti-lectin for microglia followed by a fluorescent-labeled secondary antibody (neurons in red and microglia in green), and counterstained with Hoechst® for the nuclei (in blue). (B) Photos of mixed culture in phase-contrast microscope were acquired after MNs differentiation and taken by a digital HP camera. Scale bar represents 40 µm.

1.2 Microglia restore human SOD1 (hSOD1) accumulation as well as mouse SOD1 accumulation in NSC-34/hSOD1G93A after 7 DIV

The analysis of SOD1 accumulation was performed by western blot analysis of mixed culture cell lysates. As indicated in Figure III.2, we detected two bands with low molecular weight in NSC-34/hSOD1wt and NSC-34/hSOD1G93A cells, which correspond to human and mouse forms of SOD1, with 21 and 14 kDa respectively (Kirby *et al.*, 2005), confirming the success of the transfection of hSOD1.

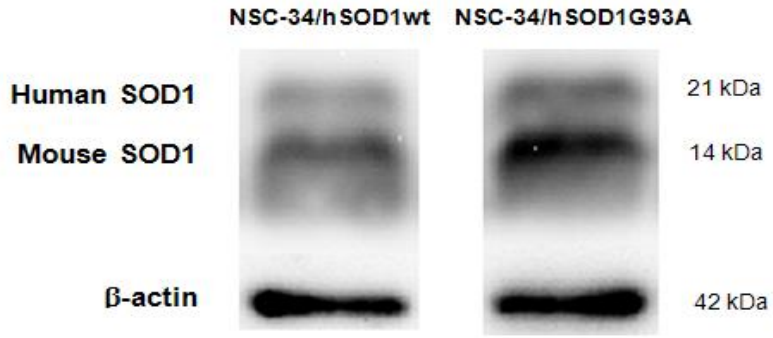

Figure III. 2 NSC-34 cells contain human SOD1 (hSOD1) successfully transfected and mouse SOD1. Cells were cultured as indicated in Methods. Total lysates were analyzed by western blot with anti-SOD1 antibody. Anti-β-actin antibody was used as internal control. Representative results from one experiment are shown.

To study the effects of microglia in SOD1 accumulation in NSC-34/hSOD1G93A cells, we performed western blot assay using rabbit anti-SOD1 antibody (1:500). Regarding human SOD1

(hSOD1), and as indicated in Table III.1 and Figure III.3B, the results showed a significant increase of protein accumulation in NSC-34/hSOD1G93A cells, when compared with respective control after 7 DIV ($p<0.01$ *vs.* respective NSC-34/hSOD1wt) and respective 4 DIV condition ($p<0.01$). Here, the presence of microglia reduced very significantly hSOD1 accumulation in NSC-34/hSOD1G93A, especially when it was added at 2 DIV ($p<0.01$). No significant alterations were seen after 4 DIV. Regarding mouse SOD1, there was a significant increase in its content in NSC-34/hSOD1G93A after 4 DIV and 7 DIV, when compared with the respective control (Table III.1 and figure III.3.C). The addition of microglia at 0 and 2 DIV reduced very significantly mouse SOD1 accumulation in NSC-34/hSOD1G93A after 7 DIV ($p<0.01$ *vs.* NSC-34/hSOD1G93A w/o microglia addition and $p<0.01$ *vs.* respective 4 DIV condition).

Table III.1 – Levels of human and mouse superoxide dismutase 1 (SOD1) are augmented in NSC-34/hSOD1G93A cells and reduced in the presence of microglia after 7 DIV.

			w/o N9	+ N9 at 0 DIV	+ N9 at 2 DIV
Human SOD1	**4 DIV**	**NSC-34/hSOD1wt**	1.00 ± 0.43	1.20 ± 0.17	0.61 ± 0.08
		NSC-34/hSOD1G93A	0.93 ± 0.08	1.75 ± 0.22	1.48 ± 0.39
	7 DIV	**NSC-34/hSOD1wt**	1.00 ± 0.90	0.92 ± 0.16	0.96 ± 0.37
		NSC-34/hSOD1G93A	2.35 ± 0.78	1.73 ± 0.43	0.38 ± 0.17
Mouse SOD1	**4 DIV**	**NSC-34/hSOD1wt**	1.00 ± 0.54	1.03 ± 0.14	1.06 ± 0.10
		NSC-34/hSOD1G93A	1.04 ± 0.34	1.47 ± 0.22	1.02 ± 0.25
	7 DIV	**NSC-34/hSOD1wt**	1.00 ± 0.86	1.26 ± 0.24	0.88 ± 0.12
		NSC-34/hSOD1G93A	1.30 ± 0.47	0.54 ± 0.03	0.58 ± 0.10

Cells were cultured as indicated in Methods. Total cell lysates were collected at 4 DIV and 7 DIV and both analyzed by western blot with antibody specific for SOD1. The intensity of the bands was quantified using image analysis software (Image Lab), normalized with respect to β-actin protein. Data are expressed as fold *vs.* NSC-34/hSOD1wt and represent the absolute values found in Figure III.3. Results are mean ± SEM from at least two independent experiments.

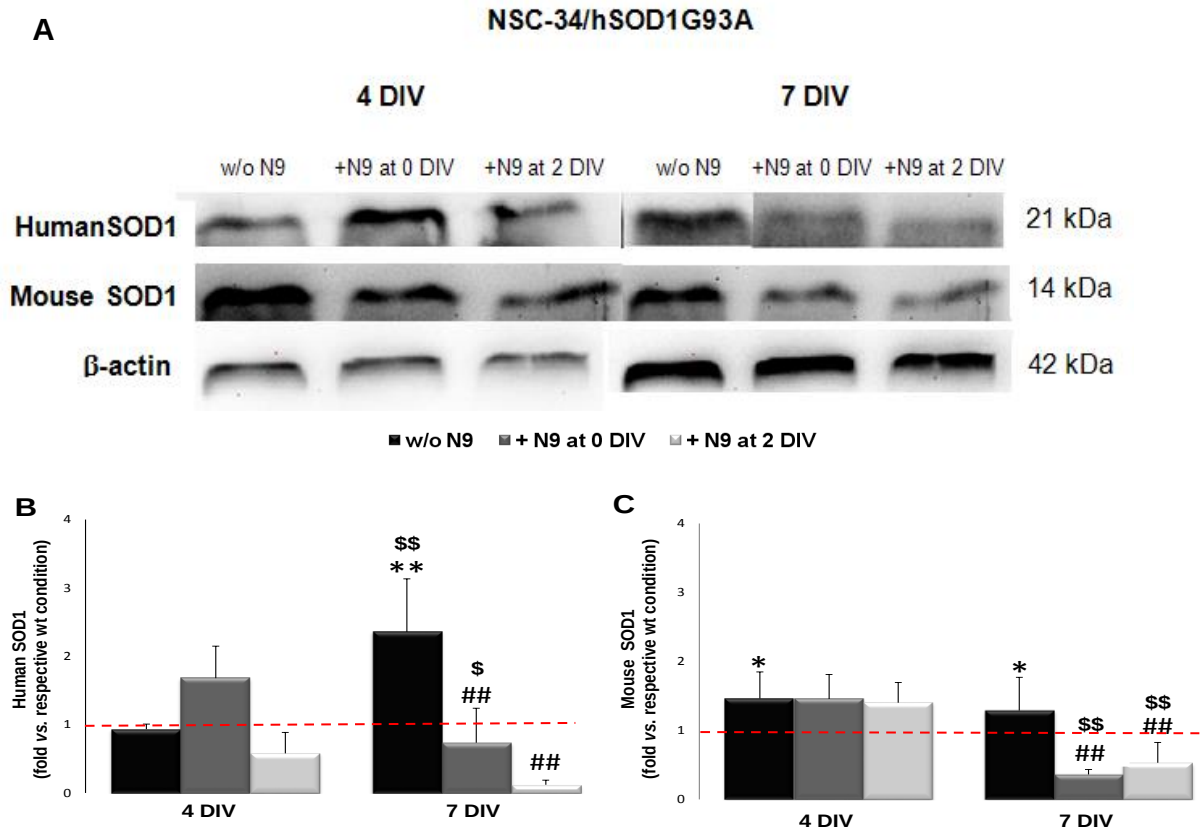

Figure III. 3 - Human and mouse superoxide dismutase 1 (SOD1) levels are increased in NSC-34/hSOD1G93A cells and reduced in the presence of microglia after 7 DIV. Cells were cultured as indicated in Methods. Total cell lysates were collected at 4 DIV and 7 DIV and both analyzed by western blot with antibody specific for SOD1. (A) Representative results from one experiment are shown. The intensity of the bands was quantified using image analysis software (Image Lab), normalized with respect to β-actin protein and expressed as fold *vs.* respective NSC-34/hSOD1wt condition, either for human SOD1 (B) or for mouse SOD1 (C). Results are mean ± SEM from at least two independent experiments. Corresponding absolute values are presented in Table III.1. **p<0.01 and *p<0.05 *vs.* respective NSC-34/hSOD1wt condition; $^{\#\#}$p<0.01 *vs.* respective NSC-34/hSOD1G93A w/o microglia; $^{\$\$}$p<0.01 and $^{\$}$p<0.05 *vs.* respective 4 DIV condition. Dotted line: respective wt (control).

1.3 Microglia differently modulate nitric oxide (NO), glutamate and Adenosine Triphosphate (ATP) extracellular levels in mixed culture with NSC-34/hSOD1G93A

It is known that abnormal accumulation of SOD1-positive inclusions in spinal MNs is a pathological hallmark in SOD1-related familial ALS (Furukawa, 2012). Moreover, these aggregates accumulate in vacuoles in the mitochondrial intermembrane space, leading to their dysfunction. Consequently, Adenosine Triphosphate (ATP) production and calcium buffering are deregulated and can increase the susceptibility of MNs to the altered calcium homeostasis associated with glutamate-mediated excitotoxicity (Ferraiuolo *et al.*, 2011). Thus, we next investigated their release by MNs from NSC-34 cell line in the presence or absence of healthy microglia.

Glutamate plays an important role in ALS, since it is responsible for the excitotoxicity event. In our results, we observed a reduction in glutamate release of NSC-34/hSOD1G93A after 4 DIV (p<0.05 *vs.* respective NSC-34/hSOD1wt) and 7 DIV, as we can see in table III.2 and figure III.4.A. After 4 DIV, the presence of microglia added at 0 DIV (prior to MN dysfunction) increased glutamate levels (although not statistically significant) but did not exert any effect when added at 2 DIV (Figure.III.4.A). However, microglia added at 2 DIV significantly increased the levels of glutamate release by mixed

microglia-NSC-34/hSOD1G93A cultures after 7 DIV (p<0.01 *vs.* respective NSC-34/hSOD1G93A and p<0.01 *vs.* respective 4 DIV condition).

Another player in ALS-mediated neurotoxicity is nitric oxide (NO), which contributes for oxidative stress and neuroinflammation. Indeed, in our model, we observed an increase of NO levels in NSC-34/hSOD1G93A cells (Table III.2 and FigureIII.4.B) either after 4 or 7 DIV (p<0.05 *vs.* respective NSC-34/hSOD1wt after 4 or 7 DIV). The presence of microglia added at 0 DIV significantly increased NO release after 4 DIV, however the opposite effect was observed in mixed cultures after 7 DIV (p<0.05 *vs.* respective NSC-34/hSOD1G93A w/o microglia).

An increase in extracellular ATP content, resulting damaged neurons was also considered an important marker for neurodegeneration (Glass *et al.*, 2010). These higher extracellular ATP levels will in turn activate microglia through purinergic receptors (D'Ambrosi *et al.*, 2009). Interestingly, here we observed an increase of extracellular ATP levels in NSC-34/hSOD1G93A cells (Table III.2 and FigureIII.4.C) after 4 DIV but mostly after 7 DIV (p<0.01 *vs.* respective NSC-34/hSOD1wt). The presence of microglia added at 2 DIV significantly reduced ATP release in mixed microglia-NSC-34/hSOD1G93A cultures after 7 DIV (p<0.01 *vs.* respective NSC-34/hSOD1G93A w/o microglia).

Table III.2 – Metabolic function are altered in differentiated NSC-34/hSOD1G93A cells, evidenced by increased production/release of glutamate, Nitric Oxide (NO) and Adenosine Triphosphate (ATP), which are modulated by the presence of microglia in mixed cultures.

			w/o N9	+ N9 at 0 DIV	+ N9 at 2 DIV
Glutamate	**4 DIV**	**NSC-34/hSOD1wt**	474 ± 89	442 ± 86	399 ± 148
		NSC-34/hSOD1G93A	310 ± 55	285 ± 74	324 ± 61
	7 DIV	**NSC-34/hSOD1wt**	539 ± 85	373 ± 74	251 ± 89
		NSC-34/hSOD1G93A	409 ± 51	326 ± 71	229 ± 12
NO	**4 DIV**	**NSC-34/hSOD1wt**	1.18 ± 0.11	1.09 ± 0.19	1.08 ± 0.13
		NSC-34/hSOD1G93A	1.82 ± 0.20*	1.95 ± 0.21	2.21 ± 0.25
	7 DIV	**NSC-34/hSOD1wt**	0.88 ± 0.05	1.10 ± 0.09	1.74 ± 0.34
		NSC-34/hSOD1G93A	2.50 ± 0.31**	1.98 ± 0.33	3.94 ± 0.12 $^{\$\$}$
ATP	**4 DIV**	**NSC-34/hSOD1wt**	25.51 ± 0.95	24.92 ± 1.07	25.49 ± 1.26
		NSC-34/hSOD1G93A	28.26 ± 1.52	26.25 ± 1.12	27.45 ± 2.09
	7 DIV	**NSC-34/hSOD1wt**	28.59 ± 0.80	28.24 ± 1.54	28.47 ± 1.38
		NSC-34/hSOD1G93A	37.15 ± 3.38*$^{\$}$	32.33 ± 2.83	28.90 ± 1.61$^{\#}$

Cells were cultured as indicated in Methods. After differentiation, extracellular media collected at 4 DIV and 7 DIV was assessed for glutamate release by L -glutamic acid kit and the absorbance was measured in a microplate reader; NO production by Griess reaction and absorbance was measured in the microplate reader; ATP release by an enzymatic assay and fluorescence intensity was quantified using a fluorimetric assay. Results are expressed in µM and represent the absolute values found in Figure III.4. Results are mean ± SEM in at least four independent experiments performed in duplicate. **p<0.01 and *p<0.05 *vs.* respective NSC-34/hSOD1wt condition; $^{\#}$p<0.05 *vs.* respective NSC-34/hSOD1G93A w/o microglia; $^{\$\$}$p<0.01 and $^{\$}$p<0.05 *vs.* respective 4 DIV condition.

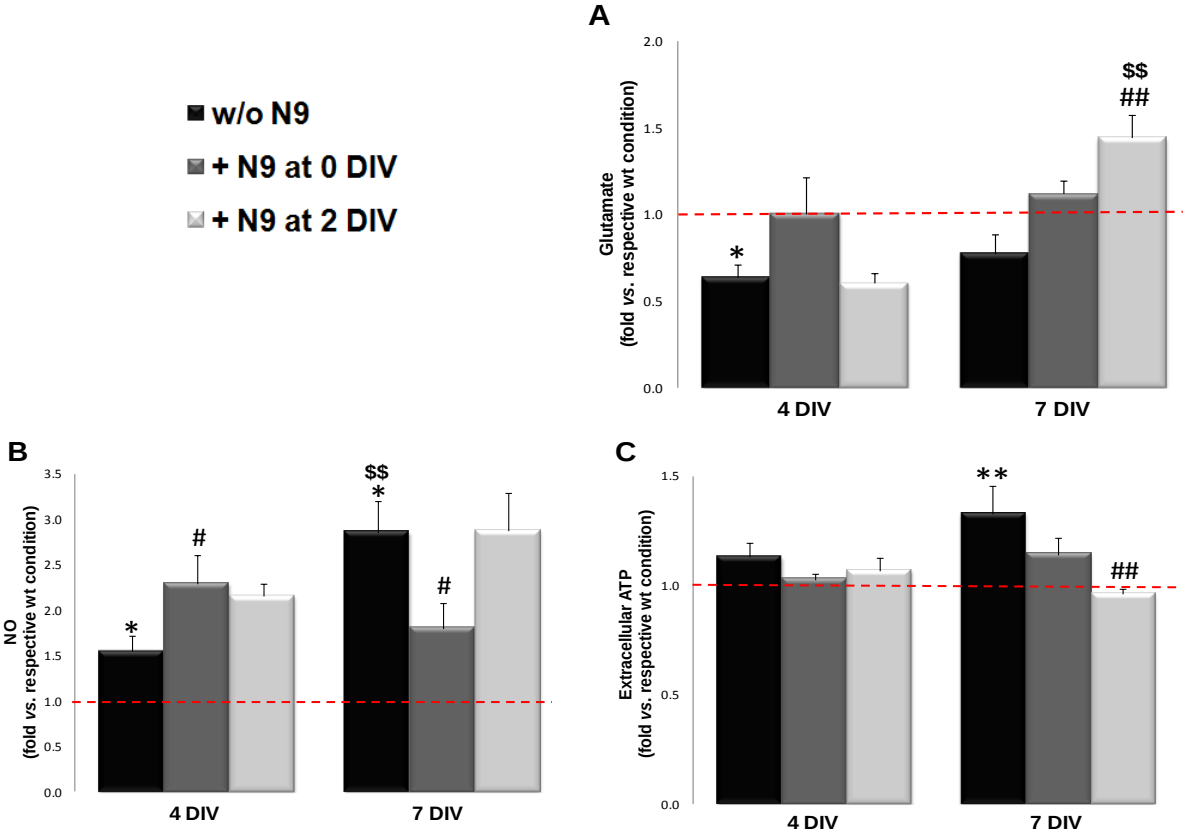

Figure III. 4 – Differentiated NSC-34/hSOD1G93A cells have altered metabolic function, evidenced by increased production/release of glutamate, Nitric Oxide (NO) and Adenosine Triphosphate (ATP), which are modulated by the presence of microglia in mixed cultures. Cells were cultured as indicated in Methods. After differentiation, extracellular media collected at 4 DIV and 7 DIV was assessed for (A) glutamate release by L-glutamic acid kit and the absorbance was measured in a microplate reader; (B) NO production by Griess reaction and absorbance was measured in the microplate reader; (C) ATP release by an enzymatic assay and fluorescence intensity was quantified using a fluorimetric assay. All the results are mean (± SEM) and expressed as fold *vs.* respective NSC-34/hSOD1wt condition from at least four independent experiments performed in duplicate. Corresponding absolute values are presented in Table III.2. **p<0.01 and *p<0.05 *vs.* respective NSC-34/hSOD1wt condition; $^{\#\#}$p<0.01 and $^{\#}$p<0.05 *vs.* respective NSC-34/hSOD1G93A w/o microglia; $^{\$\$}$p<0.01 *vs.* respective 4 DIV condition. Dotted line: respective wt (control).

1.4 Microglia prevent and restore efflux of neuroinflammatory associated markers in NSC-34/hSOD1 G93A cells

Neuroinflammation is a pathological hallmark of ALS and include the activation and proliferation of microglia. Although often considered a consequence of neuronal injury and degeneration, the neuroinflammatory response can have protective or deleterious effects on neuronal survival (Lewis *et al.*, 2012). Matrix metalloproteinases (MMPs) are important players in neuroinflammation and recent studies have associated their actions to neurodegenerative disorders. They regulate extracellular matrix structure and can affect cell signaling initiated by growth factors or death receptors (Rosenberg, 2009). In our model of mixed cultures, we were able to detect bands with 72 kDa and 92 kDa (Figure III.5A), corresponding to MMP-2 and MMP-9, respectively (Yong *et al.*, 2001). We observed an increase in MMP-2 in NSC-34/hSOD1G93A after 7 DIV (Figure III.5B) and in MMP-9 (Figure III.5C) after 4DIV (p<0.05 *vs.* respective NSC-34/hSOD1wt) and 7 DIV. Furthermore, we saw differences in activation of MMPs when microglia is present. Indeed, microglia added at 2 DIV reduced MMP-2 levels in mixed cultures of microglia with NSC-34/hSOD1G93A after 4 DIV (Table III.3 and Figure III.5.B). These reduction was very significant and significant after 7 DIV for microglia added either at 0

or 2 DIV, respectively ($p<0.01$ and $p<0.05$ *vs.* respective NSC-34/hSOD1wt). In respect to MMP-9, after 4 DIV, the addition of microglia significantly reduced MMP-9 levels ($p<0.01$ *vs.* respective NSC-34/hSOD1wt), while after 7 DIV, only microglia added at 2 DIV was able to reduce them.

Table III.3 – Activation of matrix metalloproteinases-2 and -9 (MMP-2 and MMP-9) is elevated in NSC-34/hSOD1G93A cells after differentiation, which are modulated by the presence of microglia in mixed cultures.

			w/o N9	+ N9 at 0 DIV	+ N9 at 2 DIV
MMP-2	4 DIV	NSC-34/hSOD1wt	1.00 ± 0.13	0.74 ± 0.16	1.36 ± 0.24
		NSC-34/hSOD1G93A	0.95 ± 0.17	0.84 ± 0.15	0.46 ± 0.05
	7 DIV	NSC-34/hSOD1wt	1.00 ± 0.19	0.61 ± 0.19	0.50 ± 0.11$^{\$}$
		NSC-34/hSOD1G93A	0.98 ± 0.25	0.50 ± 0.12	0.37 ± 0.06
MMP-9	4 DIV	NSC-34/hSOD1wt	1.04 ± 0.06	0.98 ± 0.09	0.99 ± 0.10
		NSC-34/hSOD1G93A	0.90 ± 0.17	0.77 ± 0.11	0.45 ± 0.19
	7 DIV	NSC-34/hSOD1wt	0.93 ± 0.09	1.76 ± 0.22$^{\$}$	1.33 ± 0.13
		NSC-34/hSOD1G93A	1.23 ± 0.23	1.48 ± 0.23$^{\$}$	0.68 ± 0.19

Cells were cultured as indicated in Methods. After differentiation, extracellular media collected at 4 DIV and 7 DIV was assessed for MMPs activity by gelatin zymography assay. The intensity of the bands was quantified using computerized image analysis (Image Lab) for MMP-2 and -9. Results are expressed as fold *vs.* NSC-34/hSOD1wt and represent the absolute values found in Figure III.5. Results are mean ± SEM from at least two independent experiments, performed in duplicate. $^{\$}p<0.01$ *vs.* respective 4 DIV condition.

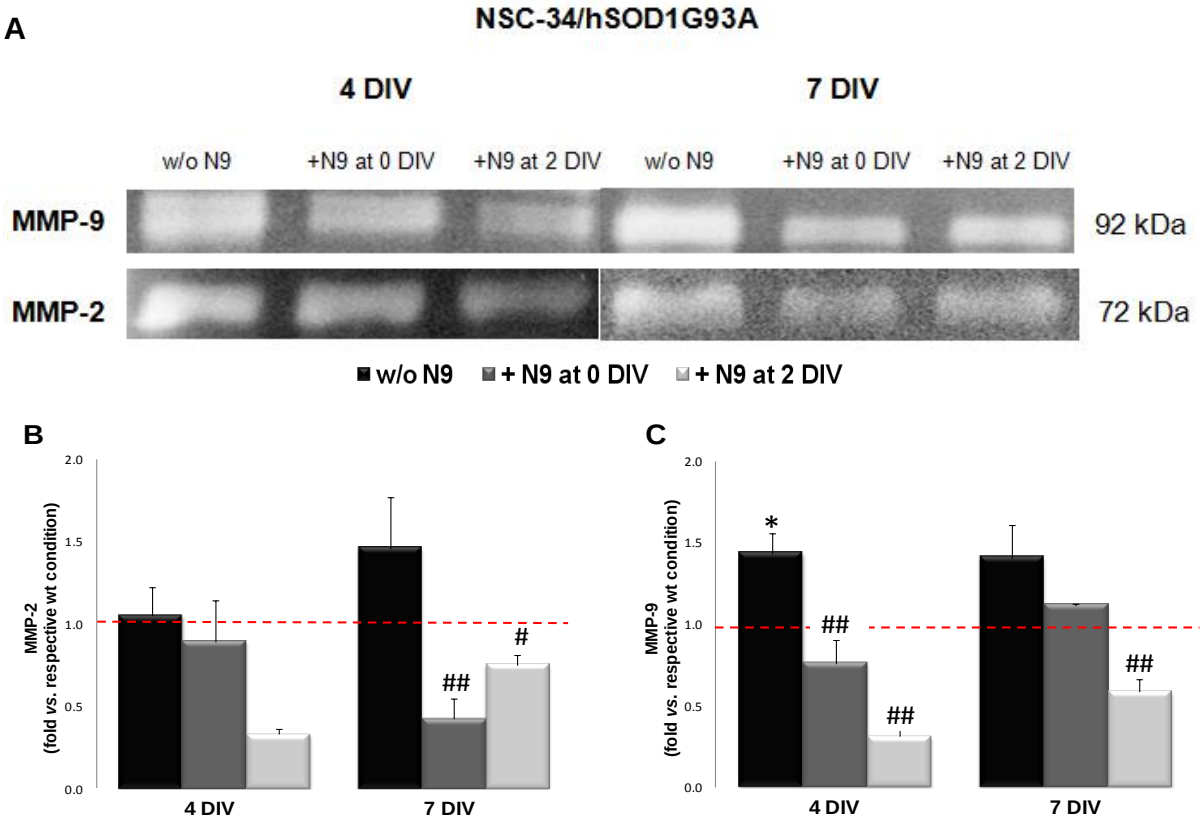

Figure III. 5 Matrix metalloproteinases-2 and -9 (MMP-2 and MMP-9) activation is elevated in NSC-34/hSOD1G93A cells after differentiation, which are modulated by the presence of microglia in mixed cultures. Cells were cultured as indicated in Methods. After differentiation, extracellular media collected at 4 DIV and 7 DIV was assessed for MMPs activity by gelatin zymography assay. Representative results from one experiment are shown (A). Similar results were obtained in at least two independent experiments. The intensity of the bands was quantified using computerized image analysis (Image Lab) for MMP-2 (B) or MMP-9 (C), and expressed as mean ± SEM fold change compared with respective NSC-34/hSOD1wt condition. Corresponding absolute values are presented in Table III.3. *p<0.05 *vs.* respective NSC-34/hSOD1wt condition; $^{\#\#}$p<0.01 and $^{\#}$p<0.05 *vs.* respective NSC-34/hSOD1G93A w/o microglia. Dotted line: respective wt (control).

1.5 Microglia increase High Mobility Group Box 1 (HMGB1) levels in NSC-34/hSOD1G93A cells after 4 DIV and reduce them after 7 DIV

Finally, a particular attention was given to High Mobility Group Box 1 (HMGB1), a ubiquitous nuclear protein released by necrotic cells or stimulated monocytes, which participate in activation of innate immune response, including chemotaxis and cytokine release (Yang *et al.*, 2013). In fact, evidence suggests a contribution of HMGB1 in many inflammatory diseases (Kim *et al.*, 2006), which may be a key point to consider in ALS pathophysiology. To study the effects of microglia in production of HMGB1 by NSC-34 cells, we performed western blot assay using mouse anti-HMGB1 antibody (1:500). After 4 DIV and 7 DIV, we observed a marked increase of HMGB1 levels (although not yet statistically significant) in NSC-34/hSOD1G93A, compared to respective control condition (Table III.4 and Figure III.6.B). Moreover, the addition of microglia at 0 DIV slightly increased HMGB1 levels after 4 DIV. Interestingly, after 7 DIV, there was a reduction of HMGB1 levels when microglia was added at 0 and 2 DIV, suggesting that this protein was released to the extracellular media.

Table III. 4 – Elevation of High-mobility-group-box-protein-1 (HMGB1) is observed in NSC-34/hSOD1G93A cells after differentiation, which is differently modulated by the presence of microglia in mixed cultures.

			w/o N9	+ N9 at 0 DIV	+ N9 at 2 DIV
HMGB1	4 DIV	NSC-34/hSOD1wt	1.00 ± 0.72	0.56 ± 0.10	0.56 ± 0.16
		NSC-34/hSOD1G93A	1.24 ± 0.30	1.05 ± 0.09	1.10 ± 0.24
	7 DIV	NSC-34/hSOD1wt	1.00 ± 0.80	1.07 ± 0.33	0.68 ± 0.18
		NSC-34/hSOD1G93A	1.89 ± 0.60	3.14 ± 0.54	0.54 ± 0.12

Cells were cultured as indicated in Methods. Total cell lysates were collected after 4 DIV and 7 DIV and both analyzed by western blot with antibody specific for HMGB1. Results were obtained in at least two independent experiments. The intensity of the bands was quantified by using computerized image analysis (Image Lab) and standardized with respect to β-actin protein. Results are expressed as fold *vs.* NSC-34/hSOD1wt and represent the absolute values found in Figure III.6.

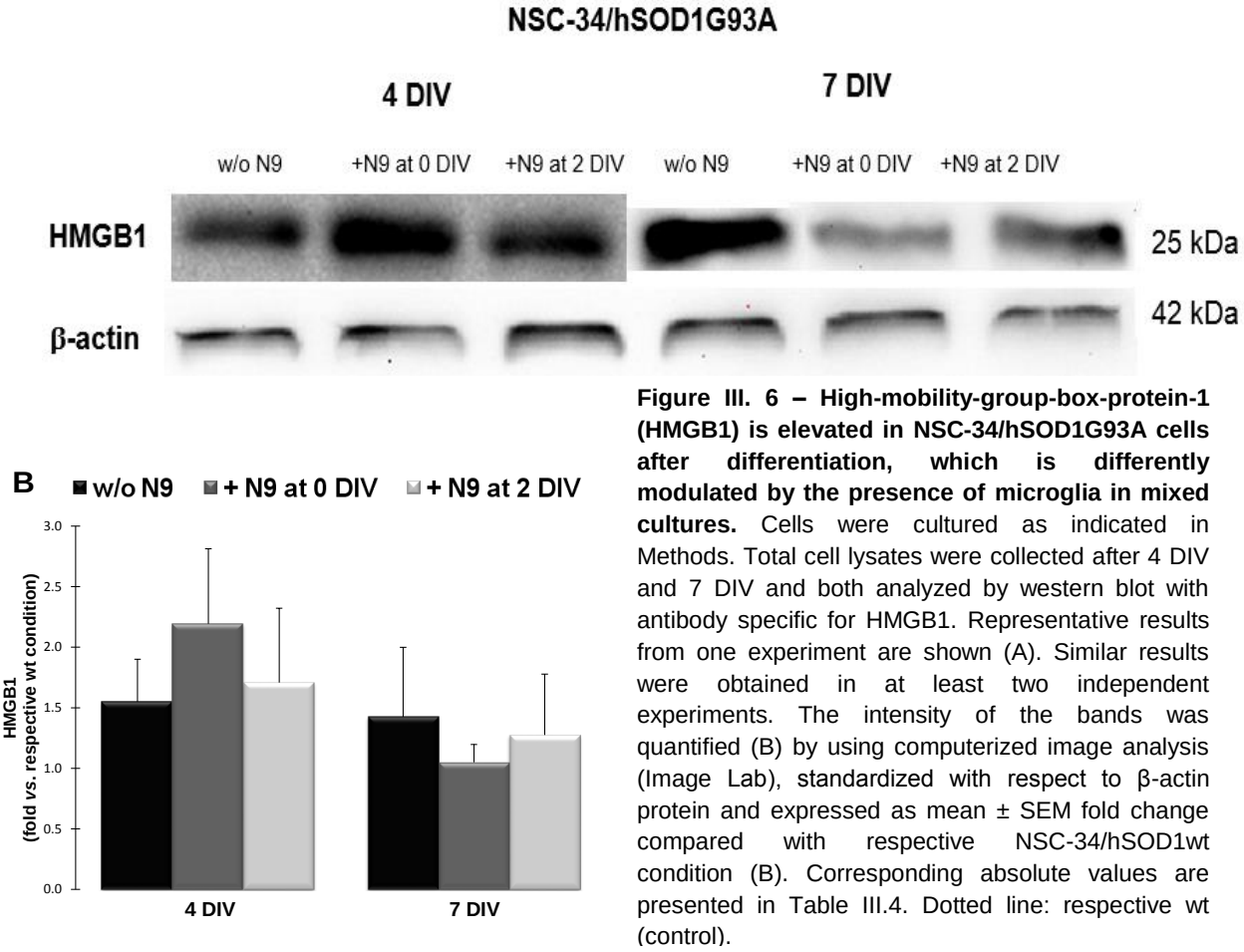

Figure III. 6 – High-mobility-group-box-protein-1 (HMGB1) is elevated in NSC-34/hSOD1G93A cells after differentiation, which is differently modulated by the presence of microglia in mixed cultures. Cells were cultured as indicated in Methods. Total cell lysates were collected after 4 DIV and 7 DIV and both analyzed by western blot with antibody specific for HMGB1. Representative results from one experiment are shown (A). Similar results were obtained in at least two independent experiments. The intensity of the bands was quantified (B) by using computerized image analysis (Image Lab), standardized with respect to β-actin protein and expressed as mean ± SEM fold change compared with respective NSC-34/hSOD1wt condition (B). Corresponding absolute values are presented in Table III.4. Dotted line: respective wt (control).

Together, these results suggest that microglia differently modulate signaling mechanisms that are involved in MN degeneration along ALS progression, which depends on the time in culture and also on the level of degeneration they face when in culture with NSC-34/hSOD1G93A.

2. Establishing organotypic slice culture as a model for study ALS

Organotypic cultures are a model that is biochemically and physiologically more similar to the *in vivo* tissue since it preserves interneuronal connections and key processes. Moreover, it is a powerful model to screen the efficacy of potential therapies (Su *et al.*, 2011). In order to approximate the *in vivo* conditions that lead to ALS development, we developed an organotypic culture composed by slices of lumbar SC from transgenic mice carrying a human mutant-SOD1 (TgSOD1-G93A). Organotypic cultures from SC of 7-days SJL WT mice were used as controls.

2.1 Implementation and characterization of SC organotypic cultures

Here, we focused our attention on lumbar segments from SC, since it is considered one of the most affected neural tissues in ALS (Chen *et al.*, 2010). We were able to implemment the proposed model and after 11 days in culture we observed an intact and functional structure of the lumbar tissue where it was possible to identify posterior and anterior horn and central canal, as as schematically represented in Figure III.7 after histological analysis.

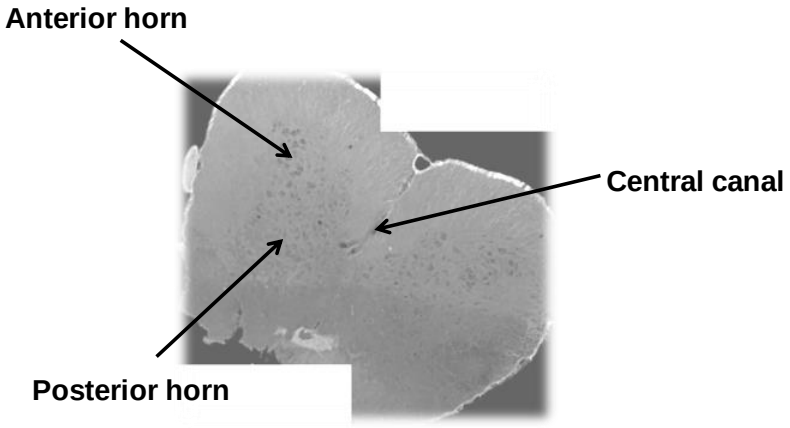

Figure III. 7- Schematic representation of a transversal section of the lumbar spinal cord. Original magnification 100x.

After the implementation of the organotypic culture model, we analyzed the parameters found to be altered on the section 1 from Results. At this moment, we have focused on SOD1 accumulation, NO and extracellular ATP levels. In addition, we also quantified the extracellular levels of NO and ATP upon an inflammatory response triggered by incubation with lipopolysaccharide (LPS). The expression of toll-like receptor 4 (TLR4), a receptor responsible for LPS recognition and the triggering of production of neurotoxic pro-inflammatory mediators and NO, was also quantified.

2.2 Mouse and hSOD1 accumulation is highly increased in transgenic mouse

The analysis of SOD1 accumulation was performed by western blot analysis of homogenates, using rabbit anti-SOD1 antibody (1:200). Preliminary results pointed a great increase, although not yet significant, of SOD1 accumulation in TgSOD1-G93A mice (Figure III.8).

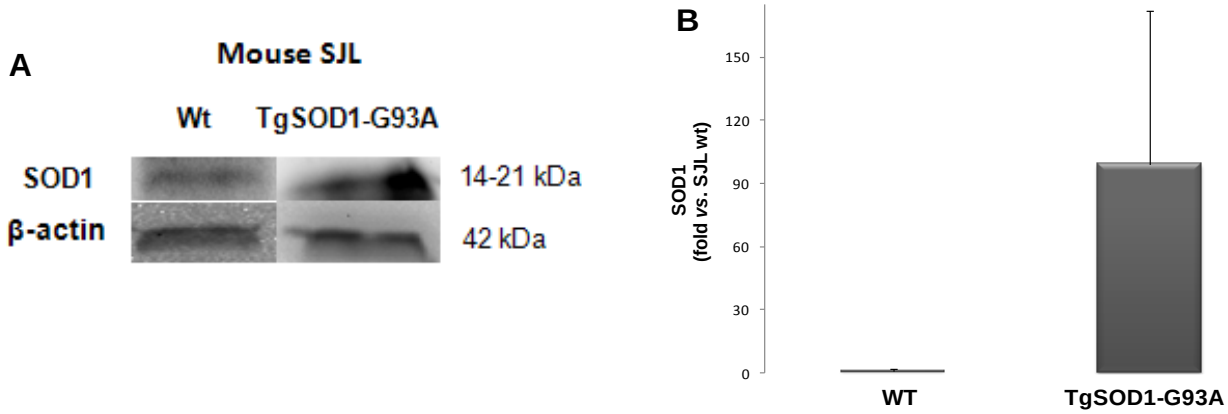

Figure III. 8 – Superoxide dismutase 1 (SOD1) levels are increased in TgSOD1-G93A mice. Lumbar spinal cord slices were cultured as indicated in Methods. Total homogenate was analyzed by western blot with specific antibody for SOD1. Representative results from one experiment are shown (A). The intensity of the bands was quantified (B) using computerized image analysis (Image Lab), standardized with respect to β-actin protein and expressed as mean ± SEM from three independent experiments.

2.3 LPS does not have a significant effect on NO release but is able to reduce extracellular ATP in TgSOD1-G93A mice

LPS exists in the outer membrane of Gram-negative bacteria and act as an endotoxin that elicit strong immune responses in animals. As depicted in Figure III.9.A no differences in NO levels were observed between wt and TgSOD1-G93A mice, either in the presence or absence of LPS. Interestingly, preliminary results suggested that TgSOD1-G93A mice have a slight reduction of extracellular ATP, which is enhanced after LPS exposure (Figure III.9B).

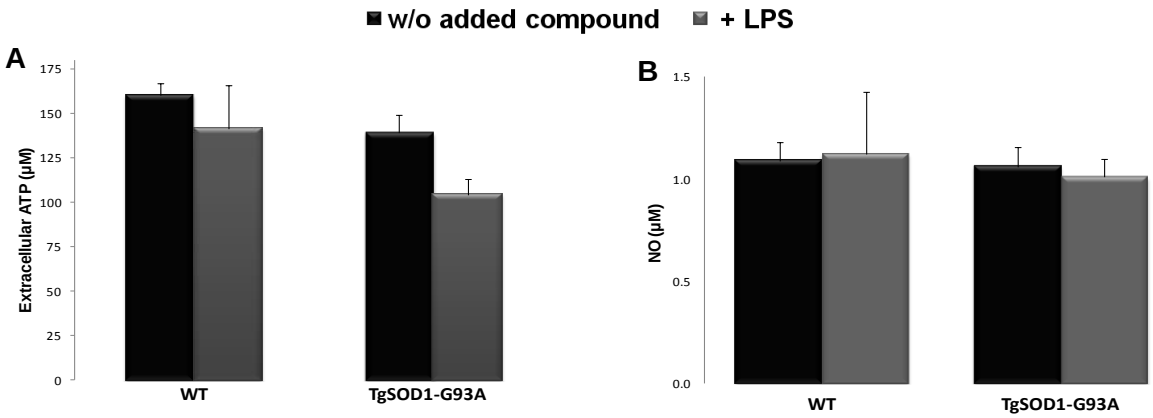

Figure III. 9 - Lipopolysaccharide (LPS) does not have any significant effect in Nitric Oxide (NO) levels but reduce extracellular levels of Adenosine Triphosphate (ATP) in TgSOD1-G93A mice. Lumbar spinal cord slices were cultured for 10 days and incubated with 1μg/ml LPS for 24 h, as indicated in Methods. After incubation, **e**xtracellular media was assessed for (A) NO production/release by Griess reaction and absorbance was measured in the microplate reader or (B) ATP release by an enzymatic assay and fluorescence intensity was quantified using a fluorimeter. Results are mean ± SEM from at least two independent experiments.

2.4 LPS reduce levels of TLR4 receptor in wt and TgSOD1-G93A mice

The analysis of levels of TLR4 was performed by western blot analysis of homogenates, using rabbit anti-TLR4 antibody (1:100). As indicated in figure III.10.A, we were able to detect one band with 95 kDa in either wt or TgSOD1-G93A homogenate samples, confirming the presence of this receptor (Yao *et al.*, 2013). As shown in figure III.10.B, incubation with LPS seemed to reduce the levels of this receptor in namely in the TgSOD1-G93A,mice, suggesting loss of microglia response in terms of its activation mediated through these receptor (Yao et al. 2013). However, these are still preliminary results, which require further confirmation.

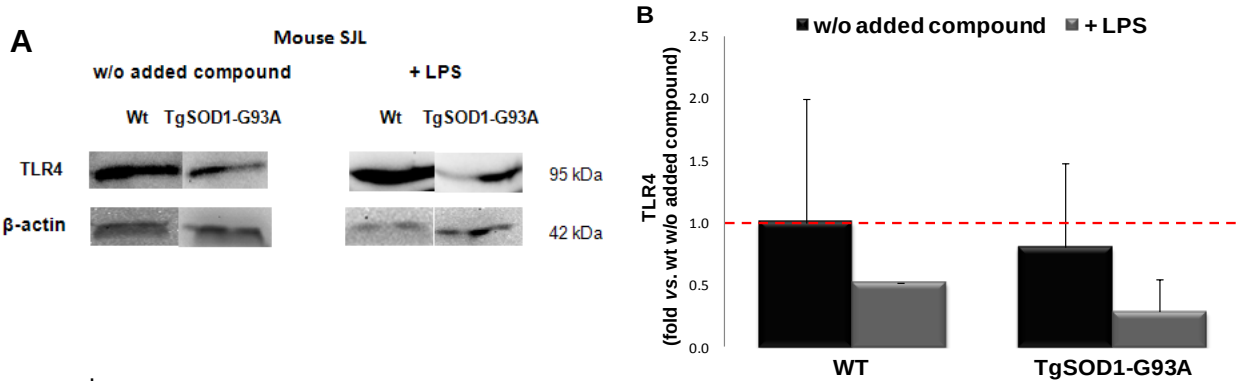

Figure III. 10 – Incubation with lipopolysaccharide (LPS) is suggested to reduce the levels of Toll-like receptor 4 (TLR4), especially in cultures from TgSOD1-G93A mice. Lumbar spinal cord slices were cultured for 10 days and incubated with 1µg/ml LPS for 24 h, as indicated in Methods. After incubation, total homogenate was analyzed by western blot with antibody specific for TLR4. Representative results from one experiment are shown (A). The intensity of the bands was quantified (B) using computerized image analysis (Image Lab), standardized with respect to β-actin protein and expressed as mean ± SEM from at least two independent experiments. Dotted line: respective wt (control).

IV. DISCUSSION

ALS is epidemiologically classified into two forms: sporadic (90%-95%) and familial (5%-10%). Among the familial cases, approximately 20% are caused by dominantly inherited mutations in the Cu/Zn superoxide dismutase-1 (SOD1) protein (Musaro, 2010). Consequently, many experimental *in vitro* and *in vivo* models including mutations in this enzyme have been developed to improve our understanding of the disease and have allowed the testing of possible therapeutic strategies. In our studies, we have used mixed cultures of Neuroblastoma Spinal Cord 34 (NSC-34) cell line with N9 cell line. NSC-34 cells present characteristics of motoneuron (MN), such as acetylcholine synthesis, storage and release, generation of potential generation, expression of neurofilament proteins and association with neuromuscular synapse-specific basal lamina glycoproteins (Tovar *et al.*, 2009). They respond to stimuli affecting voltage-gated ion channels, organization of cytoskeleton and axonal transport, very similar with primary MN, although they cannot reproduce synaptic connections when are at an undifferentiated stage (Durham *et al.*, 1993). In our work, we have used this cell line transfected with human SOD1G93A (NSC-34/hSOD1G93A) protein, where there is a change in the aminoacid 93 from one glycine to one alanine. These cells are described to produce many features of MN degeneration in ALS, like Golgi apparatus fragmentation (Gomes *et al.*, 2008) and mitochondrial deregulation (Raimondi *et al.*, 2006). Recently, in our group it was demonstrated that NSC-34/hSOD1G93A cells present accumulation of SOD1 after 4 days of differentiation (DIV), together with cell dysfunction (Vaz *et al*., 2013), which may represent the progression of MN degeneration in familiar ALS. Thus, here we considered two different time points after NSC-34 cell differentiation: (i) 4 DIV – during SOD1 accumulation (symptomatic) and (ii) 7 DIV – after SOD1 accumulation and MN cell death.

The increased number of non-neuronal cells from CNS considered to be affected in the rodent model as well as in ALS autopsies has long been considered to be a secondary response to MN degeneration (Dewil *et al.*, 2007). In addition, some studies pointed that activated microglia, considered the primary immune cells of the CNS, may contribute to MN protection or instead to MN injury and consequent cell death (Boillee *et al.*, 2006). In our model of mixed cultures we intended to see if healthy microglia was able to prevent, promote or restore MN neurodegeneration, and also to explore long term changes in microglia-MNs cross-talk. First, we implemented and characterized the mixed culture in our lab with these two types of cells, and we confirmed that N9 cells were able to maintain their capacities when moved from their proliferative medium to NSC-34 differentiation medium. In fact, Guo and colleagues (2013) have maintained N9 cells in a similar medium, also with the basis of Dulbecco's Modified Eagle Medium/F12. Differentiation of NSC-34 cells also transformed these cells into a MN-like-morphology, has been previously described (Cho *et al.*, 2011; Matusica *et al.*, 2008). They appeared with long ramifications and increased number of neurites until 4 and 7 DIV.

Stable transfection of SOD1 was confirmed by Western Blot analysis, with two bands corresponding to human and mouse forms of SOD1, with 21 and 14 kDa, respectively (Kirby *et al.*, 2005). Many SOD1 aggregates were found in fALS cases, cell culture models (Ferraiuolo *et al.*, 2011) as well as in brain and in spinal cord (SC) of the transgenic (TgSOD1-G93A) mice (Redler and Dokholyan, 2012). In our results, human SOD1 (hSOD1) accumulation was significantly increased in NSC-34/hSOD1G93A after 7 DIV, when compared with respective controls (NSC-34/hSOD1wt). This increase was much more evident than the one also found for mouse SOD1 accumulation, which can be explained by the greater susceptibility of oxidation-induced aggregation of fALS-associated SOD1 mutations harbored by transgenic mice (Rakhit *et al.*, 2002). It was also observed that microglia reduced SOD1 accumulation in NSC-34/hSOD1G93A cells, possibly because microglia is able to phagocyte SOD1 aggregates. In fact, microglia has the capacity to phagocyte cellular debris, including protein aggregates, as it was demonstrated by Chung and colleagues (1999), where microglia from mixed glial cultures could uptake, degrade, and release smaller aggregates of fibrillar or even soluble amyloide-beta peptide, present in Alzheimer's disease. Moreover, in studies with chimeric mice, it was demonstrated that non-neuronal cells that do not express mutant SOD1 can ameliorate MN degeneration and death of mutant SOD1 cells. (Clement *et al.*, 2003). Curiously, in our model, the reduction of SOD1 levels was even more evident when MNs and microglia were in contact for longer time. Since SOD1 accumulation in mutated MNs increases along ALS progression (Jaarsma *et al.*, 2008), we may speculate that healthy microglia, in some way, is trying to reduce such accumulation. It would be interesting to determine which signaling mechanisms may be mediated by SOD1 aggregates, such as phagocytic microglia ability but also signaling pathways involved in neuroinflammation.

SOD1 aggregates accumulate in vacuoles in the mitochondrial intermembrane space, leading to their dysfunction. Consequently, Adenosine Triphosphate (ATP) production and calcium buffering may become deregulated, which may increase the susceptibility of MNs to the altered calcium homeostasis associated with glutamate-mediated excitotoxicity in ALS (Pasinelli and Brown, 2006). In our model, we observed a reduction in glutamate release by differentiated NSC-34/hSOD1G93A cells in comparison with NSC-34/hSOD1wt, contradicting previous studies where high levels of glutamate in cerebrospinal fluid (CSF) of some patients with ALS were observed (Rothstein *et al.*, 1995). In addition, hyperexcitability of the motor system at early stages of ALS is described, through electrophysiological studies in humans (Vucic and Kiernan, 2006). Our result may be explained by the mitochondrial dysfunction that may have resulted from the reduction of the glucose-derived pyruvate flow through the tricarboxylic acid cycle and decreased glutathione content, which will alter glutamate metabolism (D'Alessandro *et al.*, 2011). In addition, the presence of microglia added at 2 DIV significantly increased the levels of glutamate released in mixed microglia-neuronal cultures, especially when cells are in contact for longer time (7 DIV), suggesting that microglia is activated upon contact with NSC-34/hSOD1G93A. In fact, excitotoxicity by glutamate released from activation of microglia is considered a major cause of axonal degeneration (Yawata *et al.*, 2008).

Regarding ATP, we observed higher extracellular ATP levels in NSC-34/hSOD1G93A, which may also promote activation microglia through purinergic receptors (D'Ambrosi *et al.*, 2009), probably

as an attempt to resolve the extension of the lesion. However, this is a small increase, probably derived from metabolic failure at the level of mitochondria in NSC-34/hSOD1G93A (D'Alessandro *et al.*, 2011). Moreover, our results show that interaction between microglia and NSC-34/hSOD1G93A for longer time (7 DIV) also led to a reduction of extracellular ATP levels. Indeed, ATP acts as a neuron-to-microglia alarm signal, through cell surface P2 receptors widely distributed throughout the CNS. In ALS patients (Yiangou *et al.*, 2006), as well as SOD1 G93A animals (Casanovas *et al.*, 2008), an increased immunoreactivity for P2X was found in SC microglia. This probably occurs because ATP binds to P2X receptors present in microglia, reducing their presence in extracellular media.

Neuroinflammation is a pathological hallmark of ALS, which includes the activation and proliferation of microglia. To see the influence of healthy microglia in inflammatory response, we investigated the release of nitrites/nitric oxide (NO) and metalloproteinases-9 and -2 (MMP-9 and -2) by MNs. We observed increased NO levels released by NSC-34/hSOD1G93A ($p<0.05$ *vs.* NSC-34/hSOD1wt). Conversely, studies of CSF and human postmortem CNS tissue from ALS patients showed biochemical changes that represent the effect of free radical damage or abnormal free radical metabolism (Ferrante *et al.*, 1997; Shaw *et al.*, 1995; Smith *et al.*, 1998; Tohgi *et al.*, 1999). In addition, mutated SOD1 has an imperfect folding, which disables their normal function as an antioxidant, instead it will favor an increase of superoxide anion production, which reacts with NO to produce peroxynitrite, ultimately causing tyrosine nitration (Barber and Shaw, 2010). In fact, a product of tyrosine nitration is 3-nitrotyrosine, which was widely detected in the MNs of sporadic cases with ALS (Abe *et al.*, 1997). When microglia were present with NSC-34/hSOD1G93A during 2 and 4 DIV, extracellular NO levels increased. Again, this suggests a microglia activation pattern, with consequent production and release of NO through an up-regulation of inducible nitric oxide synthase, such as the one found in microglia cocultured neurons after exposure to lipopolysaccharide (LPS) (Zhao *et al.*, 2004). When NSC-34/hSOD1G93A and microglia are maintained together until 7 DIV, it occurs a significant reduction of NO levels ($p<0.05$), suggesting a switch of microglia activation pattern.

In respect of metalloproteinases (MMPs), activity of MMP-2 increased in NSC-34/hSOD1G93A after 7 DIV and in MMP-9 after 4DIV ($p<0.05$ *vs*. respective NSC-34/hSOD1wt) and 7 DIV, confirming results performed by Fang and colleagues (2010), where MMP-2 and -9 activation was increased in SC of transgenic SOD1G93A mice. Soon and colleagues (2010) also showed that MMP-2 and -9 activity was significantly elevated in serum of SOD1G93A mice at the early symptomatic stage of ALS, decreasing thereafter during disease progression till end stages. In our model, when microglia were placed in contact with NSC-34/hSOD1G93A cells, a reduction of MMP-2 and -9 activity occurred. Thus, we proposed the intervention of inhibitors of matrix metalloproteinases (TIMPs), family of four related proteins capable of binding and inhibit MMPs activity, through tight non-covalent complexes. In CSF samples from ALS-patients, high levels of TIMP-1 and no MMP-9 activity were detected (Lorenzl *et al.*, 2003). A recent study, also performed in CSF samples from ALS-patients, showed an increase of TIMP-1 level and a decrease of MMP-9 activity (Niebroj-Dobosz *et al*., 2007). TIMPs play a critical role in maintaining tissue proteolysis homeostasis and microglia play a fundamental role in mediating tissue breakdown and repair. Therefore, Welser-Alves and colleagues (2011) found that microglia play a central role in regulating glial cell expression of TIMPs, using primary mixed glial cultures, composed

by microglia and astrocytes obtained from 0-2 day old C57Bl/6 mouse pups. So, we may suggest that in our model, microglia is producing TIMP-1, in order to inhibit MMPs activation in NSC-34/hSOD1G93A.

High Mobility Group Box 1 (HMGB1) is a non-histone structural protein located within the nucleus. Passive release of HMGB1 from the nucleus represents a signal of cell injury, which triggers the release of pro-inflammatory factors and thus activates cellular processes that stimulate tissue regeneration (Hwang *et al.*, 2013). It is involved in many inflammatory diseases, which may be a key point to consider in ALS pathophysiology. Although no different expression pattern was observed by Lo Coco and colleagues (2007) in SC tissue of SOD1-G93A transgenic mice at presymptomatic stage as compared with controls, the levels of HMGB1 and other proteins involved in HMGB1 function, such as toll-like receptors-2 and -4 (TLR-2 and TLR-4, respectively), as well as serine/threonine kinase 30 were elevated in SC tissues of patients with ALS (Casula *et al.*, 2011). We found an increase in HMGB1 content in NSC-34/hSOD1G93A, either for 4 or 7 DIV, for the first time in a model of mixed cell line culture applied for the study of ALS. Moreover, we demonstrated that the addition of microglia at 0 DIV enhanced intracellular HMGB1 levels in culture until 4 DIV. In fact it is described an intensely widely distributed HMGB1 content in the nucleus of reactive glial cells from SC tissue of SOD1-G93A transgenic mice, at the symptomatic and late stages of the disease (Lo Coco *et al.*, 2007). By contrast, we observed a reduction of HMGB1 levels when microglia was added before and after MNs have acquired signals of injury (7 DIV). A possible reason may be the advanced stage MNs injury in this case, which led to the release of HMGB1. In fact, Lo Coco and colleagues (2007) observed a reduction of HMGB1 immunoreactivity in degenerating neurons, with the progression of the disease, which reflects an extracellular release of this protein. Moreover, HMGB1 can be used to monitor ALS disease progression, as Hwang and colleagues (2013) demonstrated through the study of the serum autoantibody against HMGB1 (HMGB1 autoAb), whereupon the level of HMGB1 autoAb significantly increased in patients with ALS.

In the second part of this thesis, we aimed to set-up an organotypical culture model from the lumbar SC, in order to study whether cellular cross-talk is impaired in mice carrying the human SOD1 G93A (TgSOD1-G93A). SC organotypic culture allows preserving an *in vivo* horizontal architecture, keeping MNs in situ, with all the other cells and connections in place. Moreover, the MNs in an organotypic culture system can survive for more than three months, being a good model to study the effect of long-term treatment with toxic substances on MNs (Li *et al.*, 2008). We have started by analyzing some of the parameters evaluated in mixed cultures, such as SOD1 accumulation, ATP and NO.

Again, we observed an increase of SOD1 accumulation in TgSOD1-G93A mice, compared with SJL-WT mice (control). Puttaparthi *et al* (2003) demonstrated that the formation of SOD1-positive aggregates in TgSOD1-G93A mouse SC tissue involves proteasome mediated proteolysis. In contrast, SOD1 aggregates do not form in SC slices from wt mice or transgenic mice overexpressing SOD1 wt, following proteasome inhibition. In sum, their results establish that adult mouse SC exhibits a relative deficiency in proteasome activity compared with non-CNS tissue that may help explain the propensity of SC to form SOD1-positive aggregates.

We then tested the ability of LPS in the triggering of inflammatory response in organotypic cultures from Tg-SOD1-G93A, through the evaluation of their receptor TLR-4, ATP and NO. Regarding NO, no differences were observed between cultures of wt and TgSOD1-G93A mice, either with or without incubation with LPS. Extracellular levels of ATP were reduced in TgSOD1-G93A mice, and the presence of LPS also promoted ATP reduction both in Wt and TgSOD1-G93A mice. It is described in TgSOD1-G93A mice that mitochondrial dysfunction leads to an energy impairment, which reflects in a reduction of ATP released by mutated cells (Mattiazzi *et al.*, 2002). LPS is a potent stimulator of M1 activation of microglia (Lewis *et al.*, 2012), which have increased expression of P2X receptors (Raouf *et al.*, 2007), which binds to ATP and contributes to reduce their presence in extracellular media. In addition, we cannot exclude a possible interaction between LPS and the other types of cells present in the slice, such as astrocytes, making them potential contributors for the reduction of extracellular ATP levels.

Finally, we detect the presence of TLR4, which were slightly reduced upon LPS exposure. We were expecting an increase of TLR4 activity, once the presence of LPS activate these receptors. However this 95 kDa band detected in our western blot belongs to a non-glycosilated form of TLR4, which does not translocate to the membrane surface and, consequently, is not responsible for LPS binding. We could not detect the 130 kDa band, responsible for the glycosilated form of TLR4, which translocates to the membrane surface and binds LPS (Ohnishi *et al.*, 2003).

Together, and as schematically represented in Figure IV.1, these results show the success of the implementation of mixed cultures as well as organotypical cultures to be used in the study of the mechanisms involved in ALS. Regarding the signalling between MNs-microglia, we observed a reduction of extracelllar ATP and MMPs and a possible release of HMGB1 from mutated MNs in the presence of microglia in cullture with MNs until 7 DIV. It is also suggested that microglia is activated and modulates a series of signalling mechanisms reponsable for a reduction of NO release, SOD1 accumulation and a consequent improvement of mitochondria function in NSC-34/hSOD1G93A cells. In addition, the results suggest that slices are in a high degeneration/aging state and the presence of LPS contributes to increase mitochondrial dysfunction in organotypical culture model. We then may propose that microglia has a positive impact to counteract neuroinflammation and prevent MNs from degeneration, reinforcing the use of therapeutic strategies that can restore both the healthy state of MNs and glial cells in ALS.

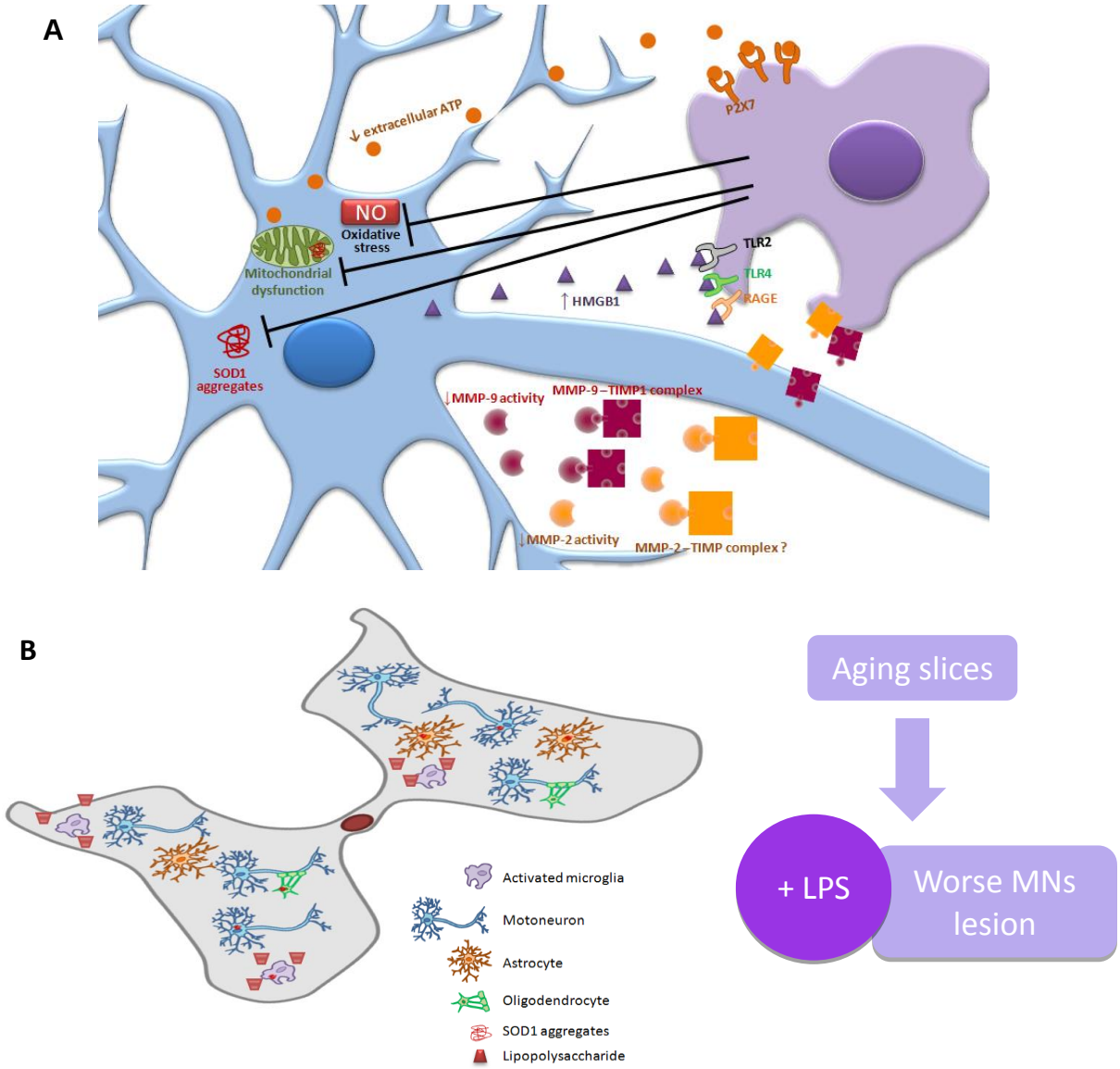

Figure IV.1 - Schematic representation of the major findings of this Master Thesis. **(A)** Mixed cultures evidence a reduction of extracelllar Adenosine Triphosphate (ATP) and metalloproteinases (MMPs) and a possible release of high-mobility group protein 1 (HMGB1) from NSC-34/hSOD1G93A cells. This can trigger microglia activation, thus promoting a set of signalling mechanisms, provoking a reduction of nitric oxide (NO) release, SOD1 accumulation and a consequent improvement of mitochondria function in NSC-34/hSOD1G93A cells. **(B)** Organotypic spinal cord cultures were composed by slices in a high degeneration/aging state characterized by reduced production of ATP, which is aggravated by the presence of lipopolysaccharide (LPS).

Future perspectives

In our study we found important clues about the role of healthy microglia in improving the degeneration of mutated motoneurons (MNs), through the use of an *in vitro* model of mixed cultures. However, further investigations are necessary to clarify the exact mechanism that microglia use to improve MNs degenerative state, in respect to the reduction of SOD1 accumulation, metalloproteinases (MMPs) activation, extracellular NO and ATP, as well as increase of extracellular glutamate and HMGB1 levels. For that, it is necessary to study markers involved in microglia activation, such as morphological alterations, phagocytic function and the production and release of inflammation-related factors, in order to better understand which phenotype microglia acquire to help MNs. Moreover, using these cell lines, it will be interesting to evaluate the reaction of injured MNs in the presence of microglia also transfected with human SOD1G93A (or even obtained from transgenic mice carrying human mutant SOD1), enabling the comparison in what regards behavior and communication established between mutated MNs and healthy or mutated microglia. Indeed, the use of cell lines brings an advantage, once they allow to study particular interactions without the influence of other cells present in surrounding environment.

Organotypic SC cultures represent a good working model to study ALS, and allow to study the interaction between MNs and all glial cells. They can survive for a long time, allowing to study the effect of long-term treatment with toxic or beneficial substances. We find that SOD1 accumulation triggers mitochondrial dysfunction, as it was demonstrated through mixed culture. However, further investigation on the disrupted mechanisms underlying the disease are necessary to perform in this model, including excitotoxicity, deregulation of endosomal trafficking, endoplasmatic reticulum (ER) stress, impaired axonal transport and cell death mechanisms. Moreover, organotypic slice cultures also has the advantage of allowing the depletion of microglia from the culture system, by using liposomes filled with clodronate, which will be ideal to assess microglia involvement along ALS progression. It will be interesting to provide continuity on testing some compounds with neuroprotective properties, such as glycoursodeoxycholic acid (GUDCA), which has been previously demonstrated in our group, to ameliorate some dysfunctions in differentiated NSC-34/hSOD1G93A.

Finally, in vivo studies using transgenic mice carrying SOD1G93A will be crucial to get a time point of each phase of the disease progression and to find biomarkers that allow to identify each of this phase.

Every effort has been taken for a better understanding of the mechanisms involved in the pathogenesis of ALS, in order to find an effective therapeutic approach, able to extend lifetime with less physical and psychological pain to the patient.

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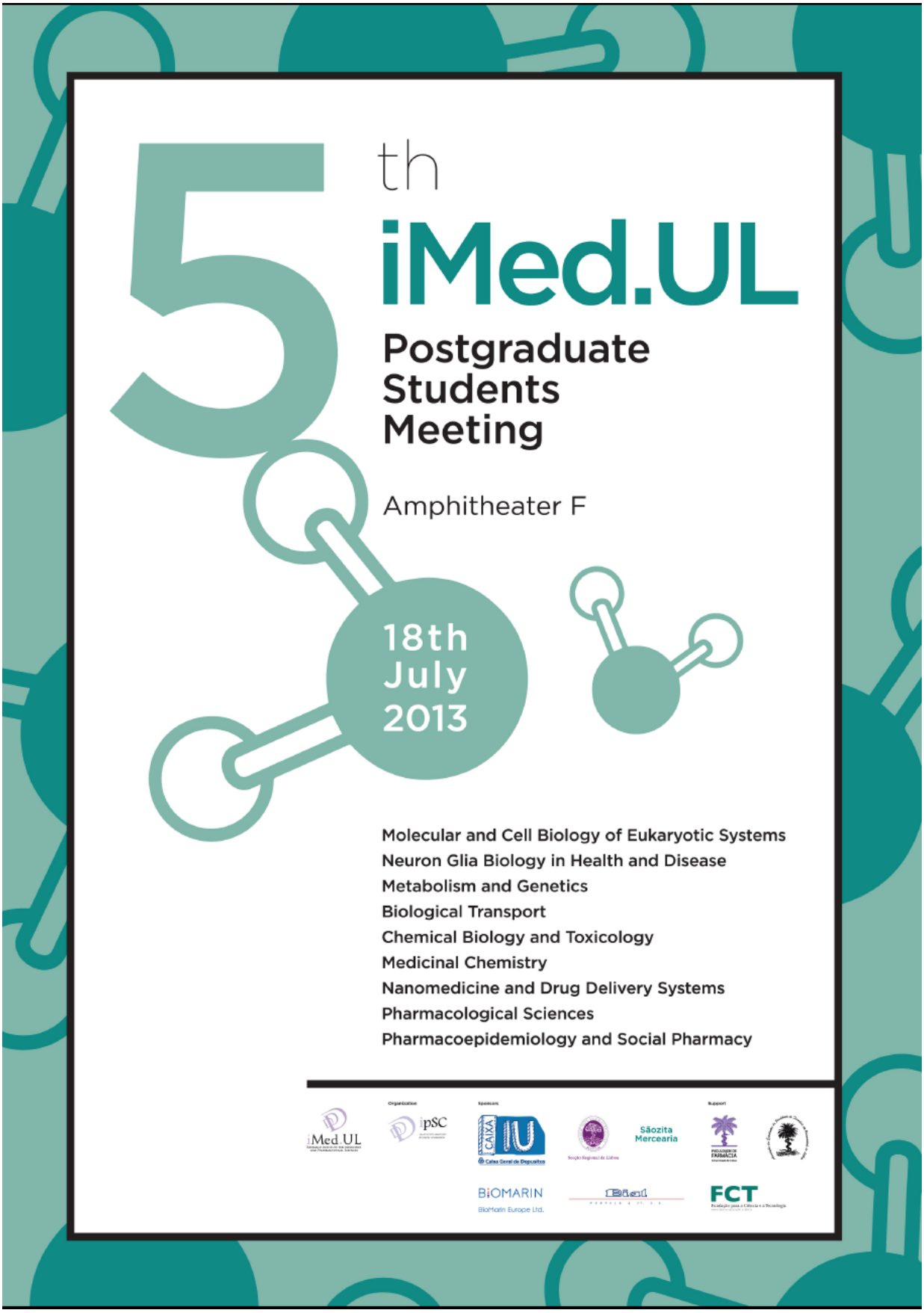
VI. ANNEX

Annex VI.1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Molecular and Cell Biology of Eukaryotic Systems
Neuron Glia Biology in Health and Disease
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Annex VI.1.1

5th iMed.UL Postgraduate Students Meeting
July 18th 2013, Lisbon, Portugal

Role of Microglia-Motor Neurons Cross-talk in ALS Modelling

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Neuron Glia Biology in Health and Disease

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease characterized by the selective loss of motor neurons (MN) in the cortex, brainstem, and spinal cord, whose sporadic form are still unknown for the underlying mechanisms of pathogenesis. Convergence of pathways involved in MN injury within multiple cell types, including microglia, were shown to favor the initiation and progression of ALS. Although initially microglia play a protective role in injured MN, slowing the disease course, at later stages of ALS progression, misfolded SOD1 or alternate signals released from injured MN may signal microglia cells to switch to a proinflammatory and neurotoxic state. Therefore, modulation of the dialogue between microglia and MN seems to be a key point to prevent ALS progression.

Here, we investigated the role of microglia in preventing or restoring MN function in ALS. We used a MN-like cell line expressing human-SOD1 with G93A mutation (NSC-34/hSOD1G93A) and the microglial cell line N9. Cells expressing human-SOD1wt were considered as controls. In this cell line, accumulation of mutated SOD1 was shown to occur at 4 DIV, together with cell dysfunction. To produce a mixed culture, we added N9 cells to mutated MNs at 0 and 2 DIV of differentiation and cultivated cells till the 7 DIV. Evaluations included HMGB1 and SOD1 cell content by Western Blot, extracellular ATP by an enzymatic fluorescent assay, NO by Griess reaction, as well asactivation of matrix metalloproteinases (MMP)-2 and -9 by gelatin zymography.

We observed a decrease in SOD1 accumulation, together with MMP-2 activation and ATP release when microglia was added at 0 and 2 DIV to NSC-34/hSOD1G93A cells. However, the activation of MMP-9 and release of HMGB1 was only evidenced to decrease when microglia were added at 2 DIV. Curiously, NO release, although not significantly, decreased when microglia were added at 0 DIV but significantly increased ($p<0.05$) in experiments where the addition of microglia were performed at 2 DIV.Together, these results show that healthy microglia have an essential role in reducing MN degeneration. Thus, any therapeutic strategy aimed at conserving/restoring both the healthy state of MNs and glial cells in ALS will contribute to delay the progression of such disorder.

Supported by FEDER (COMPETE Programme) and by National funds (FCT - projects PTDC/SAU-FAR/118787/2010 to DB and PEst-OE/SAU/UI4013/2011/2012).

Annex VI.1.2

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Modulation by Glycoursodeoxycholic Acid on an Organotypic-based Model of ALS

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Neuron Glia Biology in Health and Disease

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease characterized by motor-neuron (MN) degeneration and the compromise of other neural cell types. No pharmaceuticals have till now showed efficacy in ALS progression delay. Prior and ongoing studies in our lab using a SOD1-mutated motor-neuron (MN) cell line have shown preventive effects by glycoursodeoxycholic acid (GUDCA) over MN dysfunction by reducing apoptosis, mitochondrial failure, as well as nitric oxide (NO) release and matrix metalloproteinase-9 activation. Interestingly, GUDCA also revealed ability to delay the intracellular accumulation of SOD1 in those cells. This is not without precedent since GUDCA has already shown anti-oxidant, anti-apoptotic and anti-inflammatory properties (1). Here, we aimed to set-up an organotypic slice culture model of spinal cord (SC) from mice carrying the human SOD1 mutation (TgSOD1-G93A mice). This model is biochemically and physiologically more similar to the in vivo tissue since it preserves interneuronal connections and key processes. SC was here used once it is considered one of the most affected neural tissues in ALS. Benefits by this kind of cultures over animal models include the easy access and the precise control of the extracellular environment. Thus it is a powerful model to screen the efficacy of potential therapies (2), as we wanted to do with GUDCA.

The lumbar segment of SC from 7-days SJL (Wt) and TgSOD1-G93A mice were cut into 350 µm transverse slices with a McIlwain tissue chopper (3) and maintained during 10 DIV, the time where incubation with GUDCA at 50 µM was initiated for 24 hours. Evaluations included NO release by Griess reaction, extracellular ATP by an enzymatic fluorescent assay and DAPI for cell viability assessement.

Preliminary results with Wt and TgSOD1-G93A SC slices did not evidence significant changes in NO release between both models, although a slight decrease in extracellular ATP of TgSOD1-G93A slices was noticed with GUDCA. SC from TgSOD1-G93A exhibited a decreased cell viability. Intringuingly, although no yet significant, a preventive trend was obtained with GUDCA.

These results are a starting point for the study of the mechanisms involved in cellular degeneration in the SC of ALS models and how they can be modulated by promising compounds such as GUDCA.

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Annex VI.2


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PTW08-43

PI3K/Akt signaling pathway in the neuropathology of Krabbe disease

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Krabbe Disease (KD) is a progressive neurodegenerative disorder caused by the deficiency of the lysosomal galactocerebrosidase (GALC) enzyme and the toxic accumulation of its substrate psychosine. KD is characterized by a loss of myelin in the central and peripheral nervous systems due to defects in oligodendrocyte and Schwann cell function. Recent evidence from our laboratory suggests that there is also damage to neuronal axons and that psychosine disrupts cellular pathways associated with lipid rafts. We hypothesized that psychosine accumulation would lead to a deregulation of intracellular signaling pathways affecting key mediators of axonal integrity. Using a high-throughput quantitative Western blotting technique (Microwestern Array), we assessed the abundance and phosphorylation levels of key cellular signaling pathway components in the twitcher mouse, an authentic disease model. For this, we isolated neural stem cells from wild-type and twitcher E12 spinal cords and expanded them as neurospheres. Proliferating cells were serum-deprived and differentiated into a mixed-neuroglial culture for 7 days. Microwestern Array analysis showed a downregulation of major signaling pathways, such as the PI3K/Akt/mTOR pathway. Consistent with published data, PKCα was also downregulated in our assay. An *in vivo* evaluation of these results was performed in twitcher mice at postnatal day 30, a time point when severe peripheral axonopathy, demyelination and muscle wasting affect mutants. These analyses showed that phosphorylated (active) Akt was decreased in the twitcher neuromuscular unit, including sciatic nerves and the corresponding innervated muscles. GSK3β is a critical downstream component of PI3K/Akt signaling and Akt directly regulates its activity through Ser9-phosphorylation. Differentiated twitcher embryonic neural cultures contained a significant decrease in Ser9-phosphorylated (inactive) GSK3β, despite lack of changes in the level of total GSK3β. In corroboration with this result, GSK3β was also activated in sciatic nerve as well as muscle tissue from P30 twitcher mice. Downregulation of the PI3K/Akt pathway and its downstream effectors such as GSK3β in a psychosine-enriched environment emerges as a potentially critical factor mediating some aspects of the neuropathology in twitchers and KD. Identification of this pathway provides a new target for therapies for KD.

This work was funded by grants from the Legacy for Angels Foundation and the National Institutes of Health (RNS065808A) to ERB.

PTW08-44

Exploring motor-neuron degeneration in ALS – interaction with microglia and restoring ability by glycoursodeoxycholic acid

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Background: Amyotrophic lateral sclerosis (ALS) onset and progression has been associated with microglia activation and mutant SOD1 accumulation. However, how mutant SOD1 affects microglial activation and subsequently injure motor-neurons (MN) is still unclear.

Objective: To evaluate the: (1) suitability of NSC-34 cells transfected with human SOD1 to assess MN degeneration in ALS; (2) benefits of GUDCA in preventing and restoring MN function; (3) suitability of N9 cell line to evaluate microglia activation; (4) reactivity of microglia to NSC-34/hSOD1G93A conditioned media.
Methods: NSC-34 cells were differentiated for 1 to 4 days *in vitro* (DIV) and incubated with/without 50 μM GUDCA at 0 or 2 DIV. NSC-34 cells were stained with anti-SOD1 and N9-microglia with anti-Iba1 (morphological analysis). Evaluations included nuclear morphology (Hoechst® staining), extracellular content in glutamate (commercial kit) and in ATP (enzymatic fluorescent assay), mitochondrial viability (Mitotracker-red®), cell migration (Boyden chamber), phagocytic ability (fluorescent latex beads), NO (nitrites) and matrix metalloproteinases activity (MMP)-2 and -9 (gelatin zymography).
Results: NSC-34/hSOD1G93A evidenced accumulation of SOD1 at 3/4 DIV, reduced mitochondria viability ($p < 0.05$), increased apoptosis ($p < 0.01$), reduced release of ATP ($p < 0.05$) and glutamate ($p < 0.01$). Increased MMP-9 activation ($p < 0.05$) and nitrites generation ($p < 0.05$) were also observed. GUDCA prevented apoptosis ($p < 0.01$) mitochondrial dysfunction ($p < 0.05$), NO production ($p < 0.01$) and MMP-9 activation ($p < 0.01$), and even restored NO ($p < 0.05$) and MMP-9 ($p < 0.01$) levels. Treatment of N9 cell line with lipopolysaccharide increased the number of cells with an amoeboid morphology and with >5 ingested beads ($p < 0.01$), and decreased migration to ATP 10 and 300 μM ($p < 0.01$ for both level). When exposed for 6 h to NSC-34/hSOD1G93A-conditioned media they also showed a decreased migration to factors released by NSC-34/hSOD1wt ($p < 0.05$) or NSC-34/hSOD1G93A ($p < 0.01$). No N9 cell demise was observed after exposure to either media at 4 or 24 h incubation. However, microglia exhibited increased amoeboid morphology and decreased phagocytic ability, mainly after 24 h incubation, with NSC-34/hSOD1G93A-conditioned media.
Conclusions: Data indicate that: (1) Differentiated NSC-34/hSOD1G93A cells are adequate to explore the pathophysiological mechanisms involved in ALS; (2) GUDCA is effective in rescuing motor-neuron degeneration; (3) N9 cells respond well to induced activation; and (4) mutant SOD1 decreases microglia neuroprotective ability.

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PTW08-45

In vivo disruption of redox homeostasis caused by the synergistic action of methylmalonic acid and ammonia in brain of young rats

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Hyperammonemia is a common finding in children with methylmalonic acidemia, an inherited metabolic disease predominantly characterized by severe neurological dysfunction and biochemically by the accumulation of methylmalonic acid (MMA), especially during episodes of metabolic decompensation. Although it has been largely demonstrated that MMA is neurotoxic, the contribution of hyperammonemia to the development of the neurological symptoms in the affected patients is poorly known. In the present study we investigated the effects of intracerebroventric-

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