Maria Inês Fazendeiro Cunha

Licenciada em Biologia

Exploring deregulated signals involved in Motor neuron-Microglia cross-talk in ALS

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

Orientador: Dora Maria Tuna de Oliveira Brites,
Investigadora Coordenadora e Professora Catedrática Convidada,
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FACULDADE DE CIÊNCIAS E TECNOLOGIA
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À minha mãe,

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ABSTRACT

Amyotrophic Lateral Sclerosis (ALS) is the most common neurodegenerative disease affecting motor neurons (MNs). Neuroinflammation has shown to be a prominent pathological feature, highlighted by the presence of activated microglia, which may exert either beneficial or detrimental effects. Mutated MNs may release factors able to induce different microglial responses. However, how cells differently modulate each other remains elusive. Therefore, a better understanding of the MN-microglia signaling pathways compromised in ALS is warranted. Here, we aim (i) to uncover signaling pathways underlying MN injury and (ii) to dissect how MNs are modulating microglial response as well as the contribution of healthy microglia to rescue MN dysfunction. We focused on fractalkine-CX3XR1 axis, MFG-E8-mediated phagocytosis and HMGB1-TLR4 signaling.

For this we used a MN-like cell line (NSC-34) stably transfected with human SOD1, either wild-type (wtMNs) or with G93A mutation (mMNs), alone or in mixed cultures with N9 microglial cell line. We observed a compromised viability of microglia in the presence of mMNs, yet they were more activated, as suggested by the increase of CD11b mRNA expression. The dysfunctional mechanisms associated with increased NO and decreased glutamate production by mMNs were not recovered by the presence of healthy microglia. However, the increased activity of matrix metalloproteinase -9 observed in mMNs was decreased in the presence of microglia. In addition, mMNs presented accumulation of membrane-fractalkine and, in mixed cultures, CX3CR1 mRNA expression was up-regulated in their presence. Furthermore, we showed that mMNs expressed higher levels of MFG-E8, which were further increased in the presence of microglia. Finally, both HMGB1 and TLR4 levels were also increased in mMNs, mainly in the presence of microglia.

Together, these results highlight an impairment of microglial function caused by MN dysfunction and support the development of immunomodulatory strategies restoring both healthy state of microglia and MNs.

Keywords: Motor neuron dysfunction, neuroinflammation, microglia activation/deregulation, MN-microglia cross-talk, fractalkine -CX3CR1 axis, HMGB1-TLRs signaling pathways

RESUMO

A Esclerose Lateral Amiotrófica (ELA) é uma das doenças neurodegenerativas mais frequentes, que afeta os neurónios motores (NMs). A neuroinflamação tem vindo a ser apontada como caracteristica da ELA, sobretudo pela presença de microglia activada que pode ter efeitos benéficos ou detrimentais. Os NMs mutados podem libertar fatores capazes de induzir diferentes respostas microgliais. Contudo, as vias de sinalização intercelular não estão ainda bem esclarecidas, tornando-se necessário um melhor entendimento das vias de sinalização comprometidas entre os NMs e a microglia na ELA. Pretendeu-se: (i) descobrir as vias pelas quais os NMs sinalizam o seu comprometimento e (ii) explorar de que forma estes modulam a resposta da microglia, assim como a contribuição da microglia saudável em recuperar a função dos NMs. Focámo-nos nas vias fractalquina-CX3CR1, fagocitose mediada pelo MFG-E8 e a sinalização HMGB1-TLR4.

Utilizou-se uma linha celular de NMs (NSC-34) transfetados com a proteina humana SOD1, normal ou com mutação G93A, em mono-cultura ou em cultura-mista com uma linha celular de microglia (N9). Verificámos uma microglia comprometida na presença dos NMs mutados, mas mais ativada, com base no aumento de expressão do mRNA do CD11b. O aumento de produção de NO e diminuição de glutamato não foram recuperados na presença de microglia, no entanto a atividade aumentada da metaloproteinase-9 foi reduzida. Os NMs mutados demonstraram acumulação de fractalquina membranar e, nas culturas-mistas, a expressão do mRNA do CX3CR1 estava aumentada na sua presença. Verificou-se ainda que os NMs mutados expressam níveis superiores de MFG-E8, aumentados na presença da microglia. Mais ainda os níveis de HMGB1 e TLR4 encontravam-se igualmente aumentados nos NMs mutados, e principalmente na presença da microglia.

Os resultados obtidos evidenciam um comprometimento da função microglial causada pela disfunção dos NMs e apoiam o desenvolvimento de estratégias imunomoduladoras que recuperem a funcionalidade quer da microglia quer dos NMs.

Palavras-chave: Disfunção dos neurónios motores, neuroinflamação, ativação/desregulação da microglia, comunicação NM-microglia, eixo fractalquina-CX3CR1, vias de sinalização HMGB1-TLRs

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ABBREVIATIONS

ALS	Amyotrophic Lateral Sclerosis
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
ATF6	Activating transcription factor 6
Bcl2	B-cell lymphoma 2
BiP	Binding immunoglobulin protein
BMAA	β-N-Methylamino-L-alanine
CNS	Central nervous system
CX3CL1	Chemokine (C-X3-C motif) ligand 1
CX3CR1	Chemokine (C-X3-C motif) receptor 1
Cyt *c*	Cytochrome *c*
DMEM	Dulbecco's modified Eagle's medium
Drp1	Dynamin related protein 1
EAAT	Excitatory amino acid transporters
ER	Endoplasmic reticulum
fALS	Familial Amyotrophic Lateral Sclerosis
FBS	Fetal bovine serum
FKN	Fractalkine
FUS	Fused in Sarcoma
FWR	Forward
GFAP	Glial fibrillary acidic protein
GLAST	Glutamate aspartate transporter
GLT1	Glial glutamate transporter 1
GUDCA	Glycoursodeoxycholic acid
HMGB1	High mobility group box 1
Hsp70	Heat shock protein 70
IGF-1	Insulin-like growth factor 1
IL	Interleukin
iNOS	Inducible nitric oxide synthase
IRE1	Inositol requiring enzyme 1
LC3	Microtubule-associated protein light chain 3
LMN	Lower motor neuron
MFG-E8	Milk fat globule-EGF factor 8
Mfn1	Mitofusin 1
Mg	Microglia
MMP	Matrix metalloproteinase
MN	Motor neuron
mSOD1	Mutated Superoxide dismutase 1
NEAA	Nonessential aminoacids

NF-kB	Nuclear factor-κB
NO	Nitric oxide
OH	Hydroxyl radical
PDI	Protein disulphide isomerase
PDL	Poly-D-lysine
PERK	PKR-like ER kinase
PKR	Double-stranded RNA-activated protein kinase
PS	Phosphatidylserine
RAGE	Receptor for advanced glycation endproducts
REV	Reverse
ROS	Reactive oxygen species
sALS	Sporadic Amyotrophic Lateral Sclerosis
SOD1	Superoxide dismutase 1
TARDBP	Transactive response DNA-binding protein
TDP-43	TAR-DNA binding protein
TLR	Toll-like receptor
TLS	Translocated in liposarcoma
TNF-α	Tumor necrosis factor α
UBC	Umbilical cord stem cell
UDCA	Ursodeoxycholic acid
UMN	Upper motor neuron
UPR	Unfolded protein response
wtSOD1	Wild type Superoxide dismutase 1

I. INTRODUCTION

1. Amyotrophic Lateral Sclerosis

Amyotrophic Lateral Sclerosis (ALS) is the most common adult-onset neurodegenerative disease affecting motor neurons (MNs). It is characterized by the selective loss of MNs in the motor cortex (upper motor neurons - UMNs) as well as in the brainstem and in the spinal cord (lower motor neurons – LMNs). The disease begins focally, in the central nervous system (CNS), and spreads contiguously leading to progressive degeneration **(Figure I.1)**. In 1869 the french neurobiologist and clinician Jean-Martin Charcot first described and diagnosed the first cases of ALS and found that the symptoms vary depending on the location of the lesion (Kumar et al., 2011) resulting in a clinical presentation of muscle weakness, spasticity, atrophy and ultimately, paralysis, together with a slurred speech, dysphasia and dysarthria, culminating in death, in the majority of cases by respiratory failure. Typically the cognitive functions of the brain remain undamaged so the patient is aware of the disease progression. This relentless disease usually has a fast rate of progression being the typical age of onset around 48 years and patients usually dye within 3-5 years from symptoms onset. ALS has a worldwide incidence of 1 to 2 new cases per 100,000 individuals each year and a prevalence of 4 to 6 cases per 100,000 individuals (Chen et al., 2013).

Nowadays, ALS is considered a multifactorial disease. Although the main hallmark is MN injury other cells were shown to be actively implicated in this disorder, as surrounding glial cells. Moreover, between the numerous pathological processes that seem to contribute to MN degeneration, neuroinflammation has been highlighted mostly by the presence of activated microglia at sites of injury where these cells were shown to have a detrimental role in its evolution (Beers et al., 2006; Bowerman et al., 2013; Yamanaka et al., 2008a; Yamanaka et al., 2008b; Zhao et al., 2013; Zhao et al., 2004).

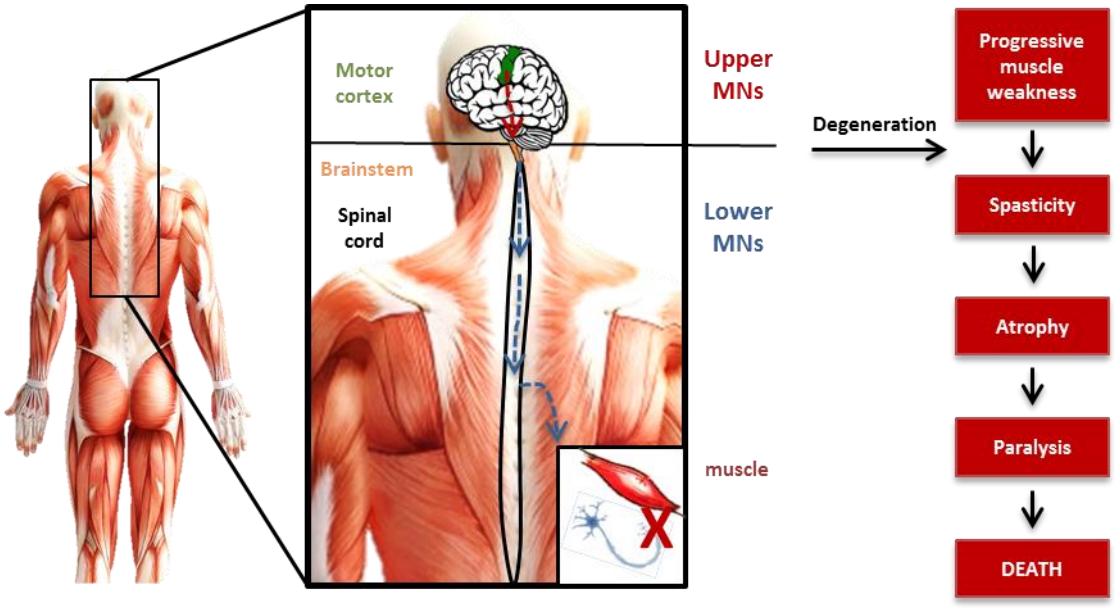


Figure I.1. ALS symptoms are a result of UMNs and/or LMNs degeneration signs. Upper motor neurons (UMNs) localize in the motor cortex and connect with lower motor neurons (LMNs) present in brainstem and spinal cord. In ALS, motor neuron (MN) degeneration begins focally in CNS and then spreads contiguously, ultimately leading to an impairment of signal transmission from MNs to muscle. Clinically this is represented by progressive muscle weakness, spasticity, atrophy, paralysis and death, mostly by respiratory failure, within 3 to 5 years from symptoms onset.

Concerning etiology, approximately 90% of ALS cases have unknown cause and are non-genetic, commonly classified as sporadic ALS (sALS), and only 5-10% of cases are genetic forms, generally known as familial ALS (fALS). Interestingly, both the non-genetic and the genetic forms of ALS are suggested to have common pathogenic mechanisms and share the clinical features (Lilo et al., 2013).

1.1. Genetics

Although only 5-10% of ALS cases have clear genetic linkage, the similarities in the clinical course and pathological findings suggest that investigating genetic forms will reveal the disease mechanisms behind non genetic forms of ALS. In fact, genetic discoveries allowed a better understanding of the pathophysiological mechanisms that contribute to the disease. ALS may have an autosomal dominant, autosomal recessive (Siddique et al., 1996) or X-linked pattern of transmission (Chen et al., 2013; Deng et al., 2011). The causative genes were identified in about 60% shedding light to superoxide dismutase 1 (*SOD1)*, transactive response DNA-binding protein (*TARDBP*), fused in sarcoma (*FUS*) and *C9ORF72*. 20% account for mutations within the gene encoding the enzyme SOD1, inherited mostly in an autosomal dominant pattern, 4-5% are results of mutations in TARDBP and FUS genes and more than 30% are related to C9ORF72 mutations. Given the range of cellular processes in which are involved the proteins these genes encode, it is generally assumed that ALS is the result of defects in a variety of independent cellular mechanisms culminating in a cascade of cellular degeneration. A single mutation can result in variable clinical phenotype and different mutations can lead to a similar phenotype revealing a high degree of heterogeneity in ALS and suggesting that the underlying mechanisms share pathophysiological pathways (Ravits et al., 2013).

1.1.1. Mutations in SOD1

In 1993, Rosen and colleagues found the first causative mutations in fALS within the gene encoding the enzyme SOD1 (Rosen et al., 1993). The gene maps to chromosome 21q22.1 and is composed of five exons encoding a 153 amino acid metalloenzyme. SOD1 is a very stable dimer ubiquitously expressed with a crucial function in cellular homeostasis. The protein binds copper and zinc ions and forms a homodimer that acts as dismutase removing toxic superoxide radicals by converting them to molecular oxygen and hydrogen peroxide, thus preventing ROS toxicity (Keller et al., 1991), and was found to localize in the cytoplasm, nucleus, lysosomes and intermembrane space of mitochondria.

So far, 177 *SOD1* mutations have been described counting for 20% of genetic forms and 2-7% of non-genetic forms (Al-Chalabi, 2014) and with few exceptions all *SOD1* mutations are dominant. The majority of the mutations in *SOD1* are missense within all five exons affecting the functional domains of the protein, like the glycine-to-alanine substitution at position 93 ($SOD1^{G93A}$) which seems to be particularly vulnerable since it is point mutated to all 6 possible residues in fALS (Turner and Talbot, 2008). Nevertheless, nonsense mutations, insertions and deletions were also reported (Shaw and Valentine, 2007).

SOD1-ALS is thought to originate as a distal axonopathy, being neuromuscular junctions the first regions of the MNs to degenerate in a process known as 'dying back' (Fischer et al., 2004). Clinically, patients with mutated SOD1 (mSOD1) present mostly with lower limb onset, with predominance of LMN features, and young age-of-onset (Gordon, 2013) but the time of onset and duration of disease may be different according to the type of mutations (Boillée et al., 2006a).

It is believed that a gain of toxic function mechanism is the main pathway for SOD1 neurotoxicity (Pasinelli and Brown, 2006). It was reported from analysis of mSOD1 transgenic mouse models that mice lacking SOD1 did not develop the disease (Shefner et al., 1999) and transgenic mice overexpressing human $SOD1^{G93A}$ develop ALS clinical features (Gurney et al., 1994). The mechanism underlying this toxicity may be, for instance, through SOD1 aggregation that is likely to be an early event in the disease as it appears at disease onset and its abundance increases along ALS progression (Wang et al., 2002).

1.1.2. Other mutations

In addition to *SOD1* mutations in the genes coding for TARDBP, FUS and C9ORF72 are closely associated with typical clinical phenotype (Chen et al., 2013).

One of the most characteristic neuropathological features of ALS is the presence of cytoplasmic inclusions in the degenerating MNs. The major component of these ubiquitinated inclusions was found to be TDP-43 (Van Deerlin et al., 2008). TDP-43 is encoded by *TARDBP* gene that is composed of six exons and maps on chromosome 1p36.22. The protein contains two RNA-recognition motifs and a glycine-rich C-terminal region that allows it to bind single stranded DNA, RNA and proteins. The C-terminal portion of TDP-43 was found to contain nuclear localization and export signal motifs allowing TDP-43 to shuttle between the nucleus and the cytoplasm (Mackenzie et al., 2010). In the brain, TDP-

43 is usually localized in the nucleus of neurons and some glial cells (Gendron et al., 2010). Although its physiological function in the nervous system is currently unknown, as it is a DNA-RNA binding protein, it is predicted to be involved in mRNA splicing and nucleo-cytosolic RNA transport (Mackenzie and Rademakers, 2008). Hence, abnormal modified (phosphorylated and truncated) TDP-43 promoting its aggregation together with cellular mislocalization of TDP-43 to the cytoplasm of affected neurons leads to the hypothesis that TDP-43 mediated neurotoxicity is likely to result from the loss of normal TDP-43 function, as well as from a toxic gain of function conferred by TDP-43 inclusions (Gendron et al., 2010). To date, several studies have report in total nearly 50 mutations in fALS and sALS (about 3% and 1.5%, respectively), mostly involving the C-terminal glycine-rich region of the protein (Lattante et al., 2013). So currently, *TARDBP* mutations seem to have comparable incidence of SOD1 mutations in sALS. In both lower MNs and glia TDP-43 positive inclusions are consistent feature of all sporadic cases and fALS without *SOD1* mutations (Tan et al., 2007).

Mutations in the gene encoding FUS / translocated in liposarcoma (TLS) have been identified in a subset of patients with familial ALS and, less common, with sporadic ALS (Liscic and Breljak, 2011). FUS is a DNA/RNA binding protein predominantly expressed in cell nuclei in physiological conditions that is involved in transcriptional regulation, RNA and miRNA processing, and mRNA transport. Similar to TDP-43, most mutations are clustered in the C-terminal region rich in arginine and glycine, which encodes also for nuclear localization signal (Lagier-Tourenne and Cleveland, 2009). Studies in postmortem tissue (brain and spinal cord) harboring FUS/TLS mutations revealed an increased cytoplasmic FUS-staining and cytoplasmic inclusions in MNs (Kwiatkowski et al., 2009; Vance et al., 2009), leading to the hypothesis that FUS mutations may contribute to ALS pathogenesis through the formation of cytoplasmic inclusions and/or the loss of the physiological nuclear functions of the protein, analogous to TDP-43. More than 30 mutations have been identified and all except one have an autosomal dominant pattern (Kabashi et al., 2011). Clinically, mutations in this gene are usually associated with age of onset younger than 40 years, survival of less than three years and onset in the upper limbs (Millecamps et al., 2010).

The striking functional and structural similarities of TDP-43 and FUS, both DNA/RNA binding proteins, suggest that abnormal RNA processing is a crucial event in ALS pathogenesis although the mechanisms underlying protein aggregation and the consequent neurodegeneration remain currently unknown.

Recently, increased evidence points to the importance of C9ORF72 gene, that represents around 40% of the genetic and 7% of non-genetic forms (DeJesus-Hernandez et al., 2011; Gordon, 2013; Renton et al., 2011). Linkage analysis of ALS and frototemporal dementia (FTD) cases revealed an important locus for the disease on chromosome 9p21 leading to deeper studies which revealed an hexanucleotide repeat located in the non-coding region of *C9ORF72* (DeJesus-Hernandez et al., 2011; Renton et al., 2011), a gene that encodes an uncharacterized protein with unknown domains or function although highly conserved across species. The repeat expansion leads to the loss of one alternatively spliced *C9ORF72* transcript and to formation of nuclear RNA foci, implying also both loss-of-function and gain-of-function mechanisms (DeJesus-Hernandez et al., 2011). Clinically, this mutation has been associated with lower age of onset, cognitive and behavioral impairment and reduced survival among

other features. Byrne et al. (2012) suggest that the *C9ORF72* repeat expansion may be inherited in an autosomal dominant way with an expanded phenotype of neurodegeneration and variable penetrance.

1.2. Sporadic ALS

Despite all the progress achieved so far, the causes of large majority of sALS remain unknown but it is thought to be probably associated with a combination of genetic and environmental factors. Environmental factors associated with ALS have been studied for many years and recent advances have shed light to the cyanotoxin β-N-Methylamino- L-alanine (BMAA). Interestingly, food containing BMAA has been found on Guam, a well-known focus of ALS/parkinsonism-dementia complex (ALS-PDC) and high concentrations of BMAA were found in the *postmortem* brain tissue (Cox et al., 2003). This non-protein neurotoxic amino acid can be misintegrated into the protein structure, in the place of L-serine (Dunlop et al., 2013) which may induce protein misfolding and cell death.

Although to date most cases are unrelated to SOD1 gene mutations, the chance that sporadic and familial ALS may converge on a common pathogenic pathway involving abnormal SOD1 species must be taken into account. For instance, SOD1 immunoreactive protein aggregates were found in spinal cord of both human fALS and sALS cases (Gruzman et al., 2007) and wild-type SOD1 (wtSOD1) was shown to acquire binding and toxic properties of mSOD1 through oxidative damage (Ezzi et al., 2007; Rakhit et al., 2004). Also, wtSOD1 can interact with Heat shock protein 70 (Hsp70) as well as chromogranin B, inducing tumor necrosis factor α (TNF-α) and inducible nitric oxide synthase (iNOS), leading to a dose dependent cell death (Ezzi et al., 2007). Moreover, oxidized wtSOD1 and mSOD1 were reported to share a conformational epitope that is not present in normal wtSOD1, and both recombinant oxidized wtSOD1 and wtSOD1 immunopurified from human sALS tissues have been shown to inhibit kinesin-based fast axonal transport, similar to SOD1-fALS (Bosco et al., 2010).

These findings imply that both genetic and non-genetic forms of ALS must share key pathogenic mechanisms such as protein aggregation, endoplasmic reticulum stress, mitochondrial failure or axonal transport dysfunction, thus triggering a compromised homeostasis of MNs, as will be further detailed.

1.3. Compromised Homeostasis of MNs

A main hallmark of ALS is MN degeneration and identification of SOD1 mutations has enabled development of animal and cell culture models from which it was possible to uncover some of the mechanisms triggering MN injury, schematically represented in **Figure I.2**. Although the precise mechanism by which SOD1-mediated toxicity triggers ALS pathogenesis remains unknown, several hypotheses have been proposed such as oxidative stress, glutamate excitotoxicity, protein aggregation, endoplasmic reticulum stress, mitochondrial dysfunction, axonal transport abnormalities, as well as microglial activation and neuroinflammation. Given the variety of mechanisms already reported to be involved in the disease, it seems, most likely that ALS results from cumulative upstream abnormalities which will lead to a common pathogenic cascade.

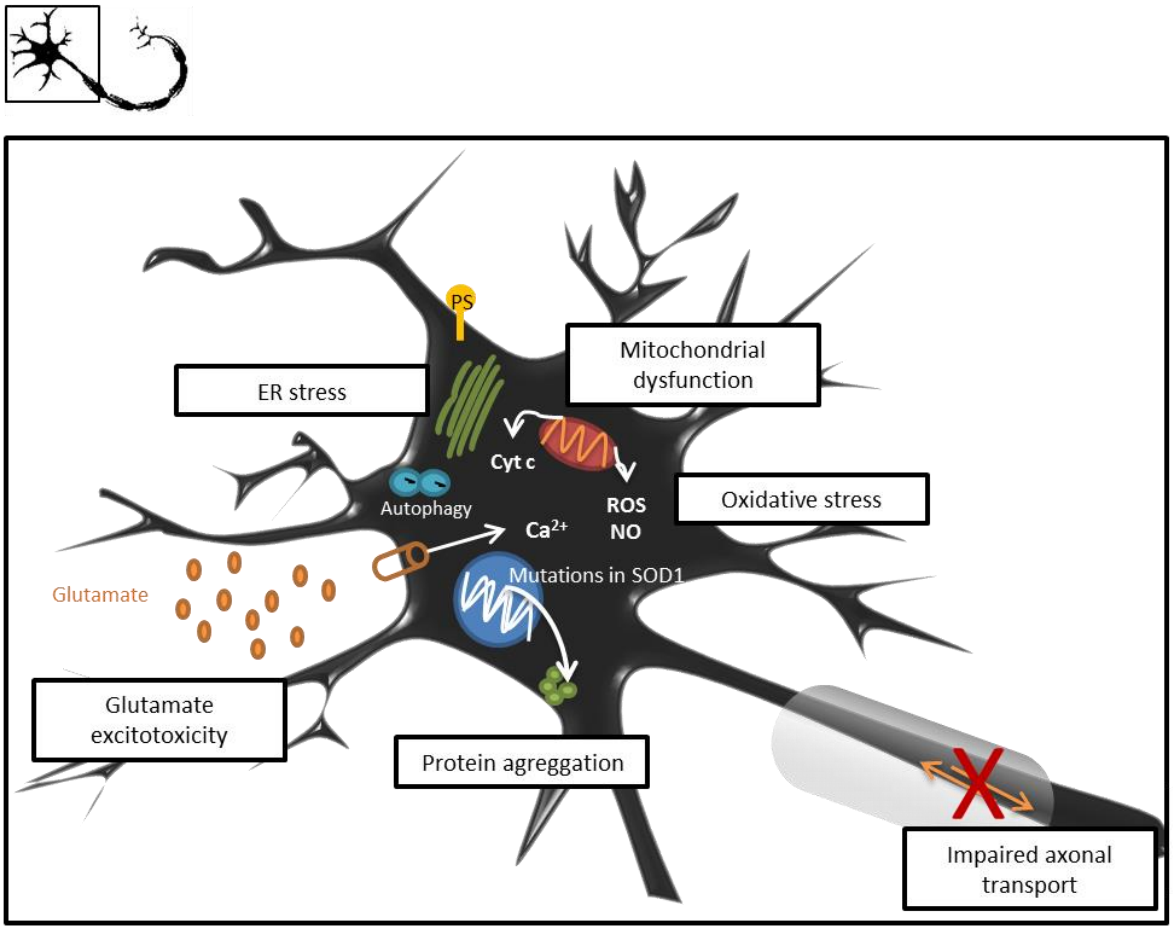


Figure I.2. Molecular mechanisms leading to compromised homeostasis of MNs in ALS. Motor neuron (MN) injury is believed to be a result of multiple dysfunctional processes. In SOD1-linked ALS, mutations in the gene will result in a protein with major propensity to aggregate with itself as well as with other proteins. These protein aggregates will cause endoplasmic reticulum (ER) stress and activate the unfolded protein response (UPR). Prolonged UPR triggers apoptosis, characterized by exposure of phosphatidylserine (PS), through ER-stress specific caspases and pro-apoptotic proteins, and cross-talk with mitochondrial intrinsic apoptotic pathway. Also mitochondrial dysfunction results in release of cytochrome c (cyt c) and production of higher levels of free radicals, as reactive oxygen species (ROS) and nitric oxide (NO), inducing apoptosis and increased oxidative stress. Increased number of autophagic vacuoles is also observed within MNs. Abnormalities in the axonal transport are reported in MNs, further resulting in accumulation of protein and organelles that cause axonal damage and, consequently, impaired signal transmission. Extracellular glutamate accumulation promoting excitotoxicity is reported in ALS. For instance, overstimulation of AMPA/kainate receptors leads to major influx of calcium (Ca^{2+}) that further contributes to disrupted homeostasis of MNs. Adapted from Ferraiuolo et al. (2011b)

1.3.1. Oxidative Stress – accumulation of free radicals

Oxidative stress, associated with the formation of reactive oxygen species (ROS), has been identified as one of the major causes of cellular injury in several neurodegenerative diseases. Oxidative stress is commonly defined as a disturbance in the balance between the production of ROS and antioxidant defenses, where biological system's ability to readily detoxify free radicals or easily repair the resulting damage is compromised. Intracellular ROS may be instigated by leakage of electrons from the mitochondrial respiratory chain, leading to incomplete reduction of molecular oxygen during oxidative phosphorylation, generating superoxide radical anion and hydrogen peroxide. Free radicals, as superoxide and nitric oxide (NO), generated by nitric oxide synthase (NOS) are also produced by immune cells and modulate the expression of the immune response (Wink et al., 2011). Increased levels of superoxide reacting with NO lead to production of peroxinitrite, a potent oxidant, and slowly decomposition of hydrogen peroxide lead to the highly reactive hydroxyl radical (OH). Both peroxynitrite and hydroxyl radical are highly reactive oxidizing agents being able to damage proteins, lipids and DNA. This injury may include changing of protein conformation, altering cellular membrane dynamics by oxidation of unsaturated fatty acids and alterations in DNA and RNA species, as reviewed in Barber and Shaw (2010).

In ALS, the role of oxidative stress has been established in several studies. In fact, Shaw et al. (1995) reported that the levels of protein carbonyl, a biomarker of oxidative stress, were increased in the spinal cord of patients with sALS and Ferrante et al. (1997) reported that those levels were also increased in the motor cortex. In addition, Beal et al. (1997) showed increased concentrations of 3-nitrotyrosine, a marker of peroxynitrite-induced oxidation, in MNs of both familial and sporadic ALS patients. Moreover, using a transgenic mouse model of ALS ($SOD1^{G93A}$), Andrus et al. (1998) proposed that the increased hydroxyl radical production associated with the $SOD1^{G93A}$ mutation may cause extensive protein oxidative injury, for instance, in SOD1 protein itself.

1.3.2. Excitotoxicity – glutamate activity

An important pathophysiological process in both forms of ALS seems to be glutamate-mediated excitotoxicity. Glutamate is the main excitatory neurotransmitter in the mammalian CNS. Whereas it is important for normal nerve cell function, it becomes toxic in elevated concentrations. This phenomenon is known as excitotoxicity and arises when there is a rupture in the equilibrium between the release and re-uptake of glutamate that leads to increased synaptic glutamate concentrations and consequent excessive stimulation of glutamate receptors, resulting in augmented intracellular calcium levels, generation of ROS, consequent neuron damage and death. This may happen either as a consequence of excessive production or by failure of glutamate transporters, as the excitatory amino acid transporters (EAAT), mostly present in glial cells. MNs are known to have a high level of glutamatergic input and to be particularly vulnerable to excitotoxic cell death (Heath and Shaw, 2002).

Multiple studies have reported the evidence of excitotoxic mechanisms in ALS. For instance, increased levels of glutamate were observed in the plasma of ALS patients (Plaitakis and Caroscio, 1987), while a decrease in glutamate levels was shown in ventral horn of spinal cord and motor cortex of postmortem CNS tissue (Tsai et al., 1991). In addition, the expression of the astrocytic glutamate

transporter EAAT2, the major glutamate transporter responsible to remove glutamate from the synaptic cleft, is reduced by 90% in the ventral horn of paralyzed transgenic $SOD1^{G93A}$ rats (Howland et al., 2002). Moreover, by using primary cultures of MNs from murine spinal cord, Roy et al. (1998) have shown that normally non-toxic glutamatergic input, particularly via calcium-permeable AMPA/kainate receptors, is a major factor in the vulnerability of MNs to the toxicity of SOD1 mutants.

1.3.3. Protein misfolding and aggregation

Protein aggregation is a hallmark of many neurodegenerative diseases, including ALS. The clumps of protein misfolded, either because of injury from ongoing cellular processes or through the heritage of an abnormal structure in genetic disorders, may affect normal MN functions and induce cell death. SOD1 inclusions constitute a primary feature of SOD1-fALS in both human ALS patients and animal models. These inclusions were reported to form before symptoms onset and to accumulate during disease progression and were found to occur in the cytoplasm, mitochondria and vacuoles of MNs, as well as axons and dendrites (Chattopadhyay and Valentine, 2009).

In addition, Wang and co-workers proposed that toxicity of aggregates is mediated by their size and that mSOD1 has an increased aggregation propensity, i.e., an increased likelihood of an unfolded protein to aggregate, toxic for ALS patients (Wang et al., 2008). In fact, aggregation is thought to be a consequence of the formation of misfolded SOD1 that escape from cellular quality-control and housekeeping mechanisms, and different ALS-associated mutations may increase SOD1 aggregation for several reasons, such as: a decrease in the negative charge of SOD1 without affecting stability or metal binding, a destabilization of protein native state, or an impairment of Cu or Zn binding by SOD1 (Shaw and Valentine, 2007). Interestingly, intracytoplasmic protein deposits strongly stained for SOD1 in the spinal cord of both sALS and fALS and, in ALS, are thought to consist of granule-coated fibrils that contain SOD1 as well as other proteins as reviewed by Chattopadhyay and Valentine (2009).

1.3.4. ER stress

Protein aggregates and inclusions containing misfolded proteins have been correlated with the activation of stress signaling pathways from the endoplasmic reticulum (ER). The endoplasmic reticulum is the organelle responsible for the native folding, post-translational modifications and trafficking of secreted and membrane proteins. ER stress occurs when misfolded proteins accumulate in the ER and ER Ca^{2+} content is depleted. ER stress is transduced by three proximal sensors of the unfolded protein response (UPR): the double-stranded RNA-activated protein kinase (PKR)-like ER kinase (PERK), the basic leucine-zipper activating transcription factor 6 (ATF6) and the inositol requiring enzyme 1 (IRE1). These sensors are maintained in an inactive state by interaction with the ER chaperone binding immunoglobulin protein (BiP). When there is increased accumulation of misfolded proteins, BiP is released and ER stress sensors become activated. Thus, to reestablish cell homeostasis, cells activate the UPR which mediates the upregulation of genes encoding ER-resident chaperones, such as BiP, glucose regulated protein 94, calreticulin, calnexin and protein disulphide isomerase (PDI) family members; decrease in protein translation in order to reduce ER protein load; and degradation of misfolded proteins by the proteasome. When protein misfolding and accumulation can no longer be

compensated for, prolonged UPR triggers apoptosis through ER-stress specific caspases and pro-apoptotic proteins, and cross-talk with mitochondrial intrinsic apoptotic pathway (Walker and Atkin, 2011). Regarding ALS, two studies reported that UPR was present in mice model SOD1^{G93A} (Atkin et al., 2006; Kikuchi et al., 2006) and that mSOD1 was present inside the ER. Atkin et al. (2008) pointed out an up-regulation of the full spectrum of UPR markers in lumbar spinal cord tissue from human sALS, therefore suggesting ER stress as common phenomenon to all ALS cases. Interestingly, Saxena et al. (2009), by using SOD1^{G93A} mice model, have observed that selectively vulnerable MNs were prone to ER stress and that the activation of UPR was correlated with microglial activation in lumbar spinal cord. Altogether, these findings highlight an important role for ER in the pathogenicity of ALS, although the underlying mechanisms remain elusive.

1.3.5. Mitochondrial dysfunction and impairment of axonal transport

Mitochondria are involved in generation of intracellular ATP and in free radicals, buffering of intracellular calcium and initiation of programmed cell death. Free radicals, as ROS and NO, are produced in mitochondria and both mitochondria and its genetic material are sensitive to oxidative stress (Duchen, 2004). Hence, impairment of mitochondrial function, for instance through an imbalance between fission and fusion processes, results in lower energy production by neurons, diminished activity and lack of healthy mitochondria at synaptic terminals.

Studies in human ALS postmortem tissue (spinal cord) have shown both biochemical and morphological mitochondrial abnormalities (Sasaki and Iwata, 1996) as well as in mSOD1 transgenic mice (Jaarsma et al., 2000). mSOD1 was shown to be toxic to mitochondria affecting bioenergetics (Mattiazzi et al., 2002), protein import (Li et al., 2010) and the conformation of apoptotic proteins (Pedrini et al., 2010). Recent studies in our lab with NSC-34/hSOD1^{G93A} cells, an in vitro model of MN degeneration in ALS, reported a significant impairment of mitochondria dynamic properties (Vaz et al., 2014). By analyzing dynamin related protein 1 (Drp1) and mitofusin 1 (Mfn1), two main proteins involved in fission and fusion processes, respectively, these cells demonstrated mitochondrial dysfunction essentially trough fission processes (Ferreira, 2013).

Axonal transport is required for multiple neural functions as neurotransmitter synthesis, release and recycling. MNs have long axons that demand orderly function of the axonal transport mechanism to keep the structure and signal transmission at synaptic terminals. The transport is mediated by two major families of motor proteins – kinesin for anterograde transport and dynein for retrograde transport (Goldstein and Yang, 2000). Impairment on the axonal transport may result in accumulation of proteins and organelles and lead to damage in axons. Altered axonal transport activity is one characteristic of ALS disease. In fact, Collard et al. (1995) reported an impaired anterograde transport of neurofilaments and tubulin before disease onset in mSOD1 transgenic mice, probably due to the observed accumulation of neurofilaments. Also, using SOD1^{G93A} transgenic mice Shi and co-workers reported a decrease in retrograde transport of dynein and suggested disruption of dynein-mediated transport as an early event in SOD1^{G93A} transgenic mice starting before disease onset (Shi et al., 2010). Thus, the literature suggests the damage to the cargoes or machinery of axonal transport as an early feature of toxicity carried out by mSOD1. Moreover, studies in our lab using NSC-34/hSOD1^{G93A} cells, have shown that

both anterograde and retrograde axonal transport are impaired due to dysfunction of kinesin and dynein, respectively (Ferreira, 2013).

1.3.6. Cell death pathways

Evidence has accumulated pointing towards a programmed cell death of MNs in ALS resembling apoptosis. Apoptosis is characterized biochemically by exposure of phosphatidylserine (PS) on the outer leaflet of the plasma membrane, alterations in mitochondrial membrane permeability, which is thought to be regulated by proteins of the B-cell lymphoma 2 (Bcl2) family, release of intermembrane space mitochondrial proteins, as cytochrome *c*, and caspase-dependent activation (Elmore, 2007). Morphologically it is characterized by plasma membrane blebbing, nuclear fragmentation, formation of apoptotic bodies and chromatin condensation in the nuclear membrane (Elmore, 2007). In human ALS *postmortem* tissue, changes in the balance of pro- and anti-apoptotic members of the Bcl2 family were reported, either for expression and intracellular localization, usually resulting in predisposition for apoptosis in mMNs when compared to controls, and increased activities of caspases 1 and 3 were reported in the spinal cord, as reviewed by Sathasivam et al. (2001). Nuclear and cytoplasmic condensation and formation of apoptotic bodies were described in morphological studies. Furthermore, in cellular models of SOD1 ALS was observed that mSOD1 cells express higher amounts of PS on cell surface and increased cleavage/activation of caspase-9 (Sathasivam et al., 2001; Shaw, 2005; Vaz et al., 2014).

Nevertheless, autophagic dysfunction is emerging as a possible contributing factor in MN degeneration in ALS. Autophagy is important to neuronal homeostasis and may serve as a neuroprotective mechanism preventing the spreading of damages. It is an intracellular mechanism involved in degradation of abnormal proteins or wasted cytoplasmic components, within lysosomes. However, upon excessive autophagy cells may undergo autophagic cell death characterized by massive accumulation of autophagosomes without nuclear condensation. Increased expression of microtubule-associated protein light chain 3 (LC3) together with other proteins, such as beclin-1, is one marker of autophagy (Caldeira et al., 2014; Glick et al., 2010). In $SOD1^{G93A}$ mice model was reported altered autophagy starting from the pre-symptomatic stage of the disease together with an increased number of LC3-labeled autophagic vacuoles in spinal cord (Li et al., 2008; Zhang et al., 2011). Induction of autophagy has also been shown in ALS *postmortem* tissue by immunohistochemical analysis of LC3 and p62, another marker of autophagy (Sasaki, 2011). It is hypothesized that mSOD1 can inhibit the autophagic machinery and lead to disrupted homeostasis (Chen et al., 2012). Still, it remains unclear whether the increased autophagic vacuoles seen within MNs are result of autophagy induction or autophagy influx impairment and whether it exerts a beneficial or detrimental role, being proposed that it could vary accordingly to disease course (Ghavami et al., 2014).

1.4. The role of glial cells in ALS

Increased evidence indicates that MN death in ALS is a non-cell autonomous process. Although it was initially considered a secondary phenomenon, an increasing number of studies support the contributory role of non-neuronal cells in the selective MN degeneration.

1.4.1. Schawn cells and oligodendrocytes

Myelination of axons allows more speed and energy efficiency of nerve conduction through the process of saltatory conduction in which there is neuronal action potential propagated between nodes of Ranvier (Waxman, 2006). This process is performed by oligodendrocytes in the CNS and Schwan cells in the peripheral nervous system.

Recently, oligodendrocytes were reported to play a relevant role in ALS since widespread degeneration was observed in the gray matter oligodendrocytes in the spinal cord of mSOD1 mice prior to disease onset (Kang et al., 2013). Even new oligodendrocytes were still formed they weren't able to maturate and therefore they were unable to mediate remyelination. Also it was suggested that oligodencrocytes injury together with demyelination and lack of metabolic support to neurons may contribute to accelerate disease progression in ALS (Philips et al., 2013).

Schwan cells, which are associated with the full length of peripheral axons that represent 90% of the volume of MNs, are also involved in non-conductive functions of MNs such as axonal development and regeneration and maintenance of neuromuscular synapses. These are processes disrupted in early stages of ALS models and thus injury in these cells may be somehow involved in ALS. For instance, Lobsiger et al. (2009) reported that decreased levels of mSOD1 within Schwann cells in a ALS mouse model promotes a significant acceleration of disease progression as well as a reduction of insulin-like growth factor 1 (IGF-1) in nerves, hypothesizing a protective role of mSOD1 within Schwann cells. Interestingly, Turner et al. (2010) stated no pathological effects of mSOD1 accumulation within Schwann cells to spinal MNs nor injurious to disease course in ALS model mice. In the other hand, Chen et al. (2010) suggested that Schwann cells are involved in the process of distal axonopathy in mouse ALS through their expression of iNOS inducing MN axonal damage at the nodes of Ranvier.

Taken together, these findings suggest an important role for myelinating glia in ALS pathogenesis.

1.4.2. Astrocytes

Astrocytes constitute the major fraction of non-neuronal cells in the CNS and are emerging as major players in MN degeneration. In fact, Nagai et al. (2007), by using an *in vitro* model of either MNs derived from mouse embryonic spinal cord or MNs from mouse embryonic stem cells co-cultured with astrocytes expressing mSOD1, have reported that astrocytes promote death of wild-type MNs mediated by the release of soluble factors specific to astrocytes. Later, it was shown by Ferraiuolo et al. (2011a) that pro-NGF (nerve growth factor) is one toxic factor compromising MN survival in those conditions.

Reactive astrogliosis is characterized by an increased immunoreactivity for glial fibrillary acidic protein (GFAP) as well as the expression of inflammatory markers such as COX-2, iNOS and neuronal NOS (Pehar et al., 2005). It constitutes a pathological hallmark of ALS reported in several regions of both genetic and non-genetic cases (Nagy et al., 1994) and transgenic $SOD1^{G93A}$ mice (Levine et al.,

1999). Additionally, dysfunction of the glutamate transporters GLAST (the rodent homologue of human EAAT1) and GLT-1 (EAAT2), the transporters responsible for most of the glutamate uptake and mainly expressed by astrocytes, has been linked with ALS (Dumont et al., 2014). Interestingly, it was recently reported a population of astrocytes with an aberrant phenotype, highly proliferative and undifferentiated in the spinal cord of symptomatic transgenic $SOD1^{G93A}$ rats. This astroglial population was also capable of inducing MN death through the secretion of soluble factors, which still remain to uncover but are suggested to be cytokines, excitotoxins or trophic factors (Diaz-Amarilla et al., 2011).

1.4.3. Microglia

Microglia are the brain immune cells that provide the first line of defense against invading microbes and can be the first to detect critical changes in neuronal activity via interactions with neurons. As the resident macrophages of the CNS, microglia are part of the innate immune response and play many significant functions in the normal brain and during neurodevelopment, by providing neurotrophic support (Benarroch, 2013). Microglia are from hematopoietic origin and are essential regulators for cell number and synapse formation during development, revealing changes within maturation process. As proposed in the literature, microglial regulation by the CNS microenvironment can change as a function of age and demands of CNS (Benarroch, 2013). In 2005, two studies showed that resting microglia are not inactive but rather in a state of surveillance. This state is represented by high motility of cells that sample their environment with continuously moving processes with a fast response to brain injury, being able to sense subtle changes through a variety of surface receptors such as purine-receptors or fractalkine (FKN or CX3CL1) receptor (Davalos et al., 2005; Nimmerjahn et al., 2005). When microglia become activated, they migrate towards the damaged cells and clear the debris and may undergo rapid morphological and functional activation, such as change from highly ramified cells towards a more amoeboid-like shape (Xiang et al., 2006), increased phagocytosis and antigen presentation. Also, microglial activation is associated with expression of a broad spectrum of factors that can modulate the functions of surrounding cells, such as chemokines, cytokines, ROS or neurotrophic factors (Nakamura, 2002) and surface markers, such as CD11b (Roy et al., 2006). The role of microglial cells has demonstrated to be very complex and the final result of their activation is likely to depend on the stimulus by which it occurs, the type of neuronal damage, the release of cytokines and the interplay with surrounding cells (Minghetti and Levi, 1998).

In 2003, Clement and colleagues, by using a model of chimeric mice in which the expression of $SOD1^{G93A}$ was induced in MNs or glial cells, report that injury to MNs requires damage from mSOD1 acting within non-neuronal cells since normal MNs in the chimeras develop ALS features. Moreover, non-neuronal cells free of mSOD1 delayed degeneration and significantly prolonged survival of mSOD1 expressing MNs (Clement et al., 2003). In accordance, an important role has been addressed by observing that wild-type microglia slowed neuron loss and disease progression in mice expressing mSOD1 suggesting that even if microglial cells are not directly involved in disease onset they do influence disease progression (Beers et al., 2006; Boillée et al., 2006b). Hence, it can be presumed that mSOD1 in MNs affects disease onset while mSOD1 in microglia leads to the propagation of disease at

later stages. More recently, it was observed that microglia is highly reactive in preclinical stages of ALS in the transgenic rat model with mSOD1^{G93A} (Graber et al., 2010) however its ablation in spinal cord close to clinical onset has not shown to protect MNs (Gowing et al., 2008). Moreover, replacement of microglial cells expressing mSOD1 using clodronate liposomes significantly slowed disease progression and prolonged survival of the transgenic ALS mice (Lee et al., 2012).

In this way, a strong possibility concerning microglia role in ALS is that microglia may assume different phenotypes along the disease course, becoming activated in the early stages in anti-inflammatory-like phenotype and then, in the later stages, become pro-inflammatory and further dystrophic, with no ability to have an active immune response and further contributing to disease progression (Appel et al., 2011; Brites and Vaz, 2014).

A general overview of the diversity of phenotypes that microglia may assume is schematically represented in **Figure I.3**, and will be further detailed in section I.2.1.

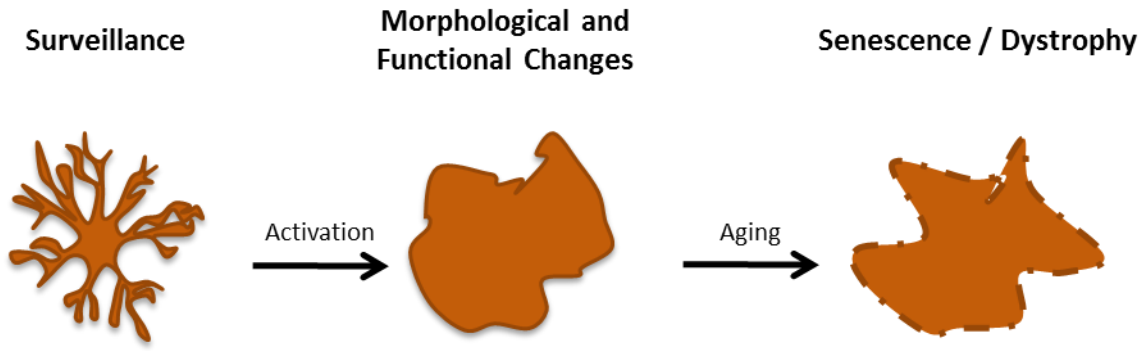


Figure I.3. General overview of microglia phenotypes. Under normal conditions, microglia remains highly ramified in a surveillant state, sampling the environment. Activation may occur through multiple stimuli causing them to change from highly ramified cells to amoeboid-like shape, with an active immune response through increased phagocytosis and antigen presentation, release of cytokines and soluble factors capable of modulate the inflammatory process. However, microglia may become senescent or dystrophic with aging, showing fragmented cytoplasmic processes and low ability to respond to external stimuli.

Microglia activation has been reported in the brain (Turner et al., 2004) and spinal cord of both patients (Henkel et al., 2004) and different mSOD1 mouse models (Beers et al., 2011), being now seen as a prominent pathological feature in ALS (Brites and Vaz, 2014).

In summary, glial cells play an important role for ALS onset/progression. A schematic interaction between those cells with MNs, and the main molecules involved in such dialogue that can be altered in ALS, are summarized in **Figure I.4**.

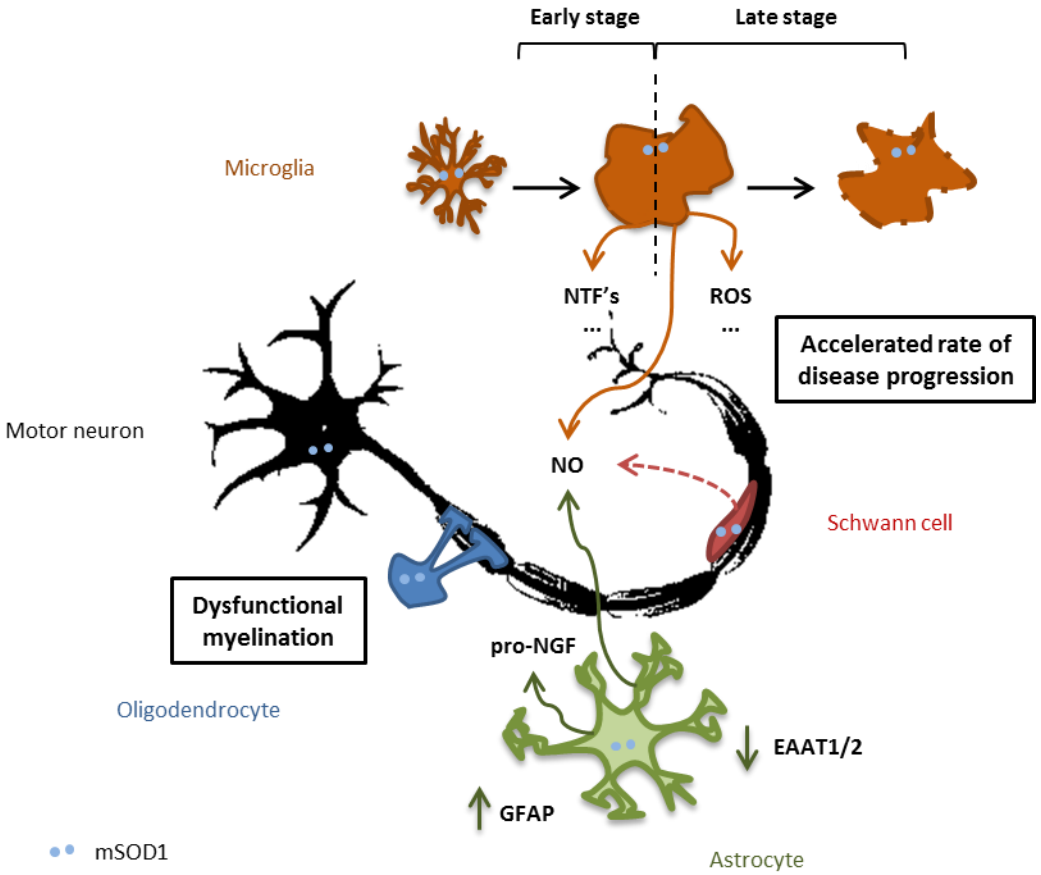


Figure I.4. The role of glial cells in ALS. ALS is a non cell-autononous disease with glial cells exerting an imperative role in its course. Injury in oligodendrocytes is translated in myelination abnormalities. The role of Schwann cells is somehow controversial; mutated SOD1 (mSOD1) in these cells is suggested to have a protective role although, at some point, they may be contributing to axonal damage of motor neurons (MNs) through increased levels of nitric oxide (NO). Astrogliosis is a feature of ALS with increased expression of glial fibrillary acidic protein (GFAP), and astrocytes are reported to release toxic soluble factors, as pro-nerve growth factor (pro-NGF), promoting injury in MNs. Also, decreased expression of the glutamate transporters, excitatory amino acid transporters 1 and 2 (EAAT1 and EAAT2, respectively) were observed, expected to be a cause of glutamate excitotoxicity. Microglia is thought to have a beneficial action in the early stages of the disease, through the release of neurotrophic factors (NTFs), whereas it's thought to contribute to an accelerated rate of disease progression in the later stages, when they become toxic, through the release of reactive oxygen species (ROS), and lately dystrophic.

2. Microglia: a key player in the pathoprogression of ALS

2.1. Variable role: from surveillant to neuroprotective or neurotoxic

As seen previously, under normal conditions microglial cells have a surveillance function essential to keep CNS homeostasis. It is widely accepted that these cells make brief, repetitive contacts with synapses and rapidly retract (Benarroch, 2013). Activated microglia are very plastic cells that may exert various phenotypes and shift their activation state in agreement with microenvironmental changes. Their main role is to initiate appropriate responses, such as inflammation. Although neuroinflammation is crucial in order to protect CNS, uncontrolled and consequent chronic neuroinflammation is harmful and leads to cellular injury (Cherry et al., 2014).

There is increasing evidence highlighting neuroinflammation as a prominent pathological hallmark in ALS, not only in animal models but also in patients. One of the most noticeable features of neuroinflammation is the presence of activated microglia at sites of MN injury. So far, studies have shown that activated microglia may have distinct phenotypic states, being able to exert either a toxic or protective effect on neurons depending on the stimulus by which they are activated. Classically activated microglia (M1) are cytotoxic due to the secretion of ROS and pro-inflammatory cytokines such as interleukin (IL)-6, IL-1β, TNF-α, interferon-γ. Alternatively activated microglia (M2), in a general way, block pro-inflammatory response and produce high levels of anti-inflammatory cytokines including IL-4 and IL-10, and neurotrophic factors like IGF-1 and tumor growth factor β (TGF-β) (Appel et al., 2011). Yet, this M2 phenotype has been further subdivided in three subgroups: M2a, M2b and M2c. M2a phenotype appears to be associated with suppression of inflammation, since it leads, to inhibition of inflammatory associated signaling molecules such as nuclear factor-κB (NF-kB) isoforms; M2b phenotype seem to be potential immunoregulator or initiator of the M2 response in general, once these cells are able to induce Th2 T cells (Cherry et al., 2014). Lastly, M2c primarily typified as a deactivated state, is now thought to be involved in tissue remodeling and matrix deposition after downregulation of inflammation (Cherry et al., 2014), and in phagocytosis, suggested to be through a Milk-fat globule EGF factor-8 (MFG-E8)-dependent manner (Brites and Vaz, 2014). The mechanisms underlying this shift from neuroprotective to neurotoxic microglia remains elusive, however strong evidence points towards the MN-microglia cross-talk **(**see **Figure I.5)**.

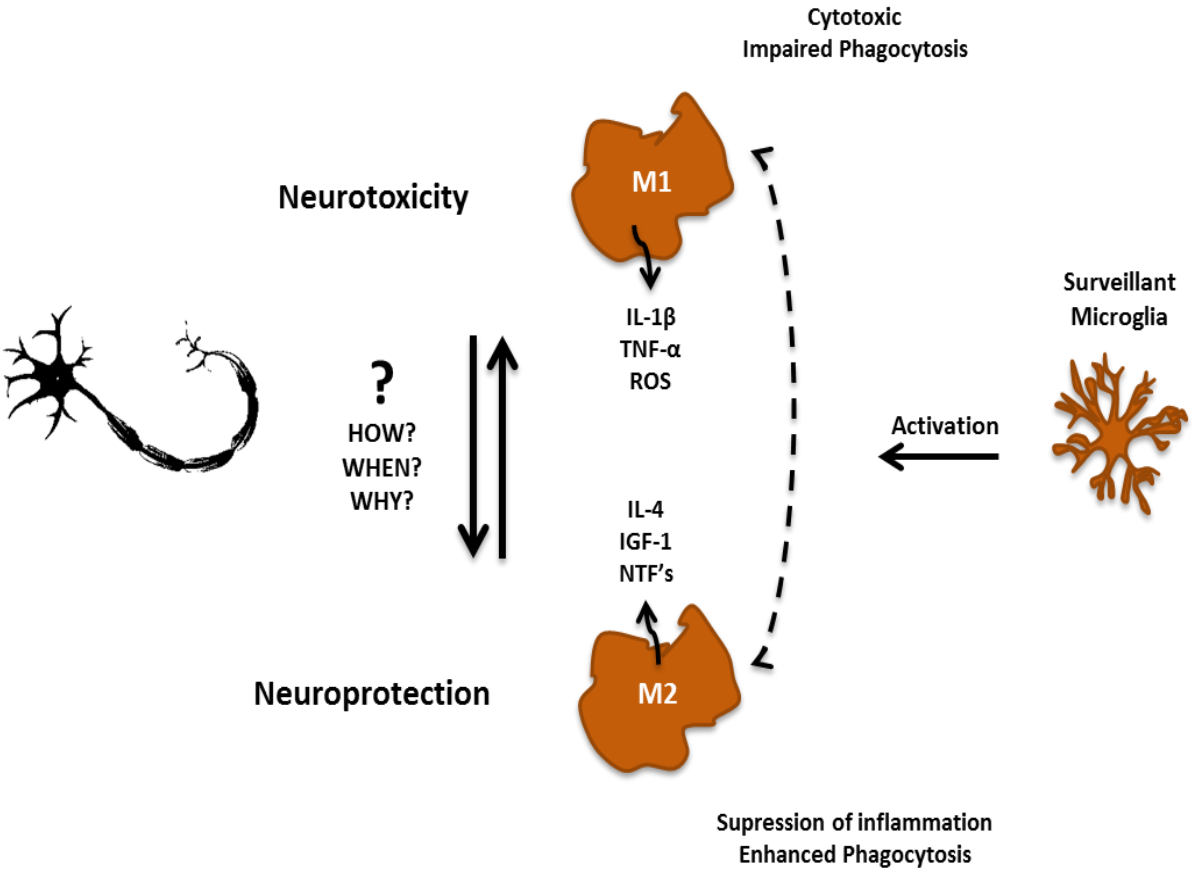


Figure I.5. Microglia's shift from surveillant to neuroprotective or neurotoxic in ALS is thought to be through interaction with MNs. Depending on the type and duration of the stimulus by which microglia are activated, these cells may turn into an anti-inflammatory phenotype, so called M2 phenotype, or into a cytotoxic phenotype (M1). M2 microglia, in general, have enhanced phagocytosis and act by the release of neurotrophic factors and anti-inflammatory cytokines such as insulin-like growth factor 1 (IGF-1) and interleukin(IL)-4, respectively, in a way to supress inflammation and help injured motor neurons (MNs). M1 microglia have impaired phagocytic ability and are cytotoxic due to secretion of pro-inflammatory cytokines, as IL-1β and tumor necrosis factor α (TNF-α), and reactive oxygen species (ROS) and promote cells' injury. It remains elusive the cause of this change of phenotypes, though in ALS MNs are believed to have a crucial role.

In addition to the activation of microglial cells, infiltrating lymphocytes at sites of MN injury may also constitute a feature of neuroinflammation in ALS pathogenesis (Henkel et al., 2009). $CD4^+$ T cells are believed to cross-talk with microglia and promote their neuroprotective or neurotoxic phenotype being a possible intervenient in the MN-microglial dialogue in ALS. Indeed, Appel et al. (2010) reported that in mSOD1 mouse model, the lack of $CD4^+$ T cells leads to faster progression of MN disease, implying a neuroprotective role of these cells. It also eliminates the early slow phase of disease together with increased pro-inflammatory and cytotoxic factors, diminished anti-inflammatory and neurotrophic factors as well as survival.

However, it is described a shift from protective to injurious T cells along with transformation of M2 to M1 phenotype along disease progression, which also implies a well-orchestrated cross-talk among microglia, neurons and T cells (Zhao et al., 2013).

2.2. Motor neuron-microglia cross-talk in ALS

2.2.1. Ligand-receptor interaction

As seen previously, it is believed that there is an immunological shift from neuroprotection to neurotoxicity with major contributes to ALS disease progression (Appel et al., 2011). Once established an imperative role of microglial cells and their mutable action, the questions now are how, when and why this shift occurs and a strong possibility is that the answer lies in the cross-talk between MNs and microglia.

Neurons may modulate microglia phenotype through the release of molecules such as the CD200 neuronal glycoprotein, suggested to suppress the pro-inflammatory-like state of microglia, and the chemokine FKN, likely to maintain the resting/ramified microglial state or promote antioxidant effects (Suzumura, 2013). CD200 has been reported to promote a down-regulation of the activated state of microglia through interaction with its receptor CD200R, predominantly expressed by myeloid cells (Deckert et al., 2006). Regarding ALS studies, by analyzing transgenic $SOD1^{G93A}$ mice during pre-symptomatic and symptomatic stages, Chen and co-workers observed a significant up-regulation of CD200R in the pre-symptomatic stage, though no alterations on CD200 were reported (Chen et al., 2004).

On the other hand, under pathological conditions, damaged neurons will release signals such as high mobility group box 1 (HMGB1) that will lead to microglial activation (Suzumura, 2013). Interestingly, in ALS context, extracellular mSOD1 was found to be able to induce activation of wild-type microglia to a pro-inflammatory-like state, in the same way the lipopolysaccharide (LPS) does (Zhao et al., 2010), by interacting with CD14, a receptor for misfolded proteins, which ligates TLR-2 and TLR-4, co-receptors, and activate a pro-inflammatory cascade, as suggested by Appel et al. (2011) **(Figure I.6)**.

The aforementioned chemokine FKN and its receptor CX3CR1, as well as the alarmin HMGB1 are two promising intervenient that are likely to play an essential role in MN-microglia cross talk in ALS, and that will be further detailed below.

2.2.1.1. Fractalkine

Fractalkine is the only member of a unique subgroup of chemokines, a family of relatively low mass proteins within the major group of cytokines that chemo-attract and activate inflammatory cells, with particular characteristics: it may exist both in a membrane-bound form or a soluble form, which may be cleaved by matrix metalloproteinases (MMPs), (Sheridan and Murphy, 2013), and binds to, so far only known specific receptor, CX3CR1, which mediates both adhesive and migratory functions of FKN (Imai et al., 1997) **(Figure I.6)**. Identified by Pan et al. (1997), FKN is located predominantly in the brain and was shown to be constitutively expressed by neurons whereas its highly specific receptor, CX3CR1, is mostly expressed in microglial cells, although it has also been reported in hippocampal neurons (Meucci et al., 2000) and astrocytes (Maciejewski-Lenoir et al., 1999). The FKN-CX3CR1 axis is reported to mediate neuron-microglial interaction, synaptic transmission and neuronal protection from toxic insults (Sheridan and Murphy, 2013).

FKN was shown to have a beneficial action in an *in vitro* model, decreasing the secretion of pro-inflammatory cytokines and ROS by microglia activated with LPS, a previous mentioned strong exogenous activator of microglial cells (Mizuno et al., 2003). Nevertheless, in human Parkinson's disease patients, the plasma levels of soluble FKN were reported to be positively correlated with disease severity and progression (Shi et al., 2011). Regarding its receptor, CX3CR1 deficiency in transgenic mouse model of ALS was reported to trigger microglial neurotoxicity and it was proposed by the authors that enhancing CX3CR1 signaling could protect neurons against toxicity (Cardona et al., 2006).

2.2.1.2 High mobility group box 1

HMGB1 is a ubiquitous nuclear protein with multiple functions able to act as a transcription factor, promoting expression of pro-inflammatory cytokines, and to bind receptors as receptor for advanced glycation endproducts (RAGE) and TLR-2 or TLR-4, triggering a pro-inflammatory cascade **(Figure I.6)**. It is known to be released from necrotic cells, retained by apoptotic cells or actively secreted by activated immune cells, such as monocytes, macrophages and microglia, in response, for example, to LPS stimulus (Zurolo et al., 2011). Lo Coco et al. (2007) described a progressive reduction of HMGB1 within MNs of $SOD1^{G93A}$ mice and a prominent immunoreactivity in the nucleus of glial cells and hypothesized that HMGB1 could be released from damaged MNs and act as inflammatory signal while contributing to proliferation and hypertrophy of glial cells. Casula et al. (2011) observed a cytoplasmic translocation of HMGB1 in activated microglia and astrocytes from spinal cord tissue of sALS patients, suggesting that extracellular HMGB1 is derived mostly from glial cells and arising the hypothesis that may exist a positive feedback loop leading to an amplified inflammatory response.

2.2.2. Phagocytosis

The clearance of pathogens, apoptotic and senescent cells from the organism is performed by immune cells trough a well conserved process termed phagocytosis. This process is characterized by the engulfment of particles and cells and is of outmost importance for the development and homeostasis of organisms, as defective or inefficient clearance may contribute to several human pathologies (Neher et al., 2013). As resident macrophages of the CNS, microglia are in charge of this process, possibly through an M2c activated state, as seen previously. MFG-E8, or lactaderin, is a protein known to mediate phagocytosis in the peripheral immune system by macrophages (Li et al., 2013). The proposed mechanism of action implies that MFG-E8 acts as a bridge between the apoptotic cell and the macrophage by binding exposed phosphatidylserine (PS) and vitronectin present in the membrane of the macrophage (Aziz et al., 2011) **(Figure I.6)**. Fuller and Van Eldik (2008) reported that microglial phagocytosis of apoptotic neurons was mediated by MFG-E8 and suggested that a dysregulation in this process could be implicated in neurodegeneration. Interestingly, Fricker et al. (2012) reported that there is phagocytosis of viable neurons by microglia activated with LPS via an MFG-E8-dependent pathway. These results suggest that, although, phagocytosis is associated with an anti-inflammatory phenotype, MFG-E8-dependent pathway may be a promising intervenient in ALS pathogenesis involved in dysfunctional phagocytosis and possibly leading to the exacerbated inflammation, likely to occur in ALS.

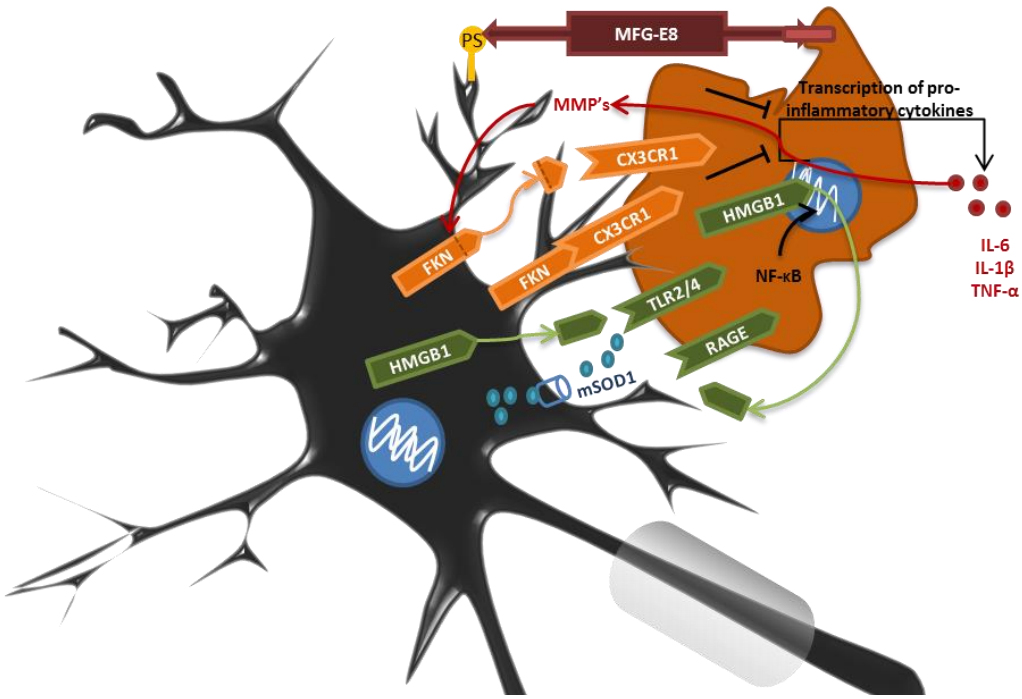


Figure I.6. Molecular pathways involved in MN-microglia cross-talk focusing on FKN/CX3CR1 axis, MFG-E8-mediated phagocytic pathway and HMGB1 signaling. Motor neurons (MNs) modulate microglia response through diverse signs. High mobility group box 1 (HMGB1) may be released from injured MNs or secreted by activated microglia, and act in receptors present in microglial cells, such as toll-like receptor 2 or 4 (TLR 2 or 4, respectively) and receptor for advanced gycation endproducts (RAGE), thus starting a pro-inflammatory cascade via activation of nuclear factor-κB (NF-kB). On the other hand, it can also act as a transcription factor in the nucleus, and promote the transcription of pro-inflammatory cytokines, such as interleukin(IL)-6, IL-1β, tumor necrosis factor α (TNF-α). The chemokine fractalkine (FKN) may exert its functions by directly bind to its receptor CX3CR1, in microglia, or it can be cleaved by matrix metalloproteinases (MMPs) which expression is induced by pro-inflammatory cytokines. Both membrane and soluble FKN are likely to attenuate the pro-inflammatory state of microglia. Concerning amyotrophic lateral sclerosis (ALS), mutated SOD1 (mSOD1) may be released from MNs and bind TLRs acting as a signal from injured cells. Apoptotic MNs expose phosphatildyserine (PS) in their membranes which can be further recognized by Milk fat globule-EGF factor 8 (MFG-E8), acting as a bridge between the phagocytic microglia and the dying MN.

3. ALS research mice models

Due to the similarities previously mentioned for non-genetic and genetic ALS, mSOD1 animal and cellular models constitute the most common tool to study the disease and they are also important to perform parallel experiments in more than one model system to better understand different aspects of a process and in order to have more accurate results.

3.1. In vitro models

In vitro models, as cell or organotypic cultures, have an increased yield and homogeneity in comparison with *in* vivo models that allows more data output in a shorter time.

3.1.1. Primary cultures and cell lines

Primary cultures refer to cells that are obtained from the tissue and maintained in culture. These may be composed of mixed cell types that can be further sorted in order to have the desired cell type to study. Primary spinal cord cultures have been used as a good model to study morphological, biochemical and electrophysiological characteristics of MNs (Tovar et al., 2009). They are obtained from 12-14 days-old rodent embryos, where the spinal cord is dissociated by mechanical and enzymatic procedures and plated on matrix-coated dishes. Although this culture system is very limited in terms of simulating *in vivo* conditions, it offers the possibility of studying, for instance, intracellular mechanisms such as whose triggering MN injury in SOD1-ALS. MNs can be induced to express various copies of the gene of interest in order to reproduce an ALS phenotype, as mSOD1^{G93A}, through microinjection of the vector into the cells identified by their specific morphology (Tradewell et al., 2011). However, cultures of MNs without the trophic support of glial cells are known to be difficult to maintain for more than 2 weeks. Moreover, the use of primary cells from embryos may fail to reproduce some features of the adult phenotype.

Cell lines constitute a good model to overcome some limited aspects of primary cultures such as yields and time of maintenance of the culture. In 1992, Cashman and co-workers developed a cell line (NSC-34) of immortalized neurons able to reproduce selected aspects of MN development. NSC-34 cell line is a hybrid cell line of neuroblastoma and spinal cord MNs from enriched primary cultures. These cells are described to have a rounded morphology within the first 24-48 hours and after they evidence morphological and physiological properties of MNs, including extension of processes, formation of contacts with cultured myotubes, support of action potentials and expression of neurofilament proteins, among other features (Cashman et al., 1992). Moreover, these cells may be able to model aspects of neuromuscular synapse formation, as referred by Martinou et al. (1991). NSC-34 cell line expressing mSOD1 is a well-accepted cellular model of ALS in which some features of MN degeneration have been described, as Golgi apparatus fragmentation or mitochondrial dysfunction (Tovar et al., 2009). More recently, it has been established in our lab that NSC-34/hSOD1^{G93A} reveals features of mitochondrial dysfunction, energy impairment, oxidative stress, as well as apoptosis and inflammation-related processes (Vaz et al., 2014). All of these are commonly described processes in transgenic mice models of the disease and in ALS patients. Studies in our laboratory have also shown that this model

demonstrated the efficacy of the anti-inflammatory and anti-oxidant bile acid, glycoursodeoxycholic acid (GUDCA) (Vaz et al., 2014).

In vitro cultures of microglia allow the study of the activation state, released factors, motility, among others. The N9 murine microglial cell line was developed by immortalization of E13 mouse embryonic cultures with the v-myc or v-mil oncogenes of the avian retrovirus MH2. It has been largely used as illustrative of mouse microglial cells since it is derived from mouse brain and has many phenotypical characteristics of primary mouse microglia in producing substantial amounts of NO and various cytokines after stimulation (Stansley et al., 2012).

3.1.2. Mixed cultures and co-cultures

Mixed cultures and/or co-cultures constitute a valuable tool for the study of specific interactions among cells being studied, discarding any external interference (Zhang and Fedoroff, 1996). Also, cells with a target mutation can be combined with healthy cells in order to better understand the role of which cell type in the disease. For instance, combining cultures of NSC-34, either transfected with wtSOD1 or mSOD1, and N9 cell line, is likely to be a valuable model to study the interaction between MNs and healthy microglia.

3.1.3. Organotypic cultures

Organotypic cultures constitute a three-dimensional culture system which has biochemical and physiological properties more similar to *in vivo* tissue (Ravikumar et al., 2012). Organotypic slice cultures can be achieved from embryos and postnatal animals and the slices are obtained from 200-400 µm-thick transversal sections that are transferred into membrane inserts to a 6-well or 12-well culture plates and can be used for more than two months (Delfs et al., 1989). The slices may be used to perform multiple analyses like immunohistochemical staining or electrophysiological recordings. This culture system constitute a good model to preserve important processes that occur in ALS, as cellular interactions between MNs and neighboring cells, once the whole slice is cultivated in an organotypic culture. Although this culture system allows dynamic studies with various drugs, it doesn't completely recapitulate what happens *in vivo* (Tovar et al., 2009). An important feature of these cultures is that they may constitute a first approach before using *in vivo* models, in order to reduce the number of animals sacrificed in experiments.

3.2. *In vivo* models

3.2.1. Transgenic mSOD1 mice

Vertebrate models allow the establishment of landmarks of disease progression and the understanding of the functional consequences of gene mutations. The laboratory mouse *Mus musculus* is widely used as a study model because of its close genetic and physiological similarities to humans, the possibility of genome manipulation and its relatively high reproductive rate.

Nowadays, the SOD1 transgenic mice constitute the most widely used model of ALS. The first model carried an ALS-causative point mutation resulting in a glycine to alanine substitution at residue

93 (G93A) and was shown to recapitulate the paralytic phenotype of ALS (Gurney et al., 1994). Therefore, this model is useful for examining the pathophysiology of MN degeneration in ALS, as briefly reviewed above, and has provided a tool for developing preclinical data on drugs that may be used to slow the course of the disease. Though G93A is currently the main study model, also G37R, G85R, G86R, among other mutations in SOD1, are used as study models of this disease, since they were shown to also recapitulate some classical features of MN degeneration (Turner and Talbot, 2008).

Still, there are also other transgenic mice being used as a model to study ALS, harboring mutations in other ALS-associated proteins, such as TDP-43 and FUS. Namely, last year, transgenic mice expressing a variant of FUS protein were create, reported to recapitulate some features of human ALS FUS, such as the presence of FUS-positive inclusions, abrupt disease onset and fast progression, and motor impairment (Shelkovnikova et al., 2013). As for TDP-43, there exist now transgenic mice carrying TDP-43 transgenes, either WT or mutated (A315T or G348C), that mimic multiple aspects of ALS, such as age-related development of motor and cognitive dysfunction, cytoplasmic TDP-43-positive ubiquitinated inclusions, axonopathy, intermediate filament abnormalities, as well as neuroinflammation (Swarup et al., 2011).

4. Therapeutic approaches – recent findings

A century after it has been first described, there is still no effective treatment for ALS. The only FDA approved drug *Riluzole* possess anti-glutamatergic properties but may only increase survival by two or three months (Bensimon et al., 1994).

Given the evidence of non-cell autonomy in ALS, therapeutic shifted its focus from neuron to their interaction with non-neuronal cells. Stem cell research aims to replace damaged cells in injurious regions. For instance, regarding the determinant role of microglia in ALS course, replacing mutant microglia for healthy microglia may serve as a source of neurotrophic factors and provide protection delaying MN degeneration and disease progression. In fact, Lee et al. (2012) reported that replacing microglial cells by bone marrow transplantation in SOD1^{G93A} mouse model slowed disease progression and prolonged survival elucidating the therapeutic potential of these cells.

In addition, treatments that may down-regulate toxic responses of innate and adaptive immune cells and up-regulate the beneficial responses could also slow ALS progression. For that, modulation of microglial phenotype is becoming an appealing neurotherapeutic strategy. Interestingly, bone marrow transplantation with stem cell factor, in transgenic SOD1^{G93A} mice, was reported to change microglia phenotype towards a neuroprotective state, resulting in increased MN function and survival (Terashima et al., 2014).

Herewith, mesenchymal stem cells (MSCs) are arising as a promising therapeutic strategy given the relative availability and their potential for autologous cellular therapy (Kassem et al., 2004). Delivery of MSCs in SOD1^{G93A} mice promotes multiple beneficial effects on disease course as improved motor function, decrease in MN loss and prolonged survival. Besides, intraspinal MSC transplants lead to improvement on neuroinflammation effects, astrogliosis and microglial activation. Moreover, MSCs are being considered to serve as vehicle to deliver neuroprotective factors to CNS. Intracerebroventricular injection of umbilical cord stem cells (UBCs), capable of differentiate into mesenchymal cells, in SOD1^{G93A} mice diminished disease progression and enhanced survival up to 10% and those effects are thought to be mediated by production and release of anti-inflammatory cytokines and chemokines. Likewise, retro-orbital delivery of UBC's improved neuromuscular transmission and intravenous administration delayed disease progression by 15%, promoted anti-inflammatory responses, decreased microglial activation and enhanced survival by 20-25% (Lunn et al., 2014).

Another strategic approach concerning therapy in ALS may involve the bile acid ursodeoxycholic acid (UDCA). UDCA was hypothesized as a therapeutic for ALS and used in a clinical trial (Min et al., 2012) due to its cytoprotective mechanisms. Its glyco-conjugated form - GUDCA, also showed anti-apoptotic, immunomodulatory and anti-oxidant effects (Fernandes et al., 2007; Vaz et al., 2010). Interestingly, recent studies in our lab with NSC-34/hSOD1^{G93A} cells, have reported that GUDCA showed beneficial effects concerning MN degeneration, such as inhibition of MMP-9 and caspase-9 increased activities, highlighting its potential use in ALS therapy in slowing disease onset and progression (Vaz et al., 2014).

A major understanding of the upstream pathogenic mechanisms underlying ALS is still lacking which greatly inhibited the development of successful targeted therapies so far, so efforts must continue to be done to accurate the most efficacious cell type, or cell types, in order to reach meaningful therapies for ALS patients.

5. Aims

With this thesis we aim to dissect the MN-microglia signaling pathways that are compromised in MN-microglia homeostasis, by using an *in vitro* model of ALS. We will focus on the production and release of mediators that are involved in cellular demise and neuroinflammation, as well as specific molecules involved in MN-microglia cross-talk that may be compromised.

Therefore, the specific aims are:

- To uncover the pathways by which MNs are signaling their own injury. For this we will use mono-cultures of a MN-like cell line (NSC-34) stably transfected with human SOD1, either wild-type (wtMNs) or with G93A (mMNs) mutation.

- To further dissect how MNs are modulating microglial immune response and the contribution of healthy microglia to rescue MN degeneration. For this we will use mixed cultures of a MN-like cell line (NSC-34) stably transfected with human SOD1, either wild-type (wtMNs) or with G93A (mMNs) mutation, and healthy N9 microglial cell line.

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), penicilin/streptomycin, L-glutamine and nonessential aminoacids (NEAA) were purchased from Biochrom AG (Berlim, Germany). RPMI-1640 medium, poly-D-lysine (PDL), trypsin-EDTA solution (1X), trypsin-EDTA solution (10X), ATP, Hoechst 33258 dye, bovine serum albumin (BSA) were purchased from Sigma-Aldrich (St.Louis, MO, USA). Geneticin 418 sulfate (G418) was obtained from Calbiochem (Darmstadt, Germany). L-glutamic acid kit and Triton X-100 were obtained from Roche Diagnostics (Mannhein, Germany). Nitrocellulose membrane was obtained from Amersham Biosciences (Piscataway, NJ, USA). Cell lysis buffer® and LumiGLO® were purchased from Cell Signaling (Beverly, MA, USA). Luminaris HiGreen High ROX qPCR Master Mix was obtained from Thermo Scientific (Waltham, MA, USA). Trizol was obtained from Invitrogen Corporation™ (Carlsbad, CA, USA). All the other chemicals were purchased either from Sigma-Aldrich or Merck.

1.2. Antibodies

Primary antibodies used in this work: goat anti-FKN [FKN (1:100)] and rabbit polyclonal anti-TLR4 (1:200) were purchased from Santa Cruz Biotechnology® (Santa Cruz, CA, USA), mouse anti-HMGB1 (1:200) from BioLegend® (San Diego, CA, USA), rabbit polyclonal anti-MFG-E8 (1:100), rat anti-CD11b (1:100), mouse anti-βIII-tubulin (1:250), mouse anti-β-actin (1:2000) was obtained from Sigma-Aldrich (St. Louis, MO, USA)

Secondary antibodies used in this work: FITC anti-mouse (1:227), Alexa Fluor® 488 anti-rabbit (1:1000) and Alexa Fluor® 594 anti-rat (1:1000) were obtained from Invitrogen Corporation™ (Carlsbad, CA, USA). Horseradish peroxidase-labelled goat anti-rabbit IgG, Horseradish peroxidas-labelled rabbit anti-goat IgG and Horseradish peroxidase-labelled goat anti-mouse IgG were purchased from Santa Cruz Biotechnology® (Santa Cruz, CA, USA).

1.3. Equipment

Fluorescence microscope (model AxioScope.A1) with integrated camera (AxioCamHRm) and optical microscope with phase-contrast equipment (Olympus, model CK2-TR) were used for cell morphology evaluation. Microplate reader (PR 2100 Microplate Reader) was used for nitrites, glutamate and protein measurement and ChemiDoc™ for immunodetection in western blot, both from Bio-Rad Laboratories (Hercules, CA, USA). In order to ensure a stable environment to optimal cell growth (37ºC 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). Guava easyCyte 5HT Base system Flow Cytometer (Merck-Milipore, Darmstadt, Germany) was used for flow cytometry studies. Eppendorf 580R (Eppendorf, Hamburg, Germany) and a Sigma 3K30 centrifuges were used for different experimental procedures. 7300 Real-Time PCR System (Applied Biosystems, CA, USA) was used for qRT-PCR.

2. Methods

2.1. Cell lines

2.1.1. NSC-34 cell line

NSC-34 cell line transfected with human SOD1, either wild type or mutated in G93A [NSC-34/hSOD1^{wt} (wtMNs) or NSC-34/hSOD1^{G93A} (mMNs), respectively] were a gift from Dr. Júlia Costa, Instituto de Tecnologia Química e Biológica (ITQB), Universidade Nova de Lisboa, Portugal and were used as currently in our lab (Vaz et al., 2014). NSC-34/hSOD1^{wt} cells were used as a control. NSC-34 cells were grown in proliferation media (DMEM high glucose, w/o pyruvate, supplemented with 10% of FBS and 1% of penicilin/streptomycin) and selection was made with geneticin sulphate (G418) at 0.5 mg/ml. Medium was changed every 2 to 3 days. Culture plates were coated with PDL before plating the cells. Cells were seeded in 6, 12 or 24-well culture plated 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 cell line was a gift from Dr. Teresa Pais, Institute of Molecular Medicine (IMM), Lisboa, Portugal and were used as currently in our lab (Cunha, 2012; Ferreira, 2013). Cells were cultured in RPMI media supplemented with 10% FBS, 1% L-glutamine and 1% penicillin/streptomycin and incubated in 6, 12 or 24-well culture plates with NSC-34 (mixed-cultures) with a ratio 1:1, and maintained at 37ºC in a humidified atmosphere of 5% CO_2.

2.2. Cell cultures

2.2.1. NSC-34 mono-culture

After 48 hours in proliferation media, differentiation was induced by changing medium for DMEM-F12 plus 1% FBS, 1% NEAA, 1% penicillin/streptomycin and 0.1% G418. Measurements were performed after 4 days in vitro (DIV), as shown in **Figure II.1A**, where mMNs showed high levels of degeneration (Vaz et al., 2014).

2.2.2. NSC-34 and N9 mixed-cultures

For mixed cultures, NSC-34 were grown and differentiated as described for mono-cultures. N9 cells were added after MN differentiation, in a pre-symptomatic-like stage of MNs, as shown in **Figure II.1B**. Cell cultures were maintained at 37ºC in a humidified atmosphere of 5% CO_2 and collected after 4 days of differentiation.

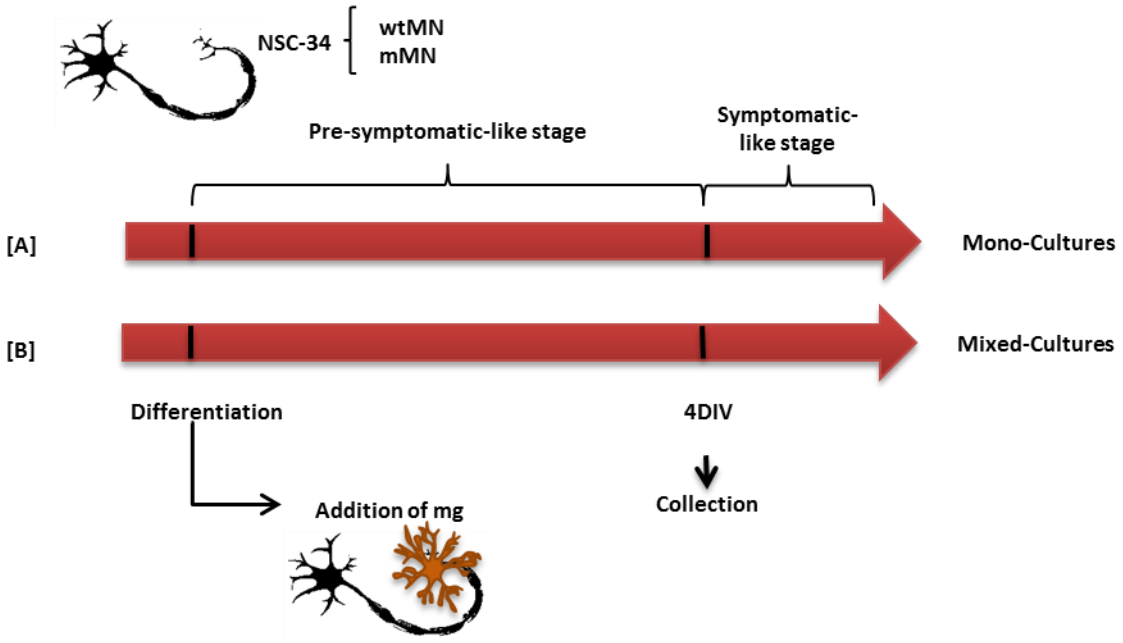


Figure II.1. Schematic representation of the experimental model of mono- and mixed cultures. (A) NSC-34 cells, stably transfected with human superoxide dismutase 1 (SOD1), wild type or with G93A mutation [NSC-34/hSOD1^{wt} (wtMNs) or NSC-34/hSOD1^{G93A} (mMNs)], were grown in proliferation media and selection was made with G418. Differentiation was induced after 48h in culture by changing medium for DMEM-F12 plus 1% FBS and 1% non-essential amino acids. Cell cultures were evaluated after 4 days in vitro (DIV), where mMNs showed high levels of degeneration (Vaz et al., 2014), considered a symptomatic-like stage. (B) In mixed cultures, microglia (N9 cell line) were added after differentiation, considered a pre-symptomatic-like stage, in order to evaluate if they could prevent MN degeneration. Cultures were evaluated on production of nitrites by Griess reaction, release of glutamate by L-Glutamic acid kit, mRNA expression by qRT-PCR, protein analysis by western blot and immunocytochemistry, and cell death by flow cytometry.

2.3. Immunocytochemistry

Immunocytochemistry was performed as usual in our lab (Vaz et al., 2014). NSC-34 cells, either alone or in mixed culture with N9 cells, were fixed with 4% (w/v) paraformaldehyde in PBS. For the immunostaining, cells were first permeabilized with 0.2% Triton X-100, for 20 min, and after incubated with blocking solution (3% BSA in PBS), for 30 min. For the immunostaining of FKN and MFG-E8 proteins, there was no permeabilization of cells. Cells were incubated overnight at 4ºC with primary antibody and then incubated with respective secondary antibody, during 2 hours at room temperature. Cell nuclei were stained with Hoechst 33258 dye (1:1000, Sigma). Fluorescence was visualized using AxioCam HR camera (Zeiss) adapted to an AxioScope® microscope. Ten random fields were acquired per sample, with 400X or 630X magnification.

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 1mM phenylmethyl sulfonyl fluoride (PMSF) for 5min, on ice and shaking. Proteins from the supernatants were precipitated by trichloroacetic acid (TCA) 9:1 washed in cold acetone containing 20mM Dithiothreitol (DTT), and solubilization in sample buffer (Brissette et al., 2012). Protein extracts were sonicated for 20seg followed by centrifugation at 14 000 *g* for 10min, at 4ºC. Protein concentration was determined using the Bradford method (Bradford, 1976) using Bio-Rad's Protein Assay Reagent. Equal amounts of protein were separated on a 10% sodium dodecyl sulfate-polyacrilamide gel electrophoresis (SDS-PAGE). After electrophoresis, proteins were transferred to a nitrocellulose membrane and immunoblot was performed. Membranes were incubated in blocking buffer [Tween 20-Tris buffered saline (T-TBS) plus 0.5% (w/v) non-fat dried milk], for 1hour at room temperature. After, membranes were incubated overnight, at 4ºC, with the following primary antibodies diluted in T-TBS with 5% BSA: goat anti-FKN (1:100, Santa Cruz Biotechnology®), mouse anti-HMGB1 (1:200, BioLegend®), rabbit anti-TLR4 (1:200, Santa Cruz Biotechnology®), rabbit anti-MFG-E8 (1:100, Santa Cruz Biotechnology®), mouse anti-β-actin (1:2000, Sigma-Aldrich). Next, membranes were washed with T-TBS and incubated for 1 hour at room temperature with respective secondary antibodies diluted in blocking solution: goat anti-rabbit HRP-linked, goat anti-mouse HRP-linked, or rabbit anti-goat HRP-linked (each 1:5000, Santa Cruz Biotechnology®). After washing with T-TBS, chemiluminescent detection was performed by using LumiGLO® reagent. Bands were visualized in Chemidoc equipment and relative intensities of protein were analized using Image Lab™ analysis software (both from Bio-Rad Laboratories, Hercules, CA, USA). Results from lysates and extracellular media were normalized to β-actin or Amidoblack protein stain, respectively.

2.5. Gelatin Zymography

Gelatin zymography was performed as usual in our lab (Silva et al., 2010). MMP-2 and MMP-9 activity in the extracellular media of NSC-34 mono-cultures and NSC-34/N9 mixed-cultures was evaluated by SDS-PAGE zymography, in 0.1% gelatin-10% acrylamide gel, under non-reducing conditions. After, the gel was washed for 1 h with 2.5% Triton-X-100 (in 50 mM Tris pH 7.4; 5 mM CaC2; 1 μM ZnCl2) to remove SDS and to renature the MMP species in the gel. In order to induce gelatin lysis,

the gel was incubated at 37ºC in the developing buffer (50 mM Tris 7.4; 5 mM CaC2; 1 µM ZnCl2), overnight. For enzyme activity analysis, the gel was stained with 0.5% Coomassie Brilliant Blue R-250 and destained in 30% ethanol/10% acetic acid/H_2O. Gelatin activity was analyzed by measuring the white band on the blue background, by using computerized image analysis (Image Lab) and normalized for total protein.

2.6. Quantification of nitrite levels

Nitric oxide levels were estimated in differentiation media of NSC-34 mono-cultures and NSC-34/N9 mixed-cultures, by quantifying the concentration of nitrites (NO_2) that are a result of NO metabolism. After a brief centrifugation, 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. Absorbance at 540 nm was determined by using a microplate reader. A calibration curve of standard nitrites was performed for every assay. Samples and standards were analyzed in duplicate and the mean value was used (Vaz et al., 2014).

2.7. Measurement of extracellular glutamate

Extracellular glutamate was determined in the differentiation media from NSC-34 mono-cultures and NSC-34/N9 mixed-cultures, by using the L-glutamic acid kit (Roche). For the reaction, a 96-well microplate was used and the absorbance was measured at 490 nm, by using a microplate reader. A calibration curve of glutamic acid was performed for every assay. Samples and standards were analyzed in duplicate and the mean value was used (Falcão et al., 2005).

2.8. Flow cytometry

After 4 days of differentiation, extracellular media was collected to 2 ml tubes and cells were detached by using a solution of trypsin 1X for 5 min at 37ºC. Next, FBS 10% was added, in order to stop the action of trypsin, and cells were collected to the tubes with extracellular media and centrifuged at 500 *g* for 5 min (Eppendorf, 5810R). The supernatant was discharged and the pellet resuspended in 400 µL of 1% BSA in PBS. The samples were added to 96-well plates and incubated with Nexin Reagent® (Anexin V-PE/7AAD), for 20 min in the dark, at room temperature. Samples were then analyzed on Guava easyCyte 5HT Base System Flow Cytometer (Merck-Millipore). 5000 events per sample were counted. This assay allows the distinction of three cellular populations: viable cells (annexin V-PE^-/$7AAD^-$), early-apoptotic cells (annexin V-PE^+/$7AAD^-$) and late apoptotic/necrotic cells (annexin V-PE^-/$7AAD^+$) (Barateiro et al., 2012).

2.9. qRT-PCR

Total RNA was extracted from 6-well tissue culture plates using Trizol Reagent (Invitrogen). RNA concentration was quantified using Nanodrop ND-100 Spectrophotometer (NanoDrop Technologies).

The sequences used as primers were: HMGB1 FWR 5'-CTCAGAGAGGTGGAAGACCATGT-3' and REV 5'-GGGATGTAGGTTTTCATTTCTCTTTC-3'; MFG-E8 FWR 5'TGACTTTGGACACACAGCGT-3' and REV 5'GTGTAGAACAACGGGAGGCT-3'; TLR-4 FWR 5'-ACCTGGCTGGTTTACACGTC-3' and REV 5'-GTGCCAGAGACATTGCAGAA-3'; CD11b FWR 5'CAGATCAACAATGTGACCGTATGGG-3' and REV 5'CATCATGTCCTTGTACTGCCGCTTG-3'; FKN FWR 5'-CTCACGAATCCCAGTGGCTT-3' and REV 5'TTTCTCCTTCGGGTCAGCAC-5'; CX3CR1 FWR 5'- TCGTCTTCACGTTCGGTCTG-3' and REV 5'-CTCAAGGCCAGGTTCAGGAG-3'; β-actin FWR 5´GCTCCGGCATGTGCAA-3' and REV 5'-AGGATCTTCATGAGGTAGT-3'. qRT-PCR was performed on a 7300 Real-Time PCR System (Applied Biosystems) using Luminaris HiGreen High ROX qPCR Master Mix (Thermo Scientific). The PCR was performed in 8-well strips, with each sample performed in duplicate, and a non-template control (NTC) was included for each amplificate (Barateiro et al., 2013).

2.10. Statistical analysis

Results of at least three different experiments were expressed as mean ± SEM for NSC-34 cultures, either alone or in mixed culture with N9 cells. Comparisons between the different parameters evaluated in NSC-34/hSOD1^{wt} and NSC-34/hSOD1^{G93A} cells, with or without microglia, were done via two-tailed Student's t-test for equal or unequal variance, as appropriate, using GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA). $p<0.05$ was considered statistically significant, $p<0.01$ very significant and $p<0.001$ highly significant.

III. RESULTS

1. Characterization of mMN-microglia mixed cultures

Our model of mixed cultures was obtained by adding healthy microglia (N9 cell line) to motor neuron-like cells (NSC-34) expressing human SOD1, either wt (wtMNs) or mutated in G93A (mMNs), before motor neuron degeneration, as described in Methods. Mixed cell cultures were collected after 4 days in culture, since at this time point, differentiated mMNs have features of neuronal dysfunction, namely mitochondrial loss of viability and MMP-9 activation as previously observed in our lab (Vaz et al., 2014).

1.1. Microglial cells are more vulnerable, yet more activated, in the presence of mMNs than wtMNs

In order to first characterize our model of mixed cultures at 4 DIV, we performed flow cytometry studies to evaluate cell viability, which was shown to be compromised in the presence of mMNs, either alone and in mixed culture with microglia ($p<0.01$) **(Fig III.1A)**. We also evaluated apoptotic and necrotic levels by using a specific reagent that comprises two dyes, Annexin V-PE and 7AAD, allowing the distinction between early apoptosis and late apoptosis/necrosis, respectively. We observed that apoptosis is likely to be the main cell death pathway in our model, since levels of early apoptosis were higher in mMNs, either alone and in mixed cultures with microglia ($p<0.05$), than wtMNs **(Figure III.1B)**, whereas necrotic levels were lower than 10% in all cases **(Figure III.1C)**. The decreased viability seen in mixed cultures of mMNs and microglia, suggests a disrupted homeostasis of these cells.

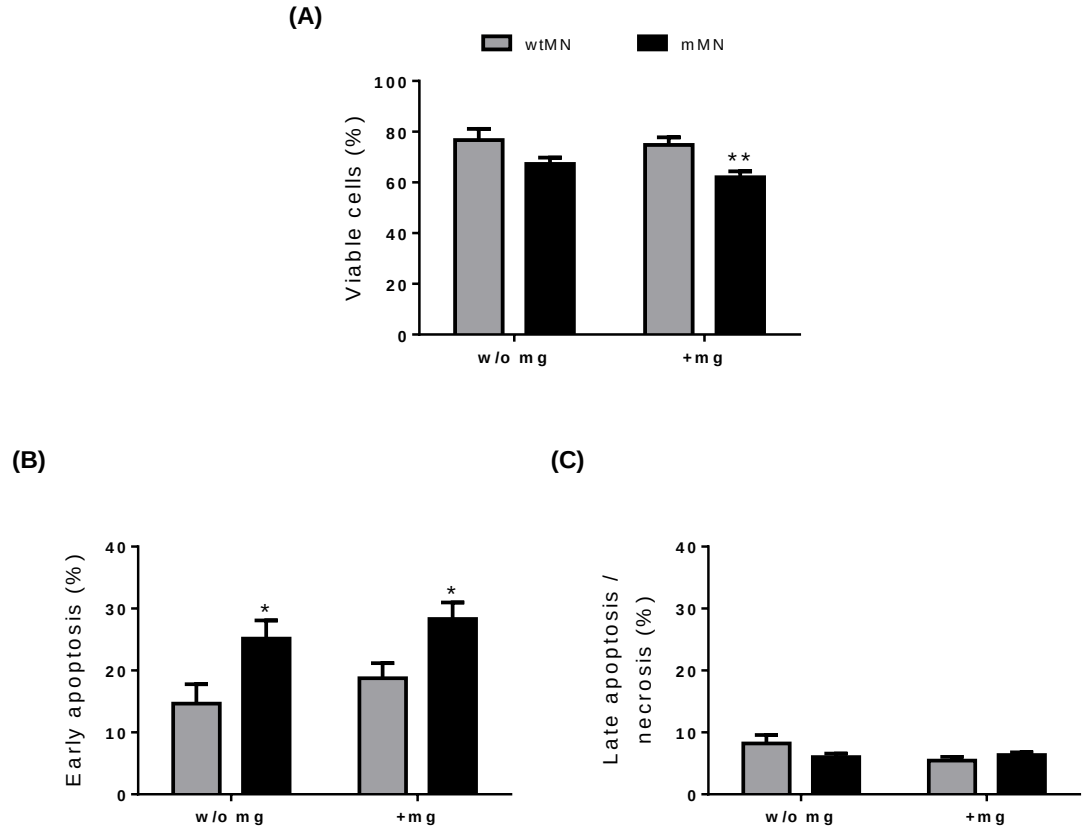


Figure III.1. Mixed cultures with mutated motor neurons (mMNs) and mMNs alone, evidence decreased viability and increased apoptosis, as compared with wild type (wt) MNs. Cells were treated as indicated in Methods. Results are expressed as percentage of (A) viable, (B) early apoptotic or (C) late apoptotic/necrotic cells from a total of 5000 events for each determination. Results are mean (± SEM) from at least three independent experiments performed in duplicate. *p<0.05, **p<0.01 *vs.* respective wtMNs.

We then analyzed the ratio MN-microglia at 4 DIV. For that, we stained the cells with anti-βIII-tubulin for MN and with anti-CD11b for microglia. By counting the number of βIII-tubulin$^+$ and CD11b$^+$ cells per total number of nuclei, we observed that there is a 30% decrease of the number of microglial cells in the presence of mMNs, which does not occur in the presence of wtMNs ($p<0.05$) **(Figure III.2A-B)**, suggesting that the presence of mutated hSOD1 is also affecting microglia viability.

In order to further characterize microglial cells, we also analyzed them in terms of morphological activation and functional changes. For the morphological analysis we evaluated the cell-body area and we noticed that microglial cells have a larger cell body in the presence of mMNs, in comparison with microglial cells in the presence of wtMNs ($p<0.05$) **(Figure III.2C)**. For the functional analysis we focused on CD11b expression, a widely described marker of microglial activation (Costantini et al., 2010; Eyo and Wu, 2013; Roy et al., 2006). As shown in **Figure III.2D**, we observed a 1.58-fold increase ($p<0.05$) in CD11b mRNA expression in the presence of mMN, even though the number of microglial cells is decreased, suggesting the presence of a more reactive microglia population.

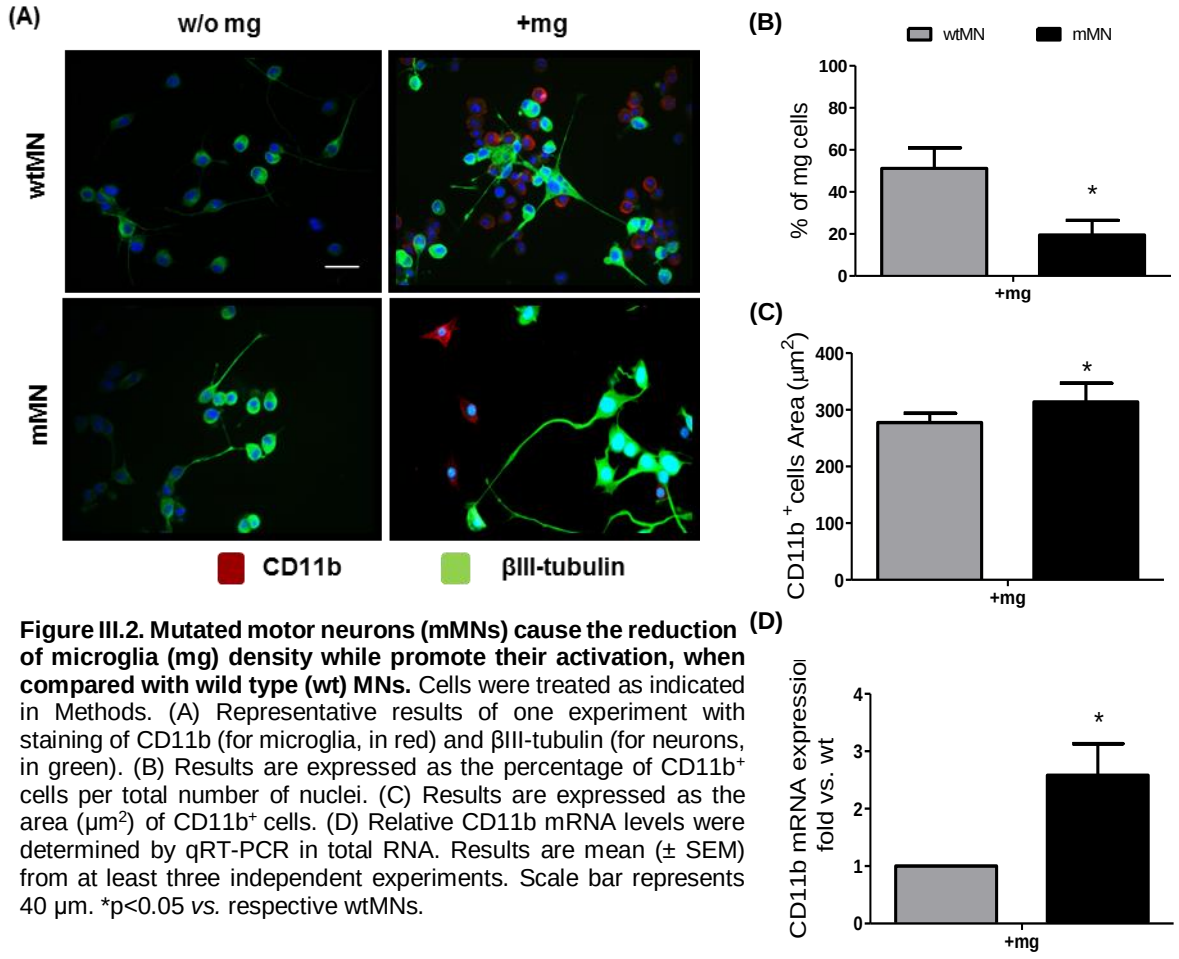


Figure III.2. Mutated motor neurons (mMNs) cause the reduction of microglia (mg) density while promote their activation, when compared with wild type (wt) MNs. Cells were treated as indicated in Methods. (A) Representative results of one experiment with staining of CD11b (for microglia, in red) and βIII-tubulin (for neurons, in green). (B) Results are expressed as the percentage of $CD11b^+$ cells per total number of nuclei. (C) Results are expressed as the area (μm^2) of $CD11b^+$ cells. (D) Relative CD11b mRNA levels were determined by qRT-PCR in total RNA. Results are mean (± SEM) from at least three independent experiments. Scale bar represents 40 µm. *p<0.05 *vs.* respective wtMNs.

1.2. Markers of neuroinflammation are differently modulated by the presence of mMNs

After having assessed an increased activation state of microglia, we considered relevant to evaluate some markers related with inflammation and dysfunction that were already reported in ALS models, as reviewed in Ferraiuolo et al. (2011b). Studies in our lab have previously reported that degeneration together with SOD1 accumulation starts to occur after 2 DIV and this differentiated mMN have higher levels of NO production than wtMNs (Vaz et al., 2014). In our model, we observed that the addition of microglia in the pre-symptomatic-like stage (before MN degeneration) doesn't have an effect on the production of NO since the increased levels in the extracellular media observed in mMNs alone ($p<0.05$) are maintained in the presence of microglia ($p<0.05$) **(Figure III.3A)**, suggesting that microglia is not ameliorating the dysfunctional effects seen in mMNs and, instead, those effects may be contributing to the compromised state of microglia.

Glutamate excitotoxicity also constitutes a feature of a compromised homeostasis of MNs in ALS, as briefly reviewed before (Van Den Bosch et al., 2006). In our model, mMNs didn't show increased production of glutamate when they were alone, instead there seems to exist lower levels of extracellular glutamate compared to wtMNs, possibly due to mitochondrial dysfunction, as suggested by D'Alessandro et al. (2011). When in mixed culture with microglial cells, glutamate levels remain lower

than wtMNs in the same conditions ($p<0.05$) **(Figure III.3.B)**, which again points towards an impaired function of microglia in recovering MN dysfunction.

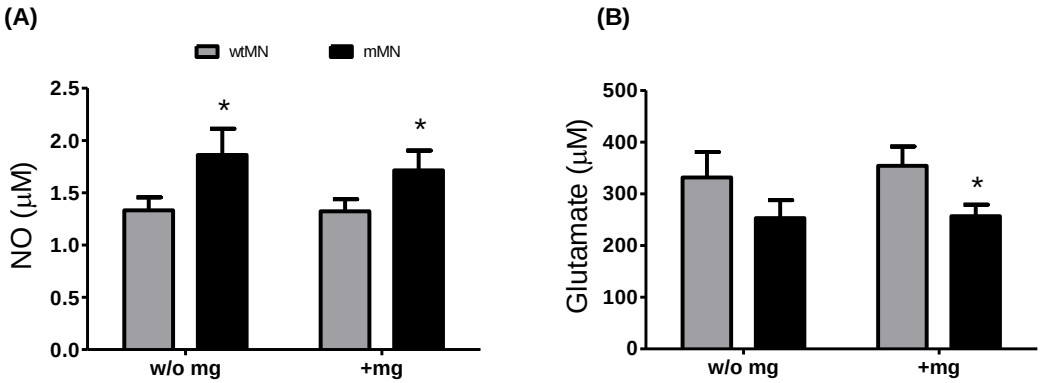


Figure III.3. Mutated motor neurons (mMNs), when compared with wild type (wt) MNs, increasingly produce NO, independently of the presence of microglia (mg), and release less glutamate, an effect aggravated by mg. Cells were treated as indicated in Methods. (A) NO production was assessed by Griess reaction. (B) Glutamate release was assessed by L-Glutamic acid kit. Results are mean (± SEM) from at least five independent experiments. *p<0.05 *vs.* respective wtMNs.

Activation of MMPs is one marker of inflammation and high levels of MMP-9 were reported in ALS MNs (Kaplan et al., 2014). In addition, studies in our lab have shown that, at 4 DIV, mMNs have increased activity of MMP-9 but no differences of MMP-2 (Vaz et al., 2014). We were able to reproduce such values in our model and, when we added microglia in the pre-symptomatic-like stage of mMNs, the activity of MMP-2 was significantly decreased (0.35-fold, $p<0.05$) as well as MMP-9 (0.44-fold, $p<0.001$), when compared to wtMNs in the same conditions **(Figure III.4)**. Together, these results suggest that healthy microglia makes an attempt to constrain the inflammatory pattern observed in the presence of mMNs, at least in terms of MMPs activation pattern.

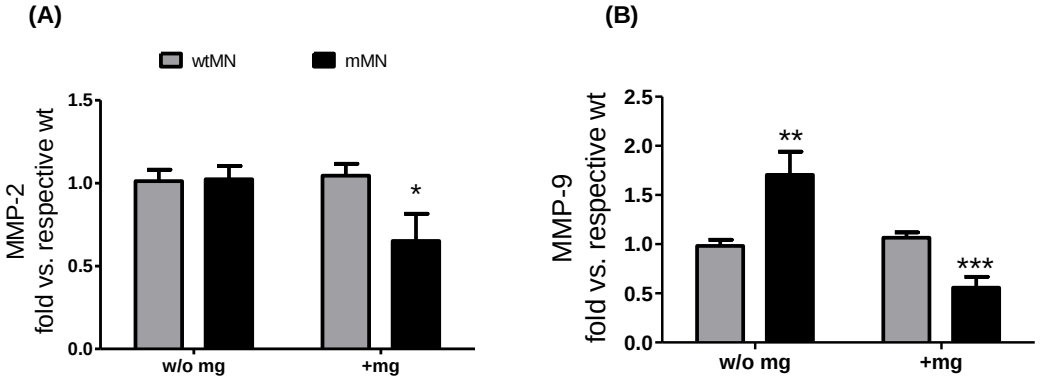


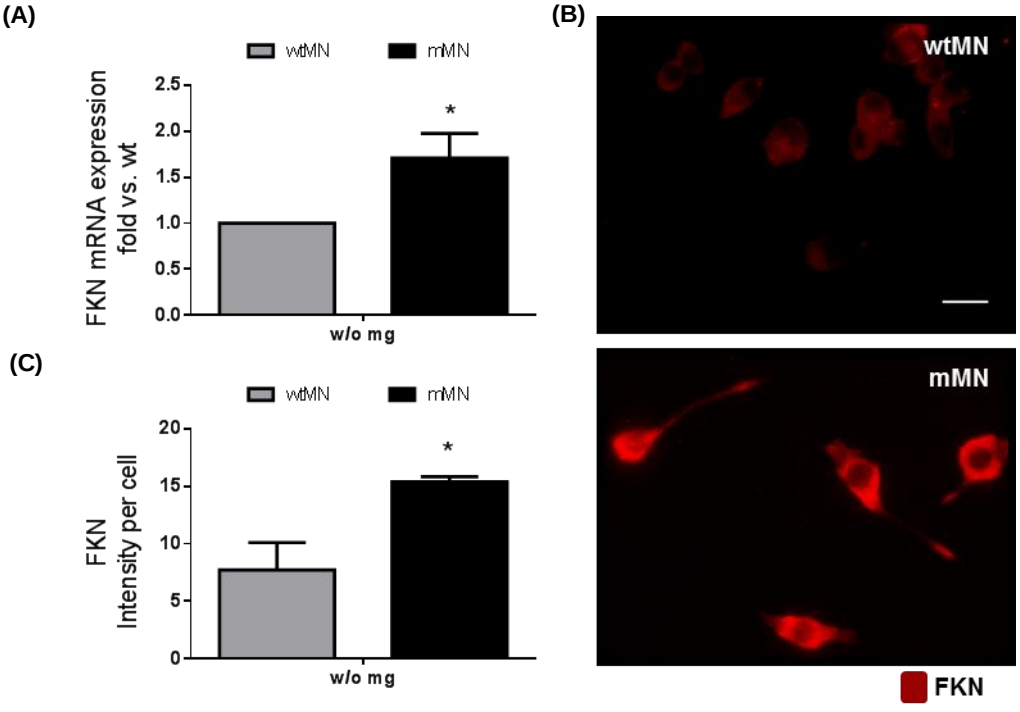
Figure III.4. Only MMP-9 is increasingly released by mutated motor neurons (mMNs) vs. wild type (wt) MNs and both MMP-2 and MMP-9 levels show to decrease by the presence of microglia (mg). Cells were treated as indicated in Methods. (A, B) MMP's activity was assessed by gelatin zymography assay. Results are mean (± SEM) from at least five independent experiments. *p<0.05, **p<0.01, ***p<0.001 vs. respective wtMNs.

2. Communication between microglia and MNs is modified when cells express mutated hSOD1 (mMNs)

In order to better explore the mechanisms behind MN-microglia cross-talk that might be altered in the presence of mMNs, we focused in molecules involved in three promising signaling pathways: the FKN – CX3CR1 axis, MFG-E8-mediated phagocytic pathway and HMGB1 – TLR4.

2.1. Deregulation of FKN/CX3CR1 signaling is observed when MNs express mutated hSOD1 (mMNs)

FKN is one kind of signal involved in the communication from MNs to microglia. Through its action on microglial receptor CX3R1, it is thought that FKN mediates microglial activation (Sheridan and Murphy, 2013). Here, we first analyzed FKN expression in mMNs alone. By evaluating FKN mRNA expression by qRT-PCR, we noticed an up-regulation of FKN gene in mMNs **(Figure III.5A)**, and, by immunocytochemistry, we also observed a significant increase in protein expression ($p<0.05$) **(Figure III.5B-C)**. Then, we further explored the different forms of FKN by Western Blot analysis both in cellular lysates and in extracellular media, and we were able to distinguish between membrane and soluble forms of FKN (respectively, 95 and 76 kDa bands) (Sheridan and Murphy, 2013; Suzuki et al., 2011). As demonstrated in **Figure III.5D**, we noticed that there is an increase in the membrane-form of FKN in cellular lysates (0.45-fold, $p<0.05$), and a decrease in the soluble-form in extracellular media (0.28-fold, $p<0.01$) in mMNs, which suggests that there is a decrease in the portion of FKN released by mMN that could be responsible for the maintenance of resting state of microglia.



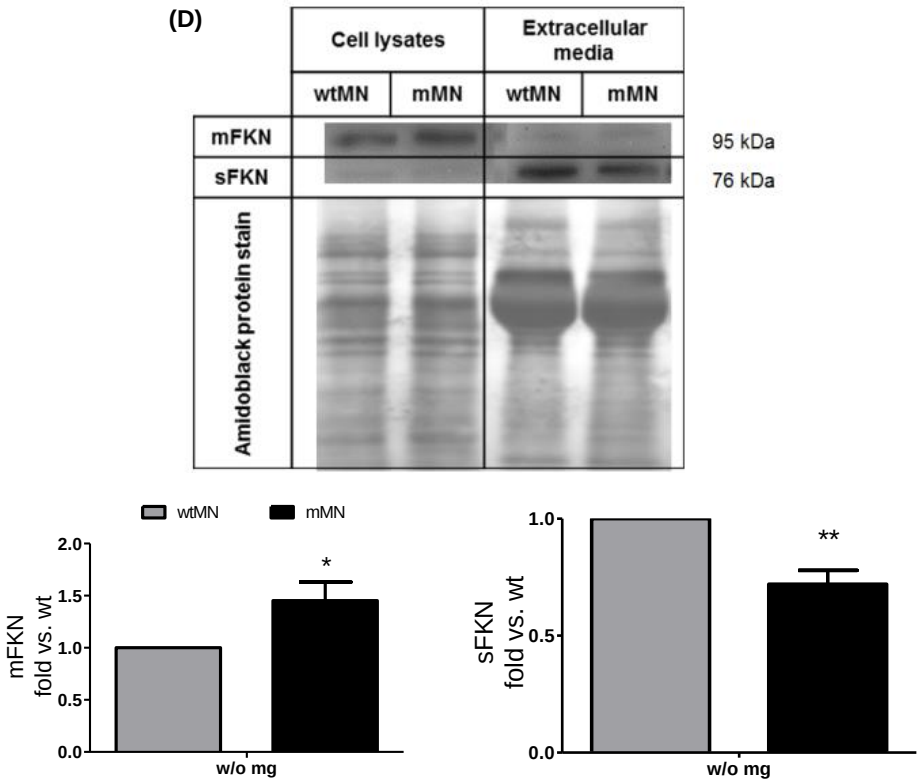


Figure III.5. Mutated motor neurons (mMNs), when compared with wild type (wt) MNs, increasingly express fractalkine (FKN) which accumulates in the membrane, instead of being released. Cells were treated as indicated in Methods. (A) Relative FKN mRNA levels were determined by qRT-PCR in total RNA. (B) Representative results of one experiment with staining of FKN (in red) and quantified in (C) Results are expressed as the intensity of fluorescence per cell. (D) Protein levels of FKN were quantified by Western Blot in total cell lysates (mFKN) and extracellular media (sFKN). Results are mean (± SEM) from at least three independent experiments. Scale bar represents 40 µm. *p<0.05, **p<0.01 *vs*. respective wtMNs.

We also analyzed FKN receptor - CX3CR1 - in mixed cultures, since it is expressed by microglial cells (Clark and Malcangio, 2014). Interestingly, we observed a 2.17-fold increase ($p<0.05$) in CX3CR1 mRNA expression when microglia was added in the pre-symptomatic-like stage of mMNs **(Figure III.6)**, despite the decreased FKN levels found in extracellular media of mMNs. Together, these results may indicate that the increased expression of CX3CR1 in microglia is not directly related with the signaling effect promoted by FKN release by MNs, which could be a possible mechanism to microglia-MN defense since FKN is thought to prevent microglia from hyper-activation and promote a neuroprotective action (Wolf et al., 2013), which will be further discussed in next section.

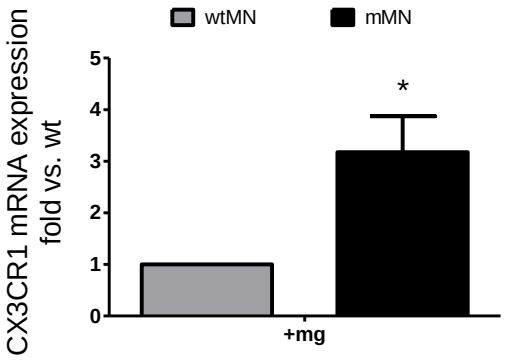


Figure III.6. Expression of CX3CR1 in microglia (mg) increases in the presence of mutated motor neurons (mMNs), when compared with wild type (wt) MNs. Cells were treated as indicated in Methods. Relative CX3CR1 mRNA levels were determined by qRT-PCR in total RNA. Results are mean (± SEM) from three independent experiments. *p<0.05 *vs.* respective wtMNs.

2.2. Neuronal MFG-E8 increases in mMNs in the presence of healthy microglia

As mentioned in the introduction section, phagocytosis is an important process in maintaining cellular homeostasis and is mainly performed by microglial cells in the CNS.

Since we and others (Ghadge et al., 1997; Guegan et al., 2001; Pasinelli et al., 2000) observed cell death preferentially by the apoptotic process, we aimed to determine if phagocytic ability of microglia could be somehow altered when exposed to mMNs, through a MFG-E8 dependent manner. For that, we analyzed protein levels of MFG-E8 by immunocytochemistry and Western Blot analysis, and we found higher levels of MFG-E8 in mMNs and that those were significantly enhanced in the presence of microglia (0.75-fold, p<0.05), when compared with wtMNs in the same conditions **(Figure III.7A-B)**. We then analyzed MFG-E8 mRNA expression and observed an up-regulation of this gene in mixed cultures with mMNs, although not statistically significant **(Figure III.7C)**. These results suggest that there may be an attempt of healthy microglia to phagocytize apoptotic neurons and the increased levels of MFG-E8 by MNs may be impairing this process, as will be further discussed in the next section.

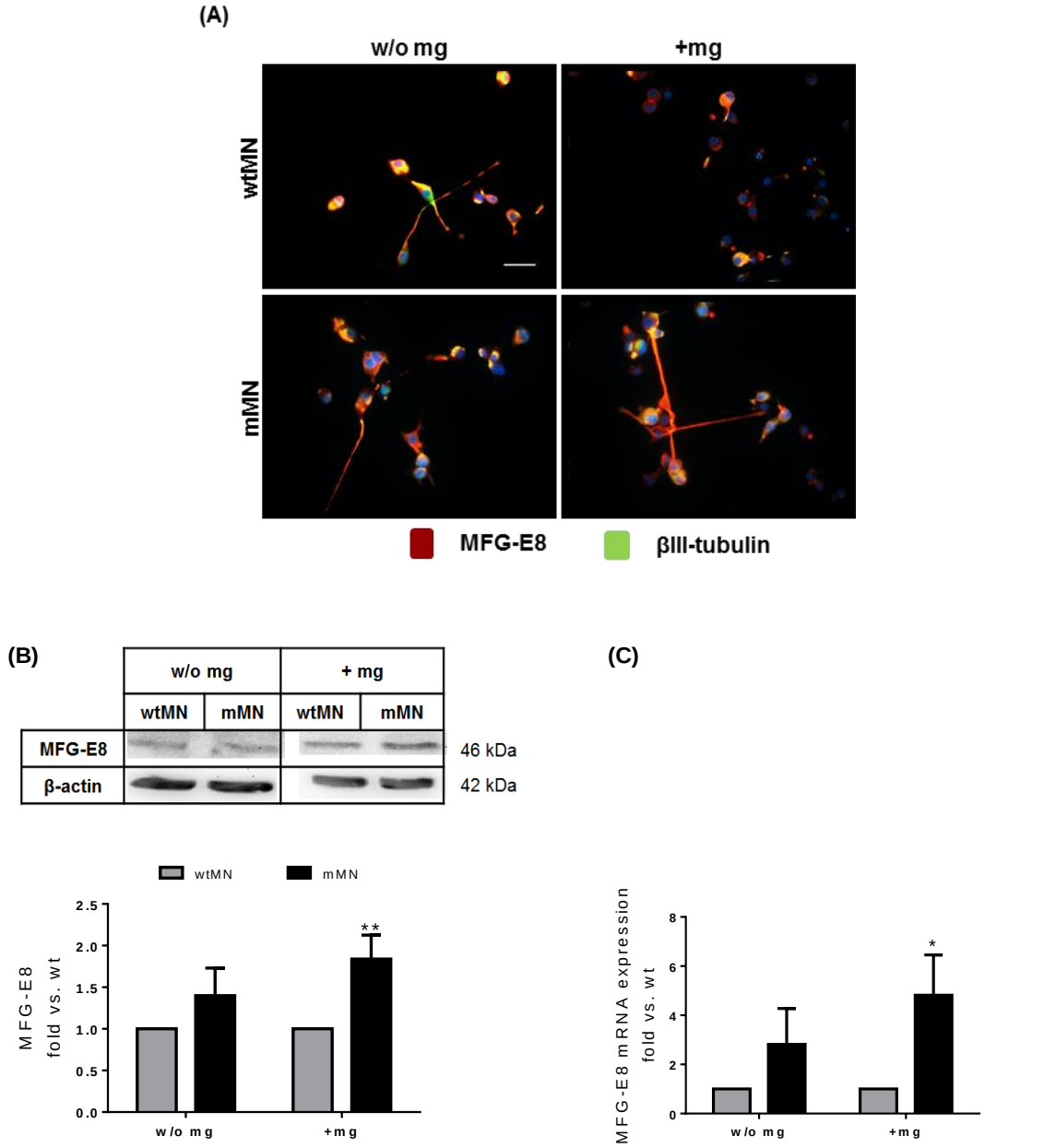
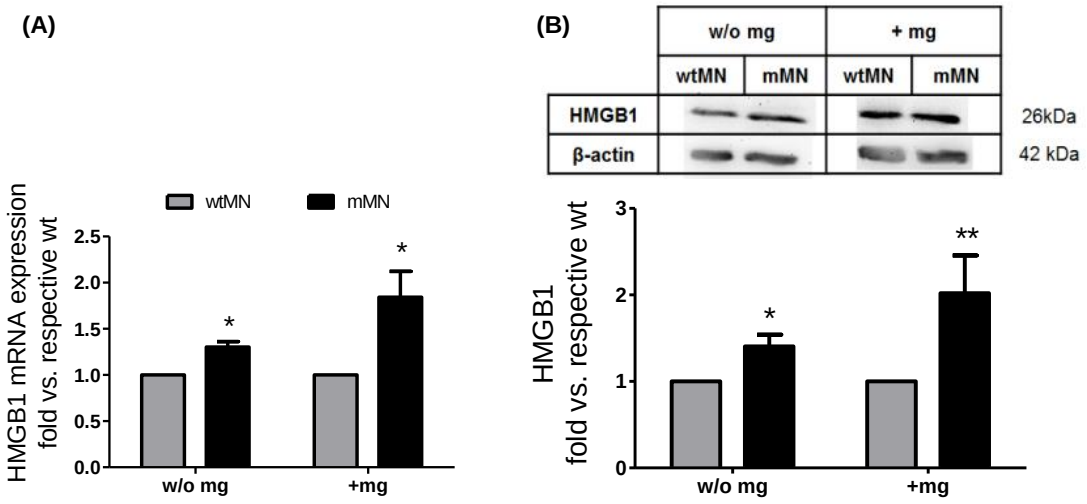


Figure III.7. Increased levels of lactaderin/MFG-E8 in mutated motor neurons (mMNs) as compared with wild type (wt) cells and mainly in the presence of healthy microglia, may compromise phagocytosis. Cells were treated as indicated in Methods. (A) Representative results of one experiment with staining of MFG-E8 (in red) and βIII-tubulin (for neurons, in green). (B) Protein levels of MFG-E8 were quantified by Western Blot in total cell lysates. (C) Relative MFG-E8 mRNA levels were determined by qRT-PCR in total RNA. Results are mean (± SEM) from at least three independent experiments. Scale bar represents 40 µm. *p<0.05, **p<0.01 *vs.* respective wtMNs.

2.3. Intracellular HMGB1 levels are higher in mMNs than in wtMNs, mainly in the presence of microglia

HMGB1 is an alarmin, considered one main player in the inflammatory process as a damage-associated molecular pattern (Erlandsson Harris and Andersson, 2004). In this sense, we intended to see if activation of microglia could be triggered via HMGB1 signaling. For this purpose, we analyzed the expression of HMGB1 in MNs either alone or in the presence of microglia. By qRT-PCR we observed a marked increase of the mRNA expression in mMNs alone (0.30-fold, $p<0.05$), which was also increased in the presence of microglia (0.84-fold, $p<0.05$) **(Figure III.8A)**. Accordingly, by Western Blot analysis, we observed an increase in the intracellular levels of this protein, both in mMN cultures alone (0.40-fold, $p<0.05$) and in mixed cultures with microglia (1.02-fold, $p<0.01$), represented by a 26 kDa band **(Figure III.8B)**. Regarding mixed cultures, we further explored which cells have higher protein levels by immunocytochemistry and we noticed that intracellular HMGB1 seems to be increased in mMNs **(Figure III.8C)**. Together, these results suggest that activated microglia may be releasing HMGB1 and the increased levels of HMGB1 in mMNs may be inducing that activation, seen through CD11b increased expression.



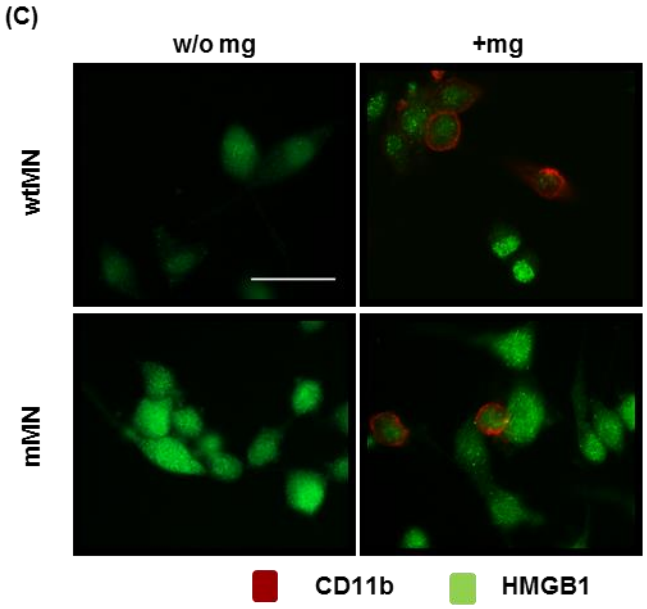


Figure III.8. Intracellular HMGB1 is increased in motor neurons expressing mutated hSOD1 (mMNs), either alone or in mixed culture with microglia. Cells were treated as indicated in Methods. (A) Relative HMGB1 mRNA levels were determined by qRT-PCR in total RNA. (B) Protein levels of HMGB1 were quantified by Western Blot in total cell lysates. (C) Representative results of one experiment with staining of CD11b (for microglia, in red) and HMGB1 (in green). Results are mean (± SEM) from at least four independent experiments. Scale bar represents 40 µm. *p<0.05, **p<0.01 *vs.* respective wtMNs.

2.4. TLR4 expression is up-regulated in mMNs, mainly in the presence of healthy microglia

Microglia express multiple receptors able to efficiently respond to external stimuli (Pocock and Kettenmann, 2007). One of them is TLR4, which, among other roles, triggers a pro-inflammatory cascade when activated (Lu et al., 2008). Since HMGB1 is one well-known ligand of TLR4 (Lee et al., 2014b), in order to uncover by which pathways microglia were being activated, we evaluated TLR-4 expression at both gene and protein level. By qRT-PCR we observed that TLR4 mRNA expression is augmented in mMNs, either alone or in mixed cultures, although not statistically significant **(Figure III.9A)**. By Western Blot analysis, we were able to take information about the two forms of the protein: the protein itself, represented by a 95kDa band; and the glycosylated form of TLR4, which is the one present in the membrane, represented by a 120kDa band **(Figure III.9B)** (Ismail et al., 2013) and with ability to recognize the inflammatory stimuli and activate the signaling pathway (da Silva Correia and Ulevitch, 2002). Interestingly, we observed that the glycosylated form is mainly increased in mixed cultures with mMNs, as well as TLR4 (1.16-fold, p<0.05) **(Figure III.9B)**. Taken together, these results suggest that there is more susceptibility to an inflammatory microenvironment in the presence of mMNs even in the presence of healthy microglia.

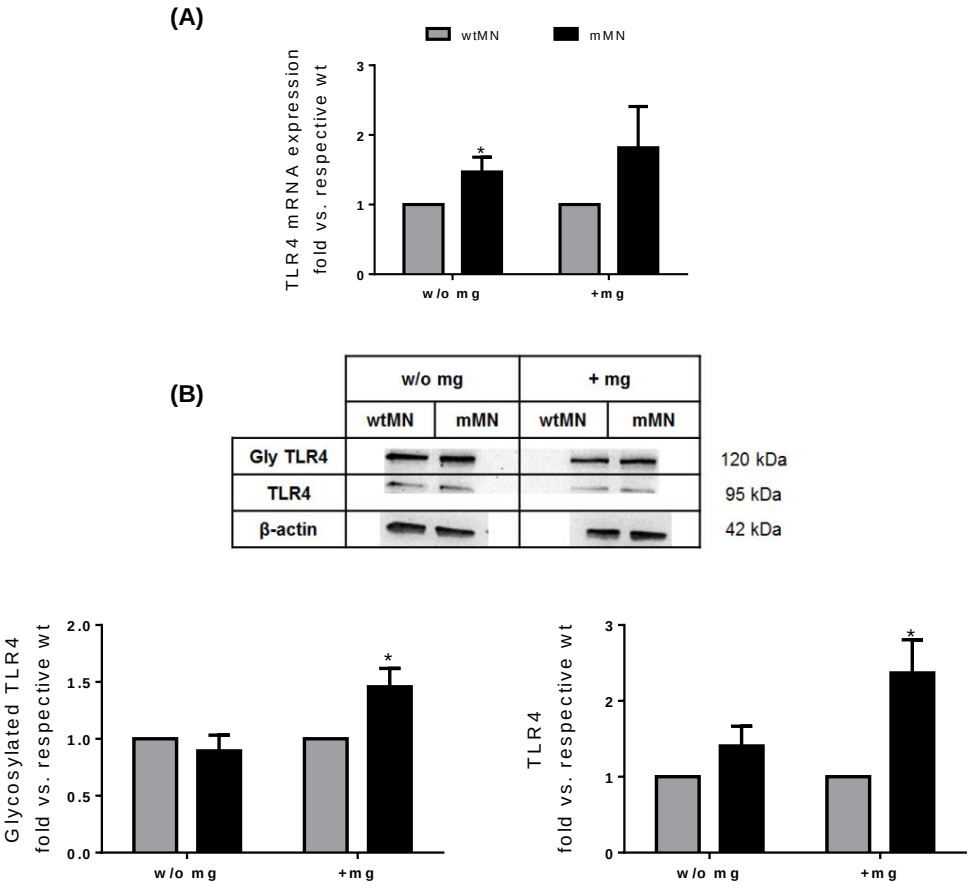


Figure III.9. TLR4 levels increased in mutated motor neurons (mMNs), as compared with wild type (wt) cells and mainly in the presence of healthy microglia (mg), may derive from the up-regulation of HMGB1. Cells were treated as indicated in Methods. (A) Relative TLR4 mRNA levels were determined by qRT-PCR in total RNA. (B) Protein levels of glycosylated (Gly) TLR4 and TLR4 were quantified by Western Blot in total cell lysates. Results are mean (± SEM) from at least three independent experiments. *p<0.05 *vs.* respective wtMNs.

IV. DISCUSSION

ALS is a neurodegenerative disease that affects thousands of people worldwide (Chio et al., 2013). Over more than a century after it was first described, there's still no effective treatment due to its established complexity. Given the variety of microglial responses when facing CNS disrupted homeostasis, and their critical role in the pathoprogression of motor neuron degeneration in ALS, one of the most recent proposals to therapy concerning neurodegenerative disorders is replacement of microglial cells (Cartier et al., 2014). In addition, modulation of microglia phenotypes from M1 (pro-inflammatory, neurotoxic) into M2 (alternatively-activated, neuroprotective) have shown to protect motor neurons in ALS mice, whereas microglia isolated from end-stage disease ALS mice have adopted an M1 phenotype and were shown to be neurotoxic, facts that support the dual phenotypes of microglia and their transformation during disease pathoprogression in these mice (Liao et al., 2012). However, what is lacking so far is the complete understanding of the interactions of these cells with the injured ones. Therefore, our study aimed to better explore the therapeutic potential of healthy microglia when exposed to MNs harboring a well-established causative mutation of ALS, a glycine-to-alanine substitution in position 93 ($SOD1^{G93A}$ or mSOD1). For this purpose, our study line consisted in mixing NSC-34 cells, a MN-like cell line, stably transfected with the human $SOD1^{G93A}$ (mMNs), and N9 cells, a microglia-like cell line, and evaluate their interaction until the time-point when these mMNs previously showed higher levels of dysfunction (4 DIV) (Vaz et al., 2014).

We started by analyzing the viability of the cells at that time-point by performing flow cytometry studies and observed that cells of mixed cultures with mMNs have lower viability and are more prone to apoptotic cell death. It is, in fact, well-known that apoptosis is one of the elected cell death pathways for motor neurons in ALS, as reviewed in Ferraiuolo et al. (2011b), although other pathways may be affected, as necroptosis or autophagy (Bandyopadhyay et al., 2014; Barmada et al., 2014; Lee et al., 2014a; Re et al., 2014). We also evaluated whether mMNs could as well induce apoptosis of the cells they were interacting with. In order to better understand which cells were, in fact, more committed, we analyzed the ratio of MN-microglia at 4 DIV and observed a significant reduction of microglial cells in the presence of mMNs. This fact give us an indication that, besides mMNs being predisposed to apoptosis, when in contact with healthy microglia they may trigger apoptotic cell death in these cells as well. Indeed, activated microglia was shown to undergo apoptosis following CNS injury (Gehrmann and Banati, 1995) and over-activation with LPS (Liu et al., 2001) and it is suggested

that neurons may influence the survival of microglial cells through released factors, as reviewed in Luo and Chen (2012). In fact, previous studies in our lab have shown that N9-microglia incubated with conditioned media from mMNs at 4 DIV have higher levels of cell death by apoptosis (Cunha, 2012), suggesting that mMNs release factors that will cause microglial over-activation which culminates in apoptotic cell death.

Microglial activation is characterized by morphological and functional changes, as turning into amoeboid-like shape and increased expression of CD11b. Therefore, in order to characterize the phenotypic-like state of microglial cells in our mixed cultures, we evaluated the cell-body area and observed that in mixed cultures with mMNs, microglial cells present a larger cell-body in accordance with an appearance of an amoeboid-like shape, characteristic of morphological activation (Xiang et al., 2006). In addition, we explored the mRNA expression of the proposed cell activation marker, CD11b, by qRT-PCR, and we observed an increase in its expression. CD11b is an integrin that acts as a bridge between cytoskeleton and cell-membrane being probably involved in cell rearrangement during morphological activation changes (Roy et al., 2008). These results highlight an activated state of microglia in the presence of mMNs.

We next explored the microenvironment in the mixed cultures. To evaluate the existence of oxidative stress, we analyzed the production of NO and our results showed no alterations with the addition of microglia. This finding suggests that mMNs dysfunction associated with NO production is not being recovered or any ameliorated by the presence of healthy microglia. In fact, if microglia is over-activated they may lose their ability to actively fight and recover microenvironment's toxicity, further contributing to it (Yu et al., 2012). Also, it is known that NO contributes to microglial activation (Roy et al., 2006). Besides nitrosative stress, also glutamate excitotoxicity has been related to MN injury in ALS, as briefly reviewed in section I.1.3.2 (Roy et al., 1998). One possibility is that this phenomenon occurs not as a consequence of excessive production by injured MNs, but as a consequence of an impaired uptake by glial cells due to deficiency in glutamate transporters (Dunlop et al., 2003). Indeed, our results with mMNs alone showed decreased levels of glutamate in the extracellular media. This feature may be caused by dysfunctional metabolism consequent of motor neuron injury (D'Alessandro et al., 2011). In this case, we also observed that glutamate levels do not change in the presence of healthy microglia in mixed cultures with mMNs, remaining decreased, again suggesting that microglia is not being able to ameliorate the dysfunctional features of mMNs. Curiously, the activity of the pro-inflammatory associated markers, MMP-2 and MMP-9, was remarkably reduced in the presence of microglia. We have previously reported that mMNs presented an increased activity of MMP-9 (Vaz et al., 2014). In addition, an increase of MMP-9 intracellular levels was also reported in mMNs and pointed as a causative factor of the selective vulnerability of motor neurons in ALS (Kaplan et al., 2014). The fact that the presence of microglia is enough to decrease those levels may suggest that there is an attempt to minimize microenvironment's toxicity, caused by mMNs degeneration. Still, one could think that if that attempt was well-succeed, those levels would achieve the same levels observed in mixed cultures with our control, wtMNs. Yet, both MMP-2 and MMP-9 activities are significantly decreased in the mixed cultures with mMNs when in comparison with wtMNs in the same conditions, which again points towards a disrupted interaction between mMNs and microglia, having the last one its functions impaired.

Accordingly, the decreased activity of MMP-9 was observed in aged cultured microglia from brain mice (Caldeira et al., 2014). Decrease of MMP-9 activity may be also a consequence of an imbalance between tissue inhibitors of metalloproteinases (TIMPs) and MMPs, in which the first ones are known to have an increased expression under inflammatory insults, as reviewed in Moore and Crocker (2012). Together, these results suggest a microglia with an attempt to fight microenvironment's pro-inflammatory conditions, yet with a compromised and impaired function.

Given the altered interaction between mMNs and microglia, we intended to further explore which signaling pathways were compromised by the presence of mMNs. It is well-known the ability of motor neurons to modulate microglial activity (Eyo and Wu, 2013). One way is through the FKN-CX3CR1 axis. This unique chemokine presented in section I.2 , is one signal of MNs to keep microglia in a quiescent-like state, preventing them from over-activation and its pro-inflammatory phenotype (Hao et al., 2013). Chapman et al. (2000) showed that cleavage of sFKN in primary cortical neurons is mediated by MMPs. In our model, we observed an increased expression of the FKN gene in mMNs alone, along with increased protein expression. However, when we were able to further dissect the two forms of the protein, the membrane-bound and the soluble form, we noticed that this increased expression of FKN is only due to the membrane-bound form but not to the soluble form, found decreased in the extracellular media from mMNs. This result suggests an impaired cleavage of the chemokine domain of FKN and that it is independent from the activity of the MMP-9, once we already saw that its activity levels are increased. We then intended to evaluate the expression of the FKN receptor, CX3CR1, in mixed cultures with microglia and we surprisingly observed a marked increase in microglia with mMNs. This phenomenon can be explained due to the fact that FKN presents itself in two forms, as mentioned, and both have active functions: the soluble one is thought to mediate migration of the immune cells to the injured area, through chemotaxis, while the membrane-bound form is thought to mediate cell-to-cell adhesion and interaction (Sheridan and Murphy, 2013). Our results propose that in our model, FKN-CX3CR1 mediated interaction is through the membrane-bound form. Curiously, Morganti et al. (2012), by using a mouse model of Parkinson's disease, reported that the soluble form is the only capable of triggering a neuroprotective environment through its action on microglia receptor, CX3CR1, and they suggested that it is because it can be internalized by CX3CR1, while the membrane form doesn't have any action on protecting from neurotoxicity since it is able to ligate the receptor but cannot be internalized. Taken together, these results suggest that one deregulated signaling pathway involved in this model of ALS is the FKN-CX3CR1 axis, which may provide a new tool to novel immunotherapy strategies.

As the resident macrophages of the CNS, an important process in order to microglia properly execute its protective functions and maintain CNS homeostasis, is phagocytosis. Cellular debris, protein aggregates as well as damaged cells, promote a toxic environment and can trigger several pathologies. MFG-E8, as detailed in section I.2.2.2, is a glycoprotein that has been highlighted for its ability to recognize PS present in the membrane of apoptotic cells, and act as a bridge between PS and the macrophage, leading to further phagocytosis of the apoptotic body (Aziz et al., 2011). With this in mind, since the most common cell death pathway in our model seemed to be apoptosis, we determined the phagocytic ability of microglia through MFG-E8 pathway when in the presence of mMNs. Here, when

we quantified the protein levels we found an increased expression in mixed cultures with mMNs but also in mMNs alone. For that reason, we performed immunocytochemistry studies, and we found that the increased expression was mainly observed in mMNs and not in microglia. Bowen et al. (2007) proposed that neurons may have phagocytic capacity, through demonstration of microspheres uptake into living neurons and the presence of integrin receptors known to be involved in macrophage phagocytosis, as CD29. They also observed that rat spinal cord neurons had processes to take up apoptotic cell debris. Considering the fact that is the number of microglial cells that is decreased and not the number of mMNs, we may assume that microglial phagocytic activity is compromised in our model of mixed cultures with mMNs. Therefore, we suggest that the overexpression of MFG-E8 molecules by mMNs may be blocking PS exposed by apoptotic MNs, thereby impairing microglia phagocytic ability through MFG-E8 pathway.

Because HMGB1 is one main player in the inflammatory process (Lee et al., 2014b), we prompted to investigate if it was involved in the activation of microglia by mMNs. As previously detailed, HMGB1 may be released from injured cells or actively secreted by activated immune cells, triggering a pro-inflammatory cascade via binding to TLR4 (Zou and Crews, 2014). Here, we observed an increased expression of HMGB1, both the gene and the protein, in mMNs either alone and in mixed cultures, wherein, we could observe by immunocytochemistry more staining in mMNs than in microglia. In accordance, Bell et al. (2006) reported that HMGB1 is released from apoptotic cells in a time-dependent manner. Then, our results suggest that the increased intracellular HMGB1 may be further released by injured mMNs contributing to microglial activation, and that it is being actively released by activated microglia.

Microglia have a broad spectrum of receptors that induce their response to the signals released from motor neurons. TLR4 is one of them and is activated by HMGB1 (Lee et al., 2014b). The activation of TLR4 is generally known to initiate a pro-inflammatory cascade leading to activation of NF-kB, further culminating in the transcription and release of pro-inflammatory cytokines (Lu et al., 2008). The expression of TLRs is reported to be up-regulated in the spinal cord of sALS cases (Casula et al., 2011). Curiously, we observed that mMNs express increased levels of TLR4. This finding suggests that neuronal TLR4 may induce the expression of mediators capable of trigger microglial activation. In fact, neurons were reported to express TLR4 (He et al., 2013; Okun et al., 2011; Tang et al., 2007; Wang et al., 2013) and their activation was shown to induce neuronal chemokines, as CXCL1, and cytokines as TNF-α and IL-6 (Leow-Dyke et al., 2012). In addition, the increased expression of TLR4 in mixed cultures with mMNs suggests that the soluble factor(s) released from mMNs leading to activation of microglial cells, are acting through TLR4 signaling pathway, shedding light to the increased HMGB1 that we previously stated. Also, a new mechanism of SOD1 toxicity was suggested in which mSOD1 may be secreted from MNs through chaperon-like proteins – chromogranins – (Urushitani et al., 2006) and act as a “danger signal” promoting the activation of microglial cells through TLRs pathway (Zhao et al., 2010). Hence, mSOD1 may be also one of those soluble factors. Moreover, TLR4 signaling was also shown to trigger apoptosis of activated microglia (Jung et al., 2005). As reviewed in section I.1.4.3, in ALS it is thought that before microglia becomes overactivated and consequently dystrophic, with no ability to efficiently respond to external stimuli, they become in a pro-inflammatory phenotype (M1), with increased release of pro-inflammatory cytokines. Therefore, the increased expression of TLR4 may be

one of those disrupted signaling pathways, preceding and further contributing to the overactivation of healthy microglia.

Taken together, the results obtained in this thesis suggest that mMNs are impairing microglia's function, impeding them to maintain cellular homeostasis and further leading to an overactivated microglia. This is suggested to be occurring through FKN-CX3CR1 axis, blocked MFG-E8-mediated phagocytic pathway and TLR4 hyperactivation, as schematically represented in **Figure IV.1**. The soluble factors released by mMNs still remain to unravel, being mSOD1 and HMGB1 strong candidates. Therefore, immunomodulatory approaches directed to the signaling pathways here addressed may constitute a promising therapeutic approach, reinforcing the use of therapeutic strategies that can restore both the healthy state of MNs and microglial cells in ALS.

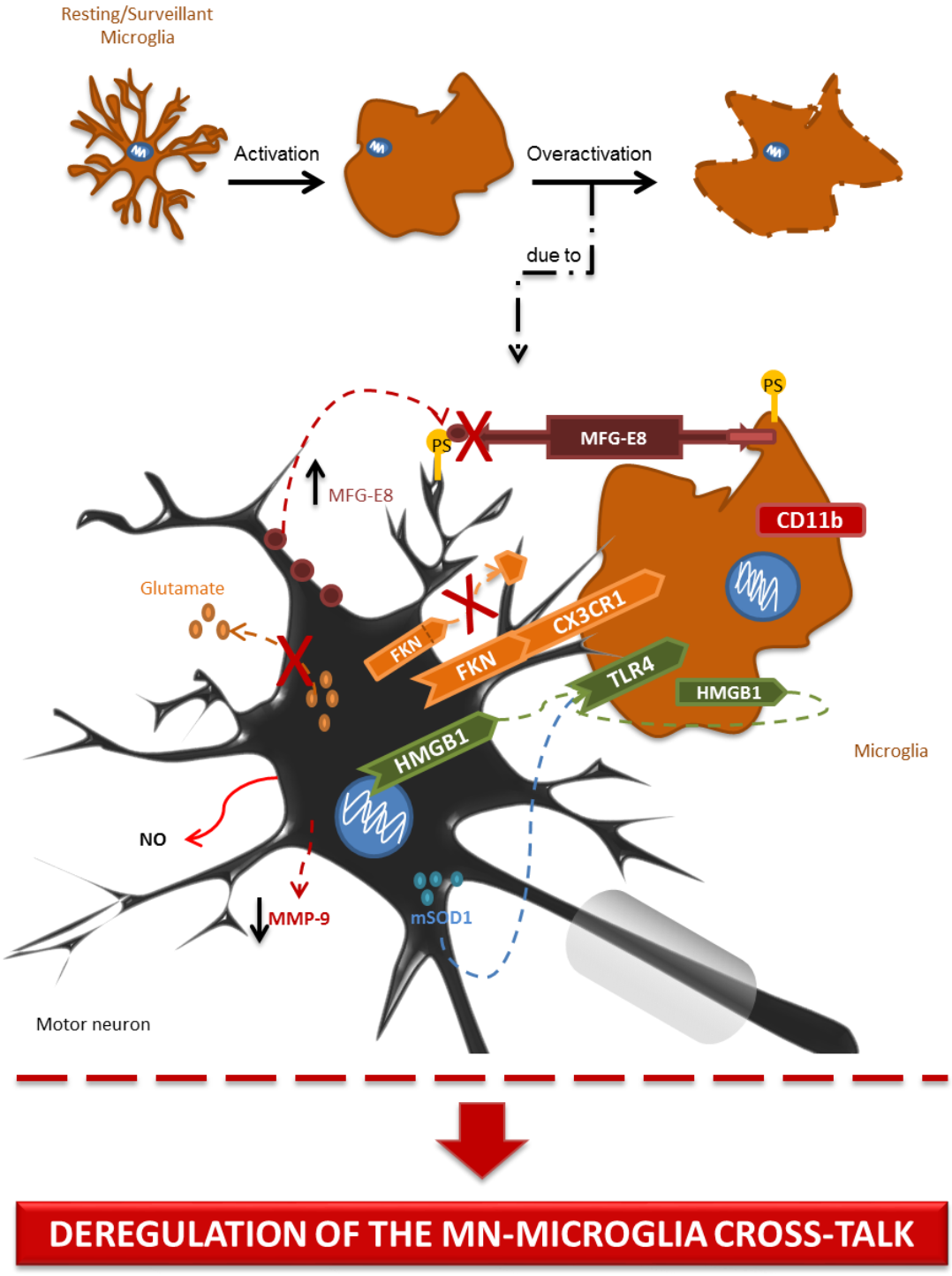


Figure IV.1. Schematic representation of the major findings of this thesis. In the presence of mutated motor neurons (mMNs), microglia become activated, exhibiting both morphological and functional alterations, evidenced by the increase in cell-body area and the increased CD11b expression. The number of microglial cells is decreased, highlighting a compromised viability of these cells and suggesting their overactivation. Apoptosis seems to be the elected cell death pathway, evaluated by the exposure of phosphatidylserine (PS). Dysfunction of mMNs regarding NO and glutamate production are not recovered by healthy microglia, although the activity of matrix metalloproteinases, namely metalloproteinase-9 (MMP-9), is strongly decreased. mMNs reveal an impairment in the cleavage of soluble fractalkine (FKN) and its receptor, CX3CR1, is increased in mixed cultures, suggesting the action of FKN through adhesion of the membrane-bound form. Milk-fat globule EGF factor-8 (MFG-E8) expression is also increased in mixed cultures and in mMNs, which may be impairing MFG-E8-mediated microglial phagocytosis. Toll-like receptor 4 (TLR4) is increased in the presence of mMNs. Whether this activation is caused by mutated SOD1 (mSOD1) release from mMNs or by high mobility group box 1 (HMGB1), which is also increased in mMNs, is still to investigate. Overall, mMNs are suggested to be causing overactivation of microglia, impairing their neuroprotective functions.

Future Perspectives

In the present study we discussed the importance of the cross talk between motor neurons and microglia in ALS, an issue that still needs to be overcome for clinical translation in cellular therapy that aims to either modulate CNS immune cells and to prevent the fast and progressive motor neuron loss in ALS patients. In this context, it will be interesting to evaluate the parameters observed to be deregulated in this culture model, namely the FKN-CX3CR1 axis, MFG-E8-mediated phagocytosis and HMGB1-TLR4 signaling, both in prior and posterior time-points, in this model as well as in transgenic mice.

It will be also important to accurate the expression of FKN in the mixed cultures, as well as the proliferation rate of microglia, and to address some other specific parameters such as mSOD1 and HMGB1 in the extracellular media, given their potential to be soluble factors triggering microglia overactivation and neuroprotective impairment here observed.

This study contributes to a better understanding on how the detrimental effects of mMNs are overriding the beneficial effects of healthy microglia. For this, it would be also interesting to assess the parameters here evaluated in a transgenic microglia, transfected with $hSOD1^{G93A}$, to further elucidate the mechanisms of action altered in mutant microglia and their modulation by intrinsic mSOD1.

Accordingly, immunomodulatory interventions may be a promising therapy in ALS, as therapeutic administration of TLR4 antagonists or CX3CR1 agonists, in order to take advantage of the neuroprotective potential of microglia.

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