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Exploring motor neuron degeneration in ALS - prevention by glycoursodeoxycholic acid and signaling to microglia

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ABSTRACT

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease that affects mainly motor neurons. Neuronal pathology involves glial cells, in particular microglia. However it is not known how these cells interact with motor neurons. This is particularly important because till now no therapy has shown efficacy in ALS treatment.

Here, we aim (i) to evaluate the suitability of NSC-34, a hybrid cell line of neuroblastoma and motor neurons, as a model of ALS, (ii) to explore the reactivity of microglia to the neuronal released factors and (iii) to assess the efficacy of glycoursodeoxycholic acid (GUDCA), which already has shown beneficial effects in several neurodevelopmental and neurodegenerative diseases.

For that, we used NSC-34 cells transfected with human superoxide dismutase 1 (hSOD1), either wild type or mutated in G93A and the microglial N9 cell line. We observed mitochondrial dysfunction, energy impairment, NO production and metalloproteinase-9 activation, with consequent apoptosis in NSC-34/hSOD1G93A cells after 4 days of differentiation, in comparison to NSC-34/hSOD1wt cells. In addition, we established GUDCA as an anti-apoptotic and anti-inflammatory agent, able to prevent all the above mentioned features. Finally, released neuronal factors induced N9 microglia apoptosis and decreased their phagocytic ability.

Overall, our results emphasize NSC-34/hSOD1G93A cells as a good ALS model, highlight GUDCA as having beneficial effects and point to microglia neuroprotective failure as a determinant mechanism of ALS pathogenesis.

Keywords: SOD1 accumulation, motor neuron degeneration, mitochondrial dysfunction, inflammation, GUDCA protective effects, microglia activation

RESUMO

A Esclerose Lateral Amiotrófica (ELA) é uma doença neurodegenerativa que afeta principalmente os neurónios motores da medula espinhal e tronco cerebral. Evidências recentes sugerem o envolvimento de outras células nervosas, em particular, a microglia. No entanto, não é ainda conhecido o modo como estas células interagem com os neurónios motores.

No presente estudo, pretendeu-se (i) avaliar de que forma a linha celular NSC-34, resultante de um híbrido entre neuroblastoma e neurónios motores obtidos da medula espinhal, pode ser usada como modelo *in vitro* de ELA, após transfecção com a superóxido dismutase-1 humana (hSOD1) normal ou com mutação G93A, (ii) explorar a reatividade das células da microglia (linha celular N9) para com os factores libertados pelos neurónios motores e (iii) investigar a eficácia do ácido glicoursodesoxicólico (AGUDC), o qual já demonstrou efeitos benéficos em outras doenças quer neurodegenerativas, quer do neurodesenvolvimento.

Os resultados obtidos indicam que aos 4 dias após a indução da diferenciação, as células apresentam disfunção da mitocôndria, falência energética, aumento da produção de óxido nítrico (NO) e morte celular por apoptose. Além disso, verificou-se a activação da metaloproteinase-9 da matriz extracelular (MMP-9), que poderá funcionar como biomarcador da doença. Estabeleceu-se também a eficácia do AGUDC como agente anti-apoptótico e anti-inflamatório uma vez que preveniu a disfunção mitocondrial, apoptose e libertação de MMP-9 e NO nas células que expressavam a hSOD1 mutada. Curiosamente, observou-se que os factores libertados pelas células NSC-34 transfectadas com hSOD1 mutada activam a microglia, reduzem a sua actividade fagocítica e induzem a apoptose nestas células.

Em resumo, os resultados aqui obtidos reforçam o potencial das células NSC-34 que expressam hSOD1 mutada como um bom modelo para o estudo *in vitro* da ELA, demonstram o efeito protector do AGUDC e apontam para a perda de mecanismos de neuroprotecção da microglia como determinante na patogenecidade da ELA.

Palavras-chave: acumulação de SOD1, degeneração de neurónios motores, disfunção da mitocôndria, inflamação, efeitos protectores do AGUDC, activação da microglia

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ABBREVIATIONS

ALDH1L1	Aldehyde dehydrogenase family 1, member L1
ALS	Amyotrophic Lateral Sclerosis
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
ATP	Adenosine triphosphate
Aβ	Amyloid-beta
CCL2	Chemokine motif ligand 2
CNS	Central nervous system
CSF	Cerebrospinal fluid
Cyt *c*	Cytochrome *c*
DMEM-F12	Dulbecco's modified Eagle's medium-Ham's F12 medium
EAAT2	Excitatory aminoacid transporter 2
ER	Endoplasmic reticulum
fALS	Familial Amyotrophic Lateral Sclerosis
FBS	Fetal bovine seruum
GFAP	Glial fibrillary acidic protein
GluR2	Glutamate Receptor 2
GUDCA	Glycoursodeoxycholic acid
Iba1	Ionized calcium binding adaptor protein
IFN-γ	Interferon-gamma
IL	Interleukin
iNOS	Inducible nitric oxidase synthase
LPS	Lipopolysaccharide
MHC	Major histocompatibility complex
MMP	Matrix metalloproteinase
mSOD1	Mutant Superoxide dismutase 1
NF-κB	Nuclear factor - κB
NMDA	N-methyl-D-aspartate
NO	Nitric oxide
PI	Propidium iodide
PS	Phosphatidylserine
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
sALS	Sporadic Amyotrophic Lateral Sclerosis

SOD1	Superoxide dismutase 1
Tc	T citotoxic cell
TGF-β	Transforming growth factor-β
Th	T helper cell
TIM-4	T cell immunoglobulin mucin 4
TLR	Toll-like receptor
TNF-α	Tumour necrosis factor α
TRAF2	Tumour necrosis factor receptor-associated factor 2
Treg	T regulatory cell
TREM-2	Triggering receptor expressed by myeloid cells-2
TUDCA	Tauroursodeoxycholic acid
UCB	Unconjugated bilirubin
UDCA	Ursodeoxycholic acid
UPR	Unfolded protein response
WB	Western Blot

I. INTRODUCTION

1. Amyotrophic Lateral Sclerosis – an overview

Amyotrophic Lateral Sclerosis (ALS), also known as Lou Gehrig's disease, is a neurodegenerative disorder characterized by the selective loss of upper and lower motor neurons, and was first described by Jean-Martin Charcot and his colleague Alexis Joffroy in 1869. As represented in Figure I.1, in motor cortex, upper motor neurons degeneration results in spasticity and hyperreflexia, while death of lower motor neurons in brainstem and spinal cord leads to weakness, muscle atrophy and, ultimately, to paralysis of voluntary muscles, culminating in death from respiratory failure, as reviewed in Tripathi and Al-Chalabi (2008). The mean age of onset is 55–60 years, and men are more affected than women. The average survival from symptom onset is approximately 3-5 years but some patients demonstrate a slower disease course (Wood-Allum and Shaw, 2010).

Muscle cramps and fasciculations seem to be the primary clinical features as they seem to precede other symptoms for years. Moreover, degeneration of motor neurons seems to begin focally and disseminates into contiguous groups of motor neurons, which implies that disease is propagated to healthy neurons in neighbouring areas, where glial cells also have a role (Ferraiuolo *et al.*, 2011). Most of ALS cases have sporadic origin (sALS), however about 10% are inherited, being designated as familial ALS (fALS). Usually, fALS is associated with autossomal dominant inheritance and about 20% of familial cases are due to mutations in superoxide dismutase 1 (SOD1) (Rothstein, 2009). Recent findings have also demonstrated the role of newly identified genes in pathogenesis of ALS (Musaro, 2010), as it will be further discuss. Interestingly, sALS and fALS share similar clinical and pathological features, therefore research regarding fALS can also be applied to sporadic cases (Bruijn *et al.*, 2004).

Despite the precise molecular pathways involved in ALS are not yet elucidated, accumulating evidence reveals that ALS is a multifactorial disease resulting not only from altered molecular mechanisms within motor neurons but also from the involvement of other cells, such as glia (Ferraiuolo *et al.*, 2011).

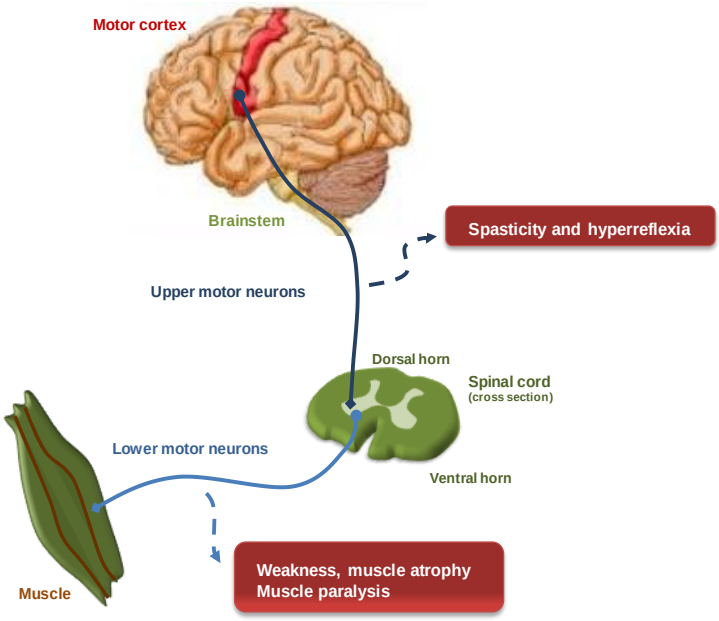

Figure I.1. Corticospinal tract is affected in ALS disease. Upper motor neurons prolong their axons from the motor cortex through brainstem to spinal cord. In ALS, degeneration of these neurons results in spasticity and hyperreflexia. On the other hand, degeneration of lower motor neurons leads to weakness and muscle atrophy and, ultimately, to muscle paralysis as dying neurons fail to make connection between spinal cord (or brainstem) and muscles. Adapted from Kiernan *et al.* (2011).

1.1 Motor neuron vulnerability in ALS

Selective death of motor neurons appears to begin focally and asymmetrically in the upper and lower limb with progressive spreading of injury to contiguous groups of motor neurons. Although the underlying mechanisms are not completely understood, special features of motor neuron physiology could raise the susceptibility to injury. First, the large cell size and long axon processes demand a robust cytoskeleton with high content of neurofilaments. In order to correctly perform neuronal transmission through all the axon, motor neurons need high metabolic rate and optimal mitochondrial function (Ferraiuolo *et al.*, 2011). Due to this intense mitochondria activity, amplified production of reactive oxygen species (ROS) is observed. High intrinsic oxidative stress is hence a putative mark of specific vulnerability of motor neurons. Moreover, with the accumulation of mutations and oxidative damage characteristic of aging, neurons may undergo special sensitivity to those mechanisms (Shaw and Eggett, 2000).

Motor neurons are extremely sensitive to glutamate accumulation, which can occur following inappropriate neurotransmission or dysfunction of the underlying mechanisms. Expression of α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA)/kainate receptors lacking the GluR2 (Williams *et al.*, 1997) is one of the causes since this subunit is impermeable to calcium influx, therefore preventing the accumulation of intracellular calcium. In addition, the low expression of calcium-

buffering proteins aggravates the described calcium homeostasis deregulation. This topic will be further discussed in the section of molecular mechanisms involved in ALS.

Furthermore, motor neurons also evidence reduced capacity for heat shock response and sensitivity to endoplasmic reticulum (ER) stress, which can predispose the cells to oxidative damage and calcium overload (Ferraiuolo *et al.*, 2011). Finally, reduced proteasome function and high expression of particular proteins, such as SOD1, possibly enhance motor neuron vulnerability to toxicity resulting from mutant proteins accumulation, a key feature of ALS disease.

1.2 Molecular mechanisms of motor neuron degeneration

Atrophy of dying motor neurons is described as the pathological hallmark of ALS (Pasinelli and Brown, 2006; Tovar *et al.*, 2009). Nevertheless, ever more studies have tryed to understand the molecular mechanisms implicated in the selective loss of motor neurons and recently some of the pathways involved have been discovered. Genetic factors, mitochondrial dysfunction, ER stress, oxidative damage, excitotoxicity, protein aggregation and axonal transport impairment, among others, seem to be interrelated and implicated in the degeneration of motor neurons in ALS.

1.2.1 Protein aggregation: SOD1 mediated toxicity

Protein aggregation has been described as one of the pathological features of ALS. So far, cytoplasmic inclusions of different mutant proteins were found to be related with fALS cases, namely mutations in TAR DNA-binding protein 43 (TDP-43), fused in sarcoma (FUS) and in copper-zinc superoxide dismutase 1 (SOD1) (Ferraiuolo *et al.*, 2011; Ince *et al.*, 2011). Furthermore, these mutant proteins are also found in sporadic cases reinforcing their role in the pathogenesis of ALS. SOD1 mutations are the most common, being associated to 20% of familial cases, which make them the basis for several *in vitro* and *in vivo* models of ALS (Gomes *et al.*, 2008; Van Den Bosch, 2011).

SOD1 is a ubiquitous protein which functions as a homodimer and its normal function is to catalyse the dismutation of superoxide radicals to hydrogen peroxide (Tripathi and Al-Chalabi, 2008). SOD1 is mainly found in cytosolic compartment but it can also be found in mitochondria. Mutations in SOD1 were first identified by Deng and colleagues (1993) and nowadays over then 100 mutations are recognized.

Conformational instability of mutant SOD1 (mSOD1), which induces the formation of harmful aggregates, are observed in SOD1 transgenic mice (Karch *et al.*, 2009; Wang *et al.*, 2003). Therefore, aggregation of the mutant protein is currently most favored as the main cause of SOD1 mediated toxicity (Tripathi and Al-Chalabi, 2008). Putative mechanisms of toxicity induced by SOD1 aggregation include sequestration of essential cellular components, reduced anti-apoptotic chaperone activity, impaired protein degradation as a cause of overwhelmed proteasome and damage of specific organelles, such as mitochondria and peroxisomes (Ferraiuolo *et al.*, 2011; Rothstein, 2009).

Loss of function is unlikely involved in ALS. In fact, it was reported that mice where SOD1 was homozygously deleted did not develop the disease (Tripathi and Al-Chalabi, 2008; Wong *et al.*, 1995). However, some ALS familial cases have evidenced reduced SOD1 dismutation activity (Liu *et al.*, 1999). On the other hand, gain of a toxic novel function of mSOD1 is also hypothesized (Pasinelli and

Brown, 2006). Indeed, Liu and colleagues (1999) have already described it, where an oxidative function is acquired by G93A mutation in SOD1.

Finally, SOD1 aggregation may be an early event in the disease as it appears by the time of disease onset and its abundance increases with disease progression. Moreover, Friedlander and colleagues (1997) have demonstrated that the effects of mSOD1 expression seem to appear primarily within motor neurons and, in later stages of the disease, the rate of progression is determined by SOD1 mutations acting on microglia.

1.2.2 Oxidative stress

The classical concept of oxidative stress describes a mechanism resulting from imbalance between generation of ROS and antioxidant activities (Sies, 1997). More recently, this concept is being redefined as “a disruption of redox signaling and control that recognizes the occurrence of compartmentalized cellular redox circuits”, as cited by Packer and Cadenas (2007), whereby ROS and reactive nitrogen species (RNS) production are increased. ROS and RNS include nitric oxide (NO), hydroxyl radical and peroxynitrite, among others, and in physiological conditions are involved in cell signaling (Aguiar *et al.*, 2012). However, in cases of excessive production, these species may cause damage to proteins, lipids, DNA and, in particular, to messenger RNA. The central nervous system (CNS) is especially sensitive to oxidative damage because of the low levels of some antioxidant enzymes expression, high content of easily oxidized substrates and high production of ROS during neurochemical reactions (Carri *et al.*, 2003).

Carri and colleagues (2003) have extensively reviewed the role of oxidative stress in the pathogenesis of ALS, specifying a series of oxidative damage markers such as intracellular levels of ROS, lipid peroxidation or protein nitration, among others. Patients with ALS show elevated markers of free radicals collected from cerebrospinal fluid (CSF), serum and urine (Mitsumoto *et al.*, 2008; Simpson *et al.*, 2004; Smith *et al.*, 1998). Accumulation of SOD1-related ALS has particular interest in this mechanism since it is involved in the endogenous anti-oxidant capacity of the cell. Whether mSOD1 mediates oxidative damage through loss or gain of function is still controversial, as mentioned in the previous section. One hypothesis defends that mSOD1 provokes aberrant oxyradical reactions (Pasinelli and Brown, 2006). Indeed, mSOD1 exposes the active copper site to aberrant substrate, developing peroxidative activity. Furthermore, mSOD1 may also cause oxidative damage by mechanisms beyond its catalytic activity. NADPH oxidase-2 (NOX2) expression is increased in SOD1 transgenic mice and in CNS of ALS patients (Wu *et al.*, 2006). Signaling required for the transcription factor nuclear erytrhroid 2-related factor 2 (NRF-2) activation (a major regulator of the antioxidant response) may be disrupted in ALS mice models and patients (Ferraiuolo *et al.*, 2011).

Finally, oxidative stress is committed with other pathophysiological processes, including excitotoxicity, mitochondrial impairment and ER stress, reinforcing the multifactorial character of ALS. In mitochondria, where most of the ROS are produced and SOD1 is greatly expressed, there is an accumulation of both, as observed in mSOD1 models of ALS. This issue will be discussed in the next section.

1.2.3 Mitochondrial dysfunction

Mitochondrial dysfunction has been extensively studied as one of the causes of motor neuron degeneration in ALS. Some of the most common features include mitochondrial depolarization, decrease of adenosine triphosphate (ATP) synthesis, impairment of calcium homeostasis and increased production of ROS. Moreover, mitochondrial damage has been attributed to the accumulation of mutant SOD1 in several *in vitro* and *in vivo* studies of ALS disease (Ferraiuolo *et al.*, 2011; Pasinelli and Brown, 2006; Volonte *et al.*, 2011).

As already mentioned, accumulation of SOD1 is implicated in pathological mechanisms of the disease. Besides being a cytosolic protein, SOD1 can be found within mitochondria and the formation of mutant SOD1 aggregates in vacuoles, in the mitochondria intermembrane space has been described in an ALS mice model (Igoudjil *et al.*, 2011). Vacuolation appears as a result of degenerating mitochondria which, ultimately, leads to cell death, as referred in Tripathi and Al-Chalabi (2008). Indeed, vacuoles are evident at early stages of the disease in transgenic mice expressing human G93A mutation in SOD1 (SOD1G93A) and increase in number and volume along disease progression (Bendotti *et al.*, 2001; Kong and Xu, 1998). In addition, reduced respiratory chain activity and ATP production is evidenced in these mice. These findings seem to be corroborated by reports on ALS patients presenting clusters of abnormal mitochondria and defects in respiratory chain complexes I and IV (Pasinelli and Brown, 2006). How mutant SOD1 aggregates can induce mitochondrial impairment is still a matter of debate but at least three hypotheses are being tested (Pasinelli and Brown, 2006). First, mSOD1 could be involved in fusing the peroxisomes and the outer membrane opening pores in mitochondria membrane, thus, allowing the release of cytochrome *c* and triggering apoptosis. Second, disruption of translocation machinery, in particular of the translocator outer membrane (TOM) complex, may result from aggregation of mutant SOD1 in the outer membrane limiting the import of functional proteins into the mitochondria. At last, abnormal interaction with other mitochondrial proteins can promote mitochondrial damage. Recently, Bcl2, an anti-apoptotic factor, has also been referred to be sequestered by SOD1 aggregates, disrupting its function (Pasinelli *et al.*, 2004).

Calcium buffering is also impaired in ALS transgenic mice. This deregulation could increase the susceptibility of motor neurons to the altered calcium homeostasis associated with glutamate excitotoxicity (Ferraiuolo *et al.*, 2011). High calcium concentrations induce production of ROS in mitochondria (Zhou *et al.* 2010) and contribute to the activation of cell death pathways as caspase-mediated apoptosis (Hajnoczky *et al.*, 2006; Roy and Hajnoczky, 2008).

Finally, mitochondria fusion and fission processes may also be altered as a result of the accumulation of mutant SOD1 in the mitochondria (Carri and Cozzolino, 2011). In fact, Ferri and colleagues (2010) have recently shown that expression of two proteins involved in mitochondrial dynamics are altered in neuronal cells expressing mSOD1, namely optic atrophy-1 (OPA-1), which is a pro-fusion factor, and dynamin-related protein-1 (Drp-1) that associates with the mitochondria, promoting its fragmentation. Mitochondria fragmentation was also demonstrated in cellular and animal models of ALS, as reviewed in Carri and Cozzolino (2011).

1.2.4 Excitotoxicity of glutamate

Glutamate is the excitatory neurotransmitter of the corticospinal tracts and certain spinal interneurons. There are three groups of glutamate receptors in postsynaptic neurons essential to a physiological neurotransmission. *N*-methyl-D-aspartate (NMDA) receptors stimulation occurs with calcium and sodium entry while non-NMDA receptors, namely AMPA/kainate and G-protein linked metabotropic receptors, allow mainly the influx of sodium (Tripathi and Al-Chalabi, 2008; Van Den Bosch *et al.*, 2006). GluR2 is a subunit present in AMPA receptors that is responsible for the resistance to calcium entry being important in glutamate excitotoxicity. During neurotransmission, excitatory signals are ended by the removal of glutamate from the synaptic cleft by glutamate reuptake transporters. When neuronal energy homeostasis or glutamate receptor expression is altered, excessive glutamate is released and accumulates in the synaptic clefts leading to excitotoxicity. Glutamate excitotoxicity is, hence, a well-recognized mechanism of neuronal death resulting from excessive activation of postsynaptic glutamate receptors, and is involved in ALS (Rothstein, 2009). Indeed, a twofold increase in glutamate levels was found in cerebrospinal fluid of ALS patients (Rothstein *et al.*, 1990). In addition, a study with 400 patients with sporadic ALS showed that the amount of glutamate was correlated with the disease severity (Spreux-Varoquaux *et al.*, 2002). Furthermore, hyperexcitability of the motor system in the presymptomatic or early stages of the disease were found by electrophysiological studies in humans (Vucic and Kiernan, 2006; Vucic *et al.*, 2008).

Clearance of glutamate after neurotransmission is critical in preventing excitotoxicity. Excitatory aminoacid transporter 2 (EAAT2), also known as glutamate transporter-1 (GLT1), is the most abundant transporter and is essential for maintaining low levels of extracellular glutamate. It is highly expressed in astrocytes, which are known to be important for rapid removal of synaptic glutamate released by motor neurons. Studies using SOD1 transgenic mice have reported that EAAT2 expression in ventral horn is reduced in presymptomatic stage and completely abolished in end-stage of the disease, as reviewed in Rothstein (2009). Overexpression of EAAT2 delayed the onset of motor neurons injury and decreased the caspase-3 activation and the formation of aggregates in SOD1G93A mice (Guo *et al.*, 2003).

On the other hand, release of glutamate is accompanied by the massive influx of calcium through permissive receptors. Calcium entry induces the production of NO and of other ROS species leading to consequent organelle damage and cell death. Motor neurons are particularly sensitive to calcium accumulation as they have low capacity to buffer calcium and lack the GluR2 subunit of AMPA receptors (Pasinelli and Brown, 2006), which enhances the vulnerability of motor neurons to excessive glutamate stimulation.

1.2.5 Endoplasmic Reticulum stress

Intracellular inclusions related to accumulation of misfolded or unfolded proteins are well known pathological hallmarks of ALS. ER is involved in protein synthesis and in its proper folding. Also, ER participates in refolding newly synthesized proteins that have an incorrect structure. Nevertheless, when misfolded proteins accumulate, an ER stress response pathway, called unfolded protein

response (UPR), is elicited in order to rapidly decrease the load of misfolded proteins. UPR involves the recognition of aberrant proteins by ER-chaperones that promotes a correct protein folding (Ferraiuolo *et al.*, 2011; Kanekura *et al.*, 2009; Walker and Atkin, 2011).

Recently, studies in cerebrospinal fluid and in spinal cord from patients that died with ALS, as well as in ALS mice models, have shown that levels of ER stress-related proteins were upregulated evidencing the contribution of ER in the pathogenesis of the disease. ER stress sensors include activating transcription factor 6 (ATF6), inositol-requiring kinase 1 (IRE1) and PKR-like endoplasmic reticulum kinase (PERK), an ER-resident type I transmembrane protein kinase (Walker and Atkin, 2011), which are actively repressed by association with the chaperone immunoglobulin binding protein (BiP) (Kanekura *et al.*, 2009). ATF6 is activated in a motor neuron like cell line (NSC-34) expressing mutant SOD1 in G93A as showed by Prell and colleagues (2012). Protein disulphide isomerase (PDI), another ER chaperone and marker of UPR, is also activated in SOD1 transgenic mice and biological samples from patients with sporadic ALS and, interestingly, evidenced to be colocalized with SOD1 inclusions (Atkin *et al.*, 2006; Atkin *et al.*, 2008). Furthermore, activation of stress sensors induces downstream pathways implicated in apoptosis, like caspase-12 activation, leading to motor neuron degeneration (Ferraiuolo *et al.*, 2011).

1.2.6 Apoptotic cell death

Apoptosis is a mechanism of programmed cell death essential for maintaining the homeostasis of different tissues, including the CNS. As previously mentioned, ALS disease can result from the disruption of several interconnected cellular mechanisms and organelles. Apoptosis seems to be also involved in motor neuron degeneration in ALS, as many of the referred mechanisms, such as mitochondrial dysfunction or ER stress, can promote the downstream cascade signaling of apoptotic cell death (Ferraiuolo *et al.*, 2011).

Transgenic ALS mice have been a useful model to explore and understand the mechanisms involved in motor neuron death, particularly mice expressing mutant SOD1. Indeed, biomarkers of apoptosis can be detected in the terminal stages of ALS in transgenic mice as well as in humans. How SOD1 promotes apoptosis, either by aggregates formation or changes in its function (mutations can transform SOD1 from an anti- to a pro-apoptotic protein) is still a matter of debate (Pasinelli and Brown, 2006).

The first evidence of SOD1 mediated apoptosis comes from the impairment of the association of cytochrome *c* with the inner membrane of the mitochondria. Cytochrome *c* is translocated from the mitochondria to the cytosol and promotes the activation of caspase-9 which initiates the apoptotic process in the mitochondria. At the same time that disease progresses in ALS mice, a reduction in intra-mitochondrial cytochrome *c* is observed (Bacman *et al.*, 2006). In addition, caspase mediated apoptosis is strictly connected with motor neuron degeneration. Caspase-1 seems to be involved at early stages, even before disease onset, and precedes the activation of caspase-9. Following activation of caspase-9, caspases-7 and -3 are stimulated, as schematically represented in Figure I.2. Caspase-7 activation coincides with the disease onset in SOD1G93A mice (Guegan *et al.*, 2001) and caspase-3 has later activation being described as the final effector of cell death (Rothstein, 2009).

Interestingly, caspase-3 is expressed both in neurons and astrocytes and are responsible for cleavage and inactivation of the glutamate transporter EAAT2 (Pasinelli and Brown, 2006), which leads to glutamate excitotoxicity as the transporter fails to remove it from the synaptic cleft (see section 1.2.4).

Despite the role as an apoptotic inducer, caspase-1 is mainly a player in inflammation (Pasinelli and Brown, 2006). As it will be further analyzed, glial cells are involved in the progression of the disease. This finding demonstrates a relationship between motor neuron degeneration and astrogliosis as well as with microgliosis. Hence, this glial activation can be, not only important for disease progression, but also is an early event in ALS.

Moreover, Bcl2, an anti-apoptotic protein, seems to be related with apoptosis in ALS. In fact, overexpression of Bcl2 preserves motor function and prolonged life span in SOD1G93A mice (Pasinelli *et al.*, 2004). SOD1 can bind to Bcl2 promoting the formation of complex aggregates. When entrapped in these inclusions, Bcl2 become non-functional and the mechanism of apoptosis repression is disrupted. Also, another suggested feature is that Bcl2 undergoes conformational modification and becomes toxic (Rothstein, 2009).

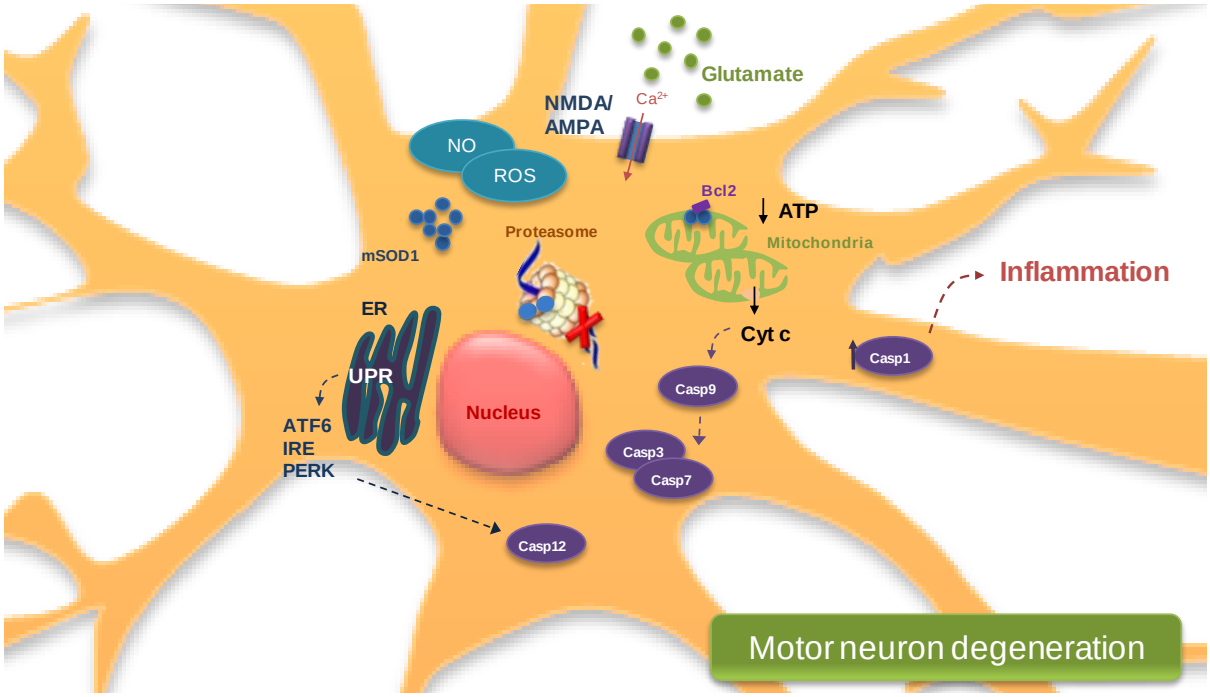

Figure I.2. Molecular mechanisms involved in ALS pathogenesis. Motor neuron degeneration results from interrelated mechanisms that ultimately induce apoptotic events. Mutant superoxide dismutase 1 (mSOD1) accumulation is the primary event. Production of nitric oxide (NO) and reactive oxygen species (ROS) is induced, and oxidative damage is increased. Mutant SOD1 impairs the proteasome who fails in correct misfolded proteins and disrupt mitochondrial function as suggested by the decrease in ATP production. Mitochondria fusion and fission can also be observed. Nonetheless, sequestration of the anti-apoptotic protein Bcl2 and release of cytochrome *c* promote caspase activation and consecutive apoptosis. Caspase-9 (Casp9) seems to be early activated followed by activation of caspases-3 (Casp3) and -7 (Casp7). Caspase-1 (Casp1) is also activated and its role is extended also to inflammatory responses. As misfolded proteins are accumulated, unfolded protein response (UPR) promotes the release of ER stress sensors which activate caspase-12 (Casp12). Glutamate excitotoxic accumulation in the extracellular space and massive influx of calcium through N-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA)/kainate receptors leads to NO and ROS production further increasing oxidative stress.

1.3 Non cell-autonomous disease – involvement of glial cell activation

Initial evidences of pathological mechanisms involved in ALS suggested that the disease is cell autonomous, resulting from different cellular dysfunctions, which ultimately triggers motor neuron death. However, accumulating evidence shows the involvement of other non-neuronal cells in pathogenesis, particular in disease progression. This concept was first proposed by Ince and colleagues in 1996 with the observation of microglia and astroglial cells activation in an *in vitro* model of ALS. In addition, activation of microglia, astrocytes and appearance of lymphocytes in post-mortem tissue of ALS patients and in spinal cord of SOD1 transgenic mice was similarly observed (Kawamata *et al.*, 1992; Philips and Robberecht, 2011). Studies with chimeric mice, where the expression of mutant SOD1 in G93A was selectively induced on motor neurons, astrocytes or microglia have demonstrated that SOD1G93A-overexpressing neurons surrounded by healthy glia remained relatively intact, although healthy motor neurons presented signs of injury when surrounded by mutant SOD1G93A - overexpressing glia (Clement *et al.*, 2003).

As reviewed in Philips and Robberecht (2011), studies in post-mortem tissues of ALS patients and in spinal cord of SOD1 transgenic mice evidenced changes in the morphology of astrocytes and microglia from a surveying phenotype to an activated state with upregulation of surface markers. CD11b, CD68 and ionized calcium binding adaptor protein (Iba1) expression were elevated in microglial cells, as well as glial fibrillary acidic protein (GFAP) and aldehyde dehydrogenase family 1, member L1 (ALDH1L1) in astrocytes. Taken together, these results evidenced that expression of mutant SOD1 in motor neurons is necessary to incite ALS but also requires toxicity of the mutant protein in the surrounding cells.

In addition, several studies with different approaches have demonstrated that mutant SOD1 expression in motor neuron determines the initial timing of disease onset and early progression in some cases, but does not have a significant contribution to later disease progression, (Ilieva *et al.*, 2009). Consequently, astrocytes and microglia must have decisive implications in disease progression after its onset. Indeed, mutant SOD1 gene excision from microglia and selective reduction in astrocytes significantly slowed disease progression (Boilleé *et al.*, 2006; Yamanaka *et al.*, 2008). However, there is still some controversia on whether astrogliosis and microgliosis can be detrimental or beneficial, what surely will depend from the levels that are induced.

1.3.1 Astrocytes

Astrocytes are one of the most abundant cell types in the adult CNS being about ten times more numerous than neurons. Astrocytic cells have ectodermal origin and are responsible for several functions of neuronal support, including regulation of extracellular glutamate, metabolic or ionic homeostatic functions and trophic support for surrounding neurons (Philips and Robberecht, 2011). Astroglial activation is a hallmark of ALS and, as already referred, is characterized by a major increase in expression of GFAP and ALDH1L1. Interaction of astrocytes with motor neurons seems to aggravate motor neuron degeneration by two different reasons; first, it activates astrocytes which are no longer able to exert their protective effect on motor neurons, and second through the release of harmful factors that will act on motor neurons (Neusch *et al.*, 2007). In fact, astrocytes modulate

neuronal integrity through the release of neurotrophic factors such as glial-cell derived neurotrophic factor (GDNF), brain-derived neurotrophic factor (BDNF), insulin-like growth factor 1 (IGF1) and vascular endothelial growth factor (VEGF). This release seems to be disrupted in ALS, since the administration of some of these compounds or their overexpression in a mouse model expressing mutant SOD1 have demonstrated to increase mice survival (Kaspar *et al.*, 2003; Park *et al.*, 2009). In addition, activated astrocytes fail to confer protection to glutamate excitotoxicity due to the loss of EAAT2 transporters, as observed in both transgenic mice and in sporadic and familial cases of ALS (Lasiene and Yamanaka, 2011), indicating the importance of astrocytic damage to motor neuron degeneration in ALS. Moreover, astrocytes expressing mutant SOD1 release factors that inhibit the expression of GluR2 in motor neurons (Ilieva *et al.*, 2009). Unlike GluR2, NMDA receptor is permeable to calcium facilitating its influx and, thus, promotes motor neuron toxicity resulting from intracellular calcium accumulation. On the other hand, upregulation of inducible nitric oxidase synthase (iNOS) and consequently the release of toxic factors, particularly ROS and NO, suggest astrocytic involvement in ALS (Philips and Robberecht, 2011). *In vitro* studies also evidenced that SOD1 astrocytes release factors which are capable of inducing motor neurons degeneration (Haidet-Phillips *et al.*, 2011).

Studies in post-mortem tissues of ALS patients have provided clues for the role of non-neuronal cells in the late stage of the disease. In the spinal cord, astrocytosis is observed in ventral and dorsal horn, as well as in the intersection of fibers with the corticospinal tract entrance in the grey matter. Activated astrocytes also appear in cortical grey matter, subcortical white matter and are not restricted to motor cortex (Philips and Robberecht, 2011).

For all that findings, astrocytes have now much interest in the design of therapeutic strategies in ALS. Interestingly, glial-restricted precursor cells have been transplanted in the spinal cord of SOD1G93A rats to evaluate the possible protective role of the wild type SOD1 astrocyte precursor (Lepore *et al.*, 2008). These cells showed to be able to differentiate into GFAP-expressing astrocytes and were capable to rescue motor neuron injury, in a way that seems dependent of elevated glutamate scavenging activity. Moreover, it was observed that this transplantation led to a significant increase in rat survival.

1.3.2 Oligodendrocytes and Schwann cells

Oligodendrocytes in CNS, and Schwann cells in the peripheral nervous system, are responsible for the myelin sheaths surrounding neurons which provide electrical insulation essential for rapid signal conduction. Schwann cells also participate in the clearance of debris and in guiding the axon after neuron damage (Ilieva *et al.*, 2009). Although there is little evidence that these cells might be involved in ALS pathogenesis, some recent studies have been trying to clarify this concept. As reviewed by Lasiene and Yamanaka (2011), abnormalities in oligodendrocytes related myelin, such as loss of compact myelin and lamellae detachment, are seen in spinal cords of pre-symptomatic SOD1 transgenic rats and aggravated at symptomatic stages. In what concerns to Schwann cells, intriguing findings were recent revealed.

A study using Cre-mediated gene excision eliminated mutant SOD1G37R from Schwann cells (Lobsiger *et al.*, 2009). Interestingly, and in opposite to what happens in other non-neuronal cells, this

elimination of mutant SOD1 specifically in Schwann cells failed to slow disease progression. Instead, a substantial acceleration of the late phase of disease was observed (Lobsiger *et al.*, 2009). Therefore, the underlying mechanism suggests a protective role to mutant SOD1 possibly due to the dismutation activity that can ameliorate some oxidative damage within the cells (Ilieva *et al.*, 2009). However, recently, Wang and colleagues (2012) found that knockdown of mutant SOD1 in Schwann cells of SODG85R transgenic mice delayed disease onset and extended survival indicating that SOD1G85R expression is neurotoxic. These results implies that different mutations confer different outcomes to cell toxicity and, in the case of Schwann cells, oxidative damage seems to be an important feature in the context of ALS.

1.3.3 Microglia

Microglia is the most important glial cell in the context of ALS as they are the typical immune cells of the CNS. There is accumulating evidence that neuroinflammation is as an imperative feature in ALS and, thus, seem to be strictly connected with microglia activation (Neusch *et al.*, 2007). Microglia expressing mutant SOD1 change from their normal surveying state to an activated one, migrate to initial sites of injury and trigger high production of extracellular superoxide and ROS (Ferraiuolo *et al.*, 2011). Indeed, like astrocytes, microglia damage has been shown to rapidly contribute to disease progression (Ilieva *et al.*, 2009). Furthermore, Boillée and colleagues (2006) have demonstrated that reduced expression of mutant SOD1 specifically in microglia in a mouse model of ALS significantly slowed disease progression. Also, studies indicated that bone marrow transplantation of SOD1G93A transgenic mice with microglia expressing wild type SOD1 altered the functional properties of microglia and prolonged mice survival (Beers *et al.*, 2006). More recently, it was observed that microglia is highly reactive in preclinical stages of ALS in the transgenic rat model with mutant SOD1G93A (Graber *et al.*, 2010) however its ablation in spinal cord close to clinical onset has not shown to protect motor neurons (Gowing *et al.*, 2008). Moreover, replacement of microglia cells expressing mutant SOD1 using clodronate liposomes significantly slowed disease progression and prolonged survival of the transgenic ALS mice (Lee *et al.*, 2012).

Such observations may underlie a clue in linking microglial activation with ALS progression. The present dissertation focuses on the comprehension of microglia properties in ALS pathogenesis using *in vitro* models, therefore, microglia features will be further dissected in the section 2 of this introduction. A schematic representation of the section 1.3 is illustrated in Figure I.3.

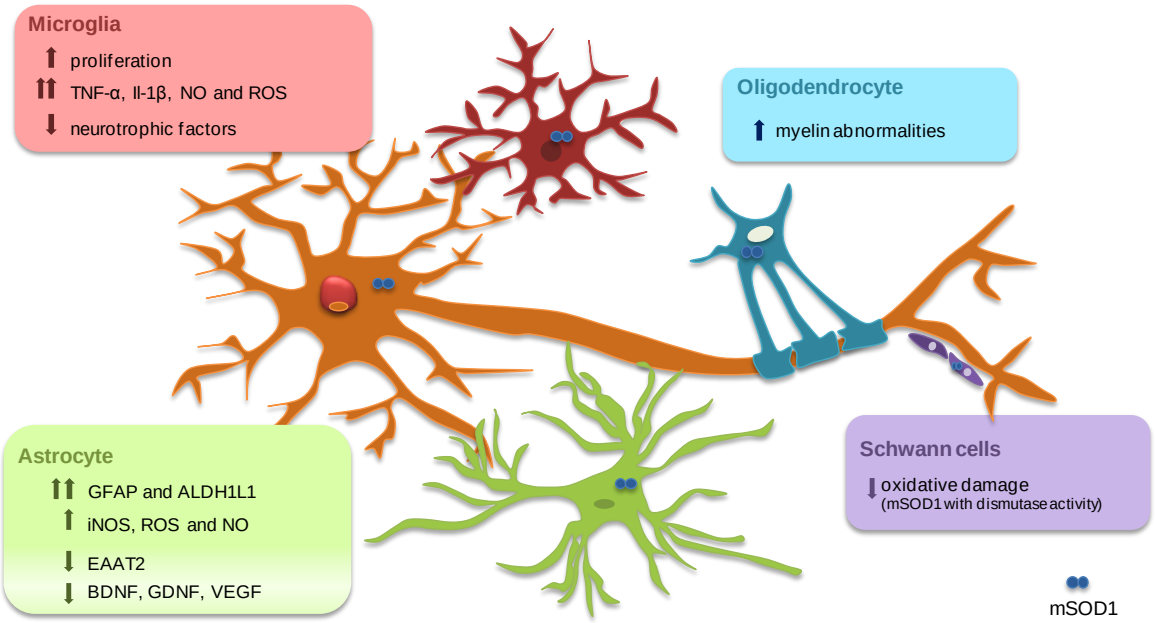

Figure I.3. ALS is a non-cell autonomous disease. Non-neuronal cells in the CNS are also involved in ALS pathogenesis. Microglia shows increase proliferation as well as production of inflammatory cytokines such as tumour necrosis factor alpha (TNF-α) or interleukin-1 beta (IL-1β) and reactive oxygen species (ROS) when mutant SOD1 (mSOD1) accumulates within the cell. In addition, reduction of neurotrophic factors accounts for motor neuron degeneration. Oligodendrocytes seem to be involved just to some extent as myelin abnormalities can be observed. Schwann cells have reduced oxidative damage when express mSOD1 with dismutase activity being able to slow disease progression. Finally, astrocytes express high amounts of reactive oxygen species (ROS) and nitric oxide (NO) and fail to support neuronal function as factors such as BDNF (brain derived neurotrophic factor), GDNF (glial-cell derived growth factor) or vascular endothelial growth factor (VEGF) are reduced. EAAT2 (excitatory aminoacid transporter-2) is inactivated by mutant SOD1 contributing to excitotoxicity due to lack of glutamate clearance from the synaptic cleft. Adapted from Ilieva *et al.* (2009).

1.4 Suitable models for ALS pathogenesis studies

Studies in biological samples from ALS patients and genetic analysis have provided important clues for the candidate intermediates involved in the pathogenesis of the disease. However, more clear and specific mechanisms have been uncovered by *in vitro* and *in vivo* models.

Since the study of the sporadic form of the disease is extremely difficult by the absence of suitable models, most recent research focuses on the genetics of familial ALS that has shown several mutations. Among them, mutations in SOD1 are the most studied. Therefore, mice expressing mutant proteins associated with fALS have been created in order to unravel the mechanisms of motor neuron loss, especially with different forms of SOD1 (Van Den Bosch, 2011). The first transgenic mice created expressed the human protein with substitution of glycine over adenine at position 93 (G93A) by its insertion in the genome (Gurney, 1994). These mice show progressive hind limb weakness leading to paralysis and death, and were able to replicate the disease progression observed in patients. Overexpression of non-mutated protein gives no phenotype, supporting the role of mutant SOD1 as cause of ALS in mice (Van Den Bosch, 2011). Further reports also showed various abnormalities in the mice before disease onset, such as behavioral motor changes (Mead *et al.*, 2011), electrophysiological dysfunctions (Bories *et al.*, 2007) and mitochondria damage (Bendotti *et al.*,

2001). Therefore, several mice expressing SOD1 mutant were created with other mutations such as G37R, G85R or D90A (Bruijn *et al.*, 1997; Jonsson *et al.*, 2006; Wong *et al.*, 1995) that evidenced similar phenotype as SOD1G93A transgenic mice.

In vitro studies addressing mechanisms implicated in ALS have also been performed in cultures of motor neurons isolated from mice spinal cord (Tovar *et al.*, 2009) and in NSC-34, a cell line that results from hybridization between neuroblastoma cells and motor neurons from mice spinal cord (Cashman *et al.*, 1992). The study of such a complex disease using *in vitro* models, which represent such a reduced and limited system, has several inconvenient but, still, can provide important informations on motor neurons physiology at cellular and molecular levels (Tovar *et al.*, 2009).

NSC-34 cells, as reported by Cashman and colleagues (1992), seem to be a good model as they evidence morphological and physiological properties of motor neurons including extension of processes, formation of contacts with cultured myotubes, support of action potentials and expression of neurofilament proteins, among other features. Moreover, these cells may be able to model aspects of neuromuscular synapse formation, as referred by Martinou and colleagues (1991). Recently, NSC-34 cells have been transfected with mutant SOD1 in G93A and some features of ALS pathogenesis are becoming uncovered, such as the formation of mutant SOD1 aggregates and Golgi apparatus disruption (Gomes *et al.*, 2010; Gomes *et al.*, 2008) and mitochondrial impairment (Raimondi *et al.*, 2006). Also, NSC-34 cells expressing SOD1G93A evidenced less proliferation and differentiation ability (Gomes *et al.*, 2008). However, NSC-34 cells retain some characteristic of neuroblastoma cells that can interfere with several cellular responses, such as the presence of the gene N-myc, an oncogene involved in cell proliferation. For the study of the pathways involved in neuronal death and its prevention by possible therapeutic agents, the effects of N-myc can disrupt the underlying mechanisms (Tovar *et al.*, 2009). Nonetheless, this *in vitro* model can be a useful tool to explore early molecular mechanisms of ALS as well as the features implicated in the disease progression.

1.5 Is GUDCA a promising therapy?

ALS is an extremely severe disease and attempts to find a novel therapeutic agent are imperative since the only drug approved for use in ALS is riluzole which only slightly prolongs survival (Pasinelli and Brown, 2006). Riluzole is a benzothiazole derivative and has been proven to ameliorate glutamate excitotoxicity through different mechanisms including inhibition of pre-synaptic release of glutamate or inactivation of neurons sodium channels that reduces glutamate transmission (Tripathi and Al-Chalabi, 2008). However, a different property seems to be associated with riluzole neuroprotection effects, as other anti-glutamate agents fail to be effective in clinical trials (Ferraiuolo *et al.*, 2011). So far, there is no successful drug for ALS treatment and search for novel therapeutic agents that could prevent motor neuron degeneration is of a great importance. In this context, we propose to study the efficacy of glycoursodeoxycholic acid (GUDCA), a conjugated species of ursodeoxycholic acid (UDCA) with glycine, on the prevention of neuronal degeneration in the cellular models of ALS since it demonstrates several neuroprotective features, as it will be further discussed.

Bile acids are acidic steroids synthesized in hepatocytes from cholesterol. UDCA accounts for up to 4% of bile acid pool and when administrated by oral intake have demonstrated to undergone

several functions in the liver, reinforcing hepatocyte function. Mechanisms of action include alteration of bile acids pool, mitochondrial integrity, immune modulation and anti-apoptotic properties, as summarized in Lazaridis *et al.* (2001). Therefore, UDCA is commonly used for the treatment of hepatobiliary disorders (Rudolph and Link, 2002). Following oral administration, UDCA is consequently submitted to hepatic conjugation either with glycine or taurine, producing, respectively, glycoursodeoxycholic (GUDCA) and tauroursodeoxycholic (TUDCA) acids, as schematically represented in Figure I.4. GUDCA is the major species, accounting to 79.8% of UDCA conjugation and, so, is the form with the highest clinical relevance (Lazaridis *et al.*, 2001). These resulting conjugated species have shown cytoprotective properties in different CNS cells, such as neurons, astrocytes or microglia (Rodrigues *et al.*, 2000; Silva *et al.*, 2012; Vaz *et al.*, 2010). Interestingly, TUDCA has already proven beneficial effects in many neurodegenerative diseases, namely in Alzheimer's, where it was able to inhibit apoptosis in an *in vitro* model of AD mutant neuroblastoma cells (Ramalho *et al.*, 2006; Ramalho *et al.*, 2008).

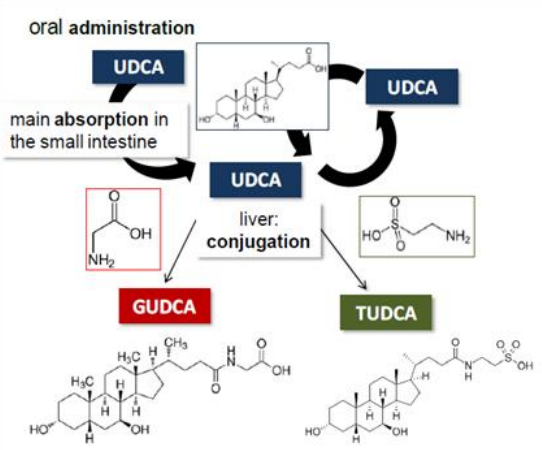

Figure I.4. Metabolism of UDCA after oral administration. The main place of absorption is in the small intestine. From there, ursodeoxycholic acid (UDCA) enters the portal vein and the enterohepatic circulation. In the liver, UDCA is conjugated mainly with glycine, leading to glycoursodeoxycholic acid (GUDCA), the conjugate form with the highest clinical relevance. A smaller amount of UDCA is conjugated with taurine, leading to tauroursodeoxycholic acid (TUDCA).

The ability of GUDCA to prevent the demise of different cells from CNS in conditions mimicking a moderate and severe hyperbilirubinemia have been extensively studied by our group. In this regard, protection against unconjugated bilirubin (UCB)-induced oxidative damage was achieved by pre-incubation of rat cortical neurons with 50 μM of GUDCA (Brito *et al.*, 2008). Later, Vaz and colleagues (2010) have demonstrated that GUDCA restore cellular antioxidant potential on immature rat neurons after UCB treatment, evidencing the ability of this bile acid to modulate oxidative stress. In the same study, it was reported that GUDCA is able to ameliorate UCB-induced mitochondrial respiratory chain dysfunction and to reverse the inhibition of cytochrome *c* oxidase activity (Vaz *et al.*, 2010). Immunosuppressive action was also showed in a study performed by Fernandes and colleagues (2007) with astrocytes exposed to UCB. Here, previous incubation with GUDCA reduced astroglial production and release of tumour necrosis factor-alpha (TNF-α) and interleukin-1 beta (IL-1β). More recently, neuroprotective effects of GUDCA were extended to the prevention of NO increase and glutamate release as well as to benefits in neuronal outgrowth dynamics and synaptic activity disruption by UCB in cortical neurons (Silva *et al.*, 2012).

Taken together the molecular and cellular mechanisms underlying motor neuron degeneration in ALS and the benefits of GUDCA as anti-apoptotic and anti-inflammatory agent, it is plausible to think

in the potential therapeutic properties of the bile acid in the treatment of ALS. Indeed, Min and colleagues (2012) have performed a clinical trial using UDCA in 80 ALS patients. Although without conclusive results, the patients were tolerant to oral administration and this pilot study could open a new view in the use of UDCA and its conjugated species in the treatment of ALS. In addition, a promising argument that GUDCA could actually be succeeded relies in the fact that other anti-inflammatory drugs have proven to be efficacious in ALS mice models treatments (Philips and Robberecht, 2011).

2. Neuroprotective *vs*. neurotoxic properties of microglia in ALS

2.1 Microglia: role in CNS

Microglia constitute about 10-20% of glial cell population and exhibit a small cell soma with fine and long processes with dynamic protrusions (Nimmerjahn *et al.*, 2005). They populate CNS parenchyma in both white and grey matter, exhibiting a higher density within the hippocampus, basal ganglia and substancia nigra (Lawson *et al.*, 1990; Tambuyzer *et al.*, 2009). Microglia origin is still a matter of discussion, however some authors believe that microglia consists of a subpopulation generated from bone-marrow cells that migrate to CNS during embryonic development and a second one derived from myeloid progenitor cells that enter the brain after birth (Ginhoux *et al.*, 2010; Ohsawa and Kohsaka, 2011; Prinz and Mildner, 2011). Microglial cells share many properties of macrophages as they belong to the mononuclear phagocyte lineage (Vilhardt, 2005) and in fact, they are considered the resident immune cells of CNS, as they act as the brain's innate immune system (Aloisi, 2001; Streit, 2002). However, it was recently shown that, at resting conditions, they differ from macrophages as they express much lower CD45 levels, which represents the only phenotypical mean available to distinguish the two populations (Greter and Merad, 2012).

They are continuously monitoring the surrounding environment with their highly branches and motile cell processes (Nimmerjahn *et al.*, 2005; Ohsawa and Kohsaka, 2011) and are the first line of defence against pathogenic organisms. When the tightly regulated CNS homeostasis is disturbed, microglia rapidly change their morphology, gene expression profile and functional behaviour (Calvo and Bennett, 2011), which is considered an activated phenotype. In fact, the traditional classification of microglia phenotypes includes a resting state, where microglia have a highly ramified morphology with small cell body in contrast to the amoeboid morphology of activated microglia. This concept implies that microglia are inactive or quiescente before an activation stimulus. However, accumulating evidence indicate that microglia activation should be considered a change in functional phenotypes rather than an awakening (Hanisch and Kettenmann, 2007). Microglia constitute, hence, a unique population and distinct from all other CNS cells and its functions will be described in the next sections.

2.1.1 Monitoring CNS environment under physiological conditions

Microglia have commonly been described as being in a quiescent or latent state under physiological CNS conditions, the so called "resting" phenotype. However, recent studies suggest that

microglia cells are highly dynamic structures in normal brain and continually survey their microenvironment (Nimmerjahn *et al.*, 2005). In healthy brain, microglia is constantly moving their highly branched cellular processes making contact with all the surfaces that lie within their environment. Indeed, recent studies based on *in vivo* two-photon microscopy in transgenic mice expressing enhanced green fluorescent protein in *CX3CR1* locus showed that microglia processes were remarkably motile, continuously undergoing cycles of *de novo* formation and withdrawal (Davalos *et al.*, 2005; Nimmerjahn *et al.*, 2005). Moreover, Nimmerjahn and colleagues (2005) have shown that the brain parenchyma is completely screened by microglia once every few hours. Besides this constant morphological alteration, microglia surveillance also involves continuous biochemical sensing and interpretation of environment. Through a variety of surface receptors, such as receptors for cytokines or chemokines, receptors for complement fragments, immunoglobulins, adhesion molecules and inflammatory stimuli, microglia can sense subtle changes (Nimmerjahn *et al.*, 2005; van Rossum and Hanisch, 2004) being able to quickly respond to any insult to CNS homeostasis.

Recent studies have suggested that interaction with neurons is required for maintaining the surveillance phenotype of microglia. In fact, neurons produce many inhibitory or calming signals, such as CD200, fractalkine (CX3CL1) or CD47, that can shape microglia responses and restrain their activation (Hanisch and Kettenmann, 2007; Tambuyzer *et al.*, 2009; Yang *et al.*, 2010). The interaction of CX3CL1 with its receptor on microglia, CX3CR1, seems to be strongly connected to motility control since disrupted CX3CL1 signaling results in about 30% reduced process speed as evidenced by observation of microglia dynamics in *ex vivo* time lapse imaging of mouse retina explants (Liang *et al.*, 2009). Also, Hoek and colleagues (2000) have shown that microglia in CD200-deficient mice exhibit a constitutively active phenotype with an increased subset of amoeboid phagocytic morphology and elevated levels of the well-known microglial markers expression, namely CD45 and complement type-3 receptor (CR3). Although the interaction with neurons is important to modulate microglial structure and function, other molecules such as chemokines, neurotransmitters or peptides, originated from both neuronal or non-neuronal cells, may also influence microglia dynamics under physiological conditions, as reviewed in Hanisch and Kettenmann (2007). Accordingly, microglia surveillance function is collectively maintained by a multitude of soluble and membrane-bound factors.

2.1.2 Defining Microglial Activation

Microglia cells are capable of reacting to a variety of stimuli by changing their phenotype to an activated state. As descendents of monocyte lineage, some of these microglial features are innate responses. However, microglia activation is an adaptive process specific for each stimulus and CNS region, and can undergo neuroprotective or neurotoxic outcomes (Carson *et al.*, 2007). It is now accepted that microglia activation encompasses incredible functional plasticity, where the specific microenvironment will determine microglial response.

For many years, the main concept of microglia activation was the morphological switch from a surveillance state with small cell soma and numerous processes to an amoeboid shape microglia. This pro-inflammatory phenotype, also called M1 state, is often associated to neurotoxic properties and can

be achieved in microglia cultures by incubating the cells with LPS or interferon-gamma (IFN-γ) (Colton, 2009; Durafourt *et al.*, 2012; Leidi *et al.*, 2009).

M1 phenotype is characterized by the expression of high levels of pro-inflammatory cytokines, such as TNF-α, IL-1β and interleukin-12 (IL-12), chemokines, proteases and redox species like nitric oxide (Durafourt *et al.*, 2012; Kraft and Harry, 2011; Nakajima *et al.*, 2003; Nayak *et al.*, 2010).

The alternative activation or M2 phenotype on microglia is inducible by multiple factors and is associated with repair and resolution of tissue homeostasis. The predominant induction signal is the production and release of anti-inflammatory cytokines like IL-4, IL-13, IL-10 and transforming growth factor-beta (TGF-β) (Colton, 2009; Glezer *et al.*, 2007; Ledeboer *et al.*, 2000; Suzuki *et al.*, 2005). Alternative activation can also result due to a switch from a pro-inflammatory to an anti-inflammatory gene profile in order to stop the response of classical activated microglia. In addition, there is another activation state of microglia regarded as anti-inflammatory, called acquired deactivation. This phenotype has a different gene profile from alternative activation but it also downregulates innate immune responses (Colton, 2009). Acquired deactivation is characterized by the inhibition of pro-inflammatory cytokine production, increased expression of scavenger receptors and increased production of anti-inflammatory cytokines, such as IL-10. This phenotype is induced by exposure to TGF-β and IL-10 or by phagocytosis of apoptotic cells.

Another microglial phenotype recently described (Streit *et al.*, 2004; Streit and Xue, 2009) is designated dystrophic or senescent microglia. As suggested by Graeber and Streit (2010), chronic activation of microglial cells could lead to overactivation followed by microglial degeneration. This is especially relevant in the context of aging-related disorders as microglia response to injury during early-life could increase the susceptibility to the development of disease in adulthood (Somera-Molina *et al.*, 2009). An hallmark of this phenotype is the fragmentation of the cytoplasm in microglia, a process called cytorrhexis (Streit and Xue, 2009), which illustrates the loss of microglia functionality. This variety of microglia phenotypes are schematically represented in Figure I.5.

Therefore, activation of microglia includes recruitment to the site of injury (migration), proliferation, morphological changes, production of inflammatory mediators, up-regulation of antigen-presenting cells capabilities and phagocytosis. This remarkable plasticity in response to injury is strictly mediated by the duration and type of stimuli received by microglia. Moreover, if stimulation is strong or more prolonged, microglia activation will comprise more dramatic changes. In the next sections these functional roles displayed by microglia will be further explored.

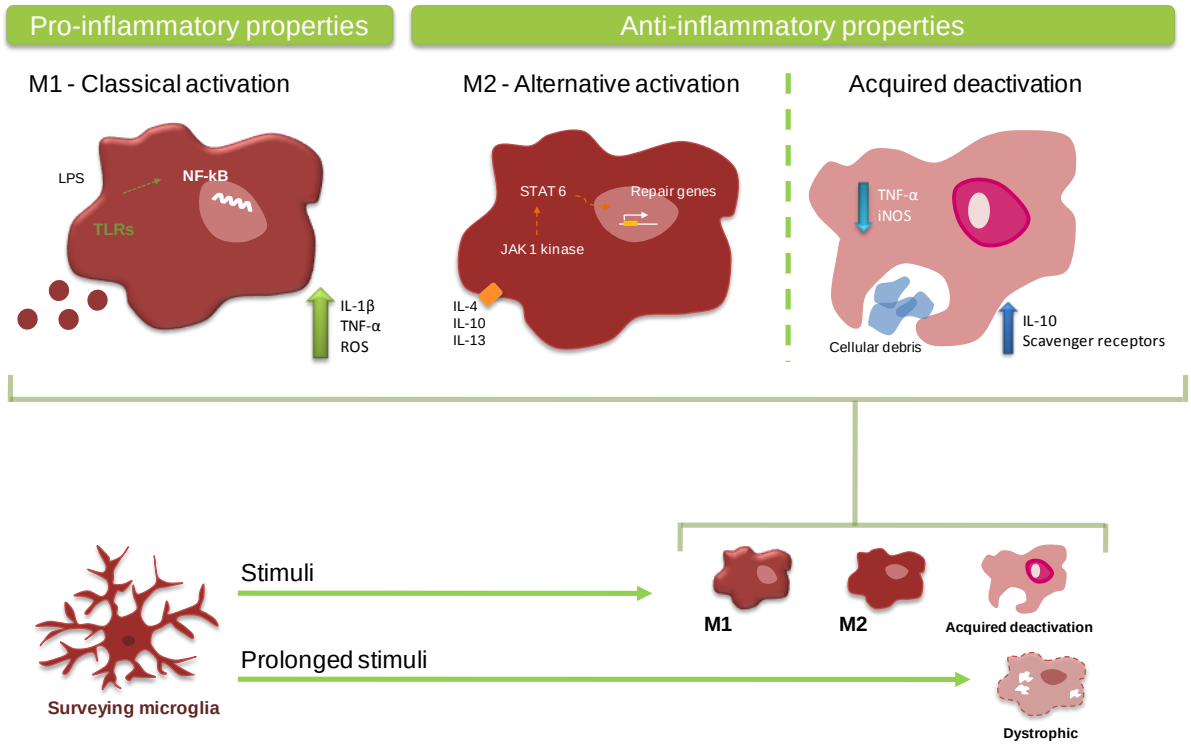

Figure I.5. Diversity of microglia phenotypes. Microglia can acquire different activation states in response to specific stimuli. LPS induce classical activation of microglia also known as M1 phenotype with increase production of proinflammatory cytokines such as tumor necrosis factor-alpha (TNF-α), among others. Toll Like Receptors (TLRs) activation can lead to the stimulation of nuclear factor κB (NF-κB). M2 or alternative activation is induced by IL-4, IL-10 or IL-13 promoting the transcription of repair genes through a signaling cascade dependent on Janus kinase 1 (JAK1) activation and signal transducer and activator of transcription 6 (STAT6) recruitment. When cellular debris are recognized by microglia, an acquired deactivation phenotype is induced. Decrease of pro-inflammatory cytokines and upregulation of IL-10 and scavenger receptors are recognized features of this activation state. Finally, when facing prolonged stimuli, microglia can became overactivated and, ultimately, dystrophic compromising their normal function.

2.1.2.1 Migration

Microglia migration has been widely studied. In fact, this ability to sense distant signals allows microglia to accumulate in sites of injury in order to restore CNS homeostasis. Migration, also called chemotaxis, is orchestrated by multiple chemotactic compounds released at the site of damage, either by injured cells or pathogenic organisms. These signals form gradients that attract microglia to the site of insult through dilution in the neighbouring areas (Calvo and Bennett, 2011). Microglia can extend their processes towards the chemoattractant molecule, which is mediated by glial calcium (Parkhurst and Gan, 2010). When reach into the damaged site, microglia undergo morphological changes becoming with an amoeboid shape and stop migrating, as demonstrated by Orr and colleagues (2009).

ATP is probably the most studied chemoattractant, however many different molecules have been proven to regulate microglia direct movement, such as other purines like ADP (Davalos *et al.*, 2005), NRG1 (Calvo and Bennett, 2011), chemokines like chemokine motif ligand 2 (CCL2) and fractalkine (Liang *et al.*, 2009), neurotensin (Martin *et al.*, 2003), bradykinin (Ifuku *et al.*, 2007), among others. ATP is known to act as neuromodulator in the CNS, regulating various physiological functions of microglia (Ohsawa and Kohsaka, 2011). Accumulating studies have shown that rapid process

extension and further migration is dependent upon ATP sensed through microglial $P2Y_{12}$ receptor (Davalos *et al.*, 2005; Ohsawa and Kohsaka, 2011), as illustrated in Figure I.6. The role of NO is also important for the control of direction of movement towards the site of injury (Parkhurst and Gan, 2010).

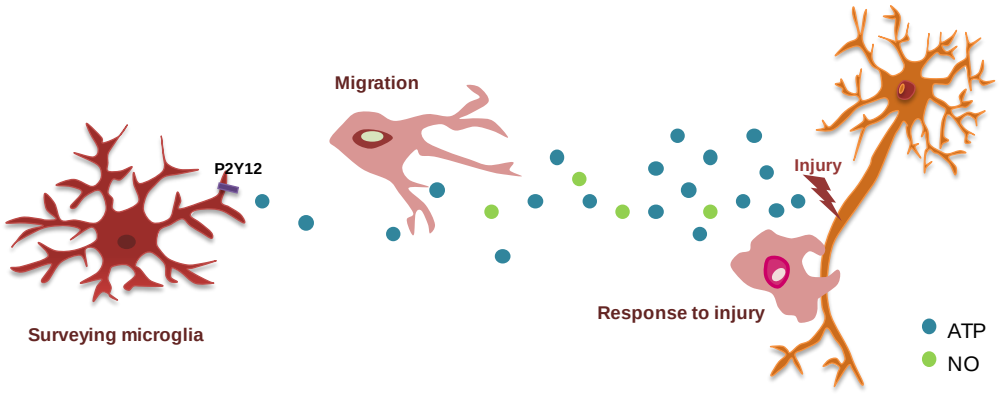

Figure I.6. ATP mediates chemotatic function of microglia. Injured neurons release large amounts of ATP creating a gradient that can be sensed by microglia in surrounding areas. ATP sensed through P2Y12 receptor induces primarily extension of processes and consecutive migration of microglial cells to the site of injury. NO has no chemoattractant potential, but seems to direct microglia movement to the damage site. Adapted from (Monk and Shaw, 2006).

2.1.2.2 Phagocytosis

Phagocytosis is an endocytic mechanism involving vesicular internalization of solid particles, such as pathogens or cellular debris, and has been widely studied in macrophages (Napoli and Neumann, 2009; Neher *et al.*, 2012). It is also performed by microglia, as they derived from myeloid lineage and share various similarities with peripheral macrophages. Phagocytosis in the CNS is initiated by the release of "find-me" signals, which recruits microglia to the site of injury. Upon recognition of cell-surface signals on target cells , microglia initiate their uptake and subsequent responses (Neher *et al.*, 2012). Phagocytosis can encompass different outcomes, either pro- or anti-inflammatory, depending on the type of receptor that has been stimulated. As described by Napoli and Neumann (2009), the uptake of apoptotic cells occur with production of anti-inflammatory cytokines like TGF-β thus restraining inflammation, while toll like receptors (TLRs) stimulation leads to the release of pro-inflammatory mediators including TNF-α and iNOS.

Phagocytic cells evidence receptors for phosphatidylserine (PS). Externalization of PS is the most common marker for the early apoptosis. Self reorganization of plasma membrane with redistribution of PS in the outer leaflet seems to be a key step on apoptotic cells and can be stimulated by several mechanisms (Neher *et al.*, 2012). This process is controlled by two enzymes, specifically aminophospholipid translocase, which removes PS from the outer leaflet and phospholipid scramblase that can promote its exposure by randomizing phospholipid distribution (Martin *et al.*, 1995; Tyurina *et al.*, 2007). In microglia, the uptake of cellular debris can be mediated by interaction with T cell immunoglobulin mucin 4 (TIM-4) and, more importantly, with the triggering receptor expressed by myeloid cells-2 (TREM-2). Despite the production of anti-inflammatory cytokines such as IL-10, TREM-2 involvement in engulfment of debris is also accompanied by downregulation of pro-inflammatory cytokine expression (Takahashi *et al.* 2007), as schematically represented in Figure I.6. Loss or ablation of this receptor leads to deficient removal of cellular debris and enhanced expression of

inflammatory mediators (Ransohoff and Perry, 2009). In addition, nucleotide leaking from necrotic cells seems to have the ability to stimulate phagocytosis, as it has been shown that uridine diphosphate (UDP) induces microglial clearance function *in vitro* through activation of P2Y6 receptor (Calvo and Bennett, 2011).

On the other hand, TLRs mediate the phagocytosis of microbial pathogens through recognition of pattern-associated molecular patterns (PAMPs) which can occur more rapidly than the uptake of apoptotic neurons. Microglia then initiates a pro-inflammatory response with release of inflammatory mediators and ROS production (Block *et al.* 2007). In addition, as represented in Figure I.7, another receptor putatively involved in the phagocytosis of pathogens is the complement receptor 3 (CR3) which recognizes the complement component iCR3b on opsonized bacteria (Napoli and Neumann, 2009). In physiological CNS, microglial phagocytosis is also required in synaptic stripping in order to remove synapses from injured neurons and also, in clearance of apoptotic neuronal cell bodies during development (Perry and O'Connor, 2010).

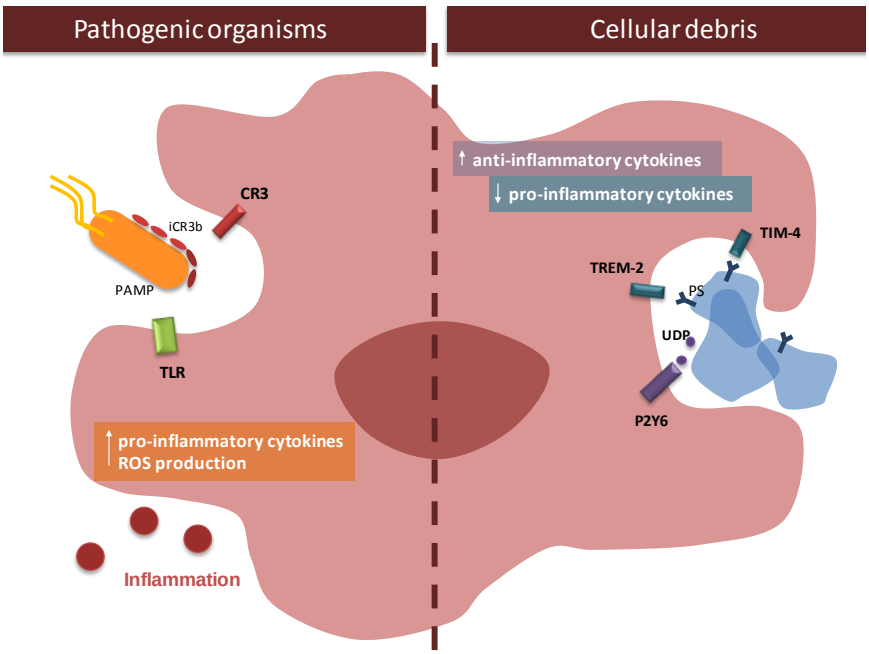

Figure I.7. Microglia phagocytosis and induced responses. Microglia is the first line of defense against pathogenic organisms once they cross the blood brain barrier (BBB). Upon infection, pattern-associated molecular patterns (PAMPs) are recognized through toll-like receptors (TLRs) which induce phagocytosis of the microorganism. Also, opsonization (a recognized mechanism of immunity control of infection) includes involvement of the pathogen with complement proteins such as iCR3b which can be recognized by the microglia complement receptor CR3. Microglia can similarly phagocyte cellular debris. Trough T cell immunoglobulin mucin 4 (TIM-4) or triggering receptor expressed by myeloid cells-2 (TREM2), phosphatidylserine (PS) in the surface of debris is recognized by microglia and internalization begins. Certain nucleotides lacking from apoptotic cells can also stimulate phagocytosis of debris upon activation of P2Y6 receptor.

2.1.2.3 Antigen presentation

Microglia express both major histocompatibility complex (MHC) I and MHC II constitutively at low levels since they are actively down-regulated by the immune inactive microenvironment of the CNS

(Tambuyzer *et al.*, 2009). While MHC I helps in mediation of cellular immunity and occurs in all cells except erythrocytes, MHC II is commonly expressed by antigen presenting cells (APC). When microglia is activated, upon infection or auto-immune disease, these complexes are up-regulated and an inflammatory response begins. Indeed, microglia expression of MHC II has been demonstrated to be involved in different forms of CNS pathology (Calvo and Bennett, 2011; Perry, 1998).

Presentation of antigens to T-cells includes the presence of co-stimulatory molecules such as CD11a, CD80, CD86 and CD40 (Chastain *et al.*, 2011; Tambuyzer *et al.*, 2009). Upon recognition of MHC II by mainly T helper cells (Th) receptor (TCR), microglia release inflammatory cytokines which enhance the stimulation of T-cells differentiation into mature CD4+ T helper lymphocytes. Depending on the type of molecules involved, differentiation can result in Th1, Th2, Th17 or T regulatory cells (Treg). Th1 is an IFN-γ producing cell contributing to inflammation whereas Th2 produces IL-4, a well-know anti-inflammatory cytokine (Chastain *et al.*, 2011). Nevertheless, counter regulatory mechanisms are indispensable to constrain microglia response in order to avoid excessive inflammation.

2.1.2.4 Production of inflammatory mediators

As described in previous sections, microglia can express several inflammatory mediators, not only cytokines but also chemokines, ROS, NO and neurotrophic factors. In this section it will be reviewed the role of some of the most common mediators in microglial functions and plasticity.

Cytokines are small proteins usually secreted by microglia under inflammatory conditions. IL-1β, IL-1, TNFα and IL-6 are pro-inflammatory cytokines and their expression can be activated by many different stimuli. LPS, through activation of TLR-4, and viruses, through activation of TLR-3, are known to stimulate IL-1, IL-6 and TNF-α secretion, as evidenced by *in vitro* studies (Lehnardt, 2010; Olson and Miller, 2004). IL-1 and TNF-α are mainly involved in the initiation of neuroinflammatory response by their ability to induce expression of adhesion molecules and chemokines that are essential for T-cell recruitment (Lee and Benveniste, 1999; Tambuyzer *et al.*, 2009). Interestingly, IL-6 is a pleyotropic cytokine with both pro- and anti-inflammatory properties (Tambuyzer *et al.*, 2009). IL-1β is a major regulator of metalloproteinases (MMPs) expression, and it can induce the production of NO, as well as the blockade of the uptake of glutamate (Kraft and Harry, 2011).

Moreover, microglial production of pro-inflammatory cytokines is frequently accompanied with the production of ROS and NO-dependent reactive nitrogen species (Kraft and Harry, 2011). ROS is mainly produced by microglia to eliminate pathogenic organisms and to mediate destruction of cellular debris. However, if microglia activation is prolonged, ROS production is too high which will aggravate inflammation. NO production can be induced by several stimuli, including LPS, as shown by *in vitro* studies (Nayak *et al.*, 2010; Zhao *et al.*, 2011). Indeed, NO production requires NF-kB-mediated gene transcription in the intracellular inflammatory cascade (Kraft and Harry, 2011). In addition, production of NO by iNOS an enzyme mainly expressed in microglia, can be upregulated in the presence of TNF-α and IFN-γ or downregulated in response to TGF-β and IL-1β (Tichauer *et al.*, 2007).

Other molecules produced by microglia are chemokines, especially when microglia is activated. These small peptides play a central role in cellular migration, being often designated as chemotactic cytokines, and intercellular communication. Among them, CCL2 and CX3CL1 are known to act in

diverse microglial responses either by microglia secretion or through recognition of their receptor on microglia. In fact, the role of chemokines in microglial dynamics seem to be more related with stimulation of receptors on microglial cells than by their own production. In particular, fractalkine (CX3CL1) is synthesized as a transmembranar glycoprotein which can be cleaved and released and will act as a chemoattractant. In fact, fractalkine is known to induce migration of microglia, as evidenced by *in vitro* and *ex vivo* studies (Liang *et al.*, 2009; Maciejewski-Lenoir *et al.*, 1999).

Finally, microglia are recognized producers of neurotrophic factors such as neurotrophins (NT), nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), glial cell-line derived neurotrophic factor (GDNF), among others. These mediators have important roles during CNS development since they promote neuronal maturation and differentiation, as well as neurite outgrowth and synapse homeostasis (Kim and de Vellis, 2005).

2.2 Models for the evaluation of microglial reactivity

Several experimental approaches can be used to explore the cross-talk between microglia and nerve or immune cells, as well as to evaluate microglial response towards toxic or external stimulus. The conditioned media model will be used in this thesis and aims to evaluate the influence of soluble factors on microglia reactivity. In fact, soluble factors released by either neurons or glial cells have proven to reciprocally influence the development and reactivity in these cells (Gomes *et al.*, 2001). Primary microglia cultures are the most approximate *in vitro* model to the *in vivo* conditions, however, they are difficult to obtain and, so, cell lines can be a good alternative. N9 cells have been described to perform several functions that are characteristic of microglia cells and, so, can be used as a suitable model for studying their dynamics. As demonstrated by Zhao and colleagues (2011), N9 microglia evidence features of the M1 phenotype after LPS stimulation, by increasing their production of pro-inflammatory cytokines, such as TNF-α and IL-1β. LPS also stimulates the production of ROS and NO in these cells (Bruce-Keller *et al.*, 2000; Chen and Chen, 2011). Moreover, N9 cell line has phagocytic ability and migration capacity towards chemoattractant molecules (Bruce-Keller *et al.*, 2000; Cui *et al.*, 2002), two well-known functions of microglial cells.

Different behaviours have been reported for microglial cells when applying *in vitro* or *in vivo* models, which may be due to the fact that these cells are always activated to same extend when they are in culture, which somewhat limits the ability to study microglial real *in vivo* functions (Farber and Kettenmann, 2005). In addition, in primary cultures, interaction with neighbouring cells, crucial for the modulation of microglial reactivity, is absent (Hanisch and Kettenmann, 2007). Hence, these inconvenients should be taking into account when manipulating microglia in culture, in order to correctly understand their behaviour.

Other useful model that can improve the study on microglial dynamics are mixed cultures. This model relies in cell-to-cell interactions such as ligand-receptor interaction and phagocytosis. Finally, organotypic slice cultures (OSCs) are heralded as the most approximate model to the *in vivo* conditions as the three dimensional architecture of the brain is maintained.

2.3 Microglia function in response to injury: neuroprotection and neurotoxicity

Microglia functions in response to injury have been largely discussed in the last 10 years giving rise to an intriguing question: is microglia beneficial or harmful to neurons? Indeed, microglia can perform both functions, as evidenced by *in vitro* and *in vivo* studies, depending of the circumstances of their activation. Studies in cell culture have demonstrated ambivalent effects of microglia on cultured neurons, either by mix or co-cultures procedures or incubation with conditioned media. Nevertheless, a different view came from *in vivo* studies where microglia is primarily neuroprotective and pro-regenerative (Streit, 2002).

The primary function of microglia should be to support neuronal function. Indeed, microglial phagocytic ability has proven to be essential for removal of tissue debris which is, in turn, imperative to regeneration of the environment after insult. Zhao and colleagues (2007) have observed this protective effect in injured tissues and also that microglia may limit damage following brain hemorrhage. In addition, after stroke and ischemia, activated microglia seem to be highly neuroprotective (Neumann *et al.*, 2006). Microglia may also play a pro-regenerative function following spinal cord injury as evidenced by Rabchevsky and Streit (1997). However, as reviewed by Gomes-Leal (2012) microglia can induce neurotoxic outcomes after the same injury.

Nonetheless, microglial involvement in neurodegenerative diseases, such as Parkinson´s disease, Alzheimer´s disease or Huntington´s disease, is now established as providing neurotoxic properties due to excessive inflammation. Microglia activation precedes neurodegeneration in Parkinson´s disease (Wu *et al.*, 2002). Furthermore, reduction of iNOS expression and inhibition of microglia activation ameliorates dopaminergic neurodegeneration, the hallmark of this disease (Liberatore *et al.*, 1999). In Huntington´s disease microglia activation is observed at onset of the disease and this activation increases along disease progression (Gomes-Leal, 2012). At last, and by opposite to activated microglia, dysfunctional microglia may also be involved in neurotoxicity in age-related neurodegenerative disorders. This is the case of Alzheimer´s disease (AD). It is now becoming established that microglia neurotoxicity outcomes are due to dysfunctional phenotype rather than activation (Ilieva *et al.*, 2009). In fact, Streit and colleagues (2009) have recently demonstrated that microglia appears dystrophic in the presence of tau protein and precedes it spread in the brain. As reviewed in Graeber and Streit (2010), microglia have also shown to play a role in amyloid-beta (Aβ) removal, although Aβ accumulates in AD brains with aging. Hence, microglia appears to be as inefficient phagocytes. Therefore, and although the role of inflammation in Alzheimer´s disease has been extensively studied, the contribution of microglia cells to the disease is still unclear (Ransohoff and Perry, 2009). The actual consensus is that the pathological environment determines microglial phenotype.

2.3.1 Microglia as main players in ALS

As described in many other neurodegenerative disorders, microglia abnormal response is also indicated to have a role in ALS. In fact, microgliosis in ALS has been recognized for the past 20 years (Lasiene and Yamanaka, 2011). Analysis of spinal cord of patients with ALS evidenced that microgliosis occur in motor cortex, motor nuclei of brainstem, along the corticospinal tract and in

ventral horn of spinal cord (Kawamata *et al.*, 1992). Using positron emission tomography imaging after injection with ^{11}C-PK11195, it was possible to demonstrate *in vivo* microglial activation in motor cortex, dorsolateral cortex and thalami of ALS patients (Turner *et al.* 2004). This study was of much interest since it showed the widespread of microglia activation and established the association between microgliosis and damage to upper motor neurons. Studies in patients, as well as in ALS mice models, have demonstrated the same features of microglia activation, as reviewed in Philips and Robberecht (2011). Microglia become activated and proliferate before motor neuron degeneration and, consequently clinical onset. During activation, production of several cytokines, such as TNF-α as well as chemokines and ROS are up-regulated, while neurotrophic factors are downregulated (Philips and Robberecht, 2011; Sargsyan *et al.*, 2005). Interestingly, overexpression of mutant SOD1 overactivates microglia when compared to wild type, reinforcing their contribution to ALS pathology (Xiau *et al.* 2007). Recently, Liao and colleagues (2012) have demonstrated that a transformation from a neuroprotective microglia phenotype to a neurotoxic one occurs in a mouse model of ALS during disease progression. The mRNA levels of mSOD1 microglia collected at early progression phase and near-end stage disease from mice spinal cords indicated that early in the disease, microglia express more M2 (neuroprotective) related markers, namely Ym1 and CD163, and in later stages M1 (neurotoxic) phenotype is acquired as NOX2 expression is elevated. Moreover, by co-culturing mSOD1 microglia with motor neurons, the authors showed that M2 mSOD1 microglia collected at early phase of the disease do not increase motor neurons death while with M1 mSOD1 microglia, the numbers of living motor neurons were decreased. These results suggest that microglia is neuroprotective in inicial phase and the transformation of mSOD1 microglia from M2 phenotype to a M1 neurotoxic state during disease progression induces motor neuron injury.

Several potential microglia-activating factors proved to be elevated in spinal cords of ALS patients, in ALS transgenic mice as well as in *in vitro* models (Sargsyan *et al.*, 2005). Chemokines such as CCL2 are up-regulated in SOD1 transgenic mice, as showed by Henkel and colleagues (2004). Similarly, TNF-α has been demonstrated to be elevated in SOD1G93A mice and in serum of ALS patients just as both of its receptors, TNFR1 and TNFR2 (Poloni *et al.*, 2000). Furthermore, the severity of motor neuron loss is interrelated with TNF-α expression (Elliott, 2001; Yoshihara *et al.*, 2002). Although it has not yet been proven, TNF-α acting through tumour necrosis factor receptor-associated factor 2 (TRAF2) seems to be a mechanism adjacent to cross-talk between microglia and neurons in ALS (Chandel *et al.*, 2001). TRAF2 activation induces two different pathways culminating in increased production of ROS by mitochondria and also NF-κB translocation into the nucleus. Elevation of ROS further induces cytochrome *c*-dependent apoptotic pathway and NF-κB activation, thus, stimulating the transcription of genes related with inflammation (Sargsyan *et al.*, 2005).

Hensley and colleagues (2003) by evaluating the mRNA content in spinal cord tissues of symptomatic and end-stage SOD1G93A mice, found that several others cytokines were elevated such as TGF-β, IL-1β, IL-4 and IL-10. Interestingly, it seems that either pro- and anti-inflammatory cytokines production was induced.

Matrix metalloproteinases- 9 and 2 (respectively, MMP-9 and MMP-2) are gelatinases responsible for extracellular matrix degradation and are implicated in inflammation (Nagase *et al.*, 2006). Secretion

by microglia in the context of neuroinflammation is involved in the recruitment of T-lymphocytes to CNS. MMP-2 and, mostly MMP-9 expression was found to be elevated in the spinal cord of SOD1G93A mice, as demonstrated by Fang and colleagues (2010). Increased expression of NO and ROS was also observed in patients and rodent models, leading to lipid peroxidation and protein carbonylation, which disrupt the integrity and function of neurons and glial cells (Beers *et al.*, 2006; Wu *et al.*, 2006). Moreover, cyclo-oxygenase-2 (COX2), which is an important enzyme involved in the synthesis of pro-inflammatory-prostanoid molecules, was also increased (Wu *et al.*, 2006).

Damaged neurons seem to initiate the inflammatory response in a way dependent on the release of mutant SOD1. Aberrant protein release occurs through a chromogranin chaperone-like process as overexpression of chromogranin A accelerates disease progression by increasing the amount of misfolded SOD1 in extracellular environment (Ezzi *et al.* 2010; Philips and Robberecht, 2011). Microglia recognition of mutant SOD1 is dependent on stimulation of a TLR receptor, namely the CD14, which leads to massive production of ROS species (Zhao *et al.*, 2010). Inflammatory microglia then release increasing levels of cytokines, which will act on motor neurons, resulting in a vicious deleterious cycle. Another player in neuron-microglia interplay seems to be the CX3CL1. The features of this activation are still to be elucidated and neurotoxic or neuroprotective outcomes can both occur. In one hand, *in vitro* studies show that CX3CL1 supports neuronal survival (Meucci *et al.*, 2000) and inhibits microglia apoptosis (Boehme *et al.*, 2000). On the other hand, Cardona and colleagues (2006) have demonstrated neurotoxic effects of fractalkine *in vivo*.

Contribution of microglia to motor neuron degeneration seems to be T-cell dependent (Philips and Robberecht, 2011). Although T-lymphocytes are not observed at early stages of the disease, microglia activation is associated and precedes infiltration of T helper (CD4+) and T cytotoxic (CD8+) cells in spinal cord. Interaction with infiltrating T-cells induces the expression of CD11c, CD86 and ICAM at the same time that enhances antigen-presentation (Chiu *et al.*, 2008; Gowing *et al.*, 2008). Interestingly, Beers and colleagues (2008) by depleting T cells in mutant SOD1 transgenic mice, observed that the disease course was worsened. Therefore, it can be speculated that T cells expressing mutant SOD1 can have a protective role in ALS in contrast to what was found for astrocytes or microglia, where mutant SOD1 excision (in astrocytes) or reduced expression (in microglia) significantly slowed disease progression (Boilleé *et al.*, 2006; Clement *et al.*, 2003; Wang *et al.*, 2009; Yamanaka *et al.*, 2008). In fact, CD4+ cells express high levels of anti-inflammatory cytokines such as IL-4 (Chiu *et al.*, 2008), thus, enhancement of M2 phenotype of microglia could be implicated in motor neurons protection.

The cross-talk between microglia and neurons in ALS is represented in Figure I.8 accordingly to what have been discussed in the present section.

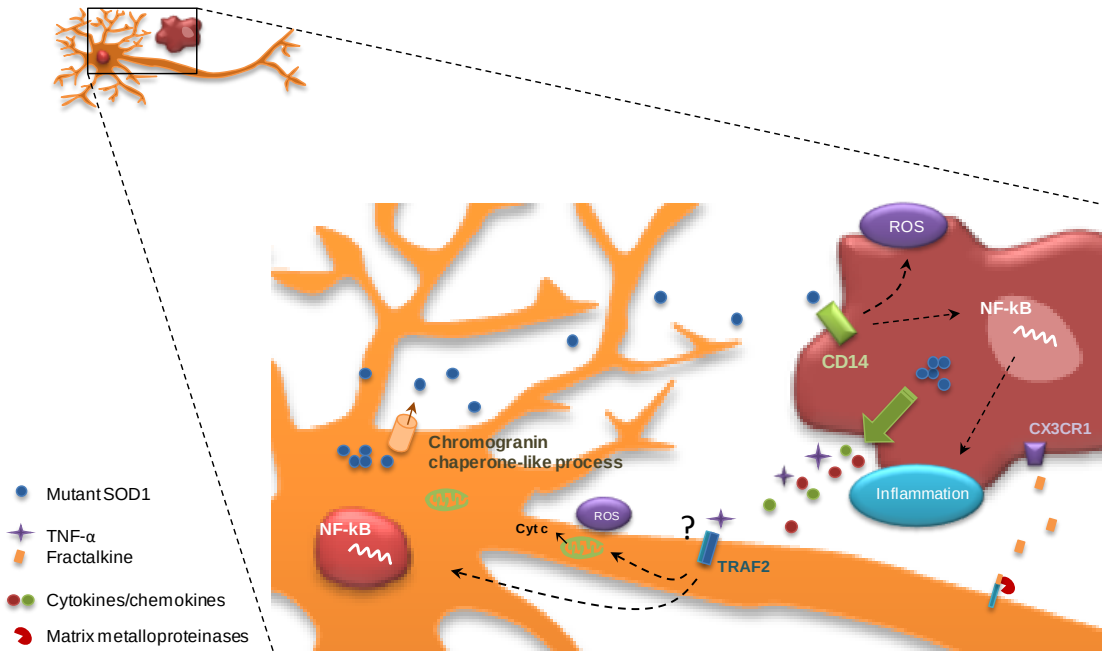

Figure I.8. Interplay between microglia and neurons in microglia in ALS. Pathogenic mechanisms involved in cross- talk between microglia and neurons seem to begin when mutant SOD1 is released from neurons through a chromogranin chaperone-like process. The mutant protein is recognized through CD14 receptor on microglia, stimulating an inflammatory response already improved by the accumulation of the same protein within microglia. In any case, CD14 activation promotes translocation of nuclear factor κB (NF-kB) into the nucleus with transcription of genes related to inflammation. Additionally, reactive oxygen species (ROS) production is elevated. One of the cytokines produced by microglia, tumour necrosis factor-α (TNF-α), possibly activates, in turns, tumour necrosis factor-associated factor 2 (TRAF2) receptor on motor neurons promoting NF-kB activation, cytochrome *c* release and ROS production. Nevertheless, CX3CR1 activation can promote microglia responses as damage neurons are proven to release its ligand fractalkine. Conversely, interaction between microglia and motor neurons might enhance motor neuron degeneration.

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

3. Aims

The general aims of this thesis are to investigate the molecular mechanisms involved in motor neuron degeneration by using an *in vitro* model of neuronal degeneration in ALS, as well as to explore the role of microglia when facing the released factors of motor neurons during ALS progression.

Therefore, the specific aims are:

1. To evaluate the suitability of the motor-neuron cell line (NSC-34) expressing human SOD1 wt or mutated in G93A as neuronal models for of "normal" behaviour and ALS disease, respectively. For this, NSC-34 cell line expressing either human wild type SOD1 or mutant in G93A (NSC-34/hSOD1wt or NSC-34/hSOD1G93A, respectively) will be differentiated and SOD1 accumulation will be assessed together with cell death and viability in both models. Mitochondria function, oxidative stress markers as well as release of soluble factors involved in inflammation were the features focused in the study, as they are known to be altered in ALS disease; NSC-34/hSOD1wt cells will be used as control condition.

2. To evaluate the ability of GUDCA in delaying or recovering motor neuron degeneration. For this, NSC-34/hSOD1G93A will be incubated with GUDCA at 0 or 2 DIV to test its ability to prevent, in the first case, and/or restore, in the second one, the effects produced by SOD1 mutation.

3. To explore non-transgenic microglia dynamics when incubated with NSC-34/hSOD1G93A conditioned media. For that, N9 microglia will be incubated with NSC-34 conditioned media and microglia responses were analyzed, namely morphology, phagocytic ability and release of inflammation-related factors.

II. MATERIALS AND METHODS

1. Materials

1.1 Chemicals

Dulbecco's modified Eagle's medium-Ham's F12 medium (DMEM-Ham´s F-12), DMEM high glucose w/o pyruvate, fetal bovine serum (FBS), Penicillin/Streptomycin, L-glutamine and nonessential aminoacids(NEAA) were purchased from Biochrom AG (Berlin, Germany); RPMI-1640 medium, trypsin-EDTA solution (1X), trypsin-EDTA solution (10X), ATP, Hoechst 33258 dye, bovine serum albumin (BSA), fluorescent latex beads 1 µm (2.5%), Coomassie Brilliant Blue R-250, propidium iodide (PI),naphtylethylenediamine ($C_{12}H_{14}N_2$), sulfanilamide ($C_6H_8N_2O_2S$), were from Sigma-Aldrich (St. Louis, MO, USA); Glycoursodeoxycholic acid (GUDCA) (minimum 96% pure) and Geneticin 418 sulfate (G418) were obtained from Calbiochem (Darmstadt, Germany); L-glutamic acid kit, Triton X-100, 6-phosphateglucose dehydrogenase andhexokinase were obtained from Roche Diagnostics (Mannhein, Germany); MitoTracker red® was acquired from Invitrogen Corporation™ (Carlsbad, CA, USA) and Giemsa solution was from Merck (Darmstadt, Germany). Nitrocellulose membrane was obtained from Amersham Biosciences (Piscataway, NJ, USA). Cell lysis buffer® and LumiGLO® were from Cell Signaling (Beverly, MA, USA). All the other chemicals were purchased either from Sigma-Aldrich or Merck.

1.2 Antibodies

Rabbit polyclonal anti-superoxide dismutase 1 (SOD1) (1:250) and horseradish peroxidise-labelled goat anti-rabbit IgG (1:1667) were purchased from Santa Cruz Biotechnology® (Santa Cruz, CA, USA); rabbit anti-ionized calcium-binding adaptor molecule 1 (Iba1) (1:250) was from Wako (Japan); Alexa Fluor® 488 goat anti-rabbit (1:1000) was obtained from Invitrogen Corporation™

(Carlsbad, CA, USA); lectin from *Lycopersicon esculentum* (tomato) (1:166), avidin-FITC-conjugate (1:50) and mouse anti-β-actin (1:1667) were from Sigma-Aldrich (St. Louis, MO, USA); horseradish peroxidise-labelled goat anti-mouse IgG (1:1667) was from Amersham Biosciences (Piscataway, NJ, USA).

1.3 Equipment

Fluorescence microscope (model AxioScope.A1) with integrated camera (AxioCamHRm) and AxioScope HBO50 microscope was purchased from Carl Zeiss, Inc. (North America); AxioScope HBO50 integrated camera (Leica, model DFC490) and optical microscope with phase-contrast equipment (Olympus, model CK2-TR) were used for cell morphology evaluation. Mini-PROTEAN P3 Multi-Casting Chamber System was used for Western Blot and zymography assay, microplate reader (PR 2100 Microplate Reader) for nitrites measurement and ChemiDocTM for mettaloproteinases gel photos were obtained fromBio-Rad Laboratories (Hercules, CA, USA). To ensure a stable environment to optimal cell growth (37°C and 5% CO_2), cell cultures were maintained in HERAcell 150 incubators (Thermo Scientific, Waltham, MA, USA) and the work performed in sterile conditions in aHoltenLamin Air HVR 2460 (Allerod, Denmark). For flow cytometry studies, we used the Guava easyCyte 5HT Base System Flow Cytometer (Merck-Millipore, Darmstadt, Germany). SNAP i.d. Protein Detection System (Merck-Millipore, Darmstadt, Germany) was used for Western blotting. A 48-well microchemotaxis chamber (Boyden chamber) and polycarbonate track-etch membranes (Neuro Probe, Inc - Gaithersburg, MD, USA) with polyvinylpyrrolidone (PVP) treatment were used for migration assay. Eppendorf 580R (Eppendrof, Hamburg, Germany) and a Sigma 3K30 centrifuges were used for different experimental procedures.

2. Methods

2.1 Cell lines

Two different cell lines were used during the experimental work: (i) NSC-34 that is a cell line resulting from hybridization between murine neuroblastoma and motor neurons obtained from mouse spinal cord (Cashman *et al.*, 1992); and (ii) N9 cell line that was obtained by immortalization of microglial cells obtained from CD1 mouse cortex (Righi *et al.*, 1989).

2.1.1 NSC-34 cell line

NSC-34 cell line transfected with human SOD1, either wild type or mutated in G93A (NSC-34/hSOD1wt or NSC-34/hSOD1G93A, respectively), were a gift from Júlia Costa, Instituto de Tecnologia Química e Biológica (ITQB), Universidade Nova de Lisboa, Portugal (Gomes *et al.*, 2008). NSC-34 cells transfected with the empty vector (pCIneo) were used as control. NCS-34 cells were grown in proliferation media (DMEM high glucose, w/o pyruvate, supplemented with 10% of fetal bovine serum (FBS) and 1% of Penicillin/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 Poly-D-Lysine (50 µM) before plating the cells. Cells were seeded in 12-well culture plates at a concentration of $5x10^4$ cells/ml and maintained at 37ºC in a humidified atmosphere of 5% CO_2.

2.1.2 N9 cell line

N9 cell line was a gift from Teresa Pais, Institute of Molecular Medicine (IMM), Lisboa, Portugal. Cells were cultured in RPMI supplemented with FBS (10%), L-glutamine (1%) and Penicillin/Streptomycin (1%), grown to confluency and splitted every 2 to 3 days. For characterization, cells were plated at concentrations of $3x10^5$ cells/ml and, in the next step, at $2x10^4$ cells/ml for incubation with NSC-34 conditioned media, as it will be further discussed. No coating was required. Cells were maintained at 37ºC in a humidified atmosphere of 5% CO_2.

2.2 Cell treatments

2.2.1 NSC-34 cells

After 48 h in proliferation media (as described), differentiation was induced by changing medium for DMEM-F12 plus FBS (1%), non-essential amino acids (1%), Penicillin/Streptomycin (1%) and G148 (0.1%), as indicated by Cho *et al.* (2011), and measurements were performed during the first 4 days *in vitro* (DIV) and at 7 DIV, as described in Figure II.1. Our aim was to evaluate the effects of hSOD1 transfection in NSC-34 cells, in order to establish a model where the disruption of molecular mechanisms observed in NSC-34 cells expressing human SOD1 mutated in G93A along differentiation could represent the progression of ALS disease.

In another set of experiments, at the time of differentiation (0 DIV) and at 2 DIV, NSC-34 were incubated either alone or with 50 µM GUDCA, to assess the ability of this bile acid to prevent, in the first case, and restore, in the second one, the effects produced by hSOD1 transfection.

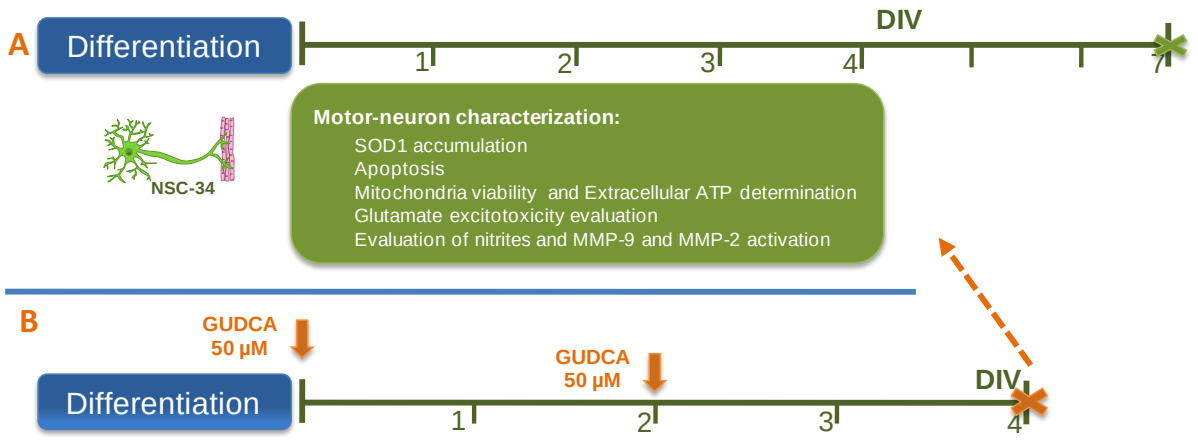

Figure II.1. Experimental procedure used in the characterization of transfected NSC-34 cell line and in the study of the neuroprotective effects of GUDCA. (A) NSC-34 cells, transfected with human SOD1, wild type or mutated in G93A (NSC-34/hSOD1wt or NSC-34/hSOD1G93A), were grown in proliferation media and selection was made with G418. After 48 h in culture, differentiation was induced by changing medium for DMEM-F12 plus FBS (1%) and non-essential amino acids (1%). During the following 4 days *in vitro* (DIV), characterization of NSC-34 cells was performed. (B) At differentiation (0 DIV) and at 2 DIV, NSC-34 were incubated either without or with 50 µM GUDCA to assess its ability to prevent, in the first case, and restore, in the second one, the effects produced by SOD1 transfection.

2.2.2 N9 cells

For N9 characterization, cells were treated with 300 ng/ml of lipopolysacharide (LPS) for 24 h in order to evaluate N9 microglia responses when facing an activation stimulus (Figure II.2).

To determine whether the soluble factors released by the motor neurons expressing wild type (wt) or mutant SOD1 in G93A have different effects on microglia, incubation with 800 µl of NSC-34 conditioned media was performed. Experimental determinations were performed after 4 and 24 h of incubation to evaluate the outcomes of a short and a prolonged exposure, respectively. For this evaluation, cells were plated at a concentration of $2x10^4$ cells/ml in order to maintain the usual proportion of microglia and neurons in the CNS, as in our previous studies with mixed neuron-microglia cultures (Silva *et al.*, 2011).

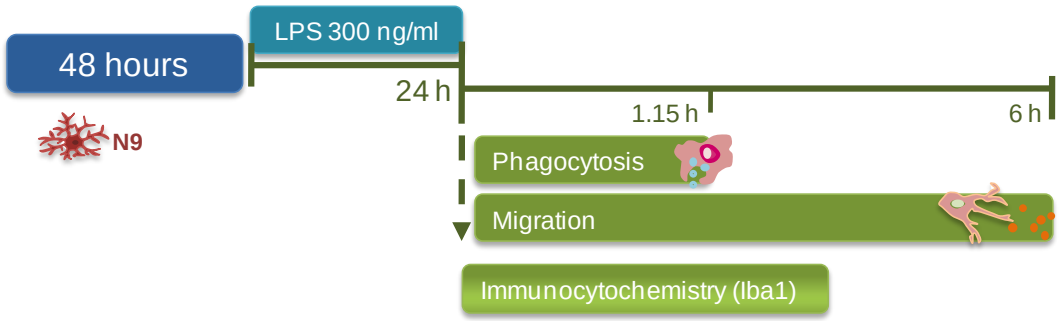

Figure II.2. Experimental procedure of N9 microglial cells characterization. Microglial cells were grown for 48 h in proliferation media before incubation. Lipopolysacharide (LPS) (300 ng/ml) treatment was then performed for 24 h and several parameters were evaluated, namely the ability to ingest fluorescence latex beads (phagocytosis), migration towards chemoattractant compounds and immunocytochemistry raised against ionized calcium-binding adaptor molecule 1 (Iba1) for morphological evaluation.

2.3 Evaluation of cell death

2.3.1 Necrosis

Necrotic-like cell death was assessed by monitoring the cellular uptake of the fluorescent dye PI [3,8-diamino-5-(3-(diethylmethylamino)propyl)-6-phenyl phenanthridinium di-iodide]. PI readily enters and stains non-viable cells, but cannot cross the membrane of viable cells. This dye binds to double-stranded DNA and emits red fluorescence (emission: 630 nm; excitation: 493 nm).

To detach the cells from the well they were treated with 450 µL trypsin (10X) diluted 1:10 in PBS during 5 min. After adding FBS (to stop the action of trypsin), cells were collected and centrifuged at 700 *g* during 5 min (Eppendorf, 5810R). The supernatant was discharged and the pellet resuspended in 200 µL of PBS. Cells were applied to the microplate and a solution of PI (75 µM) was added to each well (4 µl/well). After 15 mins, cell death was analyzed on a Guava easyCyte 5HT Base System Flow Cytometer (Merck-Millipore). 5000 events per sample were counted and the PI-stained cells were considered as necrotic.

2.3.2 Apoptosis

The number of apoptotic cells was assessed by morphological analysis of nuclei stained with Hoechst 33258 dye. Fluorescence was visualized using an AxioCam HR camera (Zeiss) adapted to an AxioSkope® microscope. Ten random microscopic fields were acquired per sample with original magnification of 400x. Results were expressed as the percentage of condensed or fragmented nuclei per total number of cells (Vaz *et al.*, 2010).

2.4 MTS assay

Cellular reduction of MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) occurs due to enzymes in functional cells. This can be considered as a marker for cellular viability. Cells were incubated during 1 h at 37°C with 100 µL of the mix MTS ([3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) and PMS (Phenazinemethosulfate) in 900 µL Dulbecco'sModified Eagle Medium (DMEM-F12) per well. The absorbance was measured at 490 nm using a microplate reader (Bio-Rad Laboratories; Hercules, CA, USA). For each experiment, the mean value of absorbance obtained from control conditions was considered as 100% of cell functionality.

2.5 Immunocytochemistry

NSC-34 cells were fixed with freshly prepared 4% (w/v) paraformaldehyde in PBS. For the immunostaining evaluation, cells were first permeabilized with 0.2% Triton X-100 and then incubated with blocking solution (3% BSA in PBS) for 30 min. Cells were incubated overnight at 4ºC with rabbit anti-SOD1 (1:250; Santa Cruz Biotechnology®). The secondary antibody used was goat anti-rabbit Alexa Fluor 488 (1:1000, Invitrogen Corporation™) and the incubation was performed for 2h at RT. Cell nuclei were stained with Hoechst 33258 dye (1:1000, Sigma-Aldrich) (Falcão *et al.*, 2005).

For the N9 cells immunostaining, the procedure was the same with minor modifications. Cells were permeabilized with 1% of Triton X-100 for 30 minutes and blocking solution used was PBS with1% BSA, 0,4% Triton X-100, 4% FBS, for 30 min. The primary antibodies used were rabbit anti-Iba1 (1:250, Wako) and lectin from (1:166, Sigma-Aldrich) and the secondary ones were Alexa Fluor 488 (1:1000, Invitrogen Corporation™) and avidin-FITC-conjudgate (1:50, Sigma-Aldrich), respectively

Fluorescence was visualized using an AxioCam HR camera (Zeiss) adapted to an AxioSkope® microscope. For both cell lines, merged images of UV and green-fluorescence of ten random microscopic fields were acquired per sample. Original magnification used was 400x. (Falcão *et al.*, 2007).

2.6 Western Blot assay

Western Blot was carried out as usual in our lab (Fernandes *et al.*, 2006). Total cell extracts were obtained by lysing cells with 1x Cell Lysis Buffer plus 1 mM phenylmethyl sulfonyl fluoride (PMSF) for 5 min, on ice and with shaking, followed by sonication during 20 seconds. The lysate was centrifuged at 14000 *g* for 10 min, at 4°C, and the supernatants were collected and stored at -80°C. Protein concentration was determined by the Bradford method (Bradford, 1976) using Bio-Rad's Protein

Assay Reagent. Equal amounts of protein were separated on a 12% sodium dodecyl sulfate-polyacrilamide gel electrophoresis (SDS-PAGE). After electophoresis, proteins were transferred to a nitrocellulose membrane and immunoblot was performed using SNAP I.D. Protein Detection System (Millipore). Briefly, membranes were incubated in blocking buffer (T-TBS plus 0.25% (w/v) non-fat dried milk) at room temperature and incubated with the following primary antibodies: rabbit anti-SOD1 antibody (1:167, Santa Cruz Biotechnology®) and mouse anti-β-actin (1:1667, Sigma-Aldrich) in T-TBS with 5% BSA) followed by the secondary antibodies goat anti-rabbit HRP-linked (1:1667, Santa Cruz Biotechnology®) and goat anti-mouse HRP-linked (1:1667, Amersham Biosciences), respectively, diluted in blocking solution. After washing membranes with T-TBS, chemiluminescent detection was performed by using LumiGLO® reagent and bands were visualized in a Hyperfilm ECL. The relative intensities of protein bands were analyzed using the Image Lab™ analysis software, after scanning with Chemidoc, both from Bio-Rad Laboratories (Hercules, CA, USA).

2.7 Evaluation of mitochondria viability by MitoTracker Red®

To stain mitochondria, cells were incubated for 30 min at 37°C with 500 nM of MitoTracker Red® solution (Barateiro *et al.*, 2012) and then fixed with 4% (w/v) paraformaldehyde. Cell nuclei were stained with Hoechst 33258 dye (1:1000, Sigma) (Falcão *et al.*, 2005). Using the fluorescence microscope, 10 pictures were taken per each slide and the original magnification used was 400x. Fluorescence was quantified by ImageJ software and normalized to the total number of cells.

2.8 Quantification of extracellular ATP

Samples were treated on ice to avoid degradation of ATP. For the determination of extracellular ATP levels, the incubation media was collected and treated with 2 M perchloric acid. Afterwards, the pH value was neutralized with 4 M KOH solution. To remove cellular debris, the samples were centrifuged (Eppendorf,5810R) during 5 min at 10,000 *g* and 4°C between the different steps. ATP levels were determined by an enzymatic assay and fluorescence intensity was quantified using a fluorimeter (F-2000 Fluorescence Spectrometer, Hitachi) at λ_{em}= 450 nm and λ_{ex} = 340 nm (Vaz *et al.*, 2010). A calibration curve of ATP was used for each assay.

2.9 Measurement of release glutamate

Glutamate content in the differentiation media from NSC-34 cell line was determined using the L-glutamic acid kit (Roche). The reaction was performed in 96-well microplate and the absorbance measured at 490 nm. A calibration curve of glutamic acid was used for each assay. All samples and standards were analysed in duplicate and the mean value was used (Falcão *et al.*, 2005).

2.10 Quantification of nitrite levels

NO levels were estimated by measuring the concentration of nitrites (NO_2), the stable end-product from NO metabolism, in differentiation media of NSC-34 cell line. 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 RT. The absorbance at 540 nm was determined using a microplate reader. A calibration curve was used for each assay. All samples were measured in duplicate and the mean value was used (Silva *et al.*, 2011).

2.11 Gelatin zymography

SDS-PAGE zymography in 0.1% gelatin-10% acrylamide gels was performed to analyze the amount of MMPs on culture media. The experiment was performed under non-reducing conditions. Afterwards, the gels were washed for 1 h with 2.5% Triton-X-100 (in 50mMTris pH 7.4; 5 mM CaCl2; 1 µM ZnCl2) to remove SDS and to renature the MMP species in the gel. To induce gelatin lysis, the gels were incubated at 37°C in the developing buffer (50 mMTris pH 7.4; 5 mM CaCl2; 1 µM ZnCl2) overnight. For enzyme activity analysis, the gels were stained with 0.5% Coomassie Brilliant Blue R-250 and destained in 30% ethanol/10% acetic acid/H_2O. Gelatin activity, detected as a white band on a blue background, was measured using computerized image analysis (Image Lab) and normalized for total cellular protein (Silva *et al.*, 2010).

2.12 Assessment of microglial phagocytic properties

To evaluate the phagocytic ability of N9 microglia, cells were incubated with 0.0025% (w/w) 1 µm fluorescent latex beads for 75 min at 37ºC and fixed with freshly prepared 4% (w/v) paraformaldehyde in PBS. Microglial nuclei were counterstained with Hoechst 33258 dye. U.V. and green fluorescence images of ten random microscopic fields (original magnification: 400X) were acquired per sample. The number of ingested beads per cell and total cells were counted to determine the percentage of phagocytic cells and the mean number of ingested beads per cell (Silva *et al.*, 2010).

2.12 Migration assay

Cell migration assays were performed in a 48-well microchemotaxis chamber (Boyden Chamber; Neuro Probe, Gaithersburg, MD, USA) according to (Nolte *et al.*, 1996) with minor modifications. The bottom wells were filled with vehicle and two concentrations of ATP (10 µM and 300 µM) for N9 characterization and, in a second approach, with conditioned media from NSC-34 cells in order to assess microglia ability to be attracted by neurons. The polycarbonate track-etch membrane with polyvinylpyrrolidone (PVP) (Neuro Probe, Gaithersburg, MD, USA) were emerged in DMEM-Ham´s F-12 during this procedure. Then, microglia cells were suspended in serum-free DMEM-Ham´s F-12 and 50 µL of cell suspension was placed into each top well (2-4 x 10^4 cells per well). The Boyden chamber was then placed in a CO_2 incubator at 37°C and microglial cells were allowed to migrate during 6h. Afterwards, the membrane was removed and the bottom side was fixed with cold methanol. Cells were stained with freshly prepared and filtered 10% Giemsa in PBS. Finally, cells on the top side of the membrane were wiped off using a filter wiper with PBS (Neuro Probe, Gaithersburg, MD, USA). A schematic representation of the chemotaxis assay is illustrated in Figure II.3.

About 10 microscopic fields (original magnification: 100X) were acquired to cover all the well using a Leica DFC490 camera adapted to an AxioSkope HBO50 microscope and the number of total cells was counted. For each experiment, at least three wells per condition were acquired.

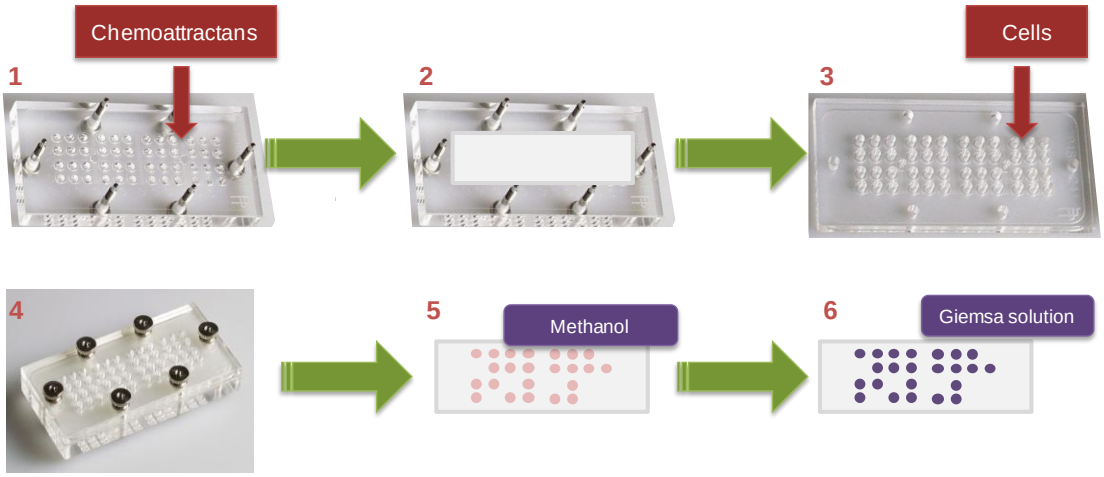

Figure II.3. Chemotaxis assay. (1) First, the chemoattractants are added to the wells of the lower chamber. (2) The membrane is added to the lower chamber and the Boyden chamber is closed with the upper chamber. (3) The cells are placed into the upper wells and the Boyden chamber is placed in the CO_2 incubator at 37ºC for 6 h (4). (5) The membrane is removed from the chamber and the lower part (where the migrating cells are) is fixed with cold methanol. (6) Finally, Giemsa solution is used to stain the cells and about 10 microscopic fields are acquired per well.

2.13 Statistical analysis

The results of three different experiments, performed in duplicate or triplicate, are expressed as mean ± SEM. Comparisons between the different parameters evaluated in wt and G93A NSC-34 cell line as well as the characterization of microglia N9 cell line were made via two-tailed Student's t-test for equal or unequal variance, as appropriate. Comparison of more than two groups in the parameters evaluated for microglia incubation with conditioned media obtained either from NSC-34/hSOD1wt or NSC-34/hSOD1G93A cells was done by one-way ANOVA using GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA) followed by multiple comparisons Bonferroni post-hoc correction. $p<0.05$ was considered statistically significant.

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III. RESULTS

1. Characterization of NSC-34 cell line: evaluation of its suitability to assess molecular mechanisms involved in motor neuron degeneration in ALS

NSC-34 cells have their origin on a fusion between murine neuroblastoma and motor neurons obtained from mouse spinal cord (Cashman *et al.*, 1992). In our model, NSC-34 cells were transfected either with wild type human SOD1 or mutated in G93A (NSC-34/hSOD1wt and NSC-34/hSOD1G93A, respectively) as well as cells transfected with the empty vector (pCIneo) which were used as the control (Gomes *et al.*, 2008). After 48 hours in proliferation media, differentiation was induced as described in Methods section. We intended to evaluate the effects of hSOD1 transfection in NSC-34 cells, in order to establish a model where the disruption of molecular mechanisms observed along the time after differentiation could represent the progression of ALS disease.

1.1 Morphological characterization and SOD1 accumulation in NSC-34 cell line either expressing human SOD1 wild type (wt) or mutated in G93A

We observed extension of ramifications and increased number of neurites, as well as reduction in cell soma after inducing cellular differentiation, right after the first day *in vitro* (DIV), as shown in Figure III.1. Moreover, the morphology was maintained during the subsequent 4 DIV, and no morphological differences could be observed between cells pCIneo, hSOD1wt and hSOD1G93A by phase-contrast microscope. In order to evaluate the efficient transfection of the cells with pCIneo vector expressing hSOD1wt or hSOD1G93A, we have performed Western blot analysis. We observed, as indicated in Figure III.2, two different bands of SOD1 in either NSC-34/hSOD1wt or NSC-34/hSOD1G93A,

corresponding to both human and mouse forms of this enzyme, while in cells expressing the empty vector (pCIneo), there was only one band corresponding to the native SOD1 of the mouse. However, we were not able to successfully separate the two bands of mSOD1 and hSOD1. To that it may have accounted the low molecular weight of both human and mouse SOD1 (classically these bands appear at 21 and 14 kDa, respectively) (Kikuchi *et al.*, 2006), making more difficult its separation. The method will be improved in the future by changing the conditions of electrophoresis (intensity of the current or voltage), as well as the percentage of acrylamide used in the resolution gel.

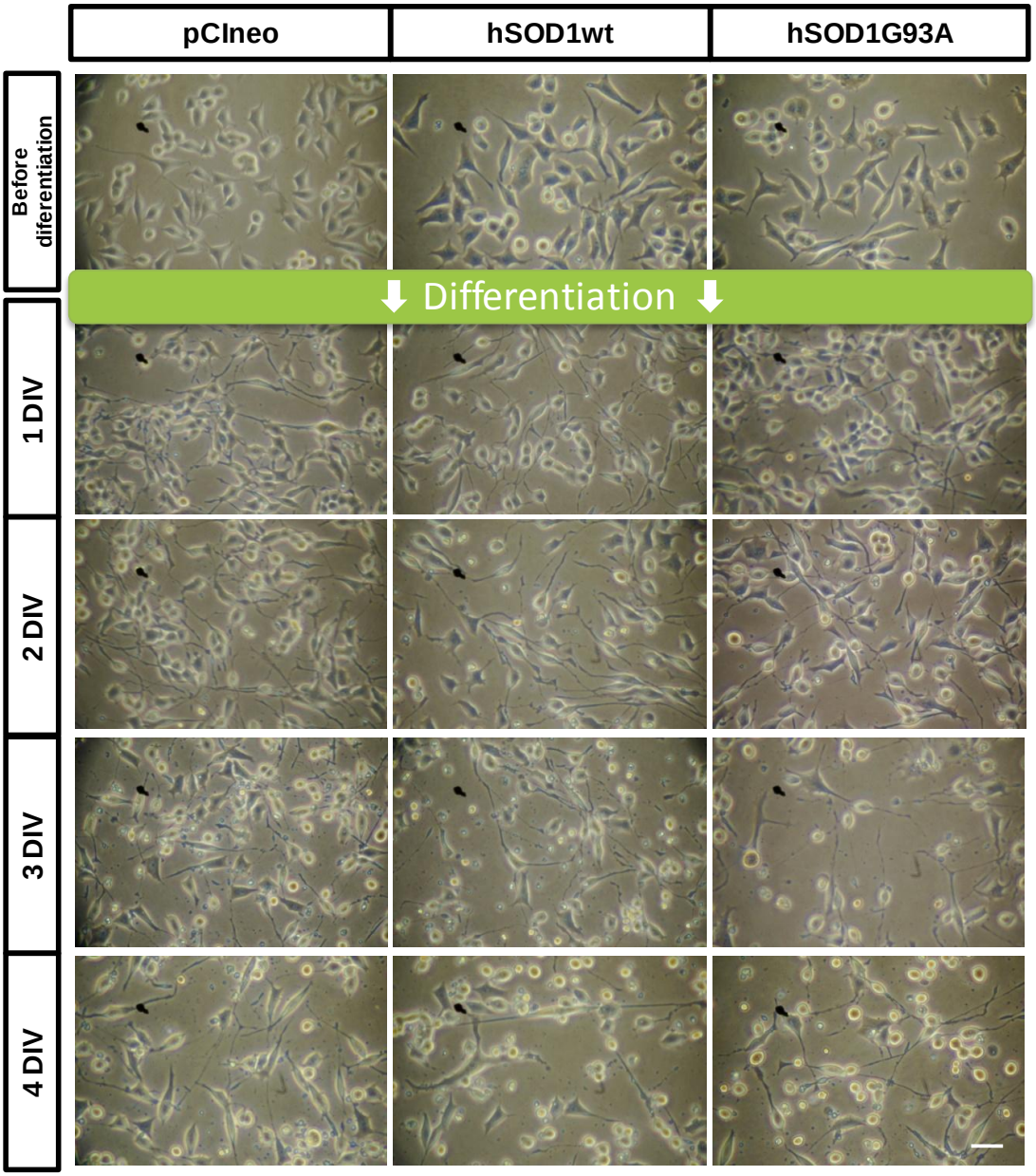

Figure III.1. Differentiation induces neuron-like morphology in NSC-34 cells. NSC-34 cells with the empty vector (pCIneo) or transfected with human SOD1, wild type or mutated in G93A (NSC-34/hSOD1wt or NSC-34/hSOD1G93A) were treated as indicated in Methods. Photos in phase-contrast microscope (original magnification of 100X) were acquired before and along differentiation, for 4 days *in vitro* (DIV). At 1 DIV, NSC-34 cells evidenced longer and numerous ramifications and reduced cell soma as compared to their morphology before differentiation. Representative results from one experiment are shown. Photos were taken by a digital HP camera. Scale bar represents 60 µm.

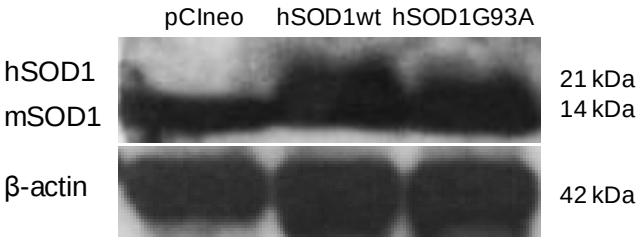

Figure III.2. NSC-34 cells demonstrate efficient transfected transfection with human SOD1. NSC-34 cells with the empty vector (pCIneo) or transfected with human SOD1, wild type or mutated in G93A (NSC-34/hSOD1wt or NSC-34/hSOD1G93A) were treated as indicated in Methods. Total lysates were analyzed by western blot with anti-SOD1 antibody. Anti-β actin antibody was used as internal control. Representative results from one experiment are shown.

Since accumulation and aggregation of SOD1 is a common feature of ALS disease, we next evaluated the cytoplasmic amount of SOD1 by immunocytochemistry using rabbit anti-SOD1 antibody (1:250). As shown in Figure III.3, NSC-34/hSOD1G93A cells clearly demonstrate accumulation of SOD1 after 3 DIV.

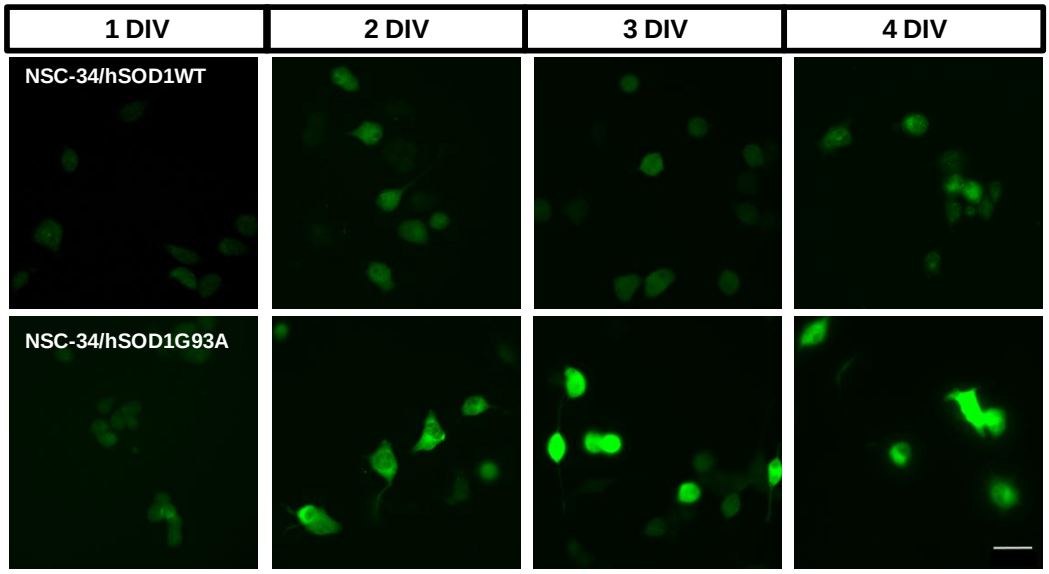

Figure III.3. Accumulation of SOD1 is noticed at 3 days *in vitro* (DIV) in NSC-34/hSOD1G93A cells. Cells were treated as indicated in methods. Accumulation of SOD1 was evaluated by immunocytochemistry using rabbit anti-SOD1 antibody, followed by a fluorescent-labeled secondary antibody. Representative results from one experiment are shown. Scale bar represents 40 µm.

1.2 NSC-34 cells expressing human SOD1 mutated in G93A evidence reduced cell viability and increased susceptibility to apoptosis

Cell viability was assessed by the capacity to reduce MTS, as indicated in Methods. NSC-34/hSOD1G93A cells evidenced loss of cellular functionality at 1 day of differentiation ($p<0.01$) and 2 DIV ($p<0.05$) when compared to NSC-34/hSOD1wt cells (Figure III.4-A). Conversely, after 3 DIV, NSC-34/hSOD1G93A showed high MTS reduction capacity that increased significantly at 4 DIV ($p<0.01$). This result can illustrate an attempt to compensate the cellular damage due to SOD1 accumulation. On the other hand, and although proliferation was not evaluated, NSC-34/hSOD1wt cells seemed to grow faster than NSC-34/hSOD1G93A cells in phase contrast microscopy

observations, accounting to the lowest relevance of cell demise. It is important to remember that NSC-34 is a cell line and so, cells do not stop their proliferation even after differentiation. As indicated in Figure III.1, we observed non neuron-like cells, characterized by the absence of ramifications, during all the days of differentiation, which may explain the less reduction of MTS by NSC-34/hSOD1wt cells. Necrosis was analyzed by flow cytometry. We did not observed considerable cell death in either cell lines as incorporation of PI occur in less than 6% of the total NSC-34 cells (see Table at Fig. III.4-B). Instead, we noticed that NSC-34/hSOD1G93A cells were more susceptible to apoptotic cell death than NSC-34/hSOD1wt ones. At 4 DIV ($p<0.01$) hSOD1G93A cells show marked increase in apoptosis as evidenced by nuclei morphological alterations after Hoechst 33258 staining (Fig. III.4-C and D).

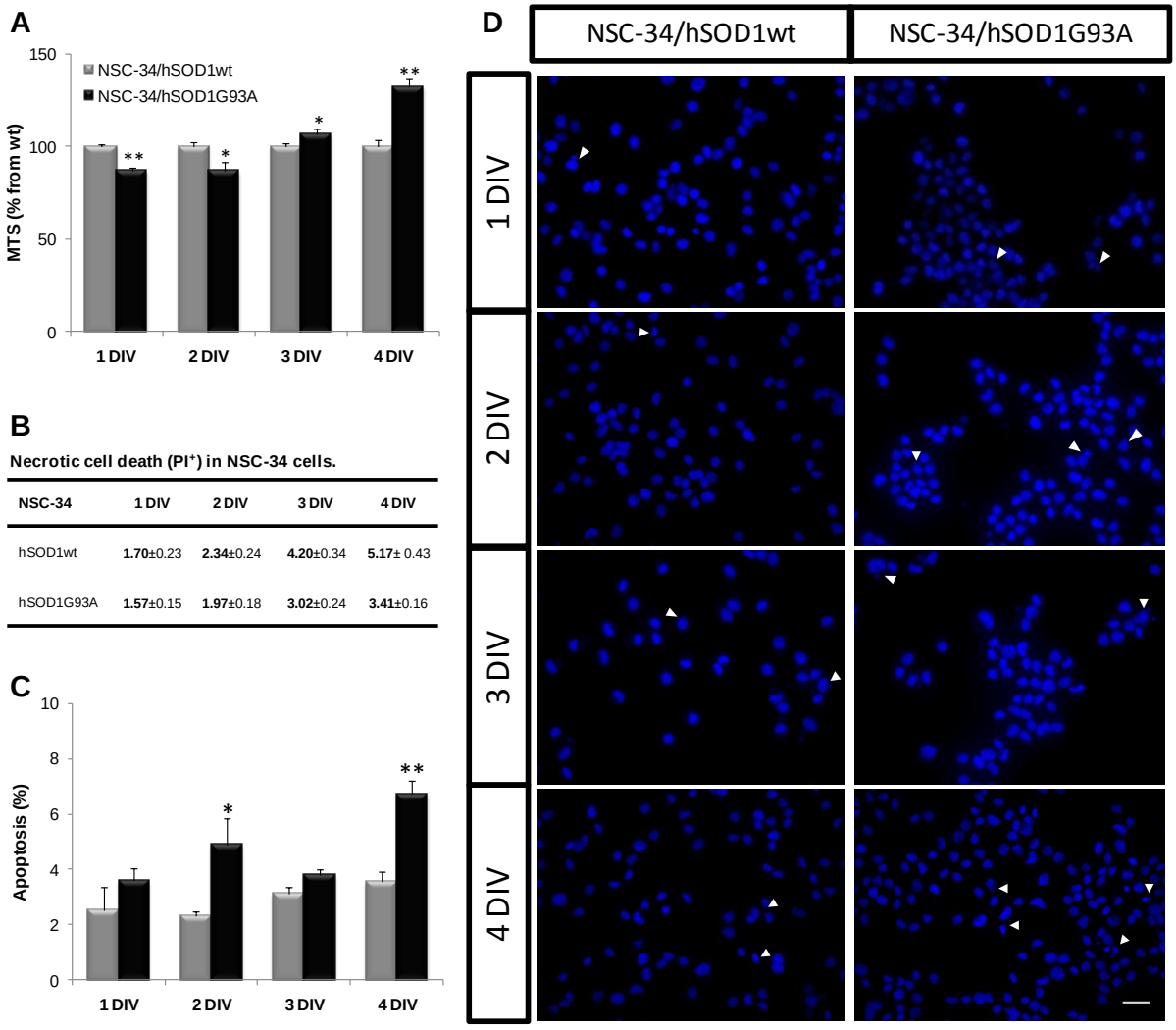

Figure III.4. Differentiated NSC-34/hSOD1G93A cells evidence increased apoptosis after 4 days *in vitro* (DIV) and reduced viability in the first 2 DIV. Cells expressing human SOD1 wt or mutated in G93A were treated as indicated in Methods. After differentiation, cellular functionality was evaluated by quantification of MTS reduction capacity and the results are expressed as percentage *vs*. NSC-34/hSOD1wt (control) (A); cells were trypsinized and incubated with propidium iodide (PI) and quantified by flow cytometry; 5000 events were counted per sample and PI^+ cells were considered to be necrotic, and expressed as percentage per total number of cells (B); cells were stained with Hoechst dye for morphological analysis of the nuclei along the 4 DIV and expressed as percentage per total number of cells (C). Representative results of apoptosis from one experiment are shown (D). Results are mean (± SEM) from at least three independent experiments performed in duplicate. Scale bar represents 40 µm. *$p<0.05$ and **$p<0.01$ *vs.* NSC-34/hSOD1wt (control).

1.3 Mitochondria functional deficits and bioenergetic failure are characteristics of NSC-34 cells expressing human SOD1 mutated in G93A

Mitochondria dysfunction and energy impairment are associated with motor neuron degeneration in ALS. To address mitochondrial properties in our model, we have performed an evaluation of mitochondrial viability by staining with Mitotracker Red®, which only stains viable mitochondria, together with the quantification of ATP release. As shown in Figure III.5-A, NSC-34/hSOD1G93A cells evidenced less stained cells in comparison to NSC-34/hSOD1wt ones during differentiation, being the red coloration markedly reduced at 3 DIV ($p<0.01$), indicating the presence of less viable mitochondria in these cells (Fig. III.5-B). A reduction in ATP efflux was also observed at 4 DIV in NSC-34/hSOD1G93A cells ($p<0.05$) (Fig. III.5-C). These results led us to the hypothesis that cells expressing the mutant protein have energy impairment. Mitochondrial dysfunction should precede this event as the major production of ATP occurs in mitochondria.

Elevation in extracellular glutamate is also considered an important marker of ALS disease. However, we were not able to observe this feature in our experimental model with NSC-34 cells. In opposite to the described excitotoxicity, we observed a reduction in glutamate release of NSC-34/hSOD1G93A cells after differentiation, from 1 to 4 DIV (Fig. III.5-D). In fact, due to mitochondria dysfunction the metabolism of glutamine may not be enough to the normal synthesis of glutamate, even more because it is also necessary for the glutathione synthesis. Thus, an altered glutamate metabolism may occur in these conditions (D'Alessandro *et al.*, 2011).

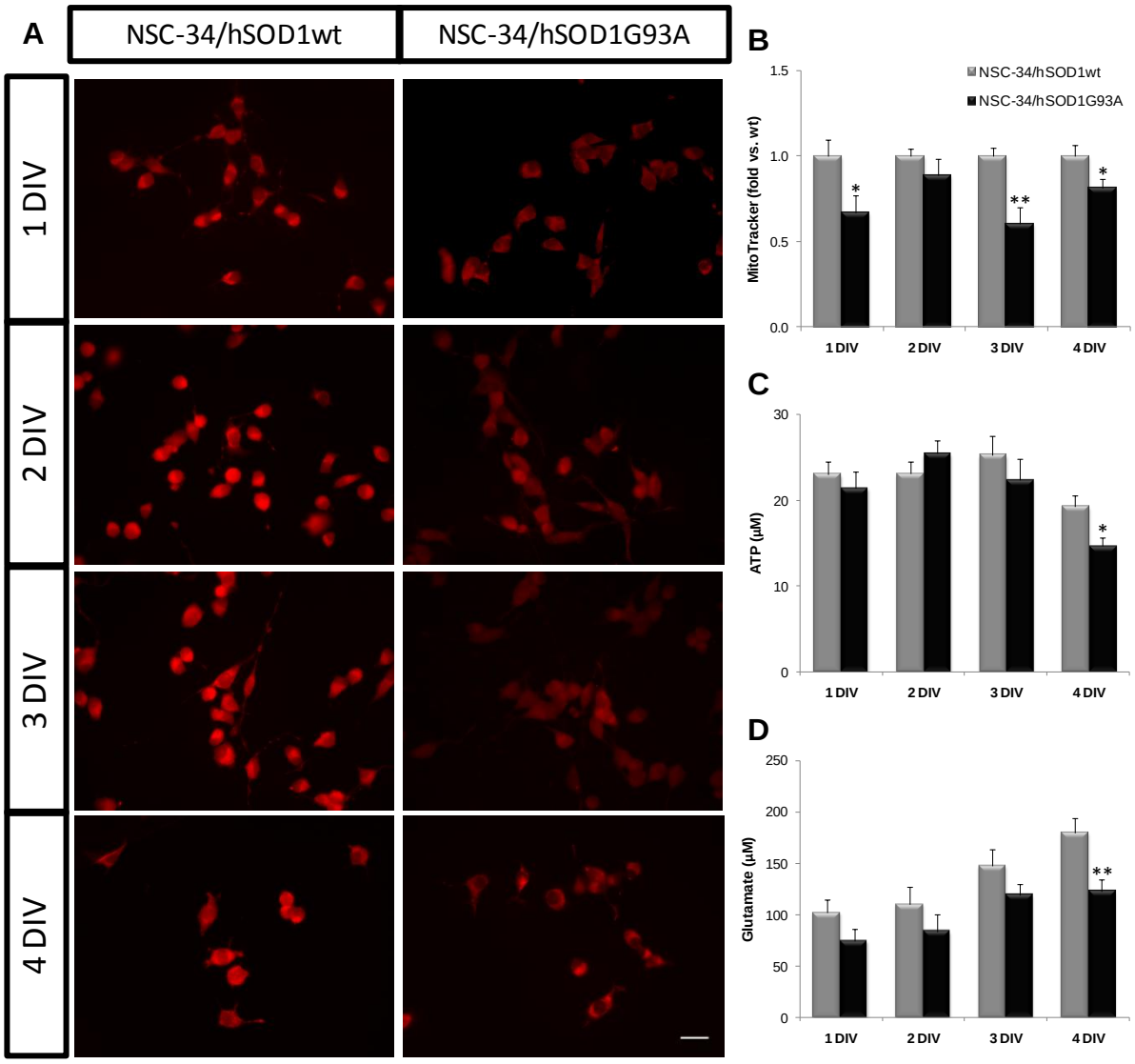

Figure III.5. Differentiated NSC-34/hSOD1G93A cells show altered energy metabolism evidenced by decreased mitochondria viability and reduced release of ATP and glutamate. Cells expressing hSOD1 wt or mutated in G93A were treated as indicated in methods. Cells were stained with Mitotracker red® for mitochondria viability (A) and expressed as fold *vs*. NSC-34/hSOD1wt (control) (B). ATP levels were measured in extracellular media by an enzymatic assay and fluorescence intensity was quantified using a fluorimeter (C). Glutamate release was assessed using the L-glutamic acid kit, as indicated in methods, and the absorbance was measured in a microplate reader (D). Results are mean (± SEM) from at least three independent experiments performed in duplicate. Scale bar represents 40 µm. *p<0.05 and **p<0.01 *vs.* NSC-34/hSOD1wt (control).

1.4 NSC-34/hSOD1G93A cells manifest increased inflammatory features

As in other neurodegenerative disorders, inflammation seems to play a major role in ALS progression (Philips and Robberecht, 2011). We further investigated the release of MMP-9 and 2, as well as NO, which are able to promote inflammatory reactions and oxidative stress that may lead to the activation of microglia. We observed a 2-fold increase in MMP-9 activation in NSC-34/hSOD1G93A cells at 4 DIV when compared to NSC-34/hSOD1wt ($p<0.05$) (Fig. III.6-A), but no significant differences were observed for MMP-2 (Fig. III.6-B).

As evidenced in Figure III.6-C, NO generation was also markedly elevated at 4 DIV ($p<0.05$) due to its increased release by the cells expressing mutant SOD1. Besides its role in inflammation, NO is also a marker of oxidative stress, another feature of ALS disease (Barber and Shaw, 2010) and a consequence of the reduced glutathione synthesis already commented.

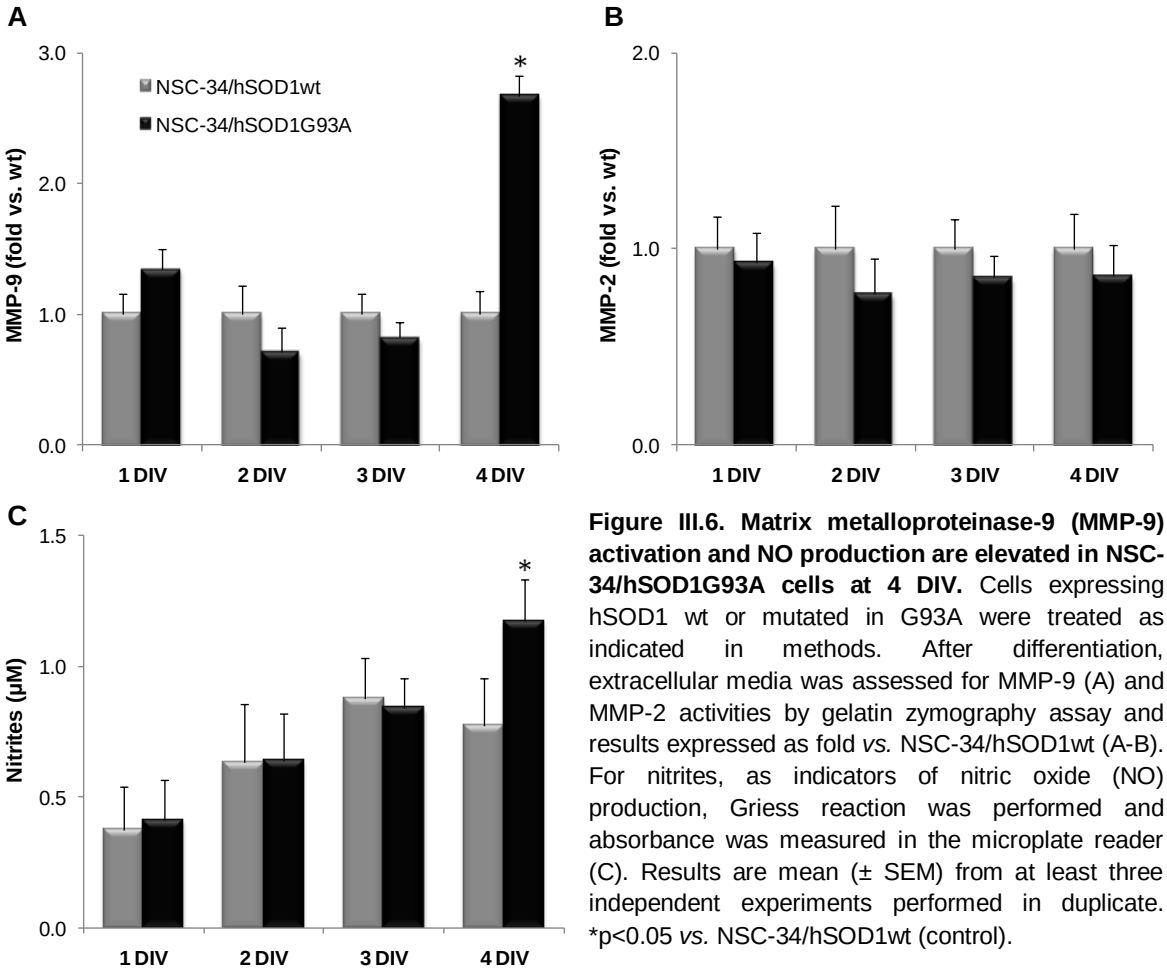

Figure III.6. Matrix metalloproteinase-9 (MMP-9) activation and NO production are elevated in NSC-34/hSOD1G93A cells at 4 DIV. Cells expressing hSOD1 wt or mutated in G93A were treated as indicated in methods. After differentiation, extracellular media was assessed for MMP-9 (A) and MMP-2 activities by gelatin zymography assay and results expressed as fold *vs.* NSC-34/hSOD1wt (A-B). For nitrites, as indicators of nitric oxide (NO) production, Griess reaction was performed and absorbance was measured in the microplate reader (C). Results are mean (± SEM) from at least three independent experiments performed in duplicate. *$p<0.05$ *vs.* NSC-34/hSOD1wt (control).

It is important to mention that differentiated cells were maintained in culture for 7 DIV, as an attempt to characterize a later stage of the disease progression. However, we did not find differences between NSC-34/hSOD1wt and NSC-34/hSOD1G93A cells at 7 DIV, possibly due to the fact that cell lines keep their continuous proliferation features, thus replacing the damaged cells that occasionally were lost along differentiation days. Therefore, we have decided to only present data referring to the first 4 DIV, i.e. time point where we observed the most significant differences between NSC-34/hSOD1wt and NSC-34/hSOD1G93A cells.

Together, the results here obtained indicate that NSC-34 cells expressing hSOD1G93A are a suitable *in vitro* model to explore features of cellular degeneration in ALS, since MMP-9 activation, oxidative stress, mitochondrial dysfunction, energy impairment, as well as altered glutamate and glutathione interplay are commonly found in ALS patients along disease progression (D'Alessandro *et al.*, 2011; Fang *et al.*, 2010; Mitsumoto *et al.*, 2008; Wiedemann *et al.*, 2002)

1.5 GUDCA reveals beneficial effects on the NSC-34 function after mutant SOD1 transfection

Glycoursodeoxycholic acid (GUDCA) has demonstrated anti-apoptotic, anti-inflammatory and anti-oxidant properties in cultured rat neurons (Brito *et al.*, 2008; Silva *et al.*, 2012; Vaz *et al.*, 2010). Therefore, and since motor neuron degeneration in ALS is associated with apoptotic events, oxidative stress, as well as with inflammation, we thought that this bile acid could be a promising compound to prevent some cellular features implicated in this pathology. Thus, we assessed the ability of GUDCA in preventing and/or recovering the functionality of NSC-34/hSOD1G93A cells, by abrogating apoptosis, loss of mitochondria viability, release of NO and enhanced MMP-9 activity. For this, NSC-34 cells were incubated either alone or with 50 μM of GUDCA at the beginning of differentiation and at two days *in vitro* (0 and 2 DIV, respectively), as indicated in Methods.

As depicted in Figure III.7-A and B, apoptosis and mitochondria viaility in NSC-34/hSOD1G93A cells were prevented by GUDCA treatment, although GUDCA failed in recovering NSC-34/hSOD1G93A cells in both conditions. Indeed, no differences were noticed between cells treated with GUDCA at 2 DIV and non-treated cells. In addition, as shown in Figure III.7-C, GUDCA was able to prevent ($p<0.01$) and recover ($p<0.05$) NSC-34/hSOD1G93A cells from oxidative stress, as it lowered the production of NO. Furthermore, GUDCA completely abolished MMP-9 activation ($p<0.01$) in both conditions (Figure III.7-D), restoring the basal conditions observed in NSC-34/hSOD1wt cells.

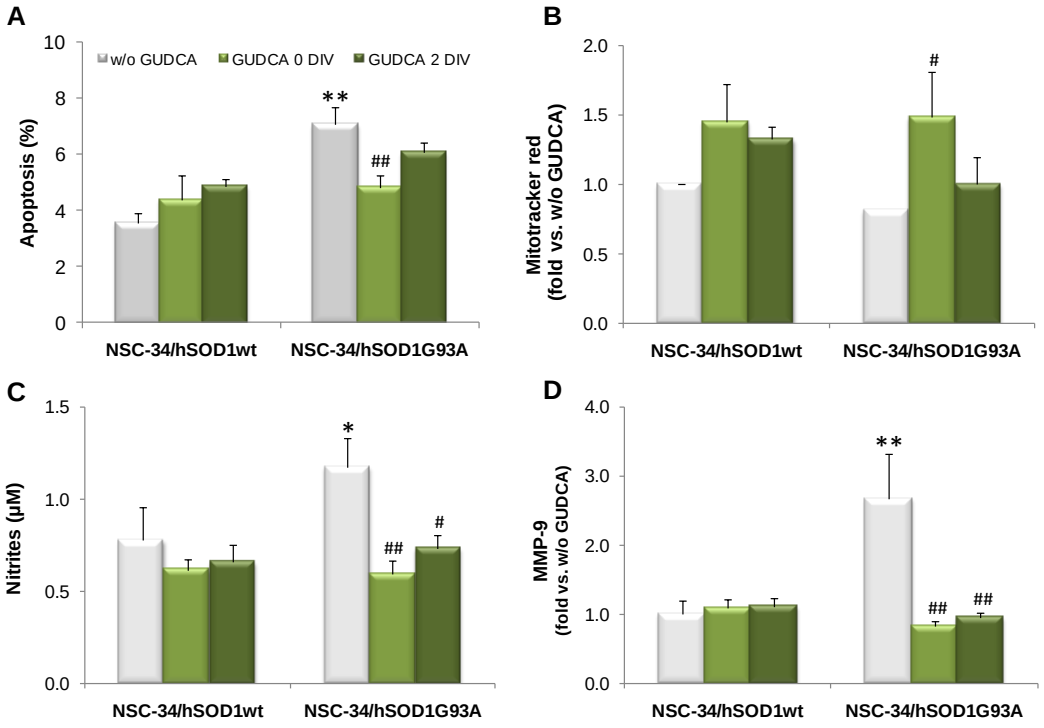

Figure III.7. Beneficial effects of GUDCA on NSC-34/hSOD1G93A cellular stress. Cells were treated as indicated in methods. Cells were stained with Hoechst dye for morphological analysis of the nuclei and apoptosis was expressed as percentage from total number of cells (A). Mitotracker red® incubation was used for mitochondrial viability and the results were expressed by fold *vs* NSC-34/hSOD1wt cells without (w/o) GUDCA (B). Evaluation of nitrites was performed by the Griess reaction (C) and matrix metalloproteinase-9 (MMP-9) activity by gelatin zymography assay (D). Results are mean (± SEM) from at least three independent experiments performed in duplicate. *$p<0.05$ and **$p<0.01$ *vs*. NSC-34/hSOD1wt; $^{\#}p<0.05$ and $^{\#\#}p<0.01$ *vs.* w/o GUDCA.

2. Evaluation of microglial response to LPS and to soluble factors released by NSC-34/hSOD1G93A

The second main goal of the present thesis was to evaluate whether microglia incubated with conditioned media obtained from NSC-34 cells expressing hSOD1 mutated in G93A will evidence different features of activation from those expressing the wild type protein. For that, N9 microglia cell line was used. N9 microglia consist in a cell line obtained from CD1 mice cortex that show features similar to microglia in primary cultures such as migration, phagocytosis and inflammation-related features (Bruce-Keller *et al.*, 2000; Cui *et al.*, 2002; Fleisher-Berkovich *et al.*, 2010).

2.1 Characterization of a murine microglia cell line (N9) dynamics when facing an inflammatory stimulus

In order to unravel the activation features of the N9 cell line, we first performed the evaluation of microglia morphological changes, as well as the migratory and phagocytic abilities after an inflammatory/activation stimulus by using 300 ng/ml of lipopolysaccharide (LPS) during 24 h of incubation (Cui *et al.*, 2002). We have chosen LPS because this agent was previously described to induce the M1 microglia, characterized by a pronounced pro-inflammatory phenotype, releasing IL-1β and TNF-α (Bruce-Keller *et al.*, 2000; Gong *et al.*, 2008; Michelucci *et al.*, 2009; Zhao *et al.*, 2011).

Morphological changes are one of the common features of microglia activation (Calvo and Bennett, 2011). Therefore, we performed morphological analysis and reactivity evaluation by immunocytochemistry. We observed that LPS induces a change in microglia morphology from a ramified to an amoeboid state, as represented in Figure III.8. The number of cell processes is decreased and microglia cell soma enlarges after treatment with LPS.

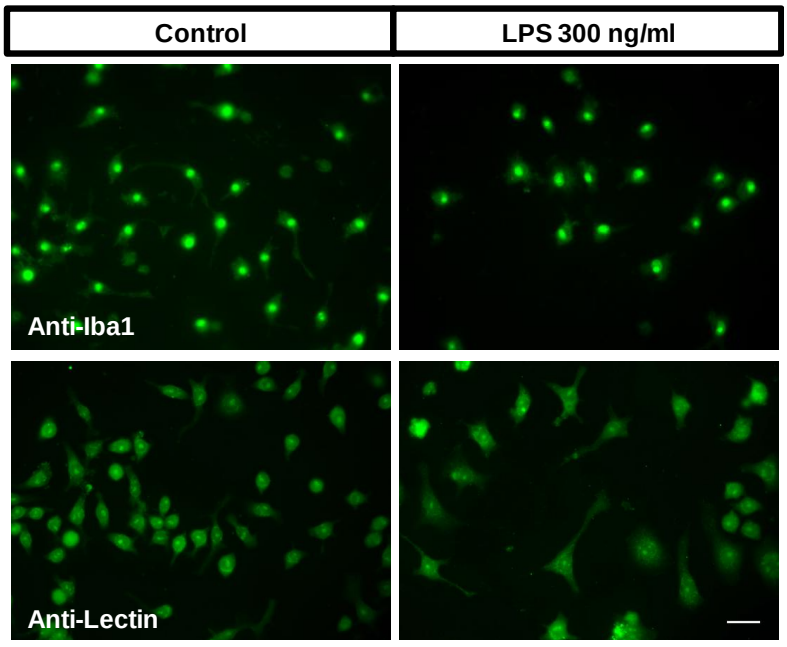

Figure III.8. Microglia evidence amoeboid morphology when incubated with LPS for 24 hours. Cells were treated as indicated in Methods. Morphology was evaluated by immunocytochemistry using rabbit anti-Iba1 or anti-lectin antibodies, followed by a fluorescent-labeled secondary antibody. Representative results from one experiment are shown. Scale bar represents 40 µm.

Phagocytosis is another property of activated microglia, being essential in clearance of cellular debris as well as in the elimination of pathogenic organisms (Napoli and Neumann, 2009). Therefore, microglial phagocytic ability was determined by assessing the capacity of the cell to ingest green latex beads. As depicted in Figure III.9, LPS stimulation slightly increased the number of phagocytic N9 cells. Interestingly, when the number of beads per cell was evaluated, we noticed that LPS-treated cells led to an increase number of beads per cell as compared with non-treated cells. Cells containing between 5 and 10 beads or even a higher number than 10 per cell suggest that, despite of no significant difference in the number of phagocytic cells between non-treated and LPS-treated cells, some of the LPS-stimulated cells have an increased avidity to engulf the beads in a higher quantity ($p<0.01$) (Fig. III.9-C).

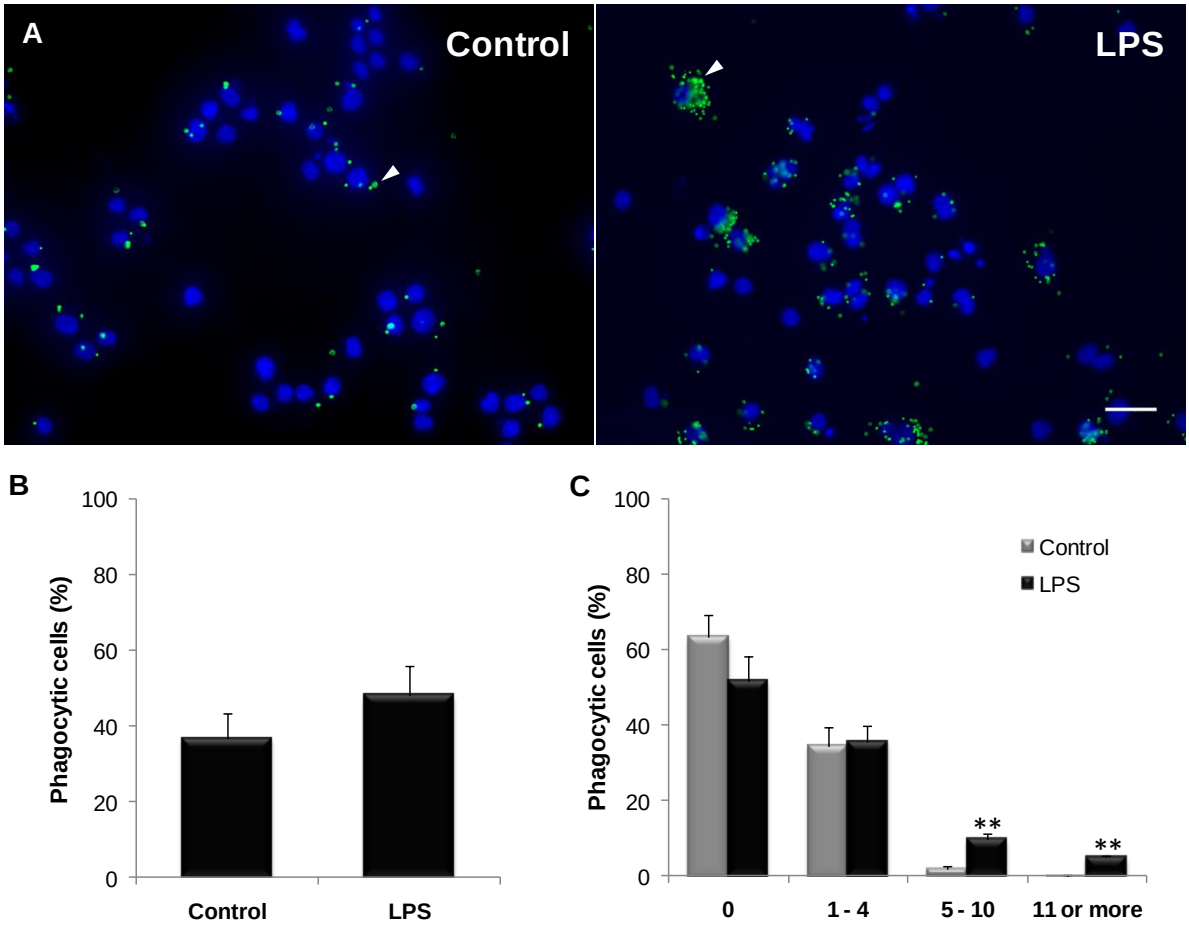

Figure III.9. Phagocytosis is increased in N9 microglia cells exposed to LPS. Cells were treated as indicated in methods. Representative results of one experiment are shown and ingested beads are indicated by arrows (A). Results are expressed as the percentage of cells showing ingested beads (B) or the percentage of cells engulfing specific numbers of ingested beads (C). Results are mean (± SEM) from three independent experiments. Scale bar represents 40 µm. **p<0.01 *vs*. non-treated cell (control).

We also decided to evaluate migration capacity of N9 microglia cells. For that, we performed a chemotaxis assay with the Boyden chamber, as indicated in Methods, and microglia was induced to migrate for two different concentrations of ATP (10 and 300 µM), a well-known microglia chemoattractant (Davalos *et al.*, 2005; Ohsawa and Kohsaka, 2011). Our results confirmed that N9

cells are attracted by ATP, as demonstrated by the almost 2-fold increase in the number of cells that migrate to ATP in comparison to those that freely migrated to basal medium (without added ATP) (Fig. III.10). In addition, we observed that the chemoattractant properties of ATP did not increase when the concentration of ATP was changed from 10 to 300 µM (Fig. III.10.B). LPS treatment showed a completely different result. In the basal conditions, the number of migrated cells previously treated with LPS was very similar those non treated with LPS (control). However, LPS-stimulated microglia loose the ability to migrate towards ATP, independently of the concentration we used.

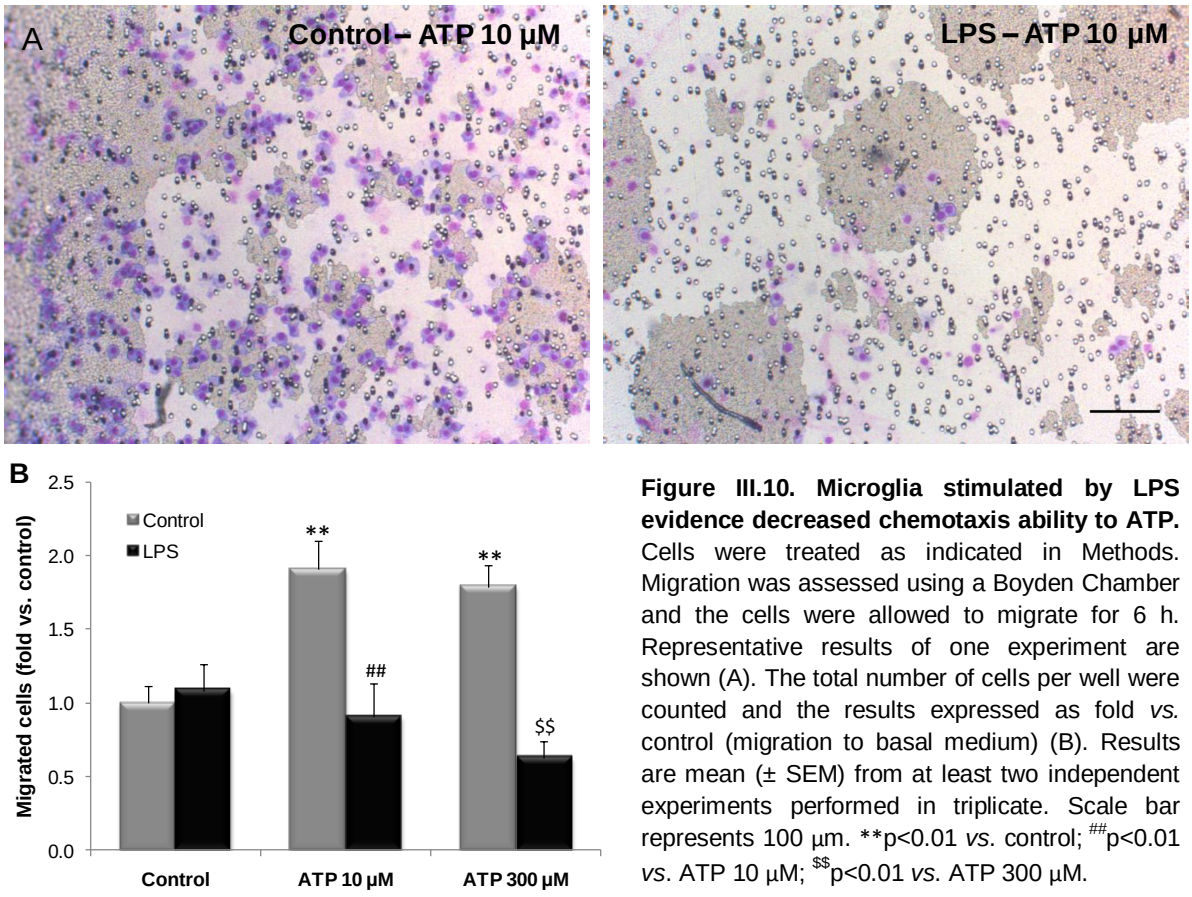

Figure III.10. Microglia stimulated by LPS evidence decreased chemotaxis ability to ATP. Cells were treated as indicated in Methods. Migration was assessed using a Boyden Chamber and the cells were allowed to migrate for 6 h. Representative results of one experiment are shown (A). The total number of cells per well were counted and the results expressed as fold *vs.* control (migration to basal medium) (B). Results are mean (± SEM) from at least two independent experiments performed in triplicate. Scale bar represents 100 µm. **p<0.01 *vs*. control; $^{\#\#}$p<0.01 *vs*. ATP 10 µM; $^{\$\$}$p<0.01 *vs*. ATP 300 µM.

Together, these results suggest that N9 microglial cells are able to undertake activation features commonly described in microglia after LPS stimulation, therefore providing clues to the role of microglia in ALS progression.

2.2 N9 microglia are not attracted by soluble factors released from motor neurons expressing hSOD1G93A

As reviewed in Calvo and Bennett (2011), microglia migrate to sites of injury in response to chemoattractants, such as ATP, or fractalkine. Therefore, we aimed to evaluate whether microglia are attracted to compounds that may have been released by motor neurons expressing hSOD1G93A during the 4 DIV. Our results showed that microglia was not attracted to NSC-34/hSOD1G93A-conditioned media, as evidenced in Figure III.11. In fact, as our previous results have shown, NSC-

34/hSOD1G93A cells evidenced decreased levels of ATP release (Figure III.5.C), one major chemoattractant for microglia. In fact, it seems that they are even repelled as a 0.36-fold significant decreased was observed when compared to microglia migration to the basal condition. It is noteworthy to point that microglia do not migrate to NSC-34 cells expressing the wild type protein either.

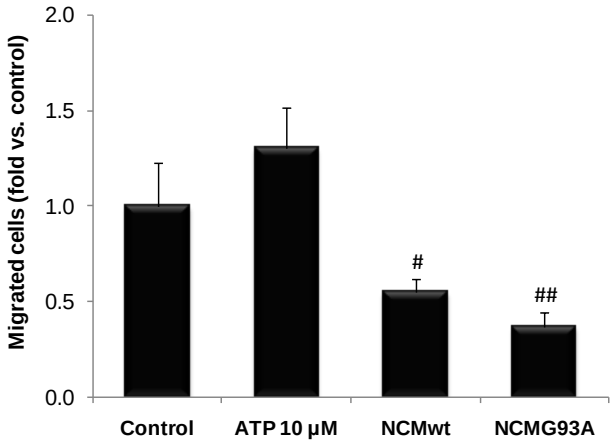

Figure III. 11. N9 microglia is not attracted to the released factors from NSC-34/hSOD1G93A. Cells were treated as indicated in Methods. Migration was assessed using a Boyden Chamber and the cells were allowed to migrate for 6 hours. The total number of cell per well were counted and the results expressed as fold *vs.* control (migration to basal medium). Results are mean (± SEM) from two independent experiments performed in triplicate. $^{\#}p<0.05$ and $^{\#\#}p<0.01$ *vs.* ATP 10 µM. NCMwt, conditioned media from NSC-34/hSOD1wt cells; NCMG93A, conditioned media from NSC-34/hSOD1G93A.

2.3 N9 microglial cell death by factors released from motor neurons are not different between NSC-34/hSOD1wt and NSC-34/hSOD1G93A in causing cytotoxicity, which only increase by the time of exposure

In order to establish an *in vitro* model that could represent the response of microglia in the pathogenesis of ALS, we explored the effects of the soluble factors released either by NSC-34/hSOD1wt or NSC3/hSOD1G93A cells on microglial dynamics. For that, we incubated microglia with conditioned media from NSC-34/hSOD1wt or NSC-34/hSOD1G93A cells (NCMwt or NCMG93A, respectively), collected at different days of differentiation. Based on the results previously indicated in section 1 of this Chapter, we hypothesized that NSC-34 cells cultured during 24 h (1 DIV) would represent the pre-symptomatic stage of the disease, as some differences in the parameters evaluated were already observed between cells expressing hSOD1wt or hSOD1G93A but not sufficient to cause significant cell death. To represent the symptomatic stage, we have chosen the 4 DIV, as we noticed more dramatic changes in NSC-34 cells expressing hSOD1G93A with increased death of NSC-34/hSOD1G93A cells. We also tested two different times of incubation in microglia, namely 4 and 24 hours, to mimic a short and a prolonged exposure to NSC-34 released factors since microglial functions can change with the duration of stimuli (Silva *et al.*, 2010).

Chronic activation of microglial cells lead to overactivation of these cells triggering ultimately senescence and degeneration (Graeber and Streit, 2010). Therefore, we evaluated whether N9 microglia evidenced increased cell death in the referred conditions as a first step in evaluating microglial reactivity to motor neurons released factors.

Cellular death by necrosis was measured by the uptake of PI and quantified by flow cytometry, as indicated in Methods. As evidenced in Figure III.12., we observed that cell death was increased from 4 h (<10%) to 24 h (~20%) incubation (Figure III.12-B), either with NCMwt or NCMG93A. Since no

differences were found between N9 microglia exposed to NCMwt and to NCMG93A, we may assume that necrosis is not the preferential cell death mechanism involved in this experimental model of ALS.

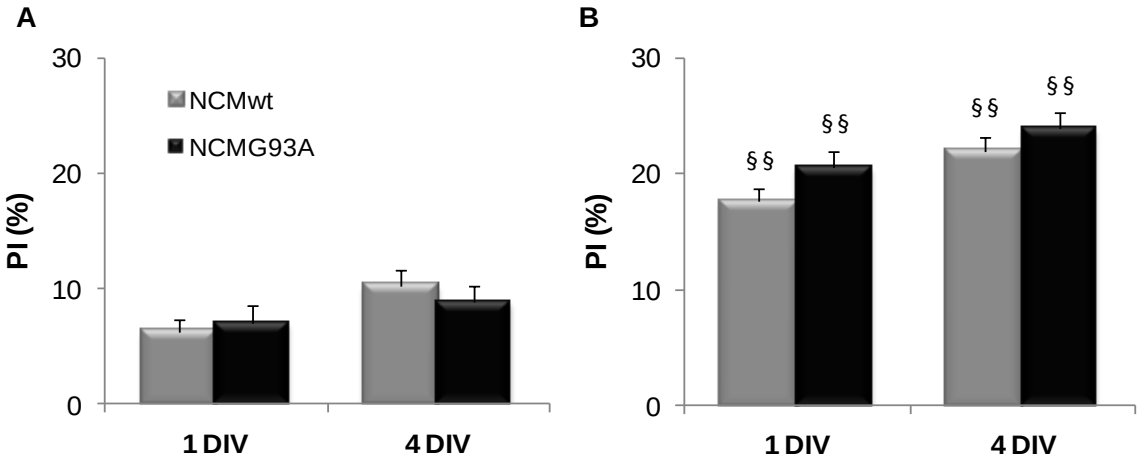

Figure III.12. Soluble factors released from NSC-34/hSOD1wt and NSC-34/hSOD1G93A equally enhance necrosis of N9 microglia in a time-dependent manner. Cells were treated as indicated in Methods. Uptake of the fluorescent dye PI (propidium iodide) was evaluated by flow cytometry after 4 (A) and 24 h (B) exposure to NSC-34-conditioned media. Results are expressed as mean (± SEM) from three independent experiments performed in duplicate. N9 treated with NCMwt was used as the control condition. §§ $p<0.01$ *vs*. 4 hours.

Apoptosis was also evaluated by nuclei morphology following Hoechst 33258 staining, and condensed or fragmented nuclei were quantified as apoptotic. In parallel with the necrosis data, apoptosis duplicated with the time of incubation from about 5% at 4 h to more than 10% at 24 h (Fig. III.13). A slight, not significant, increase was noticed in microglia incubated for 24 h with conditioned media from NSC-34/hSOD1G93A cells, suggesting that these cells are more prone to this form of programmed cell death than to other types of cellular demise.

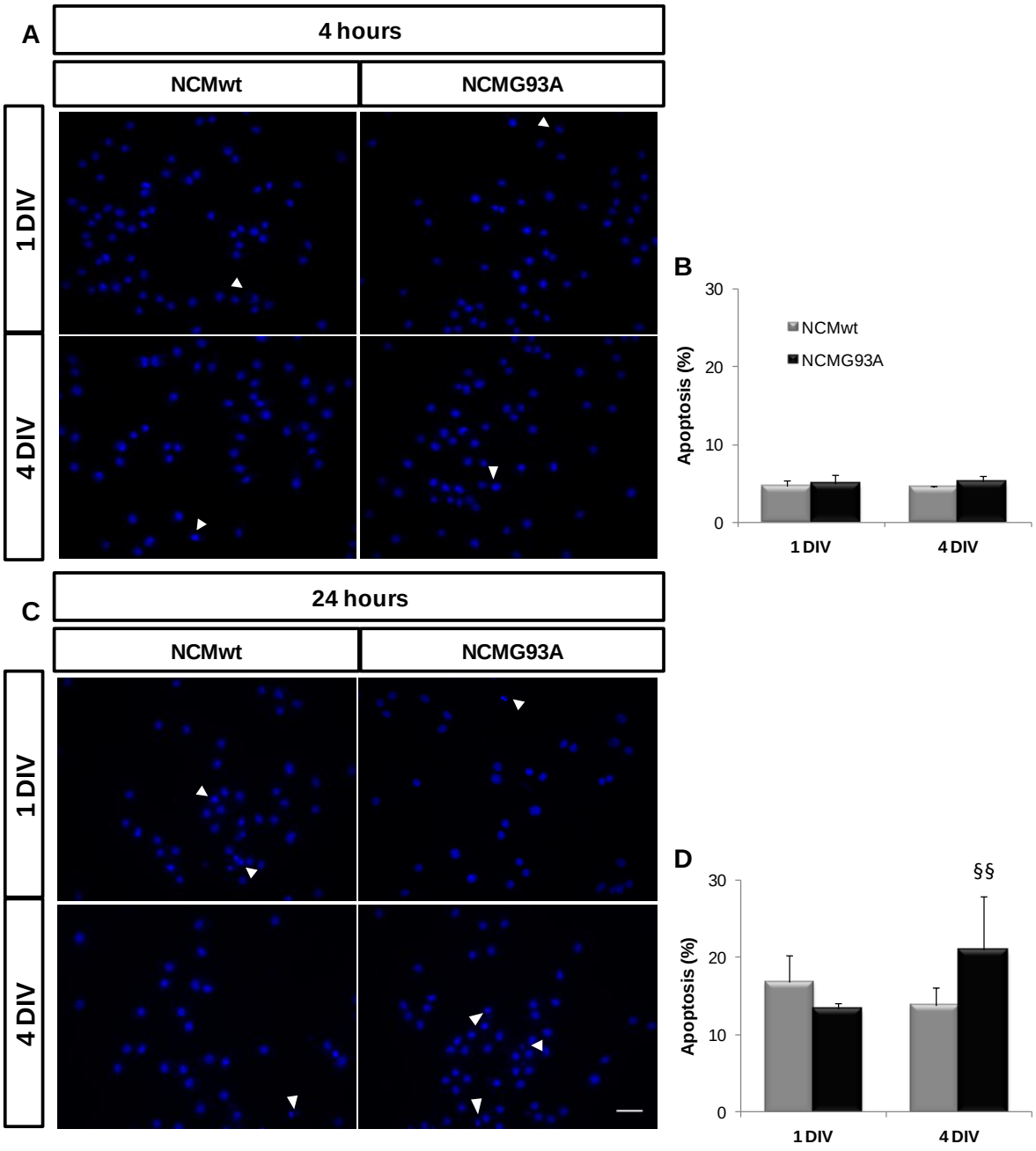

Figure III.13. Soluble factors released from NSC-34/hSOD1wt and NSC-34/hSOD1G93A equally enhance apoptosis of N9 microglia in a time-dependent manner. Cells were treated as indicated in Methods. Cells were stained with Hoechst dye for morphological analysis of the nuclei and representative results from one experiment are shown (A and C). Results are expressed as percentage per total number of cells after 4 (B) and 24 h (D) exposure to NSC-34-conditioned media and expressed as mean (± SEM) from three independent experiments performed in duplicate. N9 treated with NCMwt was used as the control condition. Scale bar represents 40 μm. §§ $p<0.01$ *vs*. 4 hours.

2.4 Microglia evidence an increased amoeboid morphology and low phagocytic ability after long exposure to NSC-34/hSOD1G93A conditioned media

We then evaluated microglia morphology and reactivity by immunocytochemistry using rabbit anti-Iba1 antibody. As demonstrated in Figure III.14, short and prolonged exposure to conditioned media collected from the two cell lines at 1 DIV did not produce a significant change on microglia

morphology, as almost all microglia cells show an elongated morphology with well-defined ramifications. However, we could notice some microglia cells with an amoeboid morphology. NCMwt from 4 DIV evidenced a combined number of cells with different morphology in both incubation periods, and consequently diverse phenotypes. Ramified, as well as amoeboid cells were observed, indicating that the soluble factors released by motor neurons are able to activate microglia at some extent. The same happened when N9 microglia were incubated for 4 h with NCMG93A from 4 DIV (Fig. III.14). Interestingly, the results obtained after the 24 h incubation demonstrated that amoeboid morphology was more frequent than in the other incubation conditions. Taken together, these results indicate that changes in microglia reactivity are probably involved in ALS progression, as microglia demonstrate enhanced activation features after incubation with NCMG93A collected at 4 DIV when compared to incubation with media from 1 DIV of differentiation.

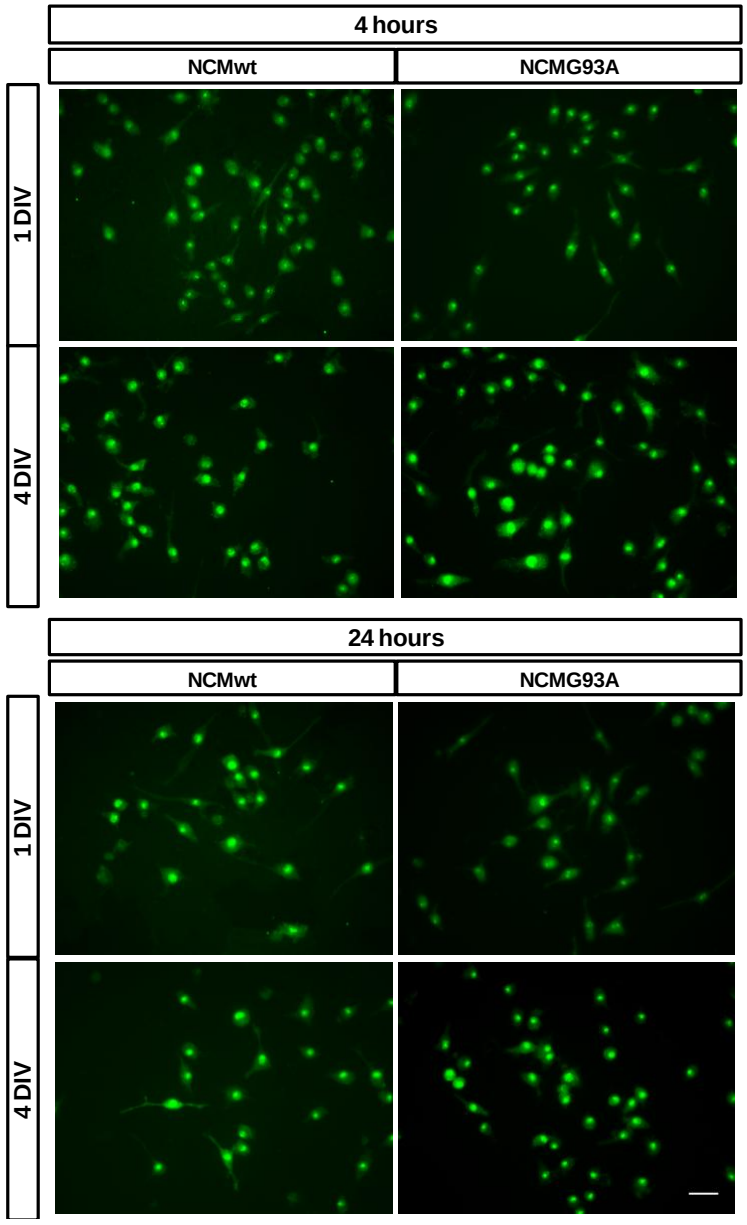

Figure III.14. Microglia demonstrate amoeboid morphology after 24 hours of incubation with NSC-34/SOD1G93A conditioned media collected at 4 DIV. Cells were treated as indicated in Methods. Morphology was evaluated by immunocytochemistry using rabbit anti-Iba1 antibody, followed by a fluorescent-labeled secondary antibody. Representative results from one experiment are shown. Scale bar represents 40 µm.

Next, we evaluated phagocytic capacity of N9 microglia after incubation with either NCMwt or NCMG93A. We observed that incubation for 4 h with both media did not produce differences in the phagocytic ability of these cells (Fig. III.15). However, microglial phagocytosis was highly reduced (5.6 % of phagocytic cells) when exposed for 24 h to NCMG93A collected at 4 DIV, although even NCMG93A from 1 DIV exhibit a similar tendency in comparison with the correspondent media obtained from NSC-34/hSOD1wt cells (control). As represented in Figure III.15, we also noticed that microglia only ingested a few number of beads in each condition evaluated, which was markedly reduced if instead 4 h we used 24 h of microglia incubation with NCM (rarely more than 2 beads were ingested by N9 microglia). Therefore, we may assume that microglia loose their phagocytic ability after exposure to mutated motor neurons, not accomplishing the relevant property of eliminating debris and dead cells, mainly the degenerated motor neurons in the present case.

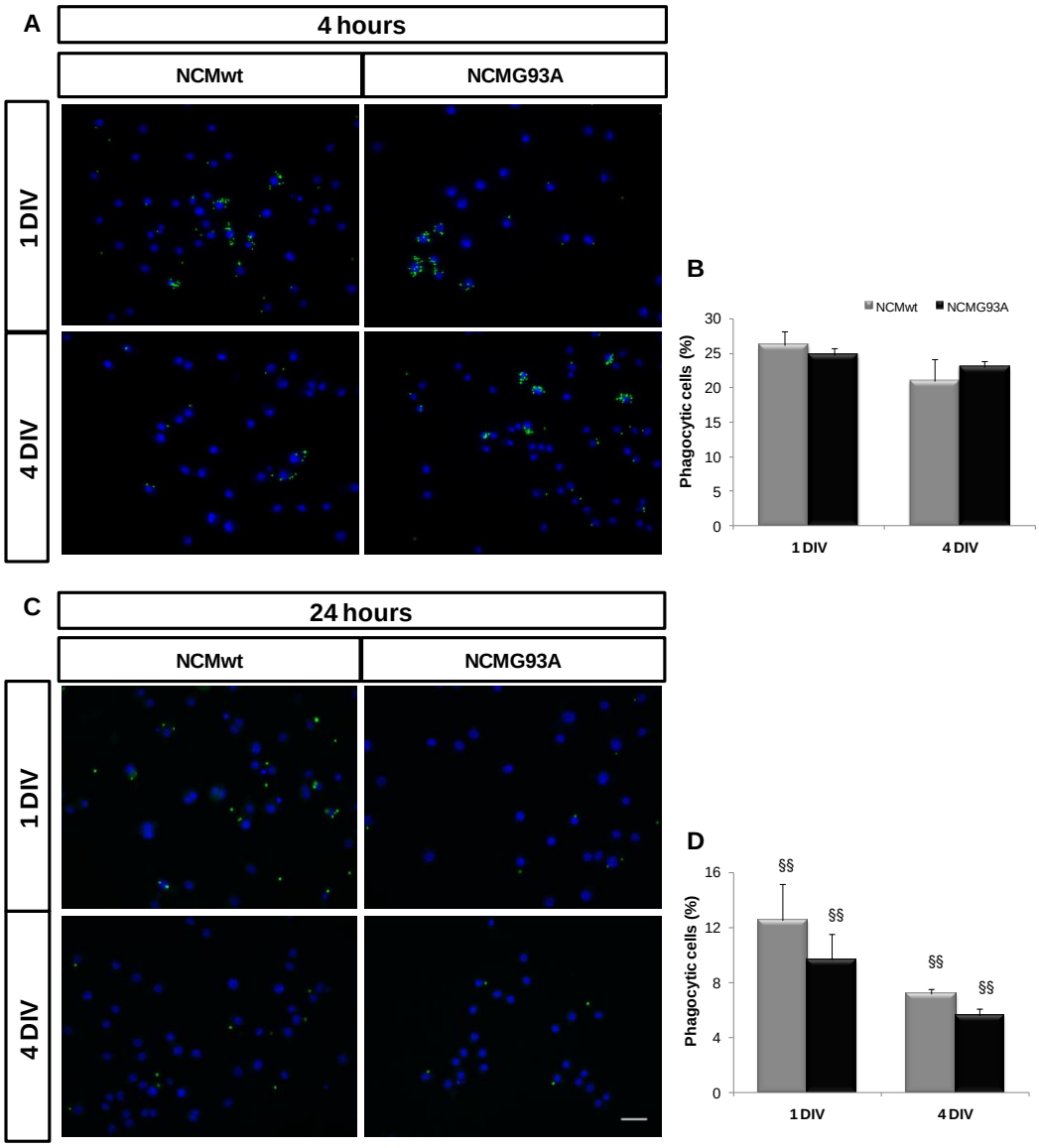

Figure III.15. Conditioned media from NSC-34/hSOD1G93A incubated for 4 DIV reduces microglial phagocytic ability. Cells were treated as indicated in Methods. Representative results of one experiment are shown for each incubation time point (4 or 24 hours, A and C, respectively). Results are expressed as number of phagocytic cells per total number of cells (B and D). Results are mean (± SEM) from three independent experiments performed in duplicate. Scale bar represents 40 μm. §§ $p<0.01$ *vs*. 4 hours.

Overall, the results here obtained suggest that at pre-symptomatic stage (NCM from 1 DIV), NSC-34/hSOD1G93A released factors do not induce a change in microglial dynamics in comparison with control conditions (NSC-34/hSOD1wt). Nonetheless, NCMG93A from 4 DIV is able to promote dysfunction in non-transgenic microglia, pointed by a decreased phagocytosis and an increased apoptosis, indicating that when motor neurons degeneration starts at the beginning of the symptomatic stage, their released factors can be deleterious to microglia. Therefore, a better understanding of microglia response to conditioned media from NSC-34 either with hSOD1wt or hSOD1G93A, in terms of autophagic ability and phagocytic capacity, as well as reactivity and inflammatory response, in particular the identification of the determinants and the targets involved, is needed and will elucidate on their relevance as diagnostic markers.

IV. DISCUSSION

Several *in vitro* and *in vivo* models have been created to study the pathological events underlying the selective degeneration of motor neurons in ALS. As studying the sporadic form of the disease (sALS) is extremely difficult, most research have been focused in familial related mutations, mainly, by using SOD1 mutants, since 20% of fALS cases are due to mutations in SOD1 (Rothstein, 2009). NSC-34 cells, which result from the fusion of neuroblastoma cells and spinal cord motor neurons (Cashman *et al.*, 1992), have been widely studied in the context of motor neurons disease, including ALS, as they evidence morphological and physiological properties of motor neurons, such as generation of action potentials, expression of neurofilament triplet proteins, as well as acetylcholine synthesis, storage and release (Cashman *et al.*, 1992; Martinou *et al.*, 1991). Recently, transfection of NSC-34 cells with SOD1 mutants have improved our understanding of the pathogenic mechanisms since the *in vitro* studies can offer a molecular based approach, corroborating several features already described in ALS patients, as well as in transgenic mice models. Gomes and colleagues (Gomes *et al.*, 2010; Gomes *et al.*, 2008) have provided important clues in this matter, by showing that transfection of these cells with hSOD1G93A protein induces Golgi apparatus disruption, as well as decreased proliferation and differentiation capacity. Therefore, in the present study, we proposed that transfection of NSC-34 cells with SOD1 mutated in G93A, a mutant form described in fALS (Rosen, 1993), could represent a suitable model to investigate the molecular mechanisms involved in motor neurons degeneration in ALS.

First, and to ensure that a motor neuron like morphology was acquired by NSC-34 cell line, differentiation was induced accordingly to Cho *et al.* (2011). Low serum conditions (1% FBS) have

already been proven to be tolerated by NSC-34 cells, as growth and maturation were properly acquired in those conditions (Cashman *et al.*, 1992; Cho *et al.*, 2011), although cell adhesion was comprised (Matusica *et al.*, 2008). After differentiation, we observed that NSC-34 cells evidenced correct processes maturation with cell soma reduction, a morphology that was maintained during the first 4 days *in vitro* (DIV), as well as cell adhesion. In addition, NSC-34 cells did not evidence significant cell death during the 4 DIV of differentiation as shown by flow cytometry analysis of PI incorporation.

Stable transfection of SOD1 was confirmed by Western Blot analysis. Indeed, we observed human SOD1 expression (21 kDa) in NSC-34/hSOD1wt and NSC-34/hSOD1G93A cells, as well as mouse SOD1 (14 kDa) and no band was detected at 21 kDa in NSC-34 cells with the empty vector (pCIneo). SOD1 aggregation is a well-known hallmark of ALS (Ferraiuolo *et al.*, 2011). In addition, Gurney *et al.* (1994) have observed formation of SOD1 aggregates in brainstem and spinal cord of mice expressing SOD1 with G93A mutation. Therefore, we looked for SOD1 accumulation or aggregation in our model. Immunocytochemistry analysis evidenced SOD1 accumulation in NSC-34/hSOD1G93A cells starting at 2 DIV and becoming more evident at 3 DIV, which is in accordance with other *in vitro* studies that have demonstrated accumulation and aggregation of SOD1 in SOD1G93A expressing cells (Cho *et al.*, 2011; Gal *et al.*, 2007; Gomes *et al.*, 2010). However, in our model, no aggregates were observed until 4 DIV. Conversely, we expected to observe molecular dysfunctions in NSC-34/hSOD1G93A cells related to SOD1 accumulation beginning at 3 DIV or beyond. However, cellular viability quantification evidenced differences between cell lines before SOD1 accumulation, as NSC-34/hSOD1G93A cells showed less MTS reduction than NSC-34/hSOD1wt ones, right after 1 DIV. At 2 DIV, MTS reduction was even lower but, surprisingly, at 3 DIV the tendency was inverted and NSC-34/hSOD1G93A cells appeared more viable than NSC-34/hSOD1wt cells. Besides the incorrect conformation of SOD1G93A, which promotes aggregates formation, this substitution of a glycine over an alanine is a dominant, gain-of function mutation (Cho *et al.*, 2011) and can cause oxidative damage before its accumulation. This could explain why NSC-34/hSOD1G93A cells are less viable in the first days of differentiation. The viability turn-over observed at 3 DIV can be interpreted as an attempt to compensate this cellular injury which is worsened by the damage due to SOD1 accumulation. On the other hand, and since we are working with a cell line, we must consider that cells keep growing even after differentiation, specially NSC-34/hSOD1wt cells, described to have a high proliferation rate (Cho *et al.*, 2011).

The presence of apoptotic features were also evaluated as expression of mutant SOD1 can induce this type of programmed cell death. In fact, motor neuron degeneration due to cytochrome *c* release induced by increased ROS production and oxidative stress was observed when SOD1G93A was expressed in NSC-34 cells or in transgenic mice (Liu *et al.*, 2002). Moreover, serum of patients with ALS evidenced elevated caspase-9 activity, which initiates mitochondrial apoptotic cascade (Ilzecka, 2011). Here, we observed increased apoptosis in NSC-34/hSOD1G93A cells at 2 DIV, possibly related to SOD1 gain-of-function, which was more notorious at 4 DIV, as fragmented and condensed nuclei almost doubled in comparison to the NSC-34/hSOD1wt cells. This is consistent with previous findings where shrunken cell soma and chromatin condensation were observed in cells

expressing mutant SOD1 (Ghadge *et al.*, 1997). Next, we investigated the mechanisms underlying apoptosis and whether it could be related to mitochondrial dysfunction. Mitochondrial viability was assessed through MitotrackerRed® staining, which only stains viable mitochondria. We noticed less viable mitochondria in NSC-34/hSOD1G93A cells, mainly at 3-4 DIV, preceding apoptosis elevation at 4 DIV. These data corroborate other studies showing that mitochondrial dysfunction is a common feature in ALS (Shi *et al.*, 2010). Menzies and colleagues (2002) have shown morphological alterations in mitochondria of NSC-34 expressing mutant SOD1, including vacuolation. They also observed that mitochondrial electron transport chain was compromised, with decreased activity of the complexes II and IV, with reproduction of the same features in SOD1G93A mice (Bendotti *et al.*, 2001; Higgins *et al.*, 2003; Kong and Xu, 1998) and in ALS patients (Afifi *et al.*, 1966; Atsumi, 1981; Wiedemann *et al.*, 1998). By evaluating the release of ATP, as an indicator of energy impairment, we observed that ATP release was significantly lower in NSC4/hSOD1G93A cells than in NSC-34/hSOD1wt at 4 DIV. The findings by D'Alessandro and colleagues (2011) can provide one possible explanation to our results. In their study, NSC-34/hSOD1G93A cells showed a reduction of glucose-derived pyruvate flow through the tricarboxylic acid cycle, which implies the existence of an altered glutamine/glutamate metabolism and energy failure in these cells.

Glutamate excitotoxicity is another mechanism involved in motor neuron degeneration in ALS (Pasinelli and Brown, 2006). In fact, a two-fold increase in glutamate levels was found in cerebrospinal fluid of ALS patients (Rothstein *et al.*, 1990) and electrophysiological studies in humans evidenced hyperexcitability of the motor system in the pre-symptomatic, or early stages of the disease (Vucic and Kiernan, 2006; Vucic and Kiernan, 2008). Therefore, we aimed to investigate whether NSC-34/hSOD1G93A cells release higher amounts of glutamate than the NSC-34/hSOD1wt cells. However, our findings did not show increased levels of glutamate released by NSC-34/hSOD1G93A cells. Instead, we have noticed a reduction in extracellular glutamate along differentiation, attaining significantly differences from NSC-34/hSOD1wt cells at 4DIV. The reduction may be explained by the mitochondrial impairment observed, as the metabolism of glutamine and glutamate are intimately linked with mitochondrial functions (D'Alessandro *et al.*, 2011).

Activity of MMPs has been widely associated with ALS progression. Indeed, Fang and colleagues (2010) by investigating the levels of MMP-9 and MMP-2 in the spinal cord and skin of SOD1G93A mice found elevated activities of these proteins. In addition, the activities of MMP-9 and MMP-2 have been found to be modified with disease progression, as serum analysis of SOD1G93A mice indicated the highest activity at the early symptomatic stage, decreasing thereafter during ALS progression till end stages (Soon *et al.*, 2010). These results point to MMP-9 and MMP-2 as biomarkers of the disease. Here, by using gelatin zymography to evaluate the activity of MMP-9 as well as MMP-2, we were able to show, for the first time by using an *in vitro* model, that NSC-34/hSOD1G93A cells present a 2.6-fold MMP-9 increased activity at 4 DIV as compared to NSC-34/hSOD1wt cells. However, MMP-2 levels evidenced to be maintained along differentiation and no significant differences were found between tested cell lines.

We then intended to understand the role of oxidative stress in motor neurons degeneration in our model. For that we evaluated the production of a specific reactive nitrogen species, the NO, and we

showed that NSC-34/hSOD1G93A cells have increased production of this species at 4 DIV, in comparison with control cells. This is not without precedent, since increased production of ROS has been associated with ALS pathology, together with lipid peroxidation and protein nitration (Carri *et al.*, 2003). Indeed, when at higher concentrations, NO rapidly interacts with superoxide and forms peroxynitrite, thus promoting protein nitration (Barber and Shaw, 2010). A marker of nitration of tyrosine residues on proteins, 3-nitrotyrosine, was found to be increased in both sALS and fALS patients, as shown in two independent studies (Abe *et al.*, 1997; Beal *et al.*, 1997). Imbalance of ROS metabolism in our model may be elicited by hSOD1G93A imperfect folding and consequent accumulation rather than by gain of oxidative function since the excessive production of NO was noticed later in differentiation. This imperfect folding can encompass modification of the active site enabling the enzyme to perform its normal function. Besides its role in oxidative stress, NO accumulation may lead to excitotoxicity caused by over-activation of NMDA receptors (Dawson *et al.*, 1991). Moreover, oxidative stress can contribute to motor neuron cell death by apoptosis, as shown by Liu and colleagues (2002). Interestingly, several studies demonstrated the association between inflammation and generation of ROS/RNS, leading to multiple organ dysfunction (Bian and Murad, 2001; Sener *et al.*, 2005). In fact, NO is generally recognized as a mediator and regulator of inflammatory responses. It was first reported that mouse macrophages produce nitrite and nitrate in response to bacterial LPS (Stuehr and Marletta, 1985). However, although high levels of NO generated in response to inflammatory stimuli can have deleterious effects, the molecule is likewise important in cellular signaling, having an important role in the amelioration of the pathogenesis of inflammation (Korhonen *et al.*, 2005). In addition, NO-induced *c*-Jun N-terminal kinases 1 and 2 (JNK1/2) phosphorylation is observed in models of neurodegenerative diseases, such as Alzheimer's and Parkinson's diseases (Katsuki *et al.*, 2006; Marques *et al.*, 2003).This finding is important since these mitogen-activated protein kinases are among the main effectors that participate in inflammatory signaling pathways (Roux and Blenis, 2004). Furthermore, NO and induction of NOS are involved in apoptosis induced by inflammatory mediators in neuronal cells (Hemmer *et al.*, 2001; Heneka *et al.*, 1998; Thomas *et al.*, 2008). Remarkably, it was recently reported that NO can also promote direct movement of microglia to the site of injury (Parkhurst and Gan, 2010).

The second aim of the present was to test the ability of GUDCA in neuroprotection, as a potential compound to slow disease progression in ALS patients. For that, we incubated NSC-34 cells either alone or with 50 μM of GUDCA at the time of differentiation (0 DIV) and at 2 DIV, to assess the ability of this bile acid to prevent, in the first case, and restore, in the second one, the effects produced by hSOD1G93A transfection. Here, we showed that apoptosis and mitochondrial dysfunction were prevented by GUDCA treatment. Our findings are consistent with previous reports by Vaz and colleagues (2010) where pre-incubation with GUDCA was proven to be anti-apoptotic on rat cultured neurons exposed to UCB and to ameliorate mitochondrial impairment. However, in the present ALS model, GUDCA was not able to restore mitochondrial function or stop the apoptotic events if the damage was already started. Anti-inflammatory properties of GUDC A were demonstrated in astrocytes exposed to UCB by preventing the release of pro-inflammatory cytokines, namely TNF-α and IL-1β (Fernandes *et al.*, 2007). Furthermore, NO production was proven to be reduced in neurons

after GUDCA treatment (Silva *et al.*, 2012). Our results evidence that GUDCA not only prevents the increase of NO but additionally is able to recovering cells from it, demonstrating its ability to delay, at least in some way, inflammation and oxidative related mechanisms, that could worsen motor neuron degeneration. In addition, we show for the first time, that MMP-9 activation is completely abrogated in both conditions. So far, two different clinical trials using UDCA were performed in ALS patients. Parry and colleagues (2010) showed that UDCA was well absorbed and did not evidence severe gastrointestinal adverse effects. Also, they demonstrated that UDCA crosses the blood-brain barrier in a dose-dependent manner. Another study with 80 patients has shown positive outcomes in the rate of disease progression (Min *et al.*, 2012). Therefore, the results obtained in our *in vitro* model of motor neuron degeneration in ALS may provide the basis for further research on GUDCA as a promising therapy to at least slow disease progression in ALS patients.

In the third part of this Thesis, we aimed to establish a model that could explore the role of microglia to the progression of ALS. For that, we first characterized N9 cell line (a microglia cell line) in order to clarify the mechanisms involved in the activation pattern of these cells. To obtain activated microglia, we incubated N9 cells with LPS, known to activate microglia, by changing their morphology and inducing the production of inflammatory-related factors, such as NO and cytokines (Nakajima *et al.*, 2003; Nakamura *et al.*, 1999; Zhao *et al.*, 2011). Therefore, N9 microglial cells were incubated with LPS for 24 hours and morphology, as well as phagocytic and migratory abilities were evaluated. Morphological analysis revealed that N9 cells while ramified in control condition change to an amoeboid morphology when stimulated with LPS. Indeed, LPS is able to induce M1 phenotype of microglia which is characterized by an amoeboid shape (Arimoto and Bing, 2003; Graeber and Streit, 2010). In their surveillance state, microglia have lower expression of surface receptors and lack phagocytic ability. However, when facing a stimulus, microglia rapidly increases the expression of activation markers and became highly phagocytic, in order to avoid the extension of the damage (Kreutzberg, 1996; Streit *et al.*, 1999). Our observations demonstrate that LPS-stimulated microglia present higher phagocytosis than control microglia, confirming that N9 cells evidence the common activation features described for primary microglial cells. Finally, we intended to evaluate N9 microglia migratory properties, a characteristic of functional microglia. Our results evidenced microglia chemotactic ability towards ATP, as demonstrated by others (Davalos *et al.*, 2005; Parkhurst and Gan, 2010). However, we found that, after LPS treatment, microglia lose their capacity to be attracted to ATP. The increased phagocytosis and morphological changes found in our model of microglia activation with LPS can disrupt their migratory capacity, since microglia migration is dependent on extension of processes towards the chemoattractant signal (Parkhurst and Gan, 2010). Furthermore, migration to the lesion sites usually precedes changes in microglia morphology to an activated and amoeboid state, where purines such as ATP can no longer attract microglia and even exert repel on them (Orr *et al.*, 2009). However, migratory properties were maintained, since N9 cells continued to migrate to basal medium, even when treated with LPS, suggesting that this activation of microglia can include changing of surface markers rather than alteration of migratory function.

The characterization of N9 microglia performed in this study allowed us to consider these cells as a useful tool to investigate microglial dynamics in neuroprotection or neurotoxicity in our *in vitro* model

of motor neuron degeneration in ALS. Therefore, the final purpose of the present study was to investigate the role of NSC-34/hSOD1G93A released factors on microglial functions and whether they can induce an activation phenotype, or rather, a dysfunctional state, as a model of ALS progression.

Microglia activation is a common feature in many neurodegenerative disorders and ALS is not an exception (Ransohoff and Perry, 2009). In fact, microgliosis was found in motor cortex, motor nuclei of brainstem, along the corticospinal tract and in ventral horn of spinal cords of ALS patients (Kawamata *et al.*, 1992). Microglia seem to act in progression rather than in the onset of the disease since reduction of mutant SOD1 expression in these cells slow disease progression (Boilleé *et al.*, 2006). However, microglia activation was found to precede motor neuron degeneration in ALS and, consequently, clinical onset (Philips and Robberecht, 2011; Sargsyan *et al.*, 2005). Also, microglia aggregates were found in the ventral horns of pre-symptomatic SOD1 transgenic rats (Graber *et al.*, 2010).

Our first goal was to unravel the ability of N9 microglia to be attracted to the factors produced by degenerating motor neurons. For this, conditioned media collected at 4 DIV either from NSC-34/hSOD1wt or NSC-34/hSOD1G93A cells was used as a chemoattractant. We observed that microglia migratory capacity was reduced in both conditions in comparison to migration to basal medium. These results indicate that not only microglia are not attracted to NSC-34/hSOD1G93A cells, but also it is repealed by their released factors. As already discussed, microglia can be repelled by chemoattractants when activated (Orr *et al.*, 2009) and, so, this could explain the results achieved. Additionally, other putative molecules released by motor neurons can be in the origin of this repulsion.

Finally, we intent to explore microglial dynamics when incubated with NSC-34 conditioned media. Therefore, we established a model of disease progression along NSC-34 differentiation, where the parameters observed at 1 DIV could represent the pre-symptomatic stage and the 4 DIV the symptomatic stage of the disease. First, we evaluated different mechanisms of cell death on N9 cells after exposure to neuronal conditioned media. Our results suggest that necrosis is not related with microglial involvement in disease progression, as this type of cell death was not significantly different after treatment with NCMG93A collected at 4 DIV, when compared to treatment with NCMwt. In addition, we noticed that the differentiation media collected from NSC-34 cells induces increased microglial death when prolonged times of exposure are used. Finally, our experiments evidenced that microglia die by apoptosis when incubated with 4 DIV NCMG93A, although the difference to NCMwt effects was not significant. This can indicate that NSC-34/hSOD1G93A cells released factors can induce toxicity to microglia by specific mechanisms.

We also found that NCMG93A from 4 DIV induces the most dramatic changes in microglia responses, which is consistent with previous findings that attributed a role for microglia in ALS progression (Boilleé *et al.*, 2006; Clement *et al.*, 2003). Moreover, a higher incubation period (24 h) was required to see microglia activation, suggesting that microglia response is dependent on continuous exposure to the factors released by hSOD1 mutated cells. We noticed increased appearance of amoeboid N9 cells after treatment with 4 DIV NCMG93A when compared to their morphology after NCMwt treatment, as well as with conditioned media collected at 1 DIV, demonstrating activation features of M1 phenotype (Graeber and Streit, 2010). However, phagocytic

ability was decreased in that condition. In contrast to what have been evidenced by LPS stimulation, NCMG93A incubation has shown to induce a different activation phenotype with changes in morphology, although without improvement of phagocytic properties in microglia, suggesting that a distinct phenotype was acquired. In our proposed pre-symptomatic stage, microglial phagocytosis is maintained suggesting that, in the beginning, microglia maintains their normal function, altering their activation features along disease progression. This is not unique, as phagocytic microglia have shown to appear adjacent to motor neurons in spinal cord in pre-symptomatic transgenic rat model of ALS (Sanagi *et al.*, 2010).

Together, our results evidence that motor neurons expressing mutant hSOD1 do release factors that activate microglia. Despite of SOD1 accumulation in NSC-34/hSOD1G93A cells, which can indicate decreased capacity to release the mutant protein, one of these factors could be the mutant SOD1 released by those motor neurons. In fact, as demonstrated by Zhao and colleagues (2010), extracellular mutant SOD1 activates microglia with morphological switch to an amoeboid shape and increased production of TNF-α and NO.

In conclusion and as schematically represented in Figure IV.1, our results suggest that NSC-34 cells expressing human mutant SOD1 in G93A are a suitable *in vitro* model to investigate the mechanisms underlying motor neuron degeneration in ALS. We observed features of mitochondrial dysfunction, oxidative stress, energy impairment as well as apoptosis and inflammation-related processes, which are commonly described in transgenic mice models of the disease and in ALS patients. All these mechanisms seem to be interrelated as they appear to be progressive and noticed at similar points during differentiation. Our model can provide not only a better understanding on the basis of these disrupted mechanisms but also address the efficacy of new therapeutic compounds such as GUDCA. Moreover, we show that microglial responses can be modulated differently when incubated with motor neurons conditioned media at different stages of differentiation, suggesting that they indeed may be linked with increased manifestations along disease progression.

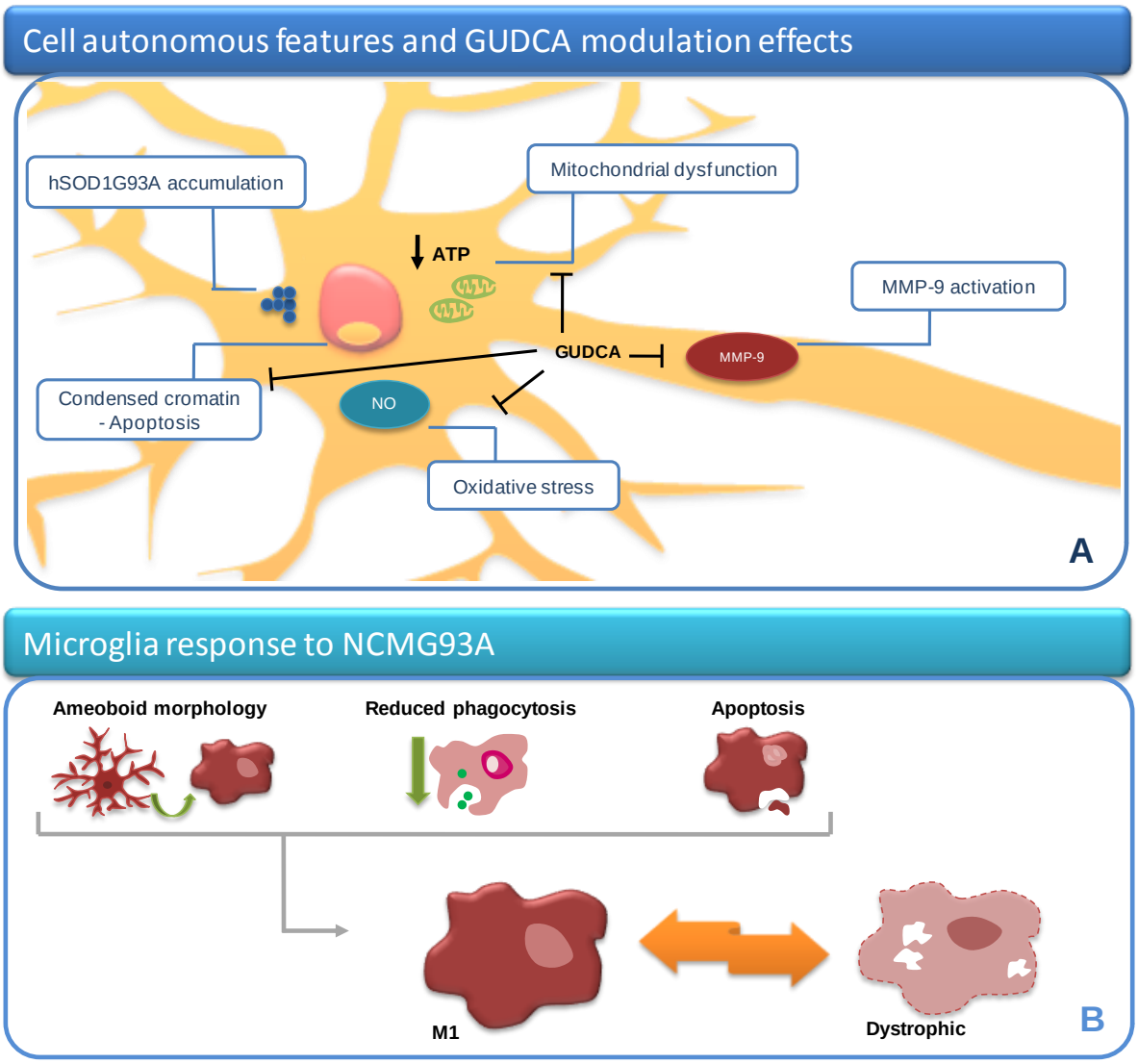

Figure IV.1. Schematic representation of the major findings in the present study. NSC-34 cells evidence mitochondrial dysfunction with decreased ATP efflux, increased oxidative stress related with nitric oxide (NO) production and matrix metalloproteinase-9 (MMP-9) activation, that courses in parallel with human SOD1 mutated in G93A (hSOD1G93A) accumulation. Apoptosis seems to be the preferential mechanism by which NSC-34 expressing SOD1G93A cells die. Moreover, glycoursodeoxycholic acid (GUDCA) is able to ameliorate mitochondrial impairment, apoptosis and release of NO and MMP-9 (A). Microglia exposed to conditioned media obtained from NSC-34 cells expressing SOD1 mutated in G93A (NCMG93A) evidence changes of morphology, from a ramified to an amoeboid shape, reduced phagocytic capacity and increased apoptosis, all markers of microglia activation. Whether microglial response indicates a pro-inflammatory phenotype (M1) or a dysfunctional state requires further investigation (B).

Future perspectives

The present study provides important clues on the molecular features involved in motor neurons degeneration in ALS through an *in vitro* model. Further investigation on the disrupted mechanisms underlying the disease, such as ER stress, altered axonal transport and autophagy, will emphasize the importance of NSC-34 cells transfected with hSOD1G93A as a useful model to the understanding of cellular and molecular mechanisms implicated in ALS pathology, while simultaneously contributing to clarify the pathogenesis of this neurodegenerative disease. Furthermore, this study by contributing, although in a simple manner, to link microglial activation to ALS pathogenesis, as it demonstrates that released factors of NSC-34/hSOD1G93A cells promotes different outcomes in non-transfected microglial responses in comparison with NSC-34/hSOD1wt cells, opens novel research approaches. However, in order to better understand the role of microglia in ALS, it is necessary to evaluate whether microglia obtained from transgenic mice carrying human mutant SOD1 will react differently from the wild type when incubated with conditioned media of NSC-34/hSOD1G93A or NSC-34/hSOD1wt cells. In addition, mixed cultures of motor neurons and microglia can provide further information on the interplay between these cells, an issue still unknown.

Moreover, *in vivo* studies using transgenic mice carrying human SOD1 with G93A mutation will guarantee an integrated understanding of the selective motor neuronal degeneration and the involvement of glial cells, while offering a temporal view of the disease progression. Pre-symptomatic biomarkers and the reasons presiding to the onset and progression of the disease should be dissected, pointing the main players involved in each stage. The final propose of unraveling the processes underlying ALS disease is, obviously, to develop an efficient therapy, cellular- or molecular-targeted. The use of different models will strengthen the protective effects of new compounds to be tested in the treatment of ALS, or at least in slowing disease progression.

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