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Dissecting neuronal dysfunction and microglia/motoneurons cross-talk in ALS: an immunofluorescence directed study

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Resumo

A esclerose Lateral Amiotrófica (ELA) é uma doença neurodegenerativa fatal, afectando de 0.4 a 1.8/100,000 habitantes. Caracteriza-se pela degeneração dos neurónios motores (NM), mas também afeta a microglia. Contudo, a contribuição desta célula na doença não está esclarecida.

Pretendeu-se: (i) explorar os processos de disfunção dos NM na ELA, nomeadamente a dinâmica mitocondrial (fusão/fissão) e transporte axonal (anterógrado/retrógrado), assim como mecanismos de morte celular; (ii) avaliar a contribuição da microglia pelo uso de culturas mistas de NM-microglia; iii) implementar o modelo de culturas organotípicas de medula espinhal (ME) de ratinhos transgénicos para ELA, para avaliar efeitos neuroprotectores pelo ácido glico-ursodeoxicólico (AGUDC).

Utilizaram-se: (i) células NSC-34, uma linha celular de NM, transfectada com superóxido dismutase humana (hSOD1) normal (WT) ou com mutação G93A; (ii) células N9, uma linha celular microglial, em cultura mista com NSC-34 (hSOD1WT ou $hSOD1_{G93A}$); (iii) culturas organotípicas de segmentos lombares de ME de murganhos com 7 dias (SJL-wt) ou transgénicos, contendo a SOD1 humana mutada (TgSOD1-G93A), incubados ou não com AGUDC aos 10 dias-*in-vitro* (DIV). Utilizaram-se técnicas de imunocitoquímica, citometria de fluxo e ensaio fluorimétrico/colorimétrico para o ATP e óxido nítrico (NO), respectivamente.

A viabilidade das células NSC-34/$hSOD1_{G93A}$ e da marcação para a βIII-tubulina diminuiu com a diferenciação. A apoptose (estádios iniciais) e a libertação de NO ($P<0.01$) e ATP ($P<0.05$) aumentou. Verificou-se disfunção da dinâmica mitocondrial por maior fissão ($P<0.05$) e menor fusão ($P<0.01$), diminuindo o transporte axonal retrógrado aos 7 DIV ($P<0.01$). Nas culturas mistas, a microglia aumentou a produção de NO e diminuiu a de ATP ($P<0.05$). As culturas organotípicas de ME foram implementadas e os ensaios com AGUDC sugerem recuperação da viabilidade celular sem alteração nos níveis de NO e ATP.

Uma melhor compreensão da falência celular na ELA e da eficácia do AGUDC podem abrir novas possibilidades terapêuticas para a doença.

Palavras-chave: Degeneração dos neurónios motores, desregulação da dinâmica mitocondrial, disfunção do transporte axonal, apoptose, efeitos neuroprotetores do AGUDC.

Abstract

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease with an incidence rate of 0.4-1.8/100,000 habitants. It is characterized by motoneuron (MN) degeneration, but also affects microglia. However, microglia contribution to ALS is not clarified.

We aimed to: (i) explore the processes leading to MN dysfunction in ALS, namely mitochondrial dynamics (fusion/fission) and axonal transport (anterograde/retrograde) changes, together with cell death mechanisms; (ii) evaluate the role of microglia in the disease by using mixed cultures of mutated MN-microglia; (iii) implement the organotypic culture model from spinal cord (SC) of ALS-transgenic mice to evaluate if the neuroprotective glycoursodeoxycholic acid (GUDCA) would have benefits.

We used as ALS models: (i) NSC-34 cells, a hybrid cell line of neuroblastoma and MN obtained from mouse SC, transfected with human superoxide dismutase 1 (hSOD1) wild type (WT) or with a G93A mutation; (ii) microglial N9 cell line in mixed culture with NSC-34, either with hSOD1WT or $hSOD1_{G93A}$; (iii) lumbar segments of SC from 7-days SJL WT or TgSOD1-G93A (mice), plus or minus GUDCA at 11 days-*in-vitro* (DIV). Immunostaining assays, flow cytometry and fluorimetric/colorimetric assays for ATP and nitric oxide (NO), respectively, were used.

NSC-34/$hSOD1_{G93A}$ cells lose βIII-tubulin and viability along the 7 DIV differentiation, evidencing early apoptotic features, particularly at 4 DIV, and release of NO ($P<0.01$) and ATP ($P<0.05$) at 7 DIV. Alterations in mitochondrial dynamics involved increased fission ($P<0.05$) and decreased fusion ($P<0.01$), decreasing retrograde axonal transport at 7 DIV ($P<0.01$). In mixed cultures, microglia contributed to NO generation while decreasing ATP production ($P<0.05$). We were successful in implementing organotypic cultures from lumbar SC of ALS mice and assays with GUDCA suggest benefits in recovering cell viability without changing NO and ATP.

Better understanding about MN and microglia failure in ALS and GUDCA efficacy may open new therapeutic strategies to the disease.

Keywords: Motoneuron degeneration, mitochondrial dynamics deregulation, axonal transport impairment, apoptosis, GUDCA neuroprotection.

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Abbreviations

ALS – Amyotrophic lateral sclerosis

AMPA - α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

ATP – Adenosine-5'-triphosphate

BBB – Blood-brain barrier

BSA – Bovine serum albumin

CNS – Central nervous system

CSF – Cerebrospinal fluid

CX3CL1 - Chemokine (C-X3-C motif) ligand 1

CX3CR1 - Chemokine (C-X3-C motif) receptor 1

DAPI – 4',6-diamidino-2-phenylindole

DIV – Days in vitro

DMEM - Dulbecco's modified Eagle's medium-Ham's

Drp1 - Dynamin-related protein 1

EAAT2 - Excitatory amino-acid transporter 2

ER – Endoplasmic reticulum

fALS – familial amyotrophic lateral sclerosis

FBS – Fetal bovine serum

FDA - Food and Drug Administration

Fis1 - Fission 1

FUS – Fused in Sarcoma

GFAP - Glial fibrillary acidic protein

GM-CSF - Granulocyte-macrophage colony-stimulating factor

GUDCA - Glycoursodeoxycholic acid

Iba1 – Ionized calcium-binding adapter molecule 1

iPSC - Induced pluripotent stem cell

LHVS - N-morpholinourea-leucine-homophenylalanine-phenyl-vinylsulfone

LMN – Lower motoneurons

LPS - Lipopolysaccharide-binding protein

M-CSF – Macrophage colony-stimulating factor

Mfn1 – Mitofusin-1

Mfn2 – Mitofusin-2

MN – Motoneurons

mSOD1 – Mutant Superoxide dismutase 1

NADPH oxidase - Nicotinamide adenine dinucleotide phosphate-oxidase

NMDA – N-methyl-D-aspartate

NO – Nitric oxide

NOS - Nitric oxide synthase

NOX - NADPH-oxidase

NT - Neurotransmitter

OPA1 - Optic atrophy 1

p38 MAPK - P38 mitogen-activated protein kinases

PAMP - Pathogen-associated molecular pattern

PBS - Phosphate-buffered solution

PDI – Protein disulphide isomerase

PDL - Poly-D-lysine

PNS - Peripheral nervous system

ROS - Reactive oxygen species

RNS - Reactive nitrogen species

sALS – sporadic amyotrophic lateral sclerosis

SC – Spinal cord

SOD1 – Superoxide dismutase 1

TDP-43 - TAR DNA-binding protein 43

TGF - Transforming growth factor

TLR - Toll-like receptor

TNF - Tumor necrosis factor

TARDBP – Transactive Response DNA binding protein

UMN – Upper motoneurons

UPR - Unfolded-protein response

I. Introduction

1. Amyotrophic lateral sclerosis (ALS): basic concepts

Amyotrophic lateral sclerosis (ALS) was initially described by French Jean-Marie Charcot, considered “the father of neurology” that in 1896 related the progressive weakness, muscle atrophy, fasciculation and muscle spasticity with lesions in both white and gray matter of the central nervous system (CNS) (Goetz, 2000). Etymologically, ALS means stiffening (Sclerosis) that begins in nerve cells from one specific side (Lateral) due to skeletal muscle atrophy (Amyotrophic) (Gowing *et al.*, 2008). ALS is described as an adult-onset neurodegenerative progressive disease, which selectively affects lower motoneurons (MN) from the ventral horn of spinal cord (SC) (Mitchell and Borasio, 2007) and brainstem, and upper MN from the motor cortex (D'Ambrosi *et al.*, 2009) **(Figure I.1)**. By affecting MN, this disease causes muscle weakness and fasciculation (twitching muscles) and hyper reflexivity of facial muscles (bulbar onset) or limbs (spinal onset), but also largely spares cognitive ability, sensation and autonomic nervous function (Redler and Dokholyan, 2012). In this pathology, injury in lower MN causes loss of movements in the limbs, neck and body, causing problems of ambulation. On the other hand, injury in upper MN causes difficulty in chewing, talking, swallowing and other quotidian actions. Interestingly, the first symptoms usually appear at a focal site and later spread along contiguous anatomic paths (Redler and Dokholyan, 2012).

In a more advanced state of the disease, the progressive neuromuscular communication failure may culminate in respiratory failure, leading to death (Ferraiuolo *et al.*, 2011). The average survival symptom onset is approximately 1 to 3 years after diagnosis (Gowing *et al.*, 2008); however, there is a small percentage of patients that have a slower disease progression (Wood-Allum and Shaw, 2010).

ALS is the most common adult-onset MN disorder (Redler and Dokholyan, 2012). The worldwide incidence of ALS is 1 to 2 per 100,000 individuals (Ferraiuolo *et al.*, 2011) and has no racial or ethnic prevalence. According to Professor Mamede de Carvalho (a reference in the study of ALS disease in Portugal, and responsible for consultation in Centro Hospitalar Lisboa-Norte - Hospital de Santa Maria), despite the absence of epidemiological studies of ALS in Portugal, it is estimated that

there are 400-500 Portuguese patients with such disease. Worldwide, men seem to be more affected than women, but this may be simply justified by the lack of attendance of female patients in the hospital still occurring in many regions, inclusive in statistical studies (Das *et al.*, 2012).

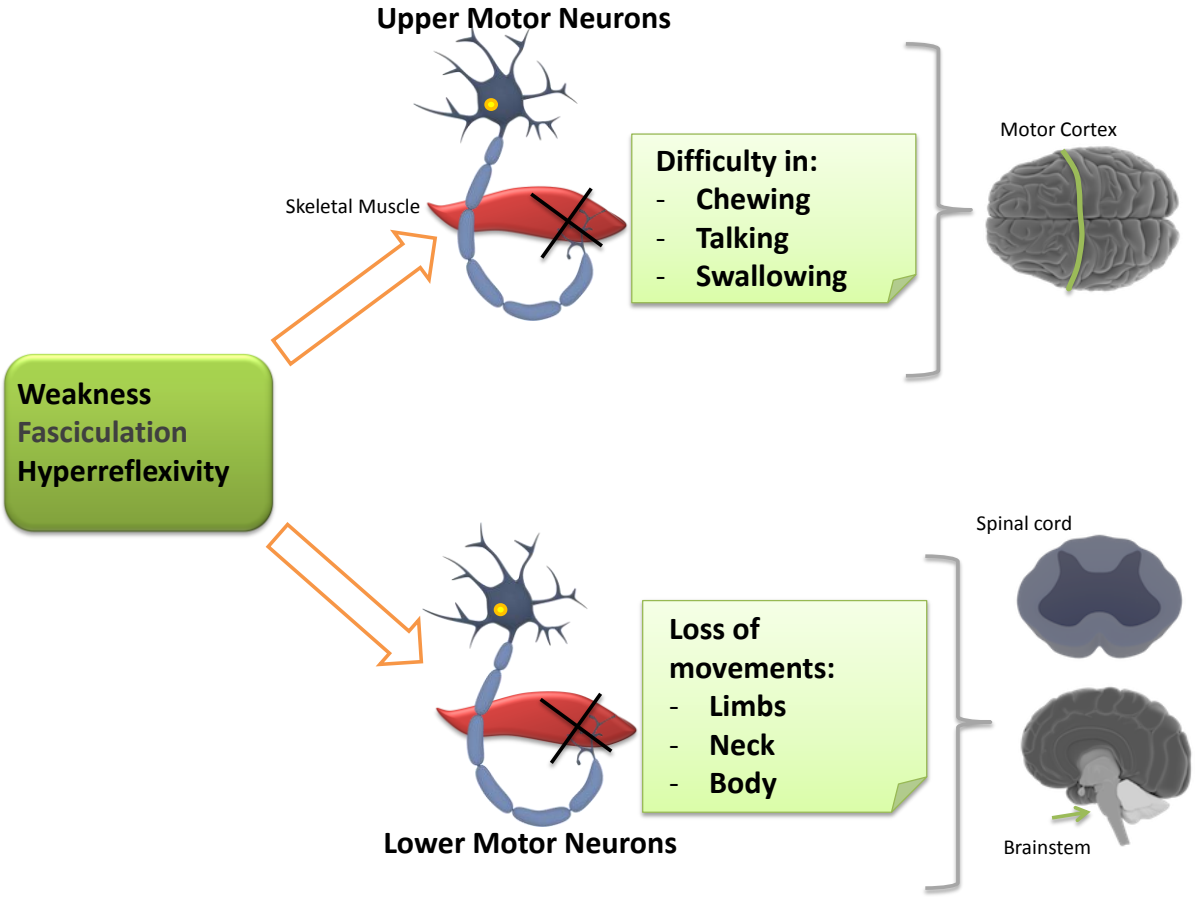


Figure I.1 – Amyotrophic lateral sclerosis (ALS) selectively affects lower motor neurons (MN) from the ventral horn of the spinal cord and brainstem and upper MN from the motor cortex. ALS is characterized by weakness, fasciculation and hyperreflexivity of the muscles. Injury in upper MN causes difficulty in chewing, talking, swallowing and other actions. Injury in lower MN causes loss of movements in the limbs, neck and body, leading to ambulation problems. The disease is characterized by a progressive neuromuscular communication failure that culminates in respiratory failure, leading to death.

1.1 The onset: Several hypotheses and no consensus

Despite the huge importance of the correct diagnosis and subsequent therapeutic strategies, there is still no consensus if ALS begins with dysfunction in the upper or lower MN. According to several authors, there are four types of ALS, taking into account the location where the disorder begins (Gordon *et al.*, 2006; Kiernan *et al.*, 2011). They are: (I) limb-onset ALS, with first symptoms in the limbs from both upper and lower motoneurons (UMN and LMN); (II) bulbar-onset ALS, presenting dysfunction in swallowing with speech and limbs features developing later in the disease; (III) the rarest form of primary lateral sclerosis, with the exclusive involvement of UMN and (IV) progressive muscular atrophy, with the exclusive involvement of LMN.

More recently, some researchers developed the “dying-forward” and “dying-back” hypothesis. In the “dying-forward” hypothesis, ALS is seen as a disorder of corticomotor neurons, which connect with anterior horn cells monosynaptically, mediating anterograde degeneration of anterior horn cells, via glutamate excitotoxicity (Kiernan *et al.*, 2011). In the “dying-back” hypothesis, ALS starts at level of neuromuscular junction (NMJ) or within the muscle cells. This last hypothesis proposes that the cause is a deficiency of a motor neurotrophic hormone normally released by postsynaptic cells and transported by retrograde transport from the presynaptic axon to the soma where it exerts its effects (Kiernan *et al.*, 2011).

Furthermore, recent studies show the involvement of spinocerebellar and sensory pathways and neuronal groups within the substantia nigra and the hippocampal dentate granule layer (Ferraiuolo *et al.*, 2011).

1.2 Genetics and features of the disease

ALS is referred to as a multifactorial disease, apparently having environmental, occupational and toxicological components (Das *et al.*, 2012), as well as evidence of a complex interaction between genetic and molecular pathways. Surprisingly, there are authors suggesting that lifetime of intensive sport or physical activity seems to be a risk factor for ALS (Kiernan *et al.*, 2011).

It is known that ALS may be sporadic (sALS) in about 90-95% of cases, or genetic/familial (fALS) in about 5-10% of cases. However, fALS and sALS are clinically and neuropathologically similar (Gowing *et al.*, 2008) and the only clinical feature that distinguishes fALS from sALS is a lower mean age of onset in the former (Andersen and Al-Chalabi, 2011).

fALS can occur more commonly by an autosomal dominant (Ince *et al.*, 2011), but also by an autosomal recessive or X-linked inheritance and is a polygenetic disease with a variable penetrance (Andersen and Al-Chalabi, 2011).

The most commonly affected gene is SOD1. In 1991, Brown and his group (Massachusetts General Hospital) found that fALS is sometimes linked to chromosome 21q22 (20% of cases of fALS), namely due to an autosomal dominant missense mutation in the SOD1 gene (that encodes cytosolic Cu/Zn superoxide dismutase 1), which is a mitochondrial and cytoplasmic enzyme, essential for the anti-oxidant defenses of the organism, since it is responsible for the detoxification of free radicals produced in the mitochondria, namely superoxide anion.

In recent years, there are described more than 160 mutations in the SOD1 gene (Sabatelli *et al.*, 2013). It is important to mention that the ability of mutant Superoxide Dismutase 1 (mSOD1) to cause neurodegeneration is not linked to a loss of dismutase function (Redler and Dokholyan, 2012). More than affecting the activity of the enzyme, mSOD1 seems to induce a gain of toxic function (Yang *et al.*, 2010) probably related to protein misfolding (Costa *et al.*, 2010), what explains that the knockout mouse SOD1 does not present symptoms of ALS (Reaume *et al.*, 1996). Additionally, conformational instability and misfolding of the SOD1 peptide result in formation of intracellular aggregates, that inhibit normal proteosomic function, disrupting axonal transport systems and vital cellular functions (Kiernan *et al.*, 2011). Recent studies showed that in fALS patients and *in vitro* mSOD1, protein instability and

the increase of aggregation rate are correlated with the decrease of survival time (Byström *et al.*, 2010; Wang *et al.*, 2008).

Another gene that can be mutated in ALS patients is the TARDBP gene (which encodes TAR DNA-binding protein 43 protein, known as TDP-43), a major constituent of the ubiquitinated protein inclusions found in surviving MN in most forms of ALS (Ferraiuolo *et al.*, 2011). TDP-43 is responsible for 4% of fALS cases and 1.5% of sALS cases (Mackenzie *et al.*, 2011) and, under physiological conditions, it functions as an RNA/DNA binding protein, being involved in alternative splicing, transcriptional regulation, mRNA stabilization and microRNA processing (Ince *et al.*, 2011).

Is also described FUS (Fused in Sarcoma), another mutated gene in ALS. FUS is situated in chromosome 16, and encodes a RNA/DNA-binding protein implicated in transcriptional regulation, alternative splicing, microRNA processing and mRNA transport (Ferraiuolo *et al.*, 2011). The FUS gene is mutated in 4% of fALS cases and in less than 1% of the sALS patients (Mackenzie *et al.*, 2010). The inheritance seems to be autosomal dominant (Vance *et al.*, 2009), but mutations have been reported in a large family originating from the Cape Verde islands showing autosomal recessive inheritance of ALS (Kwiatkowski *et al.*, 2009).

1.3 Molecular biology of motoneuron disease

Several cellular pathways have been shown to be dysregulated in tissues of patients and cell models of ALS, which lead to MN damage and death. The sequence of pathogenic events is unclear and most of them are intimately correlated (Costa *et al.*, 2010), forming a complex network that contributes to exacerbate the disease. Atrophy and death of MN, altered RNA processing, mitochondrial dysfunction, glutamate mediated excitotoxicity, protein aggregate formation, endoplasmic reticulum stress, axonal transport dysfunction, oxidative stress and neuroinflammation are some of the pathophysiological phenomena known as biomarkers of ALS **(Figure I.2)**. Mitochondrial function, axonal transport, glutamate homeostasis, oxidative stress and apoptosis will be further discussed in more detail in subsequent sections (1.3.1-1.3.6) due to their relevance for the present thesis. In fact, many of the events can be caused for and consequence of each other and they create a vicious cycle that results in motor axon disruption of neuronal equilibrium, denervation and ultimately MN degeneration in ALS (Ferraiuolo *et al.*, 2011).

Once this pathology is known as a MN disease, it is important to understand why these MN are selectively vulnerable in ALS. First of all, MN are large cells with large axonal compartment and large terminal arbors, which require an exigent metabolic capacity and a robust cytoskeleton and axonal transport efficiency (Ferraiuolo *et al.*, 2011). Moreover, MN have are highly dependent from a normal mitochondrial function, which is the main source of reactive oxygen species (ROS) that can lead to oxidative stress if dysfunctional. These neurons have particular sensitivity to excitotoxicity and dysregulation of intracellular calcium homeostasis since they have a high expression of calcium-permeable α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptors, which lack the GluR2 subunit (Williams *et al.*, 1997). They also evidence reduced expression of calcium-buffering

proteins and high dependence of synaptic glutamate re-uptake transport mechanisms. Other reasons are the reduced capacity for heat shock response and chaperone activity that MN seems to have, leading to defective correction of protein folding, increasing sensitivity to endoplasmic reticulum stress (Saxena *et al.*, 2009) and mitochondria features that predispose the cells to oxidative damage and calcium overload (Panov *et al.*, 2011). Ultimately, this defective protein folding associated with the high expression of particular proteins (e.g. SOD1), and consequently, a high vulnerability to toxicity of mutant proteins (Ferraiuolo *et al.*, 2011) contribute to the accumulation of protein aggregates and death of MN.

In patients carrying mSOD1, it may occurs the upregulation of genes promoting the MN survival during the disease process, principally those encoding phosphatidylinositol 3-kinase and phosphatase and tensin homolog-protein kinase B pathway (Kirby *et al.*, 2011). The understanding of the properties of the neurons that make them more or less resistant to the occurrence of ALS is very important to find strategies to increase defense mechanisms and promote new therapies.

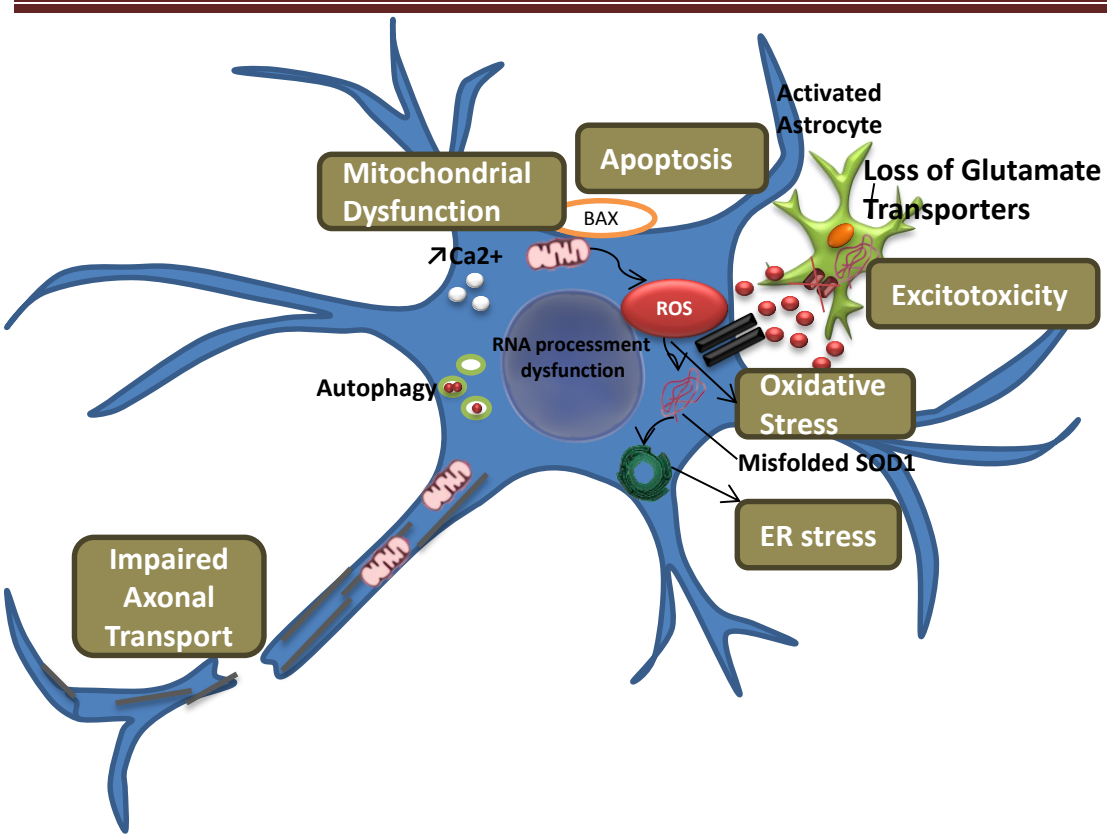


Figure I.2 – Cellular pathways that are compromised in motor neurons (MN) in amyotrophic lateral sclerosis (ALS), leading to neurodegeneration. ALS is a multifactorial disease with a sequence of pathological events that remains to be fully elucidated. Accumulation of extracellular glutamate is one of the features in the pathophysiology of ALS, leading to excitotoxicity. Moreover, dysregulation of RNA processing and the increase of oxidative stress promote the aberrant protein folding of several proteins, including superoxide dismutase-1 (SOD1), which will form aggregates leading to neurodegeneration. In addition, it leads to proteasome impairment and endoplasmic reticulum (ER) stress, that ultimately triggers the activation of autophagy or apoptotic cascades. Dysregulation of calcium homeostasis and mitochondrial impairment are also involved in MN degeneration in ALS, and can also determine the activation of apoptosis. Dysfunction of axonal transport associated with mitochondrial impairment contributes to the energetic depletion of MN. Adapted from Ferraiuolo *et al*. (2011).

1.3.1 Mitochondrial dysfunction

Mitochondria play a central role in the intracellular production of energy, calcium homeostasis and control of apoptosis. The malfunction of this organelle confers pleiotropic effects to the cells, especially to neurons with an elevated susceptibility to aging and stress (Shi *et al.*, 2010a). Mitochondria dysfunction, by swelling and vacuolization, seems to be implicated in ALS (Martin *et al.*, 2007). Hence, the study of the mechanisms underlying these phenomena may contribute to a better knowledge of the disease. In fact, mitochondria failure was indicated to include the disruption of calcium homeostasis and inadequate levels of ATP (Browne *et al.*, 2006), as well as the production of ROS in skeletal muscle biopsies from ALS patients and in mice models carrying the G93A human SOD1 mutation (mSOD1$_{G93A}$ mice). Moreover, the vacuoles are found to be aggregated in the intermembrane space of mitochondria, increasing adhesion to the outer membrane, which leads to selective dysfunction of the organelle and impedes the influx of proteins. These features will lead to a compromise in energy metabolism that will contribute to the dysfunction of MN (Ferraiuolo *et al.*,

2011), together with the activation of caspases, that can trigger apoptotic cell death. In these mSOD1 mice models, calcium buffering is also deficient in mitochondria and enhances the susceptibility of MN to the calcium homeostasis deregulation, that can be associated with glutamate-mediated excitotoxicity and with the activation of pro-oxidant and apoptotic factors such as nitric oxide synthase (NOS), phospholipases and endonucleases. It is important to notice that calcium-buffering ability is particularly deleterious to neurons and skeletal muscle, whose operation requires frequent influx of calcium to generate action potentials (Redler and Dokholyan, 2012).

On the other hand, although most SOD1 is localized in the cytosol, a fraction of mutant SOD1 (mSOD1) is associated with the mitochondria (Vande Velde *et al.*, 2008) and its accumulation seems to exacerbate mitochondrial damage (Ferraiuolo *et al.*, 2011). However, the mechanism that leads to this event is still a matter of debate, although there are some theories: (i) mSOD1 allows the release of cytochrome *c*, activating the apoptotic cascade and opening the pores of the outer membrane of mitochondria (Pasinelli and Brown, 2006); (ii) abnormal interaction of misfolded proteins and oligomers with other mitochondrial proteins can promote mitochondrial damage and apoptosis following associating with Bcl-2, a pro-survival factor (Redler and Dokholyan, 2012); (iii) aggregation of mSOD1 in the outer membrane can result in the disruption of translocation machinery, limiting the input of functional proteins into the organelle (Pasinelli and Brown, 2006); (iv) misfolded and aggregated mSOD1 also accumulate on the cytoplasmic face of the outer mitochondrial membrane and bind directly to the voltage-dependent anion channel, depolarizing the membrane and disrupting the normal functioning of complexes I and IV of the electron transport chain (Costa *et al.*, 2010; Liu *et al.*, 2009).

Other events that deserve our attention are mitochondrial fusion and fission **(Figure I.3 B)**. Mitochondria are actively transported and they can have defined subcellular distributions that can change as necessary. Indeed, this organelle keeps their shape, size, morphology, distribution and physiological function through fusion and fission processes (Shi *et al.*, 2010a). An imbalance of these two opposing events results in excessive mitochondrial fragmentation or elongation (Chan, 2012). Moreover, it is believed that mitochondrial morphology, metabolic function, membrane potential, axonal transport, fission and fusion are highly inter-dependent (Shi *et al.*, 2010a). The main constituents of the fusion machinery in mammalian cells are Mitofusin 1 (Mfn1), Mitofusin 2 (Mfn2) and Optic atrophy 1 (OPA1) **(Figure I.3 C)**. Mfn1 and Mfn2, localized in the mitochondrial outer membrane, belong to GTPase family and their depletion leads to loss of mitochondria fusion, high fragmentation, no mitochondrial tubules and decreased cellular respiration (Chen *et al.*, 2005). Moreover, in humans, mutations in Mfn2 cause Charcot-Marie-Tooth neuropathy type 2A (Zuchner *et al.*, 2004), a disease of the group of peripheral neuropathies with symptoms such as distal muscle weakness and atrophy, less severe sensory loss, and depressed tendon reflexes (Ranieri *et al.*, 2013). OPA1 is a dynamin family GTPase and localizes within the mitochondrial intermembrane space and mutations cause the most common form of hereditary optic atrophy (Alexander *et al.*, 2000).

Dynamin-related protein 1 and fission 1 (Drp1 and Fis1, respectively) are the components of mitochondrial fission machinery in mammals. Dominant-negative mutants of Drp1 inhibit mitochondrial

division and result in highly interconnected mitochondrial tubules (Smirnova *et al.*, 2001). Overexpression of Fis1 leads to mitochondrial fragmentation, release of cytochrome *c* and, therefore, apoptosis (James *et al.*, 2003). It is highly suggestive that mitochondrial fusion and fission may be influenced in the presence of mSOD1, causing disturbances at the level of mitochondrial dynamics, which are linked to disorders such as the Alzheimer's disease (Shi *et al.*, 2010a).

1.3.2 Glutamate mediated excitotoxicity

Glutamate is the major excitatory neurotransmitter (NT) in the CNS and its signal is ended by its removal from the synaptic cleft by transporters such as EAAT2 (Excitatory amino-acid transporter 2), which is mainly expressed by astrocytes (Maragakis *et al.*, 2004).

There are three groups of glutamate receptors in postsynaptic neurons essential to the physiological neurotransmission. These receptors can be divided into metabotropic and ionotropic. Metabotropic receptors are G protein-coupled and operate through signal transduction cascades. Ionotropic receptors act as ion channels and are divided into three groups: N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and Kainate receptors. NMDA receptors are stimulated by calcium and sodium symport entry, and non-NMDA receptors (generic designation for AMPA/Kainate receptors) are traditionally seen as mainly permeable to monovalent ions such as Na^+ and K^+ (Agrawal and Fehlings, 1997). The calcium permeability of AMPA receptors is broadly determined by the GluR2 subunit, responsible to making the receptor impermeable to calcium, which is extremely important in preventing glutamate excitotoxicity (Ferraiuolo *et al.*, 2011).

Excitotoxicity is a neuronal injury which can then result from the excessive activation of glutamate receptors, AMPA and NMDA, and may be caused by increased levels of glutamate in the synaptic cleft or by the increased sensitivity of the postsynaptic neurons to this NT, leading, in both situations, to an increase in intracellular calcium (Ferraiuolo *et al.*, 2011). Disruption of intracellular calcium homeostasis, with secondary activation of proteolytic enzyme systems and generation of ROS, disruption of mitochondrial function, production of ATP, promotion of transcription factors of pro-apoptotic genes or suppression of anti-apoptotic genes are key components of excitotoxicity (Ferraiuolo *et al.*, 2011) that leads to neuronal death.

In fALS and sALS patients, as well as in mutant SOD1 mice models, there are decreased levels of functional EAAT2 protein and increased circulating glutamate in the cerebrospinal fluid (CSF) (Howland *et al.*, 2002). Although the precise mechanism(s) by which EAAT2 is down-regulated in ALS are not yet understood, it is known that this gene deletion induces progressive neurodegeneration, while its overexpression was shown to delay symptom onset in ALS mouse models (Rothstein *et al.*, 2005). EAAT2 is indirectly affected when other associated processes suffer from some dysfunction, suggesting that excitotoxicity may be a secondary event in ALS pathogenesis. Indeed, when caspase-3 is activated, it results in a truncated or inactive version of EAAT2. Oxidative damage to the C-terminus of EAAT2 diminishes its ability to transport glutamate (Redler and Dokholyan, 2012). In addition, prolonged hyperstimulation by glutamate induces cell death by allowing persistent calcium

influx through the AMPA receptors, which are specifically abundant in MN (Van Den Bosch *et al.*, 2000).

It is noteworthy to mention that blocking the excitotoxic effects of extracellular glutamate is the only strategy approved by U.S. Food and Drug Administration (FDA) that has shown to be able to slow the ALS progression. This is the case of riluzole, a benzothiazole derivative, that has several effects, including the inhibition of the excitotoxic stress in neurons by slowing glutamate release (due to the inactivation of voltage-dependent Na^+ channels on glutamatergic nerve terminals), as well as the activation of a G-protein dependent signal transduction process. Moreover, riluzole seems to be able to block some of the postsynaptic effects of glutamate by non-competitive inhibition of NMDA and AMPA receptors (Van Den Bosch *et al.*, 2006; Vucic *et al.*, 2013), which showed to cause an increase in patient survival but only for few months.

1.3.3 Axonal transport dysfunction

Axon is a long and slender projection of the neuron that conducts electrical impulses and all the molecules that need to be transported (Shi *et al.*, 2010b). Since the genetic material and the majority of the protein synthesis machinery are localized to the cell body, it is necessary to exist a way to transport all materials (generically known as cargo) from the cell body to the axon terminal, and from axon terminal to cell body. The microtubules serve as rails, along the entire axon and secretory vesicles are transported to sites of release through the action of microtubule-based motor proteins. In neurons, these transport processes are collectively known as axonal transport (Siegel *et al.*, 2006).

Growth and maintenance of neuronal processes requires timely, efficient delivery of material to axonal and dendritic domains. For this, there are the anterograde and retrograde transports. The first occurs from cellular body to axon, mediated by kinesin molecular motor protein and the second occurs from axon to cellular body, mediated by dynein molecular motor protein (Ferraiuolo *et al.*, 2011).

However, sometimes the axonal transport does not work properly. In ALS, this dysfunction is described mainly due to the formation of neurofilament aggregates, which causes disruption of axonal transport that combined with mitochondrial dysfunction causes energetic depletion of distal axonal compartment of MN, thus leading to degeneration (Ikenaka *et al.*, 2012) **(Figure I.3 A)**. The disruption can occur at anterograde or retrograde level, or simultaneously in both as a consequence of decreased mobility of motor proteins or decreased binding of cargos to these motor proteins. The three main cargos indicated as biomarkers of MN degeneration by accumulation in distal axon compartment are neurofilaments, mitochondria and autophagosomes. The neurofilaments set the diameter of the axons, and its aggregation (by phosphorylation or stoichiometric imbalance) is pathological. Kinesin or dynein dysfunction leads to accumulation of neurofilamentous swellings (spheroids), as in the KIF5A and in the dynactin-1 mutant mice (Ikenaka *et al.*, 2012; King *et al.*, 2011), which are two models used in the study of axonal transport dysfunction since they have mutations commonly linked to the dysfunction of anterograde and retrograde transport, respectively.

The disruption of the anterograde and retrograde transport (Hirokawa *et al.*, 2010), leads to mitochondrial accumulation in a certain region of the cell, leading to energy depletion elsewhere, which can result in cell death (Ikenaka *et al.*, 2012).

Finally, it is known that the lysosome-autophagosome pathway is responsible by recycling intracellular compounds; therefore, its dysfunction may also cause neurodegeneration. Since this cargo is transported bi-directionally along microtubules, alterations in both types of transport will cause the accumulation of autophagosomes (Ikenaka *et al.*, 2012).

A deficiency of motor proteins associated with axonal transport can occur due to chronic exposure to neurotoxins, such as acrylamide, which has been described as being able to directly inhibit the function of kinesin, therefore the anterograde transport (Sickles *et al.*, 2002). Thus, for example, mutation with loss of function in KIF5A (kinesin subunit) causes a deficiency in binding of Kinesin I to microtubules, leading to failure of anterograde transport (Ikenaka *et al.*, 2012).

There is also evidence that mutations in SOD1 such as A4V, G85R and G93A, promotes SOD1 interaction with the complex dynein-dynactin in cell cultures and in affected tissues of ALS mice (Shi *et al.*, 2010b). The same authors suggest that mSOD1 and dynein interaction play a key role in the formation of large inclusions containing mSOD1. In the mSOD1 mice model, the impairment of axonal transport occurs at an early stage of the disease. The mechanisms behind the dysfunction in this model are still unknown, but appear to derive from an increase in tumor necrosis factors (TNFs), which is observed in mSOD1 mice, leading to the disruption of kinesin function, by a mechanism that involves the activation of p38 MAPK pathway, which has been observed in models of ALS(Shi *et al.*, 2010b).

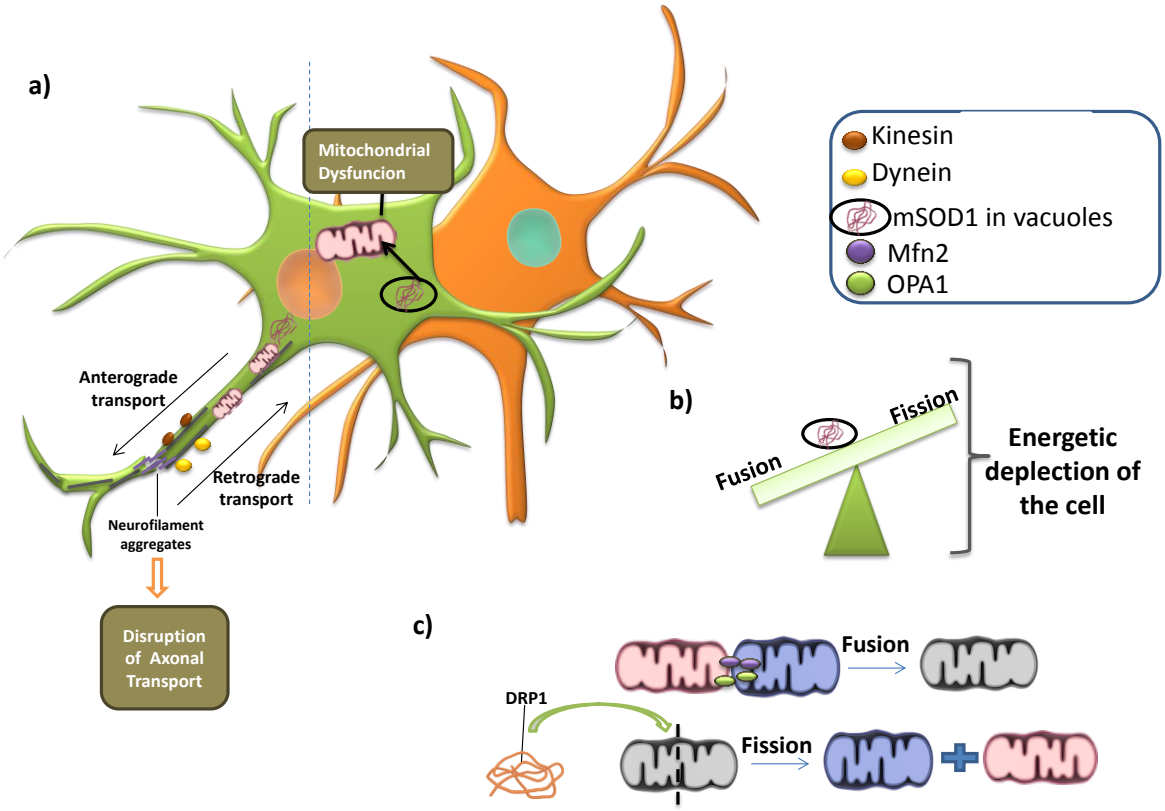


Figure I.3 – Axonal transport and mitochondrial impairment in amyotrophic lateral sclerosis (ALS).
(a) Kinesin and dynein dysfunction lead to the accumulation of neurofilaments, which causes impairment of axonal transport. Combined with mitochondrial dysfunction by accumulation of mSOD1 and consequent decrease in the influx of proteins, the transport axonal causes the energetic depletion of the cell, leading to neurodegeneration. In ALS, abnormal activation of protein kinases may cause aberrant patterns of kinesin/dynein phosphorylation, leading to its inhibition (Morfini et al., 2013). (b) It is not yet well established the influence of mutated Superoxide Dismutase 1 (mSOD1) in these events although it is proposed the existence of imbalance between fusion and fission events, resulting in mitochondrial fragmentation or elongation contributing to the energetic depletion of the cell. (c) Mitofusin 2 (Mnf2) and Optic Atrophy 1 (Opa1) are proteins responsible by mitochondrial fusion and Dynamin related protein 1 (Drp1) by the mitochondrial fission.

1.3.4 Oxidative stress

Although the production of ROS is a normal physiological event of human metabolism and have important biological functions, slight imbalances can cause oxidative stress. According to Packer and Cadenas (2007), oxidative stress consists in a disturbance of redox signaling and control that recognizes the occurrence of compartmentalized cellular redox circuits whereby ROS and reactive nitrogen species (RNS) levels tend to increase. These species include hydrogen peroxide, superoxide and hydroxyl radicals, peroxynitrite (Costa *et al.*, 2010) and nitric oxide (NO), among others. ROS and RNS are involved in several cellular functions (Dodson *et al.*, 2013). Although, in normal conditions these species play important functions, they can cause severe damage when at higher levels.

Oxidative stress is also a common feature in ALS and causes structural damage and changes in redox-sensitive signaling. Moreover, the role of oxidative stress in ALS aroused great interest because

mutations in SOD1, which encodes a major antioxidant protein, account for 20% of fALS cases (Silva *et al.*, 2011). There is also a large body of evidence of oxidative stress in sALS and fALS, as indicated by the increase of 3-nitrotyrosine levels, considered a marker of oxidative stress resultant from the elevation of peroxynitrites (Costa *et al.*, 2010).

Several studies showed that oxidative stress interacts with other pathophysiological processes that contribute to MN disease, including excitotoxicity (Rao and Weiss, 2004), mitochondrial dysfunction (Duffy *et al.*, 2011), protein aggregation (Wood *et al.*, 2003), stress of ER (Kanekura *et al.*, 2009) and changes in signaling from microglia and astrocytes (Blackburn *et al.*, 2009; Sargsyan *et al.*, 2005). Therefore, an effective reduction of oxidative stress may improve some aspects of the pathophysiology of MN degeneration. However, therapeutics directed to the regulation of the oxidative stress have not been yet effective in humans, although samples of CSF, serum and urine of ALS patients evidence markers of free radical damage (Mitsumoto *et al.*, 2008). In addition, postmortem tissue from sALS and mSOD1-related fALS cases also present elevated levels of oxidative damage to proteins, lipids and DNA (Ferraiuolo *et al.*, 2011). Some mRNA species appear to have increased susceptibility to oxidation, such as those involved in the mitochondrial electron transport chain, protein biosynthesis, folding and degradation pathways, myelination, cytoskeleton proteins, and the tricarboxylic acid cycle and glycolysis pathways (Chang *et al.*, 2008). Also mSOD1 seems to be particularly susceptible to oxidative translation modification.

In cellular models of mutant TAR DNA-binding protein 43 (TDP-43)-related ALS, the presence of this mutant protein has shown to induce oxidative stress in MN cell lines (Duan *et al.*, 2010). Finally, in other nerve cells, namely microglia, mSOD1 seems to increase NADPH oxidase (NOX)-mediated superoxide production, resulting in prolongation of ROS production (Harraz *et al.*, 2008). It was observed an increase in NOX2 expression in mSOD1 mice and in CNS of ALS patients. It seems that in mSOD1 models, as well as in CNS of ALS patients, there is a dysregulation of the erythroid 2-related factor 2 (NRF-2), which is the main regulator of the antioxidant response (Sarlette *et al.*, 2008).

It is import to note that the CNS is extremely sensitive to oxidative stress, since it has a reduced expression of antioxidant enzymes, high levels of easily oxidized substrates and high production of ROS by neurochemical reactions (Carri *et al.*, 2003).

1.3.5 Endoplasmic reticulum stress

Intracellular inclusions related to accumulation of misfolded or unfolded proteins in aggregates are hallmarks of several neurodegenerative diseases, including ALS (Vijayalakshmi *et al.*, 2011).

These events, together with oxidative stress and loss of calcium homeostasis (Rao et al., 2004a; Rao et al., 2004b), induce the ER stress. ER is an organelle responsible for maintaining cellular calcium homeostasis and synthesize/regulate the synthesis and the folding of proteins. For this, ER has resident chaperones that recognize aberrant proteins and correct their folding. This is crucial, since non-functional proteins can cause suppression of general translation and ER-associated protein degradation (Ferraiuolo *et al.*, 2011). Initially, this mechanism is cytoprotective but a prolonged activation can lead to apoptosis (Yamagishi *et al.*, 2007). According to some studies, the protein

disulphide isomerase (PDI), an unfolded-protein response (UPR) chaperone existing in ER, is activated in mSOD1 mice, where it co-localizes with mSOD1 inclusions, and in samples from sALS patients (Atkin *et al.*, 2006; Atkin *et al.*, 2008). It is suggested that ER stress is involved in the early stages of MN injury, once PDI and other UPR-induced proteins are up regulated before the disease onset in mSOD1 rodents (Atkin *et al.*, 2008). Up-regulated markers of ER stress, such as PDI, are also present in the CSF and SC of postmortem samples of ALS patients (Atkin *et al.*, 2008; Sasaki, 2010).

Interestingly, the exposure of NSC-34 cells, an hybrid cell line produced by fusion of neuroblastoma with mouse MN-enriched primary SC cells and primary spinal MN, to CSF from ALS patients led to ER stress, including expression of ER fragmentation, UPR markers and activation of caspase-12 (Vijayalakshmi *et al.*, 2011). However, it was not possible to identify the CSF constituents that are responsible for such changes.

UPR activation seems to be cytoprotective, at least in the initial phases of cellular stress. Nevertheless, an increase in survival lacking a key UPR transcription factor accompanied by increased activation of ER-associated protein degradation, enhanced autophagy and decreased mSOD1 aggregation were observed in the mSOD1 mice model (Hetz *et al.*, 2009).

1.3.6 Cell death

Apoptosis is described as the process of programmed cell death which involves a number of morphological changes such as decrease in cell volume, nucleus fragmentation, chromatin condensation and formation of small apoptotic bodies that are absorbed by the adjacent cells **(Figure I.4 A)**. The main purpose is to eliminate the damaged cell without compromising the neighboring cells and this cell fate can be derived from two different situations: (i) lack of activation of survival signals (trophic factors); (ii) induction by specific signals (Alberts *et al.*, 2008).

Programmed cell death has been one of the key areas of research in ALS in recent years (Muyderman *et al.*, 2009). Therefore, there is well-known evidence that events very similar to apoptotic mechanisms are involved in the degeneration of MN (Martin, 1999; Sathasivam and Shaw, 2005). Moreover, similar events have been detected well before the onset of symptoms in the $hSOD1_{G93A}$ mice (Kong and Xu, 1998). Furthermore, there is a massive transient increase in mitochondrial vacuoles at the onset of symptoms (Bendotti *et al.*, 2001; Kong and Xu, 1998), indicating a direct link between mitochondrial viability and MN degeneration in this disease (Takeuchi *et al.*, 2002). To corroborate these studies, overexpression of Bcl-2, an anti-apoptotic protein, extended the survival rate of mSOD1 mice, with partial rescue of MN damage (Pasinelli *et al.*, 2004; Vukosavic *et al.*, 2000).

Interestingly, recent evidence has shown that at least two different SOD1 mutations interact with Derlin-1, a key component of the cellular machinery responsible for eliminating proteins that do not fold correctly in the ER, triggering ER stress and apoptosis signal regulating kinase-1 induced cell death (Nishitoh *et al.*, 2008).

Although not a mechanism of death broadly described in ALS, necrosis is also a cell death pathway **(Figure I.4 B)**. However, unlike apoptosis, is not programmed and results in cellular injury. In this case, the cells swell to break, releasing intracellular components, what can cause inflammation (Alberts *et al.*, 2008). The $SOD1_{G93A}$ mice model is the one that has features of both apoptosis and necrosis, with ‘‘necrotic-like’’ and ‘‘apoptotic-like’’ processes dominating in different cell types and/or disease stages (Martin *et al.*, 1998; Martin *et al.*, 2007).

Finally, autophagy **(Figure I.4 C)** is a catabolic fundamental process of degradation for macromolecules and organelles, crucial for cell and tissue homeostasis (Birgisdottir *et al.*, 2013) and lysosome-dependent (Klionsky, 2007; Levine and Klionsky, 2004). Thus, this is an essential process for cell survival (Kim *et al.*, 2013). The impairment of this mechanism is implicated in chronic neurodegenerative diseases such as Alzheimer’s disease, Huntington’s disease and Parkinson’s disease (Meredith *et al.*, 2002; Nixon, 2007; Nixon *et al.*, 2005; Ravikumar *et al.*, 2004). Although the boosting of autophagy seems to retard the progression of the disease by promoting the removal of misfolded proteins it is still unclear whether autophagy activation would be beneficial or detrimental in ALS (Song *et al.*, 2012). Mutations that disrupt autophagy (as CHMP2B mutation) are found in some ALS cases, supporting the hypothesis of autophagy with a protective role in ALS (Cox *et al.*, 2010; Parkinson *et al.*, 2006). By other hand, post-mortem studies and work in animal models have shown that the number of autophagosomes is increased in SC affected by ALS (Morimoto *et al.*, 2007; Sasaki, 2011). Moreover, whether the accumulation of autophagosomes signifies decreased or increased autophagic flux has yet to be established. Furthermore, reported that treatment with lithium known to activate autophagy exacerbates ALS progression in mice models (Pizzasegola *et al.*, 2009). Trials using rapamycin as an autophagy inducer, also revealed disease progression in ALS mice (Zhang *et al.*, 2011). Controversially, other groups have reported the opposite effects with lithium and rapamycin in the same model (Fornai *et al.*, 2008; Wang *et al.*, 2012).

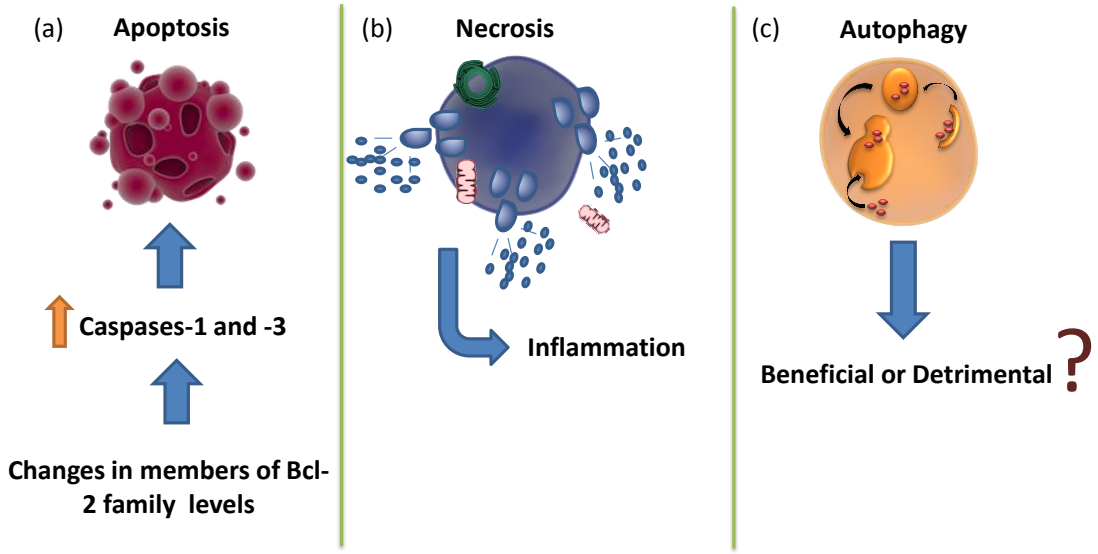


Figure I.4 – Mechanisms of cell death in amyotrophic lateral sclerosis (ALS). (a) Changes in the levels of the Bcl-2 family members of oncoproteins result in a predisposition to apoptosis, with an increase of the activation of caspase-1 and -3 and the affected motor neurons exhibit morphological features reminiscent of apoptosis. (b) Necrosis or necrosis-like, is observed in the mutant superoxide dismutase-1 (mSOD1G93A) models but it is not broadly described in ALS, although this process can trigger inflammatory features as described in ALS. (c) Autophagic activation is not well established as beneficial or detrimental in the context of the disease.

1.4. ALS is a non-cell autonomous disease: the role of glial cells

In spite of some reports defending that neuron-specific expression of mSOD1 is enough to induce ALS in transgenic mice (Jaarsma *et al.*, 2008), there is ample evidence that death of the MN is non-cell autonomous and depends on the contribution of the surrounding, glial cells such as astrocytes, oligodendrocytes, Schwann cells and microglia. Moreover, glial cells not only react to neuronal lesion, but also have a key role in the pathogenesis of the disease and are targets of hallmark cellular processes (Ince *et al.*, 2011) **(Figure I.5)**.

Gliosis, a nonspecific reactive change of glial cells in response to damage to the CNS, is an early pathologic characteristic of ALS in both SC and brain (King *et al.*, 2011). In fact, activation of microglia, astrocytes and appearance of lymphocytes is reported in the SC of SOD1 transgenic mice (Beers *et al.*, 2006; Kawamata *et al.*, 1992; Liu *et al.*, 2009; Xiao *et al.*, 2007). Furthermore, studies with chimeric mice, where the expression of mutation G93A in SOD1 was selectively induced on MN, astrocytes or microglia, have demonstrated that $mSOD1_{G93A}$-overexpressing neurons surrounded by healthy glia remained relatively intact; in contrast, healthy MN presented signs of injury when surrounded by mutant $SOD1_{G93A}$ - overexpressing glia (Clement *et al.*, 2003). One of the theories that accompany the study of this disease is that the onset is marked by protein aggregates in MN and the progression and duration are determined by the crosstalk of neighboring cells (Philips and Robberecht, 2011). Curiously, studies of Ilieva and collaborators (2009) have demonstrated that mSOD1 expression in MN determines the initial timing of disease onset and early progression in some cases, but does not have a significant contribution to later disease progression, Therefore, astrocytes and microglia seem to play a crucial role for disease progression after its onset. In addition, mSOD1

gene excision from microglia and selective reduction in astrocytes significantly slowed disease progression (Boillee *et al.*, 2006; Yamanaka *et al.*, 2008).

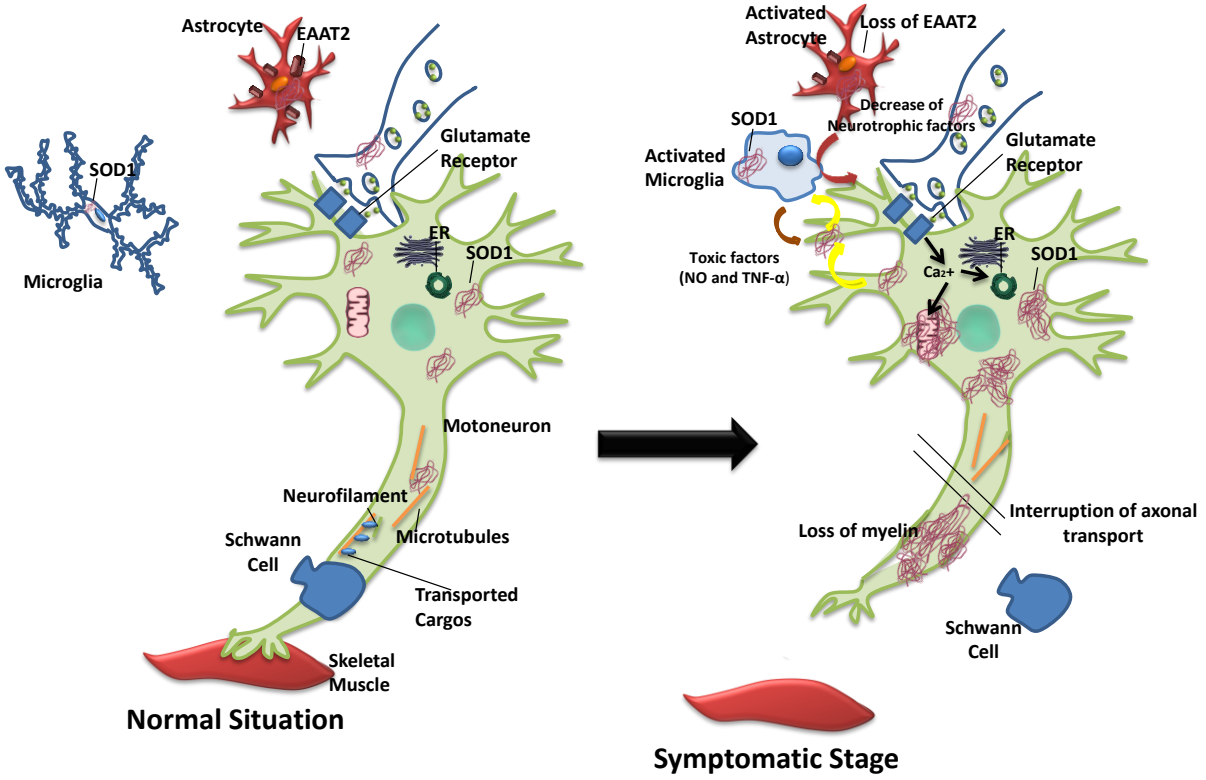


Figure I.5 – Motoneurons in amyotrophic lateral sclerosis (ALS) and the Influence of non-neuronal neighbors. Homeostatic conditions of the motor neurons are ensured by non-neuronal cells, such as astrocytes, microglia and oligodendrocytes/Schwann cells. In the symptomatic stages of ALS, there is an amplification of the initial damage that culminates in disease progression and spread. Astrocytes have shown to decrease the release of neurotrophic factors and to disturb the glutamate metabolism due to the decrease of the excitatory amino-acid transporter EAAT2. Consequently, the imbalance of Ca^{2+} will promote the reticular and mitochondrial stress, together with the ubiquitous superoxide dismutase-1 (SOD1) aggregates. After activation, microglia migrates to the injury and release toxic factors such as nitric oxide (NO) and tumor necrosis factor alpha (TNF-α), potentially triggering neuroinflammation. Moreover, myelin production by oligodendrocytes or Schwann cells is lost and the axon length decrease. The interruption of the connection to muscle and axonal transport are also indicated. Adapted from Boillée *et al*. (2006).

1.4.1. Oligodendrocytes and Schwann cells

Oligodendrocytes in CNS and Schwann cells in the peripheral nervous system are glial cells of ectodermal origin responsible for the formation and maintenance of myelin sheath in axons, thus catalyzing the electrical impulse conduction (Siegel *et al.*, 2006). Schwann cells are closely associated with MN axons, and participate in axonal development and regeneration. Until now, there are a few reports suggesting that these glial cells have a role on ALS pathogenesis. However, loss of compact myelin and lamellae detachment, indicators of myelin abnormalities, were observed in SC of pre-symptomatic SOD1 transgenic rats and aggravated at symptomatic stages (Lasiene and Yamanaka, 2011). Moreover, more pronounced marks of morphological myelin degeneration were observed in fully symptomatic stages of mSOD1 rats (Niebroj-Dobosz *et al.*, 2007).

In what concerns Schwann cells, there is still some controversy. Interestingly, and in opposite to what happens in other non-neuronal cells, the elimination of mSOD1G37R specifically in Schwann cells, not only failed to slow disease progression, but also promoted a substantial acceleration of the late phase of the disease (Lobsiger *et al.*, 2009). The underlying mechanism suggests a protective role to mSOD1 which is 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 mSOD1 in Schwann cells of mSODG85R transgenic mice delayed disease onset and extended survival indicating that mSOD1G85R expression is neurotoxic.

1.4.2 Astrocytes

Astrocytes, also known collectively as astroglia, consist in star-shaped glial cells (Siegel *et al*., 2006). They are the most abundant cells of the CNS and have an important role in the support of neurons, regulation/reuptake of neurotransmitters and ion concentrations in the extracellular space, neurovascular coupling, and maintenance of a normal blood brain barrier (BBB) and in the tripartite synapse (Fellin, 2009; Perea *et al.*, 2009; Volterra and Meldolesi, 2005).

Pathological studies have reported that the death of MN in ALS is accompanied by astrogliosis, characterized by up-regulation of glial fibrillary acidic protein (GFAP) in dorsal and ventral grey matter and in antero-lateral white matter (Ince *et al.*, 2011). It has been also indicated that the development of astrogliosis in some models occurs in pre-symptomatic phases, although it varies depending on the mutation (Vargas *et al.*, 2008).

Astrocytes are essential for the removal of glutamate from the synaptic cleft, mainly through EAAT2, which has reduced levels in sALS and fALS patients (Van Damme *et al.*, 2007). In addition, astrocytes expressing mSOD1 have shown to be more toxic than normal reactive astrocytes (Hovden *et al.*, 2013; Pehar *et al.*, 2004) and to release an insufficient amount of neurotrophic factors crucial to the neuronal balance, such as glial-derived factor, brain derived neurotrophic factor, cilliary neurotrophic factor and vascular endothelial growth factor (Dewil *et al.*, 2007; Ekestern, 2004). Moreover, astrocytes have demonstrated a disturbance of glutamate metabolism in chick SC astroglial

cultures, probably due to the up-regulation of metabotropic glutamate receptors (Anneser *et al.*, 2004), which can be the cause of the excitotoxicity in these models.

Also, astrocytes respond to toxic factors in the CSF of ALS patients by undergoing morphological transformation from flat to process bearing and elevated expression of GFAP, as well as S100A6 and S100B proteins (Shobha *et al.*, 2010). Recently, a subpopulation of astrocytes from SC of symptomatic $mSOD1_{G93A}$ rats has revealed a unique pattern of astrocytic markers, aberrant phenotypic features and an increased proliferation rate (Diaz-Amarilla *et al.*, 2011) that may represent an unknown astrocytic phenotype associated with later stages of ALS progression.

1.4.3 Microglia

In 1932, Pio del Rio-Hortega introduced the concept of microglia, the lower glial cells, as a defined cellular element of the CNS. The postulate of del Rio-Hortega states the following: 1) Microglia penetrates the brain during early development; 2) When they invade the brain, cells have amoeboid morphology and have a mesodermal origin; 3) Microglia uses vessels and white matter tracts as guiding structures for migration and enter all brain regions; 4) Here, they transform into a branched, ramified morphological phenotype in the more mature brain (known today as the resting phenotype); 5) In the mature brain, they are found almost always dispersed; 6) Each cell seems to have a defined territory; 7) In pathological situation, these cells undergo a transformation; 8) Cell that suffers this transformation acquire amoeboid morphology similar to the one observed early in development; 9) These cells have the capacity to migrate, proliferate and phagocytize.

Surprisingly, all of these 9 statements are perfectly valid today (Kettenmann *et al.*, 2011). The consensus is general: Microglial cells are the resident macrophages of the CNS (Evans *et al.*, 2013), derived from progenitors that have migrated from the periphery and are from mesodermal/mesenchymal origin (Chan *et al.*, 2007). Moreover, although microglia are a variety of macrophage, their appearance are absolutely different (Kettenmann et al., 2011).

In rodents, microglia differentiate from a primitive macrophage population produced by the yolk sac (Alliot *et al.*, 1999; Ginhoux *et al.*, 2010). Then, in colonization of the brain, its recruitment and differentiation are suggested to occurring in hematopoietic waves during the embryonic and postnatal periods (about 10 days in rodents and in the early part of the second trimester in human) (Chan *et al.*, 2007). In the adult animals there is very little exchange between blood and brain parenchyma. However, it was reported that after BBB damage, a subpopulation of monocytes can enter the brain and transform into microglia (Mildner *et al.*, 2007). Moreover, in healthy conditions of intact brain, the microglial cells exist as a stable population and may derive from two different sources: one, is derived from progenitors that are of myeloid/mesenchymal origin, but not necessarily monocytes; and the second population which represents a developmental and transitory form of fetal macrophage (Rezaie *et al.*, 2005).

In ALS, the injury by the accumulated debris, excessive aberrant protein and neurodegeneration (Harry, 2013), triggers activation of resident microglia into a phagocytic phenotype trough a strictly regulated process. After this activation, microglia migrates to the local of lesion. Here, microglia act as the first and main form of active immune defense in the CNS. For example, when activated, microglia is scavenging the CNS for damaged neurons, infectious agents and others threats (Gehrmann *et al.*, 1995).

Due to the interest of microglial cells in the ALS context, this topic will be further explored in the next chapter.

1.5 Neuroinflammation: The Breaking Point

Another hallmark of ALS is the neuroinflammation **(Figure I.6)**, which involves glial activation and infiltration of peripheral immune cells (Papadimitriou *et al.*, 2010).

In spite of the main goal of neuroinflammation to constraint the damage that can have different origins, activated microglia are capable to release cytotoxic substances such as H_2O_2 and NO. These compounds can damage neighbor cells and lead to neuronal death. Over activated microglia can also secrete proteases that catabolize specific proteins that cause cellular damage, such as TNF-α, while cytokines such as IL-1 promote demyelination of neurons. Moreover, microglia can harm neurons through NMDA receptor-mediated processes by secreting glutamate and aspartate. The purpose of these cytotoxic secretions is to destroy the infected neurons and eliminate the threat, but it can also cause serious collateral damage (Gehrmann *et al.*, 1995), leading to further neuronal dysfunction and death, originating a vicious cycle known as neuroinflammation.

By other side, neurons produce several inhibitory signals, as a CD200 (that provides an inhibitory signal for macrophage lineage in several tissues) and CX3CL1 or fractalkine (a chemokine that attract microglia and T cells) that can revert the response of microglia and prevent their neurotoxic activation (Cardona *et al.*, 2006; Hoek *et al.*, 2000; Lewis *et al.*, 2012). Evans and colleagues (2013) verified that by inducing the knockout of fractalkine receptor (CX3CR1) there was dysregulated microglial responses.

Similar to what occurs in the ER stress response, the neuroinflammatory process seems to be both protective and harmful during neurodegeneration (Liao *et al.*, 2012). Several studies reported the presence of pro-inflammatory cytokines in the CSF of ALS patients (Kuhle *et al.*, 2009) and the lack of CD4 in mSOD1 mice (Beers *et al.*, 2008), which aggravate neurodegeneration and reinforces that the inflammatory reactions have an impact on the ALS neurodegeneration (Moser *et al.*, 2013).

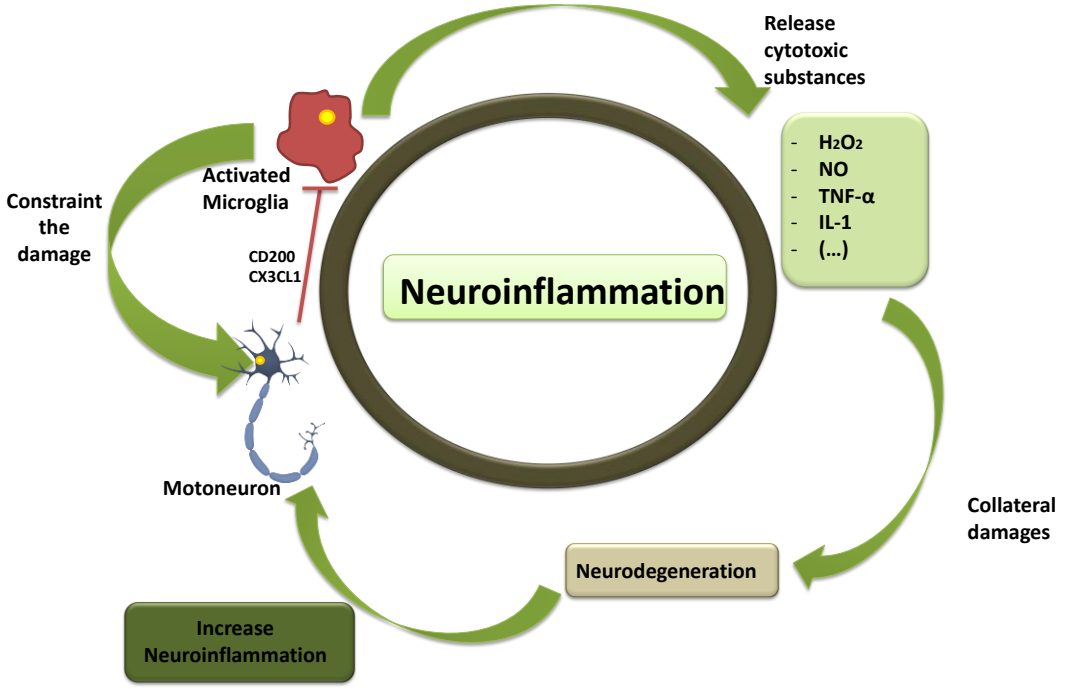


Figure I.6 – Neuroinflammation in amyotrophic lateral sclerosis (ALS): neuroprotection *vs.* neurotoxicity. In order to combat the threat, microglia release cytotoxic substances, such as tumor necrosis factor-α (TNF-α), hydrogen peroxide (H_2O_2), interleukin-1 (IL-1), nitric oxide (NO) and other causative factors that accelerate neurodegeneration. With the increase in neurodegeneration, more microglia is activated and more cytotoxic substances are released. On the other hand, neurons release CD200 and CX3CL1 that may revert microglia reactivity, thus reducing neurotoxicity.

2. Microglia: Neuroprotective or contributors for neurodegeneration in ALS?

As we have seen about neuroinflammation, it is not clear if microglia play a neuroprotective (Boillée *et al.*, 2006; Weydt *et al.*, 2004) or neurotoxic role (Lalancette-Hebert *et al.*, 2007; Simard and Rivest, 2007; Zhao *et al.*, 2010) in ALS disease. From one point of view, microglia are neuroprotective, functioning as a sensor that controls the surrounding environment and acts directly on the injury, thus playing a role in primary immune CNS response. For the other side, microglia send signals to their neighbor cells, which can exacerbate the response to injury, increase neuroinflammation and consequently neurodegeneration. Therefore, it is not consensual if microglia is an ally or an enemy in the fight against ALS pathology. Moreover, after neuroinflammation, microglia have a regenerative effect on neural tissue. These cells secrete anti-inflammatory cytokines and recruit neurons and astrocytes in the affected region. Without the support of microglial cells, remapping and regrowth of the affected areas would be an extremely slow process (Gehrmann *et al.*, 1995; Ritter *et al.*, 2006).

2.1 The different phenotypes

One of the main features of microglial cells is their plasticity, which is a crucial strategy for the proper functioning of the immune defense of the nervous system (Gehrmann *et al.*, 1995). Microglia can change their structure based on its current location and function **(Figure I.7 A)**. The different phenotypes experienced by microglia are defined based on morphological, molecular, and functional characteristics (Colton and Wilcock, 2010). In physiologic conditions, microglial cells have long branching processes and a small cellular body (Kettenmann *et al.*, 2011), a condition known as a "resting state", where the cell body of ramified microglia remains relatively static. Nevertheless, their branches are constantly moving and surveying the environment, thus contributing to the maintenance of the homeostasis, through cross-talk with astrocytes and neurons (Evans *et al.*, 2013; Philips and Robberecht, 2011). In the resting state, branches have a huge sensitivity to slight changes in physiological conditions. It is still a matter of debate which is the primary cause that induces the transformation for "resting" phenotype, although in cell culture studies, astrocyte conditioned medium increased ramification of cultured blood monocytes (Sievers *et al.*, 1994). Combining astrocyte conditioned medium with ATP or adenosine yield a phenotype with more extensive ramification, indicating that purines are not the only ramification-inducing factors of microglia (Wollmer *et al.*, 2001). Other candidates are cytokines released from astrocytes, such as transforming growth factor-β (TGF-β), macrophage colony-stimulating factor (M-CSF) and granulocyte macrophage colony stimulating factor (GM-CSF) (Schilling *et al.*, 2001). Activation of chloride channels also seems to be necessary for this morphological transformation (Kettenmann *et al.*, 2011).

In case of disturbance of nervous system homeostasis, microglial cells change their phenotype to an activated stage, which can be divided into M1 and M2 phenotypes (Gordon, 2003; Henkel *et al.*, 2009). Classical activation of microglia consists in the change to the M1 phenotype, which is mainly neurotoxic, characterized by the release of pro-inflammatory cytokines, such as TNF-α, IL-1β and interleukin-12 (IL-12), chemokines, proteases and redox species like NO (Durafourt *et al.*, 2012; Henkel *et al.*, 2009; Kraft and Harry, 2011; Nakajima *et al.*, 2003; Nayak *et al.*, 2010); and the alternative activation consist in the change to the M2 phenotype, which is mainly neuroprotective and is associated with resolution of tissue homeostasis (Hovden *et al.*, 2013), aiming the repair of damage tissues. The production and release of anti-inflammatory cytokines like IL-4, IL-13, IL-10 and transforming growth factor-beta (TGF-β) is the predominant induction signal (Colton, 2009; Glezer *et al.*, 2007; Ledeboer *et al.*, 2000; Suzuki *et al.*, 2005).

During their activation, microglia undergo through some morphological alterations, such as the thickening and retraction of branches, uptake of major histocompatibility complex (MHC) class I/II proteins, secretion of cytotoxic factors and secretion of recruitment molecules and pro-inflammatory signaling molecules.

There are several factors that may activate microglia: cell necrosis factors, glutamate receptor agonists, lipopolysaccharide (LPS), pro-inflammatory cytokines, and changes in extracellular potassium (a marker of cellular disruption). Pro-inflammatory mediators including monocyte chemoattractant protein 1 and IL-8 are present in the CSF of patients with ALS (Mantovani *et al.*, 2009).

When activated, microglia can be found in the motor cortex and SC of ALS patients and the intensity of this activation seems to be related with the severity of upper MN injury. In addition, it is often associated with infiltration of T lymphocytes (Lasiene and Yamanaka, 2011; Philips and Robberecht, 2011).

According to Beers and colleagues (2011), until the end-stage of ALS microglia presents a neuroprotective role, whereupon levels of pro-inflammatory cytokine IL-1β and TNF-α increase, and levels of NADPH oxidase are increased. Thus, in mSOD1 mice model, during initial stages of ALS, microglia has an M2 phenotype that supports neuronal survival. Nevertheless, with the advance of the disease course, microglial activation became skewed towards an M1 phenotype (Lewis *et al.*, 2012).

There is also another phenotype with anti-inflammatory profile, known as acquired deactivation, that consists in an phenotype with distinct gene profile from alternative activation but which also down-regulates the response of innate immune system. Since this state is anti-inflammatory, it inhibits the production of pro-inflammatory cytokines and increase the production of anti-inflammatory cytokines (Colton, 2009).

Finally, it was discovered the dystrophic or senescent microglia, which has an abnormal cytoplasmic structure, such as disbranched, atrophic, fragmented or unusually tortuous processes, with spheroidal or bulbous swellings (Streit, 2006). 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 The incidence of this form of microglia increases with the aging and have been reported in neurodegenerative diseases such as Alzheimer's disease or schizophrenia, indicating microglial deterioration in these diseases (Streit, 2006).

2.2 The migration to the injured tissue

The migration is a crucial event for the microglial cells to travel to the lesion sites and is triggered by the gradient formed by several chemotactic agent, such as purines, neuregulin 1 (NRG1), complement components, and chemokines such as Chemokine (C-C motif) ligand (CCL2) which is exclusively expressed in damaged neurons **(Figure I.7 B)**. Adenosine-5'-triphosphate and adenosine diphosphate (ATP and ADP, respectively) have been widely studied as a chemoattractants of microglial cells since that these nucleotides can diffuse rapidly and was been shown that they can induce membrane ruffling and attract microglia (Calvo and Bennett, 2012). This process involves the G protein-coupled receptor P2Y12 (Honda *et al.*, 2001), which when are knocked out in mice seems to decrease the ability of microglia to migrate to the site of injury (Haynes *et al.*, 2006).

2.3 Phagocytosis

Phagocytosis is a form of endocytosis involving the vesicular internalization of solid particles, such as pathological agents or cell debris (Napoli and Neumann, 2009) and is also a process undertaken by microglia, as they derived from myeloid lineage and share various similarities with peripheral macrophages **(Figure I.7 B)**.

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 initiates the uptake and subsequent responses (Neher *et al.*, 2012). Though phagocytic process, microglia is able to engulf the debris, such as damaged or apoptotic cells, namely neurons, DNA fragments or plaques, and secrete either pro- or anti-inflammatory cytokines, depending on the type of receptor that has been stimulated. As described by Napoli and Neumann (2009), the uptake of apoptotic cells occurs with production of anti-inflammatory cytokines like TGF-β thus restraining inflammation. However, microglia can recognize pathogens-associated molecular patterns (PAMPs) of pathogenic agents by toll like receptors (TLRs) pathway, leading to a release of pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α) or NOS-2 (Napoli and Neumann, 2009). Interestingly, phagocytosis is impaired in dysfunctional situations. Moreover, this phagocytic microglia interacts with astrocytes and neural cells to constrain the damage as quickly as possible, avoiding harmful effects to healthy cells (Aloisi, 2001; Gehrmann *et al.*, 1995).

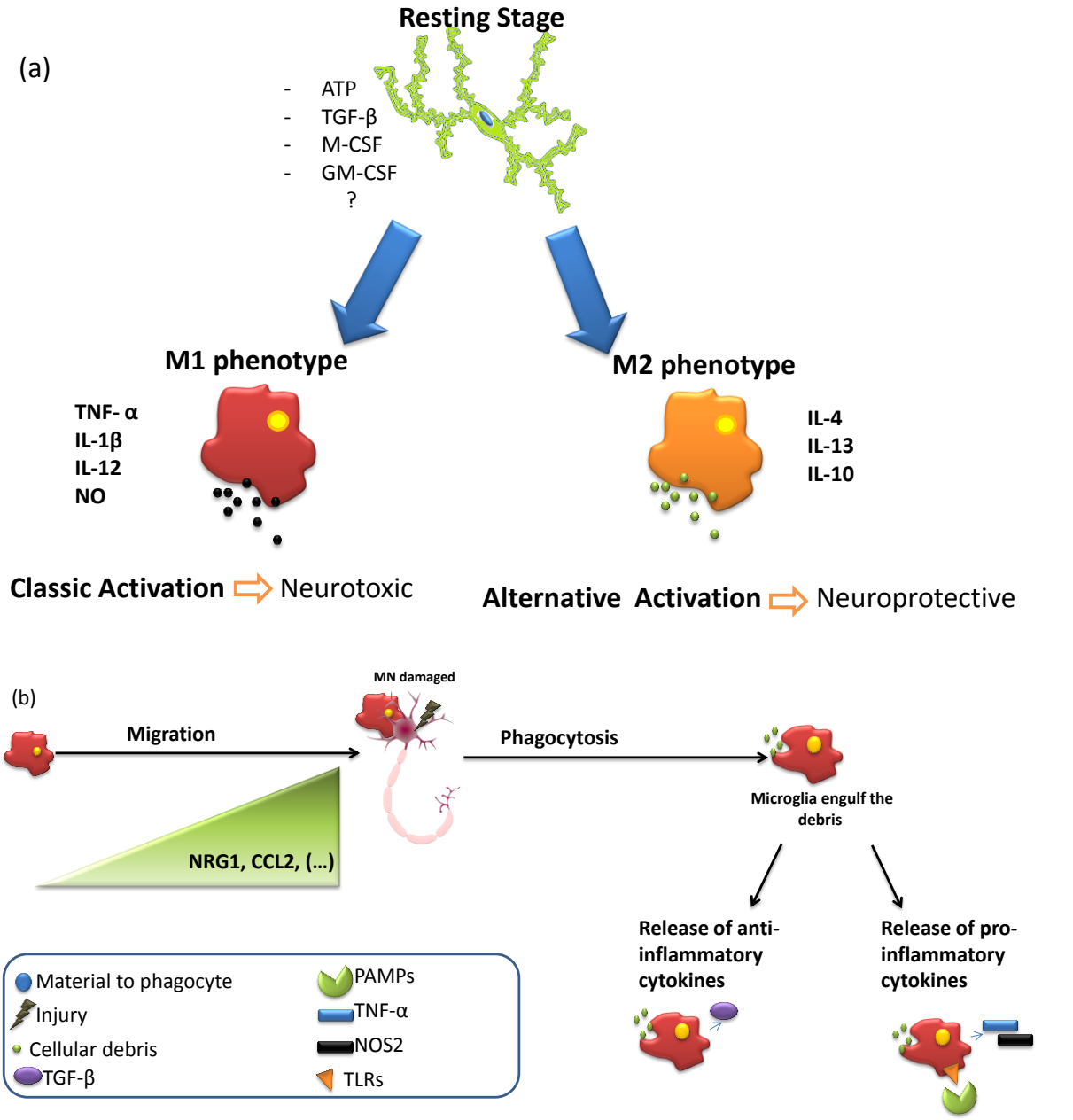


Figure I.7 – Microglial cells experiment different phenotypes, depending of the surrounding environment and neuronal injury. (a) In basal conditions, microglia acquires a resting/vigilant phenotype, with long branches in constant movement to monitor the environment. It is believed that this phenotype is induced by adenosine triphosphate (ATP), transforming growth factor-β (TGF-β), macrophage colony-stimulating factor (M-CSF) and granulocyte macrophage colony stimulating factor (GM-CSF), but this is still a matter of debate. In case of injury, microglial cells change their phenotype to an activated stage. M1 phenotype (classic activation) is mainly neurotoxic and is characterized by the release of pro-inflammatory cytokines and reactive oxygen species (ROS). M2 phenotype (alternative activation) is mainly neuroprotective and is associated with the achievement of tissue homeostasis. M2 microglia release anti-inflammatory cytokines such as IL-4, IL-13, IL-10 and TGF-β. (b) Microglial cells acquire amoeboid morphology. They response to the gradient formed by chemotactic agents, such as neuregulin 1 (NRG1) and chemokine (C-C motif) ligand (CCL2), expressed by injured MN, and migrate towards the site of lesion. After arrival at the lesion site, microglia engulf the debris upon recognition of cell-surface signals and secrete pro- and anti-inflammatory cytokines, depending on the type of receptor that has been stimulated. The uptake of apoptotic cells occurs with the production of anti-inflammatory cytokines, such as TGF-β, thus restraining inflammation. In case of pathogenic agents, microglia recognizes its pathogen-associated

molecular patterns (PAMPs) by toll-like receptors (TLRs), leading to the release of pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α), and activation of nitric oxide synthase-2 (NOS2).

2.4 The role of microglia in ALS

The activation of microglia and the infiltration of lymphocytes indicate an inflammatory event in the CNS pathology of ALS (Carpentier *et al.*, 2008). As previously described, one of the most accepted hypotheses is based on the formation of protein aggregates as a first dysfunctional cellular process of ALS that mediates the other ones. Then, microglia, astrocytes and the innate system including monocytes, macrophages and natural killer cells respond as a first line of defense. It is believed that, this response is triggered by mSOD1. Then, the adaptive immune system is activated. Throughout the progression of the disease, $CD4^{+}CD25^{+}$ regulatory T cells (Treg, secreting anti-inflammatory cytokines) and other neuroprotective cells decrease (Kipnis *et al.*, 2004), M2 microglia phenotype switch to M1 microglia phenotype and the number of CD8+(a receptor predominantly expressed on the surface of cytotoxic T cells) increase (Hovden *et al.*, 2013). In ALS, the activation of microglial cells was shown by the discovery of the signal transducer and activator of transcription-3 (STAT3) in post mortem samples of ALS patients (Shibata *et al.*, 2009). STAT3 is a signal transducer and activator of transcription that mediates cellular responses to IL-6 family members, in the development, differentiation, immunity, metabolism and cancer (Grivennikov and Karin, 2010).

Moreover, Meissner and colleagues (2010) concluded that IL-1β is an important pro-inflammatory cytokine released by microglia in $mSOD1_{G93A}$ mice that activate M1 phenotype, accelerating the progression of the disease (Meissner *et al.*, 2010). In addition, these events can be reversed by administration of the IL-1 receptor antagonist. The mSOD1 protein can also act as a powerful activator of microglia. Indeed, the presence of this mutant protein reduces the expression of neurotrophic factors such as IGF-1 and BNDF produced by microglia, which will have a deleterious role in ALS MN (Evans *et al.*, 2013). Using *in vivo* models, microglial proliferation and activation was shown to occur at earlier disease stages and to increase with ALS progression (Moisse and Strong, 2006). However, recently, the elimination of mSOD1 from microglia revealed to not change the onset but to slow disease progression by 50% (Ferraiuolo *et al.*, 2011).These findings suggest that the onset and the progression of the disease have different features, which opens new horizons for target driven therapies.

3. Different models for the study of neurodegeneration in ALS

The study of human neurodegenerative diseases evidences the impossibility to study the cellular and molecular pathological events in real time or safely and repeatedly remove tissue for analyze (Turner *et al.*, 2013). The existence of different experimental models in ALS may is considered a useful tool for providing knowledge at both cellular and molecular levels, to thereby develop more effective therapies.

In this chapter, we will just focus on the experimental models that will be used in the studies that comprise the present Thesis, namely MN-like monocultures, SC organotypic cultures and transgenic

SOD1 animal models. However, there are much more models used in ALS research, such as worms, flies, fish, mice, rats (Muyderman *et al.*, 2009) and diverse cell types (Cashman *et al.*, 1992).

3.1 Cell models

3.1.1 NSC-34 cells

NSC-34 cell line, a hybrid cell line, is a fusion of MN-enriched embryonic day 12-14 of SC cells with aminopterin-sensitive mouse neuroblastoma (Cashman *et al.*, 1992). NSC-34 cells, are considered a model of ALS when transfected with human SOD1 containing the mutation in G93A, revealing some features of MN degeneration, such as mitochondrial dysfunction (Raimondi *et al.*, 2006) and Golgi apparatus fragmentation (Gomes *et al.*, 2008). This cell line is used to dissect the complexity of mechanisms that involve mSOD1 toxicity (Atkin *et al.*, 2006; Cozzolino *et al.*, 2008; Rizzardini *et al.*, 2005).

The establishment of a cell line of immortalized neurons in culture is a major challenge and due to its intrinsic properties, such as their null capacity to proliferate when they are completely differentiated. Thus, the use of a hybrid cell line of neuroblastoma (with high proliferative capacity due to the presence of the gene N-myc, an oncogene involved in cell proliferation) together with SC MN solved the problem (Cashman *et al.*, 1992) Morphological and physiological properties of such cells reveal the suitability of the model when it is considered properties as acetylcholine synthesis, storage and release; extension of processes; generation of an action potential; formation of contacts with cultured myotubes and expression of neurofilament proteins among others (Cashman *et al.*, 1992; Tovar *et al.*, 2009).

When accessing the viability of NSC-34 after exposure to a selection of neurotoxic chemicals, it was observed that the cells respond to all of them (Durham *et al.*, 1993). The similarity in the production of the action potential to several ion channel blockers between this cell line and that of primary MN in culture, corroborate NSC-34 cells as an effective model for studying neurotoxicity in ALS. Most important is that NSC-34 cells adhere specifically to the leucine-arginine-glutamate motif of S-laminin, what shows that these hybrid cells uniquely express MN phenotypic features. This property was not revealed by most neural cell lines (Hunter *et al.*, 1991). However, NSC-34 cells are not suitable to evaluate the effect of agents on the synaptic transmission (Durham *et al.*, 1993).

It has been recently established in our lab that NSC-34/hSOD1$_{G93A}$ cells reveal features of mitochondrial dysfunction, energy impairment, oxidative stress, as well as apoptosis and inflammatory-related processes. All of these events are common processes in the transgenic mice model and in ALS patients. Studies in our laboratory have also shown that this model may be used to evaluate the therapeutic efficacy of compounds, such as the glycoursodeoxycholic acid (GUDCA), a bile acid-glycine conjugate. Based on the overall aspects mentioned, we decided to implement in our laboratory a model of mixed cultures to analyze the interaction between MN-like cells (NSC-34) and N9 (a microglial cell line), as will be discussed below.

3.1.2 Organotypic culture cells

The major problem of the most *in vitro* models is the difficulty to mimic what actually occurs in the organism. Although primary cultures and mixed cultures give valuable information on the cell function and interconnectivity, cellular processes should not be considered isolated, but rather integrated in a system that involves cell cross-talk and signals from the entire neighborhood. The neurons coexist in a complex network where the surrounding cells shape the biochemical, electrophysiological and morphological features between themselves and the remaining cells around (Tovar *et al.*, 2009). Therefore, the organotypic cultures are a closer model to *in vivo*, since we do not isolate one cell type in particular but, instead, we preserve the tissue structure when culturing the entire SC slice. According to Tovar and colleagues (2009), these sections can be cultured for 3 months, keeping up the metabolic capacity of MN, such as choline acetyltransferase and acetylcholinesterase activities (Delfs *et al.*, 1989). Moreover, the use of SC cultured slices have benefits over animal models, such as the easy access and precise control of the extracellular environment, which makes the model powerful to screen the efficacy of potential medicines (Su *et al.*, 2011).

3.2 Animal models

Mice are widely used as an animal model in biological sciences, since they react to a disease induction or treatment as humans. In general, the closer the organism is to the man in the evolutionary tree, the better will be the model. However, careful is still necessary, since even non-human primates are not entirely predictive for human outcomes in drug development studies (Kari *et al.*, 2007). In general, a model is robust when the disease (spontaneous or induced) has similarity with the human disease and this similarity is significant in the context of the study. Rodents are largely used as a study model in neurodegenerative diseases because rodents and humans use the same neurotransmitters, receptors, proteins for synaptic vesicle release and recycling and similar signaling mechanisms.

The recent discovery of ALS-associated mutations in the DNA-binding protein 43 (TDP-43) gene lead to the generation of alternative animal models of ALS, since these mutations are associated with both sALS and fALS (Xu *et al.*, 2011). Nevertheless, mutants of human SOD1 mice are still the most common model to investigate disease pathogenesis (Tovar *et al.*, 2009). The success of mice expressing mutant proteins associated with fALS (such as mSOD1) has become a powerful tool to study mechanisms of MN loss, particularly with different forms of SOD1 (Van Den Bosch, 2011). A huge advance in the ALS research was the discovery that about 20% of fALS cases were due to mSOD1 (Rosen, 1993). This discovery spurred the creation of a transgenic mice expressing human mSOD1. It was in 1994 that Gurney and colleagues developed the first transgenic mice expressing human protein with the G93A mutation, and this was the first obtained lab model based on a known cause of ALS (Gurney *et al.*, 1994). This transgenic mouse overexpressing $mSOD1_{G93A}$ was obtained by genetic engineering, inserting multiple copies of human genomic SOD1 into the mouse genome. Moreover, these mice show symptoms similar to humans such as the progressive hind limb weakness that culminate in paralysis and ultimately death, as a result of MN injury (Van Den Bosch, 2011).

Besides these first transgenic mice, several other models that overexpress human SOD1 were created, but with other mutations (G37R, G85R, G86R, D90A). Interestingly, these new mice have demonstrated identical phenotype as the $mSOD1_{G93A}$ (Bruijn *et al.*, 1997; Jonsson *et al.*, 2006; Ripps *et al.*, 1995; Wong *et al.*, 1995) but comparatively are not so often used. Indeed it was in the mSOD1 model that most studies evidenced the non-cell autonomous neurodegeneration (Boillée *et al.*, 2006), excitotoxicity (Corona *et al.*, 2007), apoptosis (Hervias *et al.*, 2006), protein aggregation (Wood *et al.*, 2003), axonal dysfunction (Sasaki *et al.*, 2005), mitochondria failure (Cassina *et al.*, 2008) and both endoplasmic reticulum (Nishitoh *et al.*, 2008) and oxidative stress (Harraz *et al.*, 2008).

The mSOD1 mouse model was also widely used to access new therapies, and minocycline is a good example of this. This drug was shown to inhibit microglial activation, a property also evidenced by other studies using two distinct mSOD1 mouse models developed in the same year (Kriz *et al.*, 2002; Van Den Bosch *et al.*, 2002; Zhu *et al.*, 2002).

In spite that mSOD1 mice and rats are accepted as optimal models to evaluate selective MN death, they have been also disappointing once no effective drugs in rodents revealed to be effective in clinical trials with humans (Van Den Bosch, 2011).

4. Therapeutic strategies: Yesterday, today and tomorrow

From the nearly two decades, knowledge about ALS has evolved considerably. Now, we know more about genetic causes in fALS cases, although not so much about sALS (Venkova-Hristova *et al.*, 2012). So far, researchers in the ALS field have joined efforts to find an effective therapeutic agent for the disease. However, to date, the only FDA approved drug is riluzole (Kiernan *et al.*, 2011).

A challenge is still to distinguish which therapies are delaying the onset of the disease versus prolonging the compensatory pre-clinical period, in which the disease process is progressing in the absence of clinical signs of motor dysfunction. For example in mSOD1 mice, it is reported that several anomalies are present before the onset of clinical symptoms (Ferraiuolo *et al.*, 2011), such as behavioral motor changes (Bories *et al.*, 2007; van Zundert *et al.*, 2008), MN electrophysiological dysfunction (Mead *et al.*, 2011), mitochondrial swelling and vacuolization (Bendotti *et al.*, 2001) and transcriptome changes in an attempt to increase MN energy provision (Ferraiuolo *et al.*, 2007).

The progression of the disease constitutes a huge therapeutic problem. For example, patient groups are very small and often include individuals in different disease stages, making difficult to obtain strong conclusions from the clinical trials. Moreover, drugs that showed benefits in animals when administered pre-symptomatically or before ALS onset, failed in humans since that diagnosis is not confirmed before symptom emergence (Hovden *et al.*, 2013). The setbacks that occur during the clinical trials led to the conclusion of a combination therapy, as a better solution. However there is still not an effective combination due to the complexity of the disease. Furthermore, despite the immune system be considered a promising target, it is possible that the action of at one specific target may be beneficial in certain ALS phases, while dangerous in others (Hovden *et al.*, 2013).

According to several researchers, a perfect combination cocktail may combine drugs that target protein aggregation, immune modulation and glutamate excitotoxicity (Hovden *et al.*, 2013). In summary, research on ALS has been characterized by same success, but also frustration.

Nevertheless, we must emphasize that scientific progress has been continuous. We are probably closer to find new hallmarks for clinical success and to further carry that success to other neurodegenerative disorders (Venkova-Hristova *et al.*, 2012).

4.1 Microglia as a therapeutic target in the future

The complexity of the cross-talk between microglia and neurons and the role of microglia in the immune system and neuroinflammation suggests that microglia is a very promising target for the therapeutic advances against ALS progression.

Indeed, studies with the transgenic mice with human mSOD1, have shown that diminishing the mutant levels of mSOD1 in microglia had little effect on the early disease phase but slowed later disease progression (Boillée *et al.*, 2006). Furthermore, elimination of microglia using clodronate liposomes, which specifically target monocyte/macrophage system (such as microglia cells), and addition of new microglia significantly slowed disease progression and prolonged survival of the transgenic ALS mice after bone marrow transplantation (BMT). So, replacement of microglia by a healthier cell may ameliorate neural cell microenvironment; however the mechanisms and functional implications of this replacement need to be further investigated (Lee *et al.*, 2012). Nevertheless, improvement by BMT may not be efficient enough since microglia are replaced by tissue-resident microglia rather than by bone marrow cells (BMCs) (Ohnishi *et al.*, 2009).

4.2 GUDCA: Beneficial effects in ALS

So far, there is no successful drug for ALS treatment and search for novel therapeutic agents that can prevent or delay MN 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 showed ability to counteract neuronal death and synaptic changes in models of neurodegeneration, such as neonatal unconjugated bilirubin encephalopathy (Silva *et al.*, 2012).

Bile acids are acidic steroids synthesized in hepatocytes from cholesterol. Primary bile acids, the major constituents of bile, are produced in the liver and when secreted into the intestine, they can be metabolized into secondary bile acids where they play crucial roles such as solubilization of lipids (Amaral *et al.*, 2009). Some bile acids are cytotoxic (Bayerdorffer *et al.*, 1993), but others not, due to small changes in the chemical structure that are found between species (Hofmann and Roda, 1984). UDCA, for example, helps in the regulation of cholesterol levels and have several functions in the liver, such as maintenance of mitochondrial integrity, immune-modulation and anti-apoptotic role. During the process of conjugation in the liver, UDCA can link with taurine or with glycine, originating tauroursodeoxycholic acid (TUDCA) or GUDCA, respectively. Once GUDCA is formed in larger amount (close to 80%) (Lazaridis *et al.*, 2001), this conjugate is of major clinical relevance derived from the utilization of UDCA in several diseases (Brites, 2002). Thus, according to some authors,

GUDCA may play protective roles in several cells of CNS, such as neurons, astrocytes and microglia (Fernandes *et al.*, 2007; Rodrigues *et al.*, 2000; Silva *et al.*, 2012; Vaz *et al.*, 2010) and has shown anti-oxidant, anti-apoptotic and anti-inflammatory properties (Fernandes and Brites, 2009) **(Figure I.8)**. In addition, the taurine-conjugated form of UDCA, tauroursodeoxycholic acid (TUDCA) has already proven beneficial effects in many neurodegenerative diseases, namely in Alzheimer's disease, 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).

Thus, driven by the promising results obtained in prior and ongoing studies from our and other groups, we decided to examine the therapeutic potential of GUDCA in our model of mouse SC organotypic cultures as an anti-inflammatory compound. Min and colleagues (2012) have recently performed a clinical trial using UDCA in 80 ALS patients. Although without conclusive results, in general the patients were tolerant to oral administration and this pilot study may open a new application for UDCA and its conjugated species for ALS management.

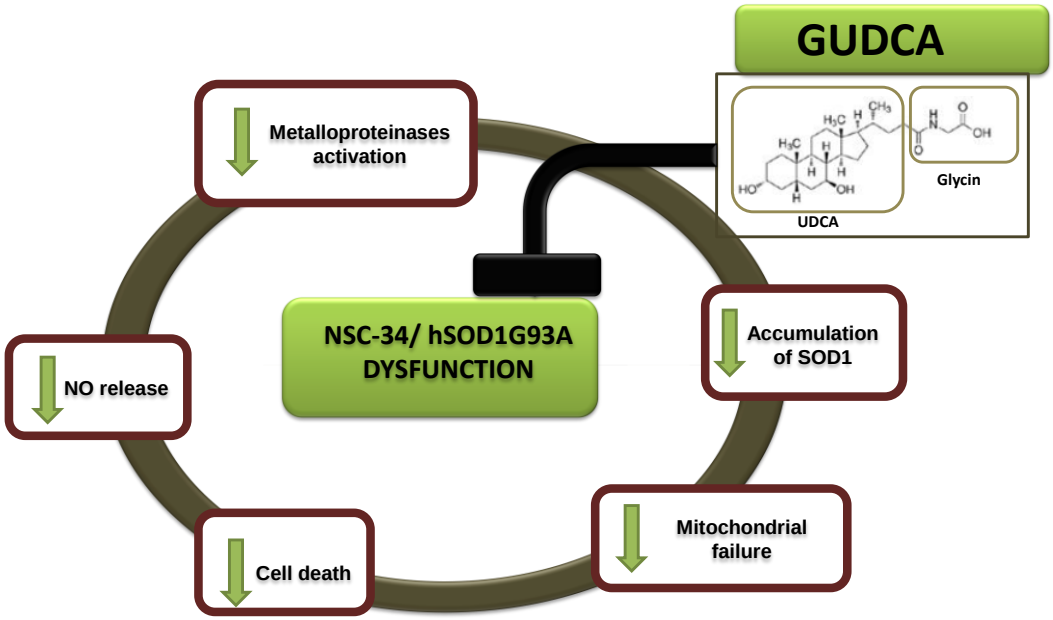


Figure I.8 – Glycoursodeoxycholic acid (GUDCA) may play a protective role in NSC-34/hSOD1G93A cells, by preventing motor neuron degeneration. GUDCA is the glycine conjugated form of ursodeoxycholic acid (UDCA), which is used for long as a therapy for liver cholestatic diseases. NSC-34/hSOD1G93A cell line was incubated with GUDCA (50 µM). Arrows in green: Protective effects of GUDCA in NSC-34/SOD1G93A cells obtained in our lab and involving the reduction of metalloproteinase activation, Superoxide Dismutase-1 (SOD1) accumulation, mitochondrial failure, cell death and Nitric Oxide (NO).

5. Aims

The aims of this thesis are to better understand some of the main molecular hallmarks involved in motor neuron (MN) degeneration in ALS, as well as to explore the cross-talk between MN and microglia in ALS, by using three different experimental models: (i) pure MN cultures (NSC-34 cell line); (ii) mixed MN-microglial cultures (NSC-34/N9 cell lines); (iii) organotypical slices from mice spinal cord (SC).

Therefore, the specific aims are:

1. To explore the processes involved in MN-mitochondrial dysfunction along ALS progression, namely mitochondrial dynamics (fusion/fission) and axonal transport (anterograde/retrograde), together with cell death mechanisms. For this, NSC-34 cell line expressing either human SOD1 wt or mutated in G93A (NSC-34/hSOD1$_{wt}$ or NSC-34/hSOD1$_{G93A}$, respectively) will be differentiated for 1-7 days (DIV) in order to select the mechanisms involved in MN mitochondrial dysfunction in ALS for each stage of SOD1 accumulation (prior or after SOD1 accumulation, respectively 1 or 4-7 DIV). NSC-34/hSOD1wt will be used as control.

2. To evaluate the contribution of microglia to MN degeneration in mixed cultures of MN-microglia. For this we will produce and characterize a mixed culture of either NSC-34/hSOD1wt or NSC-34/hSOD1$_{G93A}$ with N9 microglia and we will focus on the parameters that will be found altered in the NSC-34/hSOD1$_{G93A}$ monoculture.

3. To set-up and characterize organotypic cultures obtained from SC of ALS-transgenic mice carrying the same human SOD1 mutation (TgSOD1-G93A mice). Here, we will test the potential neuroprotective benefits of glycoursodeoxycholic acid (GUDCA).

Altogether, the final purpose of the project is to explore the mechanisms of MN degeneration in ALS and the role of microglia on them, to discover intervenient targets and drivers, while assessing modulatory effects by GUDCA. If we succeed, future research should test ways to design molecules that although having the benefits of GUDCA better achieve the brain parenchyma.

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 amino acids(NEAA) were purchased from Biochrom AG (Berlin, Germany); RPMI-1640 medium, Poly-D-lysine (PDL), trypsin-EDTA solution (1X), ATP, Hoechst 33258 dye, bovine serum albumin (BSA), naphtylethylenediamine ($C_{12}H_{14}N_2$) and sulfanilamide ($C_6H_8N_2O_2S$) were from Sigma-Aldrich (St. Louis, MO, USA); Geneticin 418 sulfate (G418), Glycoursodeoxycholic acid (GUDCA) (minimum 96% pure) were obtained from Calbiochem (Darmstadt, Germany or U.S. Canada); Triton X-100 and 6-phosphate glucose dehydrogenase and hexokinase were obtained from Roche Diagnostics (Mannhein, Germany); Neurobasal medium, B-27 Supplement (50x), Hank's balanced salt solution (HBSS) were acquired from GIBCO® (Grand Island, NY, USA). DPX mounting media for microscopy was obtained from BDH Prolabo (Poole, UK). Guava Nexin® Reagent was purchased from Merck Millipore (Darmstadt, Germany).

All the other chemicals were of analytical grade and were purchased either from Sigma-Aldrich or Merck.

1.2 Antibodies used for immunostaining

Table II.1 – Primary antibodies used and respective information.

Primary Antibody	Host	Brand	Dilution
Dynein	Mouse	Milipore, Darmstadt, Germany	1:100
Kinesin	Mouse	Chemicon	1:100
Mitofusin 2	Rabbit	AbCam, Cambridge, UK	1:50
Drp1	Rabbit	AbCam, Cambridge, UK	1:150
βIII-tubulin	Mouse	Milipore	1:500
Biotinylated tomato lectin *Lycopersicon esculentum*	-	Sigma-Aldrich, MO, USA	1:166

Table II.2 – Secondary antibodies used and respective information.

Secondary Antibody	Host	Brand	Dilution
Alexa Fluor® 488 anti-rabbit	Goat	Invitrogen Corporation™ (Carlsbad, CA, USA)	1:1000
Alexa Fluor® 488 anti-mouse	Goat	Invitrogen Corporation™ (Carlsbad, CA, USA)	1:1000
Alexa Fluor® 594 anti-rabbit	Goat	Invitrogen Corporation™ (Carlsbad, CA, USA)	1:1000
Alexa Fluor® 594 anti-mouse	Goat	Invitrogen Corporation™ (Carlsbad, CA, USA)	1:1000
Avidin-FITC-conjugated		Sigma-Aldrich, MO, USA	1:50

1.3 Equipment

Fluorescence microscope (model AxioScope.A1) coupled with AxioCam HR camera and AxioScope HBO50 microscope were purchased from Carl Zeiss, Inc. (North America) and optical microscope with phase-contrast equipment (Olympus, model CK2-TR) were used for cell morphology evaluation.

Microplate reader (PR 2100 Microplate Reader) was used for nitrites measurement and was obtained from Bio-Rad Laboratories (Hercules, CA, USA). GloMax®-Multi Detection System - Promega (Sunnyvale, CA, USA) was used to detect extracellular ATP.

To ensure a stable environment to optimal cell growth (37°C and 5% CO_2), cell cultures were maintained in HERAcell 150 incubators (Thermo Scientific, Waltham, MA, USA) and the work performed in sterile conditions in a Holten Lamin Air HVR 2460 (Allerod, Denmark).

Eppendorf 580R (Eppendorf, Hamburg, Germany) and a Sigma 3K30 centrifuges were used for different experimental procedures.

To slice tissue for organotypic cultures, was used McIlwain Tissue Chopper (Gomshall, Surrey, UK). In other procedures, were used surgical material (Fine Science Tools Heidelberg, Germany), and the Stereomicroscope Stemi DV 4 (Carl Zeiss, York, UK). For flow cytometry studies, we used the Guava easyCyte 5HT Base System Flow Cytometer (Merck-Millipore, Darmstadt, Germany).

2. Methods

2.1 *In vitro* studies

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

2.1.1 NSC-34 cell line

NSC-34 cell line transfected with human SOD1, either wild type or mutated in G93A (NSC-34/hSOD1$_{wt}$ or NSC-34/hSOD1$_{G93A}$, respectively), were a gift from Júlia Costa, Instituto de Tecnologia Química e Biológica (ITQB), Universidade Nova de Lisboa, Portugal. NSC-34/hSOD1$_{wt}$ were used as control condition. NSC-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 days. Culture plates were coated with PDL (50 µM) before plating the cells. Cells were seeded in 12-well culture plates at a concentration of 5x10^4 cells/ml and maintained at 37ºC in a humidified atmosphere of 5% CO_2.

2.1.2 N9 cell line

N9 cell line was a gift from Teresa Pais, Instituto de Medicina Molecular (IMM), Lisboa, Portugal. Cells were cultured in RPMI supplemented with FBS (10%), L-glutamine (1%) and Penicillin-Streptomycin (1%), grown to confluence and splitted every 2 to 3 days. Cells were incubated in 12-well culture plates with NSC-34 (mixed cultures) at a concentration of 2x10^4 cells/ml and maintained at 37ºC in a humidified atmosphere of 5% CO_2.

2.1.3 NSC-34 Pure Cultures

Here, NSC-34/hSOD1$_{wt}$ and NSC-34/hSOD1$_{G93A}$ were plated in a 12-well culture plates with coverslips treated with PDL. Their differentiation was promoted at 48 hours (by incubation with differentiation medium). After 48 hours in proliferation media, differentiation was induced by changing medium for DMEM-F12 plus FBS (1%), non-essential amino acids (1%), Penicillin/Streptomycin (1%) and G148 (0.1%), and measurements were performed after 1, 4 or 7 days *in vitro* (DIV), as described in **Figure II.1** and as usual in our lab.

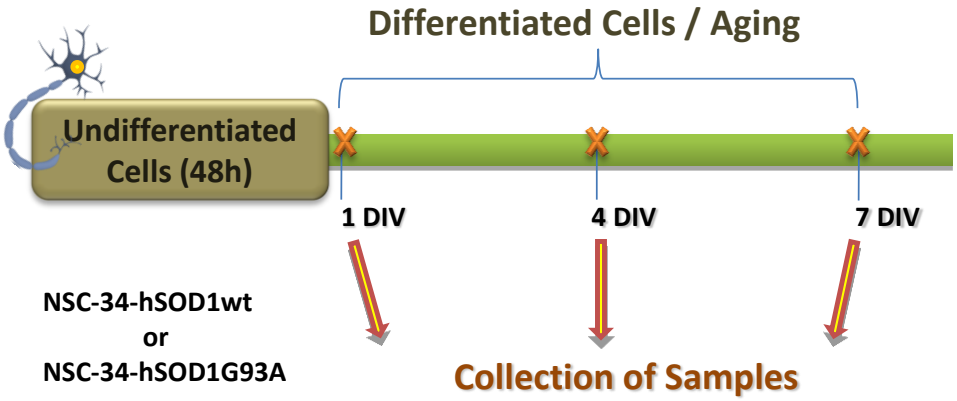


Figure II.1 - Experimental procedure used for pure culture cells of NSC-34 cells. NSC-34 cells, transfected with human SOD1, wild type or mutated in G93A (NSC-34/hSOD1$_{wt}$ or NSC-34/hSOD1$_{G93A}$) were grown in proliferation media during 48 h. After this, differentiation was induced by changing medium for DMEM-F12 with FBS (1%) and non-essential amino acids (1%). Cells were collected at 1, 4 and 7 DIV. These fixed cells are then used for immunocytochemical, cell viability assay by flow cytometry, and NO and ATP release measurement.

2.1.4 NSC-34/N9 Mixed Cultures

Here, NSC-34 cells were grown and differentiated as described for pure cultures. At 0 and 2 *days in vitro* (DIV), N9 cells were platted in mixed culture with NSC-34, as indicated in **figure II.2.** Then, cells were maintained at 37ºC in a humidified atmosphere of 5% CO_2, and collected at 4 and 7 DIV. The main aim was to evaluate the effects of microglia in NSC-34/hSOD1$_{wt}$ or NSC-34/hSOD1$_{G93A}$ if added before (0 DIV) or during MN degeneration (2 DIV) for short and long periods (collected at 4 and 7 DIV, respectively).

Comprising the need for plating NSC-34 and N9 in accordance with its proportion *in vivo*, N9 cells were plated at a concentration of 2x10^4 cells/ml and NSC-34 at 5x10^4 cells/ml, as in our previous studies with mixed neuron-microglia cultures (Silva *et al.*, 2011). With this experimental procedure, we can study neurons/microglia interaction to judge if (and when) microglia change their phenotype, if they are able to prevent the disease onset and if they can delay the disease or aggravate symptoms (neuroprotective *vs*. neurotoxic properties).

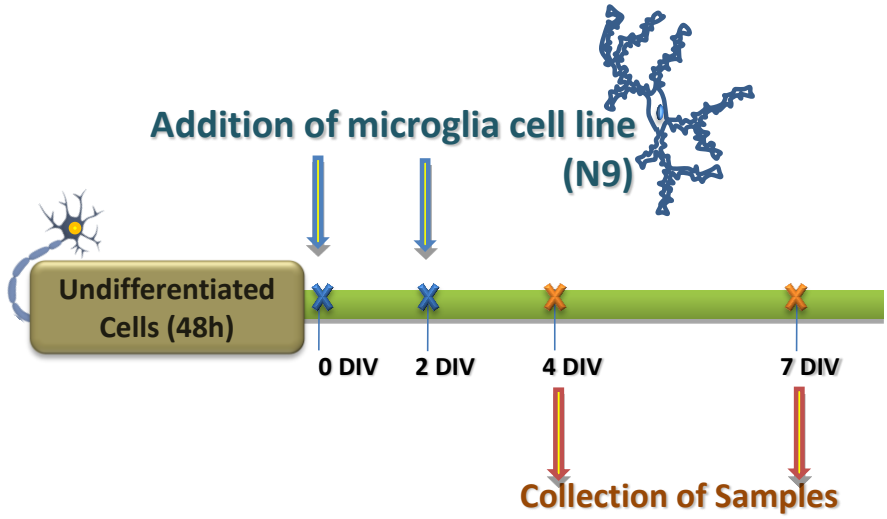


Figure II.2 - Experimental scheme of mixed culture cells with NSC-34 and N9 cell lines. NSC-34/hSOD1$_{wt}$ and NSC-34/hSOD1$_{G93A}$ cells were grown in proliferation media during 48h. After this, differentiation was induced by changing medium for DMEM-F12 with FBS and non-essential amino acids. Microglia (N9) was added at 0 and 2 DIV and cells were fixed at 4 and 7 DIV. Cells were fixed and used for immunocytochemical assays and extracellular ATP and NO release measurement.

2.2 Ex vivo studies

2.2.1 Animals

SJL (wt) and TgSOD1-G93A mice were purchased from international certified labs (Jackson Lab.) and were used to collect SC (sample tissue). SJL females and TgSOD1-G93A males were used for breeding and their progeny (SOD1 transgenic mice), maintenance and handling took place at Instituto de Medicina Molecular animal house facilities according to European Community and Portuguese guidelines and bylaws.

2.2.2 Organotypic cultures

Organotypic slice cultures were adapted from the methods previously described (Guzman-Lenis *et al.*, 2009). Briefly, 7-days SJL (Wt) or transgenic mice carrying the human protein mSOD1 (TgSOD1-G93A mice), previously genotyped, were sacrificed by decapitation and their spinal cords were dissected using surgical equipment and a magnifying glass. After dissection, the lumbar segment of their spinal cord was extracted and cut into 350 µm transverse slices with a McIlwain tissue chopper. Samples were placed in high glucose (6 mg/ml) Hank's balanced salt solution (HBSS) with 1.5% Penicillin-Streptomycin, where, they were separated and carefully transferred onto culture plate inserts and placed into a 6-well plate containing 1.5 ml of Neurobasal medium with 1x B27, 2 mM glutamine, 6 mg/ml glucose and antibiotics (1,5% Penicillin-Streptomycin), and incubated at the air-liquid interface, at 37ºC in 5% CO_2. The medium was changed the following day and, replaced three times per week until 10 DIV. At 10 DIV, slices were incubated with 50 µM GUDCA from a 5 mM stock

solution (Silva *et al.*, 2012). Incubations with Neurobasal were used as control. At 11 DIV, slices were collected and fixed in 3 ml of freshly prepared 4% (w/v) paraformaldehyde for 1 hour and rinsed in PBS (Ravikumar *et al.*, 2012). The slices were then stored at 4° C, in PBS. Extracellular media was collected for ATP and NO measurements. **(Figure II.3)**

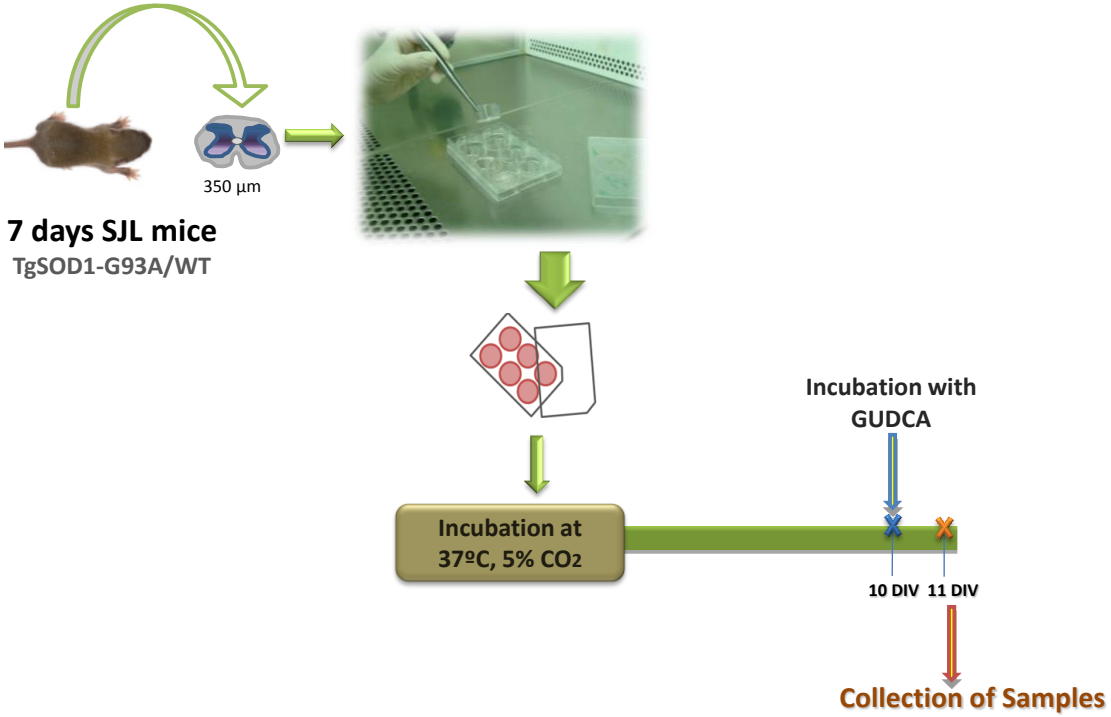


Figure II.3 - Experimental scheme of organotypic cultures of 7-days SJL (Wt) and transgenic mice carrying a human protein mSOD1 (TgSOD1-G93A mice). The lumbar segment of spinal cord was incubated in a 6-well plate and cultured during 10 days. At 10 DIV, slices were incubated with 50 µM GUDCA during 24h. Slices were fixed for later assay microscopy (immunohistochemistry) and extracellular ATP and NO release measurement.

2.3 Evaluations

2.3.1 Immunocytochemistry

NSC-34 cells, either alone or in mixed culture with N9, were fixed with freshly prepared 4% (w/v) paraformaldehyde in PBS. For the immunostaining, cells were first permeabilized with 0.2% Triton X-100, for 20 min, and then incubated with blocking solution (3% BSA in PBS) for 30 minutes. After, they were incubated overnight at 4 ºC with primary antibody, according to the specifications mentioned in **Table II.1**. The incubation with secondary antibody **(Table II.2)** was performed during 2 hours at room temperature. Cell nuclei were stained with Hoechst 33258 dye (1:1000, Sigma) (Falcão *et al.*, 2005).

Fluorescence was visualized using a fluorescence microscope (model AxioScope.A1) coupled with AxioCam HR (Zeiss). Ten random fields were acquired per sample, under 400x or 630x magnification. The integrated density of the fluorescent-labeled cells was measured using ImageJ software (National Institutes of Health, USA). The integrated density is the area above the threshold for the mean density minus the background.

2.3.2 Immunohistochemistry

Slices of 7-days SJL Wt or TgSOD1-G93A mice were placed in blocking solution (2% FBS in TBS-Triton 10%), for 1h at 25ºC. Then, cells were incubated overnight at 4ºC with primary antibody. In the second day, after rinsing twice in TBS-Tween (0.05%), for 2x10 minutes at 25ºC, cells were incubated during 1 h at 25ºC with secondary antibody. After two more washes, slices were washed with PBS and incubated with DAPI stock 1 mg/ml for 5 minutes. After two washes for 10 minutes with TBS-Tween (0,05%), slices were dehydrated with ethanol. Cells were then mounted in DPX, on a microscope slide.

Fluorescence was visualized using a fluorescence microscope (model AxioScope.A1) coupled with AxioCam HR (Zeiss). Overlapping fields were acquired (under 100x magnification) and merged in Photoshop Software Massachusetts, USA). Twelve fields were acquired per sample in two perpendicular axes, with the origin on the central canal of the spinal cord, under 100x magnification.

2.3.3 Quantifying the release of Nitric Oxide

Nitric oxide levels were indirectly quantified by measuring the concentration of nitrites (NO_2) in the extracellular media of NSC-34, either alone or in mixed culture with N9 cells, as well as in organotypic culture extracellular media. Nitrites are a stable end product of NO.

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 minutes in the dark, at room temperature. The absorbance at 540 nm was determined using a microplate reader (Bio-Rad Laboratories). A calibration curve was used for each assay. All samples were measured in duplicate and the mean value was used (Vaz *et al.*, 2010).

2.3.4 Quantification of extracellular ATP

NSC-34, either alone or in mixed culture with N9 cells, as well as in organotypic culture extracellular media were treated on ice to prevent degradation of ATP. For the determination of extracellular ATP levels, the incubation media was collected and treated with 2 M of perchloric acid. Then, the pH value was neutralized with 4 M KOH solution. To remove cellular debris, the samples were centrifuged (Eppendorf, 5810R) during 5 min at 10,000 *g* and 4°C, between the different steps. ATP levels were determined by an enzymatic assay and fluorescence intensity was quantified using a GloMax®-Multi Detection System at λ_{em} 410-460 nm and λ_{ex} 365 nm. A calibration curve of ATP was used for each assay (Vaz *et al.*, 2010).

2.3.5 Detection of Apoptosis/necrosis

After incubation of NSC-34 in12-wells culture plates at 1, 4 and 7DIV, extracellular media was collected to 2 ml tubes and cells were detached by using a solution of tripsin 1x for 5 min of at 37ºC.

After adding FBS (to stop the action of trypsin), cells were collected and centrifuged at 700 *g* during 5 minutes (Eppendorf, 5810R). The supernatant was discharged and the pellet resuspended in 400 µL of 1% BSA in PBS. The samples were added to 96-wells plates with Nexin Reagent® (Anexin V/7AAD) and incubated at 20 min, protected from the light. After dilution with 50µl 1% BSA in PBS, samples were analyzed on a Guava easyCyte 5HT Base System Flow Cytometer (Merck-Millipore). 5000 events per sample were counted. Three populations of cells can be distinguished in this assay: viable cells (annexin V-PE and 7-AAD negative), early-apoptotic cells (annexin V-PE positive and 7-AAD negative) and late stages of apoptosis or dead cells (annexin V-PE and 7-AAD positive) (adapted from (Barateiro *et al.*, 2012).

2.4 Statistical Analysis

Results of at least three different experiments were expressed as mean ± SEM for NSC-34 cultures either isolated or in mixed culture with N9. Comparisons between the different parameters evaluated in wt and G93A NSC-34 cell line and in organotypic cultures from SC of TgSOD1-G93A or WT mice were made using two-tailed Student's t-test for equal or unequal variance, as appropriate. Comparison of more than two groups in the parameters evaluated in mixed cultures with or without microglia was done by one-way ANOVA using GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA) followed by multiple comparisons Bonferroni post-hoc correction. $p<0.05$ was considered statistically significant and $p<0.01$ very significant.

III. Results

1. Isolated NSC-34 cells, a MN-like cell model

1.1 Evaluation of cellular viability of differentiated NSC-34 cells, transfected with mutant SOD1 as a model of motoneuron degeneration in ALS

NSC-34 cell line is a hybrid cell line obtained by fusion of neuroblastoma cells with motoneuron (MN)-enriched from mice spinal cord cell preparations, and expresses many of the morphological and physiological properties of MN, such as extension of processes, formation of contacts with cultured myotubes, synthesis and storage of acetylcholine (ACh), support of action potentials and expression of neurofilament proteins (Cashman *et al.*,1992). In our model, we used NSC-34 cell line that had been transfected either with wild type human SOD1 (NSC-34/hSOD1$_{wt}$) or mutated in G93A (NSC-34/hSOD1$_{G93A}$) (Gomes *et al.*, 2008). In recent work from our lab, accumulation of mutated SOD1 was shown to occur after 4 days of differentiation (DIV) in NSC-34/hSOD1$_{G93A}$ cells, together with cell dysfunction (Vaz *et al.*, 2013), which may represent the progression of MN degeneration in familiar ALS (fALS). Therefore, in our model, we considered three different time points after NSC-34 cell differentiation that could mimic three stages of MN degeneration in ALS: (i) 1 DIV – prior to SOD1 accumulation (onset); (ii) 4 DIV – during SOD1 accumulation (symptomatic) and (iii) 7 DIV – after SOD1 accumulation and cell damage.

We first characterized NSC-34/hSOD1$_{wt}$ or NSC-34/hSOD1$_{G93A}$ in terms of their morphology and viability at different days of differentiation, in order to establish a timeline of MN degeneration that mimics ALS progression. As shown in **Figure III.1**, there is an increase in the number of neurites along NSC-34/hSOD1$_{wt}$ differentiation, as well as in their extension and ramification, together with a reduction in cell soma, which was observed through βIII-Tubulin immunostaining. We next analyzed cellular loss of viability, namely necrosis and apoptosis using flow cytometry. As indicated in **Table III.1**, there is almost no variation of total cellular viability after 1 DIV but within time, although not statistically significant, there is a decrease in cell viability for NSC-34/hSOD1$_{G93A}$ after 4 DIV (~15%) or after 7 DIV (~22%). Moreover, through the immunostaining for βIII-Tubulin, it was observed that within the time after differentiation, both cell lines (NSC-34/hSOD1$_{wt}$ or NSC-34/hSOD1$_{G93A}$) have a decreased number of cells, and that the fluorescence intensity becomes weaker, indicating less βIII-

Tubulin content (**Figure III.1**). This effect was even more pronounced in NSC-34/hSOD1$_{G93A}$ cells, especially after 4 and 7 DIV.

Results of flow cytometry indicated that there is an increase in apoptosis in NSC-34/hSOD1$_{G93A}$ cells after 4 and 7 DIV when compared to respective NSC-34/hSOD1$_{wt}$ (n.s.), as indicated in **Table III.2** and in **Figure III.2**; by contrast we did not observed considerable cell death by necrosis.

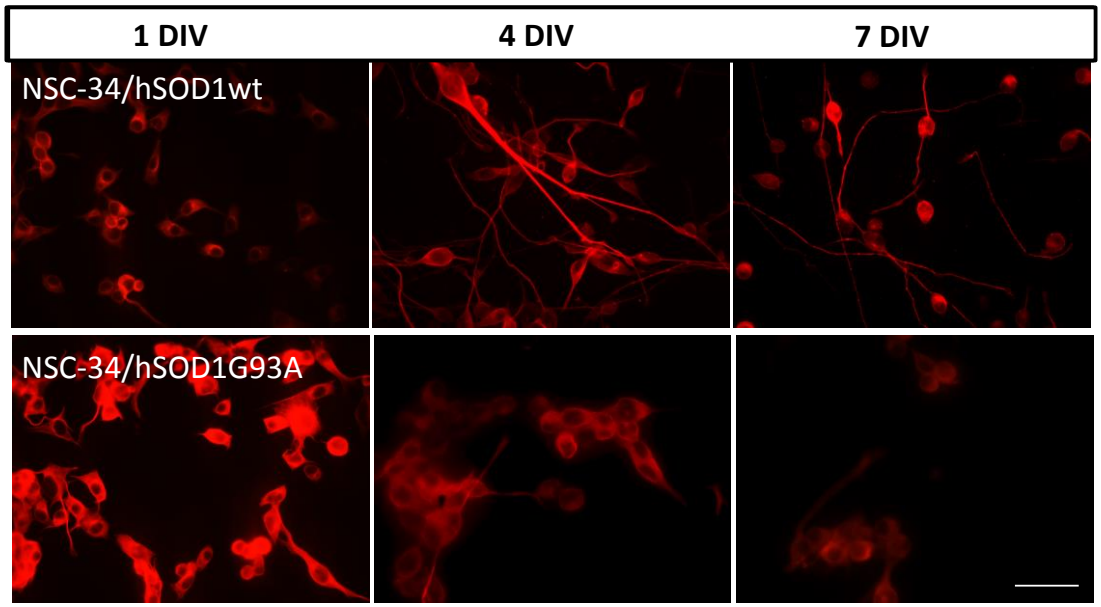


Figure III.1 – Differentiated NSC-34/hSOD1$_{G93A}$ cells have less βIII-Tubulin content, suggesting compromised cell viability. Cells were cultured as indicated in Methods. After fixation with paraformaldehyde, cells were stained with an antibody against βIII-Tubulin, followed by a fluorescent-labeled secondary antibody (in red). Scale bar represents 40 μm.

Table III.1 - Percentage of viable cells in NSC-34 cultures decreases overtime.

		NSC-34/hSOD1 wt	NSC-34/hSOD1 G93A
Viable Cells (%)	1 DIV	94.74 ± 1.27	95.36 ± 1.26
	4 DIV	88.63 ± 2.19	83.55 ± 2.80
	7 DIV	85.89 ± 1.15	75.55 ± 5.51

Results are expressed as % per total number of events. Results are mean ± SEM from at least three independent experiments.

Table III.2 - Percentage of Early Apoptosis and Late Apoptosis/Necrosis in NSC-34 cultures shown a decrease in early apoptosis leads differentiation but almost no alteration in late apoptosis/necrosis.

		NSC-34/hSOD1 wt	NSC-34/hSOD1 G93A
Early Apoptosis (%)	1 DIV	**1.90 ± 0.70**	2.29 ± 1.00
	4 DIV	**7.83 ± 1.71**	13.87 ± 2.65
	7 DIV	**10.81 ± 1.05**	14.81 ± 2.18
Late Apoptosis/Necrosis (%)	1 DIV	**2.43 ± 0.56**	3.22 ± 1.26
	4 DIV	**3.68 ± 0.78**	3.75 ± 0.55
	7 DIV	**5.47 ± 0.71**	5.11 ± 0.86

Results are mean ± SEM from at least three independent experiments and represent the absolute values of **Figure III.2**.

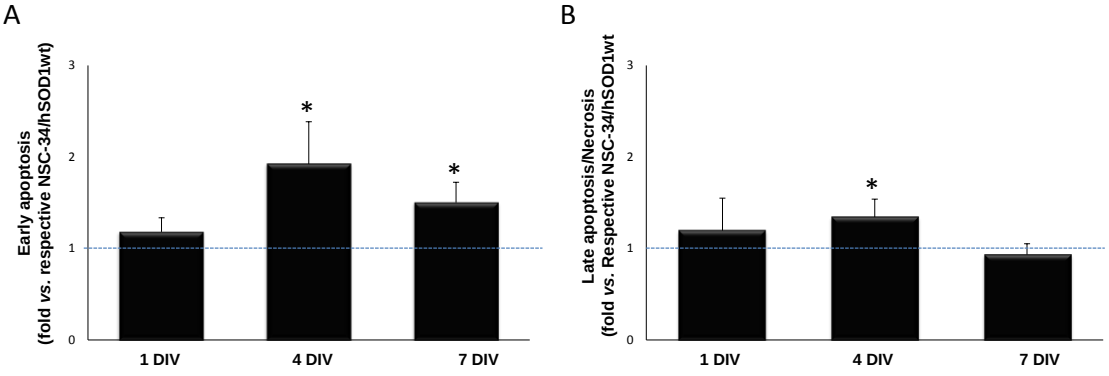


Figure III.2 – Differentiated NSC-34/hSOD1$_{G93A}$ cells have higher cell death levels, especially apoptosis after 4 and 7 days of differentiation. Cells were cultured as indicated in Methods and after differentiation they were trypsinized and labeled with Nexin Reagent®, for flow cytometry analysis. Results are expressed as fold *vs.* respective NSC-34/hSOD1$_{wt}$ condition and are mean ± SEM from three independent experiments. Corresponding absolute values are presented in Table III.2. Dotted line: Fold of respective NSC-34/hSOD1$_{wt}$ (control). $^{*}p<0.05$ *vs.* respective control.

1.2 Exploring mitochondrial dynamics/dysfunction in NSC-34/hSOD1$_{G93A}$

After having observe a decrease in the staining with βIII-Tubulin at 4-7 DIV and based on previous studies from our group demonstrating general loss of mitochondrial viability after 4 DIV (Vaz *et al.*, 2013), we further explored the mechanisms underlying mitochondrial dysfunction that could be involved in MN degeneration in ALS.

Dynamin-related protein 1 (Drp1) and Mitofusin 1 (Mfn1) are modulators of mitochondrial fission and fusion, respectively (Chapman *et al.*, 2013; Peng *et al.*, 2012; Peng *et al.*, 2013), which makes these two processes very important for mitochondrial dynamic morphology and for the healthy cellular function (Cao *et al.*, 2013). Peng and colleagues showed that in transgenic mice with the G93A human SOD1 mutation, Mfn1 and Drp1 suffer a significant increase in the anterior half of the

lumbar spinal cord before the onset of ALS, suggesting that the balanced mitochondrial morphology becomes altered by fission and fusion in MNs in this ALS model (Peng *et al.*, 2012). In order to examine mitochondrial dysfunction in NSC-34 cells, we quantified the content of these two proteins over at 1, 4 and 7 DIV, as previously, through an immunocytochemistry assay. As indicated in **Table III.3** and **Figure III.3 (A,C)**, the fluorescence intensity of Drp1 significantly decreased at 1 DIV and increased at 4 or 7 DIV in NSC-34/hSOD1$_{G93A}$ ($p<0.01$, *vs*. respective NSC-34/hSOD1$_{wt}$), with the main peak at 4 DIV, indicating enhanced fission only after at time points where SOD1 accumulation and MN degeneration occurs. Regarding Mfn1 (Table III.3 and Figure III.3B,D), we also noticed an increase in the fluorescence intensity of NSC-34/hSOD1$_{wt}$ from 1 DIV to 4 DIV, suggesting an increase in fusion process of the healthy cells, whereas the levels of Mfn1 in NSC-34/hSOD1$_{G93A}$ remains practically constant during NSC-34 differentiation ($p<0.01$, *vs*. respective NSC-34/hSOD1$_{wt}$). These results may suggest that in our model of MN degeneration, mitochondrial dysfunction occurs mainly through fission process instead of fusion.

We have also determined other hallmarks of mitochondrial dysfunction at 4 and 7 DIV, such as the release of ATP and NO to the extracellular medium. As indicated in **Table III.4** and **Figure III.4**, there was an increase in the release of ATP in NSC-34/hSOD1$_{G93A}$ after 7 DIV together with a significant increase in NO release after 4 but specially after 7 DIV ($p<0.05$, $p<0.01$ *vs.* NSC-34/hSOD1$_{wt}$). The deregulation of the production/release of these molecules may indicate not only mitochondrial impairment at the level of the respiratory chain function (Ghiasi *et al.*, 2012) but also oxidative stress, an important feature of ALS disease (Duffy *et al.*, 2011).

Table III.3 – Differentiated NSC-34/hSOD1$_{G93A}$ reveal mitochondrial dysfunction, involving fission and fusion processes.

		NSC-34/hSOD1 WT	NSC-34/hSOD1 G93A
Drp1	**1 DIV**	70.33 ± 3.95	48.60 ± 6.10*
	4 DIV	33.23 ± 12.94	79.99 ± 20.67
	7 DIV	56.95 ± 22.25	68.68 ± 5.64
Mfn1	**1 DIV**	76.71 ± 44.46	35.15 ± 6.46
	4 DIV	121.54 ± 36.76	41.99 ± 12.13
	7 DIV	103.49 ± 20.60	42.83 ± 4.58*

The integrated density of these proteins was measured using ImageJ Software and represents the area above the threshold for the mean density minus the background. Results are mean ± SEM from at least three independent experiments and represent the absolute values of Figure III.3. *$p<0.05$ *vs.* respective NSC-34/hSOD1$_{wt}$.

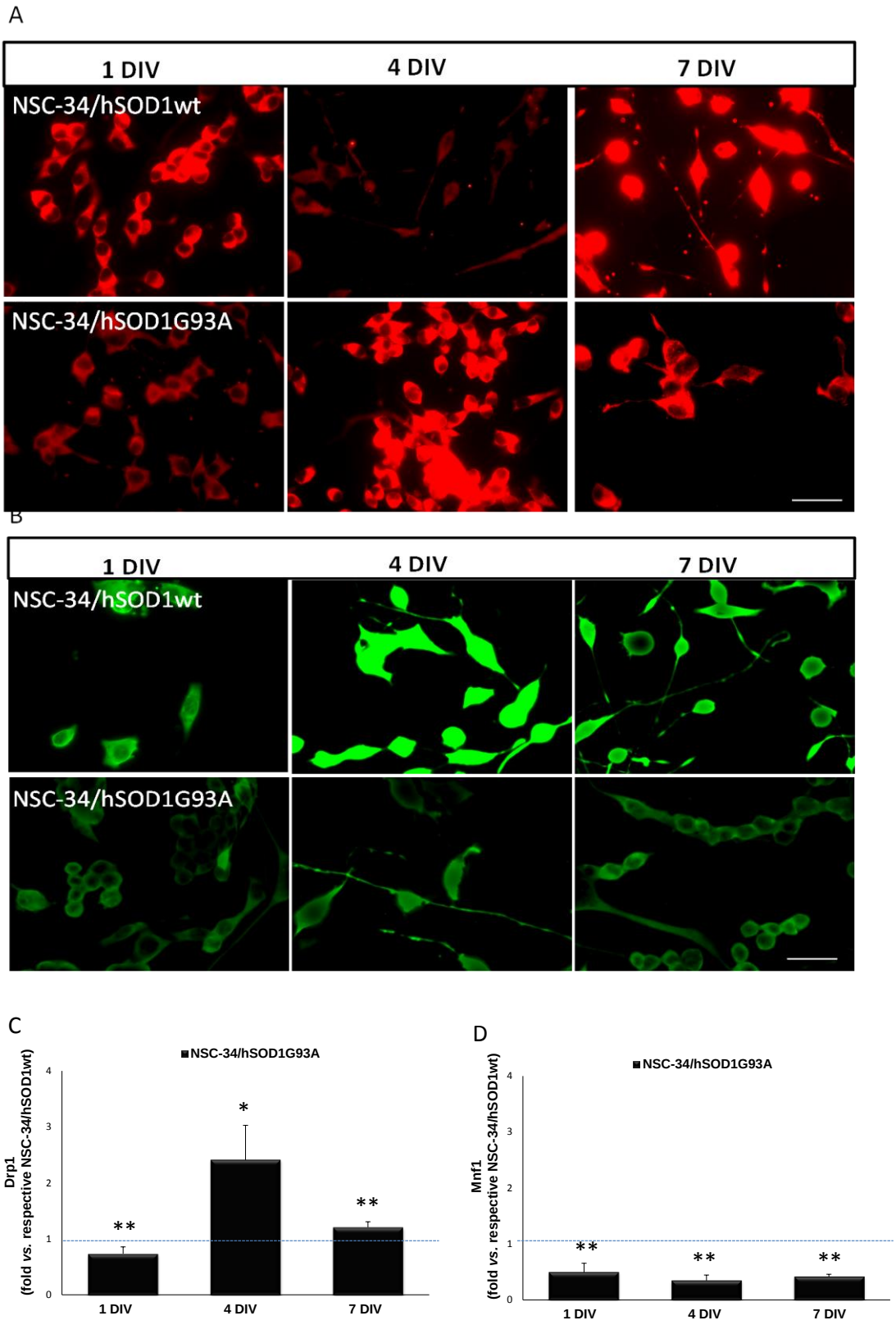


Figure III.3 – Differentiated NSC-34/hSOD1$_{G93A}$ have mitochondrial dysfunction, which involves fission and fusion processes. Cells were cultured as indicated in Methods and after differentiation they were fixed and stained with antibody against Drp1 (A) or Mfn 1 (B). Integrated density of Drp1 (C) and Mfn1 (D) was measured as described in Methods. Results are expressed as fold *vs.* respective NSC-34/hSOD1$_{wt}$ condition and are mean ± SEM from three independent experiments. Corresponding absolute values are shown in Table III.3. Dotted line: Fold of respective NSC-34/hSOD1$_{wt}$ (control). **p<0.01 and p<0.05 *vs.* respective control. Scale bar represents 40 µm.

Table III.4– Differentiated NSC-34/hSOD1$_{G93A}$ release increased levels of extracellular nitric oxide (NO) and Adenosine Triphosphate (ATP), thus reinforcing mitochondrial dysfunction.

		NSC-34/hSOD1wt	NSC-34/hSOD1G93A
NO	**4 DIV**	1.18 ± 0.11	1.82 ± 0.20*
	7 DIV	0.88 ± 0.05	2.50 ± 0.31**
ATP	**4 DIV**	25.51 ± 0.95	28.26 ± 1.52
	7 DIV	28.59 ± 0.80	37.15 ± 3.38*

Cells were cultured as indicated in Methods. After differentiation, the extracellular contents in NO was measured by the Griess reaction and ATP were determined by an enzymatic assay. Data are expressed in µM and are mean ± SEM from at least three independent experiments. These results represent the absolute values shown in Figure III.4. **p<0.01 , *p<0.05 *vs.* respective NSC-34/hSOD1$_{wt}$.

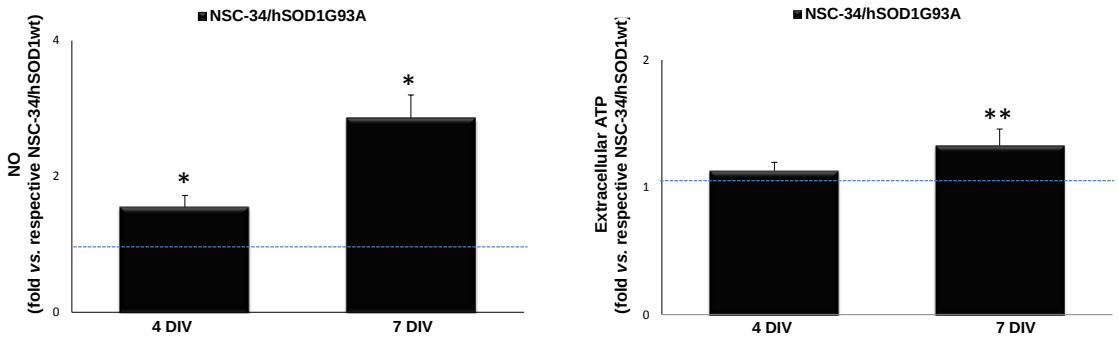


Figure III.4 – Differentiated NSC-34/hSOD1$_{G93A}$ release increased of nitric oxide (NO) and Adenosine Triphosphate (ATP), thus reinforcing mitochondrial dysfunction. Cells were cultured as indicated in Methods and after differentiation NO contents was measured by Griess reaction (A) and ATP extracellular levels were determined by an enzymatic assay (B). Results are expressed as fold *vs.* respective NSC-34/hSOD1$_{wt}$ condition and are mean ± SEM from three independent experiments. Corresponding absolute values are presented in Table III.4. Dotted line: respective NSC-34/hSOD1$_{wt}$ (control). **p<0.01, *p<0.05 *vs.* respective control.

1.3 Evaluation of Axonal transport dysfunction

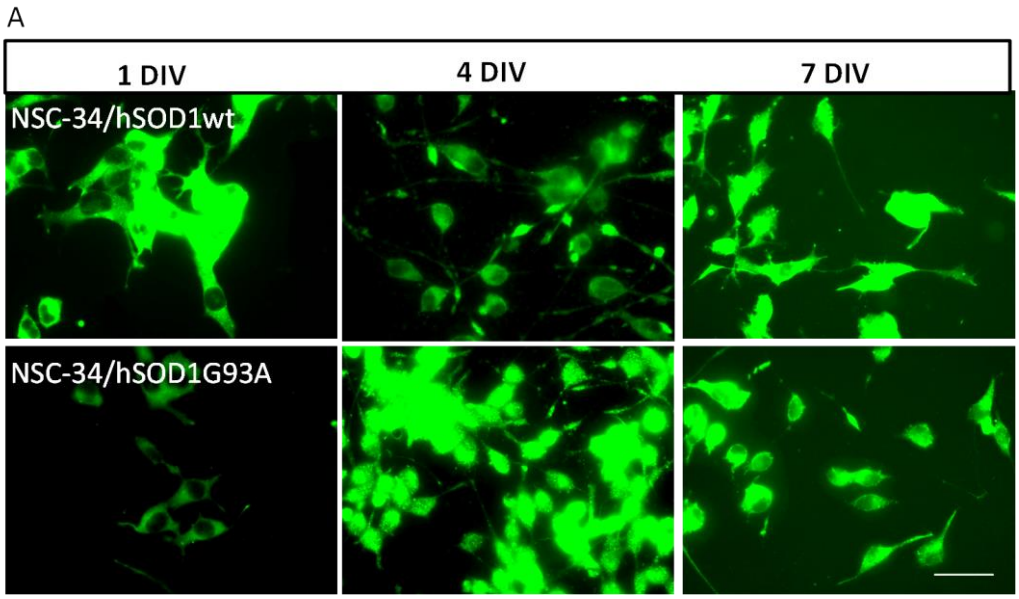
The immunostaining for βIII-Tubulin suggested that the axonal length of MN seemed to decrease along time of differentiation in NSC-34/hSOD1$_{G93A}$ cells (**Figure III.1**). This led us to deeper analyze the impairment of axonal transport, which is also referred as a hallmark of ALS (Ikenaka *et al.*, 2012). We focus on the quantification of the fluorescence of kinesin and dynein, which are the two main molecular motors in the axonal transport, respectively anterograde and retrograde (Siegel *et al.*, 2006). As indicated in **Table III.5** and **Figure III.5 (A,C)**, kinesin expression decreased in NSC-34/hSOD1$_{G93A}$ after 1 (p<0.01 *vs*. respective NSC-34/hSOD1$_{wt}$) or 7 DIV, which is consistent with anterograde axonal transport dysfunction described in ALS (Kuzma-Kozakiewicz *et al.*, 2013). The fact

that kinesin levels were hugely increased in NSC-34/hSOD1$_{G93A}$ after 4 DIV (n.s.) led us to the hypothesis that after early axonal dysfunction (1 DIV), MN activate some signaling mechanism in order to compensate the injury. However, this compensatory mechanism fails, justifying the decrease observed in NSC-34/hSOD1$_{G93A}$ after 7 DIV. In agreement, dynein levels were also decreased in NSC-34/hSOD1$_{G93A}$ after 1 and 7 DIV ($p<0.05$, $p<0.01$ *vs.* NSC-34/hSOD1$_{wt}$) but not after 4 DIV, as we can see in **Table III.5** and **Figure III.5 B,D** ($p<0.05$ *vs.* NSC-34/hSOD1$_{wt}$).

Table III.5 – Differentiated NSC-34/hSOD1$_{G93A}$ reveal axonal transport impairment

		NSC-34/hSOD1 wt	NSC-34/hSOD1 G93A
Kinesin	**1 DIV**	196.09 ± 5.08	83.27 ± 20.98*
	4 DIV	117.49 ± 17.25	251.64 ± 76.79
	7 DIV	171.60 ± 22.09	154.47 ± 39.27
Dynein	**1 DIV**	178.31 ± 5.76	111.58 ± 21.09
	4 DIV	134.31 ± 10.41	164.58 ± 24.55
	7 DIV	163.04 ± 16.60	75.29 ± 11.88*

The integrated density of these proteins was measured using ImageJ Software and represent the area above the threshold for the mean density minus the background. Results are mean ± SEM from at least three independent experiments and represent the absolute values of Figure III.6. *$p<0.05$ *vs*. respective NSC-34/hSOD1$_{wt}$.



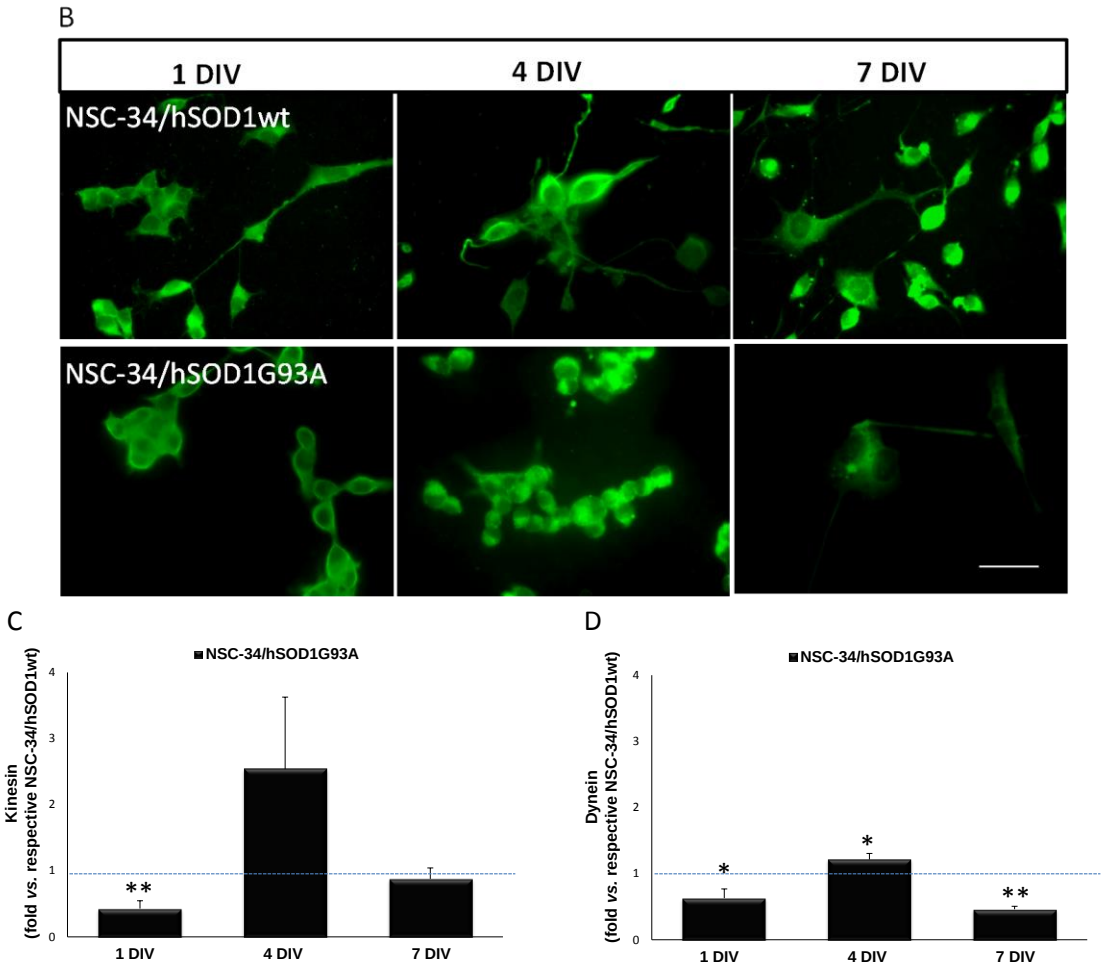


Figure III.5 – Differentiated NSC-34/hSOD1$_{G93A}$ have axonal transport impairment. Cells were cultured as indicated in Methods and after differentiation they were fixed and stained with antibody against Kinesin (A) or Dynein (B). Integrated density of Kinesin (C) and Dynein (D) was measured as described in Methods. Results are expressed as fold *vs.* respective NSC-34/hSOD1$_{wt}$ condition and are mean ± SEM from three independent experiments. Corresponding absolute values are presented in Table III.5. Dotted line: Fold of respective NSC-34/hSOD1$_{wt}$ (control). **p<0.01, *p<0.05 *vs.* respective control. Scale bar represents 40 µm.

2. Mixed Cultures

After analyzing the mitochondrial and axonal dysfunction in NSC-34-MN-like cells, we considered that it would be interesting to develop a model where cellular cross-talk with microglia was taken into account, since increasing evidence point microglia as key players for MN degeneration in ALS (Ferraiuolo *et al.*, 2011). For that we used N9 cells, which are a cell line obtained from CD1 mice cortex and that has proven to undergo microglial activation features such as migration, phagocytosis or inflammation-related features (Bruce-Keller *et al.*, 2000; Fleisher-Berkovich *et al.*, 2010). We used these N9 microglial cells in mixed culture with NSC-34, either with hSOD1$_{wt}$ or hSOD1$_{G93A}$.

2.1 Implementation and characterization of mixed cultures

At this point of the work, we abolished the 1 DIV of NSC-34 condition since we have pointed more striking alterations after 4 and 7 DIV in NSC-34/hSOD1G9A in section 1 from Results. It was possible to produce cultures from NSC-34/hSOD1$_{wt}$ or NSC-34/hSOD1$_{G93A}$ after 4 and 7 DIV in mixed culture with N9 cells, added at 0 or 2 DIV, to evaluate what happens before (0 DIV) or after (2 DIV) MN damage occurs. *In vivo*, on average the amounts of microglia and MN are near the ratio of 1/3, so NSC-34 cells were cultured at 5x10^4 cell/ml and N9 at 2x10^4 cell/ml (Silva *et al.*, 2011) and as represented in **Figure III.6,** our study model respects this ratio.

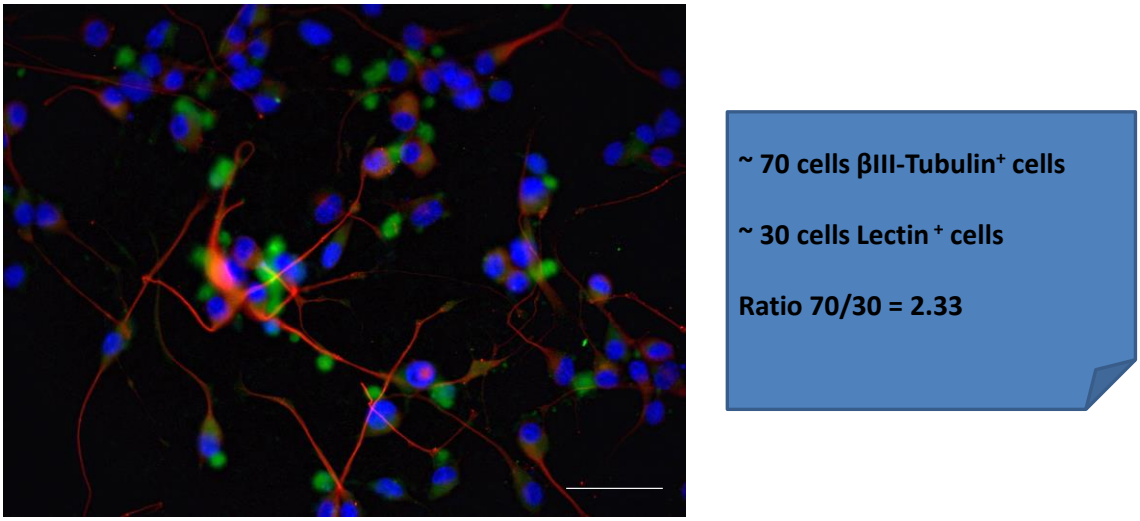


Figure III.6 – Mixed cultures of NSC-34 cell line and microglial cells from N9 cell line at 4 DIV were successfully implemented and represent the ratio 3/1 as previously described (Silva et al, 2011). Cells were cultured as indicated in Methods. (A) After fixation with paraformaldehyde, cells were double-stained with mouse anti-βIII-tubulin for neurons and with rabbit anti-lectin for microglia followed by a fluorescent-labeled secondary antibody (neurons in red and microglia in green), and counterstained with Hoechst® for the nuclei (in blue). Scale bar represents 40 µm**.**

2.2 Evaluation of mitochondrial function in mixed cultures

After the implementation of the mixed culture model, we analyzed the parameters found to be altered on the section 1 from Results. As presented in **Table III.6** and **Figure III.7**, the presence of microglia in mixed cultures reduced NO and ATP release after 7 DIV ($p<0.05$ *vs*. respective NSC-34/hSOD1$_{G93A}$ w/o microglia). Interestingly, microglia was more effective in reducing NO levels if added at the time of differentiation (0 DIV), but ATP release was only reduced if microglia was added after 2 DIV ($p<0.05$ *vs*. respective NSC-34/hSOD1$_{G93A}$ w/o microglia). These results suggest that microglia can have different signaling mechanisms that can be modulated by the factors produced/released by degenerating MN.

It will be interesting to continue the study concerning mitochondrial function (staining against Drp1, Mfn1, Kinesin and Dynein) in mixed cultures, as well as cell death pathways. Our preliminary

results suggest that the presence of N9 cells added at 0 DIV decrease cell viability and increase early apoptosis in NSC-34/hSOD1$_{G93A}$, either after 4 or 7 DIV (supplementary data S1).

Table III.6 – Differentiated NSC-34/hSOD1$_{G93A}$ cells shown altered metabolic function, evidenced by the increase of production/release of nitric oxide (NO) and Adenosine Triphosphate (ATP), which are modulated by the presence of microglia in mixed cultures.

			w/o N9	+ N9 at 0 DIV	+ N9 at 2 DIV
NO	4 DIV	NSC-34/hSOD1wt	1.18 ± 0.11	1.09 ± 0.19	1.08 ± 0.13
		NSC-34/hSOD1G93A	1.82 ± 0.20*	1.95 ± 0.21	2.21 ± 0.25
	7 DIV	NSC-34/hSOD1wt	0.88 ± 0.05	1.10 ± 0.09	1.98 ± 0.33
		NSC-34/hSOD1G93A	2.50 ± 0.31**	1.74 ± 0.34	3.94 ± 0.12$^{\$\$}$
ATP	4 DIV	NSC-34/hSOD1wt	25.51 ± 0.95	24.92 ± 1.07	25.49 ± 1.26
		NSC-34/hSOD1G93A	28.26 ± 1.52	26.25 ± 1.12	27.45 ± 2.09
	7 DIV	NSC-34/hSOD1wt	28.59 ± 0.80	28.24 ± 1.54	28.47 ± 1.38
		NSC-34/hSOD1G93A	37.15 ± 3.38*	32.33 ± 2.83	28.90 ± 1.61$^{\#}$

Results are expressed in µM and are mean ± SEM from at least three independent experiments, representing the absolute values of Figure III.8. **p<0.01, *p<0.05 *vs.* respective NSC-34/hSOD1$_{wt}$; $^{\#}$p<0.05 *vs.* NSC-34/hSOD1$_{G93A}$ w/o microglia; $$p<0.01 *vs.* respective 4 DIV.

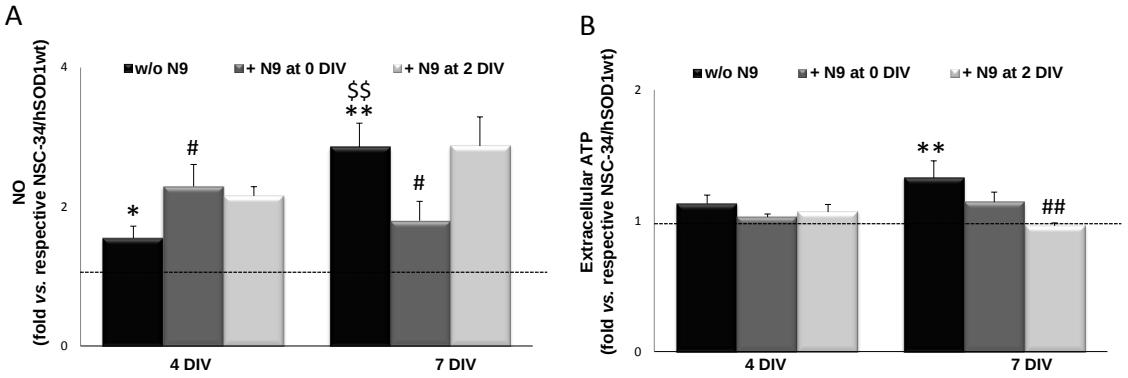


Figure III.7 – Differentiated NSC-34/hSOD1$_{G93A}$ cells have altered metabolic function, evidenced by increased production/release of nitric oxide (NO) and Adenosine Triphosphate (ATP), which are modulated by the presence of microglia in mixed cultures. Cells were cultured as indicated in Methods and after differentiation NO release was measured by Griess reaction (A) and ATP extracellular levels were determined by an enzymatic assay (B). Results are expressed as fold *vs.* respective NSC-34/hSOD1$_{wt}$ condition and are mean ± SEM from three independent experiments. Corresponding absolute values are presented in Table III.6. Dotted line: respective NSC-34/hSOD1$_{wt}$ (control). **p<0.01, *p<0.05 *vs.* respective NSC-34/hSOD1$_{wt}$ condition; #p<0.05, ##p<0.01 *vs.* NSC-34/hSOD1$_{G93A}$ w/o microglia; $$p<0.01 *vs.* respective 4 DIV.

3. Organotypic Cultures

Organotypic cultures are a model that is biochemically and physiologically more similar to the *in vivo* tissue since it preserves interneuronal connections and key processes. Moreover, it is a powerful model to screen the efficacy of potential therapies (Su *et al.*, 2011), as we wanted to do with GUDCA. Indeed, recent studies in our lab using MN cell line carrying mSOD1 have shown preventive effects by glycoursodeoxycholic acid (GUDCA) over MN dysfunction by reducing apoptosis, mitochondrial failure, as well as nitric oxide (NO) release and matrix metalloproteinase-9 activation. Interestingly, GUDCA also revealed ability to delay the intracellular accumulation of SOD1 in those cells (Vaz *et al.*, 2013). This is not without precedent since GUDCA has already shown anti-oxidant, anti-apoptotic and anti-inflammatory properties (Fernandes and Brites, 2009). Therefore, we tried to set-up an organotypic slice culture model obtained from spinal cord (SC) of 7-days mice carrying the human SOD1 mutation (TgSOD1-G93A mice) to study the mechanisms involved in cellular degeneration and to test potential neuroprotective agents, such as GUDCA. Organotypic cultures from SC of 7-days SJL WT mice were used as controls.

3.1 Implementation and characterization of SC organotypic cultures

Here, we focused our attention on lumbar segments from SC, since it is considered one of the most affected neural tissues in ALS (Staats *et al.*, 2013). We were able to implement the proposed model and after 11 days in culture we observed an intact and functional structure of the lumbar tissue where it was possible to identify posterior and anterior horn, as well as central canal, as schematically represented in **Figure III.8** after histological analysis.

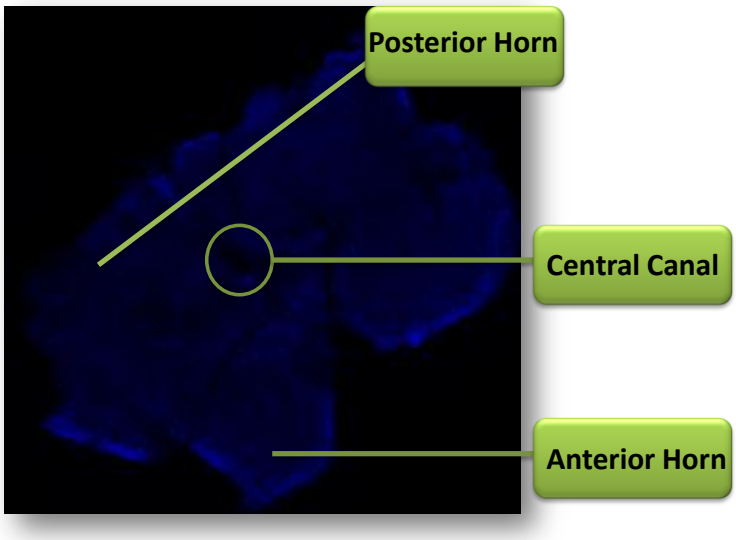


Figure III.8 – Schematic representation of a transversal section of the lumbar spinal cord. Organotypic cultures were performed from 7-days SJL (Wt) mouse and maintained in culture as described in Methods. Original magnification 100 x.

3.2 Glycoursodeoxycholic acid is able to prevent cell demise that occurs in TgSOD1-G93A SC cultures

We have just started the incubations of the SC organotypic cultures with GUDCA. As indicated in **Figure III.9**, preliminary results demonstrated decreased cell viability in cultures from TgSOD1-G93A mice ($p<0.05$ *vs*. SJL WT). GUDCA did not affect extracellular ATP and NO levels in slices from TgSOD1-G93A (**Table III.8** and **Figure III.10**) but, interestingly, although no yet significantly, prevented loss of cell viability. These preliminary results seems to be a starting point for the study of the mechanisms involved in cellular degeneration in the SC of ALS models and how they can be modulated by promising compounds such as GUDCA.

Table III.7 – Preliminary data point that spinal cord cultures from transgenic mice have decreased viability which is suggested to be recovered by Glicoursodeoxycholic acid (GUDCA).

		wt	TgSOD1-G93A
DAPI	W/o GUDCA	25.97 ± 2.32	20.26 ± 4.19
	GUDCA (50 µM)	31.91 ± 7.78	27.82 ± 3.04

Slices were stained with DAPI and the integrated density was measured using ImageJ Software and represent the area above the threshold for the mean density minus the background. Results are mean ± SEM from at least two independent experiments and represent the absolute values of Figure III.10.

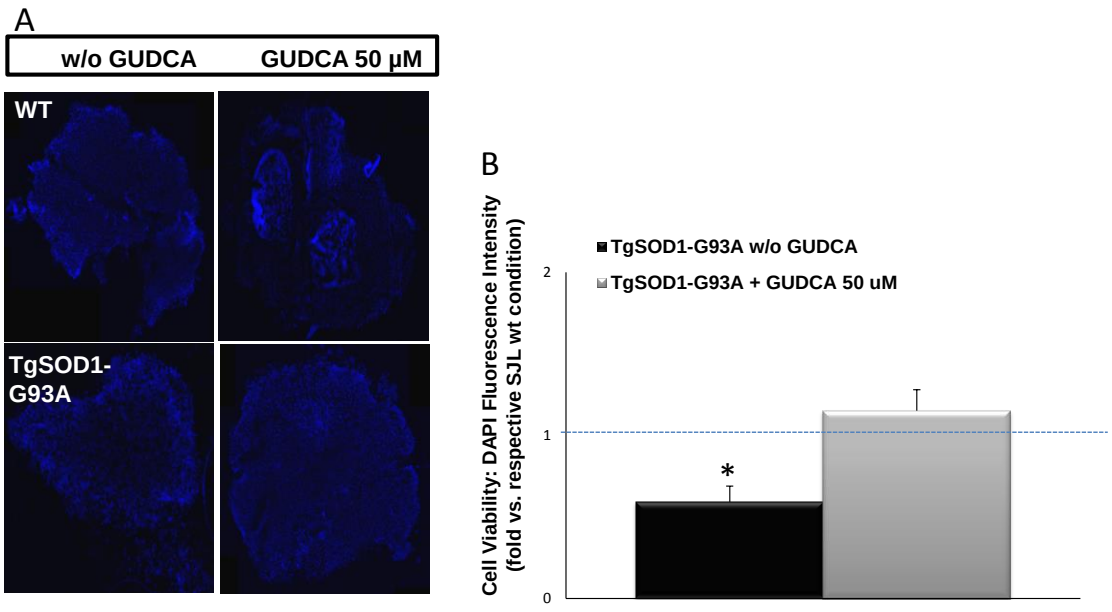


Figure III.9 – Preliminary results suggest that GUDCA may prevent cell death in organotypical spinal cord cultures from TgSOD1-G93A. Lumbar spinal cord slices were cultured for 10 days and incubated with 50 µM GUDCA for 24 h, as indicated in Methods. After incubation, slices were fixed in paraformaldehyde and nuclei were stained with DAPI (A). Total number of cells was assessed by quantification of integrated density,

considering the area above the threshold for the mean density minus the background and are presented in fold versus respective Wt condition (B). Corresponding absolute values are presented in Table III.7. Dotted line: respective SJL Wt (control). * p<0.05 *vs.* respective control.

Table III.8 – TgSOD1-G93A Spinal Cord slices do not differ from Wt ones in terms of Nitric Oxide (NO) and ATP release, even upon addition of Glycoursdeoxycholic acid (GUDCA).

		wt	TgSOD1-G93A
NO	W/o GUDCA	1.05 ± 0.10	1.11 ± 0.10
	GUDCA 50 µM	0.83 ± 0.07	1.01 ± 0.11
ATP	W/o GUDCA	25.51 ± 0.95	24.92 ± 1.07
	GUDCA 50 µM	28.59 ± 0.80	28.24 ± 1.54

Results are expressed in µM and are mean ± SEM from at least three independent experiments, representing the absolute values of Figure III.11.

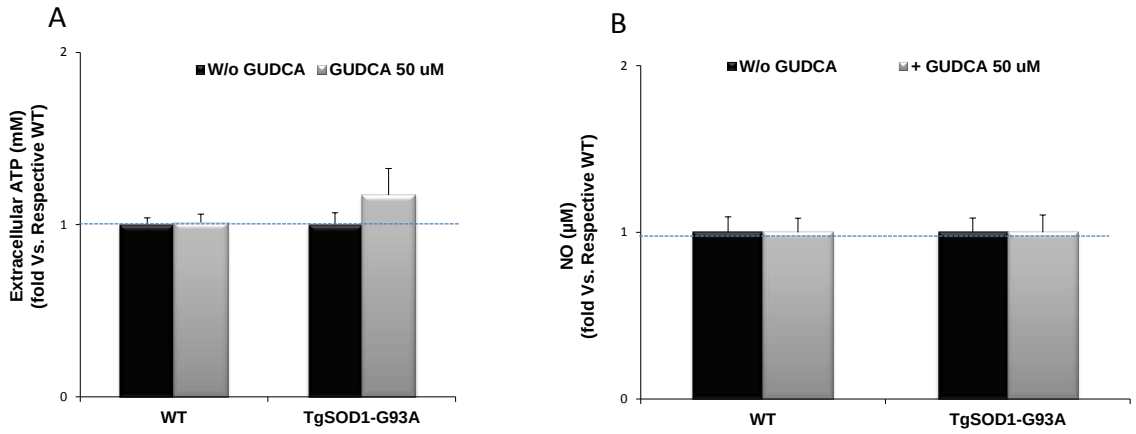


Figure III.10 – TgSOD1-G93A Spinal Cord slices do not differ from Wt ones in terms of NO and ATP release even upon addition of Glycoursdeoxycholic acid (GUDCA). Lumbar spinal cord slices were cultured for 10 days and incubated with 50 µM GUDCA for 24 h, as indicated in Methods. After incubation, extracellular media was assessed for (A) NO production/release by Griess reaction and absorbance was measured in the microplate reader or (B) ATP release by an enzymatic assay and fluorescence intensity was quantified using a fluorimeter. Results are mean ± SEM from at least two independent experiments. Dotted line: respective NSC-34/hSOD1$_{wt}$ (control).

4. Supplementary Data:

4.1 Cell viability of NSC-34 cell line seems to be less committed in the presence of N9 cell line.

Once we analyzed the cell viability and the occurrence of cell death in pure culture of NSC-34, we also decided to analyze these events upon addition of microglia, but it was not yet possible to obtain statistical significance, so all the following results need confirmation. However, we can say that in general, seems to exist a tendency to increase cell viability and decrease early apoptosis and late apoptosis/necrosis in the presence of N9 cell line, both in NSC-34/hSOD1$_{wt}$ and NSC-34/hSOD1$_{G93A}$ **(Table S.1)**. This corroborates the possibility of microglia having a neuroprotective role in the context of the disease ALS. NSC-34/hSOD1$_{G93A}$ at 4 DIV is an exception since that seems to occur a decrease in viability and an increase in late apoptosis/necrosis **(Figure S.1)**.

Table S.1 – The presence of N9 cells seems to compromise the viability of NSC-34/hSOD1$_{G93A}$ cell line

			w/o N9	+ N9 at 0 DIV
Viable Cells (%)	4 DIV	NSC-34/hSOD1 wt	88.63 ± 8.76	95.90 ± 1.41
		NSC-34/hSOD1G93A	83.55 ± 10.10	71.50 ± 6.51
	7 DIV	NSC-34/hSOD1 wt	85.89 ± 4.31	94.30 ± 2.12
		NSC-34/hSOD1G93A	75.55 ± 22.04	91.05 ± 1.34
Early Apoptosis (%)	4 DIV	NSC-34/hSOD1 wt	7.83 ± 6.85	2.85 ± 1.06
		NSC-34/hSOD1G93A	13.87 ± 9.19	7.85 ± 0.78
	7 DIV	NSC-34/hSOD1 wt	10.81 ± 4.20	4.10 ± 1.41
		NSC-34/hSOD1G93A	14.81 ± 8.17	6.50 ± 0.99
Late Apoptosis/ Necrosis (%)	4 DIV	NSC-34/hSOD1 wt	3.68 ± 2.81	1.20 ± 0.28
		NSC-34/hSOD1G93A	3.75 ± 1.91	20.55 ± 5.73
	7 DIV	NSC-34/hSOD1 wt	5.47 ± 2.47	1.00 ± 0.28
		NSC-34/hSOD1G93A	5.11 ± 2.86	1.90 ± 0.28

Results are mean ± SD from one experiment and represent the absolute values of Figure S.1.

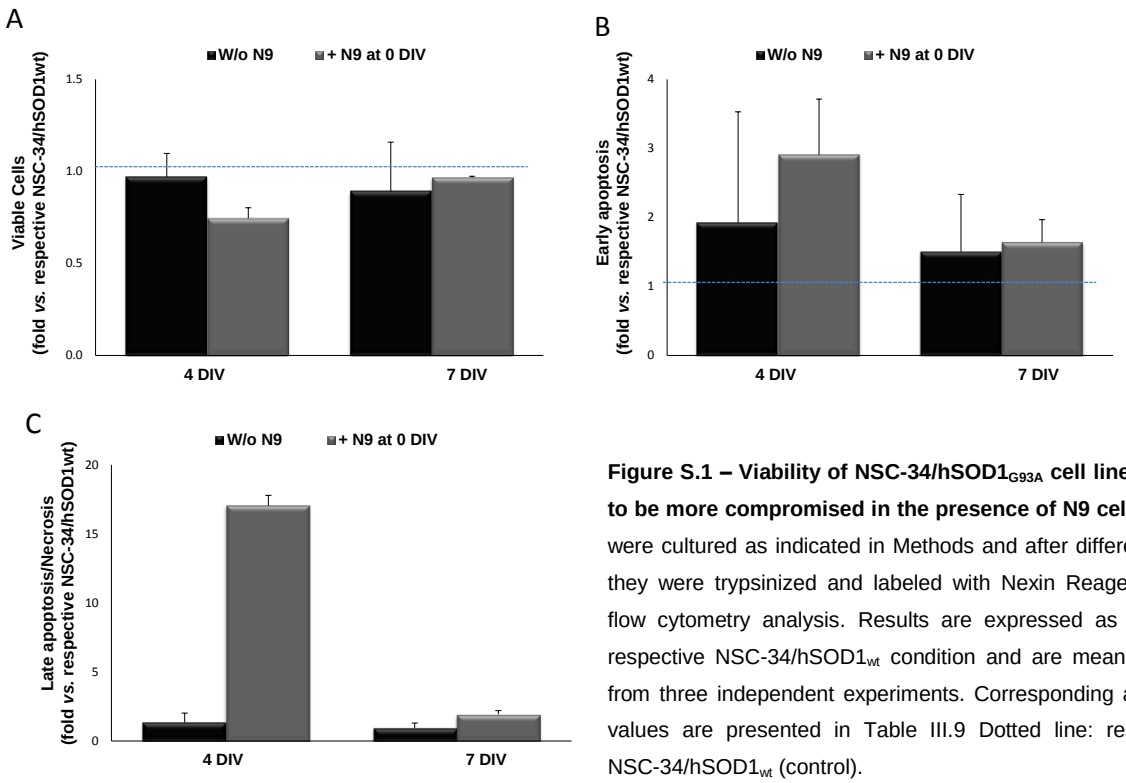


Figure S.1 – Viability of NSC-34/hSOD1$_{G93A}$ cell line seems to be more compromised in the presence of N9 cells. Cells were cultured as indicated in Methods and after differentiation they were trypsinized and labeled with Nexin Reagent®, for flow cytometry analysis. Results are expressed as fold *vs.* respective NSC-34/hSOD1$_{wt}$ condition and are mean ± SEM from three independent experiments. Corresponding absolute values are presented in Table III.9 Dotted line: respective NSC-34/hSOD1$_{wt}$ (control).

IV. Discussion

The impossibility to make real-time studies or to remove tissue samples repeatedly from the patients are limitations for the study of human neurodegenerative diseases (Turner *et al.*, 2013). Thus, the use of *in vitro, ex vivo* and *in vivo* animal models is essential. Among the familial cases in ALS, approximately 20% are caused by dominantly inherited mutations in the Cu/Zn superoxide dismutase-1 (SOD1) protein (Musaro, 2010), which provide a basis for many experimental *in vitro* and *in vivo* models including mutations in this enzyme.

This Thesis had as first aim the implementation and characterization of a mixed culture model. Until now, it is not clear what is the primary cause of motoneurons (MN) degeneration in ALS (Valori *et al.*, 2013). The MN-like cell line NSC-34 has been widely used for *in vitro* studies of ALS, once this cell line present many properties of MN, and also show morphological and physiological properties of them, like acetylcholine synthesis, storage and release; extension of processes; action potential generation; formation of contacts with cultured myotubes; expression of neurofilament proteins and association with neuromuscular synapse-specific basal lamina glycoproteins (Cashman et al., 1992; Tovar et al., 2009). Moreover, when transfected with human mutations of SOD1 (such as G93A mutation), these cells acquire some features reported in tissues of ALS patients and in transgenic mice models, such as mitochondrial dysfunction (Raimondi et al., 2006) and Golgi apparatus fragmentation (Gomes et al., 2008). Therefore, in the present study, we proposed to investigate the MN viability in general and the mitochondrial function and axonal transport in particular, in the context of the neuronal dysfunction in ALS progression. In addition, we aimed to analyze the interaction between microglia and MN in the context of the disease, in order to better understand the origin of the MN degeneration. In the last part of the study, we also tested potential neuroprotective agents in ALS models.

As presented in the Results section, we initiated the work by the study of isolated cultures of NSC-34. Recently, in our group it was demonstrated that NSC-34/hSOD1$_{G93A}$ cells presented accumulation of SOD1 after 4 days of differentiation (DIV), together with cell dysfunction (Vaz *et al.*, 2013), which may represent the progression of MN degeneration in familiar ALS. Thus, here we considered three different time points after NSC-34 cell differentiation: (i) 1 DIV – prior to SOD1 accumulation (onset); (ii) 4 DIV – during SOD1 accumulation (symptomatic) and (iii) 7 DIV – after SOD1 accumulation and MN cell death. Immunostaining assays against βIII-Tubulin could give us an idea of the behavior of NSC-34/hSOD1$_{wt}$ cells (our control condition) in culture, over the time after differentiation and evidenced that after 1 day *in vitro* (DIV), they have few branches and reduced length. After 4 DIV,

these cells seemed to present a large number of complex synaptic connections between them, indicating the presence of MN-like cells. However, after 7 DIV, it was an apparent decrease in the number of connections, as well as a decrease in the number of cells, which may be related to the stress inherent of the culture. So we decided to examine the cell viability and cell death events of NSC-34/hSOD1$_{G93A}$ comparing with NSC-34/hSOD1$_{wt}$ in each time point of differentiation. This study, either by staining against βIII-Tubulin (part of microtubules) and by flow cytometry, revealed that mutated cells have compromised viability, although with a lower extent through time in culture after differentiation. Relatively to cell death, we observed preferentially apoptosis rather than necrosis in NSC-34/hSOD1$_{G93A}$, which is in accordance with the fact of apoptosis is the most significant event of death described for ALS disease, according with the generality of authors (Ranganathan and Bowser, 2010).

After observing the occurrence of apoptosis and decreased cell viability in NSC-34/hSOD1$_{G93A}$ cells, and also because mitochondria plays a role in the apoptotic process, we decided to study more deeply the mitochondrial dysfunction. We have focus on fission and fusion processes since they are described to be implicated in neuronal injury and cell death (Barsoum *et al.*, 2006; Liot *et al.*, 2009; Yuan *et al.*, 2007). For this purpose, NSC-34/hSOD1$_{wt}$ and NSC-34/hSOD1$_{G93A}$ cells were immunostained against Drp1 and Mfn1, two key proteins in the balance between mitochondrial fission and fusion, respectively. We observed decreased levels of Drp1 after 1 DIV in NSC-34/hSOD1$_{G93A}$ ($p<0.01$ *vs.* NSC-34/hSOD1$_{wt}$) but also a significant increase after 4 DIV ($p<0.05$ *vs.* NSC-34/hSOD1$_{wt}$), indicating enhanced fusion only after at time points where SOD1 accumulation and MN degeneration occurs. The sudden increase in the expression of Drp1 from 1 to 4 DIV may be linked to increase in early apoptosis observed also at 4 DIV. In fact, it is described that the overexpression of Drp1 increases the vulnerability to mitochondrial fragmentation and neuronal cell death (Barsoum *et al.*, 2006), but how the impairment of these pathways lead to neurodegeneration is still a matter of debate (Ranieri *et al.*, 2013). By contrast, Mfn1 levels were decreased in NSC-34/hSOD1$_{G93A}$ during all time after differentiation ($p<0.01$ *vs.* NSC-34/hSOD1$_{wt}$), suggesting that in our model of MN degeneration, mitochondrial dysfunction occurs through events of fission and fusion. According to Song and colleagues (2013), this fact can be explained because mitochondrial fusion and fission are not independent and impact each, so defects in the fusion may result from increased fission rates (Song *et al.*, 2013).

Since a balance between mitochondrial fusion and fission is required for mitochondrial homeostasis, we further explored mitochondrial injury. In fact, measurement of ATP and NO levels in the extracellular media reveled an increase of both molecules in NSC-34/hSOD1$_{G93A}$ ($p<0.01$, $p<0.05$ *vs.* NSC-34/hSOD1$_{wt}$) and from 4 to 7 DIV, indicating increased production of ROS that will probably exacerbate oxidative stress. On the other hand, the increased ATP release to the extracellular media will cause depletion of cell energy because ATP may be not available to be used in the function of molecular motors that move between the soma and the axons of neurons, necessary for carrying crucial cargos to cell metabolism and homeostasis, such as mitochondria, neurofilaments and autophagosomes (Ikenaka *et al.*, 2012; Song *et al.*, 2013). Moreover, the impaired mitochondrial activity and a consequent decrease in the intracellular availability of ATP will contribute to modify

calcium homeostasis and ROS production, which may then lead to increased apoptosis (Federico *et al.*, 2012).

Impaired mitochondrial dynamics has been proposed to trigger axonal degeneration and is consistent with the "dying back" hypothesis of neuronal projections as a primary event in ALS pathogenesis (Chan, 2006; Dadon-Nachum *et al.*, 2011; Knott *et al.*, 2008). Thus, it is described the occurrence of axonal transport dysfunction as a biomarker of disease ALS, associated at mitochondrial dysfunction and cell death (Song et al., 2013). In fact, we observed that cellular processes are diminished in -34/hSOD1$_{G93A}$ throughout the differentiation, which is consistent with studies of Song and colleagues (2013) that described a reduction in neurite length and branching in neurons with G93A mutation. After 7 DIV cells seem to lose the ability to communicate with each other by shortening the axons, which may be linked to dysfunction of the carriage along them, with consequent impossibility of transporting essential molecules throughout the cell. In our model, we observed that kinesin levels diminished from NSC-34/hSOD1$_{wt}$ to NSC-34/hSOD1$_{G93A}$ at 1 and 7 DIV, which is consistent with anterograde axonal transport dysfunction. The fact that kinesin levels were hugely increased in NSC-34/hSOD1$_{G93A}$ after 4 DIV suggests that once more there is a dysfunction detected by MN-like cells which triggers the activation of a defense mechanism that will be induced to produce more kinesin to the equilibrium. Regarding dynein, involved in retrograde transport, similar to what we have seen for kinesin, their expression levels were reduced in NSC-34/hSOD1$_{G93A}$ cells after 1 and 7 DIV ($p<0.05$, $p<0.01$ *vs.* NSC-34/hSOD1$_{wt}$, respectively). This result indicates that retrograde transport is also impaired in our model, which is consistent with literature that shows dysfunction of dynein in different models of ALS (Soo *et al.*, 2011). Therefore, the dysfunction of the two types of transport does not appear to occur simultaneously at the same extent level. It appears that kinesin suffers a huge injury first but can be restored after some time, however the compromising of the level of dynein appears to occur less abruptly but the cells do not significantly recover after 4 DIV, remaining a deficit in the amount of this molecular motor at 7 DIV, which agrees with the study of axonal transport in mSOD1 mice model by Shi and colleagues (2010b), who describe the occurrence of a decreased speed of retrograde transport mediated by dynein in G93A in comparison with WT mice an early presymptomatic stage. Although Morfini and colleagues (2013) did not find the anterograde transport affected, there are several reports describing inhibition of both anterograde and retrograde axonal transport in mSOD1 mice (Bilsland *et al.*, 2010; Perlson *et al.*, 2009; Warita *et al.*, 1999). Indeed, kinesin and dynein are regulated by phosphorylation of specific subunits and an abnormal activation of protein kinases (such as p38 MAPK) are described in ALS (Bendotti *et al.*, 2004; Krieger *et al.*, 2003; Sharma *et al.*, 2010), so in 2009, Morfini and colleagues also published about the possibility of the pathogenic SOD1 in the activation of kinases.

There is increasing evidence pointing microglia, considered the primary immune cells of the CNS, as key players for MN protection or instead to MN injury and consequent cell death (Boillée *et al.*, 2006). Therefore, after the evaluations of cell viability and mitochondrial dynamics performed in cultures of NSC-34, we next aimed to evaluate these parameters in mixed cultures with microglia. For that, we implemented and characterized a model of mixed cultures with NSC-34 and N9 (microglia cell

line), in order to better understand the influence of microglia in these cellular processes and to assess whether their role is mainly neuroprotective or neurotoxic. In our model of mixed cultures we used healthy microglia and we evaluated if these cells were able to prevent, promote or restore MN neurodegeneration, and also to explore long term changes in microglia-MNs cross-talk.

First, we implemented and characterized the mixed culture in our lab with these two types of cells and we confirmed that N9 cells were able to maintain their capacities when moved from their proliferative medium to NSC-34 differentiation medium. In fact, N9 cells may be maintained in a similar medium, with the basis of Dulbecco's Modified Eagle Medium/F12 (Guo *et al.*, 2013). In addition, the ratio of cells grown initially is held for the 7 days of incubation. This last point is very important once the purpose of this study with mixed culture is to dissect the crosstalk between these two cell types, and so it is necessary to mimic as closely as possible the environment to which they are subject *in vivo*. Thus, we always kept in mind that the ratio NSC-34:N9 of 3/1 between MN and microglia should be respected in all mixed cultures (Silva *et al*., 2011).

After successfully implementation and characterization of mixed cultures, we quantified NO and ATP in the extracellular media, as in the first part with isolated NSC-34 cells.

We observed increased NO levels released by NSC-34/hSOD1$_{G93A}$ ($p<0.01$ *vs*. NSC-34/hSOD1$_{wt}$). These findings are in accordance with studies in the cerebrospinal fluid (CSF) and human postmortem CNS tissue from ALS patients that present biochemical changes reflecting production of reactive oxygen species and consequent oxidative stress (Ferrante *et al.*, 1997; Shaw *et al.*, 1995; Smith *et al.*, 1998; Tohgi *et al.*, 1999). In addition, mutated SOD1 has an incorrect folding, which impairs their antioxidant properties and favors the production of superoxide anion, and consequently peroxynitrite, ultimately causing tyrosine nitration (Barber and Shaw, 2010). In fact, a product of tyrosine nitration is 3-nitrotyrosine, which is widely detected in the MNs of sporadic cases with ALS (Abe *et al.*, 1997). Interestingly, when microglia were cultured with NSC-34/hSOD1$_{G93A}$ for longer time (2 or more DIV), extracellular NO levels were increased. These results suggest microglia activation, with consequent production and release of NO through an up-regulation of inducible nitric oxide synthase, such as the one found in microglia co-cultured with neurons after exposure to lipopolysaccharide (LPS) (Zhao *et al.*, 2004). When NSC-34/hSOD1$_{G93A}$ and microglia were maintained together until 7 DIV, it occurs a reduction of NO levels, suggesting a switch of microglia activation pattern.

Regarding ATP, we observed higher extracellular ATP levels in NSC-34/hSOD1$_{G93A}$, which may also promote activation microglia through purinergic receptors (D'Ambrosi *et al.*, 2009), probably as an attempt to resolve the extension of the lesion. Moreover, our results showed that interaction between microglia and NSC-34/hSOD1$_{G93A}$ for longer time (7 DIV) also leads to reduction in extracellular ATP levels. Indeed, ATP acts as a neuron-to-microglia alarm signal, through cell surface P2 receptors widely distributed throughout the CNS. In ALS patients (Yiangou *et al.*, 2006), as well as SOD1 $_{G93A}$ animals (Casanovas *et al.*, 2008), an increased immunoreactivity for P2X was found in SC microglia. This probably occurs because ATP binds to P2X receptors present in microglia, reducing their presence in extracellular media. Another aspect that we should keep in mind is that ATP release to the extracellular media can also function as a way to attract microglial cells, acting as a

chemoattractant for these cells, which may constitute an attempt to reverse the lesion (Corriden and Insel, 2012).

After the determinations with mixed cultures of NSC-34/N9, we proceeded to the implementation and optimization of organotypic cultures of lumbar segments of spinal cord (SC) from 7-days SJL (Wt) or TgSOD1-G93A mice. This type of cultures was chosen because they have benefits over animal models include the easy access and the precise control of the extracellular environment. Lumbar SC was here used once it is considered one of the most affected neural tissues in ALS (Chen *et al.*, 2010). Preliminary results with Wt and TgSOD1-G93A SC organotypic cultures did not evidence significant changes in NO release between both models, although a slight decrease in extracellular ATP of TgSOD1-G93A slices was noticed. SC from TgSOD1-G93A also exhibited a decreased in cell content.

Organotypic cultures are also a powerful model to screen the efficacy of compounds with potential neuroprotective effects (Su *et al.*, 2011), as we aimed to do with GUDCA, for which we had previously demonstrated their neuroprotective action in differentiated NSC-34/hSOD1$_{G93A}$ cells (Vaz *et al.*, 2013), such as in reducing apoptosis, mitochondrial failure, as well as nitric oxide release and matrix metalloproteinase-9 activation. This was not without precedent since GUDCA were already shown anti-oxidant, anti-apoptotic and anti-inflammatory properties (Fernandes and Brites, 2009).

In our model, cell dysfunction was slightly recovered by GUDCA when slices of SC were incubated with this compound, as well as the decrease of extracellular ATP. However these are still preliminary data which requires further confirmation.

The main conclusions obtained in this Thesis are shown in **Figure IV.1**. In summary, mixed and organotypic cultures were implemented successfully. Regarding isolated NSC-34-MN-like, we observed impairment of mitochondrial dynamics and axonal transport, together with an increase of NO and ATP release, as well as apoptotic cell death. The presence of microglia cultured with NSC-34/hSOD1$_{G93A}$ reduced NO and ATP release. Furthermore, we propose the possible protective properties of GUDCA, since the incubation with this compound recovered loss of cell viability and slightly reduced extracellular ATP levels in slices from TgSOD1-G93A. By uncovering different experimental models, our results contributed to the understanding of some molecular mechanisms involved in cellular failure during ALS progression, which is fundamental to develop new therapeutic strategies.

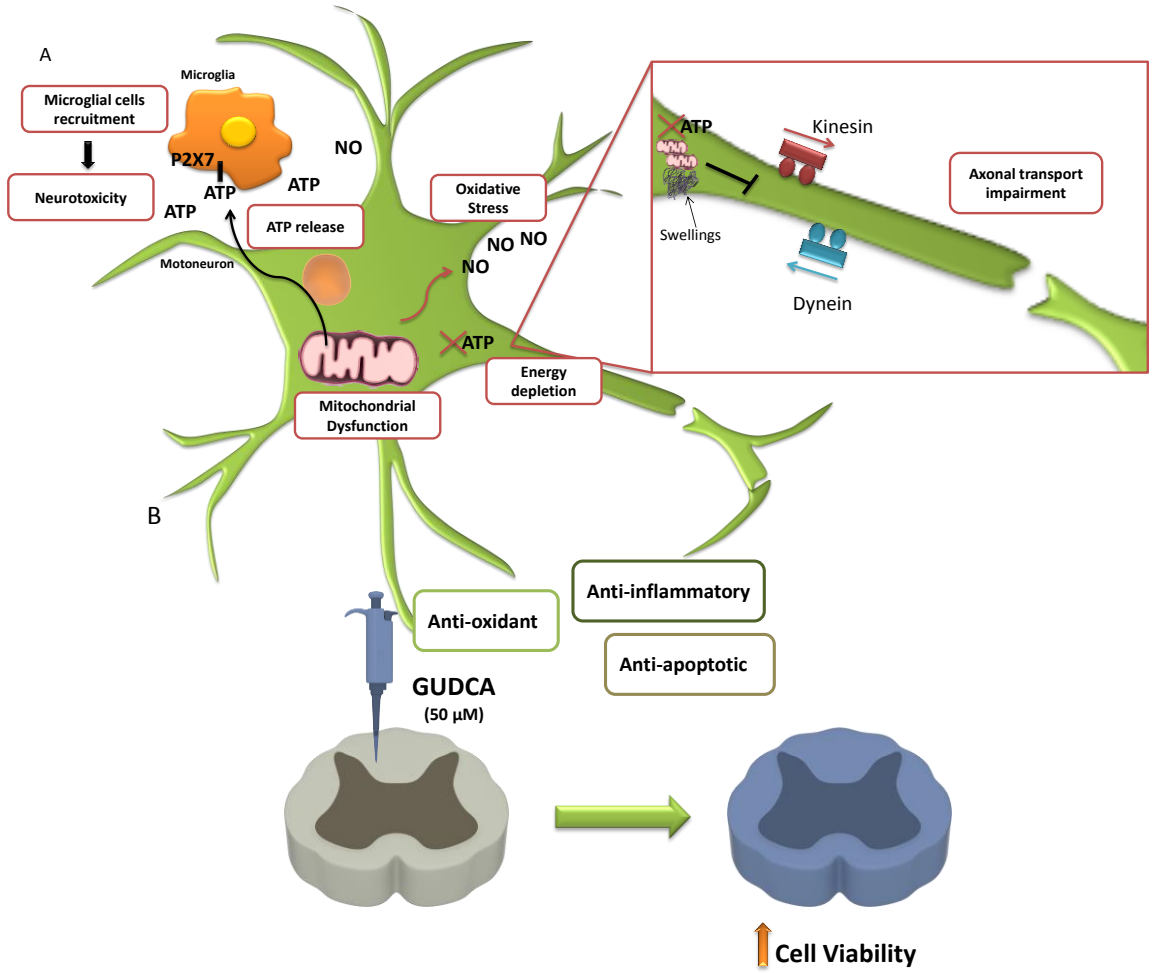


Figure IV.1 – Schematic representation of the major findings of this Master Thesis and the potential mechanisms that may be involved. (A) NSC-34 cells revealed mitochondrial dysfunction due to an imbalance between fusion and fission events. The Adenosine Triphosphate (ATP) efflux increased, which can be an alarm signal to microglia (N9 cell line), probably trough activation of the P2X7 receptor, promoting their migration to the lesion sites, where microglia can play a neuroprotective or neurotoxic role, depending on the extent of motoneuron (MN) damage. Nitric oxide (NO) release is also higher in mutated MN in comparison to normal conditions, causing oxidative stress. Moreover, the increase in the efflux of ATP is probably related with mitochondrial failure, thus justifying the altered content of molecular motors responsible for axonal transport, such as kinesin and dynein. All together, these events will contribute to an overall cell death, namely apoptosis, leading to the MN degeneration. (B) Organotypic spinal cord cultures showed that TgSOD1-G93A mice present higher levels of cellular degeneration, and preliminary data suggest that GUDCA have the ability to promote the prevention of cell death, confirming their anti-oxidant, anti-apoptotic and anti-inflammatory properties.

Future Perspectives

This study provides an increase in our knowledge regarding the pathophysiological events in the context of ALS. However, there is still a long way to go. It will be important to study more deeply the dysfunction of axonal transport, in particular at level of neurite length and branching as MNs degenerate within time. In the context of the mitochondrial dysfunction, it will be important to direct our studies for the understanding of possible signaling mechanisms that are activated upon MN injury, which seemed to help in the attempt to return to mitochondrial homeostasis. Therefore, we hope very soon to proceed with the determinations we made in isolated NSC-34 cultures, now in mixed NSC-34/N9 cultures. Furthermore, it will be interesting to study more deeply certain pathological events in organotypical cultures from lumbar SC, including mitochondrial dysfunction and axonal transport, as well as molecules involved in cellular cross-talk. In addition, further development in the study of neuroprotective effects of GUDCA would be very interesting, in view of the need to create new therapeutic strategies for ALS onset or progression, once riluzole, the only therapeutic agent approved by FDA, which inhibits the glutamate-mediated excitotoxic in neurons can only cause a modest improvement in the survival of the patients (Kiernan *et al.*, 2011). Finally, the confirmation of these pathways using transgenic mice carrying G93A mSOD1 will be crucial to get a time point of each phase of the disease progression and to find biomarkers that allow identifying each of this phase.

Currently, the biggest investment in ALS therapeutic strategies has been at the level of stem cells, with the aim of replacement the individual mutated cells by healthy ones to become less neurotoxic. In the same line of thought, (Forostyak *et al.*, 2013) described the grafting of mesenchymal stroma cells as a way to improve motor and sensory function, modifying the host microenvironment following CNS injury. In fact, they have already achieved some positive results in clinical trials. There is still much that is unknown about the primary mechanisms involved in ALS onset and progression, although it is possible that the use of these stem cells combined with the administration of some neuroprotective compound (such as GUDCA) will become a common practice therapeutic, allowing rehabilitation of the patients at motor level.

By uncovering different experimental models, our results provide a strong basis for the comprehension of the molecular events involved in cellular failure in ALS, which is fundamental to develop new therapeutic strategies.

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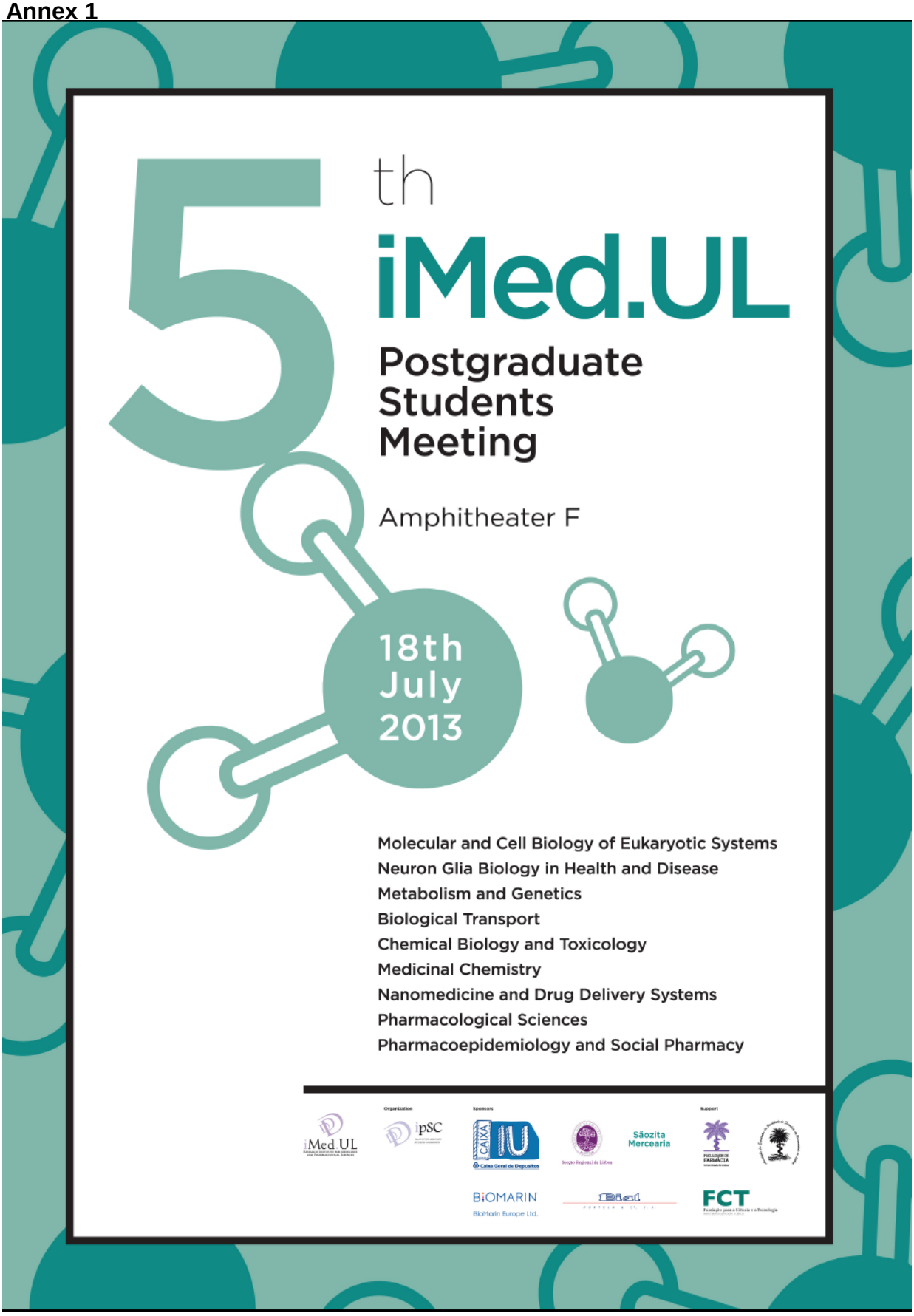
Annex 1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Modulation by Glycoursodeoxycholic Acid on an Organotypic-based Model of ALS

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Neuron Glia Biology in Health and Disease

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

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

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

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

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(1) Fernandes A, Brites D. Curr Pharm Des. 2009; 15: 2915-26.
(2) Su T, *et al*. Brain Res. 2011; 1385: 68-76.
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(b)

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

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

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Neuron Glia Biology in Health and Disease

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

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

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

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Annex 2


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PTW08-43

PI3K/Akt signaling pathway in the neuropathology of Krabbe disease

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

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PTW08-44

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

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

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

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

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